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Sexual dimorphism and heightened conditional expression in a sexually selected weapon in the Asian rhinoceros beetle

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Abstract

Among the most dramatic examples of sexual selection are the weapons used in battles between rival males over access to females. As with ornaments of female choice, the most "exaggerated" sexually selected weapons vary from male to male more widely than other body parts (hypervariability), and their growth tends to be more sensitive to nutritional state or physiological condition compared with growth of other body parts ("heightened" conditional expression). Here, we use RNAseq analysis to build on recent work exploring these mechanisms in the exaggerated weapons of beetles, by examining patterns of differential gene expression in exaggerated (head and thorax horns) and non-exaggerated (wings, genitalia) traits in the Asian rhinoceros beetle, *Trypoxylus dichotomus*. Our results suggest that sexually dimorphic expression of weaponry involves large-scale changes in gene expression, relative to other traits, while nutrition-driven changes in gene expression in these same weapons are less pronounced. However, although fewer genes overall were differentially expressed in high- vs. low-nutrition individuals, the number of differentially expressed genes varied predictably according to a trait's degree of condition dependence (head horn > thorax horn > wings > genitalia). Finally, we observed a high degree of similarity in direction of effects (vectors) for subsets of differentially expressed genes across both sexually dimorphic and nutritionally responsive growth. Our results are consistent with a common set of mechanisms governing sexual size dimorphism and condition dependence.

KEYWORDS
condition dependence, RNAseq, sexual dimorphism, sexual selection, weaponry

1 | INTRODUCTION

Sexual selection can drive rapid evolution of extreme traits, such as ornamental tails and crests used as a basis for mate choice, and horns, tusks and antlers that function as weapons in male combat (Andersson, 1994; Darwin, 1871). Ornaments and weapons of sexual selection have long fascinated biologists as they routinely attain sizes that are out of proportion with the rest of the body ("exaggerated"), and because they often diversify in form faster than other, surrounding body parts (Emlen, 2008; Emlen, Hunt, & Simmons, 2005; Emlen, Marangelo, Ball, & Cunningham, 2005; Prum, 1997). Not surprisingly, there is great interest in understanding the genetic architecture of these structures—how many genes and what types of genes or pathways underlie their rapid evolutionary transformations in form (Emlen & Nijhout, 2000; Hunt, Bussiere, Jennions, & Brooks, 2004; Kokko & Heubel, 2008; Miller & Moore, 2007; Pomiankowski & Moller, 1995; Prokuda & Roff, 2014; Radwan, 2008; Radwan, Engqvist, & Reinhold, 2016; Rowe & Houle, 1996; Tomkins, Radwan, Kotiaho, & Tregenza, 2004). One way to

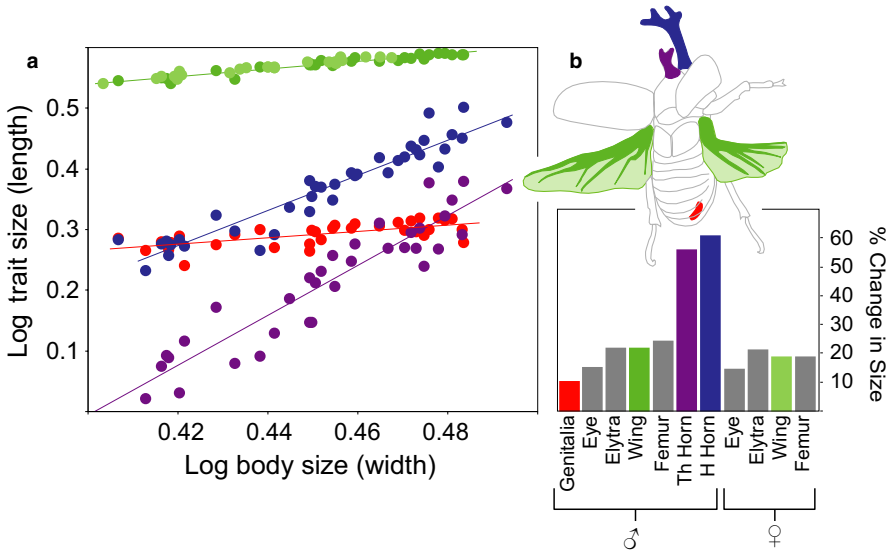

FIGURE 1 Condition-dependent growth and trait variability in the Asian rhinoceros beetle. (a) Static allometric slope for each trait investigated in the present study. Head horns (blue) and thoracic horns (purple) show relatively steeper allometric slopes than wings (green) or genitalia (aedeagus; red), indicating hypervariability of expression as expected for ornaments and weapons of sexual selection. Wings are not sexually dimorphic, and values include both males (dark green) and females (light green). Female genitalia were not sampled, and adult females in this species do not have horns, so for these traits only males are shown. Although they do not grow into measurable outgrowths, same-stage regions of epidermal tissue homologous with male horns were sampled from females to contrast with males in this study. Hypervariability of weapons in this species results from heightened condition-sensitive growth, relative to other body parts. For example, (b) manipulation of larval food availability affects head and thoracic horns (blue, purple) more dramatically than it does other body parts. As in many insects, genitalia (red) scale unusually shallowly with body size and are relatively insensitive to condition and/or larval access to nutrition. Redrawn from Johns et al. (2014)

answer these questions is to explore how these structures develop and to compare their development with that of other, non-sexually selected structures.

In the past two decades, advances in molecular tools, particularly approaches for visualizing domains of gene expression (e.g., in situ hybridization) and perturbing gene expression (e.g., RNA interference "knockdowns") in non-model organisms, have permitted researchers to explore the development of a number of exaggerated sexually selected structures, including the horns of dung and rhinoceros beetles (Emlen, Warren, Johns, Dworkin, & Lavine, 2012; Moczek & Nagy, 2005; Moczek & Rose, 2009; Wasik & Moczek, 2012), and the enlarged mandibles of male stag beetles (Gotoh et al., 2014, 2016, 2017). Collectively, these "candidate gene" studies have implicated a number of "toolkit" developmental genes in the patterning and growth of extreme structures, and established several conserved processes shared between these traits and animal appendages such as wings and legs of *Drosophila*. These studies provide critical and exciting insights into the development of sexually selected traits. In particular, they point to the influence of genes involved with both sex determination and nutrition-mediated growth as being necessary for exaggerated trait growth. However, these studies – like all those focused on candidate genes – are limited and necessarily biased in scope due to their reliance on prior knowledge of gene function from related taxa. Several recent studies have leveraged high-throughput sequencing (e.g., RNAseq) to provide transcriptome-wide screens for differential gene expression associated with extreme trait growth (Gotoh et al., 2016; Kijimoto et al., 2014; Ledón-Rettig & Moczek, 2016; Ledón-Rettig, Zattara, & Moczek, 2017; Ozawa et al., 2016; Pointer, Harrison, Wright, & Mank, 2013; Wilkinson, Johns, Metheny, & Baker, 2013), providing unbiased glimpses into the genetic architecture of sexually selected traits.

In many ways, exaggerated sexually selected structures are ideal for transcriptome-wide, differential expression approaches (Pointer et al., 2013; Stuglik, Babik, Prokop, & Radwan, 2014; Wilkinson et al., 2013, 2015). Not only are these traits often sexually dimorphic, such that similar starting tissues undergo dramatically different amounts of growth in males and females, but these traits also differ from each other in the extent of their nutritional plasticity (Moczek, 2006; Moczek & Nagy, 2005). Many exaggerated sexually selected ornaments and weapons display "heightened" condition-sensitive expression relative to other, surrounding body parts (Bonduriansky, 2007; Bonduriansky & Rowe, 2005; Cotton, Fowler, & Pomiankowski, 2004a; David, Bjorksten, Fowler, & Pomiankowski, 2000; Iwasa & Pomiankowski, 1999; Knell, Fruhauf, & Norris, 1999; Rowe & Houle, 1996; Warren, Gotoh, Dworkin, Emlen, & Lavine, 2013). Heightened conditional expression enhances the reliability of ornaments and weapons as signals, for either mate choice or rival assessment (Bonduriansky, 2007; Bradbury & Vehrencamp, 2011; Cotton et al., 2004a; Iwasa, Pomiankowski, & Nee, 1991; Maynard Smith & Harper, 2003; Pomiankowski, 1987; Searcy & Nowicki, 2005), and in insects, it manifests primarily as an elevated sensitivity to nutritional conditions experienced by larvae during development (Cotton, Fowler, & Pomiankowski, 2004b; David et al., 2000; Emlen et al., 2012; Gotoh et al., 2014; Johns, Gotoh, McCullough, Emlen, & Lavine, 2014; Warren et al., 2013). Well-fed males develop into large

adults with large ornaments or weapons, while poorly fed males mature into smaller adults bearing rudimentary structures (Emlen, 1994; Emlen & Nijhout, 2000; Gotoh et al., 2011; Iguchi, 1998; Karino, Seki, & Chiba, 2004; Moczek and Emlen 1999; Tang, Smith-Caldas, Driscoll, Salhadar, & Shingleton, 2011).

Although heightened condition sensitivity is likely to be a near-universal property exhibited by most exaggerated, sexually selected traits, the mechanistic bases for conditional expression are relatively underexplored (reviewed in Lavine, Gotoh, Brent, Dworkin, & Emlen, 2015; Shingleton & Frankino, 2013; Warren et al., 2013). Yet heightened condition-sensitive expression, like sexual dimorphism, offers an excellent opportunity for leveraging transcriptome-wide approaches like RNAseq, by contrasting gene expression across traits that differ in the magnitude of their developmental plasticity (Kijimoto et al., 2014; Ledón-Rettig & Moczek, 2016; Ozawa et al., 2016; Snell-Rood & Moczek, 2012; Snell-Rood et al., 2011).

We employed RNAseq to examine the development of two sexually selected weapons, head and thorax horns in the Asian rhinoceros beetle *Trypoxylus dichotomus*. Male *T. dichotomus* wield an elaborate "pitchfork"-shaped horn extending from the head, which, in the largest males, can exceed two-thirds the length of the body of the male. Males also have a smaller, curved, forward-projecting thoracic horn. Both horns are sexually dimorphic (Hongo, 2003, 2007; Siva-Jothy, 1987), and both are known to exhibit heightened nutrition-sensitive patterns of growth relative to other structures (Johns et al., 2014; Figure 1a). Female beetles develop small horns during the pupal period, which they later resorb (Ito et al., 2013), enabling us to contrast gene expression in horns of males with comparable "horn" epidermis in females. In addition, we contrasted gene expression in horns sampled from large and small individuals, derived from a nutritional manipulation. For comparison, we sampled developing wings from both males and females, and developing male genitals from these same animals (females in this species lack apparent genital discs, and it was not possible to include female genitalia in this study). Wings exhibit patterns of nutrition-sensitive growth typical of the body as a whole (i.e., close to an isometric static allometry), and male genitalia are relatively insensitive to nutrition (Johns et al., 2014), as is the case for many insects (Eberhard, Huber, Briceno, Salas, & Rodriguez, 1998; Eberhard, Rodriguez, & Polihronakis, 2009; Shingleton & Frankino, 2013; Tang et al., 2011).

In this study, we compare nutrition-sensitive gene expression across traits that span a wide range of nutrition sensitivities (head horns > thorax horns > wings > male genitalia) and degrees of sexual dimorphism (head horns > thorax horns > wings) in order to identify genes and developmental pathways associated with the heightened nutritionally sensitive and sexually dimorphic growth of the exaggerated male weapons. Comparing across tissue types allowed us to determine whether gene expression differences track the degree of nutrition-sensitive and sexually dimorphic growth. We predicted that gene expression differences in horns in response to nutrition would be greater than in wings and that gene expression differences in response to nutrition in wings would be greater than in genitals, following their respective degrees of nutrition sensitivity (Figure 1b). Similarly, we also asked whether patterns of differential gene expression between males and females were more pronounced in traits exhibiting a greater degree of sexual dimorphism (i.e., greater in horns than in wings?). Finally, we compared gene expression profiles resulting from sexual dimorphism and nutrition sensitivity to each other. Combined, our approach provides insight into the developmental and physiological pathways involved with heightened condition-sensitive growth and sexual dimorphism of exaggerated sexually selected animal weapons.

2 | METHODS

2.1 | Animal husbandry

Male and female *T. dichotomus* larvae were purchased from a commercial supplier and maintained ad libitum on a diet of 25% organic leaf mulch and 75% quick-fermented hardwood sawdust in 9-oz glass jars using previously established methods (Johns et al., 2014). After moulting into their third instar, high-nutrition larvae were moved into 1-gallon glass jars and were fed ad libitum, with food being replaced as the frass content reached 40% of the jar. Third-instar low-nutrition animals remained in the 9-oz jars, and food was only replaced when the frass content reached approximately 70% of the jar. This feeding regime consistently produces dramatic size differences between large and small individuals of both sexes (Emlen et al., 2012; Johns et al., 2014). Three days after the larvae had constructed their pupal cell, the four largest and four smallest larvae (by mass) of each sex were euthanized and tissues harvested (filled circles, Supporting Information Figure S1). Selecting the extremes may partially confound size differences caused by larval nutrition with naturally occurring genetic variation in body size. However, phenotypic variation in body size in this species is overwhelmingly determined by larval access to nutrition (Emlen et al., 2012; Iguchi, 1998; Johns et al., 2014; Karino et al., 2004), with only modest effects of date of oviposition (Plaistow, Tsuchida, Tsubaki, & Setsuda, 2005) and egg size (Kojima, 2015), and no detectable heritability even under laboratory conditions (Karino et al., 2004). As our goal with this experiment was to identify the transcriptional response underlying trait exaggeration, we selected individuals with the most dramatic phenotypes in order to detect the most extreme transcriptional response possible.

Head horn, thoracic horn, wing and genital tissues from four of the smallest and four of the largest male larvae were harvested at prepupal day four (the period of maximal horn, wing, and genital growth; Emlen et al., 2012; Johns et al., 2014; Zinna et al., 2016). Epidermal tissues corresponding to head and thoracic "horns" and wings were also sampled from four of the smallest and four of the largest females (Supporting Information Table S1). Tissue was dissected into RNAlater (Thermo-Fisher) and stored in a −80°C freezer until processing.

2.2 | Sequencing and de novo assembly

After removing RNAlater (several washes with RNAse-free PBS), mRNA was extracted and purified with the Ambion MagMax-96 kit (Life Technologies) for all samples. Following QC of RNA on an Agilent Bioanalyzer, 55 samples were sufficiently high quality for sequencing. Illumina sequencing (Hi-seq 2000) was performed using 50-bp paired-end sequencing at the Research Technology Support Facility core facility at Michigan State University. The initial design of four biological (individuals) replicates per condition × two conditions per sex (high and low nutrition), and either three or four tissues depending on sex (head horns, thorax horns, wings and genitalia [males only]), became slightly unbalanced due to a loss of the thoracic horn tissue from one male (Supporting Information Table S1). All samples were barcoded, with seven samples run per lane across all eight lanes. An incomplete randomized blocking design was used across each lane of the flow cell to avoid confounding lane effects with treatment effects of sex, nutritional treatment and tissue type (Supporting Information Table S1).

Sequenced reads were analysed for quality using FASTQC to check sequencing quality and the presence of adaptor contamination or GC bias (Andrews, 2010). Reads were cleaned of sequencing adapters and of low-quality reads using Trimmomatic version 0.27 (Bolger, Lohse, & Usadel, 2014). Reads shorter than 36 bp were removed from the pool for assembly. Four organisms were selected, one from each condition and sex, and a de novo transcriptome was assembled from the trimmed reads for all tissues across all the samples using Trinity v2.0.6 under the default parameters for de novo assembly (Grabherr et al., 2011; Haas et al., 2013). In tests, transcriptomes based on all individuals resulted in a large number of falsely identified transcripts due to polymorphism. The use of four individuals (one from each sex and treatment) maximized our ability to recover transcript diversity while reducing the generation of redundantly identified transcripts that were due to polymorphism. This de novo transcriptome was annotated using BLASTX against the *Drosophila melanogaster* protein database. In addition, we annotated our transcriptome against the *Tribolium castaneum* protein database, as well as a custom database combining unique identifiers from both *D. melanogaster* and *T. castaneum* (Altschul, Gish, Miller, Myers, & Lipman, 1990).

The results of the BLASTX analysis were used to filter our de novo transcriptome down using the program TRANSPS into one of three assemblies (a *Drosophila* reduced transcriptome, a *Tribolium* reduced transcriptome, and a combined *Tribolium* and *Drosophila* transcriptome) that contained only those transcripts that mapped to annotated polypeptides (Liu, Adelman, Myles, & Zhang, 2014). TRANSPS has two major functions of interest. The first is ease of annotating the transcriptome of a non-model species using BLASTX results, but the more critical functionality of TRANSPS is the ability of this program to help reduce many of the complications of transcriptome assembly such as chimeric transcripts, and redundant, partial transcripts (Martin & Wang, 2011; Surget-Groba & Montoya-Burgos, 2010).

2.3 | Assessment of transcriptomes

The four transcriptomes produced (the full transcriptome generated by the initial Trinity assembly and annotation, and the three transcriptomes reduced through the use of TRANSPS) were quantitatively analysed through the use of BUSCO (v3), which identifies single-copy orthologs from OrthoDB that are present in 90% of the species in a given group, using the Insecta OrthoDB as our BUSCO query (Simão, Waterhouse, Ioannidis, Kriventseva, & Zdobnov, 2015; Waterhouse et al., 2017).

2.4 | RNAseq analysis

All methods below were performed on both the initial de novo transcriptome and the reduced transcriptomes described above. The initial transcriptome will be referred to as the full transcriptome, whereas the reduced transcriptomes will be referred to as the *Drosophila*, *Tribolium* or *Combined* transcriptome. SAILFISH version 0.7.6 was used to map reads back to an index built from each annotated transcriptome. SAILFISH reports relative counts per transcript after the kmer projection and deconvolution steps, resulting in non-integer counts (Patro, Mount, & Kingsford, 2014), as such custom scripts in R version 3.2.3 were used to round count values to the nearest integer. Differential expression of transcripts was initially quantified using DESEQ2 v 1.10.1 in R version 3.2.3 using a multifactor design that incorporated tissue type (head horn, thoracic horn, wing and genital tissue), a categorization of body size (large vs. small), and sex (male or female) using the group function as presented in the DESEQ2 vignette (Love, Huber, & Anders, 2014, R Core Team 2013).

Despite using an incomplete block design (Supporting Information Table S1), we still examined the data for any evidence of lane effects both visually (using principal components analysis and cluster analysis) and more formally with a generalized linear model. Visually, we observed no evidence for substantial lane effects. The model fit was (~ lane + tissue + size + tissue:size). While we observed no evidence for lane effects from either visual examination of the data or statistical checks, our initial model also included sequencing lane as a factor, in order to control for potential subtle lane effects on differential expression during sequencing (Auer & Doerge, 2010). Differential expression analysis found only two genes across all samples that were differentially expressed when lane was included as a factor in models. As only 0.0046% of the total genes analysed showed differential expression due to lane effects, we excluded lane as a factor from further analyses of the data. Our final model utilized the ability of DESEQ2 to model multiple interaction terms simultaneously through the use of a grouping variable containing factors of interest, which for our experiment were the "size," "tissue" and "sex" factors (Love et al., 2014).

After fitting the model to our data, we assessed differential expression through the use of the *contrast* argument in DESEQ2, allowing comparisons of differential expression between specified reference and experimental levels. We investigated differential expression in response to nutrition by contrasting gene expression of large male tissue

(head horns, thoracic horns, wings and genitals) to their corresponding tissue in small males. We also contrasted male tissues (head horns, thoracic horns and wings) with their matching tissue in females, to analyse patterns of differential expression due to sex. Finally, because the effects of nutrition are expected to increase across traits in males (genitalia < wings < thorax horns < head horns) but not in females (e.g., the comparable "horn" tissues of females do not grow, so they should exhibit no nutrition-sensitive effects on growth; i.e., wings > thorax horns = head horns), we tested for an interaction between nutrition and sex for the three tissues for which we had both male and female samples: wings, thorax horns and head horns. Then, for head horns, we isolated the subset of these genes that displayed nutrition-sensitive DE in male but not in female horns; that is, they exhibited a sex-by-nutrition interaction in the predicted direction for putative sex-specific regulators of heightened condition sensitivity of weapon growth.

We considered transcripts with a Benjamini and Hochberg (BH) false discovery rate <0.05 as differentially expressed (Benjamini & Hochberg, 1995). It is important to note that DESEQ2 shrinks estimates of gene expression changes (by default) as a function of count abundance. This shrinkage reduces the potential for genes to have magnified expression values as a result of low counts, due to increased sampling variance for low abundance transcripts (Love et al., 2014). In addition, DESEQ2 automatically performs independent filtering of reads with low expression during the calculation of contrast results, reducing the number of transcripts examined (Love et al., 2014). Differential expression was reported as the log2 fold change in gene expression.

2.5 | Comparing directions and magnitudes for subsets of differentially expressed genes

We sought to compare whether the different sets of differentially expressed transcripts in any particular contrast were also expressed similarly *across* contrasts; for example, whether the set of genes differentially expressed in male horn tissue in response to nutrition was also similarly differentially expressed in contrasts between male and female horn tissues. To compare the extent to which gene expression profiles were showing similar patterns across different treatment contrasts, we estimated vector correlations, which are widely used in studies of multivariate evolution and geometric morphometrics (Zelditch, Swiderski, & Sheets, 2012) yet remain less commonly used in genomics (but see Kuruvilla, Park, & Schreiber, 2002). Mean centred vectors ($\boldsymbol{a}$, $\boldsymbol{b}$) containing the magnitudes of change in expression value can be compared by:

$$r_{vc} = \frac{|\boldsymbol{a} \cdot \boldsymbol{b}|}{||\boldsymbol{a}|| \times ||\boldsymbol{b}||},$$

where $\boldsymbol{a} \cdot \boldsymbol{b}$ is the dot product between the two vectors of gene expression and $||\boldsymbol{a}||$ is the magnitude (L2 norm, sometimes called length) of the vector (the square root of the sum of squared values of the vector). Vectors $\boldsymbol{a}$ and $\boldsymbol{b}$ represent the (log transformed) estimated model contrasts for relevant comparisons. If the direction of effects for changes in gene expression is similar across a particular comparison of treatments, the vector correlation will be close to 1. If the direction of effects for the subset of genes is completely different, then the vector correlation will be close to zero. Indeed, for the simple cases examined here (for comparing mean centred vectors), this is equivalent to the Pearson correlation coefficient; that is, the dot product of mean centred vectors $\boldsymbol{a}$ and $\boldsymbol{b}$ is equivalent to the covariance (sum of the cross-products), scaled by the magnitudes (each equivalent to the square root of the sum of squared deviates). In addition, we also estimated a value, α (Kuruvilla et al., 2002), for each vector comparison, defined as the L2 norm (magnitude) of centred vector $\boldsymbol{a}$ divided by the L2 norm of centred vector $\boldsymbol{b}$. α estimates the relative change in expression between the two vectors compared. When comparing vector magnitudes using this measure, an $\alpha = 1$ indicates that both vectors compared have the same magnitude, while $\alpha > 1$ indicates vector $\boldsymbol{a}$ has greater magnitude than $\boldsymbol{b}$; that is, on average, the amount of differential expression of genes in $\boldsymbol{a}$ *is* larger than that in $\boldsymbol{b}$. We modified scripts to perform both analyses from work done by Pitchers, Pool, and Dworkin (2013).

We defined experimental vectors as the log2 fold changes for each experimental pairwise comparison, across all genes that were significantly differentially expressed in response to sexual dimorphism (the "sexually dimorphic gene set," or *SD*) and then computed the vector correlation to other tissue or treatment contrasts. We also performed this analysis for the genes significantly differentially expressed in response to body size (the "nutritionally responsive gene set," or *NR*). To assess these correlations between experimental vectors within the context of all transcripts examined, the correlation coefficient (r) obtained in each comparison was compared to an empirical distribution for all the transcripts. This distribution of r values was obtained by performing an empirical resampling for the correlations between our predicted vectors and 10,000 vectors generated by randomly sampling the full set of corresponding expression contrasts to generate vectors of the same length (i.e., with the same number of genes included). We chose to use a particularly conservative, but informative approach to this resampling. While a permutation approach would provide a distribution of r under a null (no correlation) model, we instead sampled (based on the number of differentially expressed genes in the subset of interest) among all transcripts with estimated differences. This included both the subset of transcripts that were inferred to be differentially expressed as well as the remaining transcripts. As such, the distribution provides an overall estimate (independent of which genes are deemed "significant") of how similar the gene expression differences are between two sets of contrasts. Correlation coefficients of experimental comparisons that were equal to or higher than the correlation coefficients computed for the 95th percentile of random vectors were considered extreme, relative to the distribution as a whole. Thus, this provides an estimate of the extent to which the transcripts inferred to be differentially expressed are more correlated than expected compared to random subset from the full set of genes. These steps were also performed for our estimate, α, of the magnitude of differences between each vector.

TABLE 1 Assembly statistics

	# Contigs	N50	Median contig length	Average contig length	%BUSCOs present (out of 1,658 BUSCO groups searched)
Full transcriptome	48,831	2,542	1,085	1,624	96.94
Drosophila transcriptome	4,375	3,170	2,016	2,485.92	55.97
Tribolium transcriptome	5,076	3,126	2,017	2,474.68	57.65
Combined transcriptome	5,693	3,054	1,882	2,340	57.96

Note. The number of contigs in each transcriptome, along with the N50 values for each assembly, the median and average contig lengths, and the number of CEGMA genes present in each assembly (Parra, Bradnam, & Korf, 2007). There was a significant reduction in contigs after TRANSPS annotation and scaffolding; however, the N50, median length and average length of contigs all increased substantially. It is interesting to note the substantially lower % BUSCO matches from the TRANSPS set of transcripts.

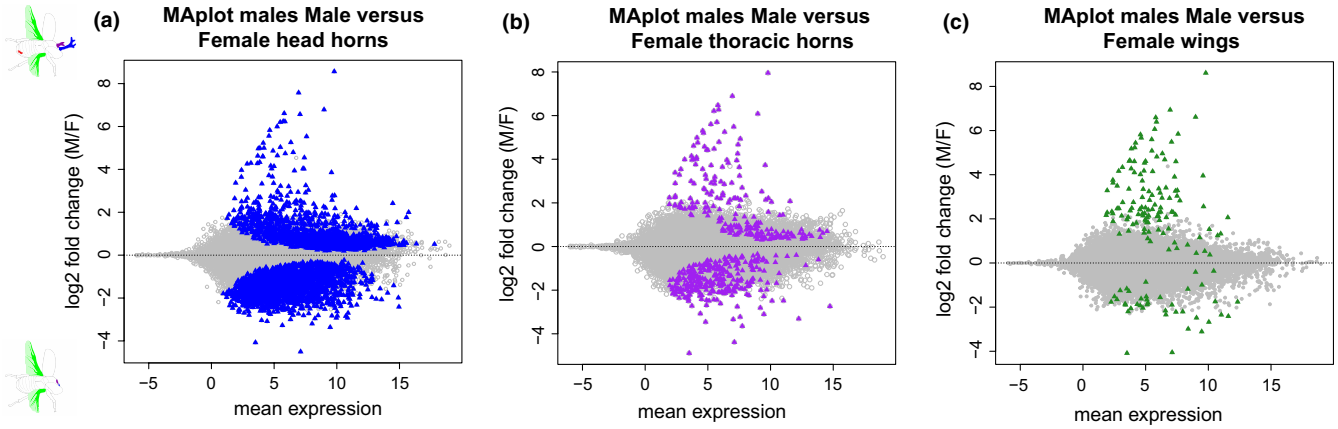

FIGURE 2 Transcripts differentially expressed based on sex. Tissues that are highly sexually dimorphic, such as head and thoracic horns, show higher levels of differential expression than wings, a tissue that is not sexually dimorphic. The *y*-axis is log2 fold change and the *x*-axis is the log2 of mean expression in the current comparison. Open circles represent genes, while filled triangles represent genes significantly differentially expressed at an adjusted *p*-value of <0.05. Hundreds of genes were differentially expressed in head horns as a consequence of sex (a). Fewer genes were DE in thoracic horns between males and females (b), and still fewer transcripts were differentially expressed in wing tissue between the sexes (c). This suggests that sex has a strong effect on differential expression in sexually dimorphic traits and that the effect is more dramatic in the most exaggerated/dimorphic trait. Note that these values represent shrunken log2 fold change estimates, as described in the text

3 | RESULTS

3.1 | De novo transcriptome assembly

After assembly, but before compression of redundant contigs into single contigs using TRANSPS, our de novo transcriptome contained 48,831 contigs. After functional annotation against polypeptides, scaffolding and removal of redundant contigs using TRANSPS, the three reduced transcriptomes (*Drosophila*, *Tribolium* and the *Combined* transcriptome) all had fewer contigs, as expected, as well as increased contig N50, median length and average length. The assembly statistics can be found in Table 1. However, despite improvements in transcript length and the removal of redundancy, our reduced transcriptomes comprised only 8%–11% of the initial assembled transcriptome. After searching each transcriptome against the Insecta BUSCO groups (comprising 1,658 groups specific to Insecta), the initial, unreduced transcriptome (the full transcriptome) retained approximately 97% of all Insecta-specific BUSCOs, while all three reduced transcriptomes saw a reduction of approximately 40% of BUSCO orthologs present (Table 1). Thus, despite the increases in transcript length and removal of redundancy due to TRANSPS, we present results from the full transcriptome, annotated against *Drosophila* polypeptides but not reduced with TRANSPS. In this full transcriptome, 36,709 transcripts were annotated using BLASTX, representing roughly 75% of sequences. Patterns of differential expression were similar across all four transcriptomes, and differential expression patterns for the three reduced transcriptomes can be found in the Supporting Information.

3.2 | Sexually dimorphic growth requires large-scale differential gene expression

When comparing differential expression patterns between corresponding male and female tissues, we observed patterns of expression generally consistent with the level of sexually dimorphic growth exhibited by each tissue type (Figure 2). Head horns, a highly sexually dimorphic structure, exhibited the highest level of differential expression, with 4,031 transcripts significantly differentially

TABLE 2 Differential Expression in each tissue comparison

Contrast	#Upregulated	#Downregulated	Total	% DE (out of 48,831 genes)	% filtered	Absolute magnitude (absolute value of the log2 fold change)					
						Minimum	1st quartile	Median	Mean	3rd quartile	Max
Large male vs. small male head horns	143	44	187	0.38	1.2	0.372	0.6313	0.8055	1.126	1.272	4.906
Large male vs. small male thoracic horns	40	1	41	0.08	1.2	0.4216	0.6058	0.7884	0.8226	0.9141	2.325
Large male vs. small male wings	8	5	13	0.03	0	0.544	1.208	2.542	2.297	3.126	4.463
Large male vs. small male genitals	19	6	25	0.05	1	0.4818	2.652	3.162	3.078	3.521	5.156
Male vs. female head horns	1,729	2,302	4,031	8.26	1.6	0.1968	0.6863	1.131	1.234	1.651	8.566
Male vs. female thoracic horns	220	305	525	1.08	5.3	0.3028	0.844	1.528	1.689	2.065	7.954
Male vs. female wings	118	39	157	0.32	1.6	0.3646	1.809	2.315	2.744	3.409	8.61
Head horn, sex × nutrition interaction	142	106	248	0.51	5.7	0.00003	0.2342	0.548	1.077	1.182	30
Thoracic horn, sex × nutrition interaction	242	126	368	0.75	13	0.00001	0.2784	0.657	1.243	1.381	30
Wings, sex × nutrition interaction	106	89	195	0.40	11	0	0.2265	0.5599	1.109	1.209	30
Male vs. female head horns, high nutrition only	808	670	1,478	3.03	9.1	0.2872	0.8019	1.408	1.632	2.243	8.725
Male vs. female thoracic horns, high nutrition only	284	478	762	1.56	5.3	0.4133	1.191	2.095	2.08	2.629	8.551
Male vs. female wings, high nutrition only	83	35	118	0.24	0	0.4091	2.348	3.463	3.53	4.363	8.497

Note. The number of genes significantly differentially expressed across each comparison. Each contrast indicates which tissues were compared (e.g., gene expression in large male horns compared to small male horns). In addition to the number of upregulated and downregulated genes in each comparison, the absolute magnitude of expression (log2 fold change) for all significantly expressed genes is also presented as well as the percentage of genes removed by DESEQ2's pre-filtering step.

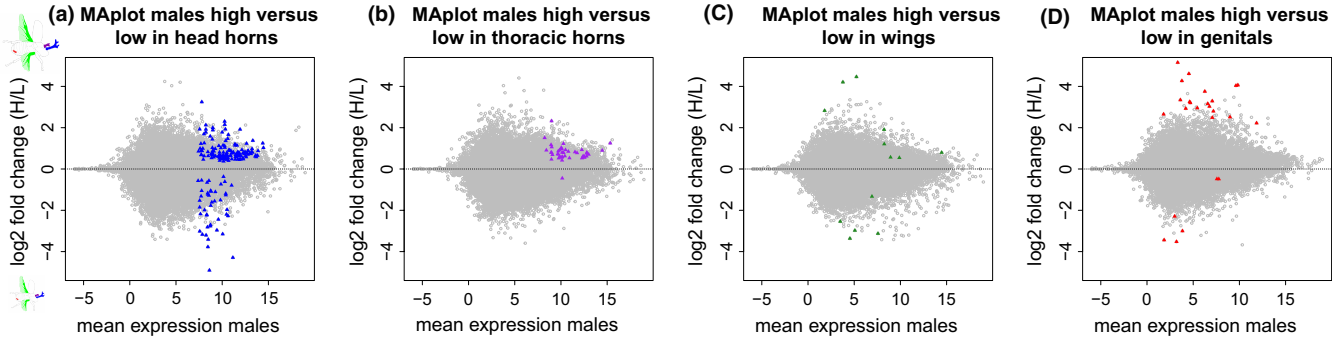

FIGURE 3 Differential expression across nutritional state in male tissues. Overall, fewer transcripts were differentially expressed in response to nutrition than were in response to sex. However, across tissues, patterns of differential expression followed expected patterns, with male head horns (a) and thoracic horns (b) showing higher levels of differential expression in response to larval food availability than wings (c) and genitals (d). In each panel, the *y*-axis is log2 fold change and the *x*-axis is the log2 of mean expression. Open circles represent genes, while filled triangles represent genes significantly differentially expressed at an adjusted *p*-value of <0.05. Note that as in Figure 2, these values represent shrunken log2 fold change estimates, as described in the text

expressed, representing 8.3% of the set of transcripts analysed (Figure 2a, Table 2). Thoracic horns, a less exaggerated but still sexually dimorphic structure, showed significant differential expression in 525 transcripts or 1.08% of the full transcriptome (Figure 2b, Table 2). Finally, wings, which are not sexually dimorphic in adults, only show differential expression in 157 transcripts, a scant 0.3% of all transcripts analysed (Figure 2c, Table 2). Fisher's exact test comparing the number of DE genes to genes not DE between the three tissues indicated these counts were statistically different from one another ($p < 0.05$), and post hoc analyses indicated that the number of genes DE between every tissue type (i.e., the number of genes DE between thoracic horns and wings) was significantly different. Perhaps more interestingly, while thoracic horns show evidence of differential expression of 525 transcripts (between males and females), approximately 40% of these genes (222) were also differentially expressed in head horns (Figure 4a). This pattern held across all of our reduced transcriptomes as well (Supporting Information Figures S2, S6 and S10, *Drosophila*, *Tribolium*, *Combined* transcriptomes, respectively. Also Supporting Information Figures S4A, S8A and S12A).

3.3 | Heightened condition-dependent growth shows a more modest pattern of differential expression in comparison with sexual dimorphism

Despite the extreme morphological differences in horn size displayed between adult males in response to differences in nutrition condition (Figure 1), surprisingly few transcripts were inferred to be differentially expressed in either head or thoracic horn tissue between large and small males (Figure 3a,b). This is not a consequence of a greater degree of difference in horn length between males and females compared with high- and low-nutrition males. The smallest males have only rudimentary horns, barely larger than having no horns (i.e., females). Thus, for horn tissues, both sexual dimorphism and high- vs. low-nutrition contrasts result in substantial differences in final adult trait size. Yet the distinction between no horn and large horn (sexual dimorphism) resulted in many more differentially expressed transcripts (8.26%) than short horn and large horn (nutrition; 0.38%).

There were differences in sample size between the nutritionally sensitive and sexually dimorphic contrasts which could have contributed to this overall pattern, as we compared expression from four tissues per group when investigating nutrition (four horns from large males, four from small males) but compared expression from eight horns per group when analysing patterns of sexually dimorphic expression (all horns from large and small males, vs. all comparable "horn" tissues from large and small females). However, even when we analysed sexually dimorphic contrasts using a reduced sample (including only the four large males and four large females, which is a comparable sample size to the nutritionally sensitive contrast), we still observed many more differentially expressed transcripts associated with sexual dimorphism than with nutrition-sensitive growth (1,478 transcripts were differentially expressed when comparing large male head horns to large female head "horns," compared with 187 differentially expressed between large male head horns and small male head horns; for thoracic horns, the corresponding numbers were 762 (sex) and 41 (nutrition); for wings, 118 (sex) and 13 (nutrition) (Table 2).

Although the number of genes that were deemed to be differentially expressed was lower than expected in males across nutritional contrasts, heightened condition-dependent growth of the male weapons was still associated with increased levels of differential expression compared with the other organs examined. More transcripts were differentially expressed in response to nutrition in both horn types than in either wings or genital tissue. Predictably, head horn tissue displayed the most differential expression in transcripts in response to nutrition in males, with 187 transcripts differentially expressed (Figure 3a, Table 2). Thoracic horns only differentially express 41 transcripts in response to nutrition (Figure 3b, Table 2), and wings only differentially express 13 transcripts between large and small males (Figure 3c, Table 2). Male genital tissue, which is characteristically insensitive to larval nutrition, surprisingly showed evidence for differential expression of 25 transcripts (Figure 3d, Table 2).

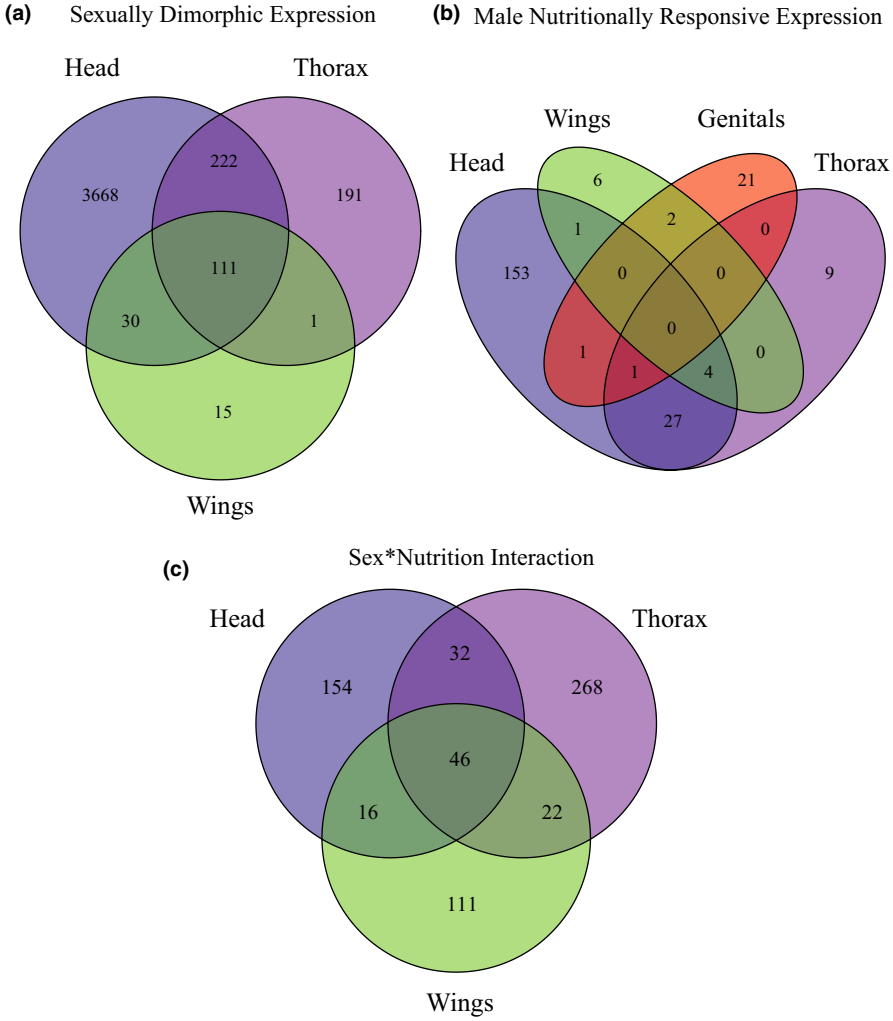

FIGURE 4 Venn diagrams showing the number of genes uniquely and co-expressed in multiple contrasts. Each diagram contains the number of genes uniquely expressed among each tissue type, in response to sexual dimorphism between males and females (a), in response to nutrition condition in males (b), or as a result of the sex-by-tissue interaction (c)

Fisher's exact tests indicated that counts of DE genes from all four tissues were significantly different ($p < 0.05$), yet unlike our analysis for sexual dimorphism, this difference was only present between head horns and other tissues, and between thoracic horns and wings. Thoracic horn DE counts were not significantly different from genital counts ($p = 0.074$), and wing and genital counts were similarly not different ($p = 0.064$). These results were consistent across all four transcriptomes, and results for the reduced transcriptomes can be found in the Supporting Information Figures (S3, S7 and S11, *Drosophila*, *Tribolium*, *Combined* transcriptomes, respectively).

As with sexual dimorphism, transcripts differentially expressed in response to nutrition were similar between male thoracic and head horns, with 27 of the 41 thoracic horn transcripts also differentially expressed in head horns (Figure 4b; also Supporting Information Figures S4B, S8B, and S12B for *Drosophila*, *Tribolium*, and *Combined* transcriptomes, respectively).

3.4 | Interactions between sex and nutrition

Because we are focusing on exaggerated weapons of sexual selection, our predictions of heightened nutrition sensitivity are sex-specific. Wings, which are not a sexually dimorphic structure, display comparable and modest nutrition sensitivity in both sexes, but growth of the weapons differs strikingly between males and females. Specifically, both head and thorax horn tissues are exquisitely nutrition-sensitive in males, but not in females. The comparable "horn" tissue in females does not grow into a discernible adult structure and is not expected to display heightened condition sensitivity. We therefore combined our samples for each tissue (four low-nutrition male, four high-nutrition male, four low-nutrition female and four high-nutrition female) and tested for an interaction between sex and nutrition. We identified 248 transcripts displaying sex-specific nutrition sensitivity (DE) in head horns, 368 in thoracic horns and 195 in wings (Table 2). Fisher's exact tests were statistically significant ($p < 0.05$), as were all post hoc comparisons between tissues. Interestingly, there was much less overlap in DE genes between tissue types when considering the interaction term (Figure 4c; also Supporting Information Figures S4C, S8C, and S12C for *Drosophila*, *Tribolium* and *Combined* transcriptomes, respectively).

When we looked specifically at head horns, our most sexually dimorphic and exquisitely condition-sensitive structure, and we singled out the subset of these transcripts that were significantly DE in male but not female horn tissues (i.e., for which the direction of the interaction made sense given our a priori predictions from tissue

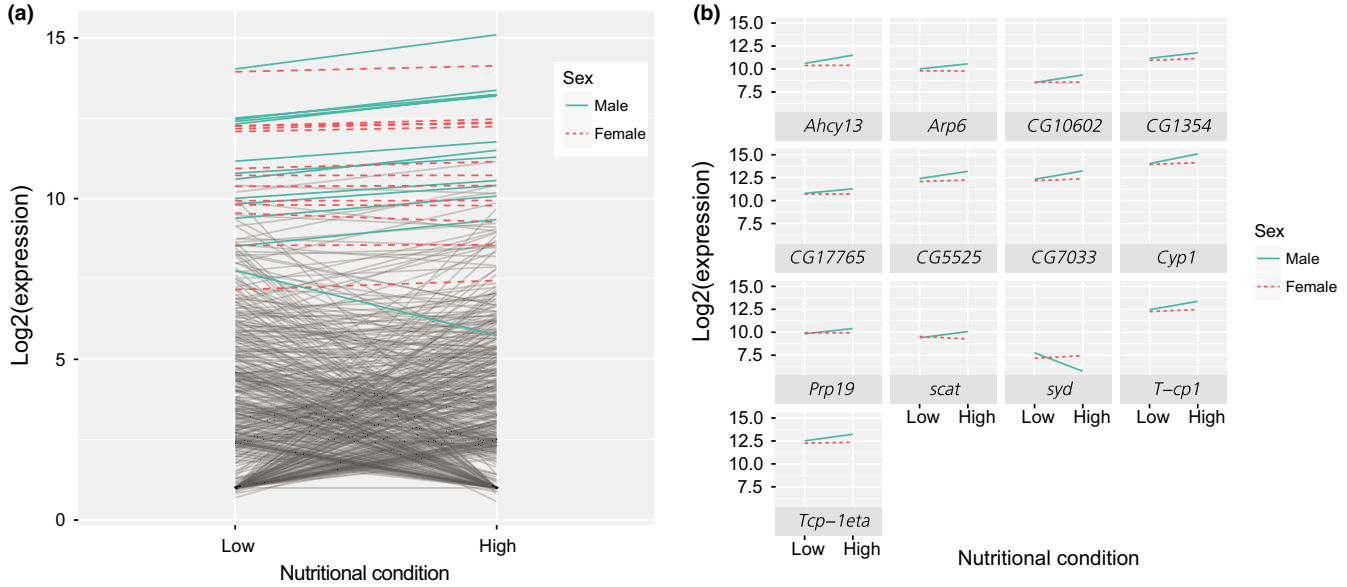

FIGURE 5 Gene expression reaction norms for genes displaying male-biased nutrition-sensitive differential expression in head horns. In this graph, the *x*-axis represents nutritional condition (low or high), and the *y*-axis plots the absolute value of the log2 of total counts per gene, with the addition of a half-count pseudo-count to normalize for genes with zero counts. In (a), black solid lines represent the background of all genes significantly DE as a result of the sex × nutrition interaction in head horns. For each gene, there are two lines—one representing the average expression in males and another representing the average expression in females. Coloured lines indicate genes significantly DE in male but not female horn tissues (i.e., the subset for which the direction of the interaction fits with our a priori predictions for the nutrition sensitivity of this tissue). Blue solid lines track the average expression of these genes in male tissue, whereas dashed red lines track the expression of these genes in female tissues. For clarity, the reaction norms of these same 13 genes are shown separately in (b). In all but one, expression increased in high-nutrition males but did not respond to nutrition in females. Gene names are indicated below each panel

nutrition sensitivity), we identified 13 genes. Interestingly, all 13 had magnitudes of expression that were high compared with the larger subset of genes DE as a result of the sex × nutrition interaction (Figure 5a). In 12 of these genes, the reaction norm of expression increased as a result of nutrition condition in males yet remained flat in females (Figure 5b). However, one gene, *Sunday driver (syd)*, actually showed a decrease in expression in male horns as a result of increasing nutritional condition.

3.5 | Changes in direction and magnitude of gene expression contrasts

While most of our comparisons had comparable statistical power (i.e., the same number of samples per contrast), the overall power of our study was low, and it is well known that the number of genes that are deemed to be differentially expressed is in part a function of the sample size (Yu, Fernandez, & Brock, 2017). Sample sizes for many genomic studies can be limiting, in particular for organisms such as these beetles where sampling and rearing can be difficult and highly space limited. We might expect that there is more evidence of shared response across tissues or "treatment" (sex vs. nutrition) by choosing a different false-positive rate. However, we utilized an alternative approach that enables the use of shared information across a subset of transcripts to compare overall direction and magnitude of vectors of effects from pairs of contrasts. To do this, we compared the overall expression profiles of two sets of transcripts. The first set contained 225 genes representing all unique genes differentially expressed across all tissues in response to nutrition (dubbed the *NR* set). The second set contained all of the 4,238 genes uniquely differentially expressed across all tissues between males and females (the *SD* set). In each set, we included genes DE in any tissue due to either nutrition or dimorphism, but in instances where genes were DE in more than one tissue, each gene was only included once. To provide overall biological context to these comparisons, we generated empirically derived distributions from random sets of genes from the relevant contrasts, which provides estimates of how similar the gene expression differences were overall (irrespective of statistical significance for individual transcripts) across pairs of contrasts. It is worth reiterating that the "*p* values" generated from the empirical resampling procedure done for this analysis do not represent a comparison from null expectations, but instead represent the degree to which these subsets of transcripts were extreme relative to random samples of all transcripts irrespective of significance.

We observed a very high degree of similarity in direction of differential transcription between sexual dimorphism and the nutritional manipulation. Within developing male head horns, changes in transcript expression associated with sexual dimorphism (large male head horns vs. female head horns) were correlated with changes due to nutrition manipulation (large male head horns vs. small male head horns), whether considering the *NR* subset ($r = 0.53$, $p < 0.05$;

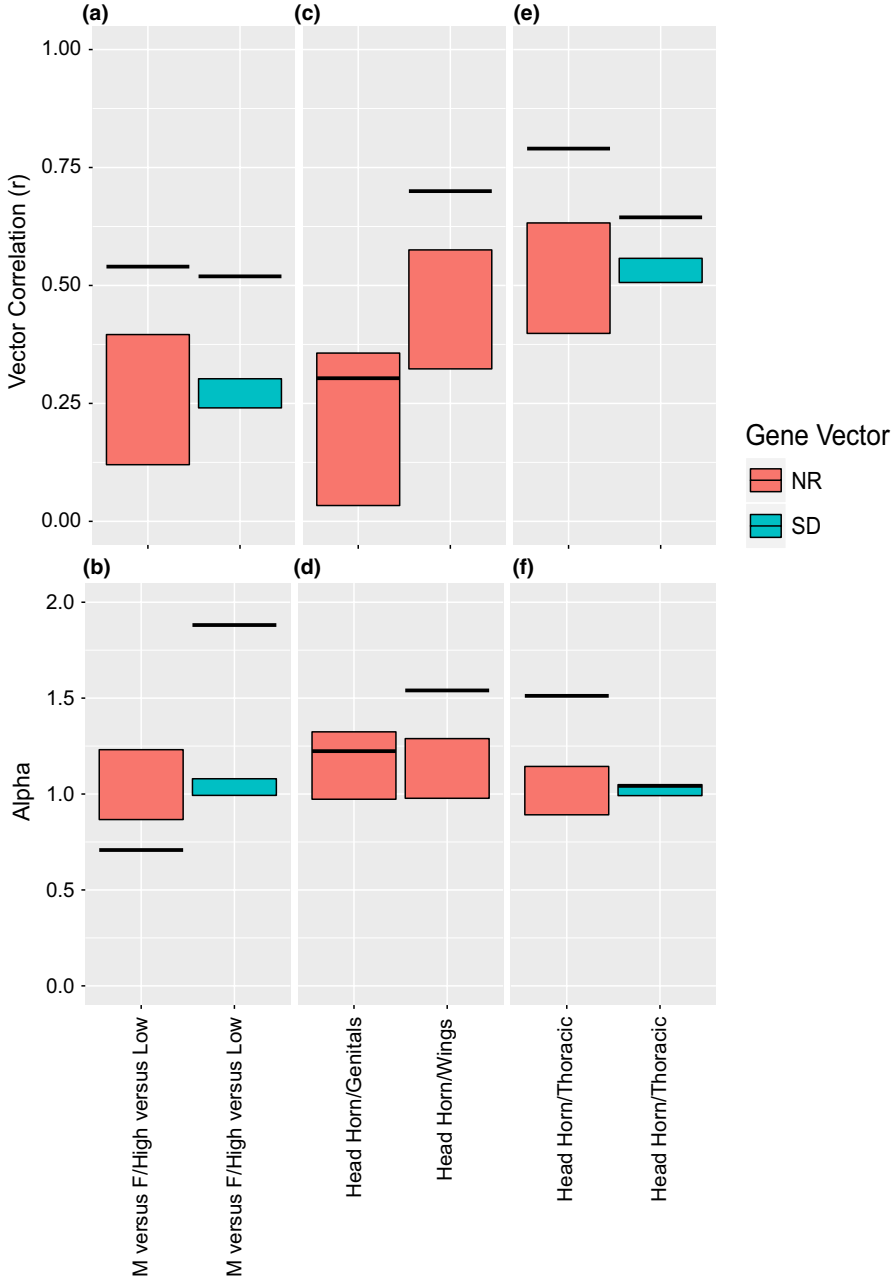

FIGURE 6 Vector correlation (*r*) and magnitude (*alpha*) between expression vectors. We tested for overall similarity in patterns of gene expression between mechanisms of sexual dimorphism and heightened condition-sensitive growth in head horns (a, b); between heightened nutrition-sensitive growth of head horns and more typical nutrition-sensitive plasticity of wings, as well as nutrition-insensitive growth of genitalia (c, d); and we compared the mechanisms associated with sexual dimorphism and heightened nutrition-sensitive growth of head and thoracic horns (e, f). For each contrast, the average direction of differential expression (vector correlation, *r*) and the average magnitude of differential expression (*alpha*) (black bars) were compared to 95 percentiles from empirical distributions generated from random resampling of our expression data (coloured boxes). The average direction (a) of differential expression associated with sexually dimorphic weapon growth (male vs. female head horn) was more similar to that of heightened nutrition-sensitive growth (high- vs. low-nutrition male horns) than expected by chance, and this was evident using the differentially expressed gene set from male vs. female head horns (*SD*, blue) and the differentially expressed gene set from high- vs. low-nutrition male horns (nutrition-responsive [*NR*], red). On the other hand, the magnitude of expression for these gene sets (b) was more dynamic, with the *NR* set having greater magnitude in nutritional contrasts and the *SD* set having greater magnitude in sexually dimorphic contrasts. Heightened nutrition-sensitive growth of horns was also more similar in both direction (c) and magnitude (d) than expected by chance when compared to nutrition-sensitive growth of wings, but neither the magnitudes nor direction of expression was similar between horns and genitalia. Finally, both the direction and magnitude of gene expression were highly correlated between head and thorax horns, for both the sexual dimorphism and nutrition-sensitive gene sets (e, f)

TABLE 3 Candidate genes with evidence of differential expression in *Trypoxylus dichotomus* head horns

FlyBase polypeptide identifier	Gene symbol	Mean expression	Log2 fold change	lfcSE	Adjusted *p*-value	Pathway	Comparison
FBpp0305137	*14-3-3zeta*	*6,179.2628*	*0.5800*	*0.1672*	*0.0417*	*Fat/Hippo*	*Large vs. Small male head horns*
FBpp0070041	*CkIIalpha*	*3,332.8707*	*0.4711*	*0.1307*	*0.0294*	*Hedgehog signalling*	*Large vs. Small male head horns*
FBpp0311456	*Thor*	*323.2838*	*−2.2181*	*0.4986*	*0.0028*	*Insulin signalling*	*Large vs. Small male head horns*
FBpp0077713	Aristaless	83.5346	−1.8794	0.5154	0.0080	Dorsal/Ventral patterning	Male vs. Female head horns
FBpp0305137	CG17870 gene product from transcript CG17870-RE	550.6857	0.6674	0.1800	0.0068	Fat/Hippo	Male vs. Female head horns
FBpp0089338	CG17870 gene product from transcript CG17870-RE	57.9032	−1.6012	0.4985	0.0236	Fat/Hippo	Male vs. Female head horns
FBpp0305137	CG17870 gene product from transcript CG17870-RE	6,179.2628	0.7111	0.1183	0.0000	Fat/Hippo	Male vs. Female head horns
FBpp0291652	Lowfat	3,752.8975	1.4151	0.4165	0.0150	Fat/Hippo	Male vs. Female head horns
FBpp0302878	Expanded	11.7482	−1.3450	0.3912	0.0135	Fat/Hippo	Male vs. Female head horns
FBpp0304253	Hippo	70.9004	1.3238	0.4344	0.0340	Fat/Hippo	Male vs. Female head horns
FBpp0292924	Dachs	285.7612	2.3866	0.4163	0.0000	Fat/Hippo	Male vs. Female head horns
FBpp0288697	Yorkie	596.1161	0.4953	0.1636	0.0356	Fat/Hippo	Male vs. Female head horns

(Continues)

TABLE 3 (Continued)

FlyBase polypeptide identifier	Gene symbol	Mean expression	Log2 fold change	lfcSE	Adjusted *p*-value	Pathway	Comparison
FBpp0288695	Yorkie	278.8974	0.4967	0.1332	0.0064	Fat/Hippo	Male vs. Female head horns
FBpp0083799	Ras association family member	81.3029	−0.7130	0.1816	0.0037	Fat/Hippo	Male vs. Female head horns
FBpp0083799	Ras association family member	909.5832	−0.8049	0.2571	0.0282	Fat/Hippo	Male vs. Female head horns
FBpp0083799	Ras association family member	635.2126	−0.8083	0.2053	0.0036	Fat/Hippo	Male vs. Female head horns
FBpp0292925	Dachs	20.3429	−1.2213	0.3142	0.0041	Fat/Hippo	Male vs. Female head horns
FBpp0085933	Four-jointed	843.7581	1.6796	0.3679	0.0005	Fat/Hippo	Male vs. Female head horns
FBpp0083687	Regulatory particle non-ATPase 7	62.1390	−1.9271	0.3853	0.0001	Hedgehog signalling	Male vs. Female head horns
FBpp0306837	Ribosomal protein L40	21,748.2549	0.6418	0.2113	0.0348	Hedgehog signalling	Male vs. Female head horns
FBpp0079606	Ribosomal protein S27A	41,672.7950	0.4638	0.1538	0.0365	Hedgehog signalling	Male vs. Female head horns
FBpp0079606	Ribosomal protein S27A	268.5435	0.7763	0.1860	0.0018	Hedgehog signalling	Male vs. Female head horns
FBpp0099945	Hedgehog	293.1701	−2.2229	0.4742	0.0003	Hedgehog signalling	Male vs. Female head horns
FBpp0078449	Proteasome beta7 subunit	2,092.4437	0.3780	0.1261	0.0379	Hedgehog signalling	Male vs. Female head horns

(Continues)

TABLE 3 (Continued)

FlyBase polypeptide identifier	Gene symbol	Mean expression	Log2 fold change	lfcSE	Adjusted *p*-value	Pathway	Comparison
FBpp0300330	Casein kinase II beta subunit	1,207.5395	0.5231	0.1147	0.0005	Hedgehog signalling	Male vs. Female head horns
FBpp0078278	Regulatory particle non-ATPase 5	2,220.8125	0.4415	0.1155	0.0050	Hedgehog signalling	Male vs. Female head horns
FBpp0078314	Dispatched	106.1610	−1.0104	0.2590	0.0039	Hedgehog signalling	Male vs. Female head horns
FBpp0078314	Dispatched	1,514.3873	−0.7458	0.1869	0.0031	Hedgehog signalling	Male vs. Female head horns
FBpp0300417	Cubitus interruptus	24.4956	−1.2575	0.3889	0.0225	Hedgehog signalling	Male vs. Female head horns
FBpp0070450	Shaggy	1,661.3376	0.5289	0.1450	0.0080	Hedgehog signalling	Male vs. Female head horns
FBpp0070041	Casein kinase IIalpha	3,332.8707	0.2853	0.0924	0.0313	Hedgehog signalling	Male vs. Female head horns
FBpp0290847	Roadkill	80.3851	−1.9112	0.5248	0.0081	Hedgehog signalling	Male vs. Female head horns
FBpp0308836	Protein phosphatase 2A at 29B	2,556.6931	0.4197	0.1348	0.0294	Hedgehog signalling	Male vs. Female head horns
FBpp0088443	Patched	265.6682	0.7602	0.1878	0.0026	Hedgehog signalling	Male vs. Female head horns
FBpp0310679	Kinase suppressor of ras	194.2759	−0.9139	0.2486	0.0074	Hedgehog signalling	Male vs. Female head horns
FBpp0111741	Shaggy	14.7655	1.4895	0.5017	0.0402	Hedgehog signalling	Male vs. Female head horns

(Continues)

TABLE 3 (Continued)

FlyBase polypeptide identifier	Gene symbol	Mean expression	Log2 fold change	lfcSE	Adjusted *p*-value	Pathway	Comparison
FBpp0087198	Engrailed	376.0268	−1.9463	0.4648	0.0017	Hedgehog signalling	Male vs. Female head horns
FBpp0076216	Eukaryotic initiation factor 4E	3,332.7406	0.3542	0.1222	0.0464	Insulin signalling	Male vs. Female head horns
FBpp0303635	CG2699 gene product from transcript CG2699-RE	462.0380	−0.3643	0.1125	0.0223	Insulin signalling	Male vs. Female head horns
FBpp0306705	CG4141 gene product from transcript CG4141-RB	189.3324	0.8117	0.2776	0.0441	Insulin signalling	Male vs. Female head horns
FBpp0302969	Forkhead box P	50.9025	−1.7854	0.3586	0.0001	Insulin signalling	Male vs. Female head horns
FBpp0292595	Broad	262.1435	−1.3043	0.4219	0.0309	Juvenile hormone signalling	Male vs. Female head horns
FBpp0304572	Broad	8.4403	−1.8039	0.5368	0.0165	Juvenile hormone signalling	Male vs. Female head horns
FBpp0311868	Juvenile hormone epoxide hydrolase 2	4,327.6938	1.0403	0.3223	0.0228	Juvenile hormone signalling	Male vs. Female head horns
FBpp0084962	Janus A	1,697.0227	0.5155	0.1400	0.0072	Sex-determination pathway	Male vs. Female head horns
FBpp0070716	Sans fille	629.1117	0.5193	0.1691	0.0324	Sex-determination pathway	Male vs. Female head horns
FBpp0308474	Wnt oncogene analog 2	22.5065	−1.4254	0.4857	0.0432	Sex-determination pathway	Male vs. Female head horns
FBpp0083066	Fruitless	626.5180	0.7130	0.2108	0.0155	Sex-determination pathway	Male vs. Female head horns

(Continues)

TABLE 3 (Continued)

FlyBase polypeptide identifier	Gene symbol	Mean expression	Log2 fold change	lfcSE	Adjusted *p*-value	Pathway	Comparison
FBpp0083067	Fruitless	62.4638	0.5051	0.1455	0.0125	Sex-determination pathway	Male vs. Female head horns
FBpp0308474	Wnt oncogene analog 2	173.0843	1.0668	0.2730	0.0039	Sex-determination pathway	Male vs. Female head horns
FBpp0087596	Wnt oncogene analog 2	11.5136	2.4833	0.5970	0.0019	Sex-determination pathway	Male vs. Female head horns
FBpp0087596	Wnt oncogene analog 2	141.8725	2.3094	0.4109	0.0000	Sex-determination pathway	Male vs. Female head horns
FBpp0088563	Transformer 2	15.3133	−1.0751	0.3459	0.0298	Sex-determination pathway	Male vs. Female head horns
FBpp0308767	Doublesex	34.2414	−2.3962	0.6165	0.0041	Sex-determination pathway	Male vs. Female head horns
FBpp0303106	Doublesex	37.8376	5.9967	0.5224	0.0000	Sex-determination pathway	Male vs. Female head horns
FBpp0303107	Doublesex	13.2532	−1.9901	0.6104	0.0211	Sex-determination pathway	Male vs. Female head horns
FBpp0308767	Doublesex	38.3376	4.6444	0.5899	0.0000	Sex-determination pathway	Male vs. Female head horns
FBpp0308767	Doublesex	12.3394	−1.5549	0.5213	0.0390	Sex-determination pathway	Male vs. Female head horns

Note. This table shows the candidate genes identified either in functional studies or that are predicted to be involved in horn growth based on their function in other organisms that are differentially expressed in our analysis. With the exception of the three genes in italics, all genes were significantly differentially expressed only when comparing male head horns to female head horns. A positive log2 fold change value represents genes that are upregulated in male head horns, whereas a negative value represents genes that are upregulated in female head horns. Gene names and FlyBase Polypeptide IDs corresponding to current FlyBase nomenclature are provided.

Figure 6a) or the *SD* subset ($r = 0.51$, $p < 0.05$). This suggests that despite many of them not being deemed significantly differentially expressed (according to our False Discovery Rate [FDR]), overall, the nutritionally sensitive genes (*NR*) and sexually dimorphic genes (*SD*) behaved similarly to each other when compared to random subsets of genes in response to both nutrition and sexual dimorphism. We observed similar patterns when performing this analysis on our three reduced transcriptomes (Supporting Information Figure S5, *Drosophila* transcriptome; Supporting Information Figure S9, *Tribolium* transcriptome; Supporting Information Figure S14, *Combined* transcriptome). This suggests that overall, the directions of changes in transcript abundance due to either sex or the nutrition treatment were quite similar.

Although the directions of differential expression were similar between the vector of large vs. small male head horns and the vector of male horns vs. female horns, the *NR* and *SD* gene subsets exhibited dramatically different expression magnitudes. The magnitudes of gene expression differences for the vector of large vs. small male head horns were larger than those of the vector of male vs. female horns for the *NR* gene subset (*NR* alpha = 0.71, $p < 0.05$; Figure 6b). On the other hand, the magnitude of expression for the *SD* subset was greater in the vector of male vs. female horns (*SD* alpha = 1.88, $p < 0.05$; Figure 6b) than in the vector of large vs. small horns.

Changes in gene expression due to heightened nutrition-sensitive growth of male head horns (large males vs. small males) appear to share a response with other tissues that also respond to nutrition. Specifically, when considering nutritional responses across tissues (with the *NR* subset), the directions of differential expression were correlated between both the head horn and wing contrasts ($r = 0.69$, $p < 0.05$; Figure 6c). However, there was no correlation between head horn and genital contrasts ($r = 0.3$, $p = 0.14$; Figure 6c). This follows the biological growth responses of these tissues—wing growth is sensitive to nutrition (Figure 1), although not to the exaggerated degree that head horns display, whereas genital growth is largely insensitive to nutrition. When investigating the magnitude of expression between tissue contrasts using the *NR* gene subset (Figure 6d), we found that head horn vectors had higher magnitudes of expression differences than wings (alpha = 1.54, $p < 0.05$), with the magnitude of expression being approximately 50% greater in head horns when compared to wings. Interestingly, magnitude of expression in head horns was not significantly larger than in genitals for the *NR* subset (alpha = 1.22, $p = 0.244$).

Finally, our vector correlation analyses suggest that changes in gene expression associated with growth of head and thoracic horns are similar. We compared the *SD* vector between head horns and thoracic horns (Figure 6e, blue) and found a high correlation ($r = 0.64$, $p < 0.05$), consistent with our finding that there are over 200 shared transcripts differentially expressed in both head horns and thoracic horns in response to sexual dimorphism. In addition, the magnitude of expression was close to 1 for these two vectors, indicating that the SD set of genes was expressed similarly between these two contrasts (alpha = 1.04, $p > 0.05$; Figure 6f) as well. We also found a high degree of correlation between head and thoracic horns for the nutrition-sensitive (*NR*) vectors ($r = 0.79$, $p < 0.05$; Figure 6e, red); however, the magnitude of expression between these two vectors indicated higher expression in head horns than in thoracic horns (alpha = 1.51, $p < 0.05$).

4 | DISCUSSION

Our results demonstrate that as the degree of sexual dimorphism between developing tissues increases, so too does the number of genes showing evidence for differential expression (Figure 2), as well as the average magnitude of expression differences (Figure 6). Or, put another way, structures that are not sexually dimorphic such as wings show only small differences in the relative number of transcripts expressed between developing males and females, while structures that are highly sexually dimorphic, such as head horns, require large-scale differences in gene expression during development in order to achieve the extreme dimorphism in trait size present in adults.

It is interesting that the thoracic horns exhibited fewer differentially expressed genes than head horns during development (Figure 3b). Both structures are sexually dimorphic, very nutritionally sensitive (Figure 1), and both are superficially similar in shape, extending out from the body wall and forking at the tip. Yet the head horn is both bigger and structurally more complex than the thoracic horn in this species, spreading as it does into a wide "pitchfork-like" extension at the tip, and having four tines rather than the two present in thoracic horns. Thus, the two male weapons differ in the amount and complexity of growth in a fashion consistent with the overall patterns of gene expression we observe.

A recent review of the evolutionary and molecular mechanisms behind the evolution of exaggerated traits in insects highlighted several coordinating molecular mechanisms predicted to be involved in the growth of sexually dimorphic, exaggerated traits (Lavine et al., 2015). The authors identified several synergistic pathways involved in exaggerated trait growth, including the insect sex-determination pathway driven by the *doublesex* gene (*dsx*), heightened sensitivity to insulin/IGF signalling, as well as hormonal control through juvenile hormone and ecdysteroid signalling (Lavine et al., 2015). In our global analysis, we find evidence for the involvement of all of these pathways during sexually dimorphic growth of head horns (Table 3).

Several downstream components of the sexual differentiation pathway in insects are differentially expressed in horns in response to sexual dimorphism, most notably *sans fille* (*snf*) (Albrecht & Salz, 1993) and several isoforms of *doublesex* (Ito et al., 2013). In addition, the transcription factor *broad* (*br*), a member of the juvenile hormone signalling pathway, was differentially expressed in response to sex (Table 3). While juvenile hormone signalling does not seem to have a major role in trait exaggeration in rhinoceros beetles (Zinna et al., 2016), the role of downstream members of the pathway such as *broad* in sexually dimorphic trait growth remains unknown. The limb-patterning gene *aristaless (al)* was also differentially expressed in dimorphic head horn growth. This gene has been shown previously

to have a role in patterning thoracic horn growth in dung beetles (Moczek & Rose, 2009). More importantly, *Hedgehog (hh)* as well as many up- and downstream targets of this pathway are differentially expressed in our system. Recent work in dung beetles demonstrated that *hh* signalling may act as a gatekeeper and regulator of polyphenic growth (Kijimoto & Moczek, 2016), and not only is *hh* differentially regulated in our system, but the upstream signals *engrailed* (*en*) and *shaggy*, along with other members of this pathway, are also differentially expressed due to sex (Table 3), suggesting that this pathway may be an important regulator of dimorphic weapon growth across scarab families.

We also found evidence for involvement of the insulin signalling pathway during sexually dimorphic growth of weapons (Table 3). This pathway has previously been shown to regulate male head horn growth in this species (Emlen et al., 2012), as well as sexually dimorphic growth of weapons in red deer (Price, Oyajobi, Oreffo, & Russell, 1994; Suttie et al., 1985), Soay and bighorn sheep (Johnston et al., 2011; Kardos et al., 2015) and shrimp (Ventura et al., 2011); condition-sensitive profiles of male cuticular hydrocarbons in flies (Kuo et al., 2012); and condition-sensitive development of male courtship song in songbirds (Holzenberger et al., 1997) and courtship locomotor behaviour in flies (Belgacem & Martin, 2006). Thus, our result provides additional validation that the insulin signalling pathway is important in sexually dimorphic growth of sexually selected signal traits.

Our analysis also provided evidence for the involvement of the Fat/Hippo signalling pathway during development of sexually dimorphic structures (Table 3; Gotoh et al., 2015; Hust et al., 2018). *four-jointed* (*fj*) is a signalling molecule that phosphorylates the extracellular cadherin domains of the receptor *fat (ft)* and is ultimately required for the signalling cascade mediated by the Fat-Dachsous (Ds) dimer (Ishikawa, Takeuchi, Haltiwanger, & Irvine, 2008; Keira, Wada, & Ishikawa, 2017). This Fat-signalling pathway is known to regulate tissue growth and planar cell polarity in *Drosophila*, and defects in this pathway cause malformations in wings (Keira et al., 2017). Importantly, perturbations of this pathway in *T. dichotomus* have been shown to affect horn allometry (Hust et al., 2018). In our analysis, *fj* and the downstream Fat-signalling molecule *dachs (d)*, as well as the gene *lowfat*, which is required to maintain Fat-Ds levels, were differentially expressed during sexually dimorphic growth of horns (Table 3).

In the current experiment, nutritionally mediated condition in male beetles had little effect on overall numbers of differentially expressed genes (Figure 3, Table 2) and generally had more modest magnitudes of differential expression than did sexual dimorphism. Our results contrast with a recent analysis by Kijimoto et al. (2014) on the effect of nutritional variation on gene expression in dung beetle tissues, which found that approximately 15% of the transcriptome was differentially expressed in response to nutrition in *Onthophagus taurus* horns. This is a significantly higher percentage of the horn transcriptome than was differentially expressed in response to nutrition in the present study (approximately 1% of transcripts).

In addition to differences in experimental design (in particular the severity of the nutritional manipulation) and power to detect significant contrasts in each study, there are also important biological differences between head horns in the two beetle species. Unlike *T. dichotomus*, male *O. taurus* are male-dimorphic for the presence/absence of horns; low-nutrition males, like females, fail to develop horns (Moczek & Emlen 1999). In contrast, all rhinoceros beetle males develop weapons to some extent, and nutritional condition only affects the final size of the adult trait, not the presence or absence of the trait (Emlen et al., 2012; Johns et al., 2014; Figure 1). Therefore, it is possible that differences in the size of head horns in *T. dichotomus* require minimal developmental specification and need only respond to nutritional condition with modulation of the amount of growth, instead of the combination of growth and differentiation presumably needed for polyphenic development of *O. taurus* horns. However, it is essential to note that a direct comparison of the severity of the nutritional manipulations in each of these studies is difficult. In our study, there is no doubt that a more severe nutritional manipulation would have elicited a stronger response (with respect to both size and differential expression), although survival of the individuals would potentially be much lower.

While we do find evidence for nutrition-sensitive changes in gene expression associated with three of the molecular mechanisms for trait exaggeration identified by Lavine et al. (2015), each pathway was represented by only a single gene (i.e., fewer genes from these pathways were DE in response to nutrition than were in response to sex). The insulin receptor substrate gene *thor*, for instance, was downregulated in large male relative to small male head horns (Table 3).

Because our predictions for nutrition sensitivity were sex-specific (e.g., we expected horn tissues in males to be sensitive to nutrition, while the corresponding "horn" tissues in females would not), we also tested for genes exhibiting a significant interaction between nutrition and sex. From this pool, we looked specifically for genes showing differential expression in male horn tissues but not in female horn tissue (i.e., for which the direction of the interaction fit with our a priori predictions). We identified 13 genes displaying male-biased nutrition-sensitive differential expression in head horns. Interestingly, all 13 were expressed at very high levels relative to the rest of the genes in this pool (Figure 5a). All but one of these genes increased in expression as a result of nutritional condition in males, but not in females (Figure 5b). Interestingly, this set of genes includes genes not previously implicated in weapon growth or even condition-sensitive growth in any organism thus far (Table 4). For instance, the gene *scattered (scat)* is involved in both the regulation of vesicle transport to the Golgi ribbon and ultimately necessary for spermatid formation in *Drosophila* (Fári, Takács, Ungár, & Sinka, 2016), and our data represent a potentially novel function for this gene during nutrition-sensitive growth of a sexually dimorphic structure. The gene *Sunday driver* (*syd*) decreased in expression with increasing nutritional condition in males, an interesting phenotype for a gene known to promote muscle development in *Drosophila* (Schulman, Folker, Rosen, & Baylies, 2014). The fact that these genes

TABLE 4 Change in average expression in 13 genes displaying male-biased nutrition-sensitive differential expression in head horns

		Δexpression (lg-sm)	
Gene	**Symbol**	**Male horn**	**Female horn**
Actin-related protein 6	*Arp6*	0.553	−0.025
Adenosylhomocysteinase at 13	*Ahcy13*	0.900	0.025
CG10602 gene product from transcript CG10602-RF	*CG10602*	0.831	0.025
CG1354 gene product from transcript CG1354-RD	*CG1354*	0.606	0.216
CG17765 gene product from transcript CG17765-RA	*CG17765*	0.502	0.002
CG5525 gene product from transcript CG5525-RA	*CG5525*	0.794	0.156
CG7033 gene product from transcript CG7033-RA	*CG7033*	0.921	0.202
CG8351 gene product from transcript CG8351-RA	*Tcp-1eta*	0.728	0.080
Cyclophilin 1	*Cyp1*	1.062	0.186
Pre-RNA processing factor 19	*Prp19*	0.579	0.005
Scattered	*scat*	0.691	−0.274
Sunday driver	*syd*	−2.014	0.289
Tcp1-like	*T-cp1*	0.913	0.210

Note. This table indicates the average change in expression (calculated as the average of the absolute value of log2 fold change per gene in large individuals minus the same value from small individuals) across nutritional condition in male and female head horns. Data correspond to panel (b) in Figure 6.

were differentially expressed in response to nutrition in male but not female horn tissue makes these high priority candidates for further study.

Overall, our results suggest that the developmental mechanisms responsible for sexual dimorphism and heightened condition-sensitive growth of rhinoceros beetle horns may be similar. When comparing expression vectors using both the sexually dimorphic (*SD*) and the nutrition-responsive (*NR*) gene sets, we find that the directions of differential expression associated with sexual dimorphism (large male head horns vs. female head horns) are highly correlated with the directions of differential expression associated with heightened nutrition-sensitive growth (large male head horns vs. small male head horns; Figure 6a). These results are generally consistent with the well-known relationship between condition dependence and degree of sexual dimorphism (Bonduriansky, 2007; Bonduriansky & Rowe, 2005; Cotton et al., 2004a; Warren et al., 2013). Indeed, these results are paralleled in other beetle species (Ledón-Rettig & Moczek, 2016; reviewed in Mank, 2017). One interpretation, and the one we favour based upon the existing literature, is that condition dependence and sexual dimorphism share some common developmental mechanisms (Bonduriansky, 2007; Gotoh et al., 2014; Oudin, Bonduriansky, & Rundle, 2015; Ledón-Rettig & Moczek, 2016). However, it may be that other, currently unknown mechanisms constrain aspects of gene expression (in terms of direction and magnitude).

Our results also suggest that the mechanisms involved with heightened nutrition-sensitive growth of the exaggerated male weapon (head horns) are similar to the general mechanisms responsible for nutrition-sensitive plasticity in the growth of the body as a whole, represented here by a more "typically" plastic trait, wings. Overall, the directions of differential gene expression in head horns (large vs. small males) were correlated with those of wings, but not with genitalia (Figure 6c). Genitalia are interesting because their growth is unusually *in*sensitive to nutrition (Johns et al., 2014), and their patterns of differential gene expression were the most distinct of the measured body structures.

Finally, our results suggest that the developmental mechanisms underlying growth of the two horn types are similar to each other (Figure 6e), a finding surprising in the light of the fact that in dung beetles, head and thoracic horn tissues generally seem to be governed by different mechanisms (Moczek & Rose, 2009) and are thought to reflect independent evolutionary gains of novel structures (Emlen, Hunt, et al., 2005; Emlen, Marangelo, et al., 2005; Emlen, Lavine, & Ewen-Campen, 2007; Moczek & Rose, 2009; Moczek et al. 2006). Interestingly, while the *SD* set of genes is correlated between head horns and thoracic horns (Figure 6e, blue bar), this gene set has the same magnitude of expression in both tissues (Figure 6f, blue bar), whereas the *NR* subset is both correlated (Figure 6e, red bar) and has greater magnitude in head horns (Figure 6f, red bar). This suggests that while these two tissues use similar mechanisms to respond to nutrition condition

and sex, head horns utilize much greater expression of nutritionally sensitive genes than do thoracic horns. It is possible that the smaller magnitude of expression of nutritionally sensitive genes in thoracic horns explains why RNAi knockdown of *dsx* in males results in complete loss of the thoracic horn but not the head horn, and why *dsx* knockdown in females results in the growth of a head horn and not a thoracic horn (Ito et al., 2013).

Our results agree with the general patterns observed from related experiments in dung beetles (Kijimoto et al., 2014; Ledón-Rettig & Moczek, 2016), in that we find fewer differences as a result of nutritional condition than we find as a consequence of sexual dimorphism, and in the broad developmental and physiological pathways we implicate. However, our results suggest that the particular genes from these pathways that are differentially expressed in dung beetle and rhinoceros beetle horns are different. Our results for differential expression in sexually dimorphic horn growth had little overlap with the set of genes differentially expressed in *Onthophagus* horns (e.g., the supplemental data from Ledón-Rettig & Moczek, 2016). It is important to note that the gene sets were annotated using slightly different methodologies in our respective studies, precluding more direct comparisons (e.g., vector correlations) without complete re-analyses of the original data sets.

Nevertheless, we interpret these differences as evidence that rhinoceros and dung beetles, scarab clades who last shared a common ancestor roughly 150 Ma (Ahrens, Schwarzer, & Vogler, 2014; Browne & Scholtz, 1999; Gunter, Weir, Slipinksi, Bocak, & Cameron, 2016; Krell, 2000; Smith, Hawks, & Heraty, 2006) and which almost certainly evolved horns independently (Arrow, 1951; Emlen & Philips, 2006; but see Emlen, Safran, Corley, & Dworkin, 2006), utilized similar developmental and physiological mechanisms (i.e., insulin/IGF signalling, *Hedgehog* signalling, limb-patterning pathways, JH signalling pathways), but co-opted different specific genes from within each pathway during the course of their respective horn evolutions. An alternative explanation for the differences is that the relative timing of growth (and the regulation of growth) may be sufficiently different among the species that neither set of snapshot studies are capturing common aspects of growth. In addition to larger sample sizes, future studies should also examine detailed time courses of the tissue development to determine the extent of similarity in trajectories or heterochronic changes.

There are several important caveats to the inferences we have made based on our experiment and analysis that may influence particular genes considered differentially expressed. As the number of individuals used for sampling tissues within each particular treatment group was modest (four individuals/sex/nutritional state), this experiment has modest statistical power. This becomes of particular concern for the analysis for the full transcriptome (with many comparisons being controlled for in the FDR) and for genes that show low expression. DESEQ2 performs a form of regularization, where estimated coefficients are shrunk when counts for a particular transcript are low (Love et al., 2014). As such, in some instances genes that have expression differences, but low mean expression may be shrunk and appear to have smaller expression differences. Furthermore, DESEQ2 also pre-filters genes whose mean expression is sufficiently low that there is no reasonable expectation of having sufficient power to be able to detect differential expression (so that fewer models are being tested, see percentage filtered column in Table 2).

In addition, we did not remove redundant isoforms outside of utilizing TRANSPS. However, we considered our "full," non-reduced transcriptome to be an overly generous and the TRANSPS reduced transcriptomes to be a very conservative method of isoform filtering. We report on the non-reduced transcriptome, but recognize that the truth probably lies somewhere between very conservative and non-existent redundancy removal. Thus, as with many studies of differential gene expression, our analyses represent a biased subset of genes with moderate (or greater) mean expression levels. While this influences our interpretation of particular genes and pathways, it is unlikely to influence our overall conclusions about the relative number of genes that are differentially expressed and their magnitudes, as we observed almost identical patterns when using the smaller set of transcripts evaluated using TRANSPS (Supporting Information Figures S2, S3, S5–S7, S10, and S11).

In short, our results demonstrate that sexually dimorphic, nutritionally responsive growth of exaggerated traits requires the coordinated action of large suites of shared genes, many of which correspond to genes implicated in exaggerated trait growth in other insects. On the other hand, "heightened" condition-sensitive growth, a widespread characteristic of the most extreme sexually selected structures and a property of both head and thoracic horns in this species, did not appear to entail mechanisms distinct from those already generating more typical levels of nutrition-sensitive plasticity in surrounding structures, though it did differ somewhat from mechanisms presumably repressing nutrition-sensitive growth in male genitalia. It is possible that heightened nutritional responsiveness of this exaggerated trait depends only on one core pathway, the insulin/IGF signalling pathway, already known to be involved with massive weapon growth in this species. It is important to note, however, that we sampled larvae during the prepupal development period, and it is possible that only sampling at one time point missed critical periods of nutrition responsiveness.

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DATA ACCESSIBILITY

The raw sequenced reads are deposited in NCBI's Short Read Archive Accession no. PRJNA493494, and the full transcriptome and annotated transcriptomes are deposited in Dryad alongside read counts and scripts used to analyse data at the https://doi.org/10.5061/dryad.f40f4qg. All scripts used in this analysis are also hosted on GitHub at the following url: https://github.com/Zinnar/Trypoxylus_RNAseq.

AUTHOR CONTRIBUTION

D.E., L.C.L., A.J. and I.D. designed the tissue harvesting and sequencing experiments. H.G. and T.N. provided specimens and animal support. A.J. and D.E. dissected tissues. I.D. performed the sequencing. R.Z. and I.D. performed the analyses. All authors contributed to writing the manuscript.

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REFERENCES

Ahrens, D., Schwarzer, J., & Vogler, A. P. (2014). The evolution of scarab beetles tracks the sequential rise of angiosperms and mammals. *Proceedings of the Royal Society of London B: Biological Sciences*, *281*(1791), 20141470.

Albrecht, E. B., & Salz, H. K. (1993). The *Drosophila* sex determination gene *Snf* is utilized for the establishment of the female-specific splicing pattern of *Sex-lethal*. *Genetics*, *134*, 801–807.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*, 403–410.

Andersson, M. B. (1994). *Sexual selection*. Princeton, NJ: Princeton University Press.

Andrews, S. (2010). *FastQC: A quality control tool for high throughput sequence data*. Retrieved from http://www.bioinformatics.babraham.ac.uk/projects/fastqc-

Arrow, G. J. (1951). *Horned beetles*. The Hague, the Netherlands: Dr W. Junk Publishers.

Auer, P. L., & Doerge, R. W. (2010). Statistical design and analysis of RNA sequencing data. *Genetics*, *185*, 405–416.

Belgacem, Y. H., & Martin, J. R. (2006). Disruption of insulin pathways alters trehalose level and abolishes sexual dimorphism in locomotor activity in *Drosophila*. *Journal of Neurobiology*, *66*(1), 19–32.

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, *57*, 289–300.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*, 2114–2120.

Bonduriansky, R. (2007). The evolution of condition-dependent sexual dimorphism. *The American Naturalist*, *169*, 9–19.

Bonduriansky, R., & Rowe, L. (2005). Sexual selection, genetic architecture, and the condition dependence of body shape in the sexually dimorphic fly *Prochyliza xanthostoma* (Piophilidae). *Evolution*, *59*(1), 138–151.

Bradbury, J. W., & Vehrencamp, S. L. (2011). *Principles of animal communication*. Sunderland, MA: Sinauer Associates.

Browne, J., & Scholtz, C. H. (1999). A phylogeny of the families of Scarabaeoidea (Coleoptera). *Systematic Entomology*, *24*, 51–84.

Cotton, S., Fowler, K., & Pomiankowski, A. (2004a). Do sexual ornaments demonstrate heightened condition-dependent expression as predicted by the handicap hypothesis? *Proceedings of the Royal Society B: Biological Sciences*, *271*, 771–783.

Cotton, S., Fowler, K., & Pomiankowski, A. (2004b). Condition dependence of sexual ornament size and variation in the stalk-eyed fly *Cyrtodiopsis dalmanni* (Diptera: Diopsidae). *Evolution*, *58*(5), 1038–1046.

Darwin, C. (1871). *The descent of man, and selection in relation to sex*. New York: Appleton and Company.

David, P., Bjorksten, T., Fowler, K., & Pomiankowski, A. (2000). Condition-dependent signaling of genetic variation in stalk-eyed flies. *Nature*, *406*(6792), 186–188.

Eberhard, W. G., Huber, B. A., Briceno, R. D., Salas, I., & Rodriguez, V. (1998). One size fits all? Relationships between the size and degree of variation in genitalia and other body parts in twenty species of insects and spiders. *Evolution*, *52*(2), 415–431.

Eberhard, W., Rodriguez, R. L., & Polihronakis, M. (2009). Pitfalls in understanding the functional significance of genital allometry. *Journal of Evolutionary Biology*, *22*(3), 435–445.

Emlen, D. J. (1994). Environmental control of horn length dimorphism in the beetle Onthophagus acuminatus (Coleoptera: Scarabaeidae). *Proceedings of the Royal Society B: Biological Sciences*, *256*(1346), 131–136.

Emlen, D. J. (2008). The evolution of animal weapons. *Annual Review of Ecology, Evolution, and Systematics*, *39*, 387–413.

Emlen, D. J., Hunt, J., & Simmons, L. W. (2005). Evolution of sexual dimorphism and male dimorphism in the expression of beetle horns: Phylogenetic evidence for modularity, evolutionary lability, and constraint. *The American Naturalist*, *166*, S42–S68.

Emlen, D. J., Lavine, L. C., & Ewen-Campen, B. (2007). On the origin and evolutionary diversification of beetle horns. *Proceedings of the National Academy of Sciences*, *104*, 8661–8668.

Emlen, D. J., Marangelo, J., Ball, B., & Cunningham, C. W. (2005). Diversity in the weapons of sexual selection: Horn evolution in the beetle genus *Onthophagus* (Coleoptera: Scarabaeidae). *Evolution*, *5*, 1060–1084.

Emlen, D. J., & Nijhout, H. F. (2000). The development and evolution of exaggerated morphologies in insects. *Annual Review of Entomology*, *45*, 661–708.

Emlen, D. J., & Philips, T. K. (2006). Phylogenetic evidence for an association between tunneling behavior and the evolution of horns in dung beetles (Coleoptera: Scarabaeinae). *Coleopterists Society Monograph*, *5*, 47–56.

Emlen, D. J., Safran, Q., Corley, L. S., & Dworkin, I. (2006). Insulin signaling and limb-patterning: Candidate pathways for the origin and evolutionary diversification of beetle 'horns'. *Heredity*, *97*, 179–191.

Emlen, D. J., Warren, I. A., Johns, A., Dworkin, I., & Lavine, L. C. (2012). A mechanism of extreme growth and reliable signaling in sexually selected ornaments and weapons. *Science*, *337*, 860–864.

Fári, K., Takács, S., Ungár, D., & Sinka, R. (2016). The role of acroblast formation during *Drosophila* spermatogenesis. *Biology Open*, *5*(8), 1102–1110.

Gotoh, H., Cornette, R., Koshikawa, S., Okada, Y., Lavine, L. C., Emlen, D. J., & Miura, T. (2011). Juvenile hormone regulates extreme mandible growth in male stag beetles. *PLoS ONE*, *6*, e21139.

Gotoh, H., Hust, J. A., Miura, T., Niimi, T., Emlen, D. J., & Lavine, L. C. (2015). The Fat/Hippo signaling pathway links within-disc morphogen patterning to whole-animal signals during phenotypically plastic growth in insects: Fat/Hippo signaling in nutrition-sensitive growth. *Developmental Dynamics*, *244*, 1039–1045.

Gotoh, H., Miyakawa, H., Ishikawa, A., Ishikawa, Y., Sugime, Y., Emlen, D. J., … Miura, T. (2014). Developmental link between sex and nutrition; *doublesex* regulates sex-specific mandible growth via juvenile hormone signaling in stag beetles. *PLoS Genetics*, *10*, e1004098.

Gotoh, H., Zinna, R. A., Ishikawa, Y., Miyakawa, H., Ishikawa, A., Sugime, Y., … Miura, T. (2017). The function of appendage patterning genes in mandible development of the sexually dimorphic stag beetle. *Developmental Biology*, *422*, 24–32.

Gotoh, H., Zinna, R. A., Warren, I., DeNieu, M., Niimi, T., Dworkin, I., … Lavine, L. C. (2016). Identification and functional analyses of sex determination genes in the sexually dimorphic stag beetle *Cyclommatus metallifer*. *BMC Genomics*, *17*, 250.

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., … Regev, A. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology*, *29*, 644–652.

Gunter, N. L., Weir, T. A., Slipinksi, A., Bocak, L., & Cameron, S. L. (2016). If dung beetles (Scarabaeidae: Scarabaeinae) arose in association with dinosaurs, did they also suffer a mass co-extinction at the K-Pg boundary? *PLoS ONE*, *11*(5), e0153570.

Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowden, J., … Regev, A. (2013). *De novo* transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nature Protocols*, *8*, 1494–1512.

Holzenberger, M., Jarvis, E. D., Chong, C., Grossman, M., Nottebohm, F., & Scharff, C. (1997). Selective expression of insulin-like growth factor II in the songbird brain. *Journal of Neuroscience*, *17*, 6974–6987.

Hongo, Y. (2003). Appraising behaviour during male–male interaction in the Japanese horned beetle *Trypoxylus dichotomus septentrionalis* (Kono). *Behaviour*, *140*, 501–517.

Hongo, Y. (2007). Evolution of male dimorphic allometry in a population of the Asian horned beetle *Trypoxylus dichotomus septentrionalis*. *Behavioral Ecology and Sociobiology*, *62*, 245–253.

Hunt, J., Bussiere, L. F., Jennions, M. D., & Brooks, R. (2004). What is genetic quality? *Trends in Ecology & Evolution*, *19*(6), 329–333.

Hust, J., Lavine, M. D., Worthington, A. M., Zinna, R., Gotoh, H., Niimi, T., & Lavine, L. (2018). The Fat-Dachsous signaling pathway regulates growth of horns in *Trypoxylus dichotomus*, but does not affect horn allometry. *Journal of Insect Physiology*, *105*, 85–94.

Iguchi, Y. (1998). Horn dimorphism of *Allomyrina dichotoma septentrionalis* (Coleoptera: Scarabaeidae) affected by larval nutrition. *Annals of the Entomological Society of America*, *91*, 845–847.

Ishikawa, H. O., Takeuchi, H., Haltiwanger, R. S., & Irvine, K. D. (2008). Four-jointed is a Golgi kinase that phosphorylates a subset of cadherin domains. *Science*, *321*(5887), 401–404.

Ito, Y., Harigai, A., Nakata, M., Hosoya, T., Araya, K., Oba, Y., … Niimi, T. (2013). The role of *doublesex* in the evolution of exaggerated horns in the Japanese rhinoceros beetle. *EMBO Reports*, *14*, 561–567.

Iwasa, Y., & Pomiankowski, A. (1999). Good parent and good genes models of handicap evolution. *Journal of Theoretical Biology*, *200*(1), 97–109.

Iwasa, Y., Pomiankowski, A., & Nee, S. (1991). The evolution of costly mate preferences II. The "handicap" principle. *Evolution*, *45*(6), 1431–1442.

Johns, A., Gotoh, H., McCullough, E. L., Emlen, D. J., & Lavine, L. C. (2014). Heightened condition-dependent growth of sexually selected weapons in the rhinoceros beetle, *Trypoxylus dichotomus* (Coleoptera: Scarabaeidae). *Integrative and Comparative Biology*, *54*, 614–621.

Johnston, S., McEwan, J. C., Pickering, N. K., Kijas, J. W., Beraldi, D., Pilkington, J. G., … Slate, J. (2011). Genome-wide association mapping identifies the genetic basis of discrete and quantitative variation in sexual weaponry in a wild sheep population. *Molecular Ecology*, *20*, 2555–2566.

Kardos, M., Luikart, G., Bunch, R., Dewey, S., Edwards, W., McWilliam, S., … Kijas, J. (2015). Whole-genome resequencing uncovers molecular signatures of natural and sexual selection in wild bighorn sheep. *Molecular Ecology*, *24*, 5616–5632.

Karino, K., Seki, N., & Chiba, M. (2004). Larval nutritional environment determines adult size in Asian horned beetles *Allomyrina dichotoma*. *Ecological Research*, *19*, 663–668.

Keira, Y., Wada, M., & Ishikawa, H. O. (2017). Chapter five-regulation of *Drosophila* development by the Golgi kinase four-jointed. *Current Topics in Developmental Biology*, *123*, 143–179.

Kijimoto, T., & Moczek, A. P. (2016). Hedgehog signaling enables nutrition-responsive inhibition of an alternative morph in a polyphenic beetle. *Proceedings of the National Academy of Sciences*, *113*(21), 5982–5987.

Kijimoto, T., Snell-Rood, E. C., Pespeni, M. H., Rocha, G., Kafadar, K., & Moczek, A. P. (2014). The nutritionally responsive transcriptome of the polyphenic beetle *Onthophagus taurus* and the importance of sexual dimorphism and body region. *Proceedings of the Royal Society B: Biological Sciences*, *281*, 20142084.

Knell, R. J., Fruhauf, N., & Norris, K. A. (1999). Conditional expression of a sexually selected trait in the stalk-eyed fly *Diasemopsis aethiopica*. *Ecological Entomology*, *24*, 323–328.

Kojima, W. (2015). Variation in body size in the giant rhinoceros beetle *Trypoxylus dichotomus* is mediated by maternal effects on egg size. *Ecological Entomology*, *40*, 420–427.

Kokko, H., & Heubel, K. (2008). Condition-dependence, genotype-by-environment interactions and the lek paradox. *Genetica*, *132*(2), 209–216.

Krell, F. T. (2000). The fossil record of Mesozoic and Tertiary Scarabaeoidea (Coleoptera: Polyphaga). *Invertebrate Systematics*, *14*, 871–905.

Kuo, T. H., Fedina, T. Y., Hansen, I., Dreisewerd, K., Dierick, H. A., Yew, J. Y., & Pletcher, S. D. (2012). Insulin signaling mediates sexual attractiveness in *Drosophila*. *PLoS Genetics*, *8*, e1002684.

Kuruvilla, F. G., Park, P. J., & Schreiber, S. L. (2002). Vector algebra in the analysis of genome-wide expression data. *Genome Biology*, *3*, RESEARCH0011.

Lavine, L., Gotoh, H., Brent, C. S., Dworkin, I., & Emlen, D. J. (2015). Exaggerated trait growth in insects. *Annual Review of Entomology*, *60*, 453–472.

Ledón-Rettig, C. C., & Moczek, A. P. (2016). The transcriptomic basis of tissue- and nutrition-dependent sexual dimorphism in the beetle *Onthophagus taurus*. *Ecology and Evolution*, *6*, 1601–1613.

Ledón-Rettig, C. C., Zattara, E. E., & Moczek, A. P. (2017). Asymmetric interactions between doublesex and tissue-and sex-specific target genes mediate sexual dimorphism in beetles. *Nature Communications*, *8*, 14593.

Liu, M., Adelman, Z. N., Myles, K. M., & Zhang, L. (2014). A transcriptome post-scaffolding method for assembling high quality contigs. *Computational Biology Journal*, *2014*, 1–4.

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*, 550.

Mank, J. E. (2017). The transcriptional architecture of phenotypic dimorphism. *Nature Ecology & Evolution*, *1*(1), 0006.

Martin, J. A., & Wang, Z. (2011). Next-generation transcriptome assembly. *Nature Reviews Genetics*, *12*, 671–682.

Maynard Smith, J., & Harper, D. (2003). *Animal signals*. Oxford, UK: Oxford University Press.

Miller, C. W., & Moore, A. J. (2007). A potential resolution to the lek paradox through indirect genetic effects. *Proceedings of the Royal Society of London B: Biological Sciences*, *274*(1615), 1279–1286.

Moczek, A. P. (2006). Pupal remodeling and the development and evolution of sexual dimorphism in horned beetles. *The American Naturalist*, *168*, 711–729.

Moczek, A. P., & Emlen, D. (1999). Proximate determination of male horn dimorphism in the beetle *Onthophagus taurus* (Coleoptera: Scarabaeidae). *Journal of Evolutionary Biology*, *12*, 27–37.

Moczek, A. P., & Nagy, L. M. (2005). Diverse developmental mechanisms contribute to different levels of diversity in horned beetles. *Evolution & Development*, *7*, 175–185.

Moczek, A. P., & Rose, D. J. (2009). Differential recruitment of limb patterning genes during development and diversification of beetle horns. *Proceedings of the National Academy of Sciences*, *106*, 8992–8997.

Moczek, A. P., Rose, D., Sewell, W., & Kesselring, B. R. (2006). Conservation, innovation, and the evolution of horned beetle diversity. *Development Genes and Evolution*, *216*(11), 655–665.

Oudin, M. J., Bonduriansky, R., & Rundle, H. D. (2015). Experimental evidence of condition-dependent sexual dimorphism in the weakly dimorphic antler fly *Protopiophila litigata* (Diptera: Piophilidae). *Biological Journal of the Linnean Society*, *116*(1), 211–220.

Ozawa, T., Mizuhara, T., Arata, M., Shimada, M., Niimi, T., Okada, K., … Ohta, K. (2016). Histone deacetylases control module-specific phenotypic plasticity in beetle weapons. *Proceedings of the National Academy of Sciences*, *113*(52), 15042–15047.

Parra, G., Bradnam, K., & Korf, I. (2007). CEGMA: A pipeline to accurately annotate core genes in eukaryotic genomes. *Bioinformatics*, *23*, 1061–1067.

Patro, R., Mount, S. M., & Kingsford, C. (2014). Sailfish enables alignment-free isoform quantification from RNA-seq reads using lightweight algorithms. *Nature Biotechnology*, *32*, 462–464.

Pitchers, W., Pool, J. E., & Dworkin, I. (2013). Altitudinal clinal variation in wing size and shape in African *Drosophila* melanogaster: One cline or many? *Evolution*, *67*(2), 438–452.

Plaistow, S. J., Tsuchida, K., Tsubaki, Y., & Setsuda, K. (2005). The effect of a seasonal time constraint on development time, body size, condition, and morph determination in the horned beetle *Allomyrina dichotoma* L. (Coleoptera: Scarabaeidae). *Ecological Entomology*, *30*, 692–699.

Pointer, M. A., Harrison, P. W., Wright, A. E., & Mank, J. E. (2013). Masculinization of gene expression is associated with exaggeration of male sexual dimorphism. *PLoS Genetics*, *9*(8), e1003697.

Pomiankowski, A. (1987). The costs of choice in sexual selection. *Journal of Theoretical Biology*, *128*(2), 195–218.

Pomiankowski, A., & Moller, A. P. (1995). A resolution of the lek paradox. *Proceedings of the Royal Society of London B: Biological Sciences*, *260*(1357), 21–29.

Price, J., Oyajobi, B. O., Oreffo, R. O., & Russell, R. G. (1994). Cells cultured from the growing tip of red deer antler express alkaline phosphatase and proliferate in response to insulin-like growth factor-I. *Journal of Endocrinology*, *143*(2), R9–R16.

Prokuda, A. Y., & Roff, D. A. (2014). The quantitative genetics of sexually selected traits, preferred traits and preference: A review and analysis of the data. *Journal of Evolutionary Biology*, *27*(11), 2283–2296.

Prum, R. O. (1997). Phylogenetic tests of alternative intersexual selection mechanisms: Trait macroevolution in a polygynous clade (Aves: Pipridae). *American Naturalist*., *149*, 668–692.

R Core Team (2013). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from http://www.R-project.org/

Radwan, J. (2008). Maintenance of genetic variation in sexual ornaments: A review of the mechanisms. *Genetica*, *134*(1), 113–127.

Radwan, J., Engqvist, L., & Reinhold, K. (2016). A paradox of genetic variance in epigamic traits: Beyond "good genes" view of sexual selection. *Evolutionary Biology*, *43*(2), 267–275.

Rowe, L., & Houle, D. (1996). The lek paradox and the capture of genetic variance by condition dependent traits. *Proceedings of the Royal Society of London B: Biological Sciences*, *263*(1375), 1415–1421.

Schulman, V. K., Folker, E. S., Rosen, J. N., & Baylies, M. K. (2014). Syd/JIP3 and JNK signaling are required for myonuclear positioning and muscle function. *PLoS Genetics*, *10*(12), e1004880.

Searcy, W. A., & Nowicki, S. (2005). *The evolution of animal communication: Reliability and deception in signaling systems*. Princeton, NJ: Princeton University Press.

Shingleton, A. W., & Frankino, W. A. (2013). New perspectives on the evolution of exaggerated traits. *BioEssays*, *35*(2), 100–107.

Simão, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., & Zdobnov, E. M. (2015). BUSCO: Assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics*, *31*(19), 3210–3212.

Siva-Jothy, M. T. (1987). Mate securing tactics and the cost of fighting in the Asian horned beetle, *Allomyrina dichotoma* L. (Scarabaeidae). *Journal of Ethology*, *5*, 165–172.

Smith, A. B., Hawks, D. C., & Heraty, J. M. (2006). An overview of the classification and evolution of the major scarab beetle clades (Coleoptera: Scarabaeoidea) based on preliminary molecular analyses. *The Coleopterists Bulletin*, *60*(sp5), 35–46.

Snell-Rood, E. C., Cash, A., Han, M. V., Kijimoto, T., Andrews, J., & Moczek, A. P. (2011). Developmental decoupling of alternative phenotypes: Insights from the transcriptomes of horn polyphenic beetles. *Evolution*, *65*(1), 231–245.

Snell-Rood, E. C., & Moczek, A. P. (2012). Insulin signaling as a mechanism underlying developmental plasticity: The role of *FOXO* in a nutritional polyphenism. *PLoS ONE*, *7*, e34857.

Stuglik, M. T., Babik, W., Prokop, Z., & Radwan, J. (2014). Alternative reproductive tactics and sex-biased gene expression: The study of the bulb mite transcriptome. *Ecology and Evolution*, *4*(5), 623–632.

Surget-Groba, Y., & Montoya-Burgos, J. I. (2010). Optimization of *de novo* transcriptome assembly from next-generation sequencing data. *Genome Research*, *20*, 1432–1440.

Suttie, J. M., Gluckman, P. D., Butler, J. H., Fennessy, P. F., Corson, I. D., & Laas, F. J. (1985). Insulin-like growth factor 1 (IGF-1) antler-stimulating hormone? *Endocrinology*, *116*, 846–848.

Tang, H. Y., Smith-Caldas, M. S., Driscoll, M. V., Salhadar, S., & Shingleton, A. W. (2011). FOXO regulates organ-specific phenotypic plasticity in *Drosophila*. *PLoS Genetics*, *7*(11), e1002373.

Tomkins, J. L., Radwan, J., Kotiaho, J. S., & Tregenza, T. (2004). Genic capture and resolving the lek paradox. *Trends in Ecology & Evolution*, *19*(6), 323–328.

Ventura, T., Manor, R., Aflalo, E. D., Weil, S., Khalaila, I., Rosen, O., & Sagi, A. (2011). Expression of an androgenic gland-specific insulin-like peptide during the course of prawn sexual and morphotypic differentiation. *ISRN Endocrinology*, *2011*, 476283.

Warren, I. A., Gotoh, H., Dworkin, I. M., Emlen, D. J., & Lavine, L. C. (2013). A general mechanism for conditional expression of exaggerated sexually-selected traits. *BioEssays*, *35*(10), 889–899.

Wasik, B. R., & Moczek, A. P. (2012). *Pangolin* expression influences the development of a morphological novelty: Beetle horns. *Genesis*, *50*, 404–414.

Waterhouse, R. M., Seppey, M., Simão, F. A., Manni, M., Ioannidis, P., Klioutchnikov, G., … Zdobnov, E. M. (2017). BUSCO applications from quality assessments to gene prediction and phylogenomics. *Molecular Biology and Evolution*, *35(3)*, 543–548.

Wilkinson, G. S., Breden, F., Mank, J. E., Ritchie, M. G., Higginson, A. D., Radwan, J., … Rowe, L. (2015). The locus of sexual selection: Moving sexual selection studies into the post-genomics era. *Journal of Evolutionary Biology*, *28*(4), 739–755.

Wilkinson, G. S., Johns, P. M., Metheny, J. D., & Baker, R. H. (2013). Sex-biased gene expression during head development in a sexually dimorphic stalk-eyed fly. *PLoS ONE*, *8*(3), e59826.

Yu, L., Fernandez, S., & Brock, G. (2017). Power analysis for RNA-Seq differential expression studies. *BMC Bioinformatics*, *18*(1), 234.

Zelditch, M. L., Swiderski, D. L., & Sheets, H. D. (2012). *Geometric morphometrics for biologists: A primer*. Cambridge, MA: Academic Press.

Zinna, R., Gotoh, H., Brent, C. S., Dolezal, A., Kraus, A., Niimi, T., … Lavine, L. C. (2016). Endocrine control of exaggerated trait growth in rhinoceros beetles. *Integrative and Comparative Biology*, *56*(2), 247–259.

SUPPORTING INFORMATION

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

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