Assessing Alternative Splicing as a Source of Genetic Variation in Biparental Burying Beetles, *Nicrophorus orbicollis*

by

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Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in

Biological Sciences

YOUNGSTOWN STATE UNIVERSITY

DECEMBER 2023

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ABSTRACT

Burying beetles have served as a model to answer diverse questions about the evolution of social behavior in insects and to understand the physiological and evolutionary mechanisms involved in the manifestation of parental care behavior. More recently, an increasing number of genomic and transcriptomic resources have become available in *Nicrophorus* spp. This study utilizes this data to assay sources of variation in the *Nicrophorus orbicollis* transcriptome with the specific aims to 1) evaluate the extent of alternative splicing in this species and 2) assess differential expression and alternative splicing in genes that are associated with distinct parental behavioral phenotypes in male and female burying beetles during breeding.

This was achieved by differential expression analysis of *N. orbicollis* transcriptomes for parents showing high and low care provisioning behavior. Two algorithms were used to increase predictive accuracy in detecting alternatively spliced genes. Genes that were alternatively spliced and differentially expressed between behavioral groups were subject to further functional characterization. An overall profile of alternative splicing was determined, indicating that many transcripts resulting from alternative splicing were the result of several types of alternative splicing events. There was also evidence for a higher occurrence of intron retention events than exon skipping events. Additionally, the occurrence of alternative splicing was not found to be more common in differentially expressed genes for any specific biological process associated with parental care behavior. Still, evidence for alternative splicing within genes relevant to variability of the parental care phenotype was presented and potential behavioral implications were discussed.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Carmen Panaitof, for her immense patience and support throughout the development of the thesis and for giving me the space to explore an avenue of research that aligned with my interests. I would also like to extend thanks to the other members of my committee, Dr. Jack Min, for teaching me the fundamentals of bioinformatics, and Dr. Jonathan Caguiat, for his feedback in the writing process. Additional thanks are extended to the entirety of faculty within the YSU Department of Chemical and Biological Sciences for everything they have taught me over the past six years.

Personally, I would like to thank Corie Hoselton for supporting me emotionally throughout my graduate studies. Finally, I must thank my family for providing me the opportunity to pursue higher education.

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INTRODUCTION

Biparental behavior in burying beetles

Biparental care, in which both parent organisms contribute to rearing their offspring, is considered the norm in most human societies (Geary, 2000). However, except for its near universality in birds (Burley and Johnson, 2002) and presence in some mammals (Gubernick and Teferi 2000), poison frogs, black rock skinks, or cichlid fishes, biparental care is rare in other species (Clutton-Brock, 1991, Dulac et al., 2014), and the exact genetic and neural mechanisms underlying these behaviors have not been fully elucidated.

While biparental care is rare in invertebrates, burying beetles (Coleoptera: Silphidae: *Nicrophorus*) exhibit this form of care and provide a unique model system for studying the mechanisms driving the shift from non-parental to parental care. Burying beetles are also a convenient behavioral model to observe in a laboratory setting due to their relatively short breeding cycle that can be replicated year-round. The *Nicrophorus orbicollis* breeding cycle has been well documented (Pukowski, 1933; Milne and Milne, 1976; Scott, 1998) and commences when the parent beetles locate a fresh small animal carcass. The carcass is prepared and rolled into a carrion ball, which will provide a food source for future larvae. Approximately 24 to 48 hours later, the female beetle will lay her eggs in the soil near the reproductive resource. After an additional 72 hours, larvae hatch and migrate toward the carrion ball. Parent beetles regurgitate partially digested carrion into their young's mouth parts for the young larvae to consume. Unlike in other burying beetle species, parental care in *N. orbicollis* is obligatory for offspring survival (Trumbo, 1992; Eggert at al., 1998; Benowitz and Moore, 2016).

To prevent giving their rare food source to unrelated conspecifics, parents have been shown to exhibit temporal kin recognition and to cannibalize larvae that arrive at the reproductive resource until approximately 8 to 12 hours before their own larvae hatch (Oldekop et al., 2007). Other parental behaviors include protecting larvae from infanticidal intruders of either sex, maintaining the brood chamber, an underground, walled-off cavity containing the carrion ball with larvae, and spreading antimicrobial secretions onto the carrion ball to slow its decay (Fetherston et al., 1994; Arce et. al, 2012).

After around 24 hours of life, larvae begin the first of several molting cycles, called instars. As they grow through the molting cycles, larvae become able to self-feed. Within seven to ten days of hatching, larvae leave the carrion ball and move into nearby soil to pupate, ending the breeding cycle and period of parental care (Scott, 1998).

Evolution of biparental care behavior

While the molecular underpinnings of *N. orbicollis* biparental behavior have not been fully elucidated, the evolutionary drive for biparental behavior in general has been well established. For parental behaviors to arise, there must be a net benefit to a parent involved in care, which is typically increased odds of offspring survival. Otherwise, from an evolutionary standpoint, it would be beneficial for a parent to abandon their brood. Similarly, for biparental care to arise, benefits to the offspring must be greater than the fitness that would be gained by either deserting parent by remating or by increasing odds of personal survival (Barta et al., 2014; Royle et al., 2016).

In *N. orbicollis*, both males and females spend time preserving the carrion ball and brood chamber. However, females have been demonstrated to feed larvae more frequently than males (Fetherston et al., 1990; Smiseth et al., 2003; Capodeanu-Nagler et al., 2018). Additionally, females often remain with the larvae until they depart the brood chamber, while males are more likely to leave before complete larval development (Scott, 1998). Increased male care occurs when necessary to compensate for loss of the female parent (Fetherston et al., 1994; Rauter and Moore, 2003; Smiseth and Moore, 2004), and presence of the male parent has been suggested to serve as a failsafe in situations where the female is incapacitated (Parker et al., 2015). There is also evidence that the primary benefit of prolonged male presence in biparental conditions is defense of larvae and their rare carcass from other insects and conspecifics (Trumbo 1991, 2006; Ratz et al, 2022).

Male departure time has been shown to be associated with advanced larval development state and small carcass size (Trumbo, 1991). Protection provided by a male must outweigh the portion of the carrion consumed by the male and the other reproductive opportunities he foregoes. However, it is believed that the rarity of the reproductive resource required for burying beetle reproduction, a fresh small animal carcass, has put evolutionary pressure on biparental conditions (Trumbo and Sikes, 2021). In many species, males can increase reproductive success

by abandonment to mate with many females (Edward and Chapman, 2011), but the ephemeral reproductive resource favors paternal investment to his current brood, as reproductive opportunities may be scarce. In fact, the ability of *Nicrophorus* spp. beetles to successfully conceal a small animal carcass represents a highly specialized facet of their evolved care behavior. One study assessed the ability of *N. orbicollis* to prepare a concealed carcass relative to another closely related beetle, *Ptomascopus morio* (Trumbo and Sikes, 2021). While *P. morio* also reproduces on small animal carcasses, it is more often a brood parasite of *Nicrophorus concolor* and does not bury its own carcasses (Sikes and Venables, 2013). The study gave a small carcass to breeding pairs of *N. orbicollis* and *P. morio* and then moved the carcass to a field three days later, without the parents, where they were shallowly buried. Of the buried *P. morio* carcasses, 75.9% were discovered by a rival beetle, while none of the *N. orbicollis* carcasses were scavenged by rivals. Successful concealment in *N. orbicollis* is attributed to the processing of carcasses into hairless, featherless carrion balls and the application of antibacterial and antifungal anal secretions to the resource that conceal the scent of rotting flesh from rivals (Trumbo, 2017; Trumbo and Dicapua, 2021). The evolution of these behaviors would be necessary as part of a strategy to monopolize an ephemeral resource, such as a small animal carcass.

Neurophysiology of *N. orbicollis* reproductive behavior

Previous attempts to characterize the underlying neuroendocrine factors driving the shift to biparental behavior used a candidate gene or candidate physiological factor that was systematically investigated for its role in the expression or modulation of behavior. This methodology relied on identifying a particular gene, such as *neuropeptide F* (Cunningham et al., 2016), or some quantifiable hormone, such as Juvenile Hormone (JH) concentration (Panaitof et al., 2004), that had prior compelling evidence from similar species to contribute to a behavioral phenotype. These candidates were then assayed in *N. orbicollis* to demonstrate if they were experimentally implicated or uninvolved with the transition to care behavior.

Early work regarding the neurophysiology of parental behavior in *N. orbicollis* discovered a correlation between JH concentration and the onset of parenting. JH, the main insect reproductive hormone, increases rapidly upon discovery of a small animal carcass with corresponding increases in ovarian mass (Trumbo, 1998). In breeding females, JH levels rise

regardless of mate presence or virginity and remain elevated during early parental care, returning to pre-breeding levels after approximately three days of parenting (Scott and Panaitof, 2004; Panaitof et al., 2004). Based on knowledge that JH could interact with the biogenic amines in honeybees (Harano et al., 2008; Sasaki et al., 2012) and that serotonin (Alekseyenko et al., 2010; Bubak et al., 2014), octopamine (Saraswati, 2004; Jones et al., 2011), and dopamine (Unoki et al., 2006; Beggs et al., 2007; Liang et al., 2012) have demonstrated links to insect behavior, the levels of these monoamines have also been compared between non-breeding and parental *N. orbicollis* (Panaitof et al., 2016). A significant elevation of dopamine, but not octopamine or serotonin, was identified in parental beetles compared to non-breeding beetles, suggesting that dopaminergic activity may be involved in the shift to parental behavior.

Another candidate molecule study focused on neuropeptide F, the homolog of vertebrate neuropeptide Y, in *N. vespilloides* during parenting (Cunningham et al., 2016). Neuropeptide Y has been demonstrated to influence the drive to self-feed (Nassel and Wegener, 2011), and thus, it was expected that expression of *npf* and its receptor *npfr* would be downregulated in parenting beetles, who must maintain a balance between self-feeding and trophallaxis. Through quantitative RT-PCR analysis of whole head, it was found that *npfr*, but not *npf*, was significantly downregulated in parenting beetles of both sexes. Authors concluded that downregulation of the receptor in head tissue allowed for decreased motivation to self-feed during parenting while also maintaining other tissue-specific functions of neuropeptide F, such as ecdysteroid production (Van Wielendaele et al., 2013) and locomotor function (Hermann et al., 2012).

Overall, studies utilizing the candidate molecule approach have provided evidence that care behavior and its malleability in *N. orbicollis* is underscored by several concurrent neuroendocrine functions. Continuing work with high-throughput genomics and transcriptomics-based methods and bioinformatics analysis can assist in identifying new candidates that could later be validated using the candidate molecule approach.

Burying beetle transcriptomics

More recently, newer, high-throughput genomics and transcriptomics-based methods can be used to study the molecular basis of parental behavior in burying beetles. These studies can now be performed due to the publication of several *Nicrophorus* spp. genomes (Cunningham et al., 2015; Benowitz et al., 2017). Recent attempts to elucidate molecular mechanisms implicated

in the biparental behavior of *N. orbicollis* and other closely related burying beetles have relied on identifying differentially expressed genes in transcriptomic studies. In observing uniparental and biparental *N. vespilloides*, it was found that several genes were differentially expressed universally in parenting beetles (Parker et. al, 2015). This included *vitellogenin*, which is associated with reproductive behavior, and *takeout*, which is associated with mating and feeding behaviors. Downregulation of *vitellogenin* and *takeout* during parenting, relative to higher expression preceding and following parental care, was confirmed via quantitative real-time PCR (qPCR). In animals, vitellogenin represents a yolk protein precursor that is necessary for oocyte development. It is associated with caste-specific behavior in eusocial insects, such as honeybees (Guidugli et al., 2005; Nelson et al., 2007; Amdam and Page, 2010) and ants (Wurm et al., 2011; Corona et al., 2013). It has also been previously demonstrated to be downregulated during parenting in burying beetles (Roy-Zokan et al., 2015) but upregulated in females following oviposition, in case a replacement clutch is required (Scott et al., 2005). Takeout has been shown to regulate circadian influences on feeding (Sarov-Blat et al., 2000; So et al., 2000) and sex-specific courtship in *D. melanogaster* (Dauwalder et al., 2002).

Using Gene Ontology, Parker et al. (2015) also identified high expression of genes involved with digesting and regurgitating food, such as *serine-type endopeptidases*, *fatty-acyl-CoA reductase*, *cytochrome p450* genes, and those involved with oxidation-reduction processes. Also, genes likely involved with producing secretions to maintain the carrion ball were upregulated, including antibacterial proteins, peptidoglycan recognition protein and lysozymes, as well as the anti-fungal protein, thaumatin. Additionally, Parker et al. (2015) observed that neither parent specialized in any given tasks associated with parental care, suggesting that differential expression of care genes between the sexes should show similar patterns. However, parental care in males is reduced when paired with a partner that is actively parenting (Featherston et al., 1990; Smiseth et al., 2005). In fact, when quantifying the mean $\log_2$ fold change in gene expression overall, it was found that biparental males exhibited differential expression levels comparable to post-caring beetles, while biparental females and uniparental females showed statistically significantly higher measures of differential expression. When providing uniparental care, males exhibited a higher degree of differential expression, similar to the profile of biparental females, demonstrating that the malleability of the male care phenotype

(Featherston et al., 1994) has underlying molecular mechanisms activated by changes in environmental conditions.

In a similar transcriptomic study in *N. orbicollis*, Won et al. (2018) identified that the genes encoding defensin and cecropin C, two antimicrobial peptides released in oral and anal secretions to preserve the carrion ball, were upregulated during parental care. Prior work isolating defensin protein from *Tribolium castaneum*, a beetle which does not provide parental care, demonstrated the efficacy of defensin against drug-resistant *Staphylococcus aureus* (Rajamuthiah et al., 2015), while work in *N. vespilloides* has identified defensin as a constituent of secretions used to preserve the carrion ball (Jacobs et al., 2016). Thus, defensin represents an example of an antibacterial peptide having a primary function in personal immunity and an evolved function in social immunity. Similar to Parker et al. (2015), there was also upregulation of lysozyme in anal secretion, which hydrolyzes bacterial cell walls. High expression of lysozyme for the purpose of preserving the reproductive resource for offspring, while its primary function is in innate personal immunity (Hoffmann, 1995), represents an example of how existing genes can develop new, social functionality through evolutionary time. The inclusion of lysozyme in anal secretions as a means of social immunity has also been demonstrated in *N. vespilloides* (Palmer et al., 2016). In this species, *lysozyme* transcripts were shown to be upregulated in parenting relative to non-parenting beetles. These studies suggest that the changes in immune function of parenting *Nicrophorus* spp. beetles may have evolved to effectively prevent decay of their rare reproductive resource for both offspring health (Arce et al., 2012) and for concealment of rotting flesh odorants from competitors.

Social evolutionary functions of genes can also manifest as diminished gene function, representing some parental sacrifice for offspring. For example, Won et al. (2018) also noted a downregulation of *phenoloxidase 2*, another factor in insect personal immunity (Rodriguez-Andres et al., 2012), in mating and parenting female burying beetles. A study with *N. vespilloides* parental females found that *phenoloxidase 2* expression increased if damage was done to the maternal beetle but not to pre-parental levels (Reavey et al., 2014). From this, it is evident that parenting burying beetles have a unique transcriptomic profile underlying several distinct behavioral facets of care.

N. orbicollis parents have been demonstrated to incur fitness costs and die younger than beetles who have mated but not parented (Trumbo and Rauter, 2014), and differential expression of immune transcripts, sacrificing personal immunity for social immunity, may represent an aspect of evolved sacrifice of parental fitness for increased offspring survival.

Another point of interest when characterizing evolved care behavior is variation within the behavior itself. At present, we have a limited understanding of the molecular basis of this behavioral variation. As there are different behavioral phenotypes of parents (uniparental male, uniparental female, biparental male, and biparental female), as well as differences in care intensity or duration, there must be some underlying changes in gene expression and gene regulation. Parker et al. (2015) demonstrated that, similar to changes in reproductive physiology, variable transcriptomic profiles become more convergent as a consequence of mate loss.

Identifying differentially expressed (DE) genes within the transcriptome of *N. orbicollis* can yield further insight to the specific factors underlying their evolved biparental behavior and the variation therein. Additionally, identification of genes undergoing alternative splicing, which has not yet been assessed in burying beetles, could further elucidate the molecular underpinnings of a complex and malleable behavioral phenotype. This thesis has the specific aims to 1) evaluate the extent of alternative splicing (AS) in burying beetles, wherein two methods of AS event and gene detection, AStalavista and rMATS, were used to increase predictive accuracy in AS gene identification and 2) compare the patterns of DE of AS genes associated with distinct parental behavioral phenotypes in male and female burying beetles during breeding, as multigenic DE has been shown to underlie aspects of the parental phenotype and variations therein (Parker et al., 2015; Benowitz et al., 2017; Won et al., 2018). AS further contributes to genetic diversity and has been demonstrated to affect behavioral phenotypes (Singh and Ahi, 2022) and thus, must be considered in determining the molecular mechanisms contributing to burying beetle biparental care behavior.

ASSESSING ALTERNATIVE SPLICING IN BURYING BEETLES

Introduction

Alternative splicing

Alternative splicing is a pre-mRNA processing event that leads to diversification of functional proteins that can be generated from one gene. In RNA splicing events, the non-protein encoding introns of a pre-mRNA transcript are removed, and protein-coding exons are joined to form a mature mRNA. Alternative splicing can occur via differential recognition of splice sites in a pre-mRNA by a spliceosome, causing inclusion or removal of varying RNA sequences (Lamond, 1993). From this, several transcript variants and their encoded protein isoforms can arise from a single gene.

There are four main types of AS events that produce these diverse isoforms: intron retention, exon skipping, alternate donor site usage, and alternate acceptor site usage. Intron retention events occur when a splice site at an exon-intron boundary is skipped, resulting in the intronic sequence being preserved in the mRNA transcript. In exon skipping, an exon is excluded from the mature mRNA by alternate exon usage during splicing. Alternate donor site and alternate acceptor site events result from recognition of cryptic splice sites, resulting in exon shortening or the incorporation of portions of introns in the mature mRNA (Stamm et al., 2005). Additionally, multiple AS events can co-occur during processing of one pre-mRNA into a mature mRNA (Park and Graveley, 2007).

Through the process of AS, several unique transcripts could be generated from a single precursor RNA. Oftentimes, splice site selection results from underlying epigenetic factors, such as gene body methylation (Flores et al., 2012; Yan et al., 2015) and chromatin remodeling (Zhou et al., 2014), which also contribute to the overall gene expression profile. Flores et al. (2012) reported that highly methylated honeybee genes were more likely to be AS, and that when comparing *A. mellifera* to humans, there was high conservation of methylated genes relative to unmethylated genes (21.5% vs 8.2%) and that AS genes were more highly conserved than non-AS genes (18.9% vs 13%), highlighting the interplay of these processes and demonstrating the role of AS as one of many contributors to evolutionarily well-conserved control of gene expression.

Transcriptomics of insect behavior

It is widely accepted that behavioral phenotypes develop through fine-tuning of several molecular factors (Kendler and Greenspan, 2006; York, 2018), but the sex-specific splicing of *fruitless* in *D. melanogaster* serves as a rare example of AS of a single gene producing distinct behavioral phenotypes. In the *fruitless* gene, there are four promoter regions, an exon subject to sex-specific splicing (S exon), five common exons, and four potential 3' exons. Transcripts starting at the first primer site contain the S exon, and presence of protein Tra, produced by female flies, conserves the entire exon, yielding the fru^F transcript. Absence of Tra in males causes more 5' donor site selection at the S exon, yielding the fru^M transcript (Demir and Dickson, 2005). In the wild, several *fru* mutations exist that disrupt sex-specific splicing and interfere with male courtship (Goodwin et al., 2000). Demir and Dickson (2005) engineered male fruit flies with only fru^F expression and discovered significant decreases in male courtship behavior toward females and increases in male-male courtship behavior. In females with only fru^M expression, fertility, receptivity, and egg laying were all significantly reduced, demonstrating the necessity of sex-specific *fru* splicing to produce appropriate courtship behavior.

Still, it is more common for transcriptomic studies to look at genes and gene variants within the entire transcriptome to identify DE patterns within several genes or ontologies that are associated with a given behavioral phenotype. In one study that evaluated the link between maternal care and offspring behavior, the orphaned offspring of the subsocial carpenter bee, *Ceratina calcarata* were found to score higher on measures of aggression. Subsequent transcriptome sequencing identified AS genes that may facilitate this behavior from several broad ontologies (Arsenault et al., 2018). Eleven DE, AS genes were in the ionotropic glutamate receptor signaling pathway or involved with carbohydrate metabolism, that were previously linked to aggression in *A. mellifera* (Alaux et al., 2009; Li-Byarlay et al., 2014; Rittschoff et al., 2018). The largest number of DE, AS genes in orphaned *C. calcarata* were linked to oxidation-reduction processes and have been demonstrated to enhance aggressive behavior (Alaux et al., 2009).

A similar study observed the role of AS events in the bumblebee, *Bombus terrestris,* social behavior (Zhu et al., 2021). The greatest number of AS events were observed in

reproductive females when compared to nonreproductive workers and drones, with the most common AS genes linked to broad metabolic functions, including the metabolism of lipids, proteins, carbohydrates, DNA replication, and ribosome production. As mentioned previously, differential AS of carbohydrate metabolic genes has been correlated with variable feeding behavior (Ament et al., 2008) and variations in aggressive behavior (Li-Byarlay et al., 2014).

As behavioral phenotypes are typically multigenic (Kendler and Greenspan, 2006; York, 2018) and work in *Nicrophorus* spp. beetles have indicated that the subtle effects of many DE genes likely contribute to biparental care and variance within (Parker et al., 2015; Benowitz et al., 2017), it is anticipated that AS will have a role in this phenotypic variability. In this study, it was predicted that subtle expression differences in parental *N. orbicollis* could take the form of AS genes that may be DE across reproductive and social contexts. This study investigates, for the first time, the extent of AS, types of AS events, and DE genes subject to AS in *N. orbicollis* male and female beetle parents.

Methods

Genomic and RNA-seq data description

Genomic data, including a *N. orbicollis* genome and corresponding general feature format annotation file (GFF), was unpublished data, provided by the lab of Dr. Allen Moore at the University of Georgia. Transcriptomic data from 40 *N. orbicollis* adults was downloaded from the National Centre for Biotechnology Institute (NCBI) database. The Bioproject ID for the RNA-seq data is PRJNA371654. RNA-seq data was obtained as part of a project exploring burying beetle differential gene expression between high and low care parents of both sexes (Benowitz et al., 2017). Burying beetle heads, consisting of brain and head fat body tissue, were the source of RNA. Sequencing was performed by Benowitz et al. on an Illumina NextSeq 500, yielding paired-end 75 base pair reads. RNA-seq data for 10 beetles from each of four groups were utilized: high care males, low care males, high care females, and low care females. Benowitz et al. quantified care intensity by observing the number of parent-larvae trophallaxis events that occurred during an 8-hour period and reported 33.9 ± 4.4 bouts of care for high care males, 11.8 ± 1.3 for low care males, 39.8 ± 5.7 for high care females, and 11.7 ± 4.5 for low care females. Behavioral observations were made 24 hours after the hatching of larvae, during

the time of most intense parental activities, and the beetles were sacrificed immediately afterwards.

RNA-seq data mapping to the genome

The *N. orbicollis* genome was indexed using Bowtie2 with default settings (Langmead et al., 2009). Following this, each of the 40 RNA-seq datasets were mapped to the reference genome using TopHat2 (Kim et al., 2013). The aligned reads files generated by TopHat2, as well as the reference GFF annotation file, were used as input for Cufflinks 2.2.1 (Trapnell et al., 2012), which assembled transcripts and quantified gene and transcript expression levels as fragments per kilobase of exon per million mapped reads (FPKM).

Differential expression analysis

The gene transfer format (GTF) Cufflinks output files for all 40 transcriptomes were merged, using Cuffmerge with default settings. For each of the 40 samples, the binary alignment map (BAM) files from TopHat2 output were used alongside the merged GTF file as input for Cuffquant, which quantified expression levels for transcripts in the merged GTF and output a binary CXB file for further analysis in Cuffdiff using default settings. The programs Cuffmerge, Cuffquant, and Cuffdiff are all contained within the Cufflinks suite (Trapnell et al., 2012).

The analysis performed by Cuffdiff identified DE at the gene and transcript level via a test statistic derived directly from the between-group $\log_2$ fold change in expression. The allowed false discovery rate was 0.05. The Cuffdiff algorithm performs geometric normalization of FPKM, which can correct for differences in between-group sequencing depth, and cross-replicate dispersion estimation, which accounts for both among-group and between-group variance in fragment counts. This methodology is identical to and adopted from normalization and dispersion models developed for DESeq (Anders and Huber, 2010). A custom Perl script was used to determine statistically significant ($q \leq 0.05$) DE genes and transcripts from Cuffdiff output for further analysis.

Identification and characterization of alternatively spliced transcripts

To account for a relatively small data set of 40 transcriptomes, as well as for difficulties in splice site determination for short read RNA-seq data, AS genes were determined by the

consensus of AS gene loci predicted by three different algorithms. The first algorithm utilized was an in-house Perl script which detected potential AS events by identifying transcripts originating from the same gene locus, as indicated in the Cuffmerge GTF. The merged GTF was also used as input for AStalavista (Foissac and Semmeth, 2007), which identifies AS events and the gene loci in which they occur. To predict AS events, the AStalavista algorithm identifies constitutive splice sites, utilized by all transcripts at a locus, as well as variable splice sites, utilized by only some transcripts, representative of AS occurrence.

AS events and gene loci were also identified by rMATS (Shen et al., 2014), which utilized the merged GTF and the TopHat2 output BAM files for all 40 transcriptomes. The rMATS algorithm was designed to deal with replicate RNA-seq data and detects AS events by assessing the frequency of exon inclusion per sample. Variations in exon usage between samples can identify alternate splice site selection and predict AS event occurrence. A custom Perl script was used to identify gene loci detected as subject to AS in all three predictive algorithms. Another Perl script allowed the list of predicted AS genes to be cross-referenced with the significantly DE genes between parental groups, as determined by Cuffdiff, to yield a list of DE-AS gene loci.

Additionally, putatively AS genes of interest were further assayed in the Integrative Genomics Viewer (IGV) to assess if alternate splice site selection frequency was commonly occurring. IGV (Robinson et al., 2011) provides a visual representation of mapped reads to the genome and Sashimi plotting of splice site selection based on the number of mapped reads that span each splice site. The spanning of alternate splice sites by a small number of reads indicates that evidence of AS at that site is not well supported within this data set.

Functional annotation of identified transcripts

The merged GTF file was input to the gtf_to_fasta tool from the TopHat2 package to retrieve FASTA sequences for each identified transcript. This FASTA file was submitted for a BLASTX (Altschul et al., 1997) search against the UniProtKB/Swiss-Prot database (Boutet et al., 2007), seeking a single hit for each sequence with a cutoff E-value ≤ 1e-5. Following this, the FASTA file, combined with its corresponding BLASTX output, was input to TargetIdentifier (Min et al., 2005), which generated an annotated list of the identified genes. A custom Perl script

was used to associate these annotations with their corresponding gene and transcript IDs, as designated by Cufflinks, for all genes, as well as for the predicted AS and DE-AS gene sets.

Gene Ontology analysis

UniProt identifiers were used to retrieve Gene Ontology (GO) terms from the BLASTX hits for all identified gene loci. Using this list of GO terms, Perl scripts were used to parse out lists of GO terms for predicted AS, DE, and DE-AS gene loci. The retrieved GO identifiers were sorted into major categories by GO Slim Viewer (McCarthy et al., 2006), referencing the Generic slim set, curated by the GO Consortium (Ashburner et al., 2000; Gene Ontology Consortium, 2023).

Results

Mapping RNA-seq data to the reference *Nicrophorus orbicollis* genome

Across all 40 sequenced male and female *N. orbicollis* transcriptomes, nearly 1.7 billion reads were processed, with 1.4 billion being successfully mapped to the reference genome using TopHat2 (Table 1). Of the mapped reads, 8.7%, or approximately 122 million reads, represented multiple alignments, corresponding to reads which mapped to two or more gene loci. The within-group degree of mapping was comparable, with the lowest proportion of mapped reads being in high care females (73.3%) and the highest proportion of mapped reads in high and low care males (87.3%). Multiple alignments were lowest in low care males (8.3%) and highest in low care females (9.3%).

Table 1. Mapped Reads and Multiple Alignments by *N. orbicollis* Parental Group

Group	Total Reads	Mapped Reads	Multiple Alignments
High care males	578,181,846	504,914,463 (87.3%)	44,744,243 (8.9%)
Low care males	571,001,258	498,614,240 (87.3%)	41,209,277 (8.3%)
High care females	248,168,776	182,020,864 (73.3%)	15,822,202 (8.7%)
Low care females	275,921,982	212,891,925 (77.2%)	19,880,422 (9.3%)
Total	1,673,273,862	1,398,441,49 (83.6%)	121,656,144 (8.7%)

Differential expression analysis

Through transcript assembly by Cufflinks, a total of 76,897 transcripts were identified from 24,399 gene loci and were subject to differential expression (DE) analysis in Cuffdiff. Of all detected genes, 1,073 were significantly DE between at least two of the four parental groups according to the statistical test implemented by Cuffdiff ($q \leq 0.05$). The Cuffdiff test statistic value reflects the significance of $\log_2$ fold change in FPKM, accounting for among- and between-group variation, and reports a q-value, which states the statistical significance of the test statistic value after correction for False Discovery Rate (FDR). An FDR cutoff of 0.05 was implemented in Cuffdiff, as relying on p-value alone in cases of multiple-testing compounds false positive results.

Functional annotation via BLASTX against the UniProtKB/SwissProt database (Boutet et al., 2007) yielded 12,353 annotated genes and 1,071 annotated DE genes. Of particular interest were those between high and low care parents of each sex. The significantly DE genes between high and low care females and males are shown in Table 2 and Table 3, respectively, and are ranked based on the absolute value of the Cuffdiff test statistic, with the first gene listed showing the highest degree of $\log_2$ fold change after adjustment for among- and between-group variance. A gene in the column "Expression in high care parent" designated that the gene was up- or downregulated in the high care parent relative to the low care parent.

Table 2. Most Highly DE Genes between High and Low Care Females

Gene ID	Gene Annotation	Function	Expression in High Care Parent
No_Trans.3346	Cytochrome b	oxidative metabolism	down
No_Trans.6723	NADH-ubiquinone oxidoreductase chain 1	oxidative metabolism	up
No_Trans.7184	NADH-ubiquinone oxidoreductase chain 1	oxidative metabolism	up
No_Trans.10385	Cytochrome P450 4g15	ecdysteroid production	down
No_Trans.199	Cytochrome c oxidase subunit 3	oxidative metabolism	up
No_Trans.6365	Cytochrome b	oxidative metabolism	up
No_Trans.6734	NADH-ubiquinone oxidoreductase chain 4	oxidative metabolism	down
No_Trans.6005	Fatty acid synthase	endogenous lipogenesis	down
No_Trans.17933	Fatty acid synthase	endogenous lipogenesis	down
No_Trans.22281	Fatty acid synthase	endogenous lipogenesis	down
No_Trans.5335	Cytochrome b	oxidative metabolism	up
No_Trans.2884	Cytochrome P450 4g15	ecdysteroid production	down
No_Trans.18042	Cytochrome b5	oxidative metabolism	down
No_Trans.8442	Fatty acid synthase	endogenous lipogenesis	down
No_Trans.18347	Acyl-CoA Delta-9 desaturase	lipogenesis	down
No_Trans.11968	Elongation of very long chain fatty acids protein AAEL008004	fatty acid biosynthesis	down
No_Trans.8107	Thrombin inhibitor rhodniin	anticoagulant	up
No_Trans.8072	fatty acyl-CoA reductase CG5065	reduction of fatty acyl-CoA to alcohol	down
No_Trans.20235	Nucleic-acid-binding protein from transposon X-element	DNA transposition	down
No_Trans.8798	Trypsin-7	protein digestion	up

Table 3. Most Highly DE Genes between High and Low Care Males

Gene ID	Gene Annotation	Function	Expression in High Care Parent
No_Trans.6677	Cytochrome c oxidase subunit 1	oxidative metabolism	up
No_Trans.5313	Cytochrome b	oxidative metabolism	up
No_Trans.16661	Lipase 1	lipid catabolism	down
No_Trans.13198	Cytochrome P450 4d2	metabolism	up
No_Trans.11503	RNA-binding protein Musashi homolog Rbp6	control of RNA processes	down
No_Trans.14327	Endocuticle structural glycoprotein SgAbd-8	cuticle component	down
No_Trans.14035	Plasma kallikrein	serine protease	down
No_Trans.19771	Juvenile hormone acid O-methyltransferase	Juvenile Hormone biosynthesis	down
No_Trans.19183	Dual specificity testis-specific protein kinase 2	spermatogenesis	down
No_Trans.20797	Protein PELPK1	development	down
No_Trans.4333	Glucose dehydrogenase	glucose catabolism	down
No_Trans.18999	Transmembrane protein 65	gap junction communication	down
No_Trans.16085	Guanine nucleotide-binding protein subunit gamma-e	G-protein coupled receptor subunit	down
No_Trans.22741	Cytochrome P450 6j1	metabolism	up
No_Trans.5015	Uncharacterized protein PF3D7_1120600	unknown	down
No_Trans.22286	Tau-tubulin kinase homolog Asator	spindle matrix organization	down
No_Trans.14461	Scavenger receptor class B member 1	steroid biosynthesis	down
No_Trans.23188	Cilia- and flagella-associated protein 298	cilia and flagella movement	down
No_Trans.19442	Galanin receptor 2b	memory, learning, food intake	down
No_Trans.17513	40S ribosomal protein SA	ribosomal subunit	down
No_Trans.6677	Cytochrome c oxidase subunit 1	oxidative metabolism	up

Summary of alternative splicing events identified by AStalavista

AStalavista was utilized to identify AS events by analyzing the merged GTF file from all 40 *N. orbicollis* transcriptomes. Analysis by AStalavista compared transcripts and identified variably utilized splice sites between predicted gene variants. The algorithm reports these events and classifies the AS events occurring to create two or more variants as either exon skipping, intron retention, alternate donor selection, or alternate acceptor selection. Events resulting in transcripts formed by multiple AS events or by other event types, such as mutually exclusive exon selection, were classified as "complex events." In the 76,897 total *N. orbicollis* transcripts, AStalavista identified 139,036 AS events (Table 4). The most common mechanism of AS was complex events, with 107,246 events detected (77.1%). Comparatively, a much lower number of events was identified for intron retention (11.1%), alternate donor (4.3%) and acceptor sites (4.9%), and exon skipping (2.6%).

A similar trend emerged when looking at the AStalavista output for the four parental groups individually (Table 5). By a large margin, the most common AS event type was complex events, ranging from 70.6% to 71.7%. Following this, the second most frequent AS event was intron retention, with frequency ranging from 15.2% to 15.5%. Alternate acceptor sites represented 5.1% to 5.6% of events; alternate donor sites represented 4.6% to 4.9% of events. Exon skipping was the least common AS mechanism, accounting for only 3.4% to 3.5% of detected events.

A custom Perl program was also utilized to compare AS events between different groups of parental beetles. From this, a relatively conserved splicing profile was identified between high and low care females, with 71.1% of events conserved, and between high and low care males, with 68.1% of events conserved (Table 6). For both sexes, the most conserved AS event type was exon skipping (80.3% in females; 77.5% in males), followed by intron retention (76.7%; 73.6%) and alternate donor site selection (71.9%; 68.2%). However, females showed higher conservation of alternate acceptor site selection events than complex events (71.7%, 69.4%), while the opposite is observed in males (65.7%, 66.7%).

Conserved events for high and low parents of the same sex were compared to assess conservation between all groups (Table 7), revealing a highly conserved set of events (82.8%), wherein only 9.2% of events were observed only in females and 8.1% of events were observed

only in males. Again, exon skipping was most highly conserved (90.2%) followed by intron retention (84.4%). Complex events (82.2%) were more well conserved than alternate acceptor (82.1%) and alternate donor site selection (81.8%).

Splice event conservation was also assessed between high or low care parents of the opposite sex (Table 8), revealing that many AS events were conserved between males and females showing the same intensity of parental care (69.6% for high care parents; 67.9% for low care parents). Exon skipping was the most highly conserved event type (78.5% in high care parents; 78.2% in low care parents) followed by intron retention (74.8%; 73.7%). Complex events showed higher conservation in high care parents (68.5%) than in low care parents (66.2%), while alternate donor site selection showed the opposite trend (68.4%; 68.9%). Alternate acceptor site selection was the least conserved event type in high care parents (66.4%) but not in low care parents (67.3%), wherein complex events were least conserved.

The conserved events for all high care parents were compared to the conserved events for all low care parents, revealing high conservation of AS events in both care intensities (83.9%), and greater than the 82.8% conserved when comparing the sexes. Overall, 8.7% and 7.5% of events were exclusive to high or low care provisioning parents, respectively. Again, exon skipping shows the highest degree of conservation (90.7%) followed by intron retention (85.4%). The next most highly conserved event types were alternate acceptor site (84.2%), alternate donor site (83.4%), and complex events (83.2%).

Table 4. Summary of AS Events Determined by AStalavista

Type of Event	Number Identified	Percentage of Total Events
Exon skipping	3,578	2.6%
Alternate donor sites	6,020	4.3%
Alternate acceptor sites	6,832	4.9%
Intron retention	15,360	11.1%
Complex events	107,246	77.1%
Total	139,036	100%

Table 5. Summary of AS Event Type for Each Parental Group Determined by AStalavista

Type of Event	Number Identified			
	HM	**LM**	**HF**	**LF**
Exon skipping	2,534 (3.5%)	2,511 (3.4%)	2,489 (3.5%)	2,479 (3.5%)
Alternate donor sites	3,491 (4.9%)	3,431 (4.7%)	3,280 (4.6%)	3,329 (4.7%)
Alternate acceptor sites	4,008 (5.8%)	3,863 (5.3%)	3,670 (5.1%)	3,643 (5.1%)
Intron retention	11,156 (15.5%)	11,217 (15.4%)	10,938 (15.2%)	11,021 (15.5%)
Complex events	50,781 (70.6%)	51,869 (71.2%)	51,721 (71.7%)	50,842 (71.3%)
Total	71,970	72,891	72,098	71,314

Table 6. Conserved AS Events between High and Low Care Parents of the Same Sex

	HF/LF			HM/LM		
	HF	**Conserved**	**LF**	**HM**	**Conserved**	**LM**
Exon skipping	275 (10.0%)	2,214 (80.3%)	270 (9.8%)	332 (11.7%)	2,202 (77.5%)	306 (10.8%)
Alternate donor site	524 (13.7%)	2,756 (71.9%)	552 (14.4%)	687 (16.5%)	2,840 (68.2%)	635 (15.3%)
Alternate acceptor site	606 (14.3%)	3,064 (71.7%)	606 (14.2%)	890 (18.8%)	3,118 (65.7%)	735 (15.5%)
Intron retention	1,401 (11.3%)	9,537 (76.7%)	1,504 (12.1%)	1,681 (13.1%)	9,475 (73.6%)	1,721 (13.4%)
Complex events	9,531 (15.7%)	42,190 (69.4%)	9,039 (14.9%)	9,797 (15.9%)	40,984 (66.7%)	10,689 (17.4%)
Total	12,337 (14.7%)	59,761 (71.1%)	11,971 (14.2%)	13,387 (15.6%)	58,583 (68.1%)	14,086 (16.4%)

Table 7. Conserved AS Events among All Parental Groups by Sex

HF+LF/HM+LM			
	HF+LF	**Conserved**	**HM+LM**
Exon skipping	117 (5.0%)	2,097 (90.2%)	110 (4.7%)
Alternate donor site	262 (8.6%)	2,495 (81.8%)	292 (9.6%)
Alternate acceptor site	281 (8.0%)	2,873 (82.1%)	347 (9.9%)
Intron retention	837 (8.1%)	8,700 (84.4%)	775 (7.5%)
Complex events	4,470 (9.7%)	37,719 (82.2%)	3,710 (8.1%)
Total	5,967 (9.2%)	53,794 (82.8%)	5,234 (8.1%)

Table 8. Conserved AS Events between High or Low Care Parents of the Opposite Sex

	HF/HM			LF/LM		
	HF	**Conserved**	**HM**	**LF**	**Conserved**	**LM**
Exon skipping	278 (9.9%)	2,211 (78.5%)	326 (11.6%)	293 (10.5%)	2,192 (78.2%)	319 (11.4%)
Alternate donor site	537 (13.4%)	2,743 (68.4%)	731 (18.2%)	561 (14.1%)	2,749 (68.9%)	682 (17.1%)
Alternate acceptor site	586 (12.6%)	3,084 (66.4%)	978 (21.0%)	652 (14.5%)	3,022 (67.3%)	814 (18.1%)
Intron retention	1,468 (11.6%)	9,452 (74.8%)	1,712 (13.6%)	1,610 (12.6%)	9,455 (73.7%)	1,762 (13.7%)
Complex events	9,863 (16.1%)	41,858 (68.5%)	9,418 (15.4%)	10,034 (16.2%)	40,957 (66.2%)	10,912 (17.6%)
Total	12,750 (15.0%)	59,348 (69.6%)	13,124 (15.4%)	13,150 (15.3%)	58,375 (67.9%)	14,516 (16.9%)

Table 9. Conserved AS Events among All Parental Groups by Degree of Care

HF+HM/LF+LM			
	HF+HM	**Conserved**	**LF+LM**
Exon skipping	114 (4.9%)	2,097 (90.7%)	100 (4.3%)
Alternate donor site	249 (8.3%)	2,495 (83.4%)	249 (8.3%)
Alternate acceptor site	301 (8.8%)	2,873 (84.2%)	239 (7.0%)
Intron retention	752 (7.4%)	8,700 (85.4%)	731 (7.2%)
Complex events	4,138 (9.1%)	37,719 (83.2%)	3,468 (7.7%)
Total	5,554 (8.7%)	53,794 (83.9%)	4,787 (7.5%)

Summary of alternative splicing events identified by rMATS

rMATS was also utilized to identify AS events in *N. orbicollis* by comparing TopHat2 mapped reads for each sample to the merged GTF of all assembled transcripts. It assessed exon inclusion and junction choice by the proportion of reads at a locus that map to a given exon or exon boundary. A summary of AS events detected by rMATS can be found in Table 10. Events classified by rMATS differ in name from the events identified in AStalavista but are synonymous, wherein skipped exons refer to exon skipping, retained intron refers to intron retention, and alternate 5' splice site and alternate 3' splice site refers to alternate donor site and alternate acceptor site, respectively (Figure 1). Additionally, rMATS quantifies the number of mutually exclusive exon events, which would be considered an example of complex events in AStalavista. In a mutually exclusive exons event, one of two exons or one of two groups of exons remains in a transcript while the other is excluded. A total of 36,183 AS events were predicted by rMATS, with the most frequently occurring event type being intron retention (35.5%) followed by exon skipping (26.9%), alternate 3' splice site (16.7%), alternate 5' splice site (15.3%), and mutually exclusive exons (5.6%).

Table 10. Summary of AS Events Determined by rMATS

Type of Event	Number Identified	Percentage of Total Events
Skipped exon	9,743	26.9%
Alternate 5’ splice site	5,532	15.3%
Alternate 3’ splice site	6,037	16.7%
Retained intron	12,854	35.5%
Mutually exclusive exons	2,017	5.6%
Total	36,183	100%

Identification of putative DE-AS genes

To accommodate for a smaller dataset of 40 transcriptomes and difficulty in identifying splice sites using short read RNA-seq data, the output of three predictive algorithms were compared to find consensus AS genes. First, a Perl script was used to identify all gene loci from which multiple transcripts were assembled, representing the total number of possible AS genes that could be detected from this analysis of the data. Genes from this list that matched gene loci containing AS events, as predicted by both AStalavista and rMATS, were parsed out as the list of putatively AS genes. Of all 24,399 gene loci, 10,785 were the source of two or more transcripts. Within these 10,785 genes, 5,868 were predicted to contain at least one AS event by both AStalavista and rMATS, representing a majority of genes identified as AS by both algorithms (Figure 1). Of the total AS genes identified by these algorithms, 4,363 were successfully functionally annotated via BLASTX.

Following this, a Perl script identified which predicted AS genes were also significantly DE, as determined by Cuffdiff, yielding a set of 311 putative DE-AS genes. The most highly DE of these DE-AS genes, sorted by the absolute value of the Cuffdiff test stat, are shown in Table 11 and Table 12 for female and male parents, respectively.

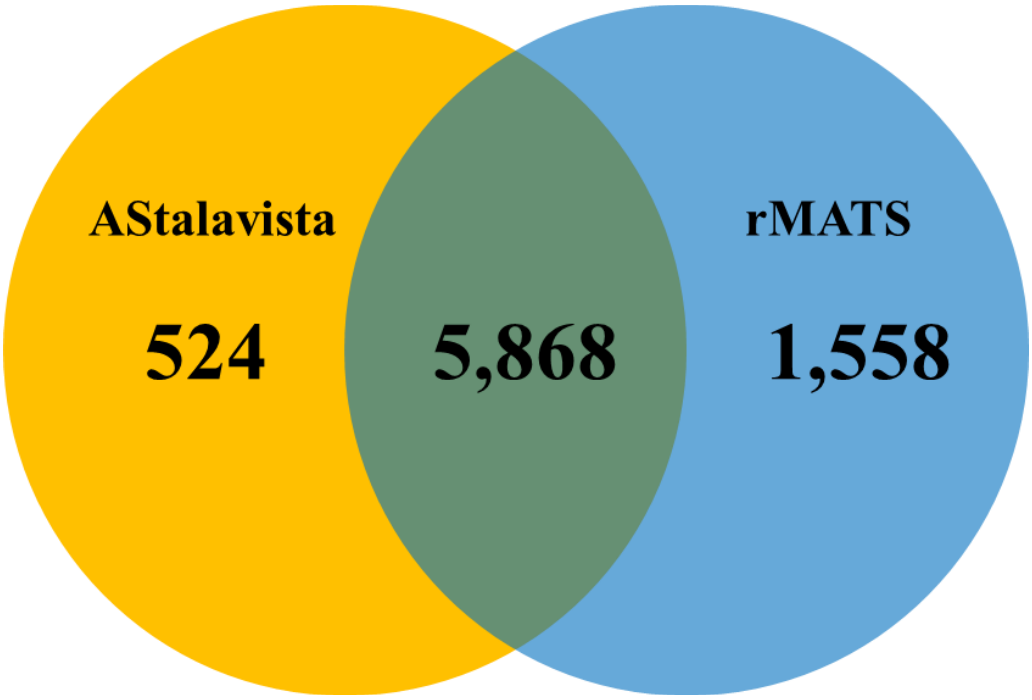

Figure 1. AS Genes Identified by AStalavista and rMATS. Overlapping genes identified by both programs are represented in green. The number of AS genes identified only by AStalavista (yellow) or rMATS (blue) are also shown.

Table 11. Most Highly DE-AS Genes between High and Low Care Females

Gene ID	Gene Annotation	Function	Expression in High Care Parent
No_Trans.199	Cytochrome c oxidase subunit 3	oxidative metabolism	up
No_Trans.17933	Fatty acid synthase	endogenous lipogenesis	down
No_Trans.2884	Cytochrome P450 4g15	ecdysteroid production	down
No_Trans.8442	Fatty acid synthase	endogenous lipogenesis	down
No_Trans.20235	Nucleic-acid-binding protein from transposon X-element	DNA transposition	down
No_Trans.2540, No_Trans.2541	Methyl farnesoate epoxidase	Juvenile Hormone biosynthesis	up
No_Trans.422	Protein CREG1	autophagy, endocytosis	up
No_Trans.18291	SET domain-containing protein SmydA-8, isoform A	methylation	up
No_Trans.6782	C-type lectin 37Da	hemocyte encapsulation (immune)	up
No_Trans.17248	Synaptotagmin-9	calcium-dependent exocytosis	down
No_Trans.2223	E3 ubiquitin-protein ligase RNF216	negative regulation of interferon production	up
No_Trans.22170	Alanine--glyoxylate aminotransferase	glyoxylate catabolism	up
No_Trans.19564	Tyrosine 3-monooxygenase	dopamine biosynthesis	up
No_Trans.17932	Fatty acid synthase	endogenous lipogenesis	down
No_Trans.1086	Protein yellow	melanin biosynthesis	up
No_Trans.1192	Uncharacterized protein ORF91	unknown	up
No_Trans.14378	Tubulin alpha-1 chain	microtubule organization	up

Table 12. Most Highly DE-AS Genes between High and Low Care Males

Gene ID	Gene Annotation	Function	Expression in High Care Parent
No_Trans.16661	Lipase 1	lipid catabolism	down
No_Trans.14035	Plasma kallikrein	serine protease	down
No_Trans.20797	Protein PELPK1	development	down
No_Trans.16085	Guanine nucleotide-binding protein subunit gamma-e	G-protein coupled receptor subunit	down
No_Trans.22741	Cytochrome P450 6j1	metabolism	up
No_Trans.5015	Uncharacterized protein PF3D7_1120600	unknown	down
No_Trans.19442	Galanin receptor 2b	learning, memory, food intake	down
No_Trans.10539	ADP-ribosylhydrolase ARH3	DNA repair	down
No_Trans.13117	Aquaporin	membrane water transport	down
No_Trans.13149	uncharacterized protein ART2	unknown	down
No_Trans.21716	Venom acid phosphatase Acph-1	secreted hydrolase	down
No_Trans.18325	Gastric triacylglycerol lipase	lipid catabolism	down
No_Trans.22477	Protein couch potato	peripheral nervous system development	down
No_Trans.868	ABC transporter G family member 23	membrane transport protein	down
No_Trans.1629	Circadian clock-controlled protein daywake	daylight sleep suppression	up
No_Trans.15264	Phenoloxidase-activating factor 1	innate immune response	down
No_Trans.11560	Elongation of very long chain fatty acids protein AAEL008004	fatty acid biosynthesis	down
No_Trans.9325	Phosphatidylinositol phosphatase PTPRQ	tyrosine phosphatase	down
No_Trans.17	Cytokine-inducible SH2-containing protein	negative regulation of cytokines in the JAK-STAT5 pathway	down
No_Trans.12087	WW domain-containing oxidoreductase	inhibits Wnt signaling, functions with p53 to promote genotoxic cell death	down

Gene Ontology analysis of DE-AS genes

To assess overall trends in the functions of DE-AS genes, Gene Ontology (GO) analysis was performed for the DE-AS gene set, the AS gene set, the DE gene set, and for all identified genes (Table 13). A total of 126,466 terms were retrieved for the whole set of 24,399 genes, with most relating to a biological process (47.6%) and fewer terms relating to molecular functions (16.0%) and cellular components (35.3%). In the 4,363 AS genes and 1,071 DE genes, 49,052 and 6,863 terms were obtained, respectively. Again, the largest fraction of terms related to biological processes (48.7%, 50.6%) with fewer terms describing molecular function (16.0%, 16.9%) and cellular components (35.3%, 32.5%). A total of 3,070 terms were obtained for the DE-AS gene set, with 51.8% in biological processes, 16.6% in molecular function, and 31.6% in cellular components.

Further analysis classified these terms using the relatively limited Generic slim set, which was curated by the Gene Ontology Consortium, containing 143 total GO terms (Figure 2). Slim sets quantify the number of "child" GO terms that fall under a more generalized "parent" term, which is useful in identifying broad trends in functionality of several genes. A comparison of term categories for biological processes in all genes, AS genes, DE genes, and DE-AS genes revealed overrepresentation of terms in DE-AS genes for developmental and regulatory functions unlikely to confer variation in the parental phenotype, such as anatomical structure development (11.6% in DE-AS genes vs 10.3% in DE genes, 10.3% in AS genes, and 9.7% in all genes), cell differentiation (8.5% vs 7.5%, 7.2%, 6.8%), and cytoskeletal organization (4.0% vs 3.0%, 2.8%, 2.7%).

However, genes that were DE, regardless of whether or not they were DE-AS, showed overrepresentation in categories relating to metabolism, such as lipid metabolic processes (4.2% of DE-AS terms and 3.7% of DE terms vs 2.5% in AS genes and 2.4% in all genes) and generation of precursor metabolites and energy (2.2%, 3.8% vs 0.9%, 1.1%). There was also higher occurrence in DE-AS and DE genes of terms related to immunity, including immune system processes (3.6%, 3.4% vs 2.6%, 2.4%) and defense response to other organisms (1.9%, 2.0% vs 1.2%, 1.1%), as well as terms that could relate to behavior, nervous system processes (3.0%, 2.9% vs 2.2%, 1.9%).

Table 13. Retrieved GO Terms for AS and DE-AS Genes

Gene Set	Biological Processes Terms	Molecular Function Terms	Cellular Component Terms	Total
All genes	60,177 (47.6%)	20,213 (16.0%)	46,076 (36.4%)	126,466
AS genes	23,897 (48.7%)	7,852 (16.0%)	17,303 (35.3%)	49,052
DE genes	3,470 (50.6%)	1,161 (16.9%)	2,232 (32.5%)	6,863
DE-AS genes	1,589 (51.8%)	511 (16.6%)	970 (31.6%)	3,070

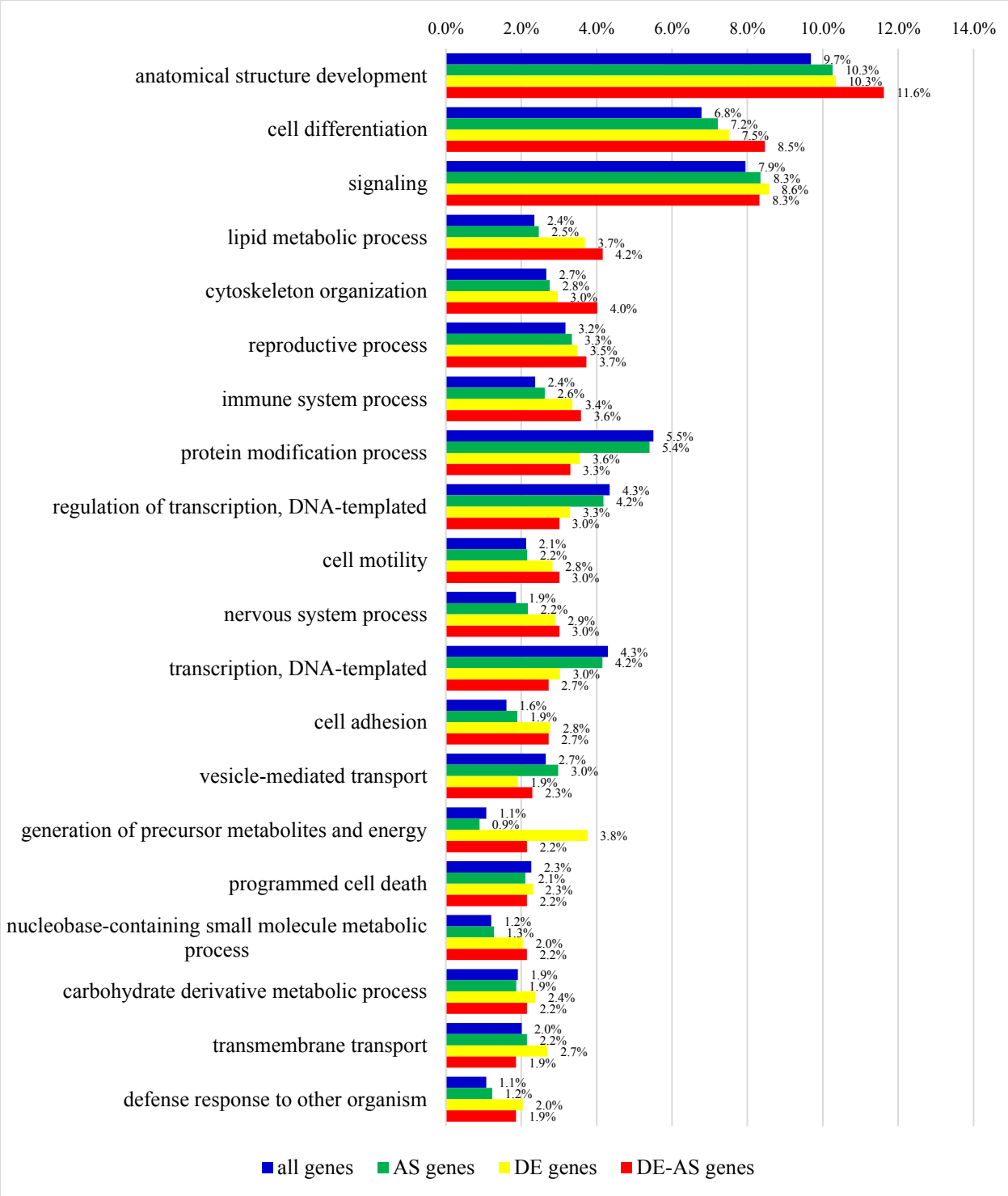

Figure 2. Top 20 GO Terms for DE-AS Genes Compared to Other Gene Groups. The 20 most highly occurring biological process GO terms from the Generic slim set for DE-AS genes (red) compared to the frequency of those terms in all AS genes (green), DE genes (yellow), and all genes (blue).

Assay of selected putative AS genes in IGV

Two DE-AS genes of interest were visualized in IGV (Robinson et al., 2011) to further confirm the characterization of these genes as AS, which was previously indicated by both AStalavista and rMATS. IGV visualized reads mapped to the genome and allowed for generation of Sashimi plots, which show the number of mapped reads that span each exon-exon boundary in the transcriptome. Thus, the resulting plot represents splice site selection and quantifies the number of reads that span each splice site. This method can reveal highly occurring AS events, which would be considered well-supported within this dataset, as well as events that have very few reads spanning a predicted AS splice junction, indicating that evidence for that predicted AS event is weak within this dataset.

AS genes subject to Sashimi plotting in IGV were selected based on evidence in previous work for the role of these genes in manifestations of traits associated with the parental care phenotype. This included a gene involved in dopamine biosynthesis, *tyrosine 3-monooxygenase* (Figure 3) as well as two genes for *takeout* that were predicted to be AS (Figures 4 and 5). Predicted events by AStalavista and rMATS within these genes are shown in Table 14.

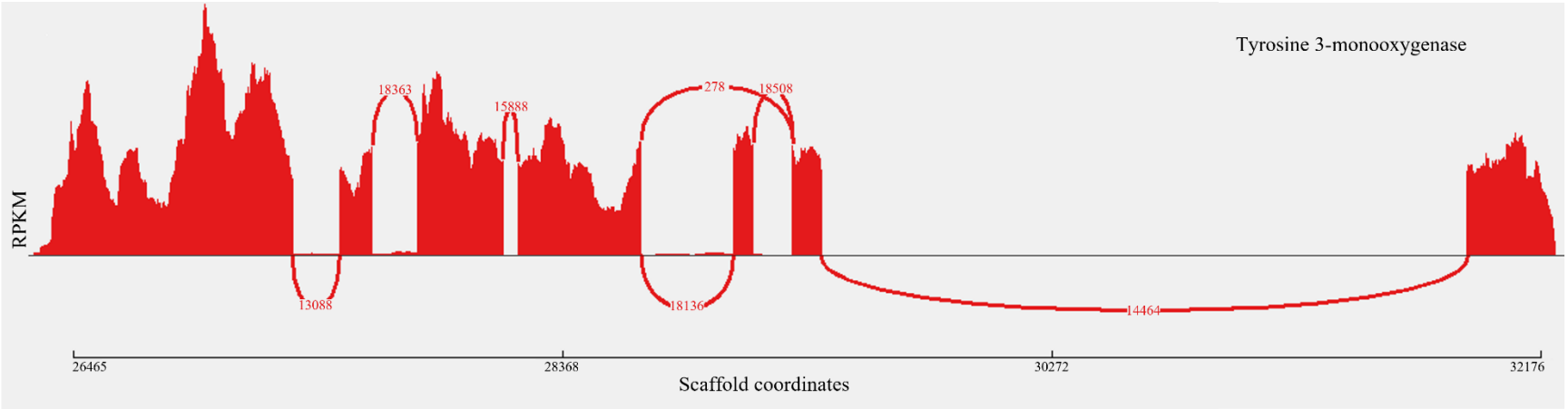

Figure 3. Sashimi plot for *tyrosine 3-monooxygenase* (No_Trans.19564).

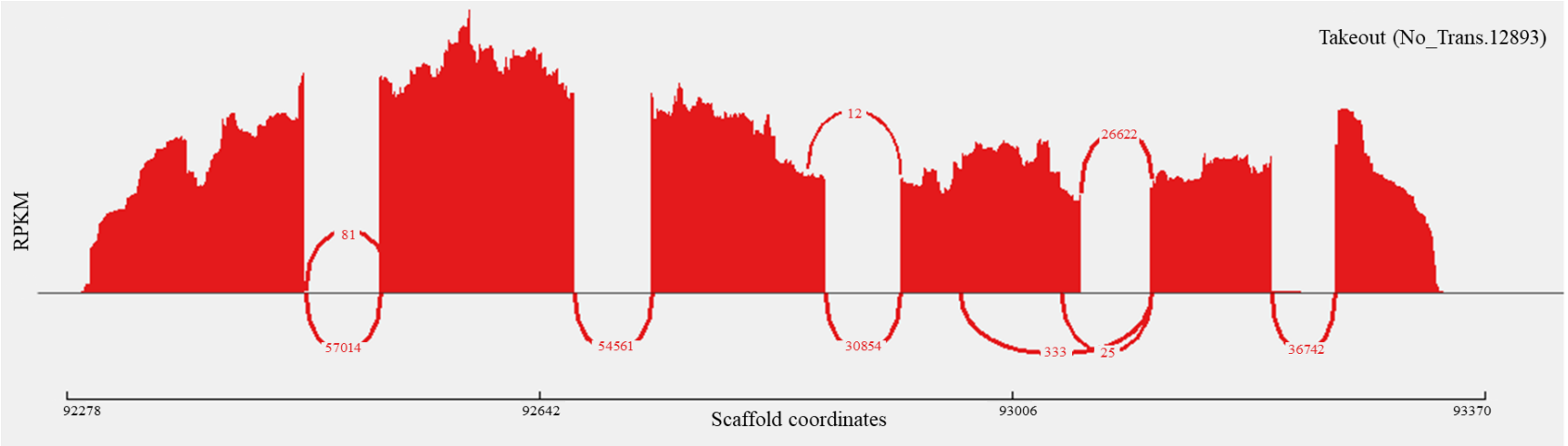

Figure 4. Sashimi plot for *takeout* (No_Trans.12893).

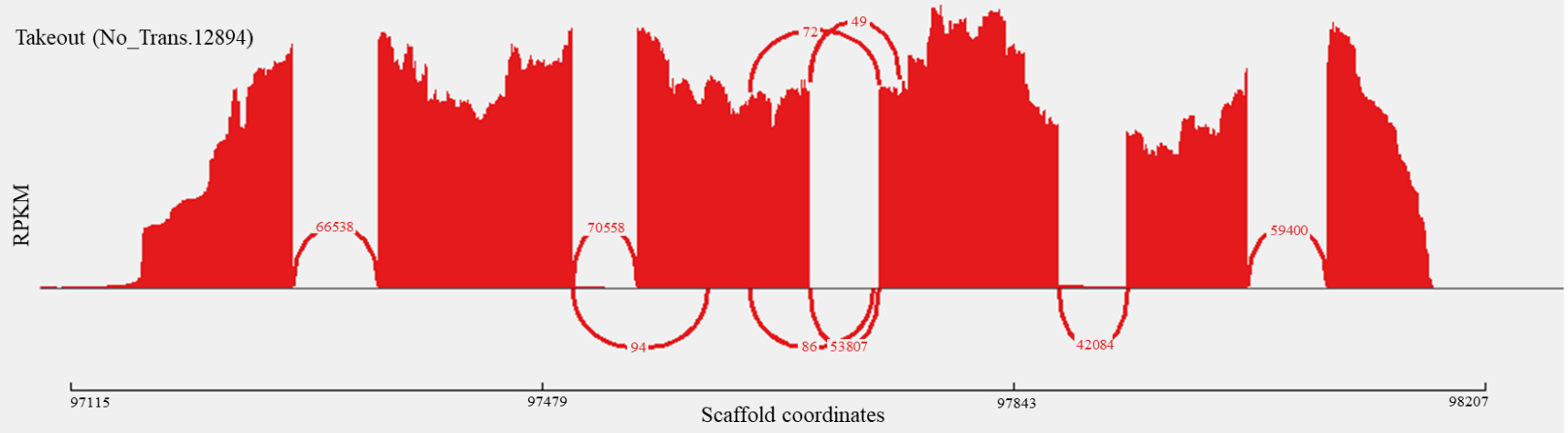

Figure 5. Sashimi plot for *takeout* (No_Trans.12894).

Table 14. Types of AS Events Predicted for Genes Modeled by Sashimi Plotting

Gene ID	Gene	Event(s) Predicted by AStalavista	Event(s) Predicted by rMATS
No_Trans.19564	Tyrosine 3-monooxygenase	Exon skipping	Exon skipping
No_Trans.12893	Takeout	Alternate acceptor site	Exon skipping Mutually exclusive exons Alternate 3' splice site
No_Trans.12894	Takeout	Alternate acceptor site	Alternate 3' splice site Alternate 5' splice site

Discussion

Read mapping to the reference genome, transcript assembly, and DE analysis

TopHat2 was utilized to map RNA-seq reads from all 40 *N. orbicollis* transcriptomes to the reference genome. Of approximately 1.7 billion total reads, 1.4 billion (83.6%) were successfully mapped to the genome, with 122 million reads (8.7%) mapped to multiple gene loci (Table 1). Typically, 5-40% of eukaryotic genes will align with multiple loci, due to the mapping of relatively short RNA-seq reads to recombined or retrotransposed genes (McDermaid et al., 2018; Dharshini et al., 2020). Low incidence of multiple mapping from *N. orbicollis* reads, 8.7% overall, was expected to contribute to higher accuracy in gene and transcript quantification (Treangen and Salzberg, 2011).

It was notable that total reads for high and low care male beetles, 578 million and 571 million, respectively, were greater than total reads for high and low care female beetles, 248 million and 276 million, respectively. Differences in sequencing depth between experimental groups were not expected to impact gene expression estimates in Cufflinks, which relies on a normalized measure, FPKM. Using FPKM normalizes expression estimates by accounting for variations in the number of mapped reads and the length of the genes being analyzed. More downstream analysis of DE genes by Cuffdiff added an additional layer of normalization through dispersion modeling of reads mapped to a locus across several samples and geometric scaling of FPKM based on variations in mapped reads between samples (Trapnell et al., 2012). These corrective algorithms are identical to those utilized in DESeq (Anders and Huber, 2010), which has been demonstrated to produce accurate expression quantifications (Conesa et al., 2016; Zhao et al., 2021).

From the 40 *N. orbicollis* transcriptomes, Cufflinks analysis identified 76,897 total transcripts from 24,399 gene loci. DE analysis of genes was performed in Cuffdiff with an FDR of 0.05 to counteract the accumulation of false-positive results from multiple testing. Cuffdiff quantified a test statistic, representing the significance of $\log_2$ fold changes in gene expression between two experimental groups after accounting for between- and among-group mapping variance, as described above. In these pairwise comparisons, gene expression was considered statistically significant between two parental groups if the q-value of the test statistic, which

accounted for the 0.05 FDR cutoff, was less than 0.05, yielding 1,073 genes that were significantly DE between at least two parental groups.

Genes were functionally annotated via BLASTX search against the UniProtKB/SwissProt database, resulting in 12,353 annotated genes and 1,071 annotated DE genes. Significantly DE genes between behavioral states were of particular interest, with analysis focusing on the 20 most highly DE genes between high and low care parents of the same sex (Tables 2-3). Overall, different metabolic profiles of high and low care parents were evident, especially pertaining to oxidative and lipid metabolic processes. Of the top 20 most DE genes in females, eight encode enzymes involved in mitochondrial oxidative metabolism, including four separate genes for *cytochrome b*, three genes for *NADH-ubiquinone oxidoreductase* chains, and a *cytochrome c oxidase s*ubunit. In males, there were three DE genes relating to oxidative metabolism, two of which were *cytochrome c oxidase* subunits and one was *cytochrome b*.

While there were clear metabolic differences between high and low care parents, it is most likely that the altered metabolic profile was a result of high parental investment and not a contributor to the manifestation of these behaviors. As high oxidative metabolism reflects high overall energy expenditure, it follows that high care provisioning parents would have higher expression of genes involved in oxidative metabolism. Interestingly, three of these genes showed downregulation in high care females, including *cytochrome b* (No_Trans.3346, No_Trans.18042) and *NADH-ubiquinone oxidoreductase chain 4*. However, the remaining five genes identified in females and all three genes identified in males were upregulated in high care parents.

In fact, higher oxidative metabolism has been observed in parenting *N. orbicollis*, wherein CO_2 production, a byproduct of oxidative metabolism, was measured for females before breeding and again on the second day of larval care (Trumbo and Rauter, 2014). Adjusting for changes in body mass, CO_2 output was 81.5% higher during parental care. Additionally, females who had raised a brood lived, on average, a 42.5% shorter life than beetles who had not, demonstrating the parental fitness loss for the benefit of their offspring. It is likely that, in part, this fitness loss is due to increased oxidative stress. High oxidative metabolism is thought to contribute to oxidative stress, which can inhibit cellular repair processes and contribute to cellular damage. Thus, measures of oxidative stress have been used to measure levels of oxidative metabolism.

For example, increased oxidative metabolism is known to occur during active care in zebra finches, as demonstrated in one study, where increased brood size was found to cause increased vulnerability to oxidative stress (Alonso-Alvarez et al., 2006). Researchers measured this by drawing blood from actively parenting finches and incubating samples with an oxidizing agent. Blood from parents with more nestlings showed increased hemocyte lysis in the presence of an oxidizing agent. Another study that assessed brood size effects on oxidative stress in zebra finches reported that two major antioxidant enzymes, superoxide dismutase and glutathione peroxidase, showed activity reductions of 28% and 24% respectively when scaled for daily energy expenditure while parenting (Wiersma et al., 2004). From this, it is evident that variations in oxidative metabolism represent a sacrifice in parental fitness during care that is exacerbated by increased intensity of care.

Further alterations in metabolism are observed for lipid metabolic processes, wherein for females, seven of the 20 most highly DE genes have functions involved in lipid metabolism. Four genes encoding for fatty acid synthase, which participates in endogenous lipogenesis, were found to be significantly DE between high and low care females. Additional lipid metabolism genes identified were an *acyl-CoA delta-9 desaturase*, which functions in lipogenesis and two genes involved in long chain fatty acid metabolism, *elongation of very long chain fatty acids protein AAEL008004* and *fatty acyl-CoA reductase CG6065*. All seven genes related to lipid metabolism were downregulated in high care females relative to low care females.

Fatty acid synthase functions in endogenous lipogenesis, the synthesis of long-chain fatty acids for storage in adipose tissue. In mammals, it is known to be subject to endocrinological regulation, with insulin stimulating fatty acid synthase expression and high cAMP concentration, which upregulates glucagon, shown to inhibit *fatty acid synthase* expression (Sul et al., 1998). In insects, fatty acid synthase is also required for cuticular hydrocarbon synthesis (Yang et al., 2020) and reproductive fitness. In a *fatty acid synthase* RNAi knockdown experiment, the small brown planthopper *Laodelphax striatellus* exhibited decreasing oogenesis, ovary mass, and expression of *vitellogenin* and its receptor (Cheng et al., 2023). As *vitellogenin* expression rises in *N. orbicollis* upon discovery of a reproductive resource and again before oviposition, the nutritional deficits associated with parental care and downregulated endogenous lipogenesis could suppress reproductive drive and prevent maternal abandonment. It follows then that,

relative to low provisioning parents, high provisioning parents, whose metabolism is elevated and nutritional input is limited, would have suppressed expression of genes involved in energy storage. Additionally, the low expression of *fatty acid synthase* may contribute to consequences of the active care phenotype, such as decreased personal immunity through reduced cuticular maintenance and decreased fecundity.

Additionally, two genes encoding for cytochrome p450 4g15 (CYP4G15), which is evidenced to have a role in lipid metabolism by anabolizing ecdysteroids from sterols (Maibeche-Coisne et al., 2000), were identified as downregulated in high provisioning females. The *CYP4* genes have been demonstrated to have a role in cuticle hydrocarbon synthesis (Dulbecco et al., 2020) and metabolism of endogenous molecules and xenobiotics, with some conferring pesticide resistance (Nauen et al., 2022). While CYP4G15 has an observed function in xenobiotic detoxification (Gao et al., 2023), investigation in *D. melanogaster* has suggested that it has an additional role in ecdysteroid production and that its expression is highest within the brain, ring gland, and fat body with lower expression in the gut (Maibeche-Coisne et al., 2000).

Overall, DE genes identified in this analysis aligned well with those identified in other transcriptomic studies of *Nicrophorus* spp. beetles. The 40 transcriptomes utilized in this work were sequenced by and subject to DE analysis in Benowitz et al., 2017, resulting in a list of 22 significantly DE genes between high and low care *N. orbicollis* females, 19 of which were annotated. Like in this study, Benowitz et al. reports DE of genes involved in lipid metabolism, five of which were *fatty acid synthase*. Both this work and Benowitz et al. also reported DE of a *fatty acyl-CoA reductase* and *CYP4G15*. Interestingly, the DE gene list of Benowitz et al. only contained one gene involved in mitochondrial oxidative metabolism, a *cytochrome c oxidase* subunit, while this work reported eight.

There were several differences between this analysis and that done by Benowitz et al. that could have resulted in identification of a distinct set of DE genes. While both studies utilized TopHat2 for read mapping and Cufflinks for transcript assembly, Benowitz et al. considered only the longest assembled transcript for each gene when assessing DE, wherein they remapped RNA-seq reads to the reduced transcriptome of 14,739 total transcripts and used RSEM (Li and Dewey, 2011) to generate gene level counts, before finally performing count normalization and DE analysis in edgeR (Robinson et al., 2010). In this work, all 76,897 transcripts from 24,399

gene loci were considered when performing DE analysis via Cuffdiff. This alternate data processing pipeline was facilitated by the use of an *N. orbicollis* genome and corresponding GFF file from the lab of Dr. Allen Moore from the University of Georgia. Use of a GFF file can facilitate read mapping to defined gene coordinates. The GFF file used here provided coordinates for 23,542 of the 24,399 genes detected in this analysis.

This methodology likely allowed for DE analysis of more lowly expressed genes within the transcriptome, as the analysis by Benowitz et al. reported 53.66% of reads for females and 55.97% of reads for males successfully mapped to the reduced transcriptome during the remapping process, whereas this study assessed DE from transcripts resulting from 83.6% of total reads mapped to the genome. The difference in results is most evident when looking at significantly DE genes between high and low care males. While this work highlights the top 20 most highly DE genes between the male parental groups, Benowitz et al. identified only one unannotated gene reaching the threshold for statistical significance in DE. However, the DE gene list for males represents a more diverse set of cellular functions than the list generated for females, in which 15 of 20 genes function in oxidative or lipid metabolism. Thus, it is possible that this methodology may sacrifice precision for breadth in assessing DE between parental groups for lowly expressed transcripts.

AS analysis by AStalavista

AStalavista was utilized to identify AS events within the 40 *N. orbicollis* transcriptomes, quantifying event frequency within five categories: exon skipping, alternate donor sites, alternate acceptor sites, intron retention, and complex events. It was found that the majority of events identified in this assay were complex events, indicating that an AS transcript resulted from several concurrent AS events. The AStalavista algorithm accepted the merged GTF file, describing all transcripts identified within the 40 transcriptomes, and modeled the manner by which transcripts from the same gene loci were spliced. It first identified constitutive splice sites, utilized in all transcripts, as well as alternate splice sites, used variably between transcripts. This algorithm produces an AS event code, describing the simplest combination of total events occurring to produce each AS transcript that originates from the same 5’ transcript start site and ends at the same 3’ cleavage site (Foissac and Sammeth, 2015). For this reason, transcripts

arising from the combination of more than one AS event will be classed generically as "complex events," contributing to this event type being highly represented relative to other event types.

Overall, of the 139,036 AS events identified by AStalavista (Table 4), 77.1% were complex events. Remaining events were mostly intron retention (11.1%), followed by alternate acceptor site (4.9%), alternate donor site (4.3%), and exon skipping (2.6%). The detected rate of complex events is slightly lower when looking at transcripts within only one parental group (Table 5), with the highest frequency being in high care females (71.7%) and the lowest frequency being in high care males (70.6%). Similarly, other categories of AS were detected in higher frequency: 3.4% to 3.5% exon skipping, 4.6% to 4.9% alternate donor sites, 5.1% to 5.8% alternate acceptor sites, and 15.2% to 15.5% intron retention. As AStalavista considers all transcripts from the same locus simultaneously and attempts to resolve the simplest splicing pattern causative of transcript formation (Sammeth, 2009), it is conceivable that the consideration of a greater number of transcripts from a single locus could increase the number of detected complex events.

Additional analysis was performed to detect the number of events that were conserved between different experimental groups. When observing conservation of AS events by behavioral phenotype (Table 8), high care males and females shared 69.6% of AS events, while low care males and females shared 67.9% of AS events. Similarly, when looking at conservation by sex (Table 6), high and low care females shared 71.1% of detected AS events and high and low care males shared 68.1% of AS events. Further analysis revealed that 82.8% of AS events were conserved in both sexes (Table 7), while 83.9% were conserved in both behavioral phenotypes, suggesting that sex is somewhat more predictive of unique AS event occurrence than intensity of parental care (Table 9).

The highly conserved AS profile observed is consistent with ideas posited in other transcriptomic studies that have indicated that the gene expression profiles of parenting *Nicrophorus* spp. beetles are highly similar, with the largest discrepancies between parental states being degree of gene expression. For example, one study indicated that the genes expressed within the transcriptome of *N. vespilloides* males involved in biparental care were highly similar to the genes expressed by uni- and biparental females and uniparental males.

However, the provisioning of care by biparental males was lower than other parental groups, which was reflected in the DE of several genes (Parker et al., 2015).

Furthermore, this work observes the AS and DE patterns within the transcriptomes of *N. orbicollis* that are all actively parenting. It would be expected that the overall gene expression profiles of these beetles were highly similar with subtle changes in AS and DE contributing to variations in degree of care provisioning behavior. In fact, the DE analysis by Benowitz et al. (2017), using this same dataset, concluded that no single gene seemingly conferred major effects on the variation in the parental phenotype and further suggested that the differential manifestations of the behavior were likely supported by minor polygenic contributions.

AS analysis by rMATS

As the accuracy of AS detection in RNA-seq data has been shown to be higher when multiple predictive algorithms are utilized (Olofsson et al., 2023), rMATS analysis was also performed to predict AS events and gene loci (Shen et al., 2014). Unlike AStalavista, the rMATS algorithm does not classify AS events as "complex events." Instead, one of the many patterns AStalavista would consider a complex event, mutually exclusive exons, is quantified by rMATS. In mutually exclusive exon selection, one exon or one group of exons is included in a transcript while a second exon or group of exons is excluded and vice-versa.

Like AStalavista, rMATS also considers transcripts in the merged transcriptome GTF when assessing AS event occurrence, but the rMATS algorithm focuses on exon and splice junction usage within the mapped reads for each biological replicate. Instead of attempting to characterize the events leading to creation of a transcript as a whole, which are often complex, rMATS classifies individual AS events that occur by identifying variable exon and splice junction usage within mapped reads. It was notable that of the 36,183 AS events identified by rMATS, the most commonly predicted AS event type was intron retention (35.5%), as intron retention tends to be the most common splicing type in unicellular organisms and plants, with lower frequency in other eukaryotes (Table 10) (Wang and Brendel, 2006; McGuire et al., 2008). However, work with *D. melanogaster* has indicated that insects may be subject to higher frequency of intron retention events (Graveley et al., 2011), and evidence suggests that intron retention is more common in insects and invertebrates than in higher eukaryotes, such as mammals (McGuire et al., 2008). Additionally, work in mice and humans has indicated that AS

event type may be tissue-dependent, with the frequency of intron retention being greater in nervous tissue than in other tissue types, such as muscle (Braunschweig et al., 2014).

Identification and characterization of putative DE-AS genes

In addition to performing AS analysis with AStalavista and rMATS, an in-house Perl script was utilized to identify all gene loci from which multiple transcripts originated. Of 24,399 total genes, 10,785 were predicted to be the source of two or more assembled transcripts, with six transcripts being the average. The largest number of transcripts, 269, were from the gene locus for *Down syndrome cell adhesion molecule* (*Dscam*, No_Trans.15976). *Dscam* has been well characterized in *D. melanogaster* and is known to encode 19,008 extracellular domains with a potential isoform number of around 38,000 (Schmucker et al., 2000). It functions in axon and dendrite development in the central nervous system by guiding self-avoidance, which describes how the numerous arbors of one neuron will spread and not cross, and by guiding tiling, which describes efficient regional organization of arbors from several neurons (Matthews et al., 2007). Expression of different *Dscam* variants in neighboring neurons allows for recognition of self and non-self arborizations (Neves et al., 2004) and would be expected to be highly represented in an AS analysis of brain tissue.

As the list of 10,785 gene loci encoding several transcripts was representative of the entirety of AS genes that could be detected within the utilized *N. orbicollis* transcriptomes, the genes in this list were cross-referenced with AStalavista and rMATS output to yield a final list of 5,868 AS genes. There were 524 gene loci predicted to be AS by AStalavista and 1,558 detected only by rMATS that were excluded from further analysis (Figure 1). The list of 5,868 genes, identified as AS by both AStalavista and rMATS, were annotated via a BLASTX search against the UniProtKB/SwissProt database to produce a list of 4,363 annotated AS genes. These genes were compared to the significantly DE genes identified by Cuffdiff, yielding a list of 311 DE-AS genes. Of particular interest were DE-AS genes between high and low care provisioning parents of the same sex. Between high and low care females, only 17 DE-AS genes were identified (Table 11); in comparing DE-AS genes between high and low care males, the top 20 most DE genes were reported (Table 12). DE-AS genes were ranked by the absolute value of the Cuffdiff test statistic, which indicates significance of $\log_2$ fold change in gene expression.

To observe general functional trends of the DE-AS genes, GO analysis was also conducted, wherein highly occurring GO term categories within the DE-AS gene set were compared to the frequency of term occurrence in DE genes, AS genes, and all genes identified. The terms retrieved for each gene set were quantified within the Generic GO slim set (Table 13), curated by the Gene Ontology Consortium, and the frequency of term occurrence for biological processes within this slim set was analyzed further (Figure 2). Overall, GO term frequency was highly similar in the AS gene set and the whole gene set, highlighting the ubiquity of AS in the genome. Similarly, the frequency of terms within the DE and DE-AS gene set were highly similar, with a few exceptions for overrepresentation of DE-AS terms related to developmental and regulatory processes, including anatomical structure development (11.6% in DE-AS genes vs 10.3% in DE genes, 10.3% in AS genes, and 9.7% in all genes), cell differentiation (8.5% vs 7.5%, 7.2%, 6.8%), and cytoskeletal organization (4.0% vs 3.0%, 2.8%, 2.7%). However, it is unlikely that these terms relate to characteristics of the parental care phenotype of interest.

However, several trends in biological processes were observable when considering term categories overrepresented both in the DE and DE-AS gene sets. Still, of the categories believed to be correlated to or resulting from parental care behavior (generation of precursor metabolites and energy, lipid metabolism, immune system processes, defense responses to other organisms, and nervous system processes), there was no category wherein DE-AS genes showed an overrepresentation of terms relative to the DE gene set. Thus, AS could contribute to diversity of function of relevant genes by the production of protein isoforms, but it is not more or less likely for a DE gene of any specific function to be AS. There was, however, one exception involving overrepresentation of DE terms for generation of precursor metabolites and energy relative to DE-AS genes (3.8% in DE genes, 2.2% in DE-AS genes, 0.9% in AS genes and 1.1% in all genes). This GO category describes genes involved in oxidative metabolism. While these genes were found to be commonly DE between parental states (Tables 2 and 3), GO analysis indicates that these genes are not common targets of AS. On the other hand, GO term frequency for genes in lipid metabolism was somewhat higher in the DE-AS gene set than in DE genes (4.2% of DE-AS terms and 3.7% of DE terms vs 2.5% in AS genes and 2.4% in all genes).

High occurrence of DE genes related to metabolism were noted previously, with 15 total oxidative or lipid metabolism genes being within the top 20 DE between high and low care

females and 4 within the top 20 DE genes between high and low care males. Within the DE-AS gene set, a *cytochrome c oxidase* subunit was upregulated in high care females, and three genes for *fatty acid synthase* were downregulated. In males, one DE-AS gene was detected relating to lipid metabolism, *elongation of very long chain fatty acids protein AAEL008004*, which synthesizes long chain fatty acids. Two other genes involved in digestive lipid metabolism were DE-AS for males, *lipase 1* and *gastric triacylglycerol lipase*. Similar to what was observed in females, all DE-AS lipid metabolism genes in males were downregulated in the high care provisioning parents.

Overall, there was a clear metabolic profile separating high care parents from low care parents, especially in females. It was not evident why this profile was less apparent when comparing male parental groups, as all transcriptomes were retrieved from uniparental beetles, and male *N. orbicollis* have been demonstrated to show both behavioral compensation (Fetherston et al., 1994) and comparable changes in gene expression as female beetles in uniparental situations (Parker et al., 2015). It would have been expected that high provisioning male beetles, which averaged 33.9 bouts of care behavior during the eight-hour observation period, would have an expression profile reflecting the high energy expenditure and sacrifice of self-feeding that was evident in gene expression for high care females, which averaged 39.8 bouts of care during the same time of observation (Benowitz et al., 2017). As females provide more care in biparental situations (Fetherston et al., 1990), mate loss alone could contribute to metabolic alteration even in low care provisioning males.

Another well documented contribution to parental sacrifice in *N. orbicollis* is alterations in immune function, as several antimicrobial peptides have evolved social immune functionality (Palmer et al., 2016) and are secreted to maintain the carrion ball for larval wellbeing and to conceal the resource from competitors (Trumbo and Sikes, 2021). Simultaneously, personal immunity of the parents is reduced (Cotter et al., 2013; Reavey et al., 2014). In this analysis, there was overrepresentation of GO terms in the DE-AS and DE gene sets for immune system processes (3.6% for DE-AS genes, 3.4% for DE genes vs 2.6% for AS genes and 2.4% in all genes) and genes conferring defense to other organisms (1.9%, 2.0% vs 1.2%, 1.1%). Immune system processes include biological processes such as activation and regulation of immune

function, whereas defense to other organisms includes processes related to innate and acquired immunity.

In the list of DE-AS genes for females, there are three upregulated genes in high care females that contribute to immune function: *yellow*, *C-type lectin 37Da*, and *E3 ubiquitin-protein ligase RNF216*. Among the most significant DE-AS genes for males, two immune genes are downregulated: *phenoloxidase-activating factor 1* and *cytokine-inducible SH2-containing protein*. Although their functions may vary, many C-type lectins serve an innate or acquired immune function (Brown et al., 2018). Two specific C-type lectins characterized in *D. melanogaster* were found to coat agarose beads and then bind to recruited hemocytes, assisting hemocytes in encapsulation of the beads (Ao et al., 2007). They were also found to agglutinate certain bacterial species, such as *E. coli* but not *S. aureus* or the yeast, *S. cerevisiae*. Thus, it was concluded that different types of C-type lectins contribute to foreign body engulfment via binding site specificity (Brown et al., 2018). One transcriptomic study in *N. vespilloides* identified upregulation of *C-type lectin* in the anal exudate of breeding females relative to non-breeding controls, indicating that members of this protein family may have an established secondary function in social immunity (Palmer et al., 2016).

Conversely, there was observed downregulation of *phenoloxidase-activating factor 1*. Phenoloxidase-activating factors have been characterized in insect innate immune response as a serine protease that is required in the activation of phenoloxidase (Yoshida et al., 1996; Kim et al., 2002). Phenoloxidase functions as an oxidoreductase for pathogen melanization, wherein pathogens are encapsulated in a melanin coat (Lemaitre and Hoffmann, 2007). Work with *N. vespilloides* has demonstrated that hemolymph phenoloxidase levels decrease after breeding in the presence of a reproductive resource, and that levels would partially recover when the parent was wounded, highlighting its role in personal immunity (Reavey et al., 2014).

Also of interest in this analysis were DE-AS genes that are involved in nervous system processes, which were also moderately overrepresented in GO term frequency for DE-AS and DE genes (3.0% and 2.9% vs 2.2%, 1.9%). This included *synaptotagmin-9*, identified as downregulated in high care females, *tyrosine 3-monooxygenase*, upregulated in high care females, and a gene *homologous to galanin receptor 2b*, downregulated in high care males.

Tyrosine 3-monooxygenase, more commonly called tyrosine hydroxylase, is an enzyme in the pathway for dopamine biosynthesis. Dopamine in insects functions in neurotransmission or, alternately, in innate immunity, for melanin synthesis via oxidation by phenoloxidase (Lee et al., 2015). As innate immunity is suppressed during parental care, it is likely that an upregulation of tyrosine hydroxylation is indicative of increased dopamine neurotransmission in high care provisioning parents. It has been demonstrated previously that brain dopamine levels are increased in parenting *N. orbicollis* relative to non-breeding controls (Panaitof et al., 2016). Other behavioral implications of dopamine neurotransmission have been demonstrated in several species, including trophallaxis in *Formica japonica* (Wada-Katsumata et al., 2011), appetitive conditioning in *D. melanogaster* (Burke et al., 2012), and social signaling and food seeking in *A. mellifera* (Huang et al., 2022). Identification of *tyrosine hydroxylase* upregulation in high care provisioning females aligns with the notion that dopamine neurotransmission might contribute to the *N. orbicollis* parental care phenotype.

Synaptotagmins are synapse proteins that detect calcium and facilitate neurotransmitter exocytosis by interacting with SNARE proteins, calcium channels, and several additional presynaptic protein complexes (Littleton and Bellen, 1995). A study in *A. ipsilon* indicated a role for synaptotagmins in facilitating reproductive behavior, wherein *synaptotagmin 1* knowndown reduced male flight in response to a sex pheromone (Bozzolon et al., 2015). Furthermore, they found a dose-dependent increase in *synaptotagmin 1* expression when administering a hormone known to initiate reproductive behavior, 20-hydroxyecdysone. Studies in mammals indicate that synaptotagmins facilitate neural network development in processes such as learning and memory (Liu et al., 2009). Six genes encoding for synaptotagmins have been identified in *D. melanogaster* and are known to be AS (Yanay et al., 2008). Overall, evidence suggests that expression of diverse synaptotagmin isoforms at the synapse coordinates nervous system function via interactions with several presynaptic protein complexes. Downregulation of *synaptotagmin-9* in high care females could be reflective of decreased expression of some neuroendocrine factor.

Also related to neuroendocrine function, one downregulated DE-AS gene in males was annotated as *galanin receptor 2b*, a member of the rhodopsin-like G-protein-coupled receptor protein superfamily. While invertebrates do not have galanin receptors, insects have orthologous

receptors for allatostatin (Hewes and Taghert, 2001). Allatostatins were first characterized in the cockroach, *Diploptera punctata,* for their role in inhibition of JH biosynthesis (Woodhead et al., 1989). Evidence also implicates allatostatin in inhibition of food intake. One study in *Blattella germanica* found that injection with allatostatin decreased food intake by 60% (Aguilar et al., 2003). Activation of allatostatin signaling in *D. melanogaster* also resulted in reduced feeding as well as decreased locomotion and increased sleep (Chen et al., 2016). Downregulation of the receptor for allatostatin in high care males would be indicative of decreased allatostatin production in the central nervous system and would be consistent with a parental phenotype marked by high food consumption for the purposes of trophallaxis and increased activity. In fact, another gene, *daywake*, which is known inhibit sleep in *D. melanogaster* (Yang and Edery, 2019), was also upregulated in high care males.

Notably, low allatostatin signaling would also reduce its inhibitory effects on JH synthesis. Previous work in *N. orbicollis* has indicated increased JH levels upon discovery of a reproductive resource, as well as during care, with levels being highest during the period of most intense care (Scott and Panaitof, 2004; Panaitof et al., 2004). Still, one of the 20 most highly DE genes between high and low care males was *Juvenile Hormone acid O-methyltransferase*, which is involved in JH biosynthesis and showed downregulation in high care provisioning males. However, increased JH biosynthesis is suggested in high care females, for which *methyl farnesoate epoxidase* was upregulated compared to low care provisioning females. Methyl farnesoate epoxidase catalyzes the terminal step of JH biosynthesis (Yagi et al., 1991), and an RNAi-mediated silencing study in *A. mellifera* demonstrated that expression of *methyl farnesoate epoxidase* is necessary for JH production (Bomtorin et al., 2014).

Sashimi plotting of AS genes of interest

To provide further validation of the DE-AS gene *tyrosine hydroxylase* and the AS gene *takeout*, the mapped reads from the 40 transcriptomes were visualized on the genome using IGV. From the mapped read data, IGV constructed Sashimi plots, which represent splice junction usage visually as well as read density at the genomic region of interest. Read density was quantified as reads per kilobase per million mapped reads (RPKM). Like FPKM, RPKM is a normalized measurement of reads that accounts for sequencing depth. However, RPKM does not

account for overlap that often occurs within two paired-reads and will count each read individually.

Genes selected for Sashimi plotting were *tyrosine hydroxylase*, which is involved in dopamine biosynthesis, and two genes for *takeout*, which has been suggested to play a role in *N. orbicollis* kin recognition (Potticary et al., 2023). All Sashimi plots show evidence of AS. For *tyrosine hydroxylase*, there were 278 reads spanning an exon skipping splice junction (Figure 3). This event was also predicted by both AStalavista and rMATS (Table 14). The Sashimi plots for *takeout* (Figures 4 and 5) also showed evidence for AS. Interestingly, two genes for *takeout* (No_Trans.12893 and No_Trans.12894) were found to lie consecutively in the genome, as annotated in the source GFF. While it is possible that this could be an error in scaffold assembly during genome sequencing, this is more likely explained by a duplication event. Evidence for duplication is further strengthened when observing the Sashimi plots, as the splice junctions involved in predicted AS events differ between these two gene loci. Duplication events are known to facilitate the introduction of novel functionality via genetic diversity, wherein the original copy of the gene tends to maintain its original function while the duplicated copy mutates and acquire new functionality (Van de Peer et al., 2009). This phenomenon has been studied once before in *N. vespilloides* in the gene *malvolio*, the duplicates of which show tissue-specific expression (Mehlferber et al., 2017). Identification of a possible duplication event and AS within *takeout* is suggestive of diversity in its function. However, no gene for *takeout* was predicted to be DE between parental states in this work.

In one *takeout* gene (No_Trans.12893), AStalavista predicted two alternate acceptor site AS events, which is consistent with the Sashimi plot (Figure 4). While rMATS also predicted the alternate acceptor site events (termed alternate 3’ splice site by rMATS), this algorithm also predicted a mutually exclusive exon and exon skipping event, which were not apparent in the Sashimi plot. For the other *takeout* gene (No_Trans.12894), AStalavista detected two alternate acceptor site events, which are evidenced within the plot (Figure 5). rMATS detected those two events as well as two alternate donor site events (alternate 5’ splice site), which are observable via the Sashimi plot as 94 reads at one splice junction and 49 reads at another seem to utilize an alternate donor site.

Recent research on the protein takeout indicates a role in the temporal kin recognition involved in *N. orbicollis* parental care (Potticary et al., 2023), whereby parents rapidly shift from infanticidal behavior to care provisioning behavior approximately 8 to 12 hours prior to larval arrival at the carrion ball (Oldekop et al., 2007). The protein takeout has been characterized in *D. melanogaster* as contributing to circadian-influenced food intake (Sarov-Blat et al., 2000; So et al., 2000) and male courtship (Dauwalder et al., 2002). Other work in *D. melanogaster* demonstrated that high *takeout* expression contributed to longer life, while male courtship behavior and oviposition were reduced (Chamseddin et al., 2012). These effects were reversed with the application of a JH analog. As takeout contains a putative JH binding domain (Sarov-Blat et al., 2000), Chamseddin et al. (2012) posited that binding of JH to takeout in the hemolymph may decrease JH-mediated reproductive behavior. Previous work in *N. vespilloides* observed downregulation of *takeout* in parenting beetles via transcriptomic methods and qRT-PCR (Parker et al., 2015). However, the qRT-PCR study performed by Potticary et al. (2023) noted higher *takeout* expression in parents who accepted early larval arrival at the reproductive resource than parents who left or committed infanticide. It could be that *takeout* levels are upregulated after mating or oviposition to mediate proper temporal kin recognition and then decline once parental behavior commences.

CONCLUSION

This is the first study to undertake a systematic analysis of the extent and event types of pre-mRNA processing by alternative splicing occurring in the burying beetle, *Nicrophorus orbicollis.* This study utilized genomic and transcriptomic resources that were only recently available for the *Nicrophorus* spp. beetles with the aims to 1) characterize the extent and types of alternative splicing events in this species and 2) assess differential expression and alternative splicing in genes that are associated with distinct parental behavioral phenotypes in male and female burying beetles during breeding.

This was achieved by performing differential expression analysis on 40 *N. orbicollis* genomes, 10 of which were from each of the following behavioral groups: high care provisioning females, low care provisioning females, high care provisioning males, and low care provisioning males. Analysis focused on differentially expressed genes between high and low care parents of the same sex, and the 20 most highly differentially expressed genes for females and males were presented. Overall, differential gene expression analysis indicated a highly variable metabolic profile between parental states, wherein several genes involved in oxidative metabolism showed upregulation in high care parents relative to low care parents. On the other hand, *fatty acid synthase*, which functions in endogenous lipogenesis, were downregulated in high care parents. The metabolic profile indicated by the differential expression analysis was consistent with higher overall energy expenditure and decreased parental self-feeding within the high care phenotype.

In characterizing the types of alternative splicing events, two algorithms were used to increase the predictive accuracy in identifying alternatively spliced genes, AStalavista and rMATS. AStalavista analysis, which predicts alternative splicing events that occur to generate alternative transcripts from a single gene locus, indicated that the majority of alternatively spliced transcripts resulted from complex events (77.1%), which is the combination of several concurrent alternative splicing events. Meanwhile, rMATS, which does not quantify complex events, as it focuses on identifying alternative splicing events at individual splice junctions, predicted the most common type of alternative splicing event was intron retention (35.5%) followed by exon skipping (26.7%). The AStalavista results showed higher incidence of complex events than observed in other model species utilizing the same algorithm (Sammeth et al., 2008) and likely resulted from trying to resolve alternative splicing in many transcripts from the same

gene locus, as there was an average of six transcripts for each gene locus that had more than a single assembled transcript. However, rMATS results, showing similar frequency of intron retention and exon skipping, were consistent with what was observed in Sammeth et al. (2008) for fruit flies and honeybees. It was somewhat unexpected to see a higher frequency of intron retention than exon skipping, as this is more common in plants. For example, intron retention makes up approximately 40% of alternative splicing events in *Arabidopsis thaliana* (Syed et al., 2012). The intron retention rate is lowest in mammals, with its frequency in humans being reported as low as 3% (Ast, 2004). Still, work in the fruit fly has suggested intron retention could represent up to 36% of alternative splicing events in this species (Kan et al., 2002), consistent with the findings presented here in *N. orbicollis*.

Overall, 5,868 genes were predicted to be alternatively spliced by AStalavista and rMATS and were subject to further functional characterization. Gene Ontology analysis revealed that genes that were differentially expressed between behavioral groups and alternatively spliced were not more likely than differentially expressed genes to be involved with any specific biological process that is associated with aspects of the parental care phenotype. Genes involved in oxidative metabolism were more likely to be differentially expressed and not alternatively spliced. However, genes involved in lipid metabolism, immunity, and nervous system processes were found to be more highly differentially expressed and differentially expressed/alternatively spliced. For a select group of these genes, potential behavioral implications, based on observations in other species, were presented. Particular focus was placed on tyrosine hydroxylase, involved in dopamine biosynthesis, due to the demonstrated increased brain dopamine levels in parental *N. orbicollis* (Panaitof et al., 2016), and on takeout, which recent research has suggested is involved in temporal kin recognition (Potticary et al., 2023). Alternative splicing events in these genes were visualizable via Sashimi plotting.

At the gene level, these types of differential expression analyses could identify novel candidate genes for which expression level between behavioral states could be further verified via qPCR analysis. Future work could seek to more fully characterize the extent of alternative splicing in *Nicrophorus* spp. by repeating analysis with RNA-seq data from a variety of life stages and tissue types. This more expansive analysis could provide better resolution of gene variants produced by alternative splicing and would allow for analysis at the transcript level.

Transcript level analysis would provide the basis for experiments wherein variant-specific qPCR primers could be used to assess tissue-specific variant expression or differential variant expression between parental care states. Additional improvements in future work could take the form of genome annotation by comparative analysis (Fiddes et al., 2018) with other, more well-annotated beetle species to minimize the introduction of spuriously assembled transcripts in RNA-seq data processing.

This work serves as an initial assay of alternative splicing events in *N. orbicollis*, as this source of genetic diversity had not yet been assessed in burying beetles. Alternatively spliced genes that were also differentially expressed between parental beetles showing variation in care behavior were identified and discussed. As phenotypic variability must be underscored by genetic diversity, it is necessary to consider the effects of alternative splicing when characterizing the molecular mechanisms that contribute to the parental care behaviors exhibited by this species and the variation therein.

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