Supplementary Materials

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

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Proc. R. Soc. B 20181631. http://dx.doi.org/10.1098/rspb.2018.1631

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SUPPLEMENTARY TABLES

Table S1. Primers used to clone *Foxo, InR1* and *InR2*, and the corresponding sequences used to synthesize *ds*RNA for *Foxo*, *InR1* and *InR2*.

Target	Primer F	Primer R	Length (bp)	Sequence cloned and used for dsRNA
Ot-Foxo	TGGATGGTTC AGAACGTGC CCTAT	ACGCCGCTCT TCTTCTCACA GATT	404	GGCGTCGATGGAAACGAGTAAATTTGAAAAACGAAGGGGGCGAGTTA AGAAGAAGGTTGATGCGATGAGAAATGGTTTACCTTTGGACCCCACTT CTAGTCCTAGTTCATCAGTCCACGAAGGGTTAGATGATTTTCCAGATTC ACCTTTAGGAAGCTTTAATCAATCAAGTCCTGATTTTCGACCAAGAACC TCATCGACAACCTCATCTTGCGGAAGATTATCCCCAATACCATCGATGA GTCACGAACCGGATTGGGGCCGAATAAACCAATACAACTCAAATTATA GCCCCGAAATGGGTGGTAATTATTCCCCGGATACTCTTGCTGGTTCTTT GGAGCAAGGGATGAAACTTCAATCCGATTACGACCTTTATATCAACGG TAGCATCCAACAGCAAC
Ot-InR1	TTTGCCGGAT AAGACACCC C	CGAACGTAG ACTTGTTTTC ATGGT	303	TTTGCCGGATAAGACACCCCATGGCATCGAAGTCGTATTGCGGAGTTG CTGACACGGGCCGGCTCTGTACTTGGTTGCTGGTTACGTGGGTGGTTTT TGGATTTTACTGCAACTTGCCGGTTACCCACGCTGGTTATATTACCGAA TACAACGGATTTACTCCATACAATAAAACATCAGGCATATGCAAGAGT GTGGATATCCGAAACTCAGGGGAGATGTTCGAGAACTTACGAGGCTGC AGAGTCGTGGAGGGTTACATCCAAATCCTTCTTTTTGACCATGAAAAC AAGTCTACGTTCG
Ot-InR2	GCATGACGA GATGTACAT GGTG	TTTGGATGGC TTGGTTCGGA	339	CCCAATGTGGAATTCGCCCTTTTTGGATGGCTTGGTTCGGACCAATTAA GAATGCAGTCTTTATCGTTTGTGTAATAAGTGATGGTTTAACGATATCC GCCGCTTCATTTTCTAAAGTTCTCGCGGTTTTCATAAGGATCGAGCTAC AATAAATCTTTCTTTGCAATTGTTCGTTCGATGATAACTCTTCAAGTGTT AAATTAGGCGGATGATTACAAGCGGAAAATAAAAATATATACAGCGT GAATTGTTCTAAATTACTTAATGTGTAATTATTAGATGATGCGTTTATA TTAAACACCATGTACATCTCGTCATGCAAGGGCGAATTCCACAGTG

Table S2. Primers used for qRT-PCR.

qRT-PCR target	Primer F (5' - 3')	Primer R (5' - 3')	Sequence length	Efficiency
Ot- actin	TCACCACCCACGCTGAAAGAGAAA	ATAAAGCTTCTGGGCAACGGAACC	185	93.5%
Ot-GAPDH	GGGCCCAAGTAACCGCAATCAA	TGGTGGCTACGGAACCTCGAAAT	108	96.8%
Ot-smo	GATCAGAAAGCTCTTCGTGTCCCGAA TTA	CGTTTCGATTGCTTACTTCCGCGATTT	99	101.6%
Ot-dsx	TGATTCCCCAATCGAAAAGCCC	GGCCAATATTGTTAATCCCCAAATTA TCTCTG	163	99.5%

Table S3. Summary statistics for genitalia measurements. Linear models were used and model selection was carried out by removing non-significant variables. Genitalia length was used as the response variable with body size, treatment and concentration of treatment (µg injected) initially used as fixed variables.

Treatment	y-intercept	Body size	Treatment	Body size* Treatment
Foxo^{RNAi}	$t = 18.773$ $p < 0.0001$	$t = 13.967$ $p < 0.0001$	$t = 4.011$ $p = 0.0001$	$t = -4.122$ $p < 0.0001$
InR1^{RNAi}	$t = 17.36$ $p < 0.0001$	$t = 12.136$ $p < 0.0001$	$t = -4.502$ $p < 0.0001$	*NA*
InR2^{RNAi}	$t = 16.872$ $p < 0.0001$	$t = 13.284$ $p < 0.0001$	$t = -2.574$ $p = 0.0123$	*NA*
InR1+2^{RNAi}	$t = 20.612$ $p < 0.0001$	$t = 14.994$ $p < 0.0001$	$t = -4.995$ $p < 0.0001$	*NA*

SUPPLEMENTARY FIGURES

Figure S1. Measurement of genitalia. Red line indicates aedeagus length (genitalia) measurement as conducted in this study.

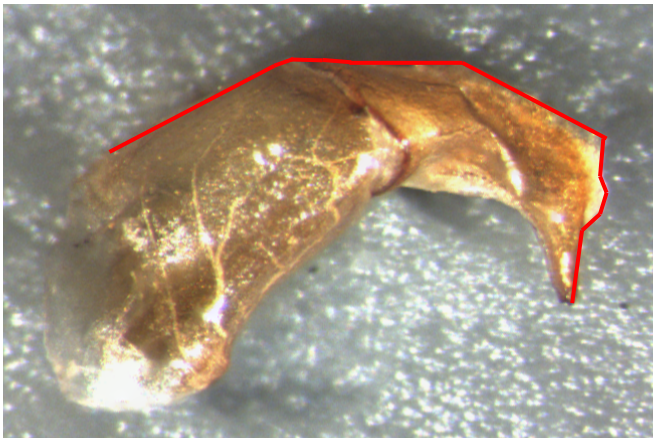


Figure S2. Molecular phylogeny of the Insulin Receptors. *Ot-InR1* clusters with *InR1* from most insect taxa (orange), including those where only a single *InR* sequence is known. *Ot-InR2* clusters with *InR2* from insects that have undergone *InR* duplication (blue), including planthoppers, red flower beetles and hymenopterans. Shown is a phylogenetic tree obtained after aligning sequences from 20 species. The protein tree was constructed using maximum-likelilhood (RAxML, bootstrap = 100) and support values for the nodes are included.

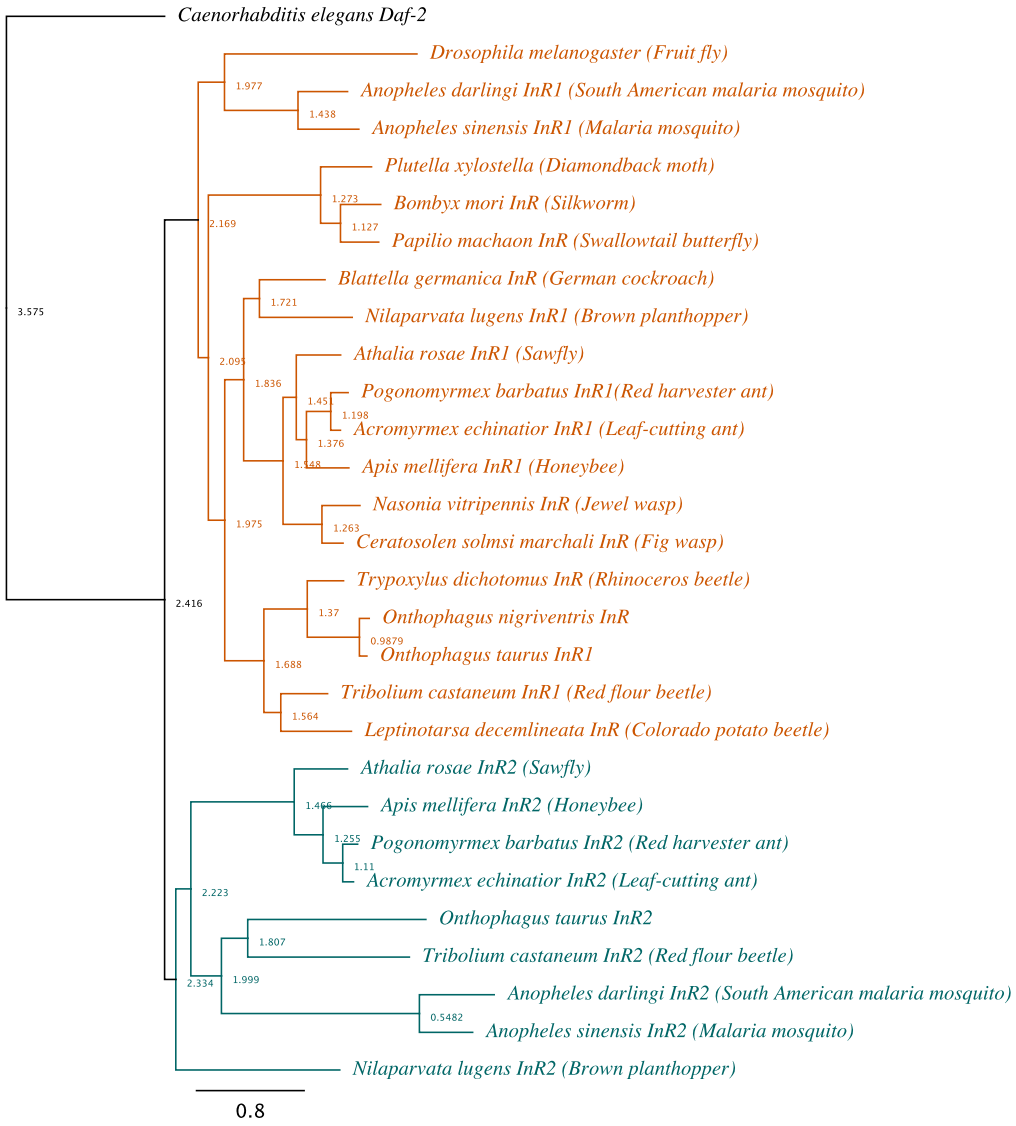


Figure S3. *InR1* and *InR2* expression levels across development and tissues. Expression levels of *InR1* and *InR2* estimated by RNAseq (data from[27,28]) for **(a)** whole body at larval, prepupal, pupal and adult stages, **(b)** horn, genitalia and brain tissue in small, low nutrition, hornless males at the pupal stage, and **(c)** horn, genitalia and brain tissue in large, high nutrition, horned males at the pupal stage. Error bars represent standard error from six replicates (individuals).

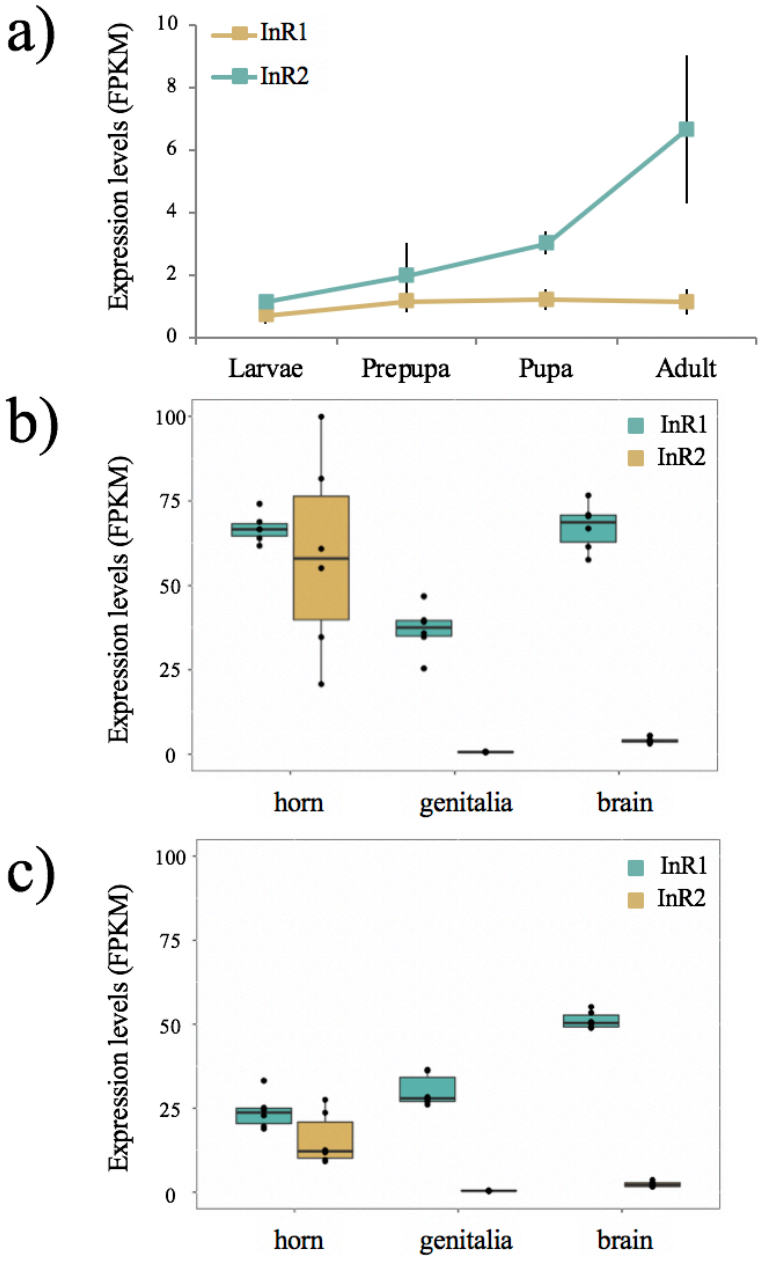


Figure S4. Horn formation and scaling in *O. taurus* is unaffected by single or double knockdown of *InR1 and InR2*. Left: allometries Right: residuals. Comparing control injected and knockdown individuals revealed no detectable effect for *InR1*^{RNAi} (a,b; residuals $p = 0.115$), *InR2*^{RNAi} (c,d; residuals $p = 0.2$) or *InR1+2*^{RNAi} (d,e; residuals $p = 0.931$) on relative horn length and horn length body size allometries. The left column (a,c,e) shows the allometries for the three treatment groups and the right column (b,d,f) shows the corresponding horn length residuals (calculated as the difference between observed and expected horn lengths for a given body size).

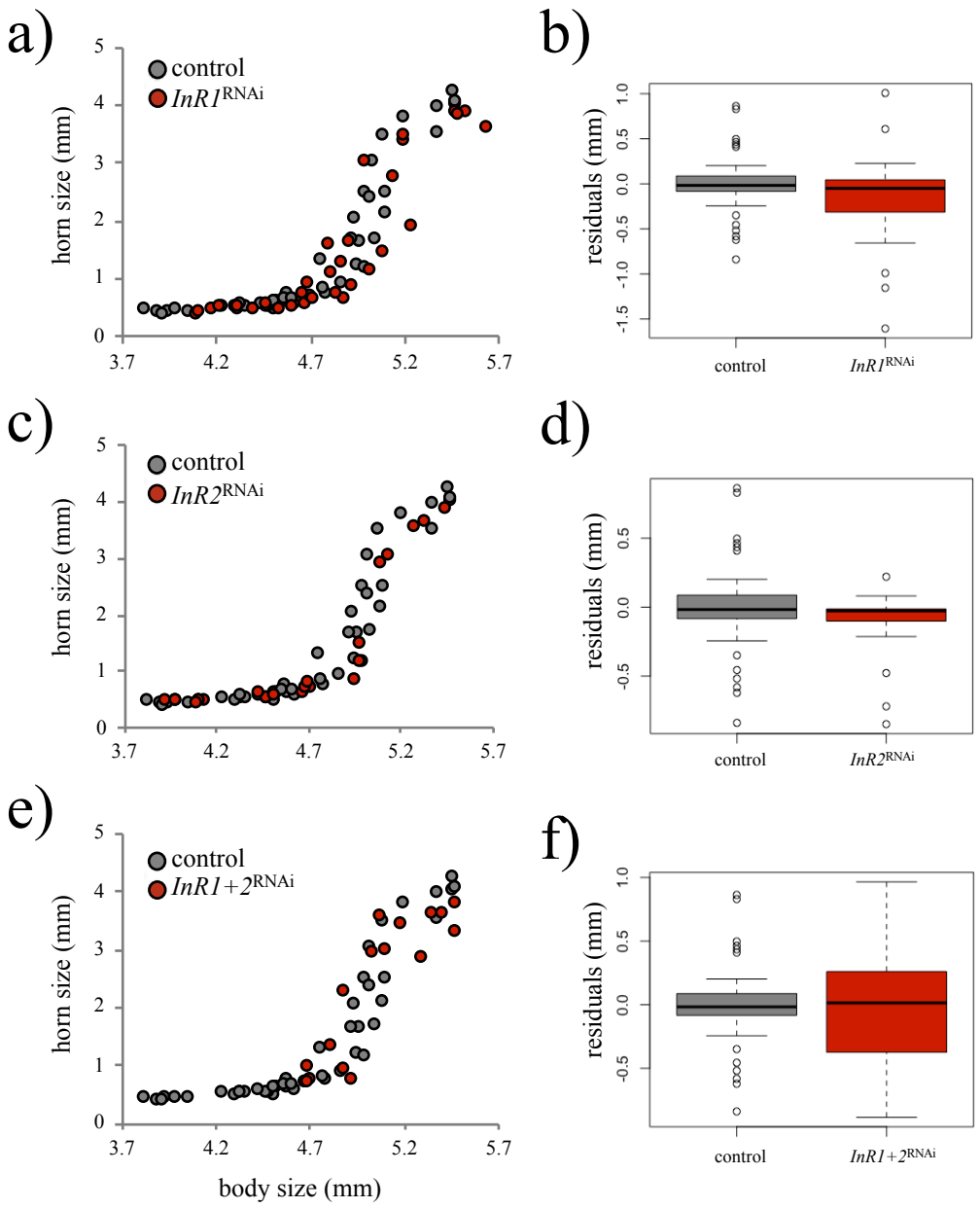


Figure S5. *InR*^{RNAi} molting defect phenotype. *InR1*, *InR2* and *InR1+2* knockdowns yielded a molting defect phenotype, preventing individuals to eclose from the larval cuticle and instead forcing to continued their development trapped within the larval cuticle. Left: control injected pupa, right: *InR1*^{RNAi} pupa.

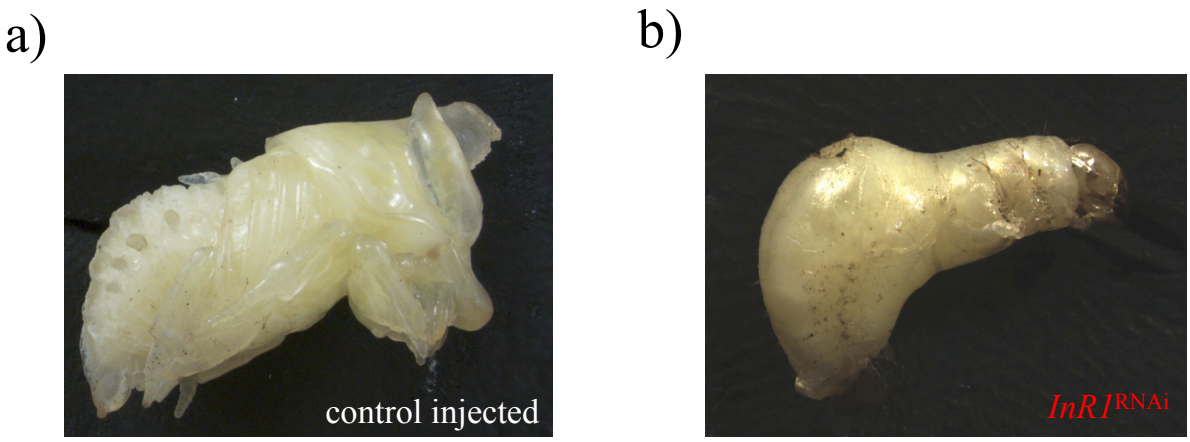


Figure S6. Pupal *dsx* and *smo* expression levels in control injected individuals as well as following *Foxo*^{RNAi}, *InR1*^{RNAi} and *InR2* ^{RNAi}. (a) qRT-PCR using whole-body pupae show an increase in *dsx* expression levels following *Foxo*^{RNAi} (34%), and modestly following *InR1*^{RNAi} (16%), *InR2* ^{RNAi} (12%). (b) *smo* expression levels increase following *Foxo*^{RNAi} (26%) and *InR1*^{RNAi} (27%), but not *InR2* ^{RNAi} (7%). Error bars represent standard error across technical replicates.

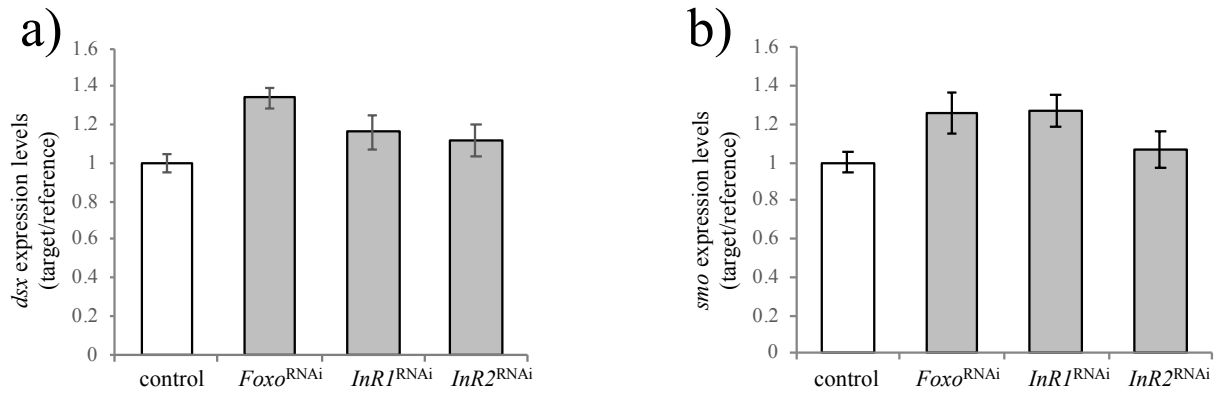
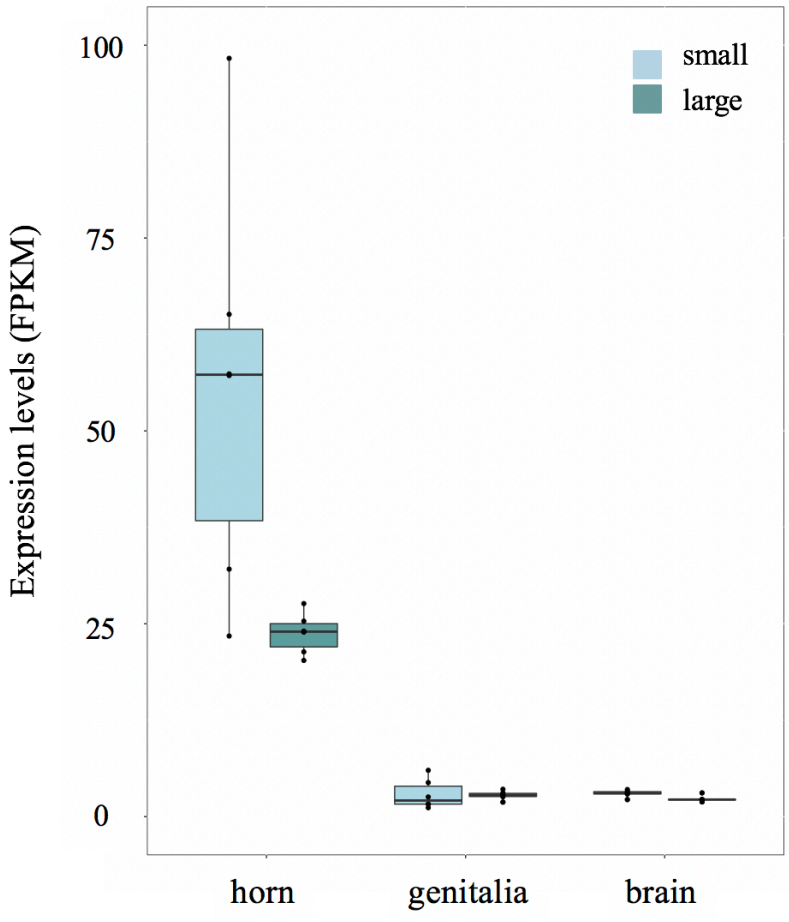


Figure S7. Expression levels of *Foxo* across tissues. Expression levels of *Foxo* as estimated by RNAseq (data from [27]) of small (light blue) and large (dark blue) male horn, genitalia and brain tissue at the pupal stage. Error bars represent standard error from six replicates (individuals).



SUPPLEMENTARY METHODS

Beetle husbandry

Onthophagus taurus beetles were collected near Bloomington, IN and Chapel Hill, NC, and reared and maintained in laboratory colonies as described previously [1]. Briefly, adult colonies were kept at 24ºC in a sand/soil mixture on a 16:8 light:dark cycle and fed homogenized cow dung twice per week. To obtain larvae, adults were bred in plastic breeding containers with packed and moist sand/soil mixture and ~0.5L of cow dung. Six females and three males were allowed to breed for six days, brood balls were collected and larvae were transferred to 12-well cell culture plates and supplied with dung as described in [2].

InR1 *and* InR2 *molecular phylogeny*

Annotated gene coding sequences and protein sequences from diverse insects were retrieved from NCBI, the gene coding sequences were translated, and the amino acid sequences were aligned using MAFFT. The alignments were used to build a molecular phylogenetic tree using RAxML in Geneious 8.1.7. Default parameters were used.

Foxo*,* InR1 *and* InR2 *cloning and sequencing*

For *Foxo*, primers were designed using a previously identified *O. taurus* fragment (GenBank accession HQ605923). *InR1* and *InR2* sequences were obtained using previously published *Tribolium castaneum InR1* and *InR2* [3] as search queries in an *O. taurus* genome and transcriptome database [4-6], and primers were designed using PrimerQuest Tool (Integrated DNA Technologies) based on these sequences. Primer sequences are listed in Table S1. For all three genes, PCR fragments (~300 bp) were cloned into a pSC-A vector using a StrataClone PCR Cloning Kit (Agilent). Cloned fragments were sequenced using BigDye Cycle Sequencing Chemistry (Life Technologies) to verify identity.

RNAi knockdown

*ds*RNA for RNAi injections was generated as previously described [7]. Briefly, the vectors containing previously cloned fragments for each target gene were purified using QIAprep Spin Miniprep Kit (Qiagen) and amplified through PCR using M13 primers. The PCR product was used as a template for *in vitro* transcription of forward and reverse RNA strands using MEGAscript T7 and T3 Kits (Life Technologies). The *ss*RNA was treated with TurboDNAse to

remove DNA template, precipitated by lithium chloride, incubated at -20 for 1 hour, centrifuged for 5 min at 4ºC, washed with 70% ethanol and resuspended in water. Forward and reverse RNA strands were mixed at a 1:1 ratio and annealed by heating to 80ºC followed by a slow cool down over 8 hours. Annealing was confirmed by gel electrophoresis, dsRNA concentration was measured using a NanoDrop, and *ds*RNA was stored at -80ºC until injection.

qRT-PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the PerfeCTa SYBR Green SuperMix (Quanta Biosciences). Primers for all genes were designed using PrimerQuest Tool (Integrated DNA Technologies) and their efficiency was tested using standard curves over four-fold dilutions of cDNA (1:4, 1:16, 1:64, 1:256).

Animals were flash frozen in liquid nitrogen and stored at -80 ºC until assayed. Total RNA was extracted using Direct-zol RNA MiniPrep kit (Zymo Research). 2 µg of total RNA was used for reverse transcription and samples were diluted 1:80. The qRT-PCR reaction was run using a LightCycler 480 II instrument (Roche Molecular Systems) and analyzed using the Light Cycler 480 software (Roche Molecular Systems). Reaction conditions were: 95º C for 3 min, then 45 cycles of 95 ºC for 15 sec, 57ºC for 45 sec, 72 ºC for 30 sec, followed by standard melt curve analysis (95ºC for 5 sec, 55ºC for 1 min and 95ºC).

Allometric measurements and analyses

Morphometric measurements were taken using a two-dimensional setup consisting of a Leica dissecting microscope, a Scion digital camera and ImageJ v1.44p software. Measurements were taken to the nearest 0.001mm.

The sigmoidal horn length-body size allometry was analyzed by separately fitting a sigmoidal 4-parameter equation (Hill equation using *Sigma Plot 12.5)* to measurements obtained from control injected and RNAi individuals. The equation contained four parameters, amplitude, slope, inflection point and *y*-intercept, and we used Welch's t-test to compare parameter means between control injected and RNAi treatment groups. For a subset of our treatments we used a residuals analysis, which is able to detect changes in allometries not reflected in changes in the specific model parameters included in the previous analysis. To do so we used the 4-parameter model previously fitted to control injected individuals as a reference to then calculate residual horn lengths, i.e. the difference between observed and expected horn length for a specific body

size [8] for all individuals, followed by Mann-Whitney U test to contrast residual horn lengths across treatment groups. For the genitalia-body size allometry we performed a linear model analysis using R statistical software. Model selection was done by carrying out ANOVAs and the stepwise removal of non-significant variables. Because there was no dosage effect (analyzed using a linear model on genitalia measurements; t= 0.023, p= 0.982) the measurements from different concentrations of *InR1*^{RNAi} were pooled for analysis. Similarly, the two concentrations of *InR2*^{RNAi} were also pooled for analysis (t = 1.273, p= 0.208).

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