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RESEARCH ARTICLE

Transcriptomic responses of females to consumption of nuptial food gifts as a potential mediator of sexual conflict in decorated crickets

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Abstract

Nuptial food gift provisioning by males to females at mating is a strategy in many insects that is thought to be shaped by sexual conflict or sexual selection, as it affords males access to a female's physiology. While males often attempt to use these gifts to influence female behaviour to their own advantage, females can evolve counter mechanisms. In decorated crickets, the male's nuptial gift comprises part of the spermatophore, the spermatophylax, the feeding on which deters the female from prematurely terminating sperm transfer. However, ingested compounds in the spermatophylax and attachment of the sperm-containing ampulla could further influence female physiology and behaviour. We investigated how mating per se and these two distinct routes of potential male-mediated manipulation influence the female transcriptomic response. We conducted an RNA sequencing experiment on gut and head tissues from females for whom nuptial food gift consumption and receipt of an ejaculation were independently manipulated. In the gut tissue, we found that females not permitted to feed during mating exhibited decreased overall gene expression, possibly caused by a reduced gut function, but this was countered by feeding on the spermatophylax or a sham gift. In the head tissue, we found only low numbers of differentially expressed genes, but a gene co-expression network analysis revealed that ampulla attachment and spermatophylax consumption independently induce distinct gene expression patterns. This study provides evidence that spermatophylax feeding alters the female post-mating transcriptomic response in decorated crickets, highlighting its potential to mediate sexual conflict in this system.

KEYWORDS
Gryllodes sigillatus, nuptial food gift, sexual conflict, sexual evolution, transcriptomics

1 | INTRODUCTION

In many insects, males offer the female a nuptial food gift prior to, during or after mating (Lewis et al., 2014; Lewis & South, 2012; Sakaluk et al., 2019; Vahed, 1998, 2007). These gifts range from prey items to male secretions, and in some cases, males even sacrifice portions of their bodies or, in the extreme, their life (Andrade, 1996; Eggert & Sakaluk, 1994; Lewis & South, 2012; Sakaluk et al., 2004). Provisioning of nuptial food gifts by males, although potentially costly (Lebas & Hockham, 2005), frequently results in a net fitness benefit for males, primarily through increased mating or fertilization success (Vahed, 1998), while the consequences of nuptial food gift consumption in females range from beneficial to detrimental (Gwynne, 2008; Lewis et al., 2014; Vahed, 2007). Although nuptial

gifts are a frequent target of sexual selection and sexual conflict in a variety of species, they have been understudied compared to other, more obvious sexual traits such as male weaponry or colourful sexual ornaments.

For a male, provisioning of a nuptial gift may enhance his probability of attracting a mate (Alcock, 1979), but also represents a route through which he might influence subsequent female behaviour or physiology. While food gifts might represent a paternal investment in the offspring or in female survival (Gwynne, 2008, Lewis et al., 2014) males might also use the gift as a more nefarious vehicle to manipulate female behaviour and physiology to their own advantage, sometimes even at a cost to the female (Sakaluk et al., 2019; Vahed, 1998, 2007). Negative fitness consequences to females can ensue if gift consumption results in decreased female longevity or receptivity to further matings that would otherwise be beneficial. This might occur if substances in nuptial gifts influence female behaviour and physiology to the benefit of the male, for example, by eliciting immediate female reproductive effort to the detriment of future female reproduction. The sexual conflict over female reproduction in gift-giving species might lead to sexually antagonistic coevolution. Indeed, it is predicted that over time the chemistry of male gifts will be selected to influence female physiology and behaviour in a manner that aligns with the fitness interests of the male. At the same time, there is a corresponding selection pressure on females to evolve counter-adaptations to such manipulations (Gershman et al., 2012, 2013; Sakaluk et al., 2006). There are a considerable number of studies investigating nuptial gifts at the organismal level, but molecular dissections of male investment into nuptial gifts and the female response to them, which might deepen our understanding of the selective pressures surrounding the evolution of nuptial food gifts, are limited (but see Al-Wathiqui et al., 2016).

The role of the nuptial gift in sexual conflict has been well-studied in the decorated cricket *Gryllodes sigillatus* (F. Walker) (Sakaluk et al., 2019). In this species, the nuptial gift takes the form of a spermatophylax, a gelatinous mass that is transferred to the female during the mating together with the ampulla, the sperm-containing portion of the spermatophore (Alexander & Otte, 1967). Once the mating is completed, the female detaches the spermatophylax from the ampulla and begins feeding on it, for approximately 40 min (Sakaluk, 1984). Once the female has consumed the spermatophylax or discards it prematurely, she removes the ampulla from her genital opening, terminating the transfer of sperm and other ejaculatory material (Sakaluk, 1984; Sakaluk et al., 2019). Thus, nuptial gift feeding deters the female from prematurely removing the ampulla, enticing females into relinquishing some of their control over insemination (Sakaluk, 1984, 1985, 1987; Sakaluk & Eggert, 1996). The spermatophylax is made up of mainly water, proteins, and free amino acids (Gordon et al., 2012; Warwick et al., 2009). Beyond benefits to females when they are water deprived (Ivy et al., 1999), there appear to be no significant nutritional benefits of nuptial gift consumption to females. Instead, the composition of the nuptial gift may enhance its gustatory appeal, resulting in increased sperm transfer (Gershman et al., 2012). Female decorated crickets exhibit polyandry and store all of the sperm they receive in their spermatheca, which is then used in direct proportion to their abundance during egg fertilization (Calos & Sakaluk, 1998; Eggert et al., 2003; Sakaluk, 1986; Sakaluk & Eggert, 1996). Even though polyandry confers indirect genetic benefits to the female (Ivy, 2007; Ivy & Sakaluk, 2005; Sakaluk et al., 2002), it greatly reduces the reproductive success of a male. The spermatophylax represents a counter-adaptation to mitigate the effects of sperm competition and to enhance paternity by increasing the amount of sperm transferred to females (Sakaluk, 1984, Sakaluk et al., 2019). In addition, male crickets may transfer compounds to the female that reduce the female's receptivity to future matings or alter female behaviour and physiology in other ways that enhance male paternity and thus fitness (Sakaluk, 2000; Sakaluk et al., 2006), as commonly found in other gift-giving insects (Arnqvist & Nilsson, 2000; Gillott, 2003).

In decorated crickets, the spermatophylax and the transfer of ejaculatory material from the ampulla both allow males direct access to female physiology (Sakaluk et al., 2019). In this study, we aimed to dissect how these two distinct routes and mating per se influence the transcriptional response of females. We conducted an exploratory RNA sequencing experiment on females for whom consumption of nuptial food gifts and receipt of sperm (i.e. ampulla attachment) was independently manipulated, aiming to inform future studies. We focused on the gut tissue and the head tissue, which respectively represent the place of first contact between the female and the spermatophylax, and the location where behavioural changes in the female are initiated. By comparing gene expression between the different treatments, we attempted to answer the following three questions: (1) How does mating influence gene expression in females? (2) Is this altered gene expression a consequence of the sperm transfer from the ampulla, feeding on the spermatophylax, or both? and (3) If there is an effect of spermatophylax feeding, is this effect caused merely by the act of feeding, or is it a consequence of the content of the spermatophylax per se? Our data suggest that, at least at the investigated timepoint, mating has no effect on gene expression in the gut, except when females are not allowed to feed during sperm transfer. In addition, we found only small numbers of significantly differentially expressed (DE) genes for the head tissue, independent of which treatments were compared. However, using a gene co-expression network analysis we show that the attachment of the ampulla and the consumption of the spermatophylax independently influence the gene expression of unique and distinct gene sets.

2 | MATERIAL AND METHODS

2.1 | Cricket husbandry

All crickets used in this experiment descended from 500 adult *G. sigillatus* collected in Las Cruces, New Mexico in 2001 that was used to initiate a laboratory culture (Ivy & Sakaluk, 2005). Crickets used for the RNA sequencing experiment, performed in 2015, were

maintained at a population size of approximately 5000 crickets at the University of Exeter, Cornwall Campus, Cornwall, UK. They were kept in ten 15-L plastic containers in an environmental chamber (Percival I-66VL) maintained at 32 ± 1°C on a 14 h:10 h light/dark cycle. They were provided with *ad libitum* cat food (Go-Cat Senior®, Purina), rat food pellets (SDS Diets), and water in glass vials plugged with cotton. Experimental crickets were removed from this colony in 2015 as newly hatched nymphs and housed individually in plastic containers (5 cm × 5 cm × 5 cm). These nymphs were used to set up an RNA sequencing experiment. Each individual nymph was provided with a piece of cardboard egg carton for shelter, water, and cat food pellets, with food and water replaced weekly. Experimental animals were checked daily for eclosion to adulthood and experiments were performed 8 days after eclosion to adulthood. In 2021, we conducted a follow-up quantitative polymerase chain reaction (qPCR) experiment, for which we used descendants from the same laboratory culture, which were reared at Illinois State University, Illinois, USA. They were reared under similar conditions but were kept at a population size of approximately 500 crickets in 19 L containers. Cat food and water were provided as before, but different rat food pellets (Tekland Global Diets, Envigo) were used. Experimental crickets were removed from the cricket culture on the day of eclosion to adulthood and subsequently kept for 8 days in small cages as described above.

2.2 | Mating and feeding treatments

For the RNA sequencing experiment, individual females were transferred to larger individual plastic containers (20 cm × 10 cm × 10 cm) under red light conditions, and randomly allocated to different combinations of mating and feeding regimes: (i) virgin (V), (ii) spermatophylax and ampulla, also referred to as fully mated (SA), (iii) spermatophylax but no ampulla, also referred to as spermatophylax (S), (iv) ampulla but no spermatophylax, also referred to as ampulla (A), and (v) ampulla and pectin gel as a simulated spermatophylax to be consumed (PA) (see also Figure 1a). Thirty-six females were assigned to each treatment, which were later pooled in groups of nine individuals to obtain four replicate RNAseq libraries per treatment. Females were allowed to acclimate to their new environment for 30 min before the mating trial was initiated. Virgin females were not provided with a male and were thus sexually naive. SA females were paired with an 8-dayold male and were allowed to mate normally. Spermatophylax females were not paired with a male but were instead offered a spermatophylax, acquired from an 8-day-old male, on the tip of a dissecting needle. The A females were allowed to mate normally, but the spermatophylax was removed before the female could begin consuming it, with females restrained in a 2 ml tube to prevent premature ampulla removal. Finally, PA females were allowed to mate, but before spermatophylax consumption could begin, PA females were instead offered a synthetic food gift on the tip of a dissecting needle. Synthetic food gifts were manufactured following the protocol outlined in (Gordon et al., 2012), and contained insect saline and pectin but none of the amino acids or proteins present in a spermatophylax (Warwick et al., 2009). Females of each treatment were observed after mating to ensure that, where applicable, females consumed the spermatophylax for at least 20 min before discarding it and similarly, the ampulla remained attached to the female reproductive tract for at least 20 min. Females were then returned to their individual containers with food, water, and shelter. For the 2021 qPCR experiment, we repeated all of the above treatments with the exception of PA and included five individuals in each treatment. Matings were staged as described above, in a mating arena of 10.5 cm × 4 cm × 7.6 cm.

2.3 | Female tissue dissections and RNA extraction

For the RNA sequencing experiment, female crickets were dissected 18–20 hours after mating behaviour was observed. This timepoint was chosen as it is close to the expected period between two matings in a natural setting for female *G. sigillatus*, which mate approximately once every 24 h (Sakaluk et al., 2002). Crickets were placed at −80°C for approximately 2 min before the dissections. The head and gut tissue were dissected and individually preserved in 200 µl of RNA*later*® (Ambion, Thermo Fisher Scientific) following standard procedures. RNA was extracted using a Trizol-chloroform extraction, after which samples were run through a PureLink RNA mini kit (Thermo Fisher Scientific) and treated with an on-column DNAse treatment. RNA was extracted from a total of 36 samples for each tissue and experimental treatment combination, after which equimolar amounts of RNA from 9 different specimens were merged to get a total of 40 pools (4 pools/group, 5 combinations of mating and feeding treatments, and 2 tissues).

For the 2021 qPCR experiment, five crickets per group were put on ice for a few min 18–20 hours after mating behaviour was observed. Heads were cut off and immediately snap frozen in liquid nitrogen, and stored at −80°C. RNA extractions were performed using a Trizol (Thermo Fisher Scientific) extraction with 1-bromo-3-chloropropane (BCP, Acros Organics), followed by a DNAse treatment using a TURBO DNA-free kit (Thermo Fisher Scientific). Samples were cleaned up with ammonium acetate precipitation.

2.4 | RNA sequencing and transcriptome assembly

Sample preparation, sequencing, and read demultiplexing were all performed by Exeter Sequencing service, University of Exeter, UK. Paired-end 100 bp reads were obtained by multiplexing the samples on four lanes of an Illumina HiSeq 2500, merging 10 pools on a lane. All reads generated for this project were uploaded onto the Bridges-2 system of XSEDE (Towns et al., 2014), and were subsequently filtered with Trimmomatic (Bolger et al., 2014) using the following thresholds: minimum quality score of 30 for bases on either end, a sliding window of 3 bases with a minimum average

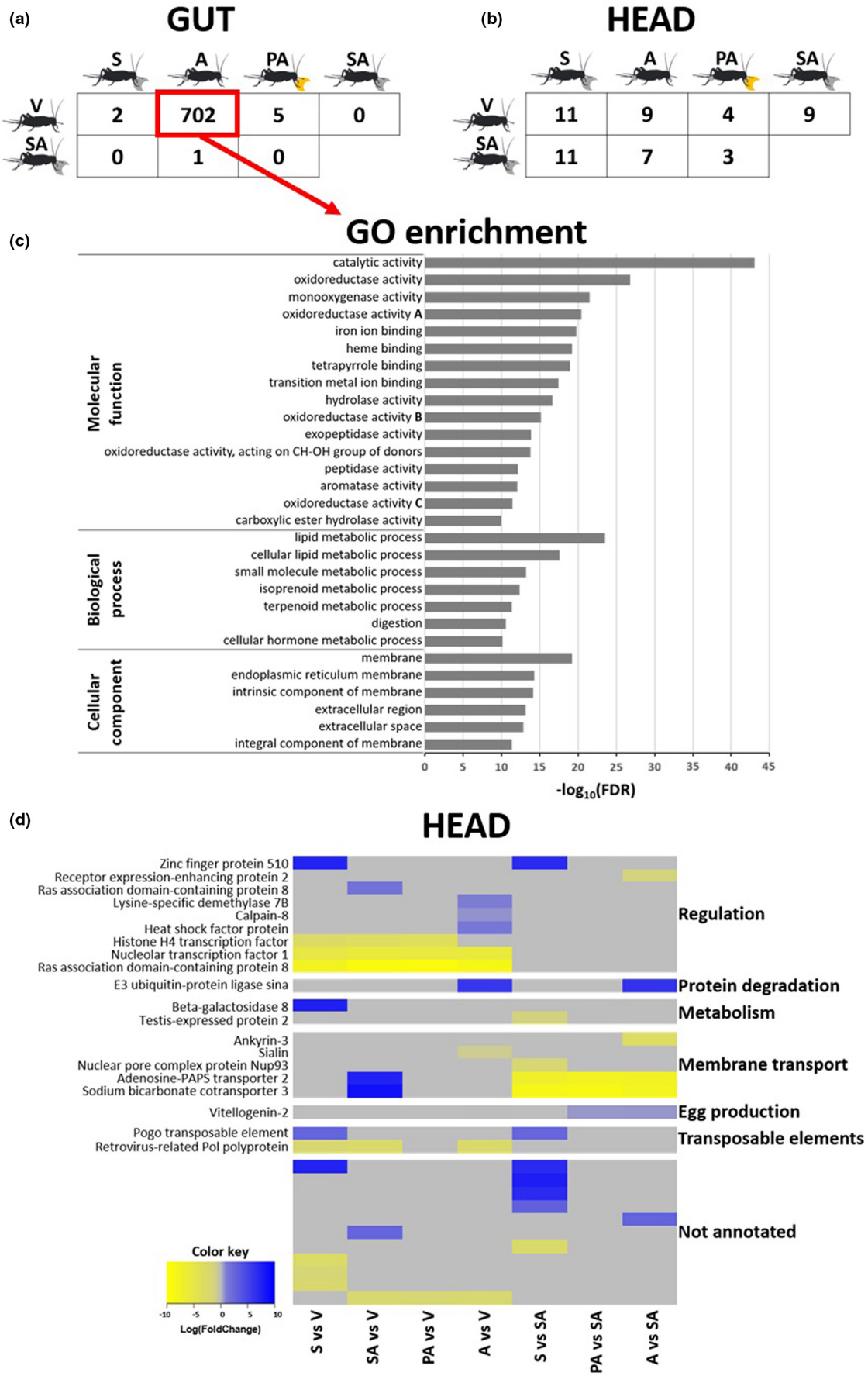

FIGURE 1 Differentially expressed genes. The number of differentially expressed genes for each relevant comparison from the gut (a) and the head (b) tissue. Differentially expressed genes were discovered with edgeR, and the numbers represent genes with an FDR <0.05 after a Benjamini–Hochberg correction for multiple testing. Each cartoon represents one of the experimental treatments, with the circle attached to the end of the abdomen representing ampulla attachment, and the larger crescent-shaped figure representing either the spermatophylax (grey) or a replacement pectin gel (yellow). (c) Gene ontology enrichment for the V vs. A comparison in the gut tissue. The most significantly enriched gene ontology terms (FDR < 10^{-10}) for genes upregulated in virgin females compared to ampulla females are shown, together with the logarithm of their false discovery rate. Gene ontology terms are grouped by their major gene ontology category. FDR = false discovery rate, as calculated by Goseq. Oxidoreductase activity A: "oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen." Oxidoreductase activity B: "oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor." Oxidoreductase activity C: "oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen." (d) Differentially expressed genes in the head tissue and their function. All genes that were differentially expressed for at least one of the relevant comparisons were included in a heatmap. Bright yellow and blue colours represent respectively lower and higher expression levels of the first treatment in the comparison, while a grey colour indicates that no statistically significant differential expression was found. Genes were clustered based on their expected function, and the gene names given during the gene annotation process were listed on the left side of the plot. V = virgin, SA = spermatophylax + ampulla = fully mated, S = spermatophylax but no ampulla, a = ampulla but no spermatophylax, PA = pectin gel and ampulla.

quality score of 30, and minimum read length of 25. Kraken2 (Wood et al., 2019) was used to identify and subsequently remove contaminating reads originating from bacteria, protozoans, and viruses. Before transcriptome assembly, reads generated for an earlier, similar but unpublished project performed on the same tissues and same cricket colony were added to our pool of reads, to increase read coverage during the assembly. These reads were subjected to the same clean-up steps as described above and were only used during the transcriptome assembly. Thereafter, transcriptomes were assembled separately for each tissue using Trinity v2.11.0 (Grabherr et al., 2011) using default settings, resulting in a head and a gut transcriptome. After the assembly was complete, all data were downloaded from the Bridges-2 system and further bioinformatics were conducted at Illinois State University. We removed duplicates and highly similar sequences using CD-hit-EST (Fu et al., 2012; Li & Godzik, 2006), with a threshold of 0.9. Subsequently, transcriptome assembly was assessed using trinitystats (Grabherr et al., 2011), bowtie2 (Langmead et al., 2009; Langmead & Salzberg, 2012), and BUSCO (Simão et al., 2015) (Table S1). Finally, transcriptomes were annotated using the Trinotate pipeline (Bryant et al., 2017). Transcripts were translated into their most likely coding regions, if any, using Transdecoder (http://transdecoder.github.io). Both the resulting protein products and all original transcripts were used to find similar sequences in the Swiss-Prot protein database (Boeckmann et al., 2003), using either BLASTP or BLASTX with a threshold of E ≤ 10^{-5} (Camacho et al., 2009). Signal peptides, transmembrane helices, and protein domains were predicted using SignalP v4.1 (Petersen et al., 2011), tmhmm v2.0 (Krogh et al., 2001), and HMERR (http://hmmer.org/) with the PFAM database (El-Gebali et al., 2019), respectively. The results, in addition to KEGG (Kanehisa et al., 2016), Eggnog (Huerta-Cepas et al., 2019), and Gene Ontology (GO) (Ashburner et al., 2000) annotations were parsed by Trinotate and stored in an SQLite database. The transcriptome assemblies generated in this project have been deposited at DDBJ/EMBL/GenBank under the accessions GJRV00000000 and GJRY00000000. All raw reads are deposited at DDBJ/EMBL/GenBank under the bioproject PRJNA784797.

2.5 | Transcriptomic analysis

Filtered sequence reads for the gut and the head were mapped back to their respective transcriptome with Bowtie2 (Langmead et al., 2009; Langmead & Salzberg, 2012), and the number of read mappings were counted using RSEM v1.3.3 (Li & Dewey, 2011). Subsequently, differential expression was analysed using edgeR (Robinson et al., 2010) in R version 4.1.2. First, genes with all sample counts under 10 or a total read count under 100 were excluded. Then samples were normalized with a TMM normalization and dispersions were calculated in the classic mode (Robinson et al., 2010). To find DE genes, we used exact tests followed by a Benjamini-Hochberg correction for multiple testing (Robinson et al., 2010), and only genes with FDR <0.05 were considered to be DE genes.

Gene co-expression patterns in the head tissue were analysed with a weighted gene co-expression network analysis (WGCNA) (Langfelder & Horvath, 2008) in R version 4.1.2. Read counts for each sample were normalized using the TPM (transcripts per million) method. All genes with an average read count under 5 were removed, after which only the top 60% of most variable genes were retained. As a result, our final dataset contained 16 667 genes. Using the scale-free topology criterion, the soft-threshold power was set to 5 for the calculations of the adjacency matrix (Zhang & Horvath, 2005). Modules of co-expressed genes were obtained with a one-step unsigned co-expression network. DynamicTreeCut (Langfelder et al., 2007) was used to detect modules of more than 30 genes with a threshold of 0.05 for separating branches in the dendrogram. We subsequently coded three different variables: "Ampulla attachment," "Spermatophylax consumption," and "Feeding." Females received a value of 0 or 1 for each of these variables, with values of 1 given if they respectively received an ampulla, fed on a spermatophylax, or fed on either

spermatophylax or pectin gel (Table S2). Subsequently, correlations were calculated between the eigengene of each module and these three variables. Gene networks were visualized in VisANT visualization software (Hu et al., 2013). GO enrichment of a test group compared to the respective transcriptome was performed with GOseq (Young et al., 2010).

2.6 | Real-time quantitative PCR

The RNA quality and RNA concentration were measured with a MultiSkan GO microplate spectrophotometer with a µDrop adapter plate (ThermoScientific), and only samples with 260/230 and 260/280 values over 2 were used for further analysis. We used five samples for each experimental group. Samples were diluted to 100 ng/µl and were converted to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific) following standard procedures. Primers for both reference genes and target genes were designed using Primer3 (Koressaar & Remm, 2007; Untergasser et al., 2012) using sequences extracted from the head transcriptome assembled in this study. All primers were ordered from Integrative DNA Technologies (IDT) and can be found in Table S3.

All real-time qPCR experiments were performed following the MIQE guidelines for qPCR experiments (Bustin et al., 2009). For each qPCR reaction, 2 µl of cDNA was added to 10 µl of Power SYBR™ Green PCR Master Mix (Fisher #4368702), 6.8 µl of H_2O, and 1.2 µl of primers at a final concentration of 300 nM. All reactions were run in duplicate on 96 well plates, using the following thermal cycling profile on a QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific): 2 min at 50 °C, 10 min at 95°C, 40 cycles of (1) 15 s at 95°C and (2) 1 min at 60°C, and a melting curve from 95 to 60°C. C_q values were exported using the default threshold. To obtain primer efficiency, a serial 5-fold dilution series up to a dilution of 1/3125 was generated based on cDNA generated as described above. Only primer pairs with an efficiency higher than 90% were used in further analyses (Table S3).

To select stable reference genes, we performed a reference gene stability analysis on 12 of our samples, equally divided over the four treatments (SA, A, S, and V). Five potential reference genes were selected based on studies in other orthopterans (Chapuis et al., 2011; Foquet & Song, 2020; Van Hiel et al., 2009; Yang et al., 2014): elongation factor 1 (*EF1*), actin 5C (*Act5C*), ribosomal protein L5 (*RIBL5*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and heat shock protein 90 (*Hsp90*) (Table S3). The obtained C_q-values of these five genes for all 12 samples were used to rank the potential reference genes based on their stability. Rankings were obtained from three different programs, geNorm (Mestdagh et al., 2009; Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), and BestKeeper (Pfaffl et al., 2004), and the overall ranking was obtained with the RankAggreg package (Pihur et al., 2007), which were all included in the endogenes pipeline (https://github.com/hanielcedraz/refGenes) and run in R (version 4.1.1). This analysis showed that Act and EF1 were the most stable reference genes (Table S4) and these two genes were used as reference genes for all further qPCR experiments.

Subsequently, we assessed the relative expression of six target genes (*Hinfp*, *Ubtf*, *Nup93*, *Vg2*, *SLC35B3*, and *Rassf8*) in the four experimental groups (SA, A, S, and V), now using all 20 samples (five samples per treatment combination). qPCRs were set up as described above, and relative expression, compared to the V group, was calculated as $2^{-\Delta\Delta Cq}$ using the $\Delta\Delta C_q$ method (Livak & Schmittgen, 2001). Statistical significance was evaluated with a two-tailed student *t*-test in R (version 4.1.2) based on non-transformed ΔC_q-values.

3 | RESULTS

3.1 | Sperm transfer-induced transcriptional changes in the gut are reversed by feeding

We first focused on post-mating gene expression in the female gut tissue, where the spermatophylax is processed after consumption, for all five treatment combinations of virgin (V), fully mated or spermatophylax + ampulla (SA), spermatophylax but no ampulla or spermatophylax (S), ampulla but no spermatophylax or ampulla (A), and finally ampulla with pectin gel (AP). There were no DE genes in the gut tissue between virgin and fully mated females (Figure 1a, Table S5). In addition, we find little evidence for a transcriptional effect of the spermatophylax in the gut tissue at the tested timepoint, as we only found two DE genes when comparing virgin females with spermatophylax females, and only one DE gene when comparing fully mated females with ampulla females (Figure 1a, Table S5). However, the comparison between virgin females and ampulla females yielded 702 DE genes (Figure 1a, Table S5). These groups only differ in the presence of the ampulla, and neither was allowed to eat during the mating. Six hundred and thirty-three of these DE genes were downregulated in ampulla females. While the most enriched GO terms were more general terms like catalytic activity and oxidoreductase activity, several significantly enriched GO terms were associated with the normal gut function (Figure 1c, Table S6). The remaining 69 genes, which were upregulated in ampulla females, did not show any significant enrichment of GO terms. Interestingly, only 5 of 702 genes were also DE when females were given a pectin gel to consume at the time of mating when compared with virgins (Figure 1a, Table S5), and only one was DE when compared with ampulla females (Table S5).

3.2 | Mating induces only small transcriptional changes in the female head tissue

In the head tissue, we found only small numbers of DE genes when performing pairwise comparisons of gene expression. For instance, only 9 DE genes were found when comparing virgin females with fully mated females, and respectively 9 and 11 DE genes were found when comparing virgins with ampulla females

and spermatophylax females, respectively (Figure 1b, Table S7). Several of these DE genes could feasibly be involved in the regulation of gene expression (Figure 1d, Table S7). These genes were especially found in the comparison between virgins and ampulla females, and virgin females exhibited a significantly lower expression for three such genes in all comparisons. Additionally, the fully mated group receiving both the spermatophylax and ampulla showed significantly lower expression levels for several genes involved in membrane transport in multiple comparisons (Figure 1d) and vitellogenin-2 exhibited a significantly increased expression in fully mated females when compared with either ampulla females or ampulla females fed a pectin gel (Figure 1d and Figure S1, Table S7).

We subsequently sought to confirm the results obtained with RNA sequencing by conducting a qPCR experiment, by selecting six genes that were DE in at least one pairwise comparison (histone H4 transcription factor, *Hinfp*; nucleolar transcription factor 1, *Ubtf*; Nuclear pore complex Nup93, *Nup93*; Vitellogenin 2, *Vg2*; adenosine 3′-phospho 5′-phosphosulfate transporter, *SLC35B3*; and Ras association domain-containing protein 8, *Rassf8*). Although our qPCR data show similar trends to our RNA sequencing data for several genes, it does not concur fully with the RNA sequencing data (Figure S1) and we were only able to confirm one of the statistically significant differences found with RNA sequencing (*Ubtf*, $t = -4.4222$, p-value <0.001).

3.3 | Ampulla attachment and spermatophylax consumption induce different transcriptional signatures in the female head

Because the pairwise analysis of differential expression for the head did not reveal strong patterns of differential expression when focusing on single genes, we analysed the gene expression patterns for the head transcriptomic data with a weighted gene co-expression network analysis. This method clusters co-expressed genes together in modules and can detect expression patterns that would otherwise be missed using a regular analysis of DE genes (Abbassi-Daloii et al., 2020). We coded each of the five treatment combinations by giving them a value of 0 or 1 for the three following traits: "ampulla attachment," "feeding (either pectin gel or spermatophylax)," and "spermatophylax consumption" (Table S2). Our analysis identified 80 modules, and the majority of these were either correlated with "ampulla attachment," or with "spermatophylax consumption," without a clear overlap between the two (Figure 2 and Figures S2 and S3, Tables S8 and S9). When comparing the modules correlated with "spermatophylax consumption" and "feeding," there was a more obvious overlap, but generally, correlations were stronger with "spermatophylax consumption" than with "feeding," suggesting that adding the samples that fed on the pectin gel diluted the correlation (Figure 3, Tables S8 and S9).

Three modules showed highly significant correlations with an absolute value of over 0.7 to one of the three studied traits (Figure 2 and Figure S4). Two of these modules, Modules 1 and 2 (Figure 2), were correlated with spermatophylax consumption highly positively (cor = −0.80, $p = 2\times10^{-5}$) and highly negatively (cor = 0.70, $p = 1\times10^{-4}$), respectively. Module 3 was highly correlated with ampulla attachment (corr = 0.74, $p = 2\times10^{-4}$). Module 1 contained 103 genes and showed GO enrichment for various terms related to muscle formation, as well as terms like "metabolic process" and "biological regulation" (Figure 3a, Table S9). Module 2, which only had 54 genes, did not exhibit any enriched GO terms but included several genes involved in either gene transcription or cytoskeleton reorganization (Figure 3b, Table S6). Finally, Module 3 contained 87 genes, which were enriched for GO terms involved in general metabolism as well as protein production (Figure 3c, Table S10).

4 | DISCUSSION

This study provides evidence that feeding on the spermatophylax, a nuptial food gift, alters the female post-mating transcriptomic response in decorated crickets. Although we expected to observe a large effect of full mating on female gene expression in either tissue at the chosen timepoint, we only observed relatively small numbers of DE genes for most comparisons in either tissue (Figure 1a,b). Nonetheless, a gene co-expression network analysis in the head tissue revealed that both the attachment of the ampulla and the consumption of the spermatophylax induce their own distinct patterns of gene expression and that it is the content of the spermatophylax per se rather than the act of feeding itself that influences gene expression (Figure 2). Additionally, we found that females that do not feed during the mating exhibit a decreased expression of a large number of genes in the gut but not in the head tissue. This might be caused by a reduction in gut function, and no such decrease was found in females that fed during mating (Figure 1a,c). This further demonstrates that spermatophylax provisioning can indeed influence female gene expression, even though in the gut tissue, this appears to result from the act of feeding rather than from the content of the spermatophylax per se.

For multiple insect species, it has been shown that female gene expression in the brain and in other tissues is influenced by mating, but also by the injection of seminal proteins and peptides (Domanitskaya et al., 2007; Kocher et al., 2008; McGraw et al., 2004, 2008; Sirot et al., 2021). Similarly, the WGCNA analysis performed in this study for the head tissue shows that several modules of co-expressed genes were correlated with ampulla attachment alone (Figure 2). Because the ampulla-receiving treatments (A, PA, and SA) are also the only treatments for which the female interacted with a male, further work would be required to satisfactorily disentangle how much of this effect is due to the transfer of sperm and seminal proteins contained in the ampulla, versus how much is due to the direct interaction with the male. Despite this, the seminal proteins and peptides in the decorated cricket, likely represent a pathway for males to influence female behaviour (Moschilla et al., 2020), even if they have yet to be characterized.

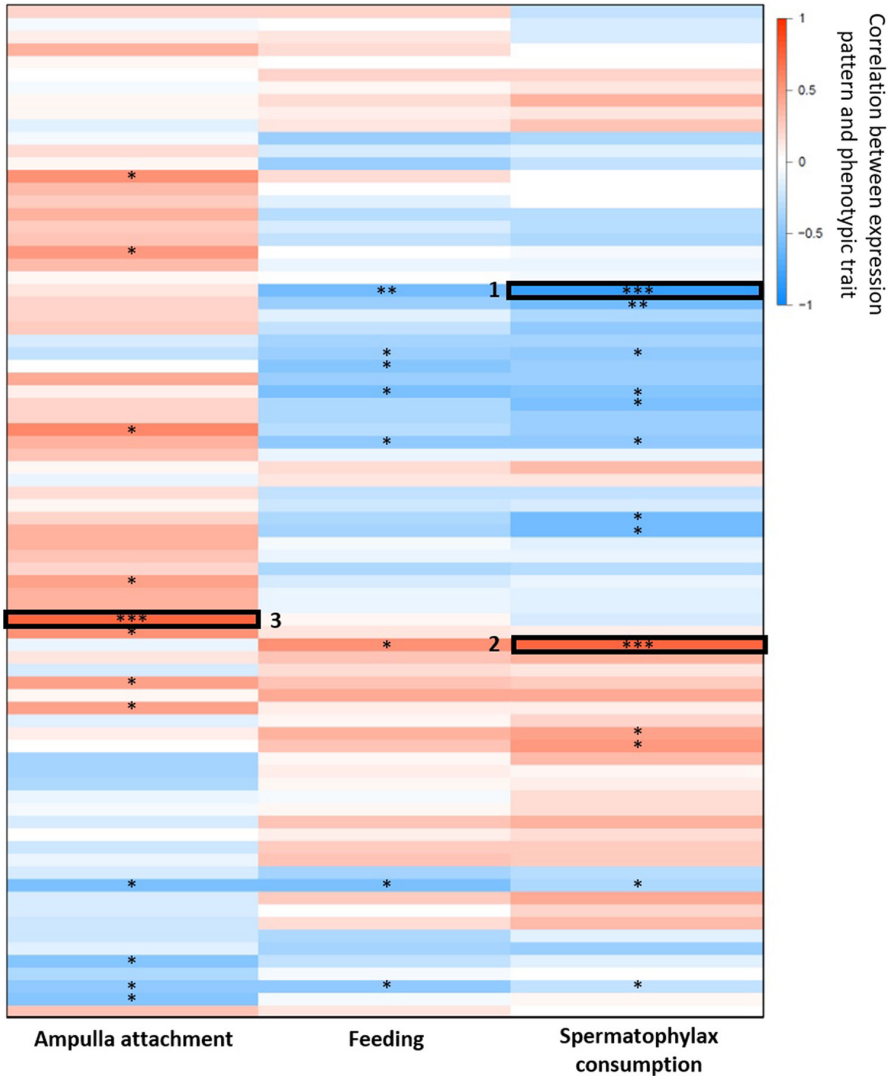

FIGURE 2 Co-expression network analysis of the head tissue. Co-expression of genes was analysed with the WGCNA-package in R, and correlations were calculated between modules of co-expressed genes and three different traits (ampulla attachment, feeding, and spermatophylax consumption). Each line represents a module of co-expressed genes. Red colours are used for highly positive correlations between the eigenvalue of a module and the investigated trait, while blue colours are used for highly negative correlations. Black rectangles mark correlations larger than 0.7 or lower than −0.7, and numbers associated with these boxes represent module numbers referred to in the main text. Significance levels of correlations: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

While seminal proteins and peptides have direct access to the female reproductive organs, the spermatophylax is orally ingested. Any spermatophylax proteins will first have to survive the gut unscathed before they can influence female behaviour and gene expression unless they act via olfactory receptors associated with the mouthparts, which seems a less parsimonious route to influence female behaviour. The role of spermatophylax in increasing sperm transfer duration is well established in decorated cricket (Sakaluk, 1984; Sakaluk et al., 2019), but its role in inducing other behavioural and physiological changes in females is less clear. When spermatophylaxes of *G. sigillatus* were fed to females of the non-gift giving cricket species *Acheta domesticus* during a mating, females took significantly longer to remate than females not fed such gift, suggesting the spermatophylax can in fact reduce sexual receptivity (Sakaluk, 2000; Sakaluk et al., 2006). Similar roles of orally ingested nuptial gifts have been described in ladybird beetles (Perry & Rowe, 2008) and scorpionflies (Engqvist, 2007). However, when *G. sigillatus* females were fed male spermatophylaxes, they did not show such an effect, suggesting that they may have evolved resistance to the male products (Sakaluk et al., 2006). Our current study shows for the first time a female response to spermatophylax consumption in *G. sigillatus* beyond the effect of extending the period of sperm transfer. The two modules that exhibit the highest correlations with spermatophylax consumption both contain a large number of genes involved in cytoskeleton reorganization, but also genes involved in regulatory functions, such as gene transcription or biological regulation (Figure 3a,b). A further study of these genes might yield important information about how males may attempt to influence female physiology through the spermatophylax, and about the female response to this manipulation.

Additionally, we found that female crickets who were not allowed to feed during the mating seemed to reduce their gut function compared with virgins (Figure 1a,c). However, this effect of ampulla attachment largely disappeared when individuals were fed a pectin gel during the mating, and completely vanished when females were allowed to feed on the male spermatophylax (Figure 1a). Of note, the response in the gut tissue to mating but not feeding is still visible at the 20 hours post-mating sampling point. However, any transcriptomic response in the gut to feeding (e.g. virgin vs. spermatophylax females) seems to be more transient, as we observed only

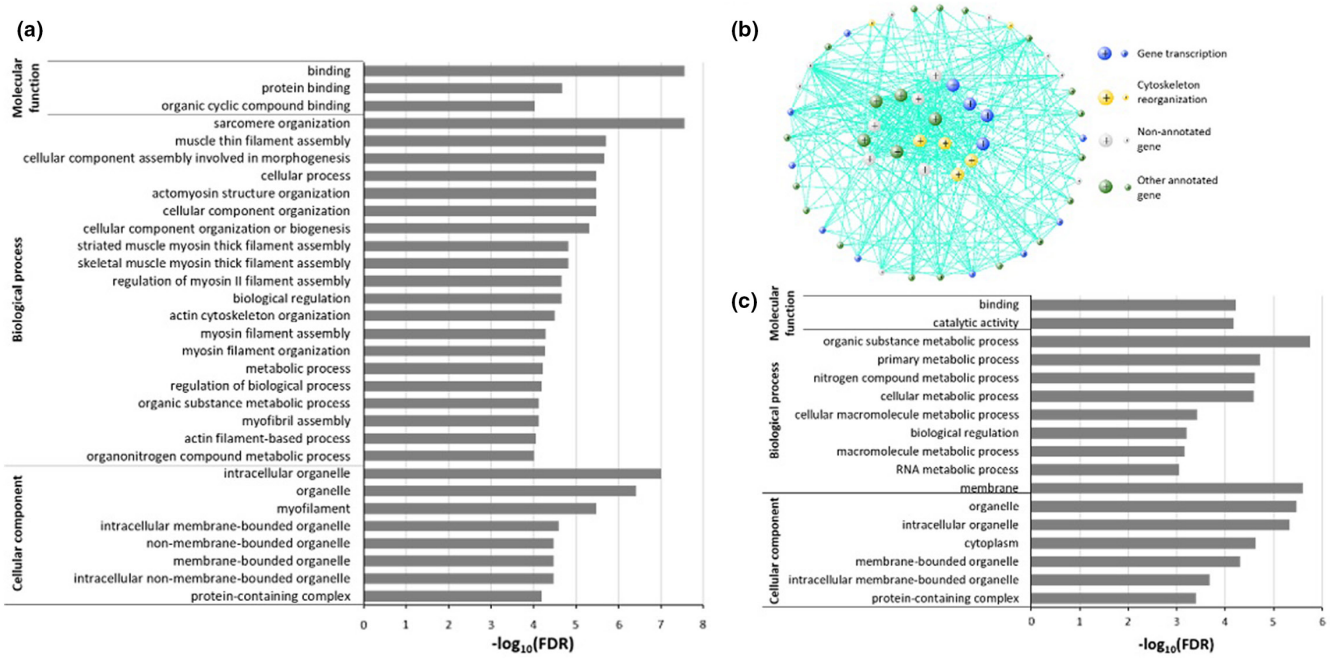

FIGURE 3 Gene ontology enrichment of highly correlated modules. (a) Gene ontology enrichment for Module 1. The most significantly enriched gene ontology terms (FDR <0.0001) for genes in the co-expression module that was highly negatively correlated with ampulla attachment are shown, together with the logarithm of their false discovery rate. Gene ontology terms are grouped by their major gene ontology category. FDR = false discovery rate, as calculated by GOseq. (b) Gene interaction network of Module 2. Connections between genes were obtained with WGCNA. Larger circles represent more highly connected genes. Plot is drawn with VISant visualization software. (c) Gene ontology enrichment for Module 3. The most significantly enriched gene ontology terms (FDR <0.0001) for genes in the co-expression module that was highly positively correlated with spermatophylax consumption are shown, together with the logarithm of their false discovery rate. Gene ontology terms are grouped by their major gene ontology category. FDR = false discovery rate, as calculated by GOseq.

small numbers of significantly DE genes in any of the comparisons where feeding took place in one of the groups. Even though the observed effect might be due to the restraining of the females, which was unique to this treatment, we consider it unlikely restraint for this time period would have such a large effect on gut gene expression. While the implication of the apparent reduction in gut function in mated but non-feeding females versus virgins is not completely clear, it might be caused by resource re-allocation, with resources being moved away from the gut tissue after mating to invest more energy in reproduction (e.g. egg production). However, currently, this remains an untested hypothesis.

In performing qPCR validation of our RNAseq results, we found that they did not entirely concur. There are several potential reasons for disagreements between the RNA sequencing data and the qPCR data. While high correlations between RNA sequencing data and qPCR are often reported (e.g. Asmann et al., 2009; Everaert et al., 2017; Griffith et al., 2010; Li et al., 2019; Wu et al., 2014), such studies often use the same samples or highly related samples as a source for both techniques, which was not feasible in our study due to the 6-year time-lag between RNAseq and qPCR data generation. This timeframe represents at least 15 cricket generations, and crickets were additionally reared in different facilities for both experiments. Even with these sample differences, we still found two genes with similar expression patterns and many genes with similar trends in the RNA sequencing data and the qPCR data (Figure S1). As such, our qPCR results do validate the general patterns of our RNA sequencing experiment, while at the same time suggesting that most of the genes found to be DE in the head tissue might not be major players in the female response to mating.

Nuptial gift provisioning is a widespread mating tactic in a number of insect species, and is likely at the heart of sexual selection and sexual conflict in these species (Gwynne, 2008; Sakaluk et al., 2019; Vahed, 2007). Dissecting the molecular responses of females to nuptial food gift feeding and mating in general will increase our appreciation of the role of nuptial gifts in these evolutionary processes. This study represents the first step in doing this in decorated cricket, a model system for understanding sexual conflict (Sakaluk et al., 2019). We demonstrated that changes to the female transcriptomic response post-mating are, in part, mediated by feeding on the spermatophylax, suggesting that provisioning of a nuptial gift is indeed a route by which male decorated crickets may alter female behaviour. These molecular-level changes are from just a snapshot in time post-mating. They suggest that further studies investigating temporal dynamics in transcriptomic profiles of females, including other relevant tissues, and functional assessments of the changes will be fruitful in connecting behavioural, physiological, and molecular interactions linked to sexual selection and sexual conflict and mediated through nuptial gifts.

AUTHOR CONTRIBUTIONS

Bert Foquet: Data curation (lead); formal analysis (lead); investigation (equal); methodology (equal); validation (lead); visualization (lead); writing – original draft (lead); writing – review and editing (lead). **James Rapkin:** Data curation (supporting); investigation (equal); methodology (equal). **Manmohan D. Sharma:** Data curation (supporting); investigation (equal); methodology (equal). **Ben M. Sadd:** Project administration (equal); resources (equal); supervision (equal); writing – original draft (supporting); writing – review and editing (supporting). **Scott K. Sakaluk:** Conceptualization (equal); funding acquisition (equal); project administration (equal); resources (equal); supervision (equal); writing – original draft (supporting); writing – review and editing (supporting). **John Hunt:** Conceptualization (equal); funding acquisition (equal); project administration (equal); resources (equal); supervision (equal); writing – original draft (supporting); writing – review and editing (supporting).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

All sequencing data, as well as the assembled transcriptomes, are publicly available at the ncbi database, under the BioProject PRJNA784797. The raw qPCR data and the gene annotations of the transcriptome are available under the following Dryad archive: doi:10.5061/dryad.rxwdbrvcp.

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SUPPORTING INFORMATION

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

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