Electronic Supplementary Materials

Neofunctionalization of embryonic head patterning genes facilitates the positioning of novel traits on the dorsal head of adult beetles

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SUPPLEMENTARY TABLES

Table S1: Primers used for qPCR in *Tribolium castaneum*

qPCR target	primer 1	primer 2	Length	efficiency
Tc-six3q F1R1	CCCATCACCAGTTTTCGTTTC	TCCTCGATTTGCGGTCTTG	109bp	105.4%
Tc-six3q F3R3	GCAACCACTCGGAGACG	CGGTGAAGTTGAGGGTGG	111bp	109.8%
Tc-otd1q F1R1	AAAGGAATCGCCCCAGATG	CCTACCAGCACGAGTACAAC	130bp	115.2%
Tc-otd1q F2R2	ACTATGCCCAGAATTACGCG	CATGGCTTGCATATGGTTCTG	92bp	103.7%
Tc-otd2q F1R1	TTTAAAGTCGGCCCCTTATCC	AAGTTGAGCCCTGGTGAAG	145bp	123.6%
*Tc-otd2*q F2R2	CTCATCTGTTTCTTTGCACCTC	AAAACCCGGAAATAATTCGCTG	108bp	92.6%
Tc-S3Eq	TTTGTATGGCGAGAAGGTGG	CTCAAAACACCATAGCAAGCC	117bp	82.7%
EGFPq	AAAGACCCCAACGAGAAGC	GTCCATGCCGAGAGTGATC	78bp	85.5%
L13Aq	ACAAGACAGAACGTGGGAAG	TTTCCTGCGGTCATATGGTG	80bp	80.9%

Table S2: Primers used to clone and synthesize dsRNA, number of individuals injected as larvae, and number of individuals surviving to adulthood.

dsRNA fragment	primer 1	primer 2	Length (bp)	#inj. larvae	#surv. adults
Tribolium castaneum					
*Tc-six3 F1R1***	TAATACGACTCACTATAGGG CGGCTGGCGCGCTTC	TAATACGACTCACTATAGGG CCCCAGCGCCCGTCC	240	20	17†
Tc-six3 F2R3	TAATACGACTCACTATAGGG GCGGAAAAAGTTCCC	TAATACGACTCACTATAGGG CGGCCGCTCGATCCC	215	20	12†
*Tc-otd1 F1R1 **	GCAGCGGCGCGAAAGGACGACA	TGCTGCCGTGGGGTGTGATGTTGC	401	36	31
Tc-otd1 F2R2 **	TAATACGACTCACTATAGGG CTTGAGGACTCAAAA	TAATACGACTCACTATAGGG TCTGGGGTTGACGCC	401	25	10†
Tc-otd2 F1R1 ***	TAATACGACTCACTATAG ATTAACTTC	TAATACGACTCACTATAG GGGGAAGC	427	35	30
Tc-otd2 F2R2 ***	TAATACGACTCACTATAGGG CAACCAGCAACAAAG	TAATACGACTCACTATAGGG TCATTTCCGCTCCCA	427	24	10†
Tc-otd1 F1R1 +Tc-otd2 F1R1			401/427	36	34
Tc-otd1 F2R2 +Tc-otd2 F2R2			401/427	18	18
Onthophagus taurus					
Ot-six3	CCTCTCCGTCACCGATAACG	CCCCCGTGGATATTCGTCTG	508	70	61
Ot-otd1	CGTGCGAAATGTCGCCAACAACTA	TACGATGCGCCCATATGATTCGGA	515	250	125
Ot-otd2	AATGTAGACAACAACAAAAGCAGC	CCAAGATTCATCTCTGGTCTTACT	506	53	34
Ot-otd1 +Ot-otd2			515/506	44	30
Onthophagus sagittarius					
Os-six3	CCTCTCCGTCACCGATAACG	CCCCCGTGGATATTCGTCTG	508bp	82	19
Os-otd1	CGTGCGAAATGTCGCCAACAACT	TGCATTTGATTTTGCGCATTCGGCT	485bp	221	99

*Templates for dsRNA synthesis were made with a primer designed for the pCR4-TOPO vector: TOPO RNAi (taatacgactcactatagggcgaatt (T7 sequence underlined)). This primer works in both forward and reverse direction.

**Templates for dsRNA synthesis were synthesized using IDTdna's Gblock service (idtdna.com).

***cDNA clones obtained from the Brown laboratory at Kansas State University

† A number of pupae from this set were sacrificed to extract RNA for qPCR experiments.

SUPPLEMENTARY FIGURES

Figure S1: Molecular phylogeny obtained after alignment of 82 protein sequences, showing three main branches containing the respective orthologues of *six2/sine oculis, six3/optix* and *six4. Tc-six3, Ot-six3* and *Os-six3* all cluster with other coleopteran *six3* sequences. Only RAxML tree is shown, as all methods yielded overall similar tree topologies.

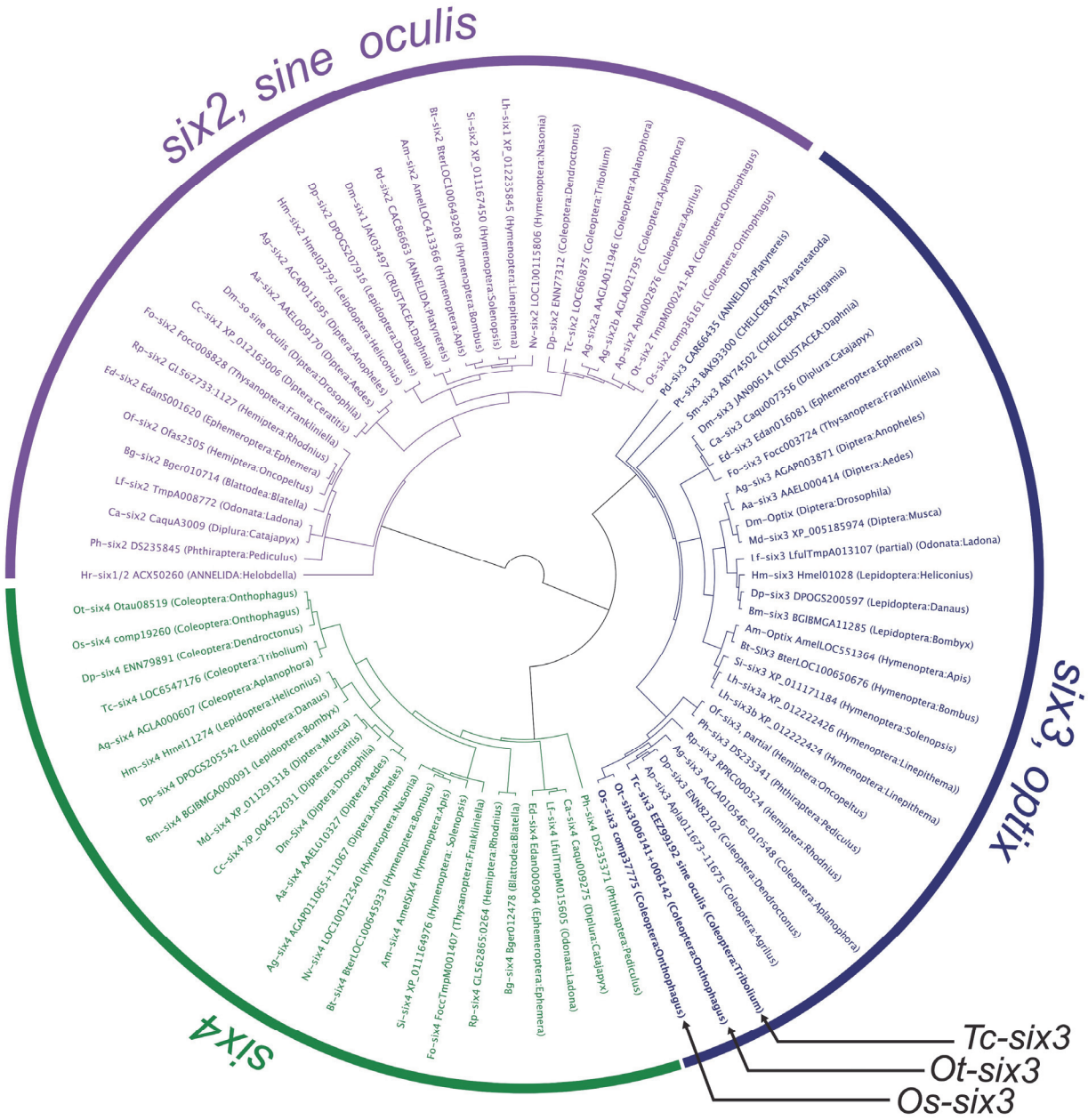


Figure S2: Molecular phylogeny of orthodenticle proteins obtained after alignment of 43 protein sequences, showing two branches containing respective orthologues of *otd1* and *otd2*. *Tc-otd1, Ot-otd1* and *Os-otd1* cluster with other coleopteran *otd1* sequences in the same main branch than fruit fly *Dm-oc*, while *Tc-otd2* and *Ot-otd2* cluster in the other branch that lacks dipteran or lepidopteran orthologues. A fragment of *Os-otd2* clustered with *Ot-otd2* in a separate analysis (not shown). Only FastTree tree is shown, as all methods yielded overall similar tree topologies.

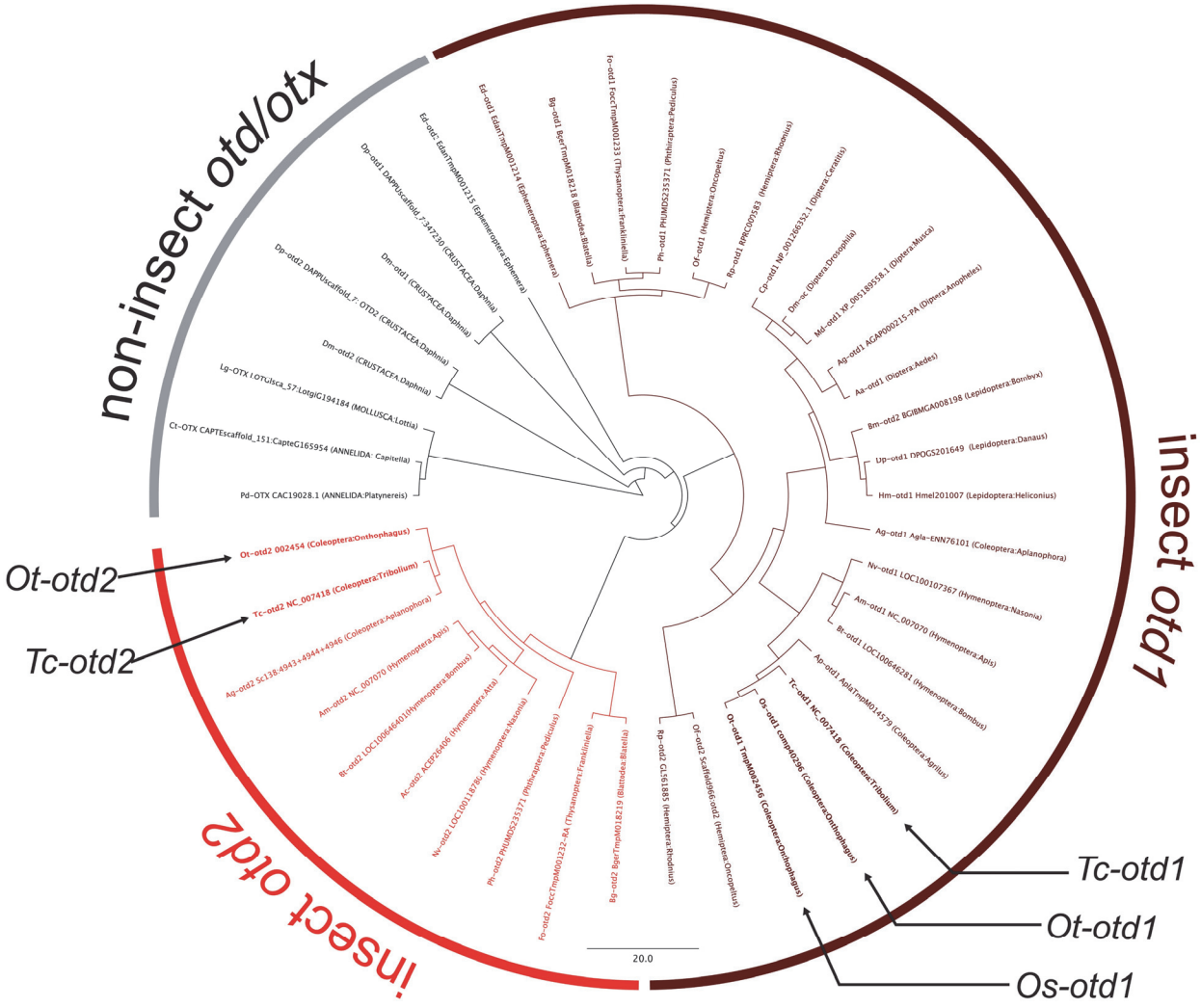


Figure S3: Expression of *six3* and *otd* orthologues during beetle development. Top row: barplots of expression levels of *Tc-six3, Tc-otd1* and *Tc-otd2* in *Tribolium* prepupae, pupae and adults, shown relative to late larvae, as estimated by qPCR of whole-body mRNA. Error bars represent standard error of three technical replicates. Middle and bottom rows: boxplots of expression levels of *Ot-six3, Ot-otd1*, *Ot-otd2* (middle row) and *Os-six3* and *Os-otd1* (bottom row) in several *Onthophagus* stages, as estimated by RNAseq of whole-body mRNA, and expressed as Fragments Per Kilobase of transcript per Million mapped reads. Boxplots show distribution across 6 biological replicates. SEL: standardized expression level; M3L: mid 3rd instar larva; L3L: late 3rd instar larva; PP1: early prepupa; PP2: late prepupa; PD1: 1 day old pupa; PD2: 2 days old pupa; PD7: 7 day old pupa; AD4: 4 days old adult.

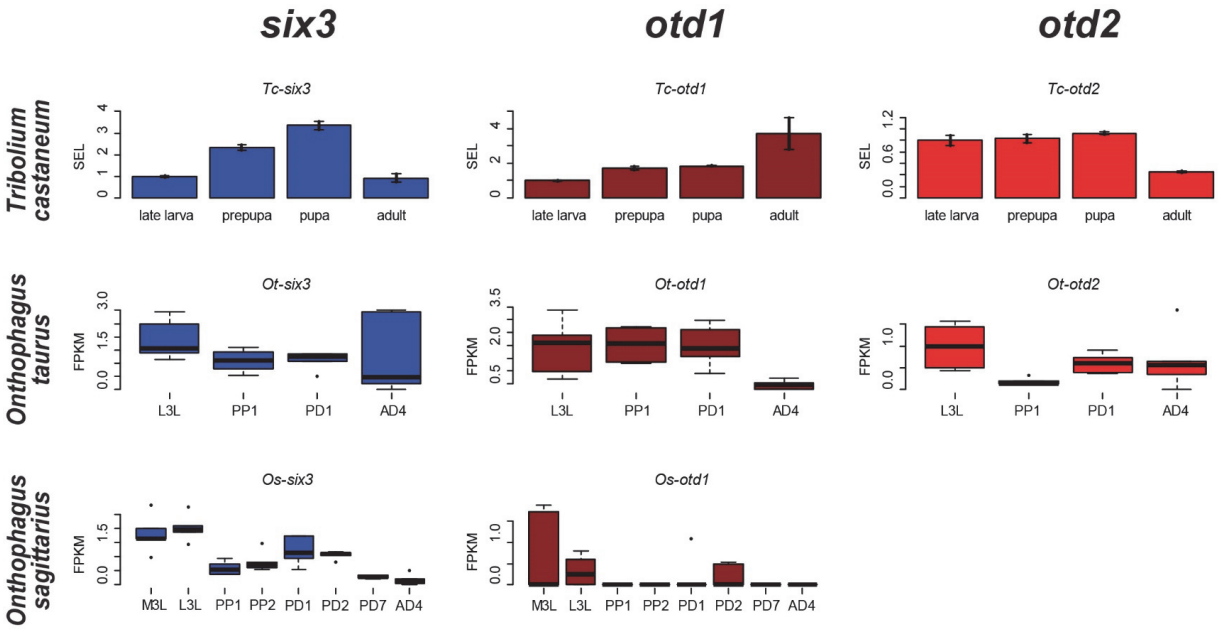


Figure S4: Phenotypes after *six3* RNAi. (*a-c*) Adult eye size as function of body size (estimated as adult thorax width) in sham controls (green circles) and *six3*^{RNAi} (blue squares) injected animals in *Tribolium castaneum (a), O. taurus* (*b*) and *O. sagittarius* (*c*). (*d*) Adult phenotype in wild type (left column), *six3F1R1*^{RNAi} (center column) and *six3F2R3*^{RNAi} (right column) *Tribolium castaneum*. Ventral view; blue arrowhead indicate eyes, which are smaller in *six3*^{RNAi} beetles.

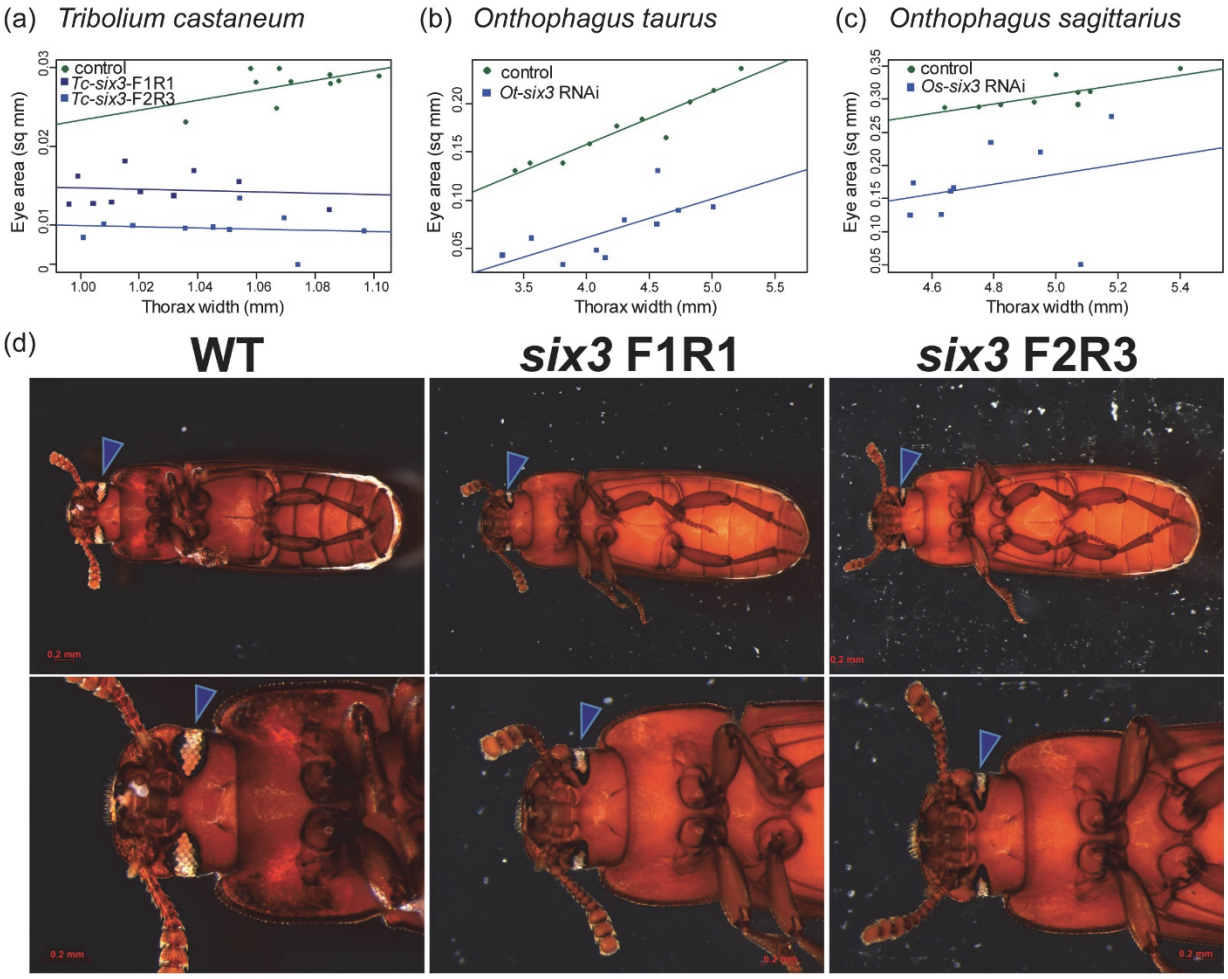


Figure S5 (next page): Metasternal and horn phenotypes after RNAi in *Onthophagus*. (*a-b*) Metasternum is drastically reduced following *otd1*^{RNAi} (dark red triangles) and *otd1+2*^{RNAi} (orange triangles) treatments, but not different from controls (green circles) in *otd2* ^{RNAi} or *six3*^{RNAi} animals (red triangles and blue squares respectively) in *O. taurus* (*a*) and *O. sagittarius* (*b*). (*c-d*) Pupal thoracic horns are reduced in *otd1* ^{RNAi} (dark red triangles) and *otd1+2* ^{RNAi} (orange triangles) treatments, but not different from controls (dark and light green circles) in *otd2* ^{RNAi} animals or *six3*^{RNAi} (red triangles and blue squares respectively) in *O. taurus* (males: *c;* females: *c'*) and *O. sagittarius* (males: *d;* females: *d'*). *(e-f*) Adult posterior head horn size is drastically reduced in *otd1* ^{RNAi} (dark red triangles), but not different from controls (green circles) in *otd2* ^{RNAi} or *six3*^{RNAi} animals (red triangles and blue squares respectively) in both *O. taurus* males (*e*) and *O. sagittarius* females (*f*). In all plots, empty symbols represent individuals with a normal metasternal phenotype (suggesting no effective *otd-1* knockdown).

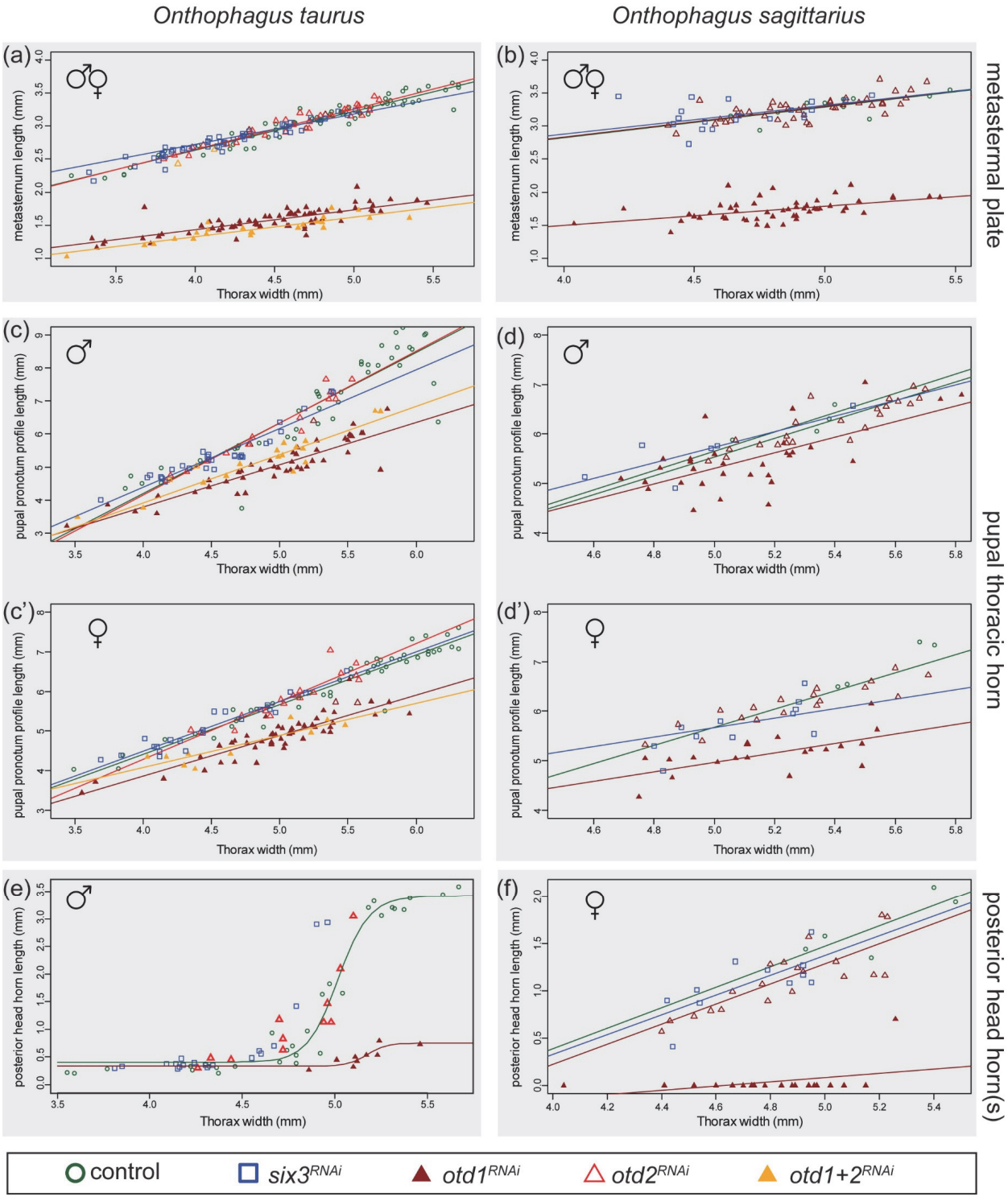

Onthophagus taurus
Onthophagus sagittarius
metastermal plate
pupal thoracic horn
posterior head horn(s)
(a)
(b)
(c)
(d)
(c')
(d')
(e)
(f)
metasternum length (mm)
pupal pronotum profile length (mm)
posterior head horn length (mm)
Thorax width (mm)
control
six3RNAi
otd1RNAi
otd2RNAi
otd1+2RNAi

Figure S6: Ectopic dorsal eyes develop after *Os-otd1* (*a-c*) and *Ot-otd1+otd2* RNAi (*d-e*). (*a*) Anterolateral view of an *O. sagittarius Os-otd1*^{RNAi} individual showing location of ectopic dorsal eye (arrowhead). (*b*) Frontal view of an *O. sagittarius Os-otd1*^{RNAi} individual and (*c*) magnified detail of the boxed area on *b)*. The area surrounding the eye in *c)* has been masked to enhance contrast. (*d*) Dorsal view of an *O. taurus Ot-otd1+otd2*^{RNAi} pupa, showing developing normal and paired ectopic eyes. (*e*) Magnified detail of the boxed area on *d)*. (*f*) Frontal view of the individual shown on *d)* immediately after adult emergence from the pupal case; arrowhead indicates ectopic eyes.

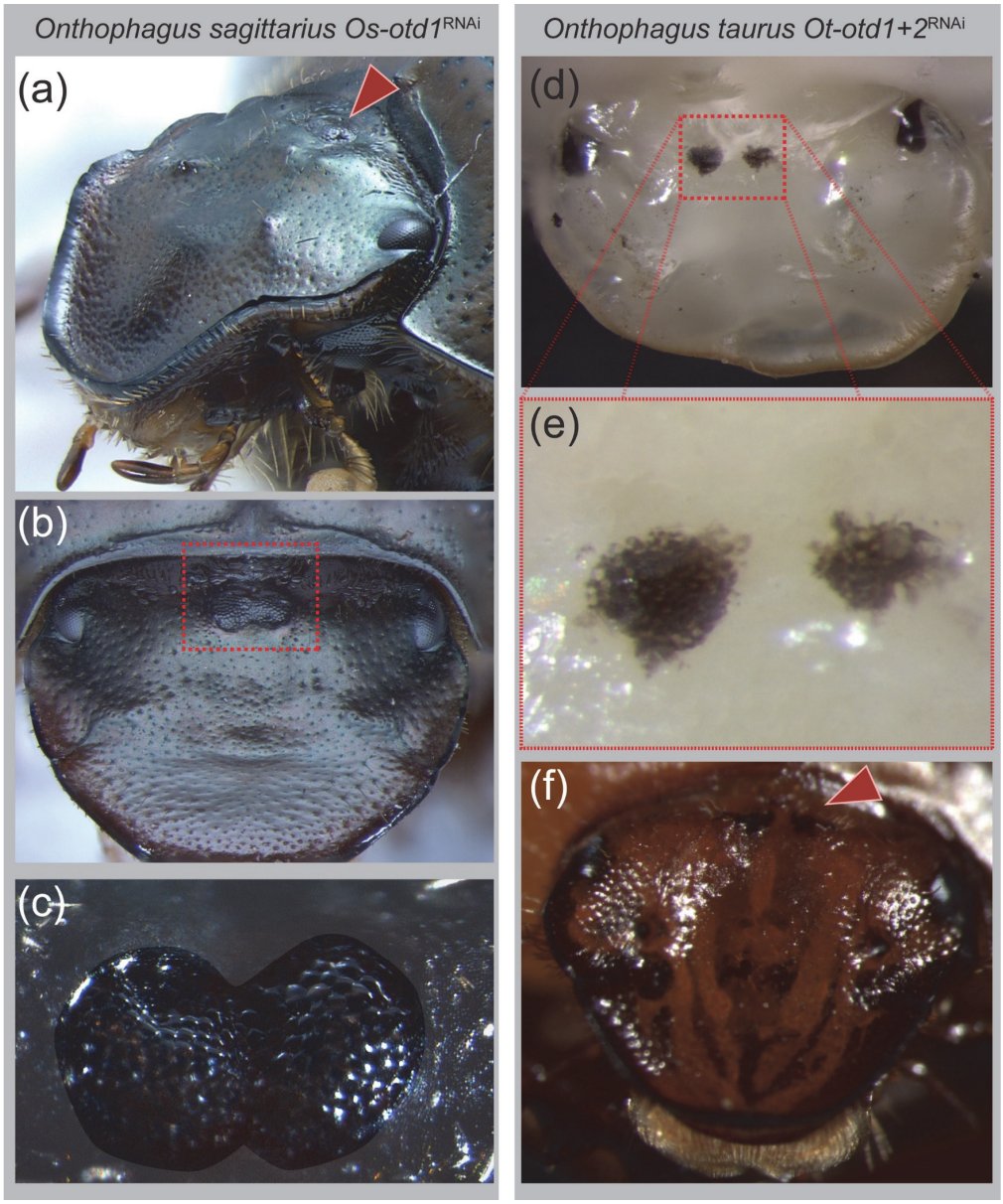


Figure S7: *Tribolium castaneum* phenotypes after *Tc-otd1* and *Tc-otd1+otd2* RNAi. (*a*) Wild type individual. (*a*') Magnified detail of the boxed area on *a)*. (*b*) *Tc-otd1*^{RNAi} individual. (*b*') Magnified detail of the boxed area on *b)*, showing slight reductions of T1 postcoxal bridge, T2 mesosternellum, T3 intercoxal process and median groove, and A3 intercoxal process (arrows). (*c*) *Tc-otd1+2*^{RNAi} individual. (*c*') Magnified detail of the boxed area on *c)*, showing more pronounced reductions of structures mentioned above, including complete disappearance of the median groove (arrows).

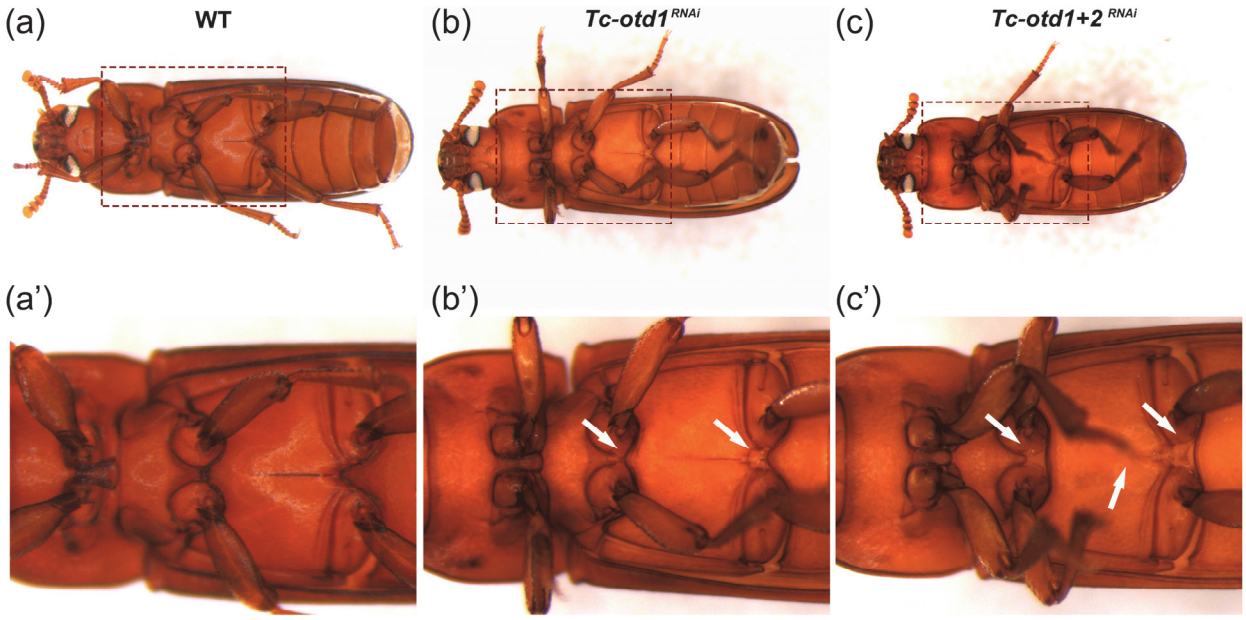


Figure S8: Tissue specific expression of *six3* and *otd* orthologues in *Tribolium castaneum* (upper right) and *Onthophagus taurus* (lower right) early pupae. Top row: boxplots of expression levels of *Tc-six3, Tc-otd1* and *Tc-otd2* in *Tribolium* pupal brain and ventral head, dorsal head, T1 dorsal epidermis, anterior ventral abdomen and posterior ventral abdomen, shown relative to whole pupae. Expression estimated by qPCR of mRNA from pooled tissues from >5 individuals, with three biological replicates per experiment; error bars represent standard error of three technical replicates. Bottom row: boxplots of expression levels of *Ot-six3, Ot-otd1* and *Ot-otd2*, as estimated by RNAseq of early pupal brain, dorsal head epidermis, T1 dorsal epidermis and posterior ventral abdominal epidermis (including genitalia). Values expressed as Fragments Per Kilobase of transcript per Million (FPKM) mapped reads. Boxplots show distribution across 12 biological replicates.

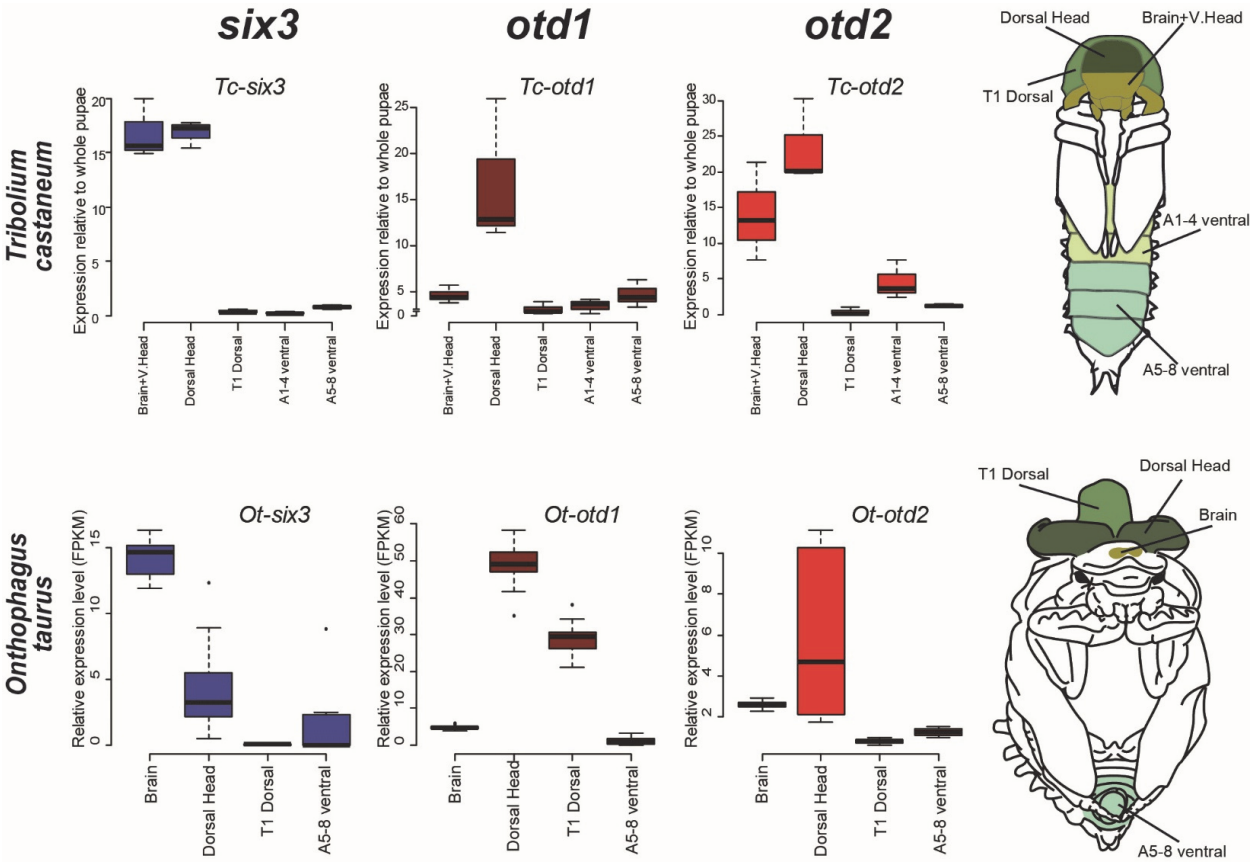
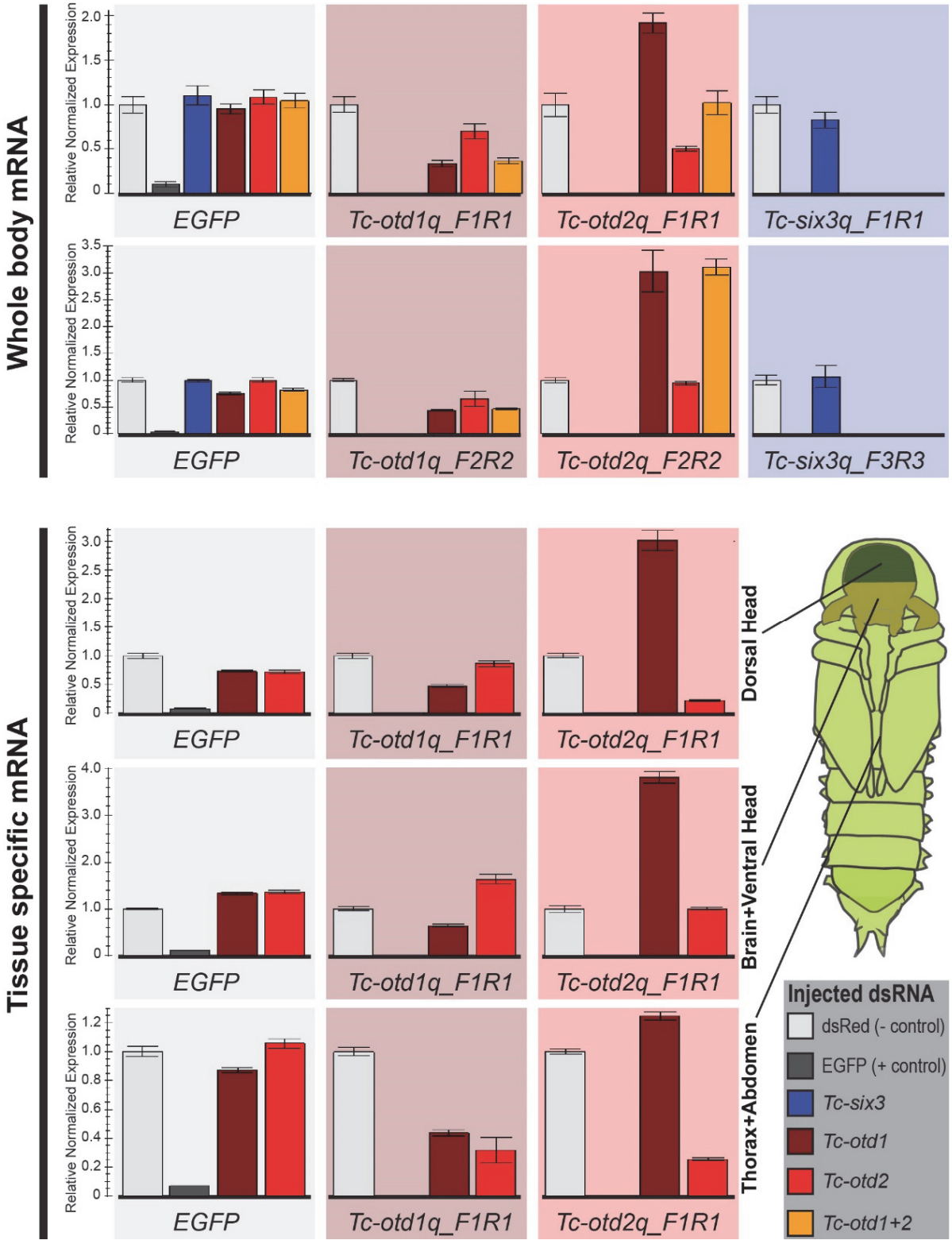


Figure S9: Whole-body and tissue specific expression levels after dsRNA injection in *Tribolium castaneum*, as estimated by qPCR. Results grouped by qPCR target; same colored bars across groups represent the same injected dsRNA. Error bars represent standard error of three technical replicates. See Supplementary Methods for details.



SUPPLEMENTARY METHODS

Animal husbandry

Horned beetles and red flour beetles used in this study were obtained and reared as previously described [1,2]. *Onthophagus* beetles were field collected near Bloomington, IN and Chapel Hill, NC (*O. taurus*) or Kaneohe, Hawaii (*O. sagittarius*), kept in a sand/soil mixture at a 16:8 h light:dark cycle at 25 °C (*O. taurus*) or 28 °C (*O. sagittarius*), and fed homogenized cow manure twice a week. Larvae used for experiments were generated by breeding 5 females and 3 males in a moist sand/soil mixture container with *ad libitum* food. After 8 days, brood balls produced by females were collected, opened and larvae transferred to 12-well plates as previously described [3]. *Tribolium castaneum* beetles were cultured on organic whole-wheat flour (+5% yeast) at 30°C with 70% humidity. The *pu11 nubbin* (*nub*) enhancer trap line [4,5] was used in all *Tribolium* RNAi and expression experiments.

Larval ablation and head fate mapping

Ablation of select epidermal cell groups underlying larval head cuticle was done using a Hyfrecator 2000 electrosurgical unit (ConMed, Utica, NY) equipped with an extra-fine epilation needle electrode (714-S, ConMed). The needle tip was used to direct a voltaic arc of 6-10 watts of electrical current to specific dorsal head regions of restrained late third instar *Onthophagus* larvae for up to 3 seconds (*O. taurus*) or 1-2 seconds (*O. sagittarius*). The sutures found at the dorsum of the head capsule were used as morphological landmarks to guide ablations. Treated larvae were returned to 12-well plates and kept at 25 °C (*O. taurus*) and 28 °C (*O. sagittarius*), imaged after pupation and adult emergence, and fixed in 70% ethanol. Because accuracy and survival varies among target regions and species, we replicated these experiments enough times to ensure a consistent result (posterior horn in *O. taurus n*=9; anterior horn in *O. sagittarius* male *n = 19*; medial posterior horn in *O. sagittarius* female *n=16*).

Genomic and transcriptomic scans for otd *and* six3 *orthologues*

Published amino acid sequences of *Tc-six3* (NP_001106938)*, Tc-otd1* (NP_001034513) and *Tc-otd2* (NP_001034526) were downloaded from NCBI and used as queries in *blastx* searches of the current electronic annotations of the latest assembly of the *O. taurus* genome [6]. Scaffolds containing matching gene models were downloaded and analyzed in Geneious v.6.1.8 (http://www.geneious.com) [7]. The gene models were used as queries in *blastn* searches of *O. taurus* and *O. sagittarius* transcriptomes assembled from data generated by Illumina HiSeq2000 RNAseq of total mRNA extracted at several stages (see below). Additionally, we used the *Ot-otd2* primers shown in **Table S2** in a PCR experiment using *O. sagittarius* genomic DNA as

template. *O. sagittarius* sequences were submitted to GenBank (*Os-otd1:* KX356386; *Os-otd2:* KX356387). *Tribolium* sequences were downloaded directly from the flour beetle genome (Tcas3.0) [8]. Phylogenetic relationships of retrieved sequences was established based on *blast* searches against NCBI insect genome databases (http://www.ncbi.nlm.nih.gov/), Ensembl (http://metazoa.ensembl.org) and the i5k pilot project (https://www.hgsc.bcm.edu/i5k-pilot-project-summary). Annotated gene coding sequences were extracted and translated, and the resulting amino acid sequences were aligned initially using MAFFT (http://mafft.cbrc.jp/) and then manually curated, discarding sequences with obvious annotation errors (i.e. missing or incorrect reading frames). The final alignments were used to infer molecular phenograms using implementations of FastTree, MrBayes, GARLI and RAxML in Geneious 8.1.7 (http://www.geneious.com). Default parameters were used in all cases. Aligned sequences and trees are available at the Dryad Digital Repository [9].

Gene expression analysis by quantitative real time PCR

Quantitative real time PCR (qPCR) was used to quantify relative expression levels of *Tc-six3*, *Tc-otd1* and *Tc-otd2* in different stages and body regions of *Tribolium*. Total RNA was isolated from last instar larvae, pre-pupae, early pupae and adult whole individuals, or from dissected tissues from early pupae including dorsal head epidermis, the remainder of the head including brain, dorsal T1 epidermis, segments A1-A4 and segments A5-A8, using Maxwell LEV simplyRNA RNA Purification Kits (Promega). Each condition was comprised of a pooled group of tissues (>2 individuals for stage specific and >10 individuals for tissue specific RNA). We made cDNA by reverse transcribing 500ng of total RNA using iScript Reverse Transcription Supermix (BioRad). The qPCR reaction was set up using SsoAdvanced Universal SYBR Green Supermix (BioRad), and run on a CFX Connect Real Time System (BioRad) and analyzed using the bundled software. Reaction conditions were 95ºC for 3 min, then 40 cycles of 95ºC for 10 sec, 60ºC for 15 sec, followed by a melting curve step at the end. Primers used are listed in **Table S1** of these supplementary materials. The melting curve step confirmed that each PCR produced only single kind of amplicon without any non-specific amplification. The amplification efficiency of each primer set, obtained using serially diluted cDNA as templates, is also listed in **Table S1**. Every reaction had three technical replicates. In addition, two biological replicates were used for tissue specific qPCR, and two biological replicates were used to determine RNAi knockdown efficiency. The knockdown efficiency results were also confirmed with a second set of qPCR primers (**Table S1**). Relative expression levels were calculated by the ΔΔCq method. A

ribosomal gene s3e was used as a reference gene for stage and tissue specific quantification, while two ribosomal genes s3e and L13A were used for RNAi knockdown confirmation.

Gene expression analysis by RNAseq

Estimation of gene expression levels for *O. taurus* and *O. sagittarius* was done via *in silico* analyses of high-throughput RNAseq datasets (WDS: whole-body developmental stages; TSP: tissue-specific early pupae) generated in the context of different projects detailed elsewhere. WDS data was obtained from late third instar larvae, early pre-pupae, first day pupae and young adult individuals (both species), plus middle third instar larvae, late pre-pupae, second and seventh day pupae (*O. sagittarius* only); whole bodies were flash-frozen in liquid N_2, ground in a Geno/Grinder tissue homogenizer (SPEX SamplePrep, Metutchen, NJ) and total RNA was extracted using RNeasy Mini spin columns (Qiagen). 6 biological replicates (one individual per replicate) were done for each stage. TSP data was obtained by dissecting brain, dorsal head epidermis, dorsal prothoracic (pronotal) epidermis or posteroventral abdominal epidermis tissues from first day pupae; individual tissues were stored in Trizol, homogenized using disposable polypropylene RNase-free pellet pestles, and total RNA was extracted using a standard phenol-chloroform protocol followed by RNeasy Mini spin column purification including on-column DNAse I digestion. 12 biological replicates (one individual per replicate) were done for each tissue.

Total RNA was quality checked using RNA ScreenTape TapeStation System (Agilent) and quantified with a Quant-iT RiboGreen Assay Kit (Thermo Fisher). RNA Stranded RNA sequencing libraries were constructed using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina, San Diego, CA) according to manufacturer's instructions. Libraries were quantified using a Quant-iT DNA Assay Kit (Thermo Fisher), pooled in equal molar amounts, and sequenced either on HiSeq2000 (WDS libraries) or on NextSeq500 (TSP libraries) instruments (Illumina, San Diego, CA). Resulting read sequences were cleaned using Trimmomatic version 0.32 [10] to remove adapter sequences and perform quality trimming. Trimmomatic was run with the following parameters, “2:20:5 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:18”. The trimmed reads where then reversed complemented using FASTX-Toolkit version 0.0.13.2 (http://hannonlab.cshl.edu/fastx_toolkit/download.html), and mapped against the *Onthophagus taurus* genome v0.5.3 gene models [6] using TopHat2 version 2.1.0[11] with the parameters “–b2-very-sensitive–read-edit-dist ”, or against reference transcriptomes assembled using TrinityRNAseq[12]. Read counting was done for each gene using htseq-count

from the HTSeq package version 0.6.1p1[13]. Read counts were normalized across samples using the DESeq2 package (version 1.10.0) in R/Bioconductor (R version 3.2.1)[14].

Cloning of Onthophagus six3 *and* otd *fragments, dsRNA synthesis and injection*

To synthesize dsRNA for horned beetle RNAi, we designed primers based on *Onthophagus six3* and *otd* sequences found in a transcriptomic database for *O. taurus* and *O. sagittarius* (**Table S2** of these supplementary materials), and amplified fragments of these genes for each species through PCR from cDNA libraries. Amplicons were subsequently cloned into pSC-A vectors using a StrataClone PCR Cloning Kit (Agilent). Correct identity of the cloned fragments was verified by sequencing using BigDye cycle sequencing chemistry (Life Technologies). dsRNA to knock down *Onthophagus six3* and *otd* genes was generated as described previously [15]. In brief, suspensions of each *E. coli* clone were boiled to free the plasmid DNA and used as template in PCR reactions using M13 forward and reverse primers. The resulting 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). After DNase I treatment, ssRNA was precipitated by lithium chloride, incubated at -20ºC for 1h, spun at 4ºC for 20 min, washed with 80% ethanol, and resuspended in water. After quantification, forward and reverse strands were mixed at a 1:1 ratio by weight, and annealed by heating to 80 °C and then cooling slowly over 5h to 35ºC. The concentration of the annealed RNA was measured, confirmed by gel electrophoresis, and stored at −80 °C until injection. Injections into *Onthophagus* larvae were carried out as described previously [15]. Doses 0.5, 1.0, 1.5 or 3.0 µg of dsRNA were diluted in injection buffer (5 mM KCl, 1 mM KPO_4 *p*H 6.9) to a total of 3 µL and injected into larvae usually during the first 10 days of the final instar. We injected a total of 417 *O. taurus* larvae (*Ot-six3*: 70, *Ot-otd1*: 250, *Ot-otd2*: 53, *Ot-otd1+2*: 44) and 221 *O. sagittarius* larvae (*Os-six3*: 82, *Os-otd1*: 319). Sham control injections were made exactly as described above, except that larvae were injected with dsRNA from a 167-bp PCR product derived from a pBluescript SK vector. Transcription reactions, DNaseI treatment, and transcript annealing were performed as described above. A total of 1 µg of dsRNA was injected into larvae during the first 10 days of the final, third instar. We made sham control injections to 147 *O. taurus* and 37 *O. sagittarius* larvae.

Tribolium castaneum six3 *and* otd *dsRNA synthesis and injection*

Details of the *Tc-otd1_F1R1*, *Tc-otd-2_F1R1* and *Tc-otd-2_F2R2* clones used in this study were previously described [16]. For *Tc-six3_F1R1, Tc-six3_F2R3* and *Tc-otd1_F2R2* respectively,

240bp, 215bp and 410bp dsRNA fragments were synthesized from DNA synthesized de novo by IDT gBlock service based on the published sequences sequences [17]. Primers are listed in **Table S2.** At least 20 last instar larvae were injected with dsRNA targeting two different regions for each gene tested. The stage was determined by the EYFP positive wing discs in the *pu11 nub* enhancer trap line. dsRNA was injected into the dorsal side of the first abdominal segment at a concentration of 1 µg/µl. Each larva can hold up to 0.7 µl of dsRNA solution at the stage described above. We phenotypically scored 144 beetles that survived to adulthood (*Tc-six3*: 29, *Tc-otd1*: 41, *Tc-otd2*: 40, *Tc-otd1+2*: 52).

Imaging and morphometric measurements

RNAi-treated and control *Onthophagus* pupae and adults were weighed on an analytical scale to the nearest mg, and imaged through a Leica dissecting microscope (Leica, Buffalo Grove, IL) mounted with a digital camera (Scion, Frederick, MD) using ImageJ [18]. Images were saved as TIF files and later analyzed using ImageJ. Thorax width was used as measure of pupal and adult body size. Length of pupal pronotum lateral profile was used to quantify pronotal horn size; head horn length was measured as described in [19]. Measurements were recorded to the nearest 0.01 mm. Images for figures were generated by imaging representative individuals at ~20-30 focal planes and using the Auto-align and Auto-blend functions in Adobe Photoshop Creative Suite 6. For *Tribolium*, adults were fixed in 95% ethanol overnight. The images were captured by Zeiss Discovery V12 with AxioCam MRc 5. Zeiss AxioVision Extended Focus module was used to obtain images with increased depth of focus. Some pictures were enhanced only for brightness and contrast with Adobe Photoshop CS5.

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