nature ecology & evolution

ARTICLES

https://doi.org/10.1038/s41559-020-1202-x

Nutrition-responsive gene expression and the developmental evolution of insect polyphenism

Sofia Casasa[1]✉, Eduardo E. Zattara[1,2]✉ and Armin P. Moczek[1]

Nutrition-responsive development is a ubiquitous and highly diversified example of phenotypic plasticity, yet its underlying molecular and developmental mechanisms and modes of evolutionary diversification remain poorly understood. We measured genome-wide transcription in three closely related species of horned beetles exhibiting strikingly diverse degrees of nutrition responsiveness in the development of male weaponry. We show that (1) counts of differentially expressed genes between low- and high-nutritional backgrounds mirror species-specific degrees of morphological nutrition responsiveness; (2) evolutionary exaggeration of morphological responsiveness is underlain by both amplification of ancestral nutrition-responsive gene expression and recruitment of formerly low nutritionally responsive genes; and (3) secondary loss of morphological responsiveness to nutrition coincides with a dramatic reduction in gene expression plasticity. Our results further implicate genetic accommodation of ancestrally high variability of gene expression plasticity in both exaggeration and loss of nutritional plasticity, yet reject a major role of taxon-restricted genes in the developmental regulation and evolution of nutritional plasticity.

Developmental plasticity, the ability of organisms to adjust development in response to changes in environmental factors, is a ubiquitous property of developmental systems[1] that has greatly diversified, ranging from modest to striking non-linear responses[1,2]. Nutrition-responsive growth is one of the most widespread environment-sensitive plastic responses of animal development, thus being intensively studied in both model and, increasingly, non-model systems[3–5]. Diverse hormonal and developmental pathways collaborate in translating nutrient availability into growth responses, including insulin/insulin-like signalling and Hippo-, Target of Rapamycin and MAP kinase pathways (reviewed in refs. [3,6]). Moreover, nutrition-responsive development varies widely across taxa, suggesting that its underlying mechanisms are evolutionarily labile and thus contribute to organismal diversification in meaningful ways.

Although research on candidate pathways has yielded important insights into the regulation and evolution of nutrition-responsive growth[7–12], this approach risks overlooking genes and pathways not traditionally considered in the context of plastic development. Examples of such unexpected candidates include *doublesex* (*dsx*), a master regulator of sex-specific differentiation, and the Hedgehog pathway, involved in establishing anterior/posterior polarity[13,14], both found to be critical regulators of nutrition-responsive horn development in beetles[11,12], suggesting that repurposing of non-canonical pathways may be an underexplored area in the evolution of nutritional plasticity. Taxon-restricted genes—that is, recently evolved genes lacking orthologues outside a given focal taxon[15]—may also play a similarly underappreciated role in the evolution of developmental plasticity. Such genes have been shown to play critical roles in development of the propelling fan of *Rhagovelia* water striders, a remarkable evolutionary innovation[16]. However, whether taxon-restricted genes indeed play an important role in the evolution of nutrition-responsive growth, or in fact any kind of plasticity, is essentially unknown. Together, full appreciation of the evolution of nutrition-responsive development and its mechanisms therefore necessitates a comparative genome-wide perspective to provide a comprehensive and non-biased assessment.

One mechanism linking regulation of developmental plasticity to its evolution is genetic accommodation (reviewed in refs. [17–20]), defined as evolutionary change due to selection on the regulation of an environmentally induced response[2]. By this mechanism, initial developmental plasticity precedes and enables subsequent evolution of a more refined plastic response, or even complete genetic canalization of initially environmentally induced phenotypes. Although several field and laboratory studies support this 'plasticity-first' scenario of adaptive evolution[21–29], its molecular mechanisms are much less understood, including whether genetic accommodation of morphological traits (for example, refs. [23,30]) is paralleled by corresponding changes in gene expression[31].

Here we use horned beetles in the tribe Onthophagini to study the evolution of mechanisms underlying nutrition-responsive development, and test the potential role of genetic accommodation in that process. Horns in onthophagine beetles are sex-biased weapons that exhibit diverse species-specific nutrition responses. We focus on three species with strikingly different nutrition responsiveness (Fig. 1): (1) *Digitonthophagus gazella*[32], in which males develop paired posterior head horns (the most common position of head horns in the tribe[33]) that exhibit a modest polyphenic response to nutrition; (2) *Onthophagus taurus*, in which males develop paired posterior head horns that exhibit a strong polyphenic response to nutrition; and (3) *Onthophagus sagittarius*, a species closely related to *O. taurus* (last common ancestor ~5 Ma, relative to last common ancestor with *D. gazella* ~37 Ma), whose males have secondarily lost nutrition-responsive posterior head horn growth and instead develop a simple, continuous ridge similar to that of females of the other two species. Phylogenetic mapping suggests that *D. gazella* head horn configuration (posterior paired horns with modest nutrition responsiveness) represents the ancestral state for the tribe, while both the increased nutrition responsiveness found in *O. taurus* and the secondary loss of posterior head horns of *O. sagittarius* males represent derived conditions[11,33].

[1]Department of Biology, Indiana University, Bloomington, IN, USA. [2]INIBIOMA, Universidad Nacional del Comahue – CONICET, Bariloche, Argentina. ✉e-mail: ascasasa@indiana.edu; ezattara@comahue-conicet.gob.ar

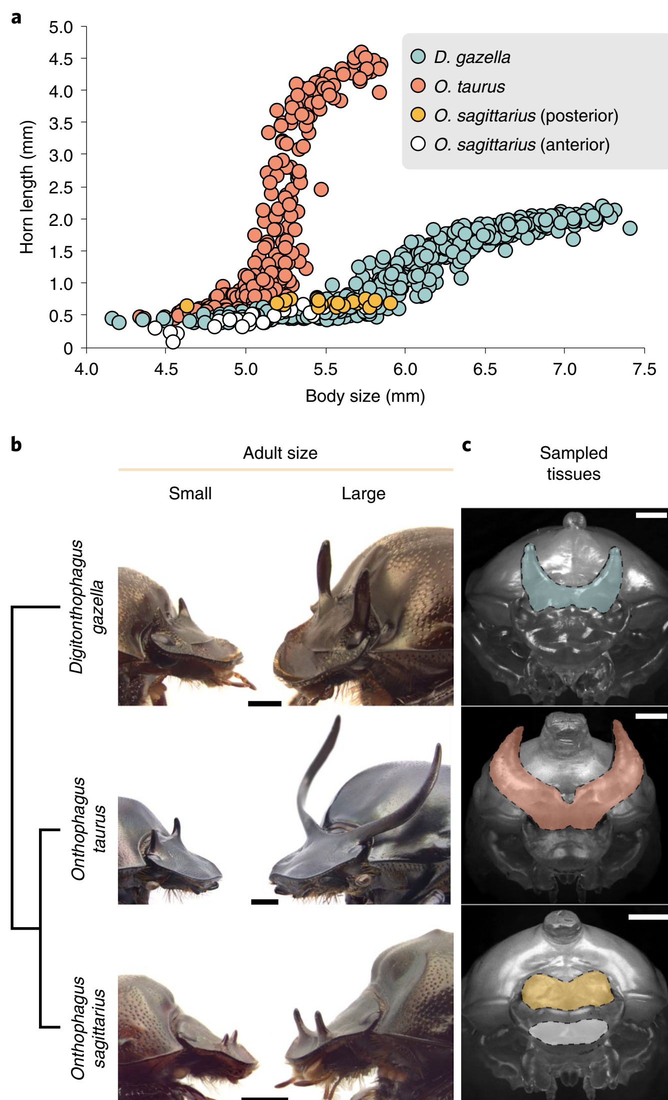


Fig. 1 | Diversity of morphological plasticity among three onthophagine species. a, Allometric relationships of posterior head horns in *D. gazella*, *O. taurus* and *O. sagittarius*. Also shown are the corresponding data for the anterior head horns unique to *O. sagittarius*. *D. gazella* exhibits a moderately sigmoidal scaling relationship reflective of the ancestral condition in the genus, whereas *O. taurus* exhibits greatly exaggerated polyphenism and *O. sagittarius* has secondarily lost nutritional plasticity in the formation of posterior horns but gained moderate responsiveness in its newly evolved anterior horns. **b**, Large, high-nutrition males (right) and small, low-nutrition males (left) of each species and their phylogenetic relationship. **c**, Pupae of *D. gazella* (top), *O. taurus* (middle) and *O. sagittarius* (bottom). RNA was extracted and sequenced from tissues marked by areas coloured as in **a**. For *O. taurus* and *D. gazella* only posterior head horn tissue was used, while for *O. sagittarius* both posterior and novel, anterior horn tissue were used. Scale bars, 1 mm.

The latter is further supported by functional studies in *O. sagittarius* showing that the transcription factor *dsx* has acquired a function in suppressing posterior horn growth in this species: when *Osagdsx* is knocked down, *O. sagittarius* develops prominent paired posterior head horns, thereby restoring the ancestral character state[11]. However, in addition to secondarily losing posterior head horns, male *O. sagittarius* have gained a pair of horns in the anterior region of the dorsal head, a location unique to this species. These anterior head horns exhibit a very modest, isometric response to nutrition. Together, the diverse degrees of nutritional plasticity present among these three species and their phylogenetic relationship to each other offer a powerful opportunity to explore the transcriptomic basis of nutrition-responsive plasticity and its evolution.

We compared genome-wide messenger RNA levels on developing horn tissues derived from high- and low-nutrition males from each of the three species to address three major aspects of nutrition-responsive developmental plasticity and its evolution. First, we aimed to better understand the gene expression mechanisms underlying diverse degrees of nutrition-responsive growth by identifying those genes whose expression is sensitive to nutrition and assessing the degree to which nutrition-responsive expression is shared, modified or lost across species. In so doing we hoped to contribute experimental data to a conceptual framework proposing that gene expression plasticity may mirror its 'higher-order', observable phenotype (that is, behaviour) or, alternatively, display a distinct pattern of gene expression[31]. Second, we sought to understand whether patterns of gene expression across species were consistent with a scenario of evolution by genetic accommodation. A key assumption of genetic accommodation is that for previous selection acting to refine a plastic response there must be variation among genotypes, which is then fine-tuned over generations into an accommodated response. We tested this assumption by comparing variance in gene expression across nutritional conditions and species showing ancestral or derived character states. Third, we sought to assess the importance of taxon-restricted genes in the regulation and evolution of nutrition responsiveness, by identifying genes putatively unique to Onthophagini and analysing their connectivity within their respective gene regulatory networks. By exploring these three questions, we hoped to gain insights into the mechanisms underlying nutrition-responsive growth and their evolution.

Results and discussion

Evolutionary changes in morphological nutritional plasticity are paralleled by matching changes in gene expression plasticity. Gene-expression changes associated with morphological plasticity are not required to evolve in the same direction or to the same degree as the morphological component of plasticity they underpin[31]. Therefore, we first tested the hypothesis that increased nutrition-responsive plasticity in morphology (Fig. 1a–c) is underpinned by increased nutrition-responsive plasticity in gene expression. Using comparative genome-wide RNA sequencing (RNA-seq), we identified 8,468 differentially expressed (DE) transcripts (11% of total transcript diversity) between moderately plastic posterior head horns of small and large males in *D. gazella*, but 12,727 (26.3%) in the nutritionally much more responsive *O. taurus* (binomial proportions test, $\chi^2 = 4924.7$, d.f. = 1, $P < 2.2 \times 10^{-16}$). In contrast, we identified only 74 (0.1%) DE transcripts in the corresponding secondarily non-plastic head region of *O. sagittarius* while the novel, modestly plastic anterior head horns in the same males exhibited 514 (0.9%) nutrition-responsive genes (binomial proportions test, $\chi^2 = 342.99$, d.f. = 1, $P < 2.2 \times 10^{-16}$). We then identified putative orthologues expressed across all three species. From this subset, we identified 946 DE genes between posterior head horns of small and large males in *D. gazella*, and 1,685 in *O. taurus* (Fig. 2a; binomial proportions test, $\chi^2 = 274.18$, d.f. = 1, $P < 2.2 \times 10^{-16}$). In contrast, in the homologous head region of the nutritionally non-responsive *O. sagittarius* only eight genes exhibited DE (Fig. 2a–c) while the novel anterior head horns exhibited 47 DE genes (Fig. 2c,d; binomial proportions test, $\chi^2 = 26.39$, d.f. = 1, $P = 2.79 \times 10^{-7}$). Thus, regardless of whether all genes were considered in the analyses or only those for which orthologues were detected across all species, more elaborate nutritional plasticity on the level of morphology, as seen in *O. taurus* compared to *D. gazella*, was associated with a much larger repertoire of genes

that exhibited nutrition-responsive expression, whereas the secondary loss of nutritional plasticity on the level of morphology—as seen in the posterior head of *O. sagittarius*—appears to coincide with a dramatic loss of gene expression plasticity.

Exaggerated growth responses evolve by enhancing plastic gene expression and by recruiting ancestrally low nutrition-responsive genes. Within the subset of common putative orthologues, we identified 492 DE genes shared between *D. gazella* and *O. taurus*, but not *O. sagittarius*. Because both *D. gazella* and *O. taurus* exhibit polyphenic development, which has been secondarily lost in *O. sagittarius*, we considered these 492 genes as part of a putative core gene set associated with polyphenic development, and henceforth refer to them as putative polyphenism genes (Fig. 2a). Focusing on this gene set, we sought to test the hypothesis that the transcriptional mechanisms of polyphenic development evolved by further increase in pre-existing, ancestral nutrition-sensitive gene expression of genes involved in growth promotion, or alternatively further decrease in ancestral nutrition-sensitive gene expression of genes involved in growth inhibition, or both. We also hypothesized that the main mechanism required to evolve exaggerated traits is growth promotion, since low-nutrition individuals are hornless regardless of species. If so, interspecific transcriptional differences should be most apparent among high-nutrition individuals. We found that, out of the 492 putative polyphenism genes, 268 (54%) exhibited a larger expression difference (that is, increased nutrition responsiveness; $\log_2$-fold change) between low- and high-nutrition conditions in *O. taurus* than in *D. gazella* (Fig. 3a top row; if the null hypothesis $(H_0)=50{:}50$, $\chi^2=0.50063$, d.f. $=1$, $P=0.4792$), whereas the remaining 46% displayed a higher degree of nutrition sensitivity in *D. gazella* compared to *O. taurus* (Fig. 3a bottom row; if $H_0=50{:}50$, $\chi^2=0.4492$, d.f. $=1$, $P=0.5027$). Importantly, both sets exhibited an increased number of genes with elevated expression in high-, compared to low-, nutrition *O. taurus* or *D. gazella*, respectively (see Supplementary Information for details; if $H_0=33{:}33{:}33$, $\chi^2=69.333$, d.f. $=2$, $P=8.80\times10^{-16}$; and $\chi^2=86.522$, d.f. $=2$, $P<2.2\times10^{-16}$). Together, these results are consistent with the hypothesis that evolution of polyphenic development is associated with both increases and decreases in gene expression plasticity, and that exaggeration of polyphenic development across species mostly involves modulation of horn growth under high-nutrition conditions.

A caveat to our interpretation is that, by using *D. gazella* as proxy for ancestral plasticity, we assume that mechanisms underlying nutrition-responsive growth did not diverge from those of the last common ancestor with *O. taurus*. However, even if such divergences did occur, they are unlikely to account fully for the specific patterns detailed above (see Supplementary Information for additional details and discussion).

Consistent with the hypothesis that genes acquired nutritional sensitivity during evolutionary exaggeration of polyphenic development in the lineage leading to *O. taurus*, we identified 1,187 genes that were DE in *O. taurus* but not in *D. gazella* or *O. sagittarius* (Fig. 2a). Of these, 859 had relatively higher expression in large individuals compared to small (positive $\log_2$-fold change; Fig. 3b top), while only 328 exhibited the opposite pattern and had instead relatively higher expression in small individuals (negative $\log_2$-fold change; Fig. 3b bottom; if $H_0=50{:}50$, $\chi^2=21.178$, d.f. $=1$, $P=185\times10^{-6}$). These proportions support the hypothesis

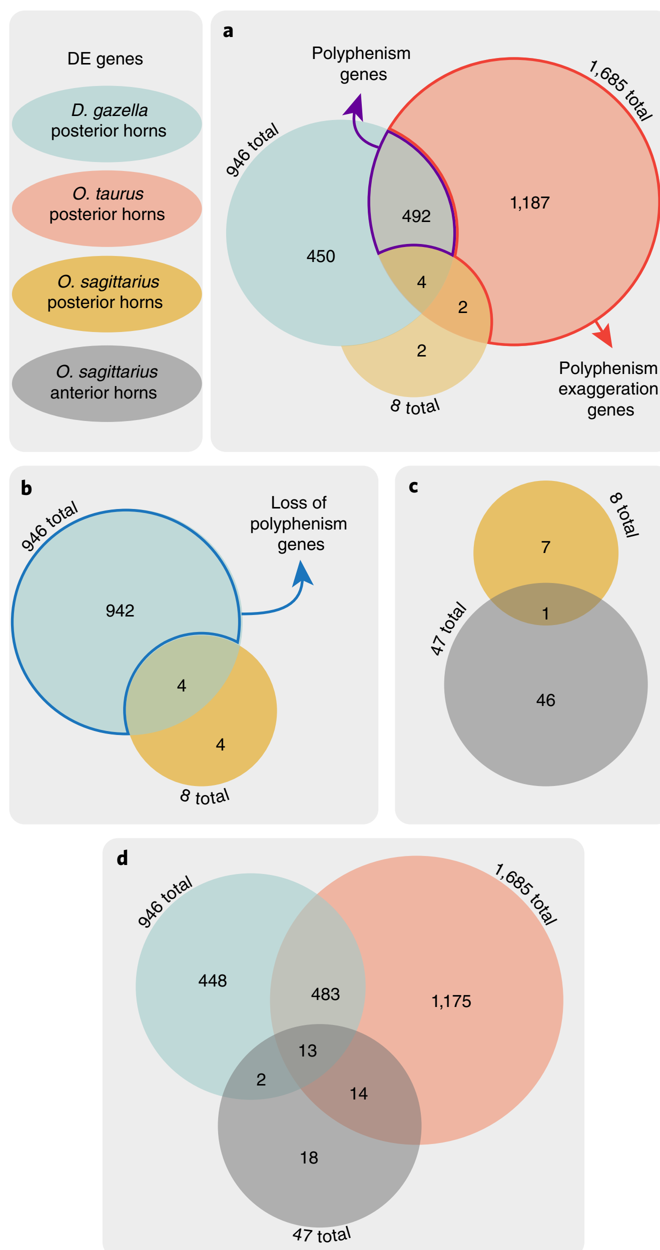


Fig. 2 | Nutrition-responsive differential gene expression as a function of nutrition across three onthophagine species. a, The number of differentially expressed genes unique to, or shared between, *D. gazella*, *O. taurus* and the posterior, secondarily non-plastic head region of *O. sagittarius*. Differentially expressed genes shared by *D. gazella* and *O. taurus* are labelled as putative polyphenism genes (purple-outlined intersection), whereas genes that are uniquely differentially expressed in *O. taurus* are labelled as putative polyphenism exaggeration genes (red-outlined subset). Totals refer to the full set of differentially expressed genes in each species. **b**, Detailed analysis of the secondarily non-plastic head region of *O. sagittarius*. Only 4 out of 946 differentially expressed genes in *D. gazella* posterior horns and 8 DE genes in the homologous region of *O. sagittarius* are shared. Assuming that gene expression in *D. gazella* reflects the ancestral condition in Onthophagini, the remaining 942 differentially expressed genes (blue-outlined subset) are considered to have secondarily lost nutrition-sensitive expression in the lineage leading to *O. sagittarius*. **c**, Only one out of 8 and 47 differentially expressed genes are shared between the posterior (hornless) and anterior (horned) head regions of *O. sagittarius*, respectively. **d**, The number of differentially expressed genes shared between the typical posterior head horns of polyphenic *D. gazella* and *O. taurus*, and the novel, moderately plastic anterior head horns in *O. sagittarius*.

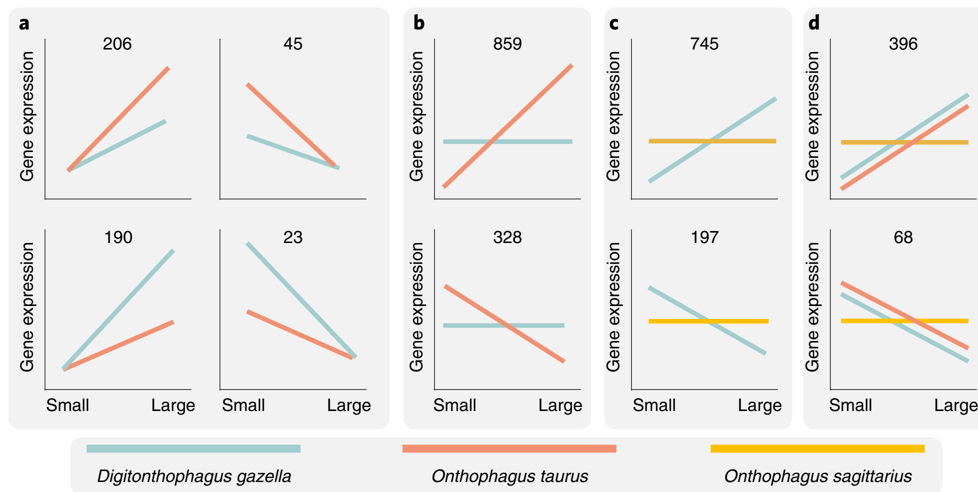


Fig. 3 | Distribution of differentially expressed genes following different patterns of response to nutrition. a–d, Schematic representation of the three categories of interest. Plots illustrate direction and relative strength of change in gene expression between small (low-nutrition) and large (high-nutrition) males for each response pattern. **a**, Of 492 putative polyphenism genes (that is, exhibiting nutrition-sensitive expression in *D. gazella* and *O. taurus* but not in *O. sagittarius*), 206 exhibited a stronger expression change in *O. taurus* than in *D. gazella* and had relatively higher expression in large males (top left), whereas 45 showed stronger expression change in *O. taurus* compared to *D. gazella* but relatively lower expression in large males compared to small (top right). In turn, 190 exhibited a weaker expression change in *O. taurus* than *D. gazella* while having higher expression in large males (bottom left), whereas 23 showed weaker expression change in *O. taurus* and lower expression in large males (bottom right). **b**, Of 1,187 putative polyphenism exaggeration genes (that is, exhibiting nutrition-sensitive expression in *O. taurus* but not *D. gazella* or the posterior head region of *O. sagittarius*), 859 showed higher expression in large *O. taurus* relative to small *O. taurus* (top) whereas 328 showed the opposite pattern (bottom). **c**, Of 942 genes associated with the loss of polyphenic development in *O. sagittarius* (that is, exhibiting nutrition-sensitive gene expression in *D. gazella* but not in *O. sagittarius*), 745 are relatively higher in large compared to small *D. gazella* (top), while 197 show the opposite pattern (bottom). **d**, Of 492 polyphenism genes conserved across *D. gazella* and *O. taurus* but which have lost nutrition responsiveness in *O. sagittarius*, 396 showed relatively higher expression in large *D. gazella* and *O. taurus* compared to small (top) while 68 showed the opposite pattern (bottom).

that most evolutionary changes in gene expression plasticity occur in putative growth-promoting genes and thus manifest under high-nutrition conditions. Collectively, our findings are consistent with the hypothesis that exaggeration of polyphenic development evolved by both enhancement of the nutritional response of ancestrally nutrition-sensitive genes and secondary recruitment of genes lacking an ancestral role in nutrition-sensitive growth.

Loss of nutritional plasticity is underlain by widespread loss of nutrition-sensitive gene expression primarily under high-nutrition conditions. The posterior head horn region in *O. sagittarius* is homologous to the region where paired horns are located in *D. gazella* and *O. taurus*, yet instead of horns, male *O. sagittarius* form only a ridge in the corresponding region, similar to those seen in female *D. gazella* and *O. taurus*. However, both phylogenetic and functional genetic evidence strongly support the hypothesis that this loss of posterior head horns is a secondary condition[11,33]. We hypothesized that the secondary loss of polyphenic development may evolve through secondary loss of ancestral nutrition-responsive gene expression. We therefore focused on the category of genes that are DE in the posterior head of *D. gazella* but not in the homologous region in *O. sagittarius*. We identified 942 genes that exhibited this pattern (Fig. 2b). Of these, 745 (79%) had higher expression in large, high-nutrition *D. gazella* (positive $\log_2$-fold change; Fig. 3c top) and 197 (21%) had relatively higher expression in small, low-nutrition individuals (negative $\log_2$-fold change; Fig. 3c bottom; if $H_0 = 50{:}50$, $\chi^2 = 40.699$, d.f. $= 1$, $P = 1.776 \times 10^{-10}$). Because we cannot discount the possibility that some of these genes may have diverged in the lineage leading to *D. gazella*, we restricted our analysis to those putative polyphenism genes that have secondarily lost nutrition sensitivity. Of those 492 genes, 396 had relatively higher expression in both large *D. gazella* and large *O. taurus* (positive $\log_2$-fold change; Fig. 3d top) while 68 had relatively higher expression in small *D. gazella* and small *O. taurus* (negative $\log_2$-fold change; Fig. 3d bottom; if $H_0 = 50{:}50$, $\chi^2 = 39.952$, d.f. $= 1$, $P = 2.603 \times 10^{-10}$). Together, our results suggest that secondary loss of polyphenic development is underlain by widespread secondary loss of nutrition-sensitive gene expression, with most changes manifest under high nutrition.

Modest nutrition-responsive growth of novel horns is underpinned by both pre-existing and novel gene expression plasticity. We explored the transcriptional basis of modest nutritional plasticity in a recently evolved novel trait, the anterior head horns of *O. sagittarius*. If nutrition responsiveness in these anterior horns evolved by drawing from the same gene set mediating nutrition responsiveness in posterior horns of other species, then DE genes in *O. sagittarius* anterior horns should overlap broadly with those DE in the posterior horns of *D. gazella* and *O. taurus*. Out of 47 DE genes in the anterior head horns of *O. sagittarius*, 15 (31.9%) are shared with *D. gazella*, 27 (57.4%) are shared with *O. taurus* and 13 (27.7%) are shared with both *D. gazella* and *O. taurus* (Fig. 2d), while only 18 (38.3%) are unique to *O. sagittarius* (if $H_0 = 50{:}50$ conserved/unique to *O. sagittarius*, $\chi^2 = 1.3219$, d.f. $= 1$, $P = 0.2502$). These results support the hypothesis that modest nutrition responsiveness of novel anterior head horns in *O. sagittarius* relies on both coopted genes showing similar expression plasticity in posterior horns of other species and genes with unique DE in this species.

Together, we found that the number of DE genes within species matched a given species' degree of morphological plasticity. While exaggeration of polyphenic development (*O. taurus*) is associated with a large number of genes newly acquiring nutrition-responsive expression compared to the modestly polyphenic *D. gazella*, *loss* of polyphenic development as in the posterior horns of *O. sagittarius* is associated with the secondary loss of nutrition-sensitive expression

in numerous genes. Furthermore, we identified a modest number of genes that secondarily acquired nutrition responsiveness in the recently evolved *O. sagittarius*-specific anterior horns. Throughout, we find that evolutionary changes in gene expression plasticity manifest primarily under high-nutrition conditions, and implicate genes associated with growth promotion. Collectively, these results suggest that diverse degrees of morphological plasticity and their evolution are underlain by matching degrees of changes in gene expression plasticity.

The evolution of *dsx* function as a case study in the regulation of polyphenic development and its evolution. Among the many genes identified by our analyses as putative polyphenism genes was *dsx*, a transcription factor previously identified as an important regulator of horn polyphenism in *O. taurus*[11]. *dsx* knockdown mutes nutrition-responsive horn formation in high-nutrition male *O. taurus* yet has no effect on the anterior horns of male *O. sagittarius*[11]. Difference in *dsx* expression between high- and low-nutrition males in *O. taurus* was larger than in *D. gazella* but absent in either horn type in *O. sagittarius* (Fig. 4a). Since *O. sagittarius* males lack posterior horns, while anterior horns exhibit only modest nutrition sensitivity, fold-change expression for each species mirrors each species' degree of morphological response to nutrition. To test whether *dsx* function in *D. gazella* similarly correlates with morphological plasticity to nutrition, we knocked down *dsx* in *D. gazella* using RNA interference. We found that although *Dgaz-dsx*^{RNAi} resulted in noticeable reduction of horn length in large males, both absolute and relative effects were significantly smaller than previously observed in *O. taurus*[11] (Fig. 4b,c; absolute: $t=4.0968$, $P=0.0001$; relative: $t=3.373$, $P=0.001$). Species-specific differences persisted even after increasing *dsx* double-stranded RNA concentration to 200–600% of that used previously in *O. taurus*. These results (1) suggest that *dsx* was already associated with nutrition-responsive development at the early stages of polyphenism evolution; (2) support the hypothesis that an enhanced *dsx* expression response to nutrition underpins evolutionary exaggeration of horn polyphenism in *O. taurus*; and (3) more generally provide a proof of principle showcasing the potential of our dataset toward identifying genes causally linked to the regulation of polyphenic development and its evolution.

Genetic accommodation of gene expression plasticity may facilitate diversification of polyphenic development. We sought to test whether genetic accommodation could have contributed to the evolution of nutrition-responsive growth in *Onthophagus*, yielding exaggerated (as in *O. taurus*) or, alternatively, canalized (lost) nutrition responsiveness (as in *O. sagittarius*) from a modestly polyphenic ancestor, similar to *D. gazella*. This hypothesis predicts higher among-individual variation in environmental responsiveness in the ancestral compared to the derived conditions. We assessed this prediction by calculating the mean absolute deviation (MAD; see Methods) of each gene and compared this measure of variation across nutritional categories within and between species. We predicted that if genetic accommodation had occurred, we would expect elevated variation in gene expression in *D. gazella* (our proxy for the ancestral condition) relative to the other two species (representing derived conditions), in particular among genes with relatively high expression in large, high-nutrition individuals. Importantly, because *D. gazella* is likely to have undergone some refinement of its own plastic response (that is, it has probably lost some gene expression variation) since diverging from the common

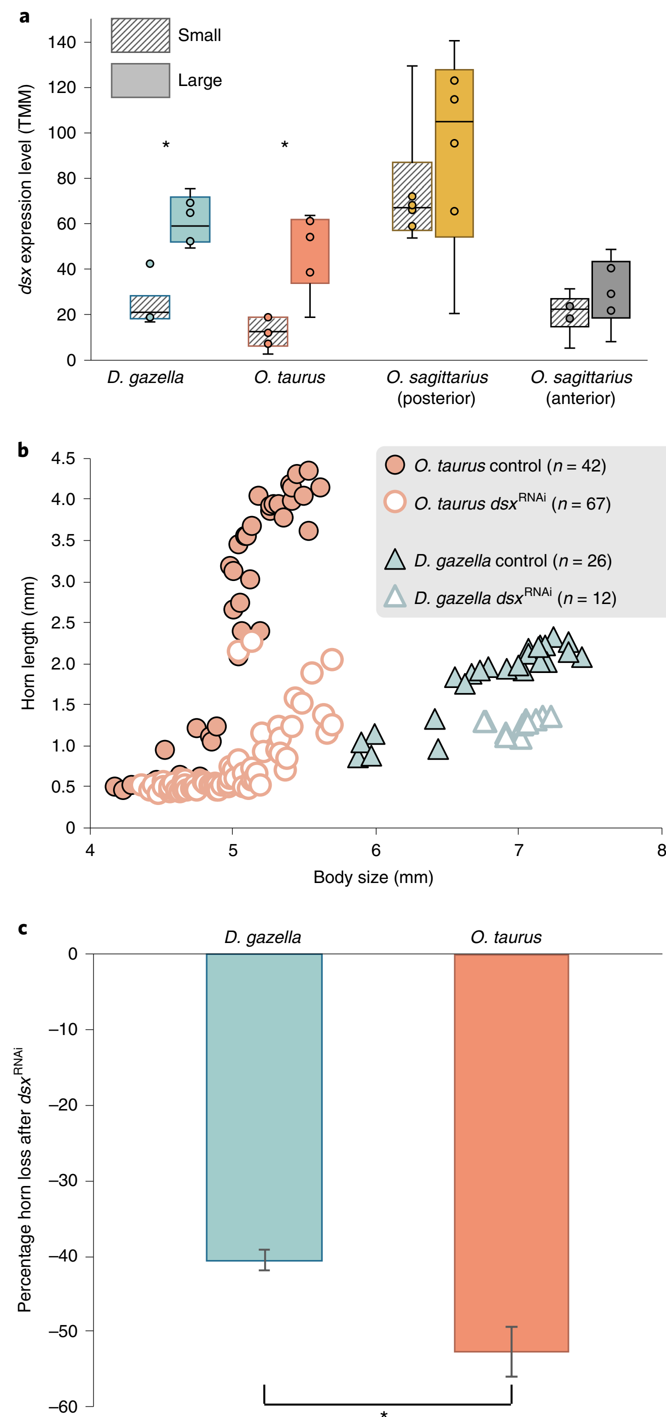


Fig. 4 | Comparative analyses of *dsx* function in the regulation of horn growth. a, Relative (TMM normalized) *dsx* expression levels across nutritional conditions (striped: small, low-nutrition males; solid: large, high-nutrition males) and species (*D. gazella*, $n=6$; *O. taurus*, $n=6$; posterior head region in *O. sagittarius*, $n=6$; anterior head region in *O. sagittarius*, $n=6$). **b**, Body size–horn length allometry for both *D. gazella* and *O. taurus*. *dsx* knockdown dramatically decreased horn length in *O. taurus* (solid red circles: *O. taurus* control, $n=42$; open red circles: *Otau-dsx*^{RNAi}, $n=67$), but only modestly in *D. gazella* (solid blue triangles: *D. gazella* control, $n=26$; open blue triangles: *Dgaz-dsx*^{RNAi}, $n=12$). **c**, Percentage horn loss after *dsx* knockdown (calculated based on horn length residuals) is significantly greater in *O. taurus* compared to *D. gazella*. The box plot shows the median (centre line), 25% and 75% quartiles (boxes), outermost values (whiskers), outliers (dots) and data points (overlapping with box and whiskers). Error bars in bar graph represent s.e.m. $*P<0.01$.

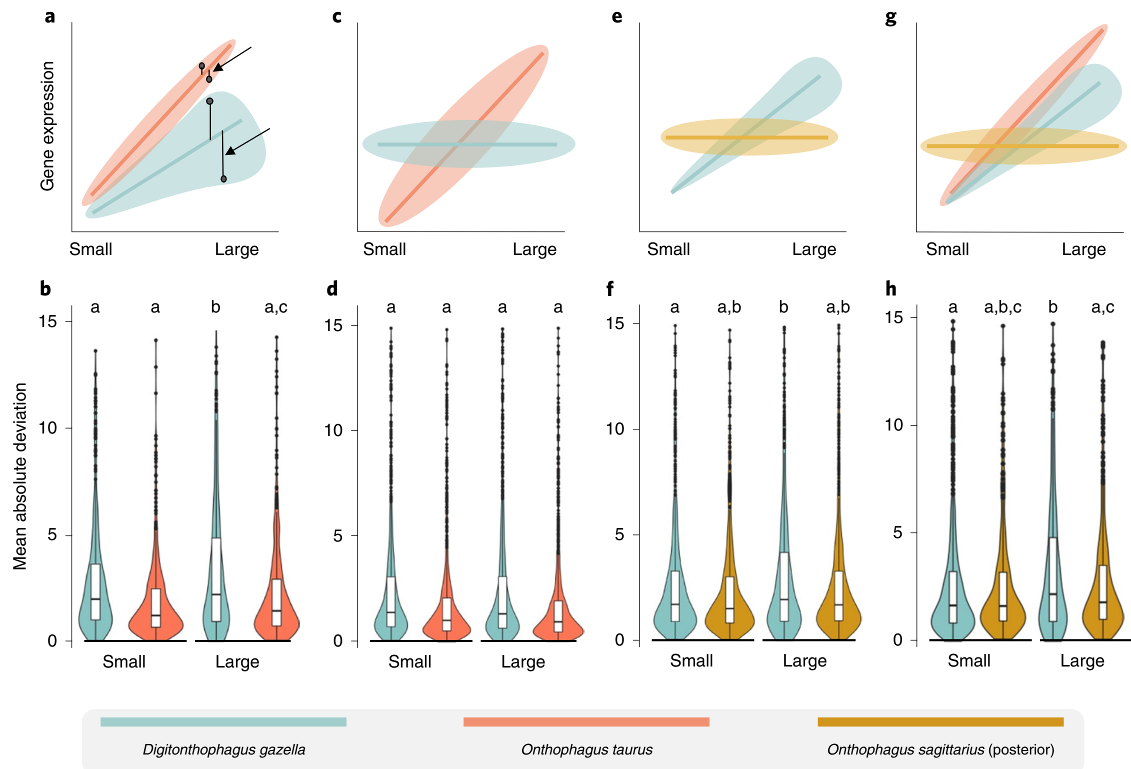


Fig. 5 | Gene expression variation (MAD) across plasticity categories. a–h, Polyphenism genes (**a**,**b**), polyphenism exaggeration genes (**c**,**d**) and genes associated with loss of polyphenism (**e**–**h**). The top row (**a**,**c**,**e**,**g**) shows a schematic description of the results, where the solid line represents mean gene expression per gene and the surrounding oval represents deviation from the mean. The bottom row (**b**,**d**,**f**,**h**) shows violin plots of mean absolute deviation (per gene) across body sizes and species. MAD differences were observed in putative polyphenism genes (**a**,**b**, $n = 492$) in which large *D. gazella* exhibited higher MAD values than small *D. gazella* and *O. taurus* for both nutritional conditions. No differences were observed for putative polyphenism exaggeration genes (**c**,**d**, $n = 1{,}187$). For genes that have lost nutrition responsiveness (**e**,**f**, $n = 942$; **g**,**h**, $n = 492$), differences were found only for those that are also putative polyphenism genes yet have lost nutrition responsiveness in *O. sagittarius*, and only in high-nutrition individuals (**g**,**h**, $n = 492$). *Y* axes for violin plots are scaled to highlight differences among samples; letters above violin plots indicate significant differences ($P < 0.0125$). Arrows in **a** indicate deviation from the mean gene expression level used to calculate MAD.

ancestor for which it serves as a proxy in this work, any differences found by our contrasts are likely to be an underestimation.

We first examined whether evolution by genetic accommodation may have contributed to the exaggerated response seen in *O. taurus*. Focusing on the 492 previously identified putative polyphenism genes, we found a significant difference in MAD values between high- and low-nutrition *D. gazella* but not between high- and low-nutrition *O. taurus* (Supplementary Table 1 and Fig. 5a,b); across species, high-nutrition *D. gazella* differed significantly from their *O. taurus* counterparts but differences were only marginally significant in low-nutrition individuals (Supplementary Table 1 and Fig. 5a,b). These results support a scenario in which genetic accommodation contributed to the exaggeration of polyphenic development in *O. taurus* by refining, and thereby reducing, ancestral heritable variation in gene expression plasticity.

While genetic accommodation predicts a reduction in expression variance of the common core of putative polyphenism genes, this is not necessarily true for genes that newly acquired nutrition responsiveness. Because these genes do not show signatures of ancestral plasticity—that is, are expressed in *D. gazella* in a nutrition-insensitive manner—we predicted that in this gene set genetic accommodation would not have been able to operate, resulting in the absence of differences in gene expression variation across species. Consistent with this hypothesis, we found no difference in MAD values for polyphenism exaggeration genes (that is, all 1,187 genes uniquely DE in *O. taurus*) within or across species and nutritional categories (Fig. 5c,d and Supplementary Table 2).

We then tested whether loss of polyphenic development in *O. sagittarius* could have occurred via genetic assimilation, a specific case of genetic accommodation in which ancestral environmental sensitivity is lost. Such a scenario would once again predict higher expression variation in *D. gazella* relative to *O. sagittarius* in genes regulating polyphenism in the common ancestor of both species. We first considered all DE genes in *D. gazella* that have lost nutrition responsiveness in *O. sagittarius*, but found no significant difference in MAD values (Fig. 5e,f and Supplementary Table 3). We then repeated the analysis for the subset of genes sharing differential expression in both *D. gazella* and *O. taurus* (that is, putative polyphenism genes) and which had also lost plasticity in *O. sagittarius*, assuming this would increase the probability of including genes functionally associated with the regulation, elaboration and loss of polyphenic development. This more focused comparison identified elevated MAD values in *D. gazella* compared to *O. sagittarius*, though this difference was significant in high-nutrition individuals only (Fig. 5g,h and Supplementary Table 4). Our results are consistent with genetic assimilation of gene expression plasticity in putative polyphenism genes during the secondary loss of polyphenic development in the evolution of *O. sagittarius*. In contrast, we found no significant differences between *D. gazella* and *O. taurus* when comparing all genes across nutritional environments (see Supplementary Table 5).

The results of the MAD analysis reflect gene expression variation as a function of expression levels, upon which selection ultimately acts[34,35]. However, to ensure that our results were not dependent on our approach to quantify variance, we repeated our analyses using coefficients of variance (CV; see Methods and Supplementary Information; CV normalizes variation against mean gene expression levels). All differences previously detected using MAD remained significant when using CV (Supplementary Tables 6–10).

Taxon-restricted genes contribute to but do not drive evolution of plastic development. Finally, we examined the potential role of genes likely to have originated within Onthophagini. We categorized 1,992 genes found within the *O. taurus* genome lacking an identifiable orthologue in any of the other genomes included in the OrthoDB9 database[36] as taxon-restricted. We hypothesized that if polyphenism evolution is fuelled preferentially through recruitment of taxon-restricted genes, then genes that we identified as nutrition sensitive should be enriched for this category. We found that the proportion of *O. taurus* taxon-restricted DE genes (22.7%) was slightly, albeit significantly, lower than the proportion of non-taxon-restricted DE genes (24.8%; binomial proportions test, $\chi^2 = 4.1474$, d.f. = 1, $P = 0.0417$; Supplementary Table 11). This result supports the hypothesis that evolution of nutrition sensitivity relies roughly equally on both repurposing pre-existing networks and recruiting lineage-specific novel genes. We then used a network approach, weighted gene co-expression network analysis (WGCNA[37]), to identify how commonly taxon-restricted genes occupy key positions within regulatory networks. We found that the proportion of highly connected taxon-restricted genes (17.3%; see Methods) was lower than that of highly connected conserved genes (19.3%; binomial proportions test, $\chi^2 = 4.2393$, d.f. = 1, $P = 0.0395$; Supplementary Table 12), suggesting that key positions in networks regulating nutrition sensitivity are occupied primarily by conserved genes and, to a slightly lesser degree, by recently evolved genes.

However, the results of our global expression analyses do not exclude the possibility that a few, select taxon-restricted genes may have evolved key roles in the regulation of nutrition-responsive growth. Instead, they highlight the need for comparative functional studies of several taxon-restricted candidate genes identified here as being both strongly DE and highly connected to other genes. The same applies to the many conserved genes whose functional significance in the context of nutrition-responsive development has yet to be explored. For example, surprising potential key regulators include the homeobox genes *araucan*, *wingless, aristaless*, *Frizzled-4, Toll-like receptor Tollo* and *spaetzle*. Components from diverse pathways ranging from Wnt signalling (*wingless, Frizzled*) to immune response (*Toll-like receptor, spaetzle*) may be playing underappreciated key roles in the regulation and evolution of nutrition-responsive growth.

Our understanding of the molecular mechanisms underlying the evolution of developmental plasticity has increased substantially in recent years[38–41]. For example, studies on plastic gene expression in the ant *Cardiocondyla obscurior* found a positive correlation between caste-specific gene expression plasticity and evolutionary rates[40]. Variation within castes, quantified using CV, showed elevated variation for highly plastic genes, similar to our findings that high-nutrition *D. gazella* exhibited higher among-individual variation than their low-nutrition counterpart. Despite recent advances, comparative assessments of gene expression plasticity among species or populations remain relatively scarce (but see refs. [39,41,42]). Our work on nutrition-responsive development of beetle horns provides one of the first broad, comparative, genome-wide characterizations of the transcriptional underpinnings of plasticity evolution. We show that the size of the gene repertoire associated with nutrition-responsive growth mirrors the degree of nutrition responsiveness evident on a morphological level, and that elaboration of morphological plasticity is underlain by both enhancement of ancestral nutrition-responsive gene expression and recruitment of previously low nutritionally responsive genes into the process. Conversely, we show that the secondary loss of nutritional plasticity on the level of morphology coincides with a dramatic reduction in gene expression plasticity. Moreover, our results implicate genetic accommodation in the evolution of transcriptional plasticity by refining ancestrally elevated variation in gene expression plasticity toward derived responses, whether they constitute exaggerated forms of polyphenic development or secondary losses thereof. Additionally, our study offers important starting points to further investigate the functional significance of genes—including those newly evolved in Onthophagini beetles—in the regulation of plasticity, in the evolution of gene networks and in their respective contributions to shaping the diversification of nutrition-sensitive development in nature. Lastly, this work lays the foundation for future studies to deepen our understanding of how conserved, pre-existing gene regulatory networks evolve nutrition responsiveness and what network features (for example, distribution of transcription factor binding sites among network members or the involvement of key genes in multiple networks) predispose some networks over others in this process.

Methods

Animal husbandry. *Onthophagus taurus* beetles were field collected near Chapel Hill, NC while *D. gazella* and *O. sagittarius* were collected at Kualoa Ranch in Kaneohe, HI; beetles were shipped to Bloomington, IN (all USA) and maintained in captivity as described previously[43]. Briefly, beetles were kept in a sand/soil mixture on a 16/8 light/dark cycle at either 24 °C (*O. taurus*) or 28 °C (*D. gazella* and *O. sagittarius*) and fed homogenized cow dung. Animals used for dissections and RNA extractions were generated by allowing five females and three males to breed in plastic containers packed with a sand/soil mixture. Brood balls were collected after 8 d and larvae were transferred to individual wells within 12-well tissue culture plates and provided with ad libitum cow manure. Cow manure of high or low quality was used to manipulate nutritional values: manure from grass-fed (high-nutrition) or hay-fed (low-nutrition) cows, both of which are encountered in nature. Larvae were checked every ~8 h to detect the onset of pupation.

Sample selection, tissue dissection and RNA extraction. Within the first 17 h after pupation, individuals were weighed and thoracic width and horn length were measured to the nearest 0.01 mm using a digital camera (Scion) mounted on a dissecting scope (Leica) using Image J software. For each species, six large and six small male individuals were selected based on pupal thoracic width (Extended Data Fig. 1). The range of thoracic width within the large and small size classes did not exceed 0.4 mm (*D. gazella*), 0.3 mm (*O. taurus*) or 0.2 mm (*O. sagittarius*) (Extended Data Fig. 1). Each pupa selected for tissue dissection was submerged and dissected in 0.05% Triton-X in phosphate buffered saline (as in ref. [44]). Posterior horn tissues were dissected from six small and six large individuals of *O. taurus* and *D. gazella*, respectively (Fig. 1). For *O. sagittarius*, tissues from two regions were dissected separately: the posterior dorsal head, a location homologous to where horns develop in *O. taurus* and *D. gazella*, and the anterior dorsal head, a location where paired anterior head horns unique to male *O. sagittarius* develop (Fig. 1). Each region was dissected from six small and six large individuals of *O. sagittarius*, resulting in 24 samples for this species. Dissected tissues were moved to ice-cold Trizol (Thermo Fisher Scientific) and stored at −80 °C until RNA extraction. Once all samples were collected, phenol-chloroform RNA extractions were carried out as in ref. [44]. Briefly, tissues in Trizol were thawed to 4 °C, homogenized with disposable polypropylene RNase-free pestles and total RNA was extracted using a standard phenol/chloroform protocol followed by RNeasy Mini (no. 74104, Qiagen) spin column purification.

Library construction and high-throughput sequencing. Total RNA quality was checked using an RNA ScreenTape TapeStation System (Agilent), and yield was quantified with a Quant-iT RiboGreen Assay Kit (Thermo Fisher). RNA stranded RNA-seq libraries were constructed using the Illumina TruSeq Stranded mRNA Sample Preparation Kit (Illumina) according to the manufacturer's instructions. Library quantification was performed using a Quant-iT DNA Assay Kit (Thermo Fisher), pooled in equal molar amounts and sequenced on an Illumina NextSeq500 platform as single-end reads using a 75-cycle High Output Kit.

Transcriptome assemblies and annotations. Reads were cleaned using Trimmomatic v.0.36 (ref. [45]) to remove adaptor sequences, perform quality trimming and discard low-quality reads. Reference transcriptomes were assembled de novo for each species using Trinity 2.4 (ref. [46]). Candidate coding sequences were predicted using Transdecoder 2.0.1 (http://transdecoder.github.io/), and the assemblies were annotated using a standard Trinotate pipeline[47] (https://trinotate.

github.io/) with the inclusion of the *O. taurus* genome as a custom database[48]. For each transcriptome, an annotation report table was exported as a text file and used to annotate differential gene expression results (see below).

Differential expression analysis. Transcript abundance estimation was based on counting reads aligned to de novo assembled reference transcriptomes. Normalized expression values, trimmed mean of *M* values (TMM), were obtained through the `edgeR` package[49] and differential gene expression from pairwise contrasts was analysed with the `DESeq2` package[50]. The false discovery rate cut-off value for differential expression was set at 0.001. A first set of contrasts was defined for within-species comparisons of gene expression between horn tissues of large and small individuals for *D. gazella* and *O. taurus*; in *O. sagittarius*, additional contrasts between and across anterior and posterior regions of the dorsal head were made. Result tables with differentially expressed genes for each contrast were merged with the transcriptome annotation table of the corresponding species (see above) using custom scripts in the R computing environment[51].

To enable comparisons across species, the annotated read count tables were merged using the best BLAST hit to gene models from the *O. taurus* genome v.0.5.3 (ref. [48]) as common key field. The resulting merge table was used to establish differentially expressed putatively homologous genes across species, and to identify up- or downregulated transcripts across large and small body sizes within each species and degree of change across species using the log-transformed fold change.

To estimate the MAD of each gene, previously calculated TMM values for each species were used (see above). TMM tables were again merged based on best BLAST hit to gene models from the *O. taurus* genome v.0.5.3 (ref. [48]) as described above. The resulting table was used to calculate the MAD of each gene as:

$$\mathrm{MAD} = \frac{\sum |x_i - \bar{x}|}{n}$$

where x_i is the TMM value, $\bar{x}$ the mean TMM value per group (small or large, per species) and $n = 6$ is the sample size. Comparisons within and across groups were done using Welch's *t*-test followed by Bonferroni correction.

To estimate the CV for each gene, TMM values for each species were used. CV was calculated as the ratio of s.d. to mean gene expression levels. For our MAD calculations, CV was calculated per group (small or large, per species) and compared within and across groups using Welch's *t*-test followed by Bonferroni correction.

Identification of taxon-restricted genes. Potential taxon-restricted genes were subset based on the OrthoDB v.9.1 database[36]. This database includes *O. taurus* as one of the nine species of beetle where orthologue clustering has been done. The species in this database most closely related to *O. taurus* is *Oryctes borbonicus*, a rhinoceros beetle in the same family as *Onthophagus* (Scarabaeidae) but a different subfamily (Dynastinae). Taxon-restricted genes for *O. taurus* were defined as those not clustered in the database and thus not sharing orthologues with *O. borbonicus*[52] or any other species. To find the expression patterns of taxon-restricted genes, a custom R script was used to subset the *O. taurus* differential expression table to contain only taxon-restricted genes. Then, to better understand the contribution of taxon-restricted within-gene networks, we used the WGCNA R package[33] (see Supplementary Information). To determine whether taxon-restricted genes were highly connected ('hub genes') within the gene networks, another custom R script was used to subset the WGCNA module membership table (see above). Hub genes were defined as those having a module membership >0.8 or <−0.8 and a gene significance >0.4 or <−0.4.

Digitonthophagus gazella dsx **dsRNA synthesis, injection and morphological analyses.** *Digitonthophagus gazella dsx* (*Dgdsx*) dsRNA synthesis and injections were performed as previously described[11,44]. Briefly, *Dgdsx* was identified using the annotations described above and verified by reciprocal BLAST to the *O. taurus* genome. *Dgdsx* sequence was retrieved from the transcriptome, and the DNA fragment was synthesized from gBlocks Gene Fragments (Integrated DNA Technologies). To synthesize dsRNA, the template was amplified by PCR using *Dgdsx*-specific primers fused with a T7 promoter sequence at the 5' end. *Dgdsx* primer sequences (from 5' to 3') were: CTCTGCGAAGGGCTCAAG and ACATTGTTATTCACCATTGTATCCC. dsRNA synthesis was carried out using the MEGAscript T7 transcription kit and purified using the MEGAclear Kit (both Invitrogen). *D. gazella* larvae injections were carried out on the third larval instar as previously described[11,53]. Either 1 or 3 µg of dsRNA was diluted in injection buffer to a total of 3 µl. Control injections were carried out with 3 µl of injection buffer.

Allometric measurements of *Dgdsx*$^{\mathrm{RNAi}}$ individuals were carried out using a two-dimensional morphometric set-up. As a measure of body size we used thoracic width, and head horns were measured as previously described[54]. For both *D. gazella* and *O. taurus* measurements (data from ref. [11]), analysis of the sigmoidal body size–horn length allometry was carried out by fitting a sigmoidal four-parameter hill equation to measurements from control injected individuals. Residuals for both control and *Dgdsx*$^{\mathrm{RNAi}}$ individuals were calculated as the difference between observed and expected horn length for a specific body size (as in refs. [8,55]). Percentage change from the predicted values was then calculated and compared between the two species using a two-tailed *t*-test.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data are available through NCBI's Short Read Archive (BioProject accession: PRJNA608082).

Received: 19 September 2019; Accepted: 9 April 2020;
Published online: 18 May 2020

References

1. Beldade, P., Mateus, A. R. & Keller, R. Evolution and molecular mechanisms of adaptive developmental plasticity. *Mol. Ecol.* **20**, 1347–1363 (2011).
2. West-Eberhard, M. J. *Developmental Plasticity and Evolution* (Oxford Univ. Press, 2003).
3. Koyama, T., Mendes, C. C. & Mirth, C. K. Mechanisms regulating nutrition-dependent developmental plasticity through organ-specific effects in insects. *Front. Physiol.* **4**, 263 (2013).
4. Gotoh, H. et al. Developmental link between sex and nutrition; *doublesex* regulates sex-specific mandible growth via juvenile hormone signaling in stag beetles. *PLoS Genet.* **10**, e1004098 (2014).
5. Koyama, T. & Mirth, C. K. Unravelling the diversity of mechanisms through which nutrition regulates body size in insects. *Curr. Opin. Insect Sci.* **25**, 1–8 (2018).
6. Mirth, C. K. & Shingleton, A. W. Integrating body and organ size in *Drosophila*: recent advances and outstanding problems. *Front. Endocrinol.* **3**, 49 (2012).
7. Emlen, D. J., Warren, I. A., Johns, A., Dworkin, I. & Lavine, L. C. A mechanism of extreme growth and reliable signaling in sexually selected ornaments and weapons. *Science* **337**, 860–864 (2012).
8. Casasa, S. & Moczek, A. P. Insulin signalling's role in mediating tissue-specific nutritional plasticity and robustness in the horn-polyphenic beetle *Onthophagus taurus*. *Proc. R. Soc. Lond. B* **285**, 20181631 (2018).
9. Xu, H. J. et al. Two insulin receptors determine alternative wing morphs in planthoppers. *Nature* **519**, 464–467 (2015).
10. Fawcett, M. M. et al. Manipulation of insulin signaling phenocopies evolution of a host-associated polyphenism. *Nat. Commun.* **9**, 1699 (2018).
11. Kijimoto, T., Moczek, A. P. & Andrews, J. Diversification of *doublesex* function underlies morph-, sex-, and species-specific development of beetle horns. *Proc. Natl Acad. Sci. USA* **109**, 20526–20531 (2012).
12. Kijimoto, T. & Moczek, A. P. Hedgehog signaling enables nutrition-responsive inhibition of an alternative morph in a polyphenic beetle. *Proc. Natl Acad. Sci. USA* **113**, 5982–5987 (2016).
13. Snell-Rood, E. C. et al. Developmental decoupling of alternative phenotypes: insights from the transcriptomes of horn-polyphenic beetles. *Evolution* **65**, 231–245 (2011).
14. Kijimoto, T. et al. The nutritionally responsive transcriptome of the polyphenic beetle *Onthophagus taurus* and the importance of sexual dimorphism and body region. *Proc. R. Soc. Lond. B* **281**, 20142084 (2014).
15. Klasberg, S., Bitard-Feildel, T. & Mallet, L. Computational identification of novel genes: current and future perspectives. *Bioinform. Biol. Insights* **10**, 121–131 (2016).
16. Santos, M. E., Le Bouquin, A., Crumière, A. J. J. & Khila, A. Taxon-restricted genes at the origin of a novel trait allowing access to a new environment. *Science* **358**, 386–390 (2017).
17. Pfennig, D. W. et al. Phenotypic plasticity's impacts on diversification and speciation. *Trends Ecol. Evol.* **25**, 459–467 (2010).
18. Moczek, A. P. et al. The role of developmental plasticity in evolutionary innovation. *Proc. R. Soc. Lond. B* **278**, 2705–2713 (2011).
19. Wund, M. A. Assessing the impacts of phenotypic plasticity on evolution. *Integr. Comp. Biol.* **52**, 5–15 (2012).
20. Levis, N. A. & Pfennig, D. W. Evaluating 'plasticity-first' evolution in nature: key criteria and empirical approaches. *Trends Ecol. Evol.* **31**, 563e574 (2016).
21. Suzuki, Y. & Nijhout, H. F. Evolution of a polyphenism by genetic accommodation. *Science* **311**, 650–652 (2006).
22. Ledón-Rettig, C. C., Pfennig, D. W. & Nascone-Yoder, N. Ancestral variation and the potential for genetic accommodation in larval amphibians: implications for the evolution of novel feeding strategies. *Evol. Dev.* **10**, 316–325 (2008).
23. Scoville, A. G. & Pfrender, M. E. Phenotypic plasticity facilitates recurrent rapid adaptation to introduced predators. *Proc. Natl Acad. Sci. USA* **107**, 4260–4263 (2010).
24. Sikkink, K. L., Reynolds, R. M., Ituarte, C. M., Cresko, W. & Phillips, P. C. Rapid evolution of phenotypic plasticity and shifting thresholds of genetic assimilation in the nematode *Caenorhabditis remanei*. *G3* **4**, 1103–1112 (2014).
25. Susoy, V., Ragsdale, E. J., Kanzaki, N. & Sommer, R. J. Rapid diversification associated with a macroevolutionary pulse of developmental plasticity. *eLife* **4**, e05463 (2015).

26. Walworth, N. G., Lee, M. D., Fu, F.-X., Hutchins, D. A. & Webb, E. A. Molecular and physiological evidence of genetic assimilation to high CO_2 in the marine nitrogen fixer *Trichodesmium*. *Proc. Natl Acad. Sci. USA* **113**, E7367–E7374 (2016).
27. Badyaev, A. V., Potticary, A. L. & Morrison, E. S. Most colorful example of genetic assimilation? Exploring the evolutionary destiny of recurrent phenotypic accommodation. *Am. Nat.* **190**, 266–280 (2017).
28. Kulkarni, S. S., Denver, R. J., Gomez-Mestre, I. & Buchholz, D. R. Genetic accommodation via modified endocrine signalling explains phenotypic divergence among spadefoot toad species. *Nat. Commun.* **8**, 993 (2017).
29. Levis, N. A., Isdaner, A. J. & Pfennig, D. W. Morphological novelty emerges from pre-existing phenotypic plasticity. *Nat. Ecol. Evol.* **2**, 1289–1297 (2018).
30. Levis, N. A., Serrato-Capuchina, A. & Pfennig, D. W. Genetic accommodation in the wild: evolution of gene expression plasticity during character displacement. *J. Evol. Biol.* **30**, 1712–1723 (2017).
31. Renn, S. C. P. & Schummer, M. E. Genetic accommodation and behavioural evolution: insights from genomic studies. *Anim. Behav.* **85**, 1012–1022 (2013).
32. Génier, F. & Moretto, P. *Digitonthophagus* Balthasar, 1959: taxonomy, systematics, and morphological phylogeny of the genus revealing an African species complex (Coleoptera: Scarabaeidae: Scarabaeinae). *Zootaxa* **4248**, 1–110 (2017).
33. Emlen, D. J., Hunt, J. & Simmons, L. W. Evolution of sexual dimorphism and male dimorphism in the expression of beetle horns: phylogenetic evidence for modularity, evolutionary lability, and constraint. *Am. Nat.* **166**, S42–S68 (2005).
34. Nourmohammad, A. et al. Adaptive evolution of gene expression in *Drosophila*. *Cell Rep.* **20**, 1385–1395 (2017).
35. Signor, S. A. & Nuzhdin, S. V. The evolution of gene expression in cis and trans. *Trends Genet.* **34**, 532–544 (2018).
36. Zdobnov, E. M. et al. OrthoDB v9.1: cataloging evolutionary and functional annotations for animal, fungal, plant, archaeal, bacterial and viral orthologs. *Nucleic Acids Res.* **45**, D744–D749 (2017).
37. Langfelder, P. & Horvath, S. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinform.* **9**, 559 (2008).
38. Daniels, E. V., Murad, R., MortazaviA & Reed, R. D. Extensive transcriptional response associated with seasonal plasticity of butterfly wing patterns. *Mol. Ecol.* **23**, 6123–6134 (2014).
39. Ghalambor, C. K. et al. Non-adaptive plasticity potentiates rapid adaptive evolution of gene expression in nature. *Nature* **525**, 372–377 (2015).
40. Schrader, L., Helantera, H. & Oettler, J. Accelerated evolution of developmentally biased genes in the tetraphenic ant *Cardiocondyla obscurior*. *Mol. Biol. Evol.* **34**, 535–544 (2016).
41. Kenkel, C. D. & Matz, M. V. Gene expression plasticity as a mechanism of coral adaptation to a variable environment. *Nat. Ecol. Evol.* **1**, 0014 (2017).
42. Alaux, C. et al. Honey bee aggression supports a link between gene regulation and behavioral evolution. *Proc. Natl Acad. Sci. USA* **106**, 15400–15405 (2009).
43. Moczek, A. P. & Nijhout, H. F. A method for sexing third instar larvae of the genus *Onthophagus* LATREILLE (Coleoptera: Scarabaeidae). *Coleopt. Bull.* **56**, 279–284 (2002).
44. Ledón-Rettig, C. C., Zattara, E. E. & Moczek, A. P. Asymmetric interactions between *doublesex* and sex- and tissue-specific target genes mediate sexual dimorphism in beetles. *Nat. Commun.* **8**, 14593 (2017).
45. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120 (2014).
46. Haas, B. J. et al. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat. Protoc.* **8**, 1494–1512 (2013).
47. Bryant, D. M. et al. A tissue-mapped axolotl de novo transcriptome enables identification of limb regeneration factors. *Cell Rep.* **18**, 762–776 (2017).
48. Zattara, E. E., Hughes, D. S. T., Richards, S., Kijimoto, T. & Mocze, A. P. *Onthophagus taurus* genome annotations v0.5.3. *Ag Data Commons* https://data.nal.usda.gov/dataset/onthophagus-taurus-genome-annotations-v053 (2016).
49. McCarthy, J. D., Chen, Y. & Smyth, K. G. Differential expression analysis of multifactor RNA-seq experiments with respect to biological variation. *Nucleic Acids Res.* **40**, 4288–4297 (2012).
50. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).
51. R Development Core Team *R: A Language and Environment for Statistical Computing* (R Foundation for Statistical Computing, 2011); www.R-project.org/
52. Meyer, J. M. et al. Draft genome of the scarab beetle *Oryctes borbonicus* en La Réunion Island. *Genome Biol. Evol.* **8**, 2093–2105 (2016).
53. Moczek, A. P. & Rose, D. J. Differential recruitment of limb patterning genes during development and diversification of beetle horns. *Proc. Natl Acad. Sci. USA* **106**, 8992–899 (2009).
54. Moczek, A. P. A matter of measurements: challenges and approaches in the comparative analysis of static allometries. *Am. Nat.* **167**, 606–611 (2006).
55. Snell-Rood, E. C. & Moczek, A. P. Insulin signaling as a mechanism underlying developmental plasticity: the role of FOXO in a nutritional polyphenism. *PLoS ONE* **7**, e34857 (2012).

Acknowledgements

We thank the Center for Genomics and Bioinformatics at Indiana University, which conducted sequencing and library construction, the Zdobnov laboratory for their guidance using OrthoDB and the National Center for Genome Analysis. We sincerely thank C. Ledón-Rettig, T. Ledón-Rettig, S. Close and W. Arnold for collecting beetles in the wild. This research was made possible through support by National Science Foundation grant nos. IOS 1256689 and 1901680 to A.P.M., as well as grant no. 61369 from the John Templeton Foundation. The opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the National Science Foundation or John Templeton Foundation.

Author contributions

S.C., E.E.Z. and A.P.M. designed the experiments. S.C. conducted the experiments (phenotyping, tissue dissection and RNA extraction). S.C., E.E.Z. and A.P.M. analysed and interpreted the data. S.C., E.E.Z. and A.P.M. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

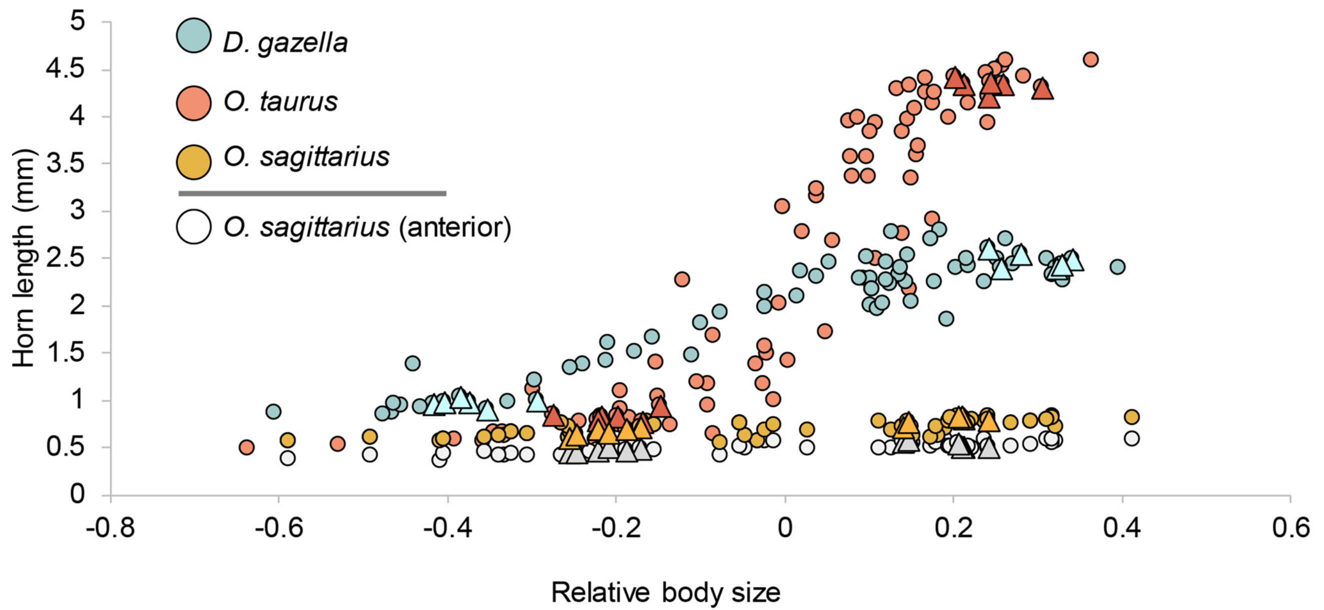
Extended data is available for this paper at https://doi.org/10.1038/s41559-020-1202-x.

Supplementary information is available for this paper at https://doi.org/10.1038/s41559-020-1202-x.

Correspondence and requests for materials should be addressed to S.C. or E.E.Z.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.© The Author(s), under exclusive licence to Springer Nature Limited 2020



Extended Data Fig. 1 | Individuals used for dissections. Relative pupal body size- horn size allometry for the three species used in this study (*D. gazella*: blue; *O. taurus*: red; *O. sagittarius*: yellow). Additionally, the novel, anterior head horn size of *O. sagittarius* is shown in white. Small and large individuals of each species that were used for dissections are shown in triangles. Relative body size was used to control for absolute body size differences across species and was calculated as the residual from the mean over the species' body size range.

nature research

Corresponding author(s): Sofia Casasa

Last updated by author(s): March 31 2020

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐	☒	A description of all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	No software was used to collect the data.
Data analysis	Trimmomatic version 0.36 , Trinity version 2.4, Transdecoder 2.0.1, Trinotate, R 3.5.1

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All supporting data is currently available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA608082. BioProject accession: PRJNA608082

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☐ Life sciences ☐ Behavioural & social sciences ☒ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	mRNA sequencing for low and high nutrition conditions using three species of onthophagine beetles.
Research sample	Low and high nutrition male Digitonthophagus gazella, Onthophagus taurus and Onthophagus sagittarius were dissected at the pupal stage. The three species were selected based on their degree of nutrition-responsiveness.
Sampling strategy	Cow manure of high or low quality was used to manipulate nutritional values: manure from grass fed (high nutrition) or hay fed (low nutrition) cows. Small and large individuals were selected based on their thorax width. The range of thorax widths within the large and small size classes did not exceed 0.4 mm (D. gazella), 0.3 mm (O. taurus), or 0.2 mm (O. sagittarius), respectively.
Data collection	Each pupa selected for tissue dissection was submerged and dissected in 0.05% Triton-X in phosphate buffered saline. Posterior horn tissues were dissected from six small and six large individuals of O. taurus and D. gazella respectively. For O. sagittarius, tissues from two regions were dissected separately: the posterior dorsal head, a location homologous to where horns develop in O. taurus and D. gazella, and the anterior dorsal head, a location where paired anterior head horns unique to male O. sagittarius develop. Dissected tissues were moved to ice-cold Trizol and stored at -80 degrees Celsius until RNA extraction.
Timing and spatial scale	Data collection for the three species started on December 2nd 2017 and endend on March 9th 2018. Individuals within the first 17 hours of pupation were selected for dissections.
Data exclusions	Individuals not within the pre-established body size ranges were excluded from the sampling.
Reproducibility	No attempts to repeat the experiments were made given the cost of RNA sequencing.
Randomization	Individuals were assigned to small or large groups based on thorax width. The range of thorax widths were species specific and pre-established.
Blinding	Blinding was not relevant given that we selected the samples for dissections based on pre-established parameters.

Did the study involve field work? ☐ Yes ☒ No

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	Male Digitonthophagus gazella, Onthophagus taurus and O. sagittarius at the pupal stage.
Wild animals	Adult field collected Digitonthophagus gazella, Onthophagus taurus and O. sagittarius were captured by inspecting dung pads. Both male and female were captured and shipped to the laboratory in plastic containers containing vermiculite to preserve moisture. After allowing them to breed and collecting the offspring used for this study, field collected animals were kept in the colonies (in a sand-soil mixture on an 16:18 light:dark cycle at 24 degrees Celsius, fed cow dung).
Field-collected samples	The study did not involve field-collected samples.
Ethics oversight	This study followed the ASAB/ABS Guidelines for the Use of Animals in Research. Animals were kept in the best possible conditions based on the biology of this species. We also adhered to the legal requirements of Indiana University and the U.S.A .

Note that full information on the approval of the study protocol must also be provided in the manuscript.