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Insulin signalling's role in mediating tissue-specific nutritional plasticity and robustness in the horn-polyphenic beetle *Onthophagus taurus*

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Organisms cope with nutritional variation via developmental plasticity, adjusting trait size to nutrient availability for some traits while enabling others to develop in a nutritionally robust manner. Yet, the developmental mechanisms that regulate organ-specific growth across nutritional gradients remain poorly understood. We assessed the functions of members of the insulin/insulin-like signalling pathway (IIS) in the regulation of nutrition sensitivity and robustness in males of the horn-polyphenic beetle *Onthophagus taurus*, as well as potential regulatory interactions between IIS and two other growth-regulating pathways: Doublesex and Hedgehog signalling. Using RNA*interference* (RNAi), we experimentally knocked down both insulin receptors (*InR1* and *InR2*) and Foxo, a growth inhibitor. We then performed morphometric measurements on horns, a highly nutrition-sensitive trait, and genitalia, a largely nutrition-insensitive trait. Finally, we used quantitative real-time polymerase chain reaction to assess expression levels of *doublesex* and the Hedgehog signalling gene *smoothened* following IIS-RNAi. Our results suggest that nutrition responsiveness of both traits is regulated by different IIS components, which transduce nutritional conditions to both Doublesex and Hedgehog pathways, albeit via different IIS pathway members. Combined with previous studies, our findings suggest that separate origins of trait exaggeration among insect lineages were enabled through the independent co-option of IIS, yet via reliance on different components therein.

1. Introduction

Variation in nutrition is one of the most fundamental and widespread challenges organisms face during development [1–3]. Organisms can meet this challenge by adjusting their phenotype through the process of developmental plasticity, thereby allowing a single genotype normally able to develop into a range of phenotypes to select trait values most adaptive given prevailing nutritional conditions [4]. Yet, different traits of the same individual may differ in what constitutes the most adaptive level of plasticity. Sexually selected traits such as weapons, for example, are often greatly exaggerated in larger individuals owing to their significance in aggressive interactions, yet muted in smaller individuals, and thus commonly exhibit extreme sensitivity to variation in nutrition [5,6]. On the other hand, legs or wings function in strict proportion to overall body size, deviations from which may carry severe fitness penalties, and thus commonly exhibit moderate nutrition sensitivity [7]. Lastly, some traits carry out functions that require a more constant absolute size regardless of overall body size or that of other structures; for example, male genitalia in insects selected to fit a wide range of female genitalia, or the central nervous system in most animals [8–10]. Such traits may exhibit minimal nutritional responsiveness during ontogeny and are said to be *robust* to nutritional perturbations. As a consequence, the degree of nutrition responsiveness—from highly plastic to robust—can vary strikingly among

different traits even within the same individual organism and in response to the same nutritional gradient.

When the growth rate of different traits varies in response to the same nutritional gradient, individuals composed of differentially responsive traits will grow not just to different overall body sizes, but will also develop different overall *shapes*. This makes the study of nutrition-dependent growth central to our understanding of the developmental regulation and evolutionary diversification of shape. Plastic responses to nutrition and their effects on scaling and shape can be investigated through the study of static allometries. Static allometries enable quantitative assessments of organ sizes relative to body sizes across individuals of the same population [11], and numerous studies now illustrate how the differential scaling of traits within species and evolutionary changes in scaling relationships among species have played critical roles in the genesis of morphological diversity (e.g. insect wings [12] and beetle horns [13]). What is less well understood, however, are the developmental mechanisms that regulate precise, organ-specific growth responses across nutritional gradients: how do organisms regulate extreme growth responses of some traits while shielding others from environmental fluctuations?

Some mechanisms regulating relative growth are starting to be elucidated in increased detail, at least in model organisms such as *Drosophila*. Chief among them is the insulin/insulin-like signalling pathway (IIS), a highly conserved pathway now recognized as mediating nutrition-responsive growth across phyla, from humans to insects [14,15]. In insects, high nutrition conditions induce the release of insulin-like peptides (ILPs) primarily from the insulin-producing cells in the brain into the haemolymph. The ILPs circulate through the haemolymph and reach target tissues where they bind to the insulin receptor (InR) and activate a phosphokinase signal transduction cascade that induces cell growth and proliferation [14]. Importantly, this pathway has been implicated in mediating differential nutrition sensitivity across organs within an individual [16]. In *Drosophila*, wings, legs and palps scale in proportion to body size [17], while other body parts such as the central nervous system [9] and genitalia [16] are far less sensitive to variation in nutrition, and differential sensitivities to nutrition have been linked to differential IIS activity. For example, genitalia achieve reduced nutritional sensitivity via the Forkhead box, subgroup O (Foxo), a growth inhibitor downstream of the InR [16]. This transcription factor is normally activated during low nutrition conditions and inactive during high nutrition conditions. However, by maintaining low *Foxo* expression levels even under low nutrition conditions, genitalia maintain a relatively constant absolute genitalia size across all nutritional environments [16]. While these findings have greatly advanced our understanding on how organisms translate a nutritional gradient into diverse and tissue-specific responses, these insights have been largely restricted to model organisms and relatively conventional types of nutritional responsiveness. By contrast, the developmental regulation of more complex nutritional responsiveness such as extreme trait exaggeration (as in weapons or ornaments) or polyphenic trait expression (i.e. presence/absence of trait expression in nutritionally cued morphs) remains poorly understood, even though both are widespread in nature. Thus, understanding the developmental underpinnings of more complex types of nutrition-responsive growth will require the use of more appropriate experimental model systems.

A recent study in the rhinoceros beetle (*Trypoxylus dichotomus*) has begun to elucidate the regulation of nutrition-responsive growth of exaggerated sexually selected traits and showed that *InR* may be involved in mediating differential nutritional sensitivities across different organs [5]. Specifically, *InR* knockdown during late larval development greatly affected the nutritional response in normally exaggerated horns, while moderately nutrition-sensitive wings were only mildly affected, and nutritionally non-responsive genitalia not at all. These results suggested that differential *InR* expression may be an important and possibly universal mechanism enabling differential growth of different organs in response to the same nutritional gradient. Furthermore, what role the IIS plays in the regulation of more complex scaling relationships such as polyphenisms is starting to be elucidated in hemipterans. Recent studies have shown that two *InR* paralogues are involved in the regulation of winged versus wingless morphs in the planthopper *Nilaparvata lugens* [18], whereas quantitative changes in *Foxo* function may be underlying the recent evolution of a novel reaction norm in the soapberry bug *Jadera haematoloma* [19]. These findings raise the possibility that this pathway may be a hotspot for the evolution of discontinuous and differentially responsive, nutrient-responsive organ growth [19]. Here, we use the polyphenic horned beetle *Onthophagus taurus* to investigate the role of IIS signalling in the development and evolution of extreme levels of nutritional responsiveness (horns) and robustness (genitalia).

Onthophagus taurus is a dung beetle common to the Mediterranean and secondarily introduced to North America and Australia. Males of this species develop, as is common for many members of this genus, into two alternative morphs depending on larval nutrition: high larval nutrition results in the development of large, horned, *major* males which rely on aggressive fighting behaviour to secure females, whereas low nutritional conditions result in the development of small, hornless, *minor* males which rely on non-aggressive sneaking behaviours and sperm competition to secure mating opportunities. The resulting horn length–body size allometry is strongly sigmoidal, and hornless and horned morphs are separated by a sharp body size threshold. Intermediate morphologies do exist in natural populations, but are comparatively exceedingly rare [20].

Recent studies in this system have shown that at least two pathways are critical regulators of body size and nutrition-dependent formation of horns. Doublesex (Dsx), the cardinal member of the somatic sex-determination pathway, promotes horn growth in large, high nutrition males [21], and dsx^{RNAi} eliminates nutrition-responsive horn growth in large males. By contrast, the Hedgehog (Hh) signalling pathway, most widely studied for its role in patterning anterior/posterior polarity, actively inhibits horn growth in small, low nutrition males, and knockdown of *smoothened* (*smo*), a key activator of Hh signalling, induces horns in male larvae otherwise fated to develop into hornless, minor males [22]. Combined, these studies demonstrated that both Dsx and Hh signalling are critical for the body size-specific induction or repression of horns, respectively, and the proper formation of the threshold body size separating alternate male morphs. However, *how* the action of either pathway is coordinated in light of a given individual's nutritional status remains unclear. Specifically, whether Dsx or Hh signalling (or both) are linked to components of IIS signalling and thus nutrition has yet to be examined.

In this study, we focused on the two insulin receptor paralogues common to most insects, *InR1* and *InR2*, as well as *Foxo*, to assess the possible functions of diverse IIS pathway members in the regulation of nutrition sensitivity and robustness. We contrasted the development of horns, a highly nutrition-sensitive trait, with that of male genitalia (specifically, the aedeagus), a nutrition-insensitive trait. Furthermore, we tested whether IIS signalling could be acting upstream of either Dsx or Hh signalling, or both, thereby providing one or both pathways with information regarding prevailing nutritional conditions during beetle development.

2. Material and methods

(a) Beetle husbandry

Onthophagus taurus beetles were collected near Bloomington, IN and Chapel Hill, NC, and reared and maintained in laboratory colonies as described previously [23].

(b) *Foxo*, *InR1* and *InR2* cloning and RNAi knockdown

Primers were designed against *O. taurus Foxo*, *InR1* and *InR2*, and corresponding fragments were cloned and sequenced to verify identity. Double Stranded RNA (dsRNA) for RNAi injections was generated as previously described [21]. Control dsRNA was generated following the same procedure using a vector sequence. The following dsRNA concentrations (dissolved in injection buffer) were used: control injections and $Foxo^{RNAi}$ (1 µg of dsRNA); $InR1^{RNAi}$ (0.5, 1, 3, 6 or 9 µg); $InR2^{RNAi}$ (0.25, 1 µg) and $InR1+2^{RNAi}$ (0.5 µg). Concentrations were varied in some cases in an attempt to improve penetrance and reduce mortality. All injections were executed during the last (=third) larval instar.

(c) Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to assess putative interactions between our genes of interest and *smo* and *dsx*. Glyceraldehyde 3-phosphate dehydrogenase and actin were used as reference genes in line with previous studies [24]. Primer sequences and fragment lengths are listed in the electronic supplementary material, table S2. Primer efficiency was tested for all genes using standard curves (see the electronic supplementary material). Whole-body samples were obtained from two developmental time points (24 h after dsRNAi injections and 24 h after pupation) to assess if effects on transcript abundance and pathway interactions are maintained across developmental stages. Three technical replicates were used for each sample. Larval samples were run separately for each individual to further control for any developmental timing effects, while pupal samples were pooled after RNA extractions. Larval samples included control ($n=5$), $Foxo^{RNAi}$ ($n=6$), $InR1^{RNAi}$ ($n=3$), $InR2^{RNAi}$ ($n_{smo}=4$, $n_{dsx}=5$) and $InR1+2^{RNAi}$ ($n=3$). Pupal samples included control ($n=7$), $Foxo^{RNAi}$ ($n=11$), $InR1^{RNAi}$ ($n=6$) or $InR2^{RNAi}$ ($n=4$). RNA was extracted using the Direct-zol RNA MiniPrep kit (Zymo Research; see the electronic supplementary material for details).

(d) Allometric measurements and analyses

dsRNA-injected adults were measured using a two-dimensional morphometric set-up. Thorax width was used as a measure of body size and horn size (head and thoracic) was measured as previously described [25]. For genitalia length, paramere and phallobase were measured as shown in the electronic supplementary material, figure S1. Following previous studies [26,27], we analysed the sigmoidal horn length–body size allometry by separately fitting a sigmoidal four-parameter equation to measurements obtained from control-injected and RNAi individuals, and using Welch's *t*-test to compare parameter means between control-injected and RNAi treatment groups. To further test the specific hypothesis that $Foxo^{RNAi}$ was linearizing the normally sigmoidal body size–horn length allometry, we used the Akaike information criterion (AIC) alongside the comparison of R^2 values to compare sigmoidal versus linear models fitted to allometric measurements obtained from control and $Foxo^{RNAi}$ individuals. Lastly, for a subset of our treatments, we also used a residuals analysis as in [24], calculating the difference between observed and expected horn length for a specific body size for all individuals, followed by a Mann–Whitney *U*-test. For the genitalia–body size allometry, we performed a linear model followed by model selection.

3. Results

(a) $Foxo^{RNAi}$ linearizes the sigmoidal body size–horn size allometry

Recall that horn development in male *O. taurus* is extremely sensitive to nutrition, resulting in the formation of two alternative horned and hornless morph separated by a sharply defined body size threshold. To investigate the role of *Foxo* in this nutritionally cued male polyphenism, we used RNAinterference-mediated transcript depletion of *Foxo* followed by morphometric assessments of horn length in relation to body size. $Foxo^{RNAi}$ profoundly altered this normally strongly sigmoidal scaling relationship by inducing relatively longer horns in small, low nutrition males while simultaneously modestly reducing horn length in several large, high nutrition males, compared to control-injected individuals (figure 1*a*,*b*). Fitting allometric (sigmoidal) models to $Foxo^{RNAi}$ and control-injected individuals revealed a significant difference in three of the parameters analysed: a reduction in amplitude ($p=0.014$), a shift in the inflection point to larger body sizes ($p=0.019$) and an elevation of the *y*-intercept ($p<0.0001$, $n_{FoxoRNAi}=52$, $n_{control}=49$; figure 1). We did not recover a significant effect on the slope ($p=0.826$).

To further assess the hypothesis that $Foxo^{RNAi}$ linearizes the normally sigmoidal body size–horn length allometry, we fitted either a sigmoidal or a linear model to each of our treatment groups and examined both R^2 and AIC values. As expected, R^2 values are maximized in control-injected animals when a sigmoid model is applied ($R^{2,sigmoid}=0.943$, $R^{2,linear}=0.671$); however, this discrepancy in fit decreases in $Foxo^{RNAi}$ males ($R^{2,sigmoid}=0.884$, $R^{2,linear}=0.759$). Similarly, AIC values indicate a superior fit of the sigmoid model to control-injected individuals ($AIC^{sigmoid}=33.73$, $AIC^{linear}=115.25$), but show that this discrepancy declines in $Foxo^{RNAi}$ males owing to both a reduction in fit of the sigmoidal model ($AIC^{sigmoid}=61.19$) and a commensurate increase in the linear model's fit ($AIC^{linear}=95.15$). Combined, these results support the hypothesis that $Foxo^{RNAi}$ significantly lessens the sigmoidal nature of the body size–horn length allometry, transforming it instead towards a more linear scaling relationship.

(b) InR^{RNAi} has no effect on the body size–horn size allometry

Mining the sequence data generated through several earlier studies [28–30] revealed the existence of two *InR* paralogues

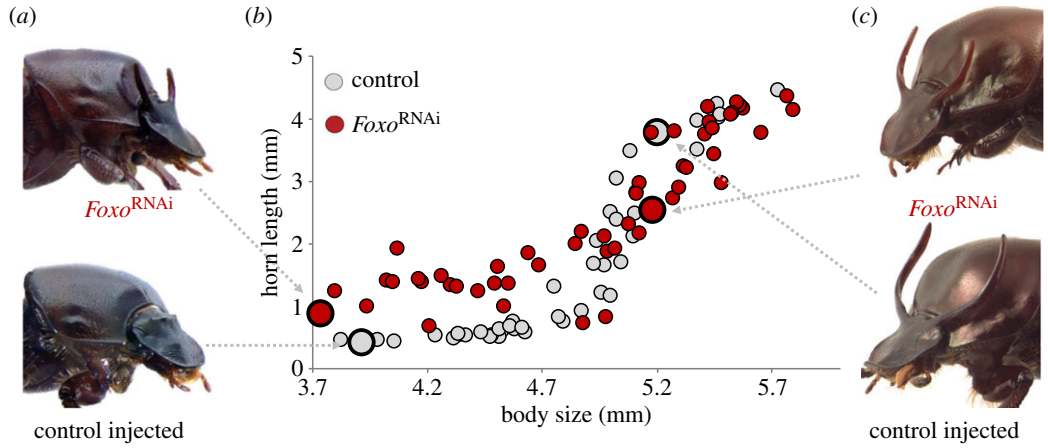

Figure 1. Effect of *Foxo*^{RNAi} on the highly nutrition-sensitive, sigmoidal scaling relationship between body size and horn length. *Foxo*^{RNAi} induced horn growth in low nutrition, normally hornless males (*a*,*b*), while modestly reducing horn growth in some high nutrition, normally fully horned males (*b*,*c*). Comparing control-injected (grey) and knockdown individuals (red) using independently fitted four-parameter Hill equations followed by Welch's *t*-tests to compare parameter estimates revealed a significant difference in amplitude, inflection point, *y*-intercept, but not slope. Comparing AIC and R^2 values between sigmoidal and linear models for both treatment groups revealed a major difference in fit for control-injected individuals, indicating that a sigmoid model most accurately captures the scaling relationship between body size and horn length. By contrast, fitting a sigmoid model to *Foxo*^{RNAi} individuals only modestly improves fit compared with a simple linear regression, suggesting that *Foxo*^{RNAi} partly transforms body size–horn length allometry from strongly sigmoidal towards a more linear relationship. (Online version in colour.)

in the *O. taurus* genome (electronic supplementary material, figure S2). More detailed analysis further revealed that expression levels of *InR1* and *InR2* differ across developmental stages (electronic supplementary material, figure S3a), and—at the pupal stage—across tissues (electronic supplementary material, figure S3b,c). *InR* duplications are common among arthropods, particulary across insects (reviewed in [31]) and a gene phylogenetic reconstruction shows that *Ot-InR1* is orthologous to the *InR* of other insects that exhibit a single *InR* copy (i.e. *Drosophila melanogaster*, the silkmoth *Bombyx mori*, the Colorado potato beetle *Leptinotarsa decemlineata*), while *Ot-InR2* clusters with the *InR2* of insects possessing duplicate *InR*s (i.e. the planthopper *N. lugens*, the red flour beetle *Tribolium castaneum*). Knockdown of *Ot-InR1* or *Ot-InR2* individually or in combination had no significant effect on the sigmoidal body size–horn size allometry (electronic supplementary material, figure S4), and analysis of residual horn lengths showed no significant effect following *InR1* ($W = 1004$, $p = 0.115$, $n_{InR1\text{RNAi}} = 34$, $n_{\text{control}} = 49$; electronic supplementary material, figure S4 a,b), *InR2* ($W = 643$, $p = 0.2$, $n_{InR2\text{RNAi}} = 22$, $n_{\text{control}} = 49$; electronic supplementary material, figure S4c,d) or *InR1* + 2 ($W = 410$, $p = 0.931$, $n_{InR1+2\text{RNAi}} = 17$, $n_{\text{control}} = 49$; electronic supplementary material, figure S4e,f) knockdown. These results are in marked contrast to earlier studies using an independent radiation of horned beetles in the subfamily Dynastinae (*T. dichotomus* [5]), which documented a major function of *InR1* in horn development (no analysis of *InR2* was conducted). Lastly, dsRNA injections in *O. taurus*, particularly at high concentrations, often resulted in a moulting phenotype similar to what has been reported for the red flour beetle *T. castaneum* [32] (electronic supplementary material, figure S5; *InR1*^{RNAi}: 46%, *InR2*^{RNAi}: 31%, *InR1* + 2^{RNAi}: 27%). Individuals with this phenotype were not able to ecdyse properly from the last larval to pupal moult and instead continued their development while remaining trapped within the larval cuticle.

(c) Foxo regulates nutrition sensitivity while InR regulates overall size of genitalia

Next, we investigated whether insulin signalling may also play a role in the regulation of nutrition insensitivity, i.e. the buffering of growth in the face of nutritional variation. To do so we focused on the development of male genitalia, in particular the aedeagus, a structure whose absolute size changes only modestly as a function of larval nutrition. Specifically, we measured aedeagus length in control-injected, *Foxo*^{RNAi}, *InR1*^{RNAi}, *InR2*^{RNAi} and *InR1* + 2^{RNAi} individuals. Our results suggest that *Foxo*^{RNAi} significantly altered the body size–aedeagus scaling relationship ($t = 4.011$, $p = 0.0001$) resulting in a further *decrease* of the slope of the genitalia–body size allometry ($t = -4.122$, $p < 0.0001$; figure 2*a*; electronic supplementary material, table S3). By contrast, *InR1*^{RNAi} resulted in a reduction of aedeagus size relative to body size across *all* body sizes ($t = -4.502$, $p < 0.0001$; figure 2*b*; electronic supplementary material, table S3), while leaving the magnitude of the nutritional response across body sizes unaltered. Qualitatively, similar results were obtained through *InR2*^{RNAi} (figure 2*c*; electronic supplementary material, table S3) and *InR1* + 2^{RNAi} (figure 2*d*; electronic supplementary material, table S3; $InR2_{\text{treatment}}$: $t = -2.574$, $p = 0.0123$; $InR2_{\text{body size}}$: $t = 13.284$, $p < 0.0001$; $InR1 + 2_{\text{treatment}}$: $t = -4.995$, $p < 0.0001$; $InR1 + 2_{\text{body size}}$: $t = 14.994$, $p < 0.0001$), suggesting that *InR1* and *InR2* have similar and body size independent growth promoting roles during genitalia development.

(d) *dsx* and *smo* expression levels may be regulated by the insulin signalling pathway

As introduced above, previous work identified two pathways critical for the expression of the nutritionally cued horn polyphenism in *O. taurus*: *dsx* signalling promotes horn formation under high nutrition conditions only, whereas *Hh* signalling inhibits horn formation under low nutrition conditions

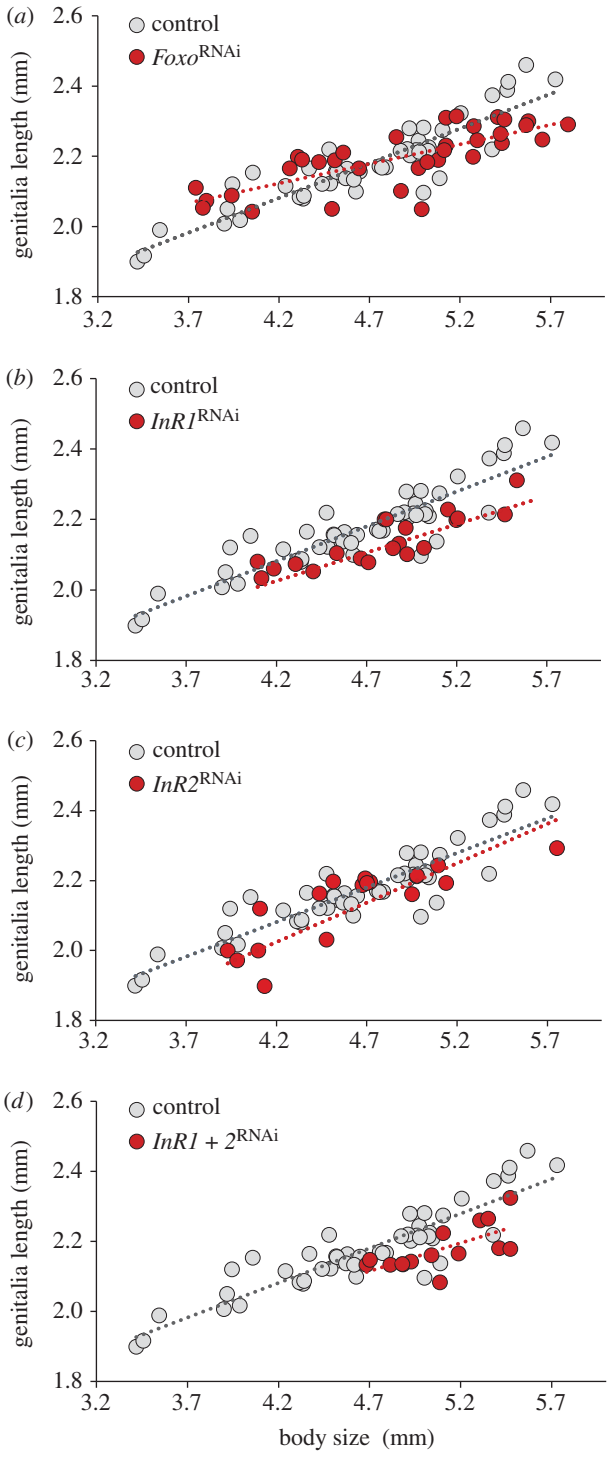

Figure 2. Effects of $Foxo^{RNAi}$, $InR1^{RNAi}$, $InR2^{RNAi}$ and $InR1 + 2^{RNAi}$ on the largely nutrition-insensitive scaling relationship between body size and male genitalia length. Shown are control-injected (grey) and knockdown (red) individuals. (*a*) $Foxo^{RNAi}$ resulted in a significant decrease in the slope of the body size-genitalia allometry, while (*b*) $InR1^{RNAi}$, (*c*) $InR2^{RNAi}$ and (*d*) $InR1 + 2^{RNAi}$ significantly reduced genitalia size across all body sizes. The same control individuals are shown across all four panels. (Online version in colour.)

only. However, exactly how nutritional variation is transduced to then affect the action of one or both pathways is unknown. To examine whether and how insulin signalling pathway members could be interacting with one or both pathways, we used qRT-PCR to assess expression levels of *dsx* and the Hh signalling gene *smo* across various knockdown backgrounds. Larval *dsx* expression decreased following $InR2^{RNAi}$ ($p = 0.029$; $n_{InR2RNAi} = 5$, $n_{control} = 5$; figure 3*a*), and a marginally significant decrease was also detected following $InR1 + 2^{RNAi}$ ($p = 0.083$; $n_{InR1+2RNAi} = 3$, $n_{control} = 5$). However, no effect was detected for $InR1^{RNAi}$ ($p = 0.675$; $n_{InR1RNAi} = 3$, $n_{control} = 5$) or $Foxo^{RNAi}$ ($p = 0.499$; $n_{FoxoRNAi} = 6$, $n_{control} = 5$), suggesting that *InR2*, but not *InR1* or *Foxo*, promotes *dsx* expression levels in larval *O. taurus*. By contrast, larval *smo* expression levels decreased after $Foxo^{RNAi}$ ($p = 0.01$; $n_{FoxoRNAi} = 6$, $n_{control} = 5$; figure 3*b*), but not when *InR1* ($p = 0.725$; $n_{InR1RNAi} = 3$, $n_{control} = 5$), *InR2* ($p = 0.693$; $n_{InR2RNAi} = 4$, $n_{control} = 5$) or *InR1* + 2 ($p = 0.885$; $n_{InR1+2RNAi} = 3$, $n_{control} = 5$) were downregulated, suggesting that *Foxo*, but not *InR1* or *InR2*, may promote *smo* expression levels in wild-type larvae.

A similar approach at the pupal stage yielded, in part, strikingly different results. While $Foxo^{RNAi}$ did not affect *dsx* expression 24 h after larval injection, in the resulting pupae *dsx* expression exhibited an increase by 34% (electronic supplementary material, figure S6a). Similarly, pupal *smo* expression increased following both $Foxo^{RNAi}$ and $InR1^{RNAi}$, whereas larvae had exhibited a decrease or no change in expression, respectively (electronic supplementary material, figure S6b). These results suggest that the nature of interactions between *dsx*, *smo* and members of IIS may vary throughout development and/or that later developmental stages may be affected through compensatory adjustments in expression dynamics as a result of RNAi perturbations. More generally, these results raise the possibility that IIS may provide nutritional information critical for the subsequent nutrition-dependent action of body-part specific growth regulators.

4. Discussion

Static allometries are the product of developmental mechanisms matching relative growth of body parts to overall body size, and evolutionary changes in these mechanisms underlie the wide diversity of scaling relationships observed in nature [12]. Understanding the mechanisms that relate the growth of parts to that of the entire organism and to the evolutionary diversification of organismal shape has been a major objective of a long-standing research programme at the interface of developmental biology and physiology. A large number of studies have now established the IIS pathway as an important regulator in the fine tuning of allometric scaling. However, comparatively fewer studies have examined the significance of the IIS pathway in organisms that show more extreme growth responses to nutritional conditions (e.g. [5]), or organisms whose growth responses are nonlinear, thereby enabling the widespread formation of alternative morphs or casts. Here, we use the polyphenic beetle *O. taurus* to investigate the potential functions of multiple members of the IIS pathway in the regulation of disparate growth responses by investigating two body regions that exhibit highly disparate levels of nutrition-responsive growth: (i) head horns characterized by the highly nonlinear, explosive and bimodal development, and (ii) the nutritionally canalized and largely unresponsive male copulatory organ. Our results suggest that different IIS pathway members regulate nutrition responsiveness in different body parts, and propose a candidate mechanism for the evolutionary transition from linear to sigmoidal scaling

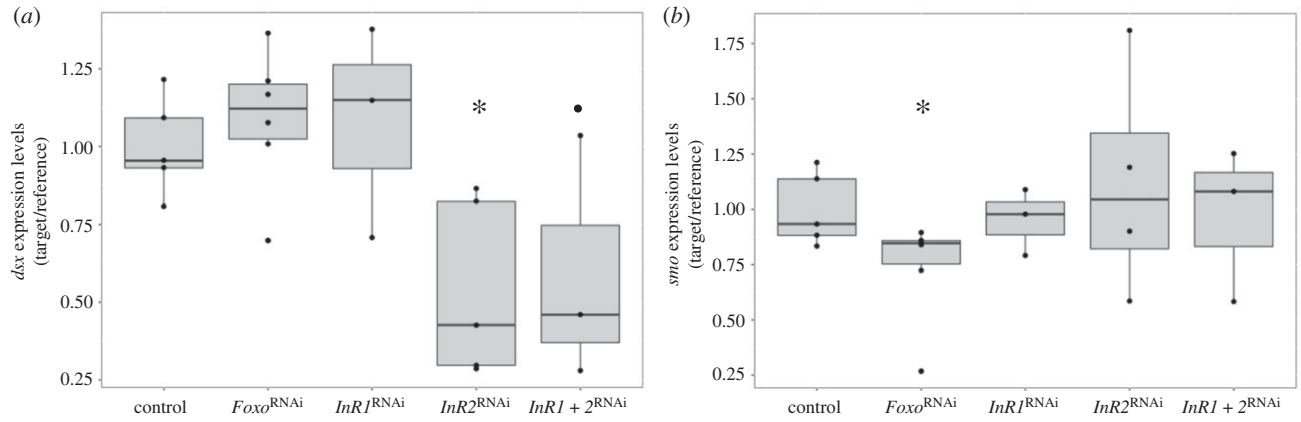

Figure 3. Larval *dsx* and *smo* expression levels in control-injected individuals and following $Foxo^{RNAi}$, $InR1^{RNAi}$, $InR2^{RNAi}$ and $InR1 + 2^{RNAi}$. (*a*) qRT-PCR using whole-body larvae shows that *dsx* expression levels are unaffected by $Foxo^{RNAi}$ or $InR1^{RNAi}$, but significantly reduced following $InR2^{RNAi}$ and marginally significantly reduced following $InR1 + 2^{RNAi}$. This suggests that *InR2*, but not *InR1*, promotes *dsx* expression in wild-type individuals. (*b*) *smo* expression levels are significantly reduced following $Foxo^{RNAi}$, but unaffected by $InR1^{RNAi}$, $InR2^{RNAi}$ and $InR1 + 2^{RNAi}$, suggesting that *Foxo* promotes *smo* expression in wild-type individuals. Box plots show 25% and 75% quartiles (boxes), medians (lines dividing the boxes), outermost values (whiskers), outliers (dots) and data points (dots overlapping with boxes and whiskers). $^{*}p < 0.05$, $^{\bullet}p < 0.1$.

relationships. By comparing our findings to previous work, our results raise the possibility that separate origins of trait exaggeration among horned beetle lineages were enabled through the independent co-option of the same signalling pathway, yet via reliance on different components therein.

(a) *Foxo*'s role in the development and evolution of sigmoidal horn–body size scaling

$Foxo^{RNAi}$ resulted in a significant increase in horn lengths of small, normally hornless males, suggesting that *Foxo* inhibits horn growth in low nutrition wild-type individuals. This is in marked contrast to a previous study in *Onthophagus nigriventris*, where $Foxo^{RNAi}$ had no effect on thoracic horn development [24]. However, our results are broadly consistent with separate previous studies, identifying *Foxo* as a growth inhibitor active when nutrition is scarce [16]. Similarly, $Foxo^{RNAi}$ resulted in a modest decrease in horn lengths of a subset of larger males, in particular those whose body sizes place them close to the horned side of the size threshold. This result is also consistent with prior findings in *Drosophila* and mammals, which identified *Foxo* as a growth sensitizer, upregulating *InR* under low nutrition conditions and priming tissues ready to proliferate in case further nutrition becomes available [33,34]. Combined, these results support the hypothesis that $Foxo^{RNAi}$ therefore lessens the sigmoidal nature of the normally strongly biphasic body size–horn length allometry, thereby transforming it towards a more linear scaling relationship. While this allometric transformation is incomplete, it nevertheless raises the possibility that recruitment of *Foxo*-mediated inhibition of horn growth at low nutrition coupled with growth sensitization at intermediate to high nutritional conditions could have played critical roles in the evolutionary transition from ancestral linear allometries to derived sigmoid scaling relationships [35]. This hypothesis is further supported by a previous study, which recovered strikingly high *Foxo* expression levels in horn tissue (particularly in small males), compared to genitalia or brain tissue (electronic supplementary material, figure S7; [29]). This raises the possibility that an increase in *Foxo* expression levels could have been a key step in the evolutionary transition from a linear to a sigmoidal allometry. Comparative functional analysis of *Foxo* and other putative growth regulators in species with varying degrees of male polyphenism could shed further light on the regulation and evolution of nonlinear scaling relationships.

(b) *Foxo* regulates both plasticity and robustness in a tissue-specific manner

Previous work in *Drosophila* implicates *Foxo* in the regulation of tissue-specific nutritional plasticity [16]. In most traits studied, to date, low nutrition results in the upregulation of *Foxo* and subsequent growth inhibition. However, in *Drosophila* genitalia, *Foxo* expression remains very low even when nutrition is scarce, which in turn is thought to reflect one of the mechanisms maintaining the shallow, largely nutrition-insensitive allometry of fly genitalia. By maintaining low *Foxo* expression levels, genitalia in low nutrition animals are able to 'ignore' their nutritional status and grow to similar sizes as those of medium or high nutrition individuals [16]. Similarly, experimental upregulation of *Foxo* in genitalia and wings increased nutrition sensitivity by decreasing trait size in small individuals [16]. In *O. taurus*, *Foxo* expression levels are much higher in head horns compared to other traits (electronic supplementary material, figure S7; [29]), and our $Foxo^{RNAi}$ results are consistent with findings in *Drosophila*: by decreasing *Foxo* expression levels in the highly plastic head horns, we were able to decrease nutritional plasticity. However, this was also true, albeit to a lesser degree, for *O. taurus* genitalia. Here, the already shallow slope of the aedeagus body size allometry was further decreased. Even though male genitalia of both wild-type *Drosophila* and *O. taurus* share similar allometric slopes when plotted on a log–log scale (*Drosophila:* [16]; *O. taurus*: S. Casasa 2018, unpublished data), $Foxo^{RNAi}$ has no effect in flies [16] yet results in a significant further decrease of the allometric slope in *O. taurus* (figure 2*a*). This additional slope reduction in $Foxo^{RNAi}$ genitalia suggests that (i) even modest *Foxo* expression levels may be sufficient to instruct a shallow, but nevertheless nutrient-sensitive allometry in *O. taurus*, and (ii) that the observed effect in *O. taurus* genitalia, but its absence in *Drosophila*, could be owing to a greater overall

nutrition sensitivity in *O. taurus*. Together, our results suggest that low-to-moderate *Foxo* expression levels may be contributing to the nutritional robustness of genitalia. Further studies exploring interacting nutrient-sensing systems (i.e. TOR or Hippo signalling) could help us better understand the mechanisms of nutritional insensitivity in genitalia.

(c) *InR* regulates aedeagus but not horn growth

In an important earlier study, Emlen *et al.* [5] reported a highly significant decrease in horn length in the rhinoceros beetle *T. dichotomus* (Dynastinae) following $InR1^{RNAi}$. The same study also reported a modest reduction of adult wing size, but no effect on male aedeagus length, following the same manipulation. Based on these results, Emlen *et al.* [5] proposed that the IIS pathway in general and differential *InR* expression in particular constitute a central regulator of relative trait size and nutritional plasticity during insect development, and proposed it as a critical facilitator of honest signalling underlying the evolutionary origin and maintenance of exaggerated secondary sexual traits across animals. By contrast, we were unable to detect any measurable effect of $InR1^{RNAi}$, $InR2^{RNAi}$ or $InR1 + 2^{RNAi}$ on the horn length–body size allometry in *O. taurus*. At the same time, we did recover a highly significant reduction in male genitalia size. Recall that injection of high dsRNA concentrations of either construct resulted in a lethal moulting phenotype that could have masked potential horn phenotypes. However, this appears unlikely because lower concentrations were sufficient to yield a highly repeatable reduction in genitalia size alongside successful and complete eclosion.

Trypoxylus dichotomus (Dynaestine) and *O. taurus* (Scarabeinae) belong to different subfamilies of scarab beetles which independently evolved exaggerated horns and horn-like structures [36,37], though only the latter also evolved pronounced male polyphenisms. Our results raise the possibility that as both lineages independently evolved exaggerated horns, they each did so by independently recruiting the IIS into the regulation of relative horn size, yet by using different pathway components for different sets of traits: dynastine beetles may rely on differential expression of at least one of the insulin receptors to promote different degrees of nutrition-responsive growth across traits including horns (high levels, high sensitivity and exaggeration) and genitalia (low levels, insensitive, no exaggeration). By contrast, scarabaeine beetles may use the insulin receptor only to facilitate nutrition-insensitive growth of male genitalia and instead use *Foxo*-mediated differential inhibition in both horns and genitalia to enable different types of nutrition-responsive growth—polyphenic in the case of horns and largely nutritionally insensitive in the case of genitalia. A partly similar scenario appears to emerge from recent findings in hemipterans: while two *InR* paralogues are involved in the regulation of alternative wing polyphenic morphs in the planthopper *N. lugens*, evolutionary changes in Foxo function seem to underlie divergences in wing polyphenisms between populations of the soapberry bug *J. haematoloma* [18,19]. Our results thus generally support the broader significance of IIS in the evolutionary diversification of nutrition-responsive growth, but may call into question the claim that conserved insulin signalling is a universal mechanism of simple trait exaggeration. Instead, IIS may be a common pathway recruited into the sensitivity of nutrition responsiveness, but the precise mechanisms (i.e. IIS components) involved may differ greatly across taxa.

Our results also suggest the possibility of developmental interactions between *InR* and moulting hormones, as indicated by the moulting phenotype detected following high-dosage $InR1/2^{RNAi}$ injections noted earlier. Previous studies in insects documented interactions between IIS and two major moulting hormones, ecdysone and juvenile hormone (JH; [38,39]). Interestingly, ecdysone has also been implicated in the regulation of *Drosophila* imaginal disc growth [40], and JH contributes to the regulation of nutrition-responsive growth in stag beetle mandibles [41]. Comparative studies across taxa and pathways are needed to further disentangle the developmental and evolutionary routes to differential nutritional plasticity across trait types.

(d) Insulin/insulin-like signalling pathway interacts with both *dsx* and *smo* to regulate nutrition-sensitive growth

Recall that previous work identified both *dsx* and Hh signalling as critical regulators of nutrition-responsive growth in *Onthophagus*. While the male isoform of *dsx* promotes horn formation under high nutrition conditions only [21], Hh signalling via *smo* actively inhibits horn growth under low nutrition conditions only [22]. One of the major questions raised by these results concerns how either pathway may be functionally linked to nutritional conditions experienced during growth. An indirect hint was obtained from subsequent work, which pursued a genome-wide screen to identify direct and indirect *dsx* target genes [29]: conspicuously, absent among the otherwise enormous diversity of genes and pathways that both possessed Dsx-binding motifs in their promotor region and responded in their expression to experimental *dsx* downregulation were any members of the IIS. This raised the possibility that IIS may instead be operating upstream of *dsx* and perhaps *smo* as well. Lastly, results presented here show that $Foxo^{RNAi}$, but not $InR1/2^{RNAi}$, partly phenocopies horn phenotypes induced by both *dsx* and *smo* RNAi. To test the hypothesis that IIS pathway members may be regulating *dsx* and/or *smo* to promote or inhibit horn growth, respectively, we assessed *smo* and *dsx* expression in various RNAi backgrounds using whole-body RNA extractions. While this approach does not allow us to assess organ-specific pathway interactions, our results nevertheless provide a roadmap for further study into the regulation of *dsx* and *smo* by components of IIS signalling in the regulation of nutrition-responsive growth. We find that $Foxo^{RNAi}$ (but not $InR1^{RNAi}$, $InR2^{RNAi}$ or $InR1 + 2^{RNAi}$) results in a reduction of *smo* expression, suggesting that *Foxo* promotes *smo* expression in wild-type males. Because *dsx* inhibits *smo* at high but not low nutrition [29, E. Zattara 2018, unpublished data], this proposed interaction between *Foxo* and *smo* may therefore only be phenotypically relevant at low nutrition and explain why the experimental downregulation of either gene results in the induction of horns in small, low nutrition males only (figure 4).

Conversely, we also find that $InR2^{RNAi}$ (but not $Foxo^{RNAi}$, or $InR1^{RNAi}$) results in a reduction of *dsx* but not *smo*, consistent with a role of one of the insulin receptor paralogues in promoting *dsx* expression and thereby horn growth. In contrast to our $Foxo^{RNAi}$ results, however, $InR2^{RNAi}$ does *not* phenocopy dsx^{RNAi} horn phenotypes, rendering these findings in need of further study. Collectively, our results thus

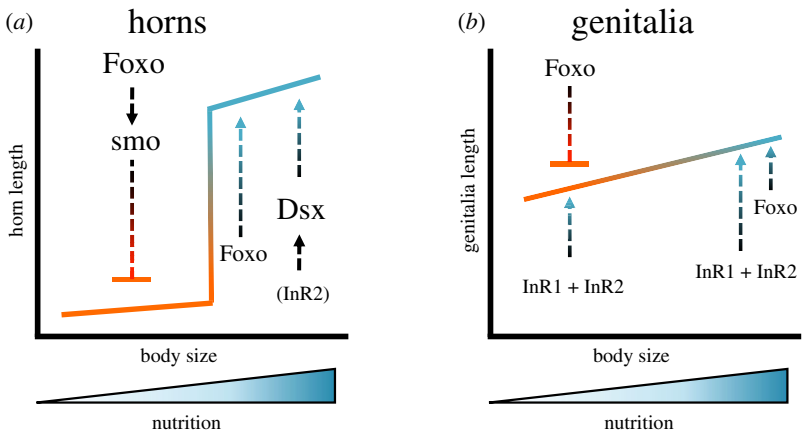

Figure 4. Proposed model for the regulation of horn and genitalia allometries by insulin signalling and interacting pathways. (*a*) Regulation of high levels of nutritional responsiveness during horn formation across a nutritional gradient. Low nutrition conditions result in the upregulation of *Foxo*, promoting *smoothened* (*smo*) expression in the process (directly or indirectly), which in turn inhibits proliferation of horn tissue, resulting in a hornless male phenotype (this study and [22]). In these individuals, *Foxo*^{RNAi} results in the disinhibition and induction of horn growth. In intermediate-sized males, *Foxo* acts as a growth sensitizer resulting in disproportionate growth responses and the reduction in horn growth in *Foxo*^{RNAi} individuals. Lastly, high nutrition conditions result in *Foxo*-independent expression of the male isoform of *doublesex* (*dsxm*) and the promotion of horn proliferation [21]. Results presented here provide partial support that *InR2* expression promotes *dsxm* expression under these conditions (thus *InR2* is presented in parentheses). (*b*) Regulation of low levels of nutritional responsiveness during genitalia growth across a nutritional gradient. *Foxo* inhibits genitalia growth under low nutrition condition, but promotes genitalia growth under high nutrition conditions. As a consequence, *Foxo*^{RNAi} males possess relative larger genitalia under low nutrition condition, but relatively smaller under high nutrition conditions, resulting in a shallower genitalia–body size allometry. By contrast, *InR1* and *InR2* promote genitalia growth regardless of nutritional conditions (figure 2), and *InR1/2*^{RNAi} results in reduced genitalia sizes across the entire range of body sizes. How *InR1/2* and *Foxo* interact during genitalia formation remains to be determined. Effect size is indicated by font size, and blue arrows and orange bars indicate interactions (growth induction or inhibition, respectively) supported by RNAi or qRT-PCR data (this study and [21,22]). (Online version in colour.)

provide important evidence, supporting that IIS signalling may provide essential nutritional information to the Hh (via Foxo) and possible Dsx (via InR2) pathways which then transduce this information to instruct organ-specific growth responses (figure 4).

In conclusion, our results suggest that in the horn-polyphenic beetle *O. taurus*, both nutrition sensitivity and insensitivity may be regulated by diverse components of the IIS pathway and their interactions with *dsx* and Hh signalling across different body parts. The growing number of studies implicating diverse IIS components in both hemi- and holometabolous insects supports the hypothesis that this pathway may be a hotspot for the evolution of nutrient-sensitive trait growth [5,18,19], including, as suggested by the results of this study, the evolutionary transition from linear to strongly sigmoidal allometries. However, the specific components of IIS and their interactions with other pathways (e.g. TOR, Hippo, Dsx and Hh) will require further investigation to disentangle the developmental and evolutionary routes to differential nutritional plasticity across trait types in different lineages.

Ethics. All animal procedures adhered to the ABS Guidelines for the Use of Animals in Research. Animals were kept in the best possible conditions based on the biology of this species. The study involved a common species of insect for which no review by an institutional or governmental regulatory body was required.

Data accessibility. All data are available either as the electronic supplementary material or deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.35g4j2q [42].

Authors' contributions. S.C. and A.P.M. designed the study. S.C. performed the molecular work and morphometric measurements. S.C. and A.P.M. analysed the data and drafted the manuscript.

Competing interests. We have no competing interests.

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