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INVESTIGATION

Oviposition but Not Sex Allocation Is Associated with Transcriptomic Changes in Females of the Parasitoid Wasp Nasonia vitripennis

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ABSTRACT Linking the evolution of the phenotype to the underlying genotype is a key aim of evolutionary genetics and is crucial to our understanding of how natural selection shapes a trait. Here, we consider the genetic basis of sex allocation behavior in the parasitoid wasp Nasonia vitripennis using a transcriptomics approach. Females allocate offspring sex in line with the local mate competition (LMC) theory. Female-biased sex ratios are produced when one or a few females lay eggs on a patch. As the number of females contributing offspring to a patch increases, less female-biased sex ratios are favored. We contrasted the transcriptomic responses of females as they oviposit under conditions known to influence sex allocation: foundress number (a social cue) and the state of the host (parasitized or not). We found that when females encounter other females on a patch or assess host quality with their ovipositors, the resulting changes in sex allocation is not associated with significant changes in whole-body gene expression. We also found that the gene expression changes produced by females as they facultatively allocate sex in response to a host cue and a social cue are very closely correlated. We expanded the list of candidate genes associated with oviposition behavior in Nasonia, some of which may be involved in fundamental processes underlying the ability to facultatively allocate sex, including sperm storage and utilization.

KEYWORDS sex allocation transcriptomics behavior gene expression local mate competition

Linking the evolution of the phenotype to the underlying genotype remains a key aim of evolutionary genetics. To fully understand how natural selection is shaping a given trait, it is necessary to understand the constraints placed on adaptation at the phenotypic level by the underlying genetic architecture (Sokolowski 2001; Orr 2005). In some cases, one or a relatively few genes have provided a good understanding of the genetic basis of a given behavioral trait. One of the best examples is the roversitter polymorphism in the foraging strategy of Drosophila melanogaster larvae, which is attributable to two alleles of the foraging (for) gene and is a cGMP-dependent protein kinase (de Belle and Sokolowski 1987; Osborne et al. 1997). However, it has been argued that intensive efforts to explain the genetic basis of quantitative traits by identifying underlying genes have yielded rather little (Travisano and Shaw 2013) and that genomic methods such as QTL analysis may leave important small-effect loci undiscovered (Rockman 2011; Travisano and Shaw 2013). For some behavioural traits, the underlying genetic architecture can be very complex with, for example, epistatic effects and pleiotropy to consider (Orr 2005; Radwan and Babik 2012). These complexities of genetic architecture and the polygenic basis of many traits have led to the suggestion that we should focus on evolutionary processes at the phenotypic level instead (Rockman 2011). However, gene-focused and process-focused approaches could be reconciled by studying the genetic basis of quantitative traits where our understanding of phenotypic evolution is already extensive.

Here, we contrast the transcriptomic responses detected at the level of the whole body for two different behaviors related to sex allocation. Our knowledge of how natural selection shapes sex allocation is perhaps more developed than for any other behavioral trait (West 2009). There is
a large body of theoretical literature, from the early works of Düsingu and Fisher describing how frequency-dependent selection acts on sex allocation (Fisher 1930; Edwards 2000) to the pioneering work of Hamilton (1967) and Trivers and Willard (1973). There have been many refinements to sex allocation theory since (reviewed by West 2009), including the recent work of Macke et al. (2011) that explored the ability of individuals to adjust the sex ratio depending on the environment in which they had evolved.

The parasitoid wasp Nasonia vitripennis has been the focus of much research on sex allocation behavior. Females of this species allocate the sex of their offspring in line with the local mate competition (LMC) theory (Hamilton 1967; Werren 1980, 1983), which predicts a female-biased sex ratio when mating occurs between the offspring of one or a small number of females on a patch. Briefly, natural selection favors mothers who bias the sex ratio toward their daughters, thereby minimizing competition among her sons for mates and increasing the number of mates available for those sons (Taylor 1981). As the number of females (foundresses) contributing offspring to a patch increases, less female-biased sex ratios are favored. LMC theory has been extended, using N. vitripennis as a model, to incorporate a variety of more complex situations, including when the level of LMC on a patch is asymmetrical between broods (Shuker et al. 2005), when females are related to their cofoundresses (Shuker et al. 2004) and, from a mechanistic point of view, which cues females use to infer the levels of LMC their sons will experience (King et al. 1995; Shuker and West 2004). Moreover, females can rapidly alter their pattern of sex allocation when the environment changes (e.g., when other females arrive on a patch) (Shuker et al. 2007). In short, we know a lot about sex allocation behavior in Nasonia vitripennis at the phenotypic level but little regarding the genetic basis of the behavior, or about how genetic architecture may constrain the evolution of this trait.

Genetic studies of sex ratio in Nasonia vitripennis have been largely quantitative. Early work by Orzack and colleagues demonstrated genetic variation for the ability to facultatively adjust sex ratio in line with theoretical predictions (Orzack and Gladstone 1994). More recently, Pannebakker et al. (2008) performed a mutation accumulation study, demonstrating that sex ratio has a mutational heritability of 0.001–0.002. The authors calculated that the genetic variation observed in natural populations of N. vitripennis is lower than expected given their mutational parameters, suggesting that other selective pressures may be acting against mutations in sex ratio genes, i.e., sex ratio genes may be pleiotropic and have influence over other fitness-related traits. Building upon this hypothesis, Pannebakker et al. (2011) identified quantitative trait loci (QTL) associated with sex ratio, which exhibited some overlap with clutch size QTL. These data suggest that perhaps some of the same genes influence both sex ratio and clutch size and have identified genomic locations for further study.

More recently, patterns of differential gene expression between ovipositing and resting N. vitripennis females have been uncovered (Pannebakker et al. 2013). In particular, oviposition is associated with focusing gene expression away from certain aspects of metabolism during egg-laying. Of the 332 differentially expressed genes identified, the majority were associated with greater expression in resting females. In that experiment, females were alone when ovipositing (i.e., they were single “foundresses”) or resting. Here, we extend this experiment to test whether facultative sex allocation under LMC also leaves a transcriptomic footprint. We explore whole-body gene expression changes under two sets of environmental cues. These cues are used by females to assess the level of LMC experienced by offspring and therefore to allocate sex (Shuker and West 2004). First, we examine gene expression when females oviposit in the presence of cofoundresses (a “social” LMC cue); second, we examine gene expression when females oviposit on either fresh or parasitized hosts, where the presence of eggs is a “host” cue used to facultatively change sex allocation. We therefore determine whether the same genes are upregulated during oviposition when the cues known to influence sex allocation are also available.

MATERIALS AND METHODS

Study species

Nasonia vitripennis (Hymenoptera, Chalcidoidea) is a generalist parasitoid of large dipteran pupae including species of Calliphoridae. Depending on host species, females oviposit between 20 and 50 eggs in an individual host, with male offspring emerging just before females (after approximately 14 d at 25°C) (Whiting 1967). Male individuals are brachypterous and are unable to fly, remaining close to the emergence site where they compete with each other for emerging females, including their sisters. Females disperse after mating to locate new hosts.

The focal females used in this experiment were from the AsymC strain. This line was originally isolated in 1986 by curing the wild-type strain LabII of Wolbachia and is known to be free of sex-ratio distorters (Gherna et al. 1991; Werren 1991). Wasps have been maintained on Calliphora vomitoria or C. vicina hosts at 25°C, 16L:8D light conditions ever since. For some experimental treatments cofoundresses were required. These data were taken from the red-eye mutant STDR strain, allowing us to track the offspring of a single AsymC female using eye color. The STDR strain is maintained under conditions identical to the AsymC strain.

Importantly, the genome of the AsymC strain has been sequenced and annotated by the Nasonia Genome Working Group (Werren et al. 2010), permitting the direct mapping of transcriptomic data from this experiment onto available genomic resources.

Experimental design

To control for possible host and other maternal effects, we isolated approximately 450 single (2-d-old, mated) wild-type AsymC females from the mass cultures into individual glass vials and provided each with three hosts. We used females from the resulting F1 generation in the experiment, one female per “grandmother.” These experimental females were then provided with a single host for 24 hr as a pretreatment to facilitate egg development. Pretreatment hosts were discarded and each female was then given a piece of filter paper soaked in honey solution for a further 24 hr.

For the transcriptomics experiment, we used a 2 × 3 factorial experimental design, giving a total of six treatment combinations with N = 70 replicates per treatment combination (total N = 420 experimental females). Females were placed into either of two “foundress number” groups: (i) single foundress or (ii) 10 foundress (i.e., one experimental female plus nine red-eye mutant STDR cofoundresses, which allowed us to follow the sex ratios produced by experimental females). Females were then subdivided into one of three “host treatment” groups, in which females were provided with: (i) no hosts; (ii) three fresh hosts; or (iii) three previously parasitized hosts. Group iii hosts had been parasitized for the preceding 24 hr by individual STDR females. Given that females can rapidly alter their sex ratios as the environment changes (Shuker et al. 2007), we tested for genes that will alter their expression rapidly upon exposure to these known sex allocation cues (cofoundress number and parapheritized hosts). After a period of 3 hr (the experiment was run between 0900 and 1200, at 25°C, 16L:8D light conditions), AsymC females were placed into 1.5-ml microcentrifuge tubes, flash-frozen in liquid nitrogen, and stored at −80°C prior to RNA extraction. STDR cofoundresses were discarded and the hosts were retained in the same temperature and light conditions to allow the offspring to develop and eclose normally.
RNA extraction
Prior to RNA extraction, experimental females exposed to identical treatment combinations were pooled into groups of 10. This resulted in seven pooled samples for each of the six treatment combinations (i.e., in terms of pooled sample, N = 42). Key tissues for the behaviors being tested are not known; therefore, RNA was isolated from whole bodies using the TRIzol Plus RNA purification kit in conjunction with the PureLink RNA mini kit (Life Technologies, Paisley, UK) according to the manufacturer’s instructions. Optional steps for “On-Column PureLink DNase Treatment During RNA Purification” were followed. Concentration and integrity of RNA samples were checked using a Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE) and a Bioanalyzer system (Agilent Technologies, Santa Clara, CA).

Library preparation and sequencing
Total RNA samples were prepared for sequencing using the Illumina TrueSeq RNA Sample Prep Kit (Illumina, San Diego, CA) following the Illumina TrueSeq Sample Preparation v2 (Low Sample) protocol. Briefly, mRNA molecules were purified from total RNA samples using oligo-dT attached magnetic beads and fragmented using divalent cations at 94°C. First strand cDNA synthesis was carried out using reverse transcriptase and random hexamer primers. Second strand synthesis was carried out using RNA polymerase I and RNase H. Overhangs resulting from fragmentation were converted to blunt ends and 3′ ends were subsequently adenylated. Sanger indexing adapters were ligated to the fragments that were then purified and PCR-amplified to create the final cDNA libraries for sequencing. Libraries were sequenced on the HiSeq2000 according to the manufacturer’s instructions. Raw sequence reads are available from the Gene Expression Omnibus database at NCBI (Accession: GSE74241).

Mapping, filtering, and annotation of sequence reads
Reads were aligned to Nasonia Official Gene Set version 2 (OGS2, January 2012, http://arthropods.eugenes.org/EvidentialGene/nasonia/genes/) using GSNAP software v2013-02-05. Reads that mapped ambiguously, i.e., to more than one feature, were discarded. Annotation and Gene Ontology information were obtained from the annotation for OGS2 generated by the Nasonia Genome Working Group (http://www.hymenopteragenome.org/nasonia/?q=evidential_gene_data).

Read counts per sample were obtained using HTseq version 0.5.4p1 using “intersection-strict” mode, whereby the whole read must map to a single transcript to be counted.

In terms of the dataset, we initially obtained 1,296,283,832 reads, of which 95.75% (1,241,156,436) mapped uniquely to OGS2 (see Supporting Information, Table S1 for individual library mapping statistics). Because of the “intersection-strict” mode applied to HTSeq, some of the reads that map ambiguously to a gene were removed and we were left with 155,799,857 reads that mapped to 20,388 genes. Transcripts present at extremely low abundance are a common source of noise in this type of study. Therefore, genes with less than 100 reads across all 42 samples were excluded from further analysis (i.e., a mean of less than three reads per replicate). Removing these very low abundance transcripts left us with 155,652,340 reads from 12,230 genes for the analysis of differential expression (i.e., this last step removed 40.01% of the genes but only 0.09% of the reads).

Statistical analysis
Tests for differential expression were carried out using DESeq (version 1.12.1) (Anders and Huber 2010) implemented in the R environment (version 3.0.2, R Core Team 2013). As a first processing step, effective library size was estimated using robust regression. These size factors were then used to bring raw read counts per gene to a common scale (normalization). DESeq tests for differential expression within a generalized linear model framework using the negative binomial error distribution to model transcript abundance. Given the 2 × 3 factorial design of this experiment, we made use of the generalized linear model framework to determine which genes were significantly differentially expressed as a result of either of the main effects (foundress number and host type), and also to explore any interaction between the main effects. When there was significant differential expression in association with a main effect, follow-up pairwise tests were performed in DESeq to explore differences between treatment groups. The significance of differential expression is estimated for each gene and then corrected for multiple testing using the Benjamini and Hochberg (1995) false discovery rate (FDR) adjustment. Genes were considered to be differentially expressed if, after FDR adjustment, P < 0.05.

Correlations in expression changes between host treatment groups were examined using linear regression implemented in the R environment (version 3.0.2, R Core Team 2013).

Enrichment analysis
Enrichment analysis of Gene Ontology (GO) terms was carried out to determine whether groups of differentially expressed genes are associated with any particular function(s). The analysis was performed using the Blast2GO suite (Conesa et al. 2005). The package uses Fisher’s exact test to detect annotation differences between two sets of sequences and corrects for multiple testing using FDR (Benjamini and Hochberg 1995). Groups of differentially expressed genes were compared to Nasonia Official Gene Set version 2 to determine whether these groups contained an overrepresentation of any GO terms.

Data availability
Table S1 contains the mapping statistics for each of the 42 sequenced libraries. Table S2 contains a list of all genes differentially expressed in association with the main effect “host treatment”. Table S3 contains a list of all genes differentially expressed between host treatment A (no hosts) and host treatment B (fresh hosts). Table S4 contains a list of all
genes differentially expressed between host treatment A (no hosts) and host treatment C (parasitised hosts). Table S5 contains a list of all the genes differentially expressed in response to oviposition in both this study and the study carried out by Pannenbakker et al. (2013). Table S6 contains a list of genes differentially expressed in association with host treatment that reside with an oviposition QTL significant at the genome-wide level (Pannenbakker et al. 2011). Table S7 contains a list of genes differentially expressed in response to host treatment that are also differentially expressed in N. vitripennis females in response to bacterial infection (Sackton et al. 2013). File S1 shows the methods used to carry out additional statistical analysis using only genes known to be differentially expressed in the head of N. vitripennis females based on Hoedjes et al. 2015). Gene expression data are available at GEO with the accession number: GSE74241.

RESULTS
Oviposition, i.e., the presence or absence of a host, led to significant changes in gene expression, whereas cues associated with sex allocation did not. In fact, facultative sex allocation cues were not associated with any differential expression in female N. vitripennis at the whole-body level.

From a total of 12,230 genes tested, 1359 were differentially expressed in response to host treatment that are also differentially expressed in N. vitripennis females in response to host treatment (Figure 1). Pair-wise analyses revealed that in females ovipositing on fresh hosts, 1170 genes (844 of which were upregulated) were differentially expressed relative to the control group (FDR P < 0.05) (Table S3). When ovipositing on previously parasitized hosts, 822 genes (540 of which were upregulated) were differentially expressed relative to the control group (FDR P < 0.05) (Table S4).

In terms of sex allocation behavior, there were no differentially expressed genes between those females ovipositing on fresh hosts and those ovipositing on previously parasitized hosts, so that the effects of oviposition itself on gene expression were consistent across host type. There were also no detectable gene expression changes in Nasonia vitripennis females in response to the presence of cofoundresses. Thus, neither superparasitism nor the presence of cofoundresses, both of which influence sex allocation, produced gene expression changes detectable at the level of the whole body.

Notably, 11 genes exhibited a greater than four-fold log2 change in expression (a common measure of differential expression). Six of these highly expressed genes were upregulated in females ovipositing on either fresh or previously parasitized hosts as compared to “resting”
confirmed that oviposition behavior, regardless of the LMC context in which that oviposition takes place, is associated with changes in gene expression. Below we discuss some interesting candidate genes for oviposition and perhaps sex allocation at a more fundamental level.

We have expanded on the set of candidate genes associated with oviposition behavior first generated by Pannebakker et al. (2013). Those authors used a tag-based transcriptome sequencing approach (“DeepSAGE”) to identify 232 genes (322 “tags”) that were differentially expressed between ovipositing and resting *N. vitripennis* females. Our RNA-seq approach revealed 1359 candidates, of which 46 overlapped with their list (Table S2 and Table S5). For instance, Pannebakker et al. (2013) found *glucose dehydrogenase* (gd; OGS2 ID: Nasvi2EG0010910) to be upregulated in ovipositing females. The GLP protein, expressed in the spermathecal duct, is associated with the storage and utilization of sperm in *Drosophila melanogaster* (Lida and Cavener 2004). We confirmed that *gd* is indeed upregulated in ovipositing females (Table S2, Table S3, Table S4, Table S5) and discovered that a total of eight *glucose dehydrogenase* genes were differentially expressed in ovipositing vs. resting females. Given that female *N. vitripennis* need to control sperm release very precisely to allocate sex, we suggest that this gene family may have a role in play in adaptive sex allocation behavior, and we are currently developing functional genomic tests of this hypothesis.

A QTL analysis by Pannebakker et al. (2011) detected three sex ratio QTLs, one of which was significant at the genome level. Given that sex allocation was not associated with changes in gene expression, it is difficult to make a direct comparison between the results of that study and the results we present here. However, we did find that 104 of the genes differentially expressed during oviposition reside within the QTL significant at the genome-wide level (Table S6). These genes did not vary in expression in response to LMC cues used by females. However, we have confirmed their involvement in oviposition, and further study will reveal whether these genes turn out to be “sex ratio” genes insofar as they are involved in the more fundamental processes underlying the ability to facultatively allocate sex.

Interestingly, we found that the female-specific isoform of the transcription factor *doublesex* (*dxs*) was found to be upregulated in ovipositing females. Differential splicing of male and female *dxs* mRNA generating sex-specific proteins that regulate downstream somatic sexual dimorphism was first described in *Drosophila melanogaster* (Baker and Wollner 1988). Since then, the *doublesex/mab-3 related* (*Dmr*) family of transcription factors has been shown to be involved in sex-specific differentiation in all animals studied (Kopp 2012). In *Drosophila*, genes of the sex determination hierarchy, including *dxs* and *fruitless* (*fru*), control the development and differentiation of sex-specific tissues, thereby establishing gender-specific physiology, neural circuitry, and of course behavior (Villela and Hall 2008; Yamamoto and Koganezawa 2013). Dxs also influences sex-specific reproductive behaviors, such as male courtship and female receptivity in *D. melanogaster* (Rideout et al. 2007, 2010; Zhou et al. 2014), sex-specific morphology, i.e., wing shape in *Nasonia* spp. (Loehlin et al. 2010), and mimicry in swallowtail butterflies (*Papilio polytes*) (Kunte et al. 2014). Here, we have evidence to support that *doublesex* may be involved in oviposition, another sex-limited behavior.

In addition, we found that three genes annotated as major royal jelly protein-like (*mrjpl9*; Nasvi2EG014218, *mrjpl6*; Nasvi2EG021396 and *mrjpl5*; Nasvi2EG014323) and one annotated as yellow *x-2* (Nasvi2EG010358) were differentially expressed in association with “host treatment.” During their first 3 d, honey bee larvae are fed with royal jelly (RJ), a substance secreted by nurse bees and involved in the development of queen larvae (Snodgrass 1925; Buttstedt et al. 2014). The nine most abundant proteins in RJ became known as the major royal jelly proteins, MRJPs, now known to have evolved from the yellow
gene family (Ferguson et al. 2011; Buttstedt et al. 2014). Evidence suggests that Yellows and MRJPs have diverse and context-dependent roles in development and reproduction (Drapeau et al. 2006) and, notably, *Nasonia* has the largest number of yellow/MRJPs so far found in any insect, with a particular expansion of mrjps (Winet et al. 2010). Their function in *Nasonia* is still unknown, and Winet et al. (2010) note their broad expression across tissues and life stages. Our results here highlight its association with oviposition in *Nasonia vitripennis*.

Enrichment analyses can sometimes provide insight into the possible functions of differentially expressed genes. In the present case, no GO terms were overrepresented, which may be due to the polygenic nature of this trait. However, looking to other studies helped to assign function to some of our candidates. For example, several of our differentially expressed genes are annotated as venom proteins and serine proteases and are likely to be linked to the process of oviposition itself (de Graaf et al. 2010) (Tables S2, Table S3, Table S4). Also, we found that 27 genes differentially expressed in *Nasonia vitripennis* individuals responding to bacterial infection (Sackton et al. 2013) (Table S7) are also differentially expressed in response to oviposition. These comparisons suggest that some of the genes upregulated as females lay eggs could in fact be “immunity genes” that allow females to guard against bacterial infection posed to them by their dipteran hosts. We note that glucose dehydrogenase genes have also been linked with immune response in the silkworm (Sun et al. 2012), and further study will confirm whether they serve an immune response and/or a sex allocation function in *Nasonia*.

In addition, we found that several of the genes differentially expressed in response to oviposition are annotated as heat shock proteins (HSPs) (Table 1, Table S2, Table S3, Table S4). HSPs, first described in 1962, are highly conserved cellular stress proteins present in all organisms that have known functions in reproduction (Neuer et al. 2000). For instance, HSP expression is a crucial process during oogenesis in a number of species, including *Drosophila melanogaster* (Ambrosio and Scheid 1984). Heat shock proteins act as chaperones (mediating folding, assembly, and transport of other proteins) and also in response to cellular stresses, including temperature changes and the presence of viral and bacterial infections (Neuer et al. 2000); it is possible that HSPs may be expressed in response to the stresses that a female experiences while interacting with hosts during oviposition.

Previous studies have successfully used transcriptomic approaches to identify candidate genes for sex-specific behaviors. For example, a study by Immonen and Ritchie (2012) identified genes in *Drosophila melanogaster* females that were differentially expressed after only 15 min of exposure to a male courtship song. Gene expression changes were observed in the heads of females in association with stimulation by a courtship song. Gene expression changes can be induced by short-term exposure to stimuli. However, it also suggests that behavioral transcriptomic studies may pick up immune genes as those most likely to display rapid changes in expression even in a tissue-specific study.

In summary, we did not observe any transcriptomic changes in response to two essential LMC cues, foundress number, and prior parasitism of a host. Future studies targeted to gene expression in specific tissues, for example, the brain, may reveal candidate genes for sex allocation not picked up in this analysis. Using a whole-body RNA-seq approach, we instead revealed some potentially useful mechanisms, highlighted above, that may be involved in sex allocation behavior at a more fundamental level.

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