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ORIGINAL PAPER

Sex- and developmental-specific transcriptomic analyses of the Antarctic mite, *Alaskozetes antarcticus*, reveal transcriptional shifts underlying oribatid mite reproduction

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Abstract

The oribatid mite *Alaskozetes antarcticus*, one of the most abundant terrestrial invertebrates in Antarctica, survives extreme temperature fluctuation and desiccation, and thrives in the short growing season characteristic of this polar environment. Several aspects of the mite's ecology and physiology are well studied, but little is known about its reproduction. In this study, we utilize sex- and development-specific next-generation RNA-sequencing (RNA-seq) analyses to identify differentially regulated transcripts underlying reproduction of *A. antarcticus*. Pairwise comparisons between males, females, and tritonymphs revealed more than 4000 enriched transcripts based on different transcriptional levels among sexes and developmental stages. More than 500 of these enriched transcripts were differentially upregulated over 1000-fold. Many of the highly enriched and sex-specific transcripts were previously uncharacterized or have no known orthology. Of the transcripts identified, gene ontology-based analyses linked the transcriptional distinctions to differences in reproduction, chemosensation, and stress response. Our comparative approach allowed us to determine sexually dimorphic transcript expression in *A. antarcticus*. We anticipate that this study will provide a baseline to better understand the mechanisms that underlie reproduction in both polar and non-polar oribatid mites.

Keywords RNA-seq · Reproduction · Testis-specific serine/threonine protein kinases · Mite · Antarctic reproductive biology

Introduction

The Antarctic oribatid mite *Alaskozetes antarcticus* is among the most common terrestrial invertebrates in the sub-Antarctic and maritime Antarctica. It is one of the largest terrestrial arthropods in Antarctica, measuring approximately 1 mm in length and weighing 200–300 µg. As an herbivore and detritivore, *A. antarcticus* feeds on organic debris including penguin guano, algae, and lichens (Strong 1967; Goddard 1977, 1980, 1982; Block and Convey 1995). Large aggregations, with hundreds to thousands of individuals, contain all developmental stages, with adults comprising approximately 30% of the individuals present (Block and Convey 1995). Many studies of this mite have focused on environmental stress tolerance (Young and Block 1980; Block and Convey 1995; Benoit et al. 2008; Everatt et al. 2013).

Most studies on acarine reproduction have focused on ticks due to their medical importance and their large size

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in comparison to other mites. Reproduction in females has been examined more thoroughly than in males, and most work on females has focused on hormonal regulation and the production of vitellogenin (Roe et al. 2008; Cabrera et al. 2009). Male-associated studies have examined specific factors that increase in ticks following blood feeding and those that impact female blood feeding (Weiss and Kaufman 2001, 2004; Roe et al. 2008). For non-tick acarines, studies have been limited to morphological-based observations of male and female reproductive processes (Pound and Oliver Jr. 1976; Mother-Wagner and Seitz 1984; Walzl 1992; Norton 1994), impact of *Wolbachia* on reproduction (Breeuwer 1997; Weeks and Breeuwer 2001), and studies on the basic reproductive output of specific mite species (Norton 1994).

Females of *A. antarcticus* may develop up to 14 eggs at a time but commonly as few as 4–6 eggs can be found when females are dissected (Block 1980; Convey 1994b, c). Egg maturation appears to occur only during the adult's second year (Convey 1994a; Block and Convey 1995). A synchronous burst of egg production occurs early in the austral spring and summer, with low levels and less synchronous egg production persisting into late summer and winter (Convey 1994a; Block and Convey 1995). Female mites collected from sub-Antarctic islands had a higher reproductive investment than those from maritime Antarctica (Convey 1998), suggesting that harsher environments reduce investment in reproduction. Only a single molt can be achieved each year; thus, mite maturation requires at least 4–5 years (Convey 1994a, b). Transfer of sperm occurs through an indirect process, where males deposit a stalked spermatophore on the substrate and females take up the spermatophore through their genital aperture (Norton 1994; Block and Convey 1995), a strategy that is similar to that of other oribatid mites (Søvik 2002; Søvik and Leinaas 2003a, b; Pfingstl 2013), except for those that undergo asexual reproduction (Maraun et al. 2003; Cianciolo and Norton 2006; Brandt et al. 2017). Though some specific aspects of reproduction have been examined for *A. antarcticus* and other oribatid mites, transcriptional aspects underlying reproduction in these mites have not been examined.

In this study, we utilized RNA-seq to examine the molecular mechanisms underlying Antarctic mite reproduction. Males and females were examined, along with tritonymphs, to establish female-, male-, and tritonymph-specific transcript libraries. Although these studies revealed few female-specific transcripts, many male-specific transcripts were identified. Transcripts with distinctly sex-specific expression were validated through polymerase chain reaction (PCR) and quantitative PCR. These sex-specific libraries were also compared to other mites using two methods: 1. examination of overlapping differences in transcript levels between male and female mites, and 2. orthology analysis with gene sets from mites with sequenced genomes. This study presents a sex-specific analysis of this Antarctic oribatid mite, along with comparative analyses of putative sex-specific gene sets among multiple mite species. We anticipate that this study will provide the groundwork for future studies focusing on Antarctic and oribatid mite reproduction.

Materials and methods

Mite collections and RNA extraction

Antarctic mites were collected from Humble Island, near Palmer Station, Antarctica (64°45′59″S, 64°05′60″W) in January 2017 and maintained in the laboratory at 4 °C under long day length (20-h light:4-h dark), conditions typical of summer at Palmer Station. Mites were provided access to algae (*Prasiola crispa*) and other organic debris collected with the mites. To ensure standardization, mites were held under these conditions for two weeks before examination. Males, females, and tritonymphs (final juvenile stage) were separated based on described morphological characteristics (Block and Convey 1995). Male and female were denoted by the characters of the genital areas. Males have a smaller, more rounded, genital area with six or more setae. Females have a larger, more oblong, genital area with only two setae (Block and Convey 1995). Females were examined for the presence of developing eggs within the body cavity. Select males from each cohort (Five mites per sample) were placed within Petri dishes, which were examined for the presence of deposited spermatophores (males were only used if examined mites deposited spermatophores). Samples were frozen at − 70°C until used. Each sample consisted of 40–50 mites.

RNA was extracted by homogenization (BeadBlaster 24, Benchmark Scientific) in Trizol (Invitrogen), based on the manufacturer's protocol and modifications based on other acarid studies (Rosendale et al. 2016). Extracted RNA was treated with DNase I (Thermo Scientific) and cleaned with a GeneJet RNA Cleanup and Concentration Micro Kit (Thermo Scientific) according to the manufacturer's protocols. RNA concentration and quality were examined with a NanoDrop 2000 (Thermo Scientific). Two independent biological replicates were generated for the male, female, and tritonymph samples.

Poly(A) libraries were prepared by the DNA Sequencing and Genotyping Core at Cincinnati Children's Hospital Medical Center. RNA was quantified using a Qubit 3.0 Fluorometer (Life Technologies). Total RNA (150–300 ng) was poly(A) selected and reverse transcribed using a TruSeq Stranded mRNA Library Preparation Kit (Illumina). An 8-base molecular barcode was added to allow multiplexing, and following 15 cycles of PCR amplification each library was sequenced on a HiSeq 2500 sequencing system (Illumina) in Rapid Mode. For each sample, 30–40 million paired-end reads of 75 bases in length were generated. Raw RNA-seq data have been deposited at the National Center for Biotechnology Information (NCBI) Sequence Read Archive (Bioproject: PRJNA428758).

De novo contig assembly and annotation of *A. antarcticus*

RNA-seq reads were trimmed for quality (Phred score limit of 0.05) and sequences with ambiguities were removed. In addition, five and eight nucleotides were removed from the 5′ and 3′ ends, respectively, and sequences shorter than 45 bases were removed. Reads before and after cleaning and trimming were examined with FastQC (S. Andrews https://www.bioinformatics.babraham.ac.uk/projects/fastqc) to verify each set had Phred scores near 40. Contig sets were generated with a combination of CLC Genomics Workbench (Qiagen), Trinity (Grabherr et al. 2011), and Velvet-Oases (Schulz et al. 2012). The minimum contig length for each program was set at 150 base pairs; settings used were based upon a previous study (Rosendale et al. 2016). Following assembly, each contig set was further processed individually or as a combined set. Protein-coding gene sets were predicted using Transdecoder (Haas et al. 2013). Sequence redundancy was reduced by removing sequences with minimum similarity cut-off of 95%, 93%, and 90% using CD-HIT-EST (Huang et al. 2010; Fu et al. 2012). BUSCO (Simão et al. 2015) against the arthropod dataset was utilized to evaluate the completeness of each assembly.

The highest quality contig set was searched (BLASTx) against the NCBI non-redundant (nr) database specified for arthropods. In addition, assembled sequences were searched against the SwissProt protein database and reference protein sets for the fruit fly (*D. melanogaster*) and two acarines (*Ixodes scapularis* and *Tetranychus urticae*). If a positive match was identified through BLAST, gene ontology (GO) terms were assessed with Blast2GO (Conesa et al. 2005) by merging results from the GO mapping tool (*e* value of 1×10^{-5}) and InterProScan. Along with these general analyses, we performed more targeted analyses on gene categories that are likely to have differential expression based on previous sex-based RNA-seq for other invertebrates (Benoit et al. 2016; Schoville et al. 2018). Transcription factors were identified by methods previously described and utilized in other arthropod systems that focused on the identification of specific motifs (Benoit et al. 2016; Schoville et al. 2018). Gustatory and ionotropic receptors were identified by BLASTx comparisons of those annotated from *I. scapularis* and *Metaseiulus occidentalis* (Gulia-Nuss et al. 2016; Hoy et al. 2016), which are critical for chemosensation in mites and could have differential expression between sexes and developmental stages.

Expression and functional analyses

RNA-seq analyses were conducted using two distinct pipelines. The first method used was CLC Genomics, as previously described (Benoit et al. 2014; Rosendale et al. 2016). Briefly, reads were mapped to the contigs with a cut-off of at least 80% of the reads matching at 90% identity, with a mismatch cost of 2. Each read was permitted to align to only 20 contigs. Expression values were based upon total read counts in each sample, calculated as transcripts per million reads mapped. A Baggerly's test (a proportion-based statistical test) was used to test significance among samples. A multiple comparison correction was performed (false discovery rate, FDR; Benjamini and Hochberg 1995). Contigs were considered to have differential expression if the fold change was greater than 2.0 and the FDR p value was < 0.05. In addition, contigs needed to have at least 5 mapped reads per sample to be retained in further analyses. Lastly, contigs were considered sample specific if enrichment was noted in relation to both the other datasets.

The second method for examining transcript expression involved RNA-seq tools available through the Galaxy software package (www.https://usegalaxy.org/, Afgan et al. 2016) with Salmon, using the suggested settings. Differential expression between contigs was examined with the DeSeq2 package (Love et al. 2014). A generalized linear model assuming a binomial distribution followed by false discovery rate (FDR) approach was used to account for multiple tests (Benjamini and Hochberg 1995). Cut-off values for significance, enrichment, and sample specificity were determined as described in the CLC-based analyses.

After analysis of the RNA-seq data, transcripts identified as sex specific or development specific by both pipelines were used for subsequent analyses. Pathways enriched within males, females, or tritonymphs were identified with a combination of DAVID (Huang da et al. 2009a, b), Blast2GO enrichment analyses (Conesa et al. 2005), CLC gene set enrichment analysis (GSEA), and gProfiler (Reimand et al. 2016). Due to the taxonomic limitations of DAVID and gProfiler, sets of enriched transcripts were compared by BLASTx to the *I. scapularis* and the *D. melanogaster* RefSeq protein datasets to identify homologous sequences. BLAST hits (*e* value < 0.001) from these two species were submitted to DAVID for analysis.

Comparative analyses among mite species

While few studies have examined sex-specific gene expression in mites, a recent project focused on sexual conflict in bulb mites, *Rhizoglyphus robini* (Stuglik et al. 2014; Joag et al. 2016), and a recent study examined sex differences in the American dog tick, *Dermacentor variabilis* (Rosendale et al. 2016). These mites are highly divergent from *A.*

antarcticus as both *R. robini* and *D. variabilis* are within another order (Sarcoptiformes). RNA-seq datasets for the bulb mite (NCBI Bioproject: PRJNA330592) and American dog tick (NCBI Bioproject: PRJNA305720) were acquired for further analyses. Males and females (Stuglik et al. 2014; Joag et al. 2016) from both the fighter and scrambler phenotypes of the bulb mite were used in the analyses based upon previous descriptions (Joag et al. 2016). The acquired data were analyzed as described for *A. antarcticus*, with the exception of using a single de novo library built with Trinity (Grabherr et al. 2011; Haas et al. 2013). Contigs enriched at least 20-fold in males or females were compared to those from *A. antarcticus.* Sequences with BLAST matches at *e* value < 0.001 were considered orthologs and used in our comparisons to establish putative male- and female-specific contig sets for the mites.

Putative orthologs of male- and female-associated genes identified from *A. antarcticus* were compared to predicted protein sets from five Acari species: *I. scapularis* (Gulia-Nuss et al. 2016), *M. occidentalis* (Hoy et al. 2016), *Sarcoptes scabiei* (Rider et al. 2015), *T. urticae* (Grbić et al. 2011), and *Varroa destructor* (Cornman et al. 2010). tBLASTp analyses (*e* value < 0.001) were performed using CLC Genomics Workbench (Qiagen). Protein sequences were defined as orthologs if they were reciprocal-best BLASTp hits having an *e* value < 10^{-10}. Overlap was compared between these five analyses to produce putative sex-specific transcript sets.

PCR and qPCR analyses

Select genes of interest that were highly enriched in males or females were examined by PCR using samples collected independently from those utilized in initial RNA-seq analyses. Total RNA was extracted from males, females, and tritonymphs as before and used as a template for Superscript III reverse transcriptase according to the manufacturer's protocol (Invitrogen). PCR was performed with gene-specific primer pairs (Online Resource 1) using a DNA polymerase kit (Promega). The PCR conditions were 95 °C for 3 min, 35 cycles of 30 s at 95 °C, 52–56 °C for 1 min, and 1 min at 70 °C using an Eppendorf Mastercycler Pro Series. Four independent (biological) replicates were conducted for each sex.

qPCR analyses were conducted based on previously developed methods (Rosendale et al. 2016). RNA was extracted as before for independent biological replicates. Complementary DNA (cDNA) was generated with a DyNAmo cDNA Synthesis Kit (Thermo Scientific). Each reaction used 250 ng RNA, 50 ng oligo (dT) primers, with the reaction buffer containing dNTPs and 5 mmol l^{-1} $MgCl_2$, and M-MuLV RNase H+ reverse transcriptase. KiCqStart SYBR Green qPCR ReadyMix (Sigma Aldrich), along with 300 nmol l^{-1} forward and reverse primers, cDNA diluted 1:25, and nuclease–free water was used for all reactions. Primers were designed with the use of Primer3 based on contigs obtained from the transcriptome analysis (Online Resource 1). qPCR reactions used an Illumina Eco quantitative PCR system. Reactions were run according to previous studies (Rosendale et al. 2016). Four biological replicates were examined for each sex. Expression levels were normalized to alpha-tubulin using the ΔΔCq methods as previously described (Schmittgen and Livak 2008). Fold change was compared between males and females, followed by calculation of Pearson's correlation coefficient (*r*).

Results

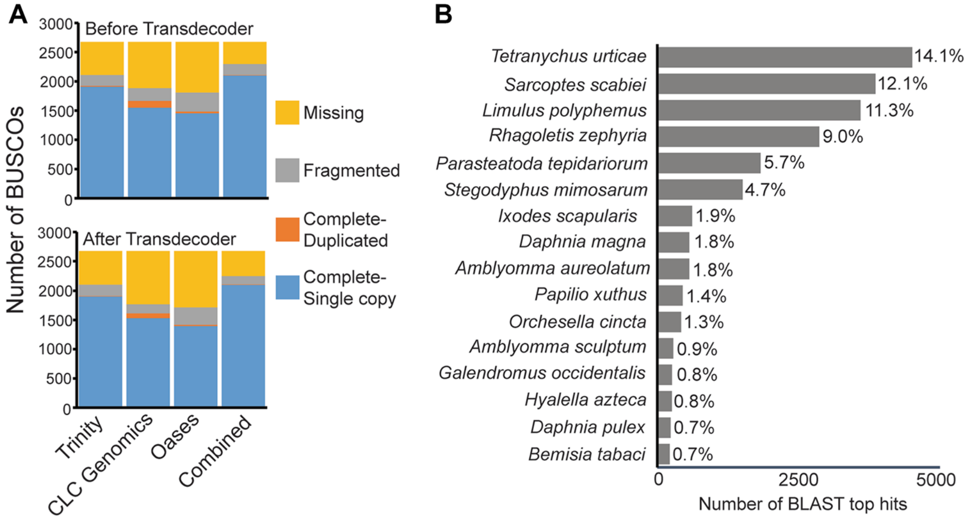
De novo assembly

Six cDNA libraries, generated from tritonymphs, females, and males (two independent biological replicates), yielded a combined 341,103,288 reads after removal of low-quality reads. Benchmarking Universal Single-Copy Orthologs (BUSCO)-based analysis of the assemblies revealed that Trinity generated the highest quality individual assembly. Combining multiple assemblies led to an improvement of ~ 10% (Fig. 1). Transdecoder had a minimal impact on the number of matches to BUSCO genes (Fig. 1), indicating that this step used to obtain protein-coding genes had little influence on the quality of the de novo library. These combined analyses yielded a final set of 51,418 contigs. Annotation using BLASTx and the NCBI nr database for arthropods revealed that *A. antarcticus* contigs had highest similarities to the spider mite, *T. urticae*, and the scabies mite, *S. scabiei* (Fig. 1). The total number of contigs with matches was only ~ 50%, based on an *e* value of < 0.001, which is similar to the number of matches reported for other acarine species (Cabrera et al. 2011; Bu et al. 2015; He et al. 2016; Rosendale et al. 2016). Of the sequences with BLAST hits, 12,140 were annotated with GO terms and used for subsequent GO enrichment studies.

RNA-seq analyses

Differential transcript levels were determined between males, females, and tritonymphs using two independent pipelines (Fig. 2, Online Resource 2-S). Of importance, the two pipelines yielded 95–98% overlap at our significance cut-off (two-fold difference, combined TPM among all samples of at least 5, and a FDR correction-based *p* value < 0.05). There was a high correlation in transcript levels for specific genes between each of the replicates for each sex and the tritonymphs (Pearson correlation coefficient over 0.94 in all cases). Between 43 and 45% of the reads from each

Fig. 1 Quality metrics of de novo assembly from *Alaskozetes antarcticus*. **a** BUSCO-based results among Trinity, CLC Genomics, Oases, and a combined set. **b** Top BLAST hits for the combined contig set (Color figure online)



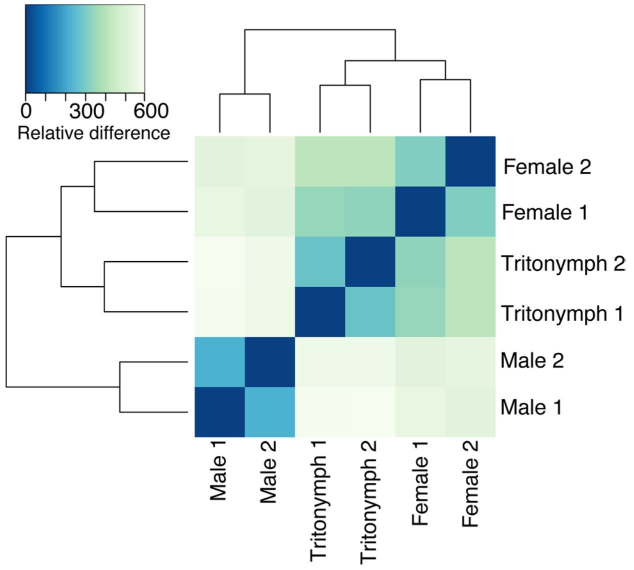


Fig. 2 Hierarchical clustering RNA-seq gene expression patterns for male, female, and tritonymphs of *Alaskozetes antarcticus* based on sample distance (Euclidean distance matrix) of log transformed expression from transcripts based on DeSeq (Love et al. 2014) (Color figure online)

RNA-seq set were mapped to assembled contigs for both analysis methods. At the 20-fold level or greater for stage specificity, the overlap between the two methods exceeded 99%, thus we used the Galaxy-based pipeline for the remaining analyses. Transcripts with significant enrichment were examined at 20-, 100-, 500-, 1000-, and 5000-fold differences between developmental stages. For females, compared to males and nymphs, 143 transcripts were enriched 20-fold and 31 were enriched 1000-fold, but no transcripts were enriched over 5000-fold (Fig. 3). These transcripts included matches to female-associated genes, vitellogenin and multiple peroxinectins (Online Resource 3). Sixty-eight of the transcripts did not have matches to sequences from other arthropods.

Our analysis identified 3836 transcripts that were 20-fold enriched in males compared to females and juveniles, and 45 transcripts were enriched 5000-fold or more. Many factors previously associated with male reproduction were identified as enriched compared to the females and tritonymphs (Online Resource 2). Lastly, transcripts with enrichment in tritonymphs compared to adults revealed 228 enriched 20-fold, 11 enriched over 1000-fold, and none over 5000-fold (Fig. 3). Many of these contigs matched factors associated with cuticle development and growth. Similar to females, ~ 30% of the male and tritonymph sets did not have matches to other arthropods (Online Resources 2-4). This high level of species-specific transcripts is common in acarine transcriptome studies (Gibson et al. 2013; Rosendale et al. 2016), which likely represent novel oribatid and/or *A. antarcticus*-specific transcripts.

GO enrichment analyses revealed many categories differentially regulated between our sample groups. For males, there were over 200 GO categories with altered levels (Fig. 4, Online Resource 5) at the 20-fold enrichment level and nearly 80 with 500-fold enrichment. These GO categories included the monooxygenase and oxidation–reduction process, metal/ion/small molecular binding, nucleotide binding, and transferase and kinase activity (Online Resource 5). GO category enrichment shifted for both tritonymphs and females and showed only a few enriched categories (Fig. 4; Online Resource 6–7). Tritonymph transcriptomes demonstrated a distinct enrichment in categories associated with chitin metabolism (Online Resource 6), while females showed a distinct enrichment of GO categories associated with response

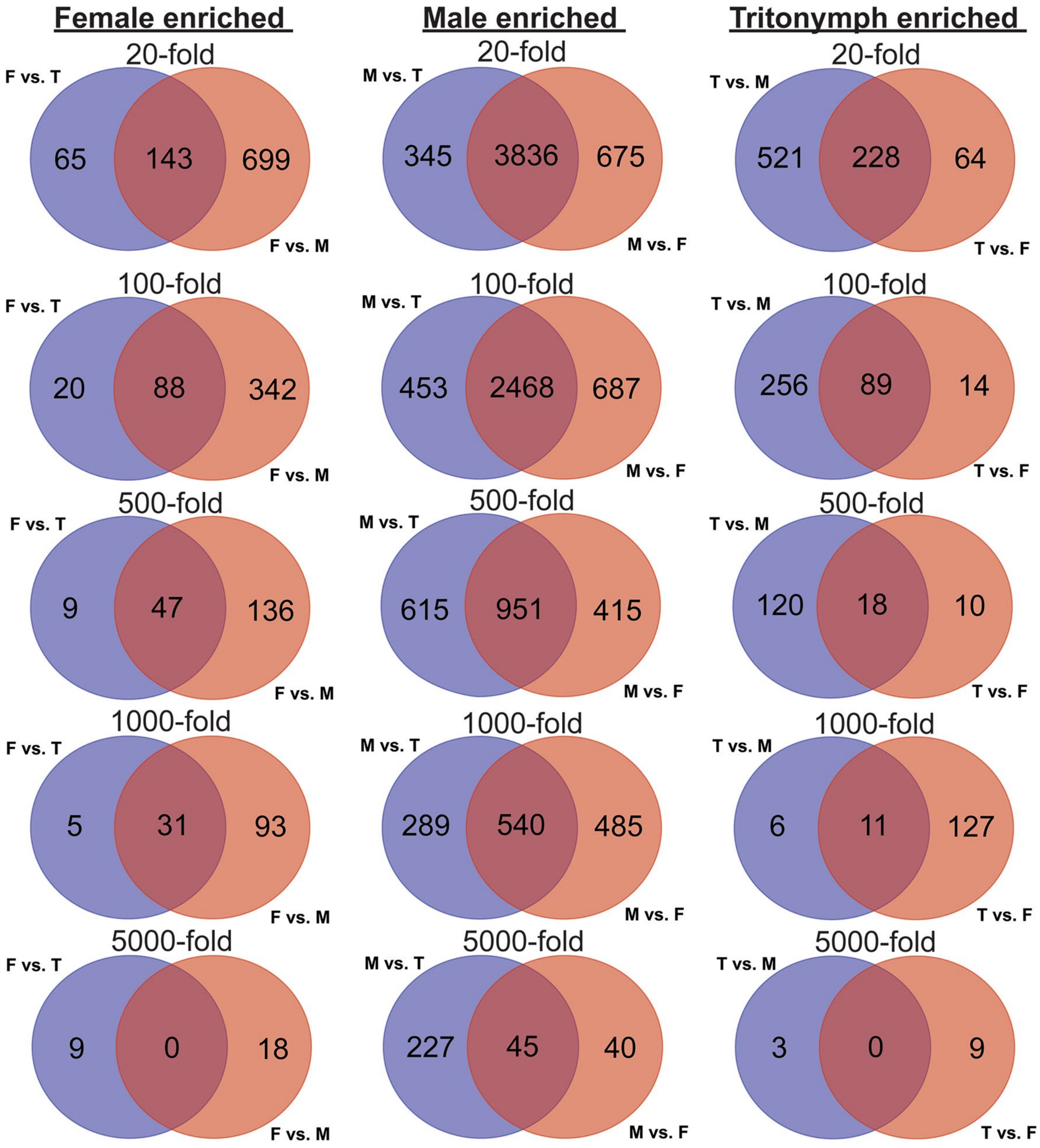


Fig. 3 Contigs with enriched expression in females, males, and tritonymphs of *Alaskozetes antarcticus* at varying expression levels. T versus F, significant between tritonymph and female; M versus F, significant between male and female; T versus M, significant between tritonymph and male. Specific details of contig expression are presented in Online Resources 1–3 (Color figure online)

to oxidative stress. Transcription factor analyses revealed very few with sex-biased expression (Fig. 5, Online Resources 8,9). Along with transcription factor analyses, we examined expression differences between contigs associated with chemical reception, gustatory (GRs) and ionotropic receptors (IRs) (Fig. 5). No GRs or IRs were specifically enriched in any stage compared to the two others, but many of the IRs showed differential expression between males and females (Fig. 5).

PCR and qPCR validation

Validation of the RNA-seq data was performed using both PCR and qPCR with independent biological replicates. Seven genes with female- and male-specific gene expression, the known functions of which included sperm-associated processes and vitellogenin, were examined. PCR-based analyses validated that male- and female-specific contigs have significant enrichment in each sex (Fig. 6). qPCR-based analyses correlated directly with the RNA-seq

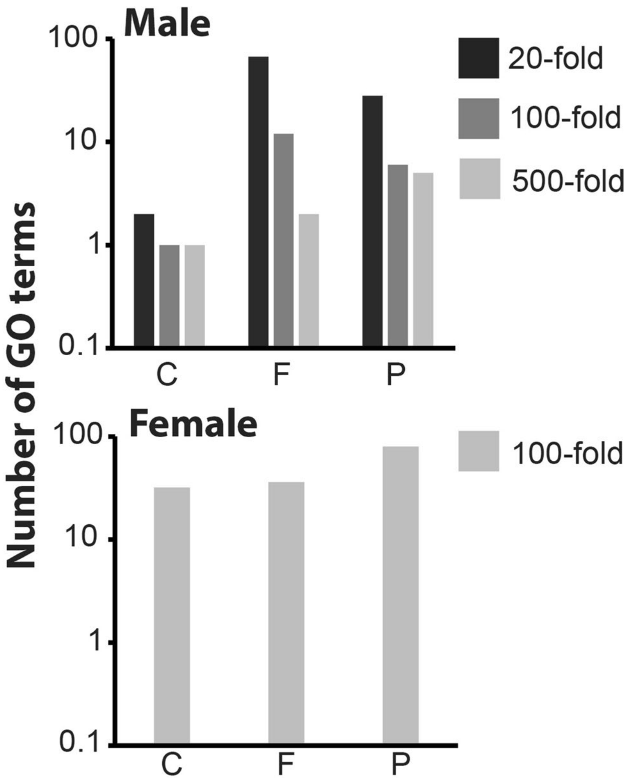


Fig. 4 Gene ontology (GO) enrichment in males (top) and females (bottom) of *Alaskozetes antarcticus*. C, cellular component; F, molecular function; P, biological process. Specific details of GO analyses are presented in Online Resources 4–6

analyses (Pearson correlation coefficient = 0.931, Fig. 6). Thus, results from our RNA-seq studies were validated by both PCR and qPCR analyses.

Comparative analyses among sex-specific transcriptomes of different mite and tick species

We compared our results from the Antarctic mite with male- and female-enriched gene sets of the American dog tick, *D. variabilis*, and a mite, *R. robini* (fighters and scramblers; Stuglik et al. 2014; Joag et al. 2016). The number of female-enriched genes conserved between the three sex-specific comparisons was low (Fig. 7). This set included a superoxide dismutase, glycogen phosphorylase, and nesprin (Online Resource 10). Male-associated transcripts had much greater overlap, with over 140 transcripts enriched in all three species surveyed for sex-specific expression (Fig. 7, Online Resource 11). This included many previously identified testis-specific (19 total) and uncharacterized contigs (50 total). Distinct enrichment in males for contigs identified as testis-specific serine kinase (TSSK) was noted between the mites. Importantly, there may be limitations to these comparative RNA-seq studies due to differences in experimental design, variation in reproductive biology among the mites, and/or the phylogenetic differences between species.

Orthology-based analyses identified putative male- and female-specific gene sets that overlapped between the Antarctic mite and five other acarine species with sequenced genomes (Figs. 8, 9, Online Resources 12, 13). Male-specific orthology analyses were conducted with both male-enriched gene sets from only *A. antarcticus* and a smaller set generated by comparisons between the bulb mite, American dog tick, and Antarctic mite. Among five acarine genomes, orthologs were identified for 851 and 112 contigs from the Antarctic-specific and the smaller comparative acarine sets, respectively (Fig. 8). Along with enrichment of TSSK, this ortholog set includes matches to many proteins previously identified as critical to male reproduction, underlying sperm development and production of seminal proteins (Online Resource 12).

Female orthology analyses were conducted only with the Antarctic mite since few overlapping enriched transcripts were found in analyses comparing the bulb mite, American dog tick, and Antarctic mite. Only 27 putative orthologs were identified between the female-specific set from *A. antarcticus* and the genome-associated protein sets from the other acarines (Fig. 9). This putative female-enriched mite transcript set included transcripts known to be critical for egg production such as vitellogenin, peroxinectin, chitin-associated proteins, transporters, and cuticle hydrocarbon synthesis (Online Resource 13).

Discussion

This study, using RNA-seq to examine the molecular mechanisms underlying reproduction in A. *antarcticus*, generated a rather complete de novo contig library, featuring over 80% of the BUSCO benchmarking genes for arthropods. Analyses of overlapping expression between females, males, and tritonymphs established distinct sex-specific contig sets. Expression patterns of specific genes from our RNA-seq analyses were validated using RT-PCR and qPCR. Targeted bioinformatic analyses of transcription factors and chemosensory genes have identified specific components that may underlie differences in male and female biology. Lastly, we provide a comparative analysis of male- and female-specific RNA-seq analyses among three members of the Acari and an orthology-based survey including five species with sequenced genomes to identify male and female contig sets that overlap among the acarines. These results suggest putative molecular mechanisms associated with reproduction in oribatid mites, which will be critical for understanding basic reproductive mechanisms in mites.

Female-enriched transcripts for the Antarctic mite included contigs associated with biological processes such

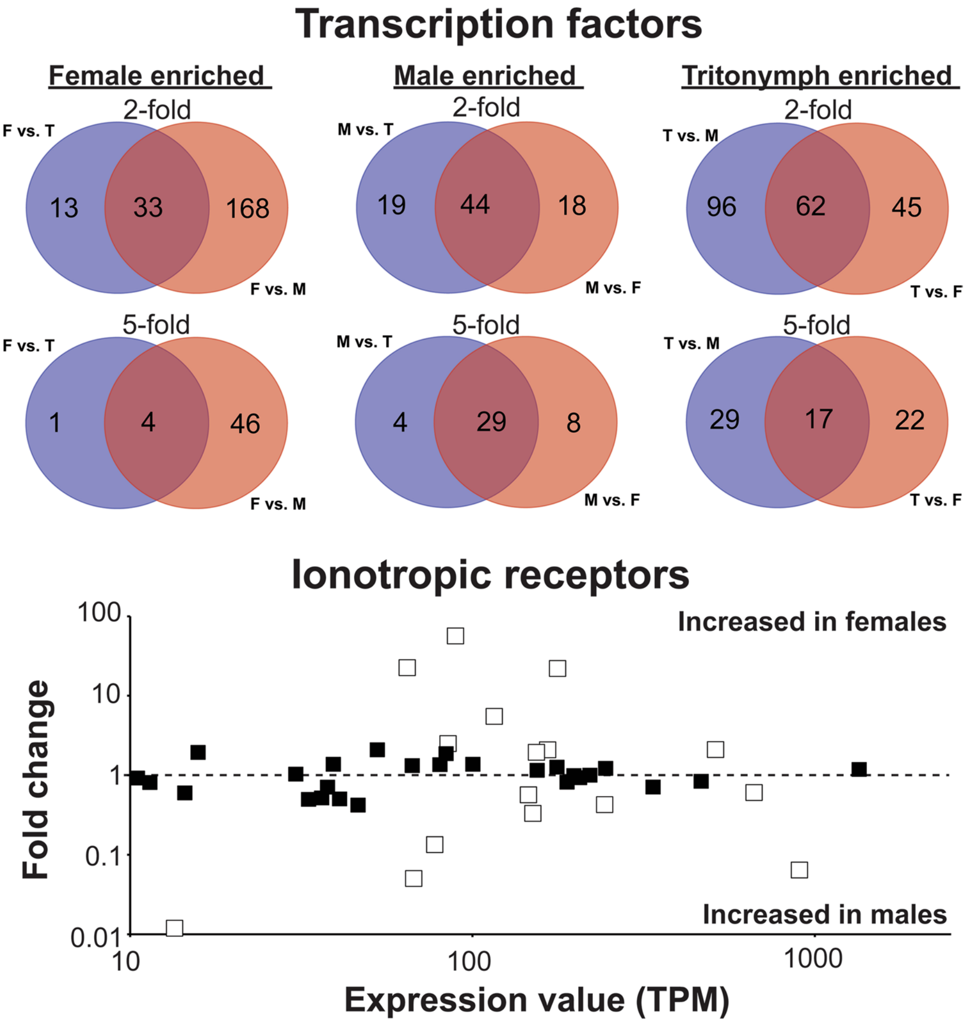


Fig. 5 Specific categories of interest that could vary between male and female *Alaskozetes antarcticus.* (top) Contigs with enriched expression for genes identified as likely transcription factors in females, males, and tritonymphs at varying expression levels. T versus F, significant between tritonymph and female; M versus F, significant between male and female; T versus M, significant between tritonymph and male. Specific details of contig expression are presented in Online Resource 7-S8. (bottom) Ionotropic receptor differential expression between males and females. Open squares denote contigs with statistically different expression between the sexes following a false detection rate of $p < 0.01$ (Baggerly's test) (Color figure online)

as chitin metabolic processing, antioxidant responses, and amino-sugar metabolic processes. These specific GO categories are all critical for egg production (Urbanski et al. 2010; Petrella et al. 2015; Chauhan et al. 2016; Leader et al. 2017; Papa et al. 2017). Other specific transcripts of interest were differentially regulated in female mites. First, two unique enriched transcripts identified as vitellogenin underscore the active process of oocyte development occurring within the female (Roe et al. 2008; Cabrera et al. 2009, 2011). Four peroxinectins were also enriched in the females; these proteins play roles both in immunity as well as in development of ovarian follicle cells during *Drosophila* oogenesis (Tootle and Spradling 2008; Tootle et al. 2011; Park et al. 2014). The female-enriched set generated from a comparison of expression data from all available mite and tick databases included very few genes. Vitellogenin-like genes were enriched in Antarctic and bulb mites, but they are absent in ticks, most likely because expression of these genes does not increase until after blood feeding (Roe et al. 2008; Cabrera et al. 2009; Seixas et al. 2018). Of note is the fact that nearly 30% of transcripts enriched for females did not match proteins for other arthropods or only matched hypothetical/uncharacterized proteins, indicating that many of the underlying molecular aspects associated with reproduction in the Antarctic mite are novel or may be mite specific.

Male-enriched transcripts were more prevalent: over 3500 transcripts showed greater than 20-fold enrichment in males compared to females or nymphs. The combined generation of sperm and seminal fluid likely requires expression of many male-specific transcripts (Findlay et al. 2008, 2009; Takemori and Yamamoto 2009; Avila et al. 2011; Sonenshine et al. 2011; Scolari et al. 2016). There were many specific GO categories that were either enriched or showed reduced representation in males, a feature common to several other RNA-seq studies on arthropods examining male specificity (Koutsos et al. 2007; Graveley et al. 2011; Petrella et al. 2015; Scolari et al. 2016). The enriched categories of monooxygenase and oxidation–reduction process, metal/ion/small molecular binding, nucleotide binding, and transferase and kinase activity all have been identified in

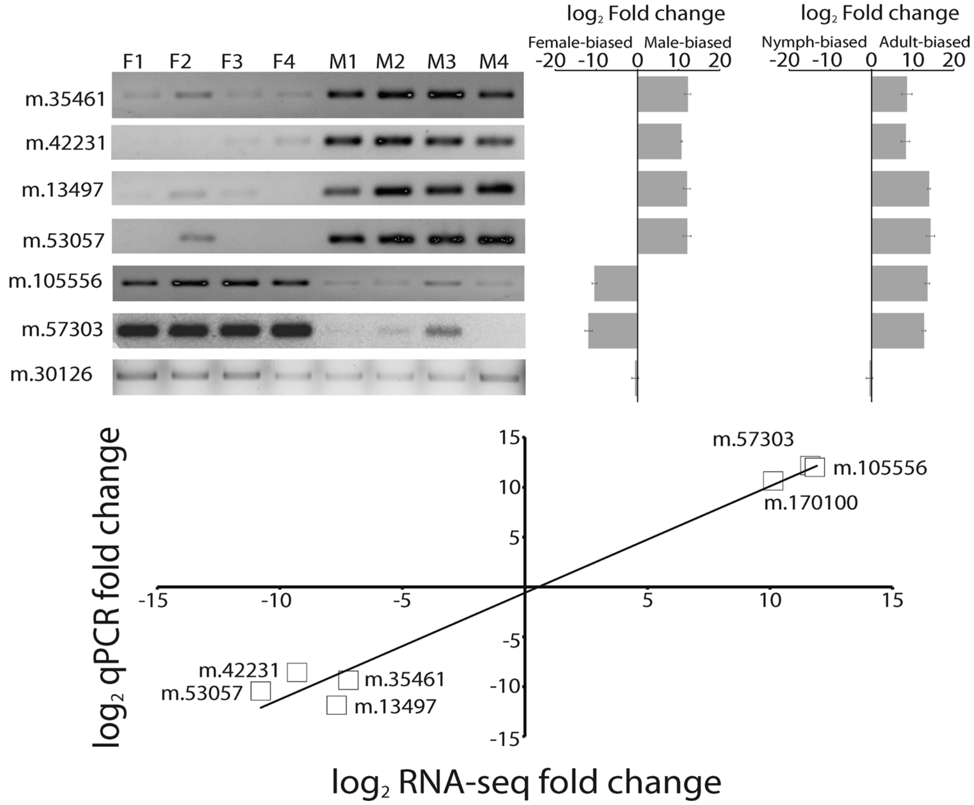


Fig. 6 PCR- and qPCR-based validation of RNA-seq analyses between male and female *Alaskozetes antarcticus*. (top) PCR-based gel image examination of male and female specificity and $\log_2$ fold changes were based on RNA-seq results (Online Resource 2-S4). (bottom) qPCR validation of select genes compared to $\log_2$ fold changes were based on RNA-seq results (Online Resource 2-S4). PCR and qPCR were based on four replicates. m0.35461, Transmembrane and coiled-coil domains 1; m0.42231, argonaute-2-like; m0.13497, *Alaskozetes*-specific male protein; m0.53057, *Alaskozetes*-specific male protein 2; m0.105556, *Alaskozetes*-specific female protein; m0.53057, chemosensory receptor; m0.170100, vitellogenin

other arthropod studies focusing on male-associated tissues (Mikhaylova et al. 2008; Sonenshine et al. 2011; Scolari et al. 2012, 2016). Male-enriched genes of interest include zonadhesin, a sperm-specific membrane protein associated with cell adhesion (Gao and Garbers 1998; Tardif et al. 2010), and multiple genes associated with sperm motility. Of interest is the fact that the sperm are not yet motile when RNA-seq was conducted and will remain non-motile when deposited in the external spermatophore (Block and Convey 1995). This suggests that either the sperm are primed for motility once female insemination occurs or also express these factors when sperm are held in a stationary state. Lastly, many water/solute and ion transporters were enriched in males, suggesting a role in male-specific processes. Of particular interest is the fact that over 50% (~ 1900) of the enriched male contigs have no match to other arthropods or only match hypothetical/unassigned proteins. This number is likely the combined result of the high number of species-specific genes associated with male reproductive processes (Reinhardt et al. 2009; Takemori and Yamamoto 2009; Sonenshine et al. 2011; Scolari et al. 2012, 2016) and the large number of unidentified genes in the genomes and transcriptomes of other Acari (Grbić et al. 2011; Gibson et al. 2013; Chan et al. 2015; Gulia-Nuss et al. 2016).

Comparison of male-enriched genes among the Acari revealed an overall enrichment for genes associated with signal transduction, peptidyl-serine phosphorylation, serine/threonine kinase activity, and ATP binding. The increase in ATP binding is the result of multiple members of the ATP-binding cassette (ABC) transporter family being enriched in male mites. Specific ABC transporters are critical components of spermatogenesis (Ban et al. 2005; Xia et al. 2007). These transporters are likely essential for the ATP-dependent transport of substrates across biological membranes (Higgins 1992). In previous sperm studies, an ABC transporter was implicated in the removal of cholesteryl esters, fatty acid esters, and triacylglycerols from the cell (Ban et al. 2005). Enrichment of over 50 contigs for ABC transporters in male Antarctic mites (18 enriched in both male bulb and Antarctic mites) suggests a critical role in male physiology, possibly including sperm and seminal fluid generation.

Enrichment for both peptidyl-serine phosphorylation (PSPs) and serine/threonine kinase (TSSK) is indicative of increased expression for multiple testis-specific PSPs and TSSKs in male mites; both TSSKs and PSPs are linked to sperm viability (Spiridonov et al. 2005; Xu et al. 2008a; Li et al. 2016). Mutation or reduced expression of TSSKs can lead to low sperm abundance (McReynolds et al. 2014), likely through a negative impact on the centriole and microtubule structures during spermatogenesis (Xu et al. 2008b). Serine/threonine kinase transcripts are one of the most abundant transcripts in male reproductive organs of ticks (Sonenshine et al. 2011). Thus, the processes of serine and

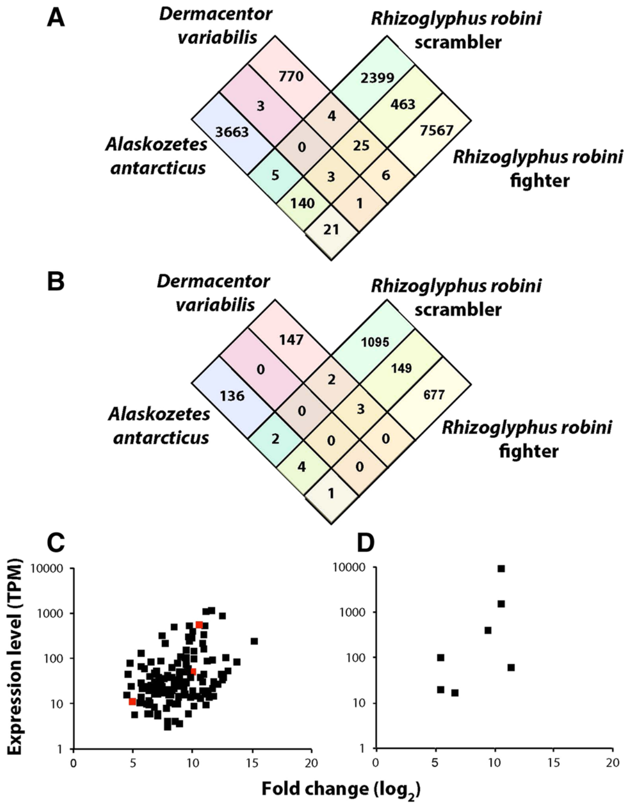


Fig. 7 Comparative analyses among male- and female-enriched transcripts from *Alaskozetes antarcticus* with male and female RNA-seq analyses from the bulb mite *Rhizoglyphus robini*, and the American dog tick, *Dermacentor variabilis*. **a** Overlap in contigs with increased expression in male mites. **b** Overlap in contigs with increased expression in female mites. **c** Comparison of expression levels in relation to fold change between contigs identified in **a**. Black symbols are enriched in Antarctic and bulb mite males and red symbols are highly enriched in tick and mite males. **d** Comparison of expression levels in relation to fold change between contigs identified in **b** (Color figure online)

threonine protein phosphorylation are likely essential for successful spermatogenesis in mites as well.

We also performed targeted studies on contigs that were identified as transcription factors (TFs) and those involved in chemical sensation. Over 70 transcription factors had differential expression between male and female mites. We documented increased male expression of *double-sex*, a major gene in sexual differentiation (Salz 2011); other TFs enriched in males or females could provide novel targets to examine mechanisms underlying the regulation of sex-specific genes in mites. Expression analysis of specific chemosensory genes revealed no IRs or GRs that were specifically enriched in females, males, or nymphs when all three datasets were compared. There were nearly 20 IRs, and no GRs, with differing expression between males and females, but these are not uniquely expressed, as they were also detected at significant levels in the tritonymphs. This overall lack of sex- and developmental-specific enrichment among GRs is surprising but is perhaps due to the fact that specific chemical receptors have tissue-specific expression, which may be masked by our RNA-seq analyses targeting whole mites. Thus, differential expression of IRs between males and females and associated sex-specific differences warrant future study.

Reproduction in *A. antarcticus* has been linked to distinct environmental conditions (Block and Convey 1995); specifically, females accumulate eggs during late summer and winter, leading to a burst of oviposition during spring and early summer (Convey 1994b). The underlying mechanism for this increased oviposition is unknown. Our identification of female-associated transcript sets could be critical for assessing specific factors prompting increased spring-associated egg deposition. For example, if expression of female-enriched genes, such as vitellogenin, respond solely to increases in temperature, then thermal increases in spring may be responsible for increased oviposition. Other environmental factors such as photoperiod or water availability could also contribute to this increase in egg deposition. Our studies provide resources for examining gene expression changes associated with reproduction during the transition from the austral winter to spring.

A unique aspect of reproduction in oribatid mites is that asexual reproduction has developed independently for multiple species (Maraun et al. 2003; Cianciolo and Norton 2006; Brandt et al. 2017). Of interest, asexual oribatid mites seem resistant to accumulation of deleterious non-synonymous mutations, a feature attributed to large population sizes (Brandt et al. 2017). Our identification of male-associated factors in *A. antarcticus* could provide the framework to examine if there is a rapid breakdown in male-associated genes in asexual oribatid species compared to their sexual counterparts. Specifically, we would expect rapid accumulation of deleterious non-synonymous mutations and eventual loss of genes associated with sperm generation and spermatophore development. Full genome sequencing will be necessary for these analyses as male-associated transcripts likely have no or reduced expression in asexual lineages.

Research on Antarctic organisms, specifically terrestrial arthropods, has predominantly focused on mechanisms underlying stress tolerance, basic aspects related to phenology, and, in rare cases, on nutrition dynamics (Convey et al. 2003; Benoit et al. 2008; Michaud et al. 2008; Everatt et al. 2013; Teets et al. 2013; Zmudczyńska-Skarbek et al. 2015; Chown and Convey 2016). Few studies have examined molecular aspects related to reproduction in Antarctic terrestrial arthropods (Pearse et al. 1991; Convey 1994c; Block and Convey 1995). Here, we established male- and female-specific transcript sets for the widely-distributed Antarctic species *A. antarcticus*, sets that include large-scale and targeted experiments using comparative RNA-seq

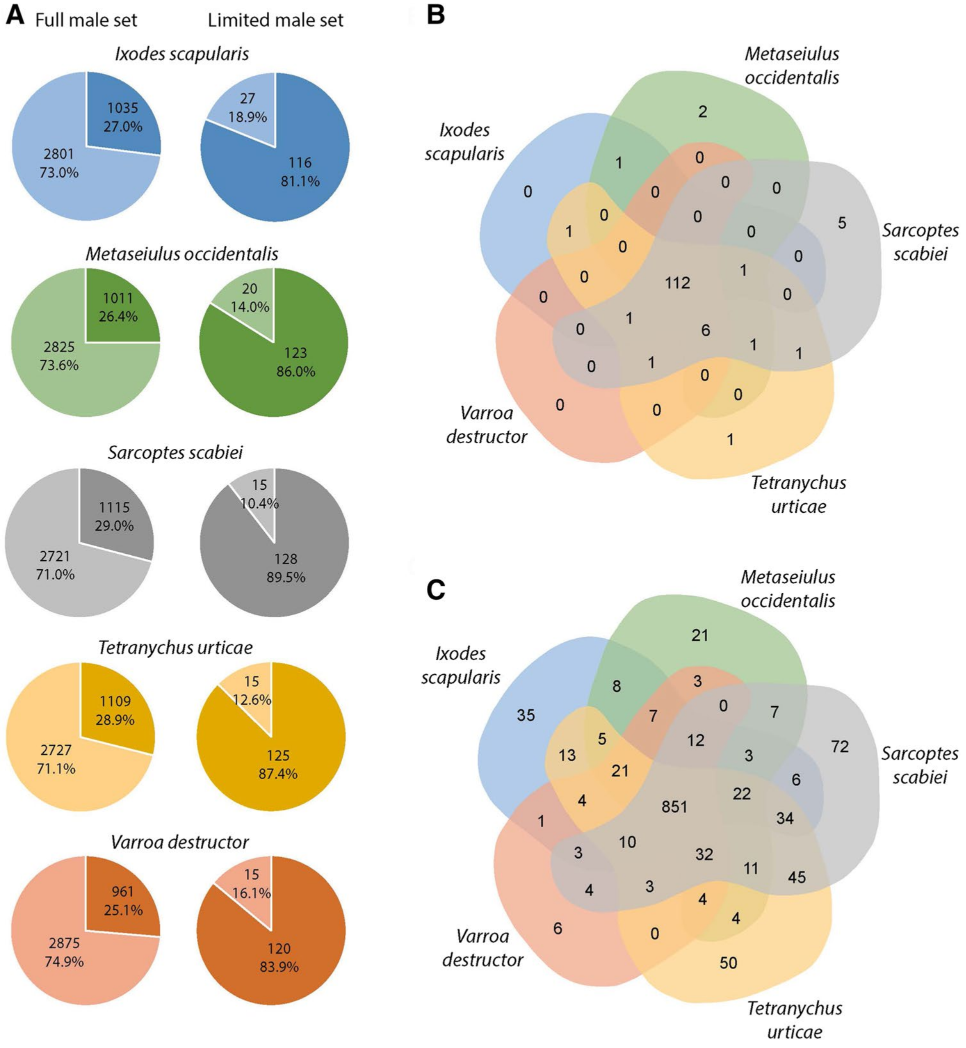


Fig. 8 Comparative orthology analysis of male-associated genes in *Alaskozetes antarcticus,* compared with the genomic gene sets for *Ixodes scapularis* (Gulia-Nuss et al. 2016), *Metaseiulus occidentalis* (Hoy et al. 2016), *Sarcoptes scabiei* (Rider et al. 2015), *Tetranychus urticae* (Grbić et al. 2011), and *Varroa destructor* (Cornman et al. 2010). **a** Pie charts depict the positive and negative BLAST hits between *A. antarcticus* and the other acarines; dark color is positive, and light color denotes no hit (negative). Full male set is the complete set of male-enriched transcripts from *A. antarcticus*, limited male set is following reduction based on analyses in Fig. 7. **b** and **c** Venn diagram illustrating common orthologs among all species. b, limited male set; c, full male set (Color figure online)

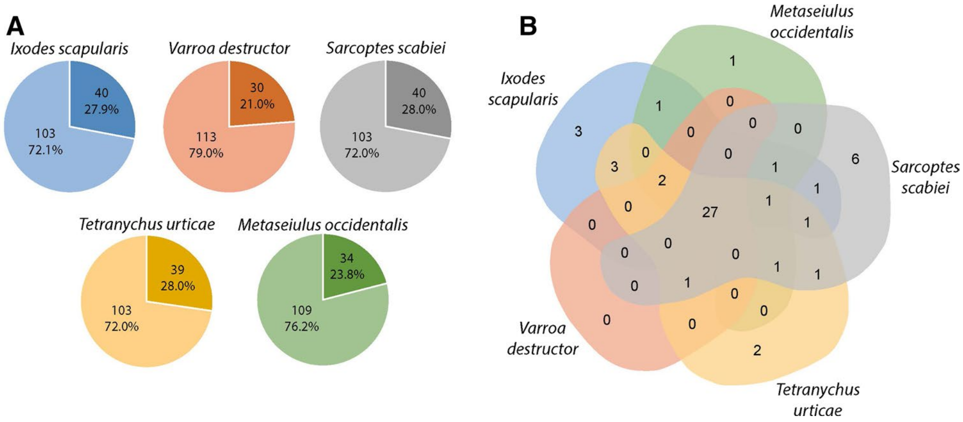


Fig. 9 Comparative orthology analysis of female-associated genes in *Alaskozetes antarcticus* compared with the genomic gene sets for *Ixodes scapularis* (Gulia-Nuss et al. 2016), *Metaseiulus occidentalis* (Hoy et al. 2016), *Sarcoptes scabiei* (Rider et al. 2015), *Tetranychus urticae* (Grbić et al. 2011), and *Varroa destructor* (Cornman et al. 2010). **a** Pie charts depict the positive and negative BLAST hits between *A. antarcticus* and other acarines; dark color is positive, and light color denotes no hit (negative). **b** Venn diagram illustrating common orthologs among all species (Color figure online)

analyses. Comparative transcript sets underlying female and male biology for mites were identified from our Antarctic mite sex-specific transcriptional studies, thus generating the first putative mite-specific gene sets underlying reproduction. This information provides the groundwork for future genomic and transcriptomic studies investigating interactions between the environment and reproductive potential of *A. antarcticus* and may prove useful in future studies examining reproductive physiology of other acarines, specifically oribatids.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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