Multi-level analysis of reproduction in the Antarctic midge, *Belgica antarctica*, identifies female and male accessory gland products that are altered by larval stress and impact progeny viability

Geoffrey Finch[1], Sonya Nandyal[1], Carlie Perrieta[1], Benjamin Davies[1], Andrew J. Rosendale[1,2], Christopher J. Holmes[1], Josiah D. Gantz[3,4], Drew Spacht[5], Samuel T. Bailey[1], Xiaoting Chen[6], Kennan Oyen[1], Elise M. Didion[1], Souvik Chakraborty[1], Richard E. Lee, Jr.[2], David L. Denlinger[4,5] Stephen F. Matter[1], Geoffrey M. Attardo[7], Matthew T. Weirauch[6,8,9], and Joshua B. Benoit[1,*]

[1]Department of Biological Sciences, University of Cincinnati, Cincinnati, OH

[2]Department of Biology, Mount St. Joseph University, Cincinnati, OH, USA

[3]Department of Biology, Miami University, Oxford, OH

[4]Department of Biology and Health Science, Hendrix College, Conway, AR

[5]Departments of Entomology and Evolution, Ecology and Organismal Biology, The Ohio State University, Columbus, OH

[6]Center for Autoimmune Genomics and Etiology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA;

[7]Department of Entomology and Nematology, University of California, Davis, Davis, CA 95616, USA;

21 [8]Divisions of Biomedical Informatics and Developmental Biology, Cincinnati Children's Hospital Medical
22 Center, Cincinnati, OH 45229, USA.

23 [9]Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH 45267, USA.

24 [*]Author for correspondence

25 Joshua B. Benoit

26 Department of Biological Sciences,

27 University of Cincinnati, Cincinnati, OH

28 Email: joshua.benoit@uc.edu

29 Phone: 513-556-9714

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Abstract

The Antarctic midge, *Belgica antarctica*, is a wingless, non-biting midge endemic to Antarctica. Larval development requires at least two years, but adult life lasts only two weeks. The nonfeeding adults mate in swarms and females die shortly after oviposition. Eggs are suspended in a gel of unknown composition that is expressed from the female accessory gland. This project characterizes molecular mechanisms underlying reproduction in this midge by examining differential gene expression in whole males, females, and larvae, as well as in male and female accessory glands. Functional studies were used to assess the role of the gel encasing the eggs, as well as the impact of stress on reproductive biology. RNA-seq analyses revealed sex- and development-specific gene sets along with those associated with the accessory glands. Proteomic analyses were used to define the composition of the egg-containing gel, which is generated during multiple developmental stages and derived from both the accessory gland and other female organs. Functional studies indicate the gel provides a larval food source and thermal and dehydration buffer, all of which are critical for viability. Larval dehydration stress directly reduces production of storage proteins and key accessory gland components, a feature that impacts adult reproductive success. Modeling reveals that bouts of dehydration may significantly impact population growth. This work lays a foundation for further examination of reproduction in midges and provides new information related to general reproduction in dipterans. A key aspect is that reproduction and stress dynamics, currently understudied in polar organisms, are likely to prove critical for determining how climate change will alter survivability.

Keywords: Antarctic midge, reproduction, stress biology, accessory glands, thermal buffering

1 INTRODUCTION

The Antarctic midge, *Belgica antarctica,* is long-lived, wingless, and the only insect endemic to maritime Antarctica(Convey & Block, 1996; Sugg, Edwards, & Baust, 1983). It has a patchy distribution along the western coast of the Antarctic Peninsula and South Shetland Islands, where it may form large aggregations into the thousands under favorable conditions (Convey & Block, 1996; SUGG et al., 1983). The larval period lasts two years; growth and development take place during the short austral summer, and larvae overwinter encased in ice (Usher & Edwards, 1984). Larvae commonly reside in damp areas and are non-selective feeders by consuming dead plant and animal matter, algae, and microorganisms (Edwards, & Baust, 1981; Strong, 1967). Larvae are extremely tolerant of numerous stresses including cold, dehydration, and UV exposure (JBenoit, Lopez-Martinez, Elnitsky, Lee, & Denlinger, 2009; Benoit, Lopez-Martinez, Michaud, et al., 2007; Lopez-Martinez et al., 2009; Lopez-Martinez, Elnitsky, Benoit, Lee, & Denlinger, 2008; Teets et al., 2008). Adult emergence is a fairly synchronous event occurring in early summer (Edwards, & Baust, 1981), and there is some evidence for protandry (Edwards, & Baust, 1981; Harada, Lee, Denlinger, & Goto, 2014). The wingless adults mate in swarms formed on rocks and other features of the substrate (Edwards, & Baust, 1981; Harada et al., 2014). Environmental stressors in Antarctica can be severe and highly variable over short distances and time periods (Convey, 1997; Kennedy, 1993), and swarming may play a role in locating and taking advantage of intermittent, favorable microhabitats (Convey & Block, 1996; Hahn & Reinhardt, 2006; Sugg et al., 1983). Mating swarms present a potential obstacle for establishing long-lasting colonies in a laboratory setting, as mating is likely facilitated by large-scale, synchronous adult emergence (Harada et al., 2014).

Adult females that emerge in the laboratory lay, with a few exceptions, a single egg mass, each containing about 40 eggs (Convey & Block, 1996; Edwards, & Baust, 1981; Harada et al., 2014; Sugg et al., 1983). Nevertheless, multiple matings are common, and multiple oviposition events have been

86 reported (Edwards, & Baust, 1981; Harada et al., 2014; Sugg et al., 1983). Specific underlying materials
87 transferred from the male to the female during copulation are unknown in *B. antarctica*. Eggs are
88 encased in a hygroscopic gel that has been suggested as a potential food source for developing larvae
89 (Edwards, & Baust, 1981; Harada et al., 2014). This gel is likely secreted by the female accessory gland
90 during oviposition, but the protein components of the gel are unknown. Little additional information is
91 available on reproduction in these extremophiles (Edwards, & Baust, 1981; Harada et al., 2014), and
92 chironomid reproduction in general is poorly studied beyond the basic descriptions of their reproductive
93 anatomy and impaired reproduction during exposure to toxic substances (Sibley, Ankley, & Benoit, 2001;
94 Vogt et al., 2007; Wensler & Rempel, 1962).

95 By contrast, reproduction in other dipteran species has been examined extensively (Alfonso-
96 Parra et al., 2016; Avila, Sirot, LaFlamme, Rubinstein, & Wolfner, 2011; J.B. Benoit, Attardo, Baumann.,
97 Michalkova, & Aksoy, 2015 ; A. G. Clark et al., 2007; Dottorini et al., 2007; Izquierdo et al., 2019; K. P. Lee
98 et al., 2008; McGraw, Clark, & Wolfner, 2008; Meier, Kotrba, & Ferrar, 1999; Papa et al., 2017; Polak et
99 al., 2017; Ravi Ram & Wolfner, 2007; Villarreal et al., 2018). In most Diptera, insemination does not
100 involve injection of a spermatophore; rather, male seminal fluid is usually transferred directly into the
101 female reproductive tract, often with the addition of a mating plug to reduce male-male competition as
102 is seen in mosquitoes and *Drosophila* (Giglioli & Mason, 1966; Lung & Wolfner, 2001; Mitchell et al.,
103 2015; Scolari et al., 2016). Some flies do utilize a spermatophore, which may facilitate quicker mating
104 while also creating a barrier to multiple inseminations (Kotbra, 1996). In the tsetse fly, *Glossina*
105 *morsitans,* a dipteran that uses a spermatophore, proteins making up the spermatophore are secreted
106 from the male accessory gland during copulation (Attardo et al., 2019; Scolari et al., 2016). The
107 spermatophore is deposited such that the spermatozoa are funneled efficiently to the openings of the
108 spermathecal ducts, allowing only one spermatophore to maintain this connection at a time (Attardo et

109 al., 2019; Scolari et al., 2016). In mosquitoes, accessory gland-specific proteins, along with the steroid
110 hormone, 20-hydroxy-ecdysone, are transferred to the female during copulation, producing a mating
111 plug (Dottorini et al., 2007; Mitchell et al., 2015; Rogers et al., 2008). In *An. gambiae* the mating plug
112 has multiple effects that promote copulation, enhance egg production, and even trigger egg laying
113 (Dottorini et al., 2007; Gabrieli et al., 2014; Mitchell et al., 2015; Thailayil, Magnusson, Godfray, Crisanti,
114 & Catteruccia, 2011). First-male precedence and last-male precedence have both been reported in
115 multiple species within the Diptera (Dixon, Coyne, & Noor, 2003; Gwynne, 2012; Price, 1997; Shutt,
116 Stables, Aboagye-Antwi, Moran, & Tripet, 2010), but it remains unknown how fertilization priority is
117 established in *B. antarctica*. Depending on the mating strategy of the species, contents of the
118 spermatophore may include a large amount of seminal fluid proteins (SFPs) and other factors or contain
119 primarily the sperm itself (Avila et al., 2011; Lung & Wolfner, 2001; Rogers et al., 2008; Scolari et al.,
120 2016). These seminal fluid proteins are suspected or demonstrated to have a variety of functions,
121 including induction of refractoriness in the female, counteracting protease activity of female secretions
122 with protease inhibitors, defending spermatozoa against microbial assault, and neutralizing reactive
123 oxidative species (Alfonso-Parra et al., 2016; Avila et al., 2011; Lung & Wolfner, 2001; Ravi Ram &
124 Wolfner, 2007; Shutt et al., 2010), all which can compromise sperm function and impair their ability to
125 fertilize. The particular cocktail and amounts of SFPs utilized reflects the selection pressure from life-
126 history differences, conspecific competition, and diverse reproductive strategies between species and
127 even within species (Hopkins et al., 2019; Hopkins, Sepil, & Wigby, 2017; Izquierdo et al., 2019; Papa et
128 al., 2017).

129 Secretions from the female accessory glands also play important roles in insemination and
130 oviposition in dipterans, as well as other insects. In the house fly, *Musca domestica,* female accessory
131 gland secretions enhance sperm viability (Degrugillier, 1985; Leopold & Degrugillier, 1973). In some

132 species, substances secreted from the female accessory gland are expelled with the eggs at oviposition
133 and often function as an adhesive, anchoring eggs to the substrate (Lococo & Huebner, 1980). Accessory
134 gland secretions may also provide protection from diverse biotic and abiotic stressors. In the medfly,
135 *Ceratitis capitata*, the primary components of the accessory gland secretion deposited during
136 oviposition are ceratotoxins, which act as potent antibacterial agents (Marchini, Bernini, Marri,
137 Giordano, & Dallai, 1991). Similarly, accessory gland secretions from the sand fly, *Phlebotomus papatasi*,
138 have antimicrobial effects that protect the eggs, developing embryos, and adult female reproductive
139 tract from microbial invasion (Rosetto et al., 2003). Some male seminal fluids also contain antimicrobial
140 peptides, probably for similar reasons as in the female (Lung, Kuo, & Wolfner, 2001; Poiani, 2006).
141 Female accessory gland proteins can also be a source of nutrition for developing progeny, either while
142 growth occurs in the mother or as a secreted food source to nourish external progeny (Benoit, Kölliker,
143 & Attardo, 2019; Benoit et al., 2015 ; Kaiwa et al., 2014; Kaulenas, 2012).

144 In this study, we use RNA-seq, proteomics, and functional analyses to examine the reproductive
145 physiology of *B. antarctica*. Specifically, male and female accessory glands are examined to identify
146 factors related to male accessory protein generation and synthesis of egg components during
147 oviposition. Proteomic analysis of the gel secretion is used to identify its components, while
148 comparative genomic analyses are used to identify orthologs of specific reproduction-associated genes
149 in mosquitoes and midges. Functional studies reveal that stress impinging on late instar larvae impacts
150 synthesis of gene products associated with reproduction, lowering both male and female reproductive
151 success. Furthermore, we determine that the gel likely acts to prevent egg dehydration and serves as
152 thermal buffer, preventing overheating of the eggs. Population growth modeling reveals that impaired
153 fecundity from larval stress may reduce reproduction below population replacement levels. Our analysis
154 shows that reproduction in the Antarctic midge is directly impacted by larval stress, and identifies novel

155 roles for products manufactured by the female accessory gland. These studies confirm that an
156 understanding of reproductive biology is critical for establishing how these Antarctic extremophiles are
157 able to survive and proliferate in the challenging polar environment.

158

159 **2 MATERIALS AND METHODS**

160 2.1 Midge collections

161 Antarctic midges were collected from islands near Palmer Station (64°46'S, 64°04'W) in January 2007
162 and January 2017. Males and females were separated based upon the major morphological characters
163 described previously (Convey & Block, 1996; Sugg et al., 1983; Usher & Edwards, 1984), homogenized
164 on-site, and stored in Trizol (Invitrogen) at -70 °C for shipment to the University of Cincinnati. Female
165 and male accessory glands were also dissected (N = 20-30) and stored in Trizol similar to whole body
166 stages.

167 Larvae were collected from the same location as adults. Larvae within organic debris were
168 returned to Palmer Station, and larvae were extracted into ice water with a modified Berlese funnel.
169 Following recovery, larvae were stored with substrate from their natural habitat (rocks, soils, moss, and
170 the alga *Prasiola crispa*, which serves as a food source for *B. antarctica*) at 2-4 °C. Larvae were shipped
171 to the University of Cincinnati and stored under similar conditions until they were used in studies
172 examining the impact of larval stress or gel presence on adult fertility or egg viability, respectively.

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175 2.2 RNA extraction and processing

176 RNA was extracted from the midges by homogenization (BeadBlaster 24, Benchmark Scientific) in Trizol
177 reagent (Invitrogen), using manufacturer's protocols with slight modification based on other studies of
178 invertebrates (Hagan et al., 2018; Rosendale, Dunlevy, McCue, & Benoit, 2019). Extracted RNA was
179 treated with DNase I (Thermo Scientific) and cleaned with a GeneJet RNA Cleanup and Concentration
180 Micro Kit (Thermo Scientific) according to manufacturer's protocols. RNA concentration and quality
181 were examined with a NanoDrop 2000 (Thermo Scientific).

182 Poly(A) libraries were prepared by the DNA Sequencing and Genotyping Core at the Cincinnati
183 Children's Hospital Medical Center. RNA was quantified using a Qubit 3.0 Fluorometer (Life
184 Technologies). Total RNA (150-300 ng) was poly(A) selected and reverse transcribed using a TruSeq
185 Stranded mRNA Library Preparation Kit (Illumina). An 8-base molecular barcode was added to allow for
186 multiplexing and, following 15 cycles of PCR amplification, each library was sequenced on a HiSeq 2500
187 sequencing system (Illumina) in Rapid Mode. For each sample, 30-40 million paired-end reads at 75
188 bases in length were generated. Raw RNA-seq data have been deposited at the National Center for
189 Biotechnology Information (NCBI) Sequence Read Archive: Bio-project PRJNA576639. Along with the
190 RNA-seq samples collected for this study, larval (control, dehydration, and cryoprotective dehydration)
191 samples were acquired from Teets et al.(Teets et al., 2012) under the NCBI Bioproject PRJNA174315.
192

193 RNA-seq reads were trimmed for quality (Phred score limit of 0.05) and sequences with
194 ambiguities were removed. In addition, five and eight nucleotides were removed from the 5' and 3'
195 ends, respectively, and sequences shorter than 45 bases were removed. Reads before and after cleaning

196 and trimming were examined with FastQC for quality (S. Andrews

197 http://www.bioinformatics.babraham.ac.uk/projects/fastqc) to verify the quality of each set.

198

199

200 2.3 Gene expression analyses

201 RNA-seq analyses were conducted using two distinct pipelines. The first method utilized was CLC

202 Genomics (Qiagen), as previously described (Hagan et al., 2018; Rosendale et al., 2019). Briefly, reads

203 were mapped to contigs with a cutoff of at least 80% of the read matching at 90% identity with a

204 mismatch cost of 2. Each read was permitted to align to only 20 contigs. Expression values were based

205 on total read counts in each sample calculated as transcripts per million reads mapped. EdgeR was used

206 to test significance among samples. A multiple comparison correction was performed (false discovery

207 rate, FDR). Genes were considered to be differentially expressed if the fold change was greater than 2.0

208 and the p-value was < 0.05. In addition, genes were required to have at least 5 mapped reads per

209 sample to be retained for further analyses. For whole-carcass expression analyses, genes were

210 considered sample-specific if enrichment was noted in relation to both of the other whole-carcass

211 datasets. For accessory gland specific analyses, genes were considered tissue specific if they were

212 enriched relative to the relevant whole-carcass dataset (female or male). In addition, a *de novo*

213 assembly was conducted on the female accessory gland RNA-seq datasets using Trinity (Grabherr et al.,

214 2011) based on standard methods to determine if bacterial symbionts were present in this organ and

215 could be transferred to the egg while in the gel.

216 The second method for examining transcript expression involved utilization of RNA-seq tools

217 available through the Galaxy software package (www.https://usegalaxy.org/) (Afgan et al., 2018;

218 Goecks, Nekrutenko, & Taylor, 2010) using Salmon with the suggested settings. Differential expression
219 between genes was examined with the DeSeq2 package (Love, Huber, & Anders, 2014). A general
220 linearized model assuming a binomial distribution followed by a false discovery rate (FDR) approach
221 were utilized to account for multiple testing. Cut-off values for significance, enrichment and sample-
222 specificity were the same as those used in analysis conducted with CLC Genomics.

223 Transcripts identified as sex- or development-specific were examined using the CLC-based
224 pipeline as there was over 95% overlap between each RNA-seq analysis method. Pathways enriched
225 within males, females, and larvae were identified with a combination of Database for Annotation,
226 Visualization and Integrated Discovery (DAVID (Huang da et al., 2009)), Blast2GO enrichment
227 analyses(Conesa et al., 2005), CLC gene set enrichment analysis (Clark & Ma'ayan, 2011), and g:Profiler
228 (Raudvere et al., 2019). Due to the taxonomic limitations of DAVID, sets of enriched transcripts were
229 compared by BLASTx to the *An. gambiae* and to the *D. melanogaster* RefSeq protein datasets to identify
230 homologous sequences. Blast hits (e-value < 0.001) from these two species were submitted to DAVID.
231 There was considerable overlap between the results, and only the CLC-based methods were used in
232 subsequent analyses.

233 Lastly, we utilized weighted correlation network analysis (WGCNA) to identify specific modules
234 of genes that have similar expression profiles across developmental stages and accessory glands
235 (Langfelder & Horvath, 2008). WGCNA was implemented in an R software package used to construct
236 correlation networks and describe correlation between gene expression across samples in RNA-seq or
237 microarray data. Genes sharing similar patterns of expression across samples were clustered into
238 modules to identify groups of biologically significant genes that were particular to one of the sample
239 groups. For this analysis, RNA-seq data were screened for genes of zero variance prior to WGCNA,
240 leaving 13,424 genes for signed network construction. The minimum module size allowed was 20 and

241 the soft power was set to 14 as determined by the package's scale-free topology function. Modules
242 exhibiting the highest Pearson correlation coefficient were selected for further analysis to determine
243 function and relationship to developmental stages and accessory glands. Modules identified as enriched
244 in a specific developmental stage or tissue were examined for enriched GO categories with the use of
245 g:Prolifer and DAVID, as described earlier.

246

247 2.4 Comparative analyses with other chironomid midges and *Anopheles* mosquitoes

248 This is the first study to examine genome-wide, sex- and stage-specific expression in a midge. However,
249 a recent study examines sex-specific expression in four species of anopheline mosquitoes, a clade not
250 distantly related to midges, along with expression specific to the male and female reproductive tracts
251 (MRT and FRT, respectively (Papa et al., 2017)). The species covered in this study were *An. gambiae, An.*
252 *minimus, An. albimanus,* and *An. arabiensis.* In addition, male accessory glands enriched genes for *B.*
253 *antarctica* were directly compared to those from Anopheles male accessory glands(Izquierdo et al.,
254 2019). Predicted gene sets from this study that had enriched expression in specific stages and organs
255 were used in comparative analyses with *B. antarctica.* The genomes of four species of chironomid
256 midges were also acquired: *Clunio marinus* (Kaiser et al., 2016)*, Parochlus steinenii* (Kim et al., 2017)*,*
257 *Polypedilum vanderplanki* (Gusev et al., 2014)*,* and *Polypedilum nubifer*(Gusev et al., 2014)*.* Studies that
258 resulted in these sequenced midge genomes did not include analyses of differential expression between
259 sexes.

260 For comparative analyses between midges, predicted gene sets for *B. antarctica* were compared
261 with genomes of each midge species, and results of these four analyses were pooled to establish
262 putative sets of genes common to all five species. This analysis also resulted in identification of

263 differentially expressed genes unique to *B. antarctica*. Similarly, predicted gene sets from *B. antarctica*
264 were compared with each species of *Anopheles* mosquito. Antarctic midge gene sets were compared to
265 *Anopheles* whole carcass gene sets, as well as reproductive tract-specific gene sets (Papa et al., 2017).
266 Lastly, we compared the expression of genes within the male accessory glands to orthologous gene sets
267 that are uniformly expressed in the male accessory glands of mosquitoes (Izquierdo et al., 2019). The
268 common gene sets produced by these analyses were then subjected to ontological analyses, using
269 gProfiler, to establish sex-specific enriched pathways. tBLASTp analyses (e-value < 0.001) were
270 performed using CLC Genomics Workbench (CLC bio Qiagen). Protein sequences were defined as
271 orthologs if they were reciprocal-best BLASTp hits having an e-value < 10^{-10}. Overlap was compared
272 between these analyses to produce putative sex-specific transcript sets.

273 Transcription factors (TFs) and their predicted DNA binding motifs were identified based on
274 methods used for other invertebrate genomes (Attardo et al., 2019; Benoit et al., 2016; Olafson et al.,
275 2019). In brief, putative TFs were identified by scanning the amino acid sequences of all proteins for
276 putative DNA binding domains using the HMMER software package (Eddy, 2009) and a compilation of
277 Pfam DNA binding domain models (Weirauch & Hughes, 2011). Experimentally determined DNA binding
278 motifs were then inferred from other species (*e.g., Drosophila*) based on amino acid identity, using
279 previously established rules (Weirauch et al., 2014). Using this collection of inferred DNA binding motifs,
280 we examined enrichment of each motif within the 500 and 2000 bp promoter regions of genes with
281 increased expression in each sex, larvae, and accessory glands. These results were then compared to the
282 expression profiles of each TF to determine specific TF candidates that might regulate sex and
283 reproduction associated genes.

284

285 2.5 PCR and qPCR analyses

286 Select genes of interest that were highly enriched within males and females were examined by PCR.
287 Total RNA was extracted from males, females, larvae, female accessory glands, and male accessory
288 glands as described previously in the RNA-seq section and used as a template for Superscript III reverse
289 transcriptase according to the manufacturer's protocols (Invitrogen). PCR was performed with gene-
290 specific primer pairs (Table S15) using a DNA polymerase kit (Promega). The PCR conditions were 95 °C
291 for 3 min, 35 cycles of 30 sec at 95 °C, 52-56 °C for 1 min, and 1 min at 70 °C using an Eppendorf
292 Mastercycler Pro Series. Three independent (biological) replicates were conducted for each sex or
293 tissue stage.

294 qPCR analyses were conducted based on previously developed methods (Rosendale, Romick-
295 Rosendale, Watanabe, Dunlevy, & Benoit, 2016). RNA was extracted as described previously for
296 independent biological replicates. Complementary DNA (cDNA) was generated with a DyNAmo cDNA
297 Synthesis Kit (Thermo Scientific). Each reaction used 250 ng RNA, 50 ng oligo (dT) primers, reaction
298 buffer containing dNTPs and 5 mmol•l^{-1} $MgCl_2$, and M-MuLV RNase H+ reverse transcriptase. KiCqStart
299 SYBR Green qPCR ReadyMix (Sigma Aldrich, St Louis, MO, USA) along with 300 nmol l^{-1} forward and
300 reverse primers, cDNA diluted 1:20, and nuclease-free water were used for all reactions. Primers were
301 designed using Primer3 based on contigs obtained from the transcriptome analysis (Table S15). qPCR
302 reactions were conducted using an Illumina Eco quantitative PCR system. Reactions were run according
303 to previous studies(A. J. Rosendale et al., 2016). Four biological replicates were examined for each sex,
304 and three biological replicates were examined for each accessory gland. Expression levels were
305 normalized to *rpl19* using the ΔΔCq method as previously described(Joshua B Benoit et al., 2018; A. J.
306 Rosendale et al., 2016). Fold change was compared between larvae, males, females, and accessory
307 glands followed by a Pearson correlation coefficient (r).

308

309 2.6 Proteomics analysis of accessory gland-derived gel

310 Samples were analyzed at the Proteomics and Metabolomics Laboratory at the University of Cincinnati.
311 Two proteomic samples were collected by removing eggs from the gel with a micropipette and
312 dissolving the gel in 1X PBS with 0.1% Tween. Samples (4 µg) were run on a 1D SDS PAGE gel and silver
313 stained to confirm the presence of proteins; at least 15 distinct proteins could be visualized. Based on
314 this initial characterization, gel proteins (6 µg) were run 2 cm into a 1D 4-12% Bis-Tris Invitrogen NuPage
315 gel using MOPS buffer. Lanes were excised, reduced with 10 mM dithiothreitol, alkylated with
316 Iodoacetamide and digested with trypsin according to the standard protocol (Heaven et al., 2016;
317 Turnier et al., 2018). The resulting peptides were concentrated with a speed vac centrifuge and
318 resuspended in 0.1% formic acid. Each sample (2 µg) was used in subsequent analyses. Nanoscale LC-
319 electrospray ionization-MS/MS (nanoLC-ESI-MS/MS) analyses were performed on a TripleTOF 5600
320 (Sciex, Toronto, ON, Canada) coupled to an Eksigent (Dublin, CA) nanoLC ultra nanoflow system. Protein
321 from each gel sample was loaded and analyzed as described (Heaven et al., 2016; Turnier et al., 2018).
322 The data were recorded using Analyst-TF (v.1.6) software and searched against the *B. antarctica* genome
323 (Kelley et al., 2014) using the Protein Pilot program (Sciex). Gel proteins were compared to those with
324 differential expression in specific tissues from our RNA-seq studies. Protein, carbohydrate, and lipid
325 content were examined through spectrophotometric assays based upon methods described in
326 Rosendale et al. (2019)

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330 2.7 Thermal buffering by the accessory gland gel

331 The gel has been suggested to serve as a source of nutrients for newly emerged larvae (Convey, 1992;
332 Convey & Block, 1996; Harada et al., 2014). In addition to this role, we examined whether the gel
333 increases thermal buffering capabilities of the egg compared to eggs directly deposited on the local
334 substrate. To examine the effect, we placed an Omega thermocouple within six gels and immediately
335 adjacent to these six gels at a field location near Palmer Station. Temperature was measured every
336 minute over the course of three days.

337 To establish whether the gel prevents egg death caused by thermal stress, eggs with and
338 without gels were exposed to 20 °C for two hours. Females were allowed to lay their eggs onto filter
339 paper disks (Whatman) which were placed in 50 ml centrifuge tubes before being transferred to a 4 °C
340 water bath. Temperature was then ramped up to 20 °C over the course of four hours 4 °C per hour
341 before slowly being reduced back to 4 °C over the same course of time. These samples were compared
342 to those that were held statically at 4 °C without the gel. Following treatment, all eggs were maintained
343 at 4 °C and monitored for larval emergence.

344

345 2.8 Role of the accessory gland gel in dehydration

346 To determine if the accessory gland gel could prevent egg dehydration, we subjected midge eggs to
347 dehydrating conditions with and without the gel's presence. All eggs were removed from the gel with a
348 fine metal probe and half were carefully reinserted into the gel. Three groups of eggs with or without
349 the gel were moved to 75% RH at 4 °C for 12 hours. Following this treatment, eggs with no gel were
350 placed back into the gel. Viability was determined by counting the number of larvae that emerged from
351 the total number of eggs.

352

353 2.9 Impact of larval dehydration stress on fecundity

354 To determine if larval dehydration stress impacts adult fecundity, we performed combined RNA-seq
355 analysis (described previously) and exposure of larvae to dehydration stress, both standard and
356 cryoportective (Teets et al., 2012). RNA-seq studies were conducted on larvae that were quickly
357 dehydrated (30% water loss) and on those that had undergone a slower form of dehydration,
358 cryoprotective dehydration (30% water loss). The resulting data sets were then examined to find genes
359 with increased expression in male or female accessory glands.

360 To determine whether mating is directly impacted by dehydration stress, groups of 100 fourth
361 instar larvae (final larval instar) were held at 75% RH until they lost 40% of their water content.
362 Following dehydration, larvae were returned to the standard rearing conditions and monitored every 12
363 hours for the presence of pupae or newly emerged adults. Each adult was removed and stored
364 separately at 98% RH, 4°C until mating, which occurred no later than four days after treatment. The
365 following mating pairs were examined: males from dehydrated larvae vs. control females, females from
366 dehydrated larvae vs. control males, and both males and females from dehydrated larvae. Males and
367 females that failed to copulate were removed from the experiment.

368

369 2.10 Statistics

370 Replicates are independent biological samples. Sample sizes are listed in each method section or the
371 figure legend. Significance is indicated within each figure and/or in the figure legend. Statistical tests are

372 listed within the respective section in the methods or in the figure legends. All statistical analyses were
373 performed using JMP version 11 (SAS) or R-based packages.

374

375 2.11 Population level effects

376 To explore population level effects of dehydration, gel surrounding the egg mass, and thermal stress we
377 used a Leslie matrix approach (Lefkovitch, 1965). Here, the dominant eigenvalue of the matrix is the
378 population growth rate (λ). We simplified life history to egg to larvae to adult despite there being four
379 larval instars, potentially occurring over several years. For control populations we used a mean fecundity
380 of 42.9 (eggs laid), an egg survival rate of 0.82, and larval survival rate of 0.78. To determine the effects
381 of larval dehydration on population growth, we used fecundities (eggs laid) of 34.6, 35.6, and 21.5 for
382 male, female, and male and female dehydration, respectively; egg and larval survivorship were assumed
383 to be the same as control populations. To determine the effects of gel on population growth we used all
384 parameters (as for control populations), but reduced larval survival to 0.5, based on the experimental
385 data from our studies. Similarly, to investigate effects of thermal stress we reduced egg survivorship to
386 0.63, but left all other parameters at control values. We also investigated a worst case scenario with
387 male and female dehydration, no gel, and thermal stress. All values were derived from the previously
388 described experiments. We determined the dominant eigenvalue of each matrix using the function
389 "eigen" in R.

390

391

392

393 **3 RESULTS**

394 3.1 Description of mating and reproductive organs in *B. antarctica*

395 Beyond copulation and sex ratios (Edwards, & Baust, 1981; Harada et al., 2014; Sugg et al., 1983), little is
396 known about mating and reproductive aspects for *B. Antarctica.* In this study, the reproductive organs
397 were observed by dissection. Following copulation (Fig. 1A), a spermatophore containing sperm and
398 other, likely accessory gland products, are transferred as a bundle to the females (Fig. 1A, inset).
399 Females deposit a gel around the fertilized eggs, and it is within this gel that embryogenesis occurs (Fig.
400 1B). The source of the gel appears to be the female's accessory gland (Fig. 3C) because the gland is
401 depleted followed egg laying (Fig. 1D,E). The male reproductive organs, including the testes and
402 accessory glands, are depicted in Fig. 1F. The general organization and structure of the male and female
403 reproductive anatomy is similar to that of another midge, *Chironomus plumosus* (Wensler & Rempel,
404 1962) and mosquitoes (Masci et al., 2015), with the exception that the female accessory gland of *B.*
405 *antarctica* is dramatically enlarged. It is this enlarged gland that we consider to be the source of the
406 gel deposited around the eggs.

407

408 3.2 RNA-seq analyses of *Belgica* reproduction

409 Differential transcript levels were determined for males, females, and larvae using two independent
410 pipelines using the *B. antarctica* genome as a reference (Kelley et al., 2014). The two pipelines yielded
411 95% overlap at our significance cut-off (two-fold difference, combined transcripts per million (TPM)
412 among all samples of at least 5, and a correction-based P-value < 0.05). Between 60% and 71% of the
413 reads from each RNA-seq set mapped to predicted genes. At the 4-fold or greater level for stage-
414 specificity, overlap between the two methods was over 99%; thus we used the CLC Genomics (Qiagen)

415 pipeline (Attardo et al., 2019; Rosendale et al., 2019) for the remaining analyses (Table S1). When
416 expression differences were compared among all developmental stages, each sex and associated
417 reproductive organs were most similar, followed by similarity between adults, while the larval stages
418 were the most unique (Fig. 2). When the *de novo* assembly was examined for presence of putative
419 microbial symbionts, we detected no substantial matches (Table S2), indicating the gel does not likely
420 serve as a source of potential microbial symbionts as in other insects (Kaiwa et al., 2014).

421 Individual comparisons between stages revealed 392 male-enriched genes, 1825 female-
422 enriched genes, and 862 genes enriched in larvae (Fig. 2, Table S3-S5). Specific gene ontology (GO)
423 categories were associated with each stage including carboxylic acid biosynthesis in males, DNA repair in
424 females, and aminoglycan metabolism in larvae (Fig. 3; Table S3-S5). When accessory glands were
425 specifically examined, 20 genes were enriched in the female accessory gland and had significantly higher
426 expression in females compared to other stages (Fig 4A; Table S6). A similar analysis for the male
427 accessory gland identified 25 enriched genes. GO categories associated with the female accessory gland
428 were associated with glycosylation and mucin biosynthesis. Notably, similar GO categories were
429 associated with gene products of the male accessory gland (Fig. 4B; Table S7).

430 Beyond this first analysis, weighted correlation network analysis (WGCNA, Langfelder &
431 Horvath, 2008) was used to identify clusters (modules) of highly correlated genes between
432 developmental stages and within specific reproductive organs (Fig. 5; Table S8-S12). Specific modules of
433 enriched expression were identified in each tissue and developmental stage (Fig. 5B,C). When GO
434 analyses were conducted for each developmental stage, unique GO categories were associated with
435 larvae, females, and males (Fig. 5). Female accessory glands had few enriched GO categories based on
436 WGCNA results; enriched categories included phosphatase binding, response to stimulus, and ion
437 channel activity. Male accessory glands also had a low number of GO categories associated with

438 enriched modules; these included metallopeptidase activity and integral components of the membrane.
439 These results provide correlated stage- and tissue-specific expression modules. For larvae, males, and
440 females, GO categories from stage specific modules showed overlap with our previous analysis (Fig. 3),
441 but unique categories were also identified (Fig. 5).

442

443 3.3 Proteomic analysis of accessory gland derived gel

444 Proteomic analysis of the gel surrounding the eggs revealed 24 associated proteins (Fig. 6). Three
445 proteins comprised a majority of the gel (Fig. 6B; Table S13), including a vitellogenin-like protein
446 (IU25_12621), larval serum protein (IU25_03947), and an apolipophorin (IU25_03809). The two
447 analyzed gel samples had a highly-correlated protein content (Pearson correlation = 0.92, Fig. 3C).
448 Expression analyses revealed that transcripts for most of the gel proteins are not directly generated in
449 the female accessory gland (Fig. 3D), but instead are produced in other female organs (e.g. IU25_12621)
450 or within the larvae (e.g. IU25_03947 and IU25_03809). qPCR validation of the protein gel components
451 confirmed that transcripts for each of the gel proteins are expressed in tissues identified in the RNA-seq
452 studies (Fig. 5E). Additional qPCR analyses for genes not involved in producing major components of the
453 gel served as validation for results obtained from other RNA-seq samples (Fig. 5E).

454

455 3.4 Transcriptional regulation of reproductive-associated factors

456 To compare potential mechanisms regulating gene expression between samples, we predicted putative
457 transcription factors (TFs, Fig. 7A, Table S14) and their DNA binding motifs. We next performed TF
458 binding site motif enrichment analysis in the upstream regulatory regions of our differentially expressed

459 gene sets (see Methods). This analysis revealed that fourteen TFs had significantly enriched binding
460 sites in at least one gene set (Fig. 7B). Of these, one TF showed enhanced binding in the 500bp
461 regulatory regions of male-enriched genes. The remaining thirteen showed enhanced binding in female-
462 enriched genes; we limited our analysis to the set of seven TFs with enhanced binding in both 500 and
463 2000bp regions of the female- and/or FeAG-enriched gene sets. Five members of this set showed higher
464 transcript levels in either the female or female accessory gland compared to other tissues and
465 developmental stages (Fig. 7C). The increased gene expression in the respective tissues or stage along
466 with enriched binding sites suggest that these TFS are key regulatory elements for female reproduction
467 in *B. antarctica*. Transcription factors include forkhead box protein 1 and mothers against
468 decapentaplegic (Mad) homolog 1. In *Drosophila* Mad has been identified as a participant in the
469 signaling pathway of *decapentaplegic* (dpp), a morphogenetic protein that plays a role in regulating the
470 development of egg polarity and early embryonic development (Xie & Spradling, 1998). Several
471 transcription factors identified in our screening remain uncharacterized with no assigned biological
472 functions.

473

474 3.5 Comparative analyses between anopheline mosquitoes and chironomid midges

475 Sex- and tissue-specific gene sets from *B. antarctica* were compared with predicted gene sets from four
476 species of *Anopheles* mosquitoes and four species of chironomid midges (Figs. 8 and 9). Additionally, *B.*
477 *antarctica* enriched gene sets were compared with gene sets enriched in male and female reproductive
478 tracts of the same four *Anopheles* mosquitoes (Papa et al., 2017) (Fig. 8 and 9) and in comparison to the
479 male accessory gland enriched genes from five *Anopheles* species (Izquierdo et al., 2019)

480 Genes enriched in females of *B. antarctica* showed the highest degree of conservation among all
481 species examined by a large margin. All five species of midges had 1396 genes in common, 1401 were
482 shared with all four *Anopheles* species, and 1267 were common to all 9 species examined (Fig. 8). Gene
483 ontology analysis of this set revealed enrichment in functions related to RNA, DNA, chromatin binding,
484 nuclease activity, chromosome and organelle organization, transport of RNA and proteins in and out of
485 the nucleus, RNA processing, and cell cycle regulation. No female-enriched genes detected in *B.*
486 *antarctica* were unique when compared to the other eight species examined. *Belgica* females have 122
487 genes with no orthologues in any of the other four midge species. However, this gene set did not show
488 significant enrichment in any GO functional categories.

489 Among males, 345 genes were common among midge species, and 337 genes were shared
490 between *B. antarctica* and all four mosquito species (Fig. 8). Genes common to all midge species were
491 enriched in functions associated with anion transport, alpha-amino acid catabolism, and carboxylic acid
492 transmembrane transport. As with females, no genes were identified in the male-enriched gene set that
493 were unique to *B. antarctica*. There were 16 genes unique to *B. antarctica* among the five midge
494 species, but, again, they were not significantly enriched in any single functional category.

495 The larva-enriched gene set was compared to the midge and mosquito gene sets. Over 600
496 genes were identified with orthologues common to all examined midge and mosquito species (Fig. 8).
497 The gene set common to all midge species was enriched in functional categories associated with chitin
498 metabolism, iron ion binding, and cytochrome P450-driven metabolism. Two identified genes were
499 unique to larvae of *B. antarctica* (Fig. 8; Table S5): one is uncharacterized, and the other putatively
500 encodes polyubiquitin B. Gene ontology analysis comparing larvae of *Belgica* with other midges revealed
501 88 unique genes, but no functional categories were significantly enriched for this gene set.

502 Gene sets specifically enriched in male and female reproductive tracts of each mosquito species
503 were compared to sex-specific gene sets in *B. antarctica* (Fig. 9; Table S6-7). Comparison of the female-
504 enriched gene set with the female reproductive tract (FRT)-specific gene set yielded 241 genes with
505 putative FRT orthologues. This set was particularly enriched in GO categories related to mitotic cell cycle
506 processes and regulation of gene expression. For example, several orthologues in this set code for
507 cyclins, cyclin dependent kinases, zinc-finger proteins, transcription factors, and transcription
508 termination factors. Additionally, a large number of genes in this set (~44%) mapped to apparently
509 uncharacterized orthologs. The comparison between male-enriched and Male Reproductive Tract (MRT)
510 specific genes yielded a common set of 48 genes. This set is dominated by CLIP-domain serine proteases
511 and cytochrome p450s, and also contains three glucose-dehydrogenases (Fig. 9). When *B. antarctica*
512 MAG-enriched genes are compared to those for mosquitoes, only four overlapping genes were
513 identified based on moderate or high expression reported in a previous study(Izquierdo et al., 2019) of
514 the mosquito MAG (Fig. 9C); these were identified as venom allergen, carboxypeptidase, serine
515 protease, and a gene of unknown function.

516

517 3.6 Dehydration reduces larval serum protein and possibly subsequent egg production

518 Since specific female accessory gland components are expressed in larvae, we asked whether larval
519 dehydration impacts expression of adult female accessory gland components (Fig. 10). To do so, we re-
520 analyzed results from a previous RNA-seq study (Teets et al., 2012) on dehydration and cryoprotective
521 dehydration of *B. antarctica* (Fig. 10). One of the major female accessory gland proteins (IU25_03947)
522 had significantly reduced expression when larvae experienced either dehydration or cryoprotective
523 dehydration (water loss specifically induced by cold temperatures) (Fig. 10A-B). This protein is a

524 component of the accessory gel and, as a hexamerin, serves as a protein reserve (Burmester, 1999;
525 Burmester, Massey Jr, Zakharkin, & Benes, 1998; Telfer & Kunkel, 1991). The dehydration-evoked
526 suppression in transcripts associated with reproductive-associated factors possibly results in reduced
527 amounts of materials invested in the progeny at birth and reduced egg output (Fig. 10D-F). These results
528 suggest that stress experienced as larvae may have a direct impact on female reproduction, most likely
529 acting through reduced expression of larval serum protein.

530

531 3.7 Dehydration reduces specific male accessory gland components and impacts fertilization

532 We next examined the impact of larval dehydration stress on male fertility. To do so, we analyzed
533 expression of accessory gland components in adult males after larval dehydration or cryoprotective
534 dehydration (Fig. 11A). Thirteen genes enriched in male accessory glands were differentially expressed
535 in the adult male following larval dehydration compared to fully-hydrated individuals (Fig. 11A).
536 Enrichment of glutathione transferase activity and phosphatidylcholine metabolic processes were
537 detected. Male body mass was not altered following dehydration stress, but mating was substantially
538 compromised (Fig. 11B-C). When males dehydrated as larvae mated with unstressed females, fewer
539 eggs were deposited, and additive reductions were noted if dehydrated males mated with females that
540 were dehydrated as larvae (Fig. 11C). These results indicate an impact on male fertility brought about
541 by dehydration stress during larval life.

542

543

544

545 3.8 Role of gel as a nutrient source

546 Analyses of major gel protein components (over 3% of the total proteins of the gel) was performed.
547 Amino acid content of the major protein components revealed that all essential amino acids can be
548 provided within the gel (Fig. 12A). Predicted glycosylation and phosphorylation sites of the proteins
549 suggest that these proteins could provide phosphate and sugar resources for the developing larva as the
550 gel is consumed (Fig. 12). Spectrophotometric analysis of the gel revealed that 81%, 14%, and 5% of the
551 caloric content consisted of proteins, carbohydrates, and lipids, respectively. Larvae were denied access
552 to the gel to determine its impact on larval growth (Fig. 12B). Fewer of the gel-less larvae were alive
553 after one month (Fig. 12B), and those remaining were nearly 30% smaller than larvae that hatched from
554 eggs with free access to the gel (Fig. 12C). Importantly, these larvae were provided access to algae and
555 other organic debris with and without gel, indicating that non-gel food resources do not fully
556 compensate for loss of the gel as a food source. These results underscore the value of the gel for
557 successful larval development.

558

559 3.9 Thermal and dehydration buffering by the gel

560 We performed experiments to determine whether the gel impacts dehydration resistance in developing
561 eggs. When gel-less eggs were held in desiccating conditions (75% relative humidity) at 4 °C for 12 h and
562 subsequently treated with water, no viable eggs were detected , even when the eggs were
563 subsequently covered with gel to promote growth (0% for all three replicates). By contrast, viability of
564 eggs encased in gel exceeded 80% under in the same conditions, suggesting that the accessory gel
565 protects eggs against dehydration-induced mortality. These results indicate that the gel is likely critical
566 for maintaining water homeostasis within the egg and possibly for the developing embryo as well.

567 We also examined whether the gel acts as a thermal buffer. First, temperature changes were
568 examined within the gel (next to the eggs) and on the surface immediately adjacent to the eggs (Fig.
569 13A). The gel buffered temperature changes during the course of the day by reducing both the
570 maximum temperature and the rate of temperature change (Fig. 13). Gel-less eggs exposed to 20 °C
571 displayed reduced viability when compared to eggs encased in gel and to those held constantly at 4 °C
572 (Fig. 13). These results indicate that the accessory gland gel provides both thermal and dehydration
573 protection to the eggs.

574

575 3.10 Population modeling

576 Dehydration of males, females, and both males and females together reduced population growth
577 relative to control populations (Fig. 14). The combined effect of male and female dehydration had the
578 largest effect, reducing population growth by nearly 21%. Lack of gel also negatively impact population
579 growth, resulting in a 13% reduction relative to controls. The effect of thermal stress on egg viability
580 would reduce population growth by 8%. In spite of these scenarios, populations would still be expected
581 to increase in abundance under each specific stress. Under the "worst case scenario" where fecundity is
582 reduced by male and female dehydration, larval survival is reduced by thermal stress, and there is a lack
583 of gel reducing egg survival, population growth rate is negative ($\lambda = -0.95$), indicating declining
584 population size. We note that all these growth rates are fairly liberal because of the relatively short time
585 over which larval survival was observed (12 days) relative to the time spent as larvae in nature. In
586 addition, it is likely that other factors, such as starvation, freezing, or pathogen attack, could occur,
587 resulting in additive impacts on population growth.

588

589 **4 DISCUSSION**

590 This study examined reproductive biology of the Antarctic midge with the goal of establishing key
591 molecular mechanisms associated with male and female biology. Combined RNA-seq and proteomics
592 established the transcriptional components of reproduction and protein constituents deposited in the
593 gel surrounding the egg. Specifically, we examined whether the gel that encases the eggs alters egg
594 viability and larval survival and examined the impact of larval dehydration exposure on adult fertility.
595 Little is known about midge reproductive biology and we have summarized the major findings of this
596 study in Figure 15. Thus, this study will hopefully provide a foundation for the fields of Antarctic and
597 midge reproductive biology. Lastly, we modeled the impact of multiple reproductive factors on
598 population growth, each of which exert minor effects but in combination could yield a negative growth
599 rate. Our results highlight the importance of understanding the reproductive biology of this Antarctic
600 insect, a species restricted to a limited geographic region and a specific habitat.

601 The large proportion of apparently conserved, female-specific or female-enriched genes that are
602 uncharacterized (≈44%) suggests there are many aspects of *Belgica* female physiology that remain
603 poorly understood. However, the results of the GO analysis of the female-enriched gene set are
604 consistent with results reported for *Drosophila* and other insect systems (Attardo et al., 2019; Olafson et
605 al., 2019; Panfilio et al., 2019; Swanson, Wong, Wolfner, & Aquadro, 2004). In an analysis of genes
606 differentially expressed between germline-naive and gonadectomized females in *Drosophila*, Parisi *et al*.
607 found enrichment in terms associated with metabolism (Parisi et al., 2004) . In fact, genes associated
608 with energy storage and utilization, such as digestive proteases or lipid storage genes, showed increased
609 expression in germline-naive males and females. This suggests that enrichment of such genes would be
610 expected in the larval gene set, even if adult *Belgica* were active eaters which they are not. Indeed, the
611 larva enriched gene set is replete with putative trypsins, chymotrypsin like proteins, and lipases (Cao &

612 Jiang, 2017; Graveley et al., 2011; McKenna et al., 2016; Pauchet et al., 2010; Venancio, Cristofoletti,
613 Ferreira, Verjovski-Almeida, & Terra, 2009). Oogenic gene expression has also been linked to nutrient
614 sensing in *Drosophila* (Shim, Gururaja-Rao, & Banerjee, 2013; Terashima & Bownes, 2005). Enrichment
615 of terms associated with metabolism, including components of ribosomes as protein-building
616 machinery, identified in *B. antarctica* is most likely associated with oogenesis-related biosynthesis. This
617 could include mobilizing lipids and proteins from the adult fat body during vitellogenesis, as well as
618 synthesis of components of the egg gel excreted from the accessory gland. Transcription factor analyses
619 revealed a subset of genes likely to have critical roles in female biology and thus warrant further more
620 directed studies. One identified was mothers against decapentaplegic (Mad) homolog 1, which likely is
621 likely critical for development (Newfeld, Chartoff, Graff, Melton, & Gelbart, 1996; Xie & Spradling, 1998).

622 Many enriched GO terms can also be tied to gametogenesis and embryogenesis or *Belgica*
623 females. Terms associated with cell cycle control and DNA/RNA/chromatin handling are among the main
624 examples. Beyond meiotic divisions that result in formation of gametes, which of course takes place in
625 the male as well, female may have to exercise more precise control over the timing of oocyte
626 maturation, deploying cell-cycle modulators, transcription regulation machinery, and/or RNA silencing
627 factors (Lefebvre & Lécuyer, 2018; Qazi, Heifetz, & Wolfner, 2003; Soller, Bownes, & Kubli, 1999). TF
628 analyses revealed a NF-kappa relish-like transcription factor as a potential regulator of transcript levels
629 for the female-enriched gene set. Indeed, NF-kappaB transcription factors play well-established roles in
630 transcriptional regulation of cytokines, molecules involved in cell cycle control, and development
631 (Gilmore, 2006; Kim & Kim, 2005; Sosic & Olson, 2003). Curiously, this TF has its highest expression in
632 males despite a paucity of binding sites in the regulatory regions of the male-enriched gene set.
633 However, expression in the male accessory gland is limited and lower than that of the female or female

634 accessory gland. The meaning of these findings remains unclear, but indicate a potential critical role
635 related to accessory gland function in *Belgica*.

636 The female also produces developmental factors that play a role in successful embryogenesis,
637 such as mRNAs, hormone biosynthesis precursors, and general cytoplasmic components (sperm are
638 quite cytoplasm-poor relative to eggs) (Swevers, Raikhel, Sappington, Shirk, & Iatrou, 2005). Among the
639 most highly enriched genes in females relative to males (>1000 fold higher) are a handful of genes
640 known to be involved in embryonic patterning in insects, such as *nanos*, *oskar*, and multiple innexins,
641 components of invertebrate gap junctions involved in intercellular communication during
642 embryogenesis (Bauer et al., 2005; De Keuckelaere, Hulpiau, Saeys, Berx, & Van Roy, 2018; Güiza, Barria,
643 Saez, & Vega, 2018; Quinlan, 2016; Richard & Hoch, 2015). This lends support to the notion that the
644 female-enriched gene set is comprised largely of genes regulating and supporting oogenesis and
645 embryogenesis. In addition, paternal DNA must be de-compacted after fertilization, meaning that sperm
646 nucleotide binding proteins must be degraded (Doyen et al., 2015). The enzymes/proteins that
647 participate in this process are likely already present in the egg, and therefore maternally contributed
648 (Tirmarche, Kimura, Dubruille, Horard, & Loppin, 2016). This is followed by histone/chromatin assembly
649 which is dependent on maternally provided histones and nucleosome assembly factors (Loppin,
650 Dubruille, & Horard, 2015; Tirmarche et al., 2016). Male-contributed mitochondrial DNA must also be
651 degraded (DeLuca & O'Farrell, 2012; Sato & Sato, 2011). This further accounts for the prevalence of
652 terms associated with histones and chromosome organization. Finally, in *Drosophila,* nurse cells
653 surrounding the oocyte are known to polyploidize regions of their nuclear DNA to enhance transcription
654 and support provisioning of the oocyte (Bastock & St Johnston, 2008; Kaulenas, 1992; Orr-Weaver,
655 2015). This provides yet another physiological link to DNA replication and chromosome organization, but

656 chironomids lack nurse cells. It is uncertain whether other cells of the ovary act in a similar manner to
657 *Drosophila* nurse cells (Bastock & St Johnston, 2008).

658 The dominant GO terms associated with the male-enriched gene set are 'anion transport' and
659 'carboxylic acid biosynthesis.' These are both terms that can be linked to spermatogenesis and may also
660 be linked to the differing metabolic needs of males, females, and larvae: larvae eat, digest, and grow;
661 females conduct vitellogenesis, oogenesis, and embryogenesis; and males likely devote the bulk of their
662 energy store to spermatogenesis and mating (Perry, Harrison, & Mank, 2014). The two terms may be
663 interrelated. For example, genes involved in synthesis and transport of pyruvate, a carboxylate anion
664 and the starting substrate of the tricarboxylic acid (TCA) cycle, could be associated with either GO term.
665 The functional role of pyruvate metabolism in sperm viability is not known, but mitochondrial activity is
666 demonstrated to be important for fertility, and a mitochondrial pyruvate transporter that is almost
667 uniquely expressed in the male germline has been identified in placental mammals (Vanderperre et al.,
668 2016). However, inhibition of pyruvate transporters in the mitochondrial membrane does not appear to
669 affect sperm motility, lending support to the notion that sperm motility is fueled primarily by glycolysis
670 (not import of pyruvate into mitochondria) (Vanderperre et al., 2016); alternatively, amino acid
671 metabolism may play a role in motility, as it has been associated with sperm motility in *An. gambiae*
672 (Izquierdo et al., 2019). TCA-based energy production must play a role in some other aspect of sperm
673 viability (Vanderperre et al., 2016). The fact that a mitochondrial pyruvate transporter is important in
674 sperm viability suggests that pyruvate transport and metabolism contribute to the significance of the GO
675 terms 'tricarboxylic acid cycle,' 'monocarboxylic acid metabolic process,' and 'anion transport.'
676 Additionally, sperm are thought to take up lactate and pyruvate through general monocarboxylic acid
677 transporters (MCTs) in humans (Ramalho-Santos et al., 2009; Rato et al., 2012; Vanderperre et al.,
678 2016). Perhaps there is an analogous uptake of energetically important organic acids in the seminiferous

679 tubules of *Belgica* and other insects. Finally, pyruvate may be useful in supplying Acetyl-CoA for the
680 histone acetylation that is essential for chromatin condensation during spermatogenesis (Ramalho-
681 Santos et al., 2009; Rato et al., 2012; Vanderperre et al., 2016).

682 Overall, males showed far fewer genes with enriched expression relative to the female than vice
683 versa. Of the 12 genes that are >1000 fold higher in the male compared to other stages, three are
684 putative metalloendopeptidases. These may be involved in the proteolytic processing of amyloid
685 precursor proteins (APPs), which are integral components of sperm membranes in humans, though their
686 specific roles have not been determined (Silva et al., 2015). Also of interest in this small set of highly
687 enriched genes are a few genes commonly, though not exclusively, associated with immunity: a leucine
688 rich immune protein TM, a toll protein, and an apparent homolog of the transcription factor NF-X1
689 (Brucker, Funkhouser, Setia, Pauly, & Bordenstein, 2012; De Gregorio, Spellman, Rubin, & Lemaitre,
690 2001; Irving et al., 2001; Palmer & Jiggins, 2015). This transcription factor binds X-box motifs to regulate
691 many eukaryotic genes and is generally thought to be a negative regulator of transcription (Stroumbakis,
692 Li, & Tolias, 1996). The homolog of this transcription factor identified in *D. melanogaster*, named
693 shuttlecraft (stc), is essential for normal embryonic development and is expressed most highly in the
694 embryo CNS. Moreover, expression of this gene is highest in the ovary, and the promoters of many
695 maternally contributed genes known to be crucial in embryonic development contain X-box motifs, such
696 as *oskar, torpedo, pumilio,* and vitelline membrane proteins (Stroumbakis et al., 1996). Therefore it is
697 tempting to suggest that, unlike in *Drosophila*, it is for some reason the male who produces this critical
698 TF, transferring it to the female during copulation in order to trigger or facilitate proper development of
699 their progeny. In support of the notion that the NF-X1 gene product is transferred to the female during
700 copulation, expression of the gene is roughly 24-fold higher in the male accessory gland than in the
701 whole carcass.

702 Gene ontology analysis of the larvae-enriched gene set revealed a preponderance of terms
703 related to peptidase, hydrolase, and detoxification activity. These GO terms are related to digestion and
704 detoxification of ingested materials by the growing larvae – neither of which would be relevant issues
705 for the non-feeding adults. One cytochrome p450s (CYP6Z2), up-regulated in larvae, has been implicated
706 in chemical resistance in *Anopheles* mosquitoes(Malik et al., 2016) and therefore may be critical for
707 larval survival in their potentially toxic microhabitat, such as seabird guano (Rial et al., 2016).

708 Besides the ingestion of food, larvae of *B. antarctica* are also longer-lived and must face the
709 seasonal challenges of permanent residence in Antarctica (Edwards, & Baust, 1981; Sugg et al., 1983). In
710 particular, larvae must survive freezing and desiccation during the austral winter along with potential
711 thermal stress during summer (Benoit et al., 2009; Benoit, Lopez-Martinez, Michaud, et al., 2007; Lopez-
712 Martinez et al., 2008; Michaud et al., 2008; Rinehart et al., 2006; Teets, Kawarasaki, Lee, & Denlinger,
713 2013). Genes enhancing survival under these conditions, such as heat shock proteins (Benoit et al.,
714 2009; Benoit, Lopez-Martinez, Michaud, et al., 2007; Lopez-Martinez et al., 2008; Michaud et al., 2008;
715 Rinehart et al., 2006; Teets et al., 2013), are expected to be up-regulated by larvae. Based on previous
716 studies in *Belgica* and other species that are desiccation and freeze tolerant, genes of importance could
717 include oxido-reductase related enzymes (e.g. cytochrome p450s), Late Embryogenesis Abundant (LEA)
718 proteins, protein repair methyltransferases, hemoglobins, aquaporins, and enzymes involved in
719 trehalose metabolism (Benoit et al., 2009; Benoit, Lopez-Martinez, Michaud, et al., 2007; Dunning et al.,
720 2013; Lopez-Martinez et al., 2008; Lv et al., 2010; Michaud et al., 2008; Rinehart et al., 2006; Ronges,
721 Walsh, Sinclair, & Stillman, 2012; Teets et al., 2013; J. Zhang, Marshall, Westwood, Clark, & Sinclair,
722 2011). Many of these categories are indeed enriched in the larvae and are likely substantial factors in
723 the stress resistance abilities of *B. antarctica*. Note that the presence of hemoglobin in insect
724 hemolymph is unique to chironomids (Gusev et al., 2014; Kaiser et al., 2016; S.-M. Lee, Lee, Park, & Choi,

725 2006) and furthermore it is thought to be unique to the larval stage. This could partially explain the
726 prevalence of iron binding as a GO term enriched in the larvae and conserved among the midge species
727 analyzed (Gusev et al., 2014; Kaiser et al., 2016; Lee et al., 2006).

728 Most prevalent among GO terms enriched in the larval gene set are those associated with
729 aminoglycan metabolism. This set includes, for example, multiple putative N-
730 acetylgalactosaminyltransferases, which catalyze the initial glycosylation of serine and threonine
731 residues (Tran & Ten Hagen, 2013). Mucin type O-glycosylation occurs commonly on proteins with
732 extracellular domains, comprising a portion of the extracellular matrix, and it is thought to be important
733 in intercellular communication and adhesion (Tran & Ten Hagen, 2013). In *Drosophila*, enzymes
734 responsible for building glycosylated proteins, such as mucins, are critical for embryonic development,
735 particularly for the CNS (Tian & Hagen, 2007; Tran & Ten Hagen, 2013; Zhang, Zhang, & Ten Hagen,
736 2008). Maternal and zygotic O-linked glycans have also been implicated in proper respiratory
737 development. These O-glycans are termed *pgant* in *Drosophila* (Tian & Hagen, 2007; Tran & Ten Hagen,
738 2013; Zhang et al., 2008). One essential gene, *pgant4*, is involved in regulating gut acidification, but this
739 is not the only such gene required in the digestive system (Tran & Ten Hagen, 2013; Tran et al., 2012).
740 The larva enriched gene set in *Belgica* includes putative homologs of *pgant4* and *pgant6*, as well as the
741 essential genes CG30463 and C1GalTA, both involved in mucin type O-linked glycosylation. However,
742 larva-enriched GO terms, as mentioned above, are dominated by hydrolysis and catabolic processes,
743 including glycosaminoglycan catabolism. This may be a sign of the breakdown of extracellular materials
744 associated with growth and development or an indication of a diet rich in aminoglycans.

745 Composition of male accessory glands has been a major focus in numerous insects. These
746 products influence fertilization rates, subsequent female receptivity to courtship, and are critical in the
747 composition of the spermatophore (Alfonso-Parra et al., 2016; Avila et al., 2011; Dottorini et al., 2007;

748 Gabrieli et al., 2014; Izquierdo et al., 2019; McGraw et al., 2008; Mitchell et al., 2015; Ravi Ram &
749 Wolfner, 2007; Rogers et al., 2008; Sirot, Wong, Chapman, & Wolfner, 2015; Thailayil et al., 2011;
750 Villarreal et al., 2018). Many of the male accessory gland genes from *Belgica* have orthologs based on
751 predicted genes from midge and mosquito genomes, but few overlapping orthologs were identified that
752 are expressed in the male accessory gland of *B. antarctica* compared to the male reproductive tissues of
753 mosquitoes (Izquierdo et al., 2019; Papa et al., 2017). This is not surprising since a similar lack in
754 orthology among male accessory gland products has been observed between *Drosophila* and *Glossina*
755 and other higher flies (Attardo et al., 2019; Scolari et al., 2016). We did not conduct biological
756 examination of specific roles for accessory gland proteins from *B. antarctica*, such as whether their
757 transfer during mating impacts refractoriness of females, as noted in many species (Avila et al., 2011;
758 Baldini, Gabrieli, Rogers, & Catteruccia, 2012; Ravi Ram & Wolfner, 2007; Sirot et al., 2015). There is an
759 enrichment for genes associated with glycoprotein synthase, which is unsurprising as glycoproteins are
760 common constituents of seminal fluid (Avila et al., 2011; Poiani, 2006). In addition, there were specific
761 serine proteases, immune factors, and products involved in response to oxidative stress. These are
762 similar to those observed in other fly species(Avila et al., 2011; Baldini et al., 2012; Ravi Ram & Wolfner,
763 2007; Sirot et al., 2015; Tian et al., 2017) and likely serve to preserve sperm viability but could impact
764 other aspects such as female biology (Abraham et al., 2016; Denis et al., 2017). These factors are likely
765 critical to male success, as this midge will mate on multiple occasions. It is important to note that
766 females will deposit eggs with and without fertilization (Harada et al., 2014), thus suggesting that factors
767 supplied in the spermatophore are not essential for ovulation and oviposition as in other fly species
768 (Avila et al., 2011; Baldini et al., 2012; Ravi Ram & Wolfner, 2007; Sirot et al., 2015).

769 Our gene expression analysis is one of the few that has explicitly examined gene expression
770 within the female accessory gland. Many, but not all, products present within the gel were expressed in

771 the female's accessory gland. A comparison of our results to expression pattern in mosquito female
772 tracts (Papa et al., 2017) revealed few genes with overlapping expression(Papa et al., 2017). Few studies
773 in other flies explicitly examined products generated by the female's accessory gland. Even in
774 *Drosophila*, the female accessory gland (paraovaria) remains one of the most understudied organs
775 (Attardo et al., 2014). In tsetse flies, this organ provides nourishment to developing intrauterine larvae
776 (Attardo et al., 2019; Benoit et al., 2015 ; Attardo et al., 2014), but there are no similarities, beyond
777 standard housekeeping genes, between this organ in tsetse flies and *B. antarctica*. Of interest,
778 regulation of transcript expression within the female accessory gland seems to be conserved (Attardo et
779 al., 2014), suggesting that regulatory aspects may be similar but drive expression of specific genes
780 related to unique functions of this organ in different flies. Transcription factor analyses identified a
781 single gene (IU25_08656) that has enriched levels of both binding sites upstream of female accessory
782 gland enriched genes and is itself expressed in the female accessory gland. This is an uncharacterized
783 zinc finger proteins, but is likely to have a critical role in female reproductive function for *B*. *antarctica*.

784 The three major protein components of the gel are vitellogenin, larval serum protein, and
785 apolipophorin, all which are reasonable components of a gel whose primary function includes fueling
786 the development of larvae upon hatching. *Belgica* larvae exhibit "drinking behavior" shortly before
787 hatching, suggesting that up to this point embryonic development is fueled by nutrient reserves present
788 in the egg at the time of oviposition (Harada et al., 2014). Upon hatching, the gel is ingested by the larva,
789 making it the first meal fueling their development. Larval serum protein likely acts as an amino acid
790 reservoir, while vitellogenin and apolipoproteins could provide fatty acid, sugar, and protein reserves.
791 The gel may also contribute other elements in smaller amounts, such as pre-synthesized developmental
792 hormones in an inactive form, hormone precursors, proenzymes, and enzyme cofactors that are
793 important for continued development (Harada et al., 2014). Larval serum protein is a storage protein

794 belonging to the family of hexamerins (Burmester, 1999; Burmester et al., 1998; Telfer & Kunkel, 1991).
795 Genes for such proteins are often highly expressed in the final larval instar preceding pupation
796 (Burmester, 1999; Burmester et al., 1998; Telfer & Kunkel, 1991) and serve as a nutrient reserve for
797 developing pupae and newly emerged adult. During the sweeping morphological changes that
798 accompany metamorphosis in holometabolous insects like *Belgica*, such storage proteins are extracted
799 from the larval fat body, transferred into the hemolymph, and subsequently re-sequestered in the newly
800 formed adult fat body(Burmester, 1999; Burmester et al., 1998; Keeley, 1978; Larsen, 1976; Telfer &
801 Kunkel, 1991). The following scenario is the likely fate of the larval serum protein found in the egg gel:
802 Initially re-sequestered by the adult fat body and shuttled to the female accessory gland with
803 vitellogenin, or possibly sequestered in the female accessory gland during adult development after
804 pupation without a layover in the fat body. Importantly, lack of expression in the female adult indicates
805 these proteins must be synthesized in the larva for subsequent use by the adult.

806 Along with establishing components of the female accessory gland derived gel, a major goal of
807 this study was to identify functional roles of this gel and to determine if the gel components are
808 impacted by stress in the developing larvae. Based on our results, one of the main functions of the gel is
809 to provide a nutritional resource, which leads to a higher larval survival. Products of the accessory
810 glands of females have been documented as food sources for insect species among many orders(Attardo
811 et al., 2019; Benoit et al., 2015 ; Denlinger & Ma, 1974; Kaiwa et al., 2014; Ma, Denlinger, Jarlfors, &
812 Smith, 1975). Production of gel substances surrounding eggs have been noted in other species, such as
813 in stink bugs, where the gel serves as a source of nutrition, protects the eggs, and provides a vehicle to
814 transfer microbial symbionts (Kaiwa et al., 2014). Similar to our study, removal of the gel inhibits
815 juvenile development in stink bugs, but in contrast, removal of the gel around the stink bug eggs has
816 little impact on hatching success. In addition to providing a critical nutritional source and preventing

817 dehydration, we show that the egg gel acts as a thermal buffer that limits temperature extremes. Our *de*
818 *novo* assembly of the female accessory gland failed to detect signatures of microbial symbionts within
819 the gel, indicating that the gel does not serve as a mechanism of bacterial transfer to the developing
820 larvae. This lack of microbial presence could very well limit microbial exposure of the midge until after
821 larvae emerge from the gel. Accessory gland products also have been demonstrated to possess
822 antimicrobial properties(Avila et al., 2011; Otti, McTighe, & Reinhardt, 2013; Otti, Naylor, Siva-Jothy, &
823 Reinhardt, 2009); this is a possibility for *B. antarctica* because immune peptides are present in the gel.

824 Larval dehydration stress had a major impact on fertility of both adult males and females. The
825 most likely cause of reduced fecundity in males and females is a direct reduction in larval serum protein,
826 a hexamerin that acts as a storage protein in larvae (Burmester, 1999; Burmester et al., 1998; Telfer &
827 Kunkel, 1991). This hexamerin represents one of the highest expressed transcripts in developing larvae,
828 so its accumulation during the juvenile stages is likely a critical resource for production of eggs and
829 accessory gland components. This effect is likely even more pronounced in *B. antarctica* because adults
830 do not feed or even readily drink water (Benoit, Lopez-Martinez, Elnitsky, Lee, & Denlinger, 2007). Along
831 with acting as a nutritional source, the larval serum protein generated in juvenile stage of females is
832 likely incorporated into the accessory gland gel, suggesting that a reduction in this product may directly
833 impact gel composition. Larval nutritional status has a direct impact on fecundity in numerous insect
834 systems (Aguila, Hoshizaki, & Gibbs, 2013; Aguila, Suszko, Gibbs, & Hoshizaki, 2007; Rosa &
835 Saastamoinen, 2017), including other midges (Sibley et al., 2001). To the best of our knowledge, larval
836 dehydration has not previously been examined in relation to subsequent adult fecundity. Other stressful
837 conditions, such as chemical exposure, have impaired both larval development and subsequent adult
838 reproduction in a non-biting midge, *Chironomus riparius*(Vogt et al., 2007).

839 This study provides an encompassing view of reproductive biology of the Antarctic midge, from
840 molecular mechanisms to the impact of larval stress exposure on adult fecundity. Key findings are
841 summarized in Figure 15. This is followed by population growth modeling to establish how these factors
842 directly impact persistence of this insect in its limited Antarctic range. Population modeling revealed
843 that each factor (dehydration stress, lack of gel, thermal buffering), by itself, has a small impact on
844 population growth but combined factors likely result in negative population growth. The limited
845 reproductive window of 2-3 weeks makes understanding both male and female reproduction critical for
846 understanding how this midge survives in Antarctica. Studies on the reproductive biology of flies have
847 been limited largely to *Drosophila* and disease vectors (sand flies, mosquitoes, etc.), and our results
848 expand into Chironomidae to provide the groundwork for future studies with this dipteran system.

849

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856

857 **AUTHOR CONTRIBUTION**

858 This study was designed and conceived by J.B.B. The manuscript was written by G.F. and J.B.B. RNA-seq
859 studies were conducted by G.F., A.J.R., B. D., and S.T.B. Molecular and physiological studies were

conducted and interpreted by S. N., C. P., G. F., C.J.H, E.M.D., and J.B.B. Transcription factor analyses were contributed by X.C. and M.T.W. and interpreted by J.B.B. and G.F. Additional data and statistical analyses were conducted by K.J.O., G.M.A., and J.B.B. Samples were collected by J.D.G., D.S., R.E.L., and D.L.D. Population modeling was conducted by S.F.M. Figures were prepared by G.F. and J.B.B. Illustrations were by G.M.A. All authors were responsible for editing the manuscript and have approved publication.

DATA AVAILABILITY STATEMENT

All data generated for this project have been submitted to NCBI or made available in the supplemental files.

878 **FIGURE LEGENDS**

879 **Figure 1: Antarctic midge, *Belgica antarctica*, during reproduction.** A. Mating pair, male on left. Inset,
880 spermatophore transferred to females immediately after copulation. Image is posterior end of female
881 and white material is the spermatophore. B. Female depositing eggs and accessory gland-derived gel. C.
882 Accessory gland (left middle, b) and ovaries (top left and right, a) of gravid females 3 days after adult
883 eclosion. D. Female accessory glands (left, a) and ovaries (top and right, b) following egg and gel
884 deposition. E. Egg mass following the completion of deposition. F. Male reproductive tract, a. testes, b,
885 accessory gland, and c, common duct.

886

887 **Figure 2: Gene expression heat map of Antarctic midge, *Belgica antarctica*, during development,**
888 **between sexes, and for accessory glands.** Hierarchical clustering of RNA-seq gene expression patterns
889 for males, females, larvae, and accessory glands based on sample distance (Euclidean distance matrix) of
890 differentially expressed contigs.

891

892 **Figure 3: Genes uniquely enriched for the Antarctic midge, *Belgica antarctica* in males, females, and**
893 **larvae and associated gene ontology enrichment.** A. Gene enriched in males (left) and gene ontology
894 (right), B. Gene enriched in females (left) and gene ontology (right), C. Gene enriched in larvae (left) and
895 gene ontology (right). Each box represents a specific category and color represent major GO groups.

896

897 **Figure 4: Genes uniquely enriched in the Antarctic midge, *Belgica antarctica*, female and male**
898 **accessory glands and associated gene ontology enrichment.** A. Genes enriched in female accessory

899 glands (left) and gene ontology (right), B. Genes enriched in male accessory gland (left) and gene
900 ontology (right). GO conducted with g:Prolifer (Raudvere et al., 2019).

901

902 **Figure 5: Weighted gene co-expression network analysis (WGCNA) across developmental stages and**
903 **for male and female accessory glands of the Antarctic midge, *Belgica antarctica*.** A. Average linkage
904 hierarchical clustering dendrogram of the genes. Modules, designated by color code, are branches of the
905 clustering tree. B. Correlation of module eigengenes to developmental and accessory gland traits. Each
906 row corresponds to a module eigengene and columns are traits. *, represents values with a significant
907 positive correlation for Pearson r ($P < 0.05$). C. Unsupervised hierarchical clustering heatmap (bottom)
908 and dendrogram (top) of module eigengenes and traits. Gene ontology (GO) analysis of eigengenes
909 associated with larvae (D), males (E), and females (F). GO conducted with g:Prolifer(Raudvere et al.,
910 2019) and visualized with REVIGO(Supek, Bošnjak, Škunca, & Šmuc, 2011).

911

912 **Figure 6: Proteomic analysis of female accessory gland derived gel material from the Antarctic midge,**
913 ***Belgica antarctica*.** A. Female depositing eggs with gel and protein components of two gel samples
914 without eggs. B. Identification of proteins that represent at least 3% of total protein composition of the
915 accessory gland gel. C. Congruence of protein abundance and content between two gel samples. D.
916 Heatmap for transcript levels of gel-specific genes among larvae, females, males, and accessory glands.
917 E. qPCR validation of RNA-seq data. All tested genes have Pearson correlation coefficients over 0.85. Gel
918 specific genes have a Pearson correlation of 0.87.

919

920 **Figure 7: Transcription factors (TFs) and TF binding sites associated with reproduction in the Antarctic**
921 **midge, *Belgica antarctica*.** A. Relative abundance of transcription factors encoded by genomes of
922 midges and mosquitoes. TF families towards the top contain more TFs in *B. antarctica*. B. Enrichment
923 for specific TF binding site motifs in regulatory regions (2000 bp; 500 bp data not shown due to overlap
924 with 2000 bp) of genes expressed highly in specific stages and accessory glands. Groups of TFs are
925 separated by their motif enrichment profiles across samples. Those highlighted in orange are
926 significantly enriched within the specific stage or tissue. Scale for heatmap is set at relative abundance
927 on a Z scale of -2 to 2 across each row. C. Transcript levels of select TFs with significant motif enrichment
928 in the promoters of genes expressed in specific tissues (orange font color in panel B).Scale for heatmap
929 is set at relative abundance on a Z scale of -2 to 2 across each row. MAG, male accessory gland; FeAG,
930 female accessory glands.

931

932 **Figure 8: Comparative analysis of female, male, and larvae-specific gene sets with mosquitoes and**
933 **midges to Antarctic midge, *Belgica antarctica***. A. Female-specific genes compared to midges (left),
934 mosquitoes (middle), and genes with enriched expression in the female reproductive tract (FRT) of
935 mosquitoes (right) (Papa et al., 2017). B. Male-specific genes compared to midges (left), mosquitoes
936 (middle), and genes with enriched expression in the male reproductive tract (MRT) of mosquitoes
937 (right)(Papa et al., 2017). C. Larvae-specific genes compared to midges (left) and mosquitoes (right).
938 Protein sequences were defined as orthologs if they had reciprocal-best BLASTp hits with an e-value <
939 10^{-10}.

940

941 **Figure 9: Comparative analysis of accessory gland gene sets with mosquitoes and midges to the**
942 **Antarctic midge**. A. Female accessory gland genes compared to midges (left), mosquitoes (middle), and
943 genes with enriched expression in the female reproductive tract of mosquitoes (right) (Papa et al.,
944 2017). B. Male accessory gland genes compared to midges (left), mosquitoes (middle), and genes with
945 enriched expression in the male reproductive tract of mosquitoes (right) (Papa et al., 2017). C. Overlap
946 between genes expressed in male accessory glands between mosquitoes and *B. antarctica*. Left, highly
947 enriched in *Anopheles* male accessory gland. Right, enriched in *Anopheles* male accessory gland.
948 Enrichment for *Anopheles* male accessory gland genes is based on values from Izquierdo et al. (2019).
949 Protein sequences were defined as orthologs if they had reciprocal-best BLASTp hits with an e-value <
950 10^{-10}.

951

952 **Figure 10: Expression changes in gel-associated proteins in larvae following dehydration stress.** A.
953 Transcript level changes in larvae for gel proteins following dehydration stress. RNA-seq studies were
954 acquired from Teets et al. (2012). Orange denotes significance between control and dehydrated larvae
955 based on RNA-seq analyses. B. Heat map of transcript levels for gel-associated proteins during
956 dehydration (D) and cryoprotective dehydration (CD) compared to control (C) that are components of
957 the gel proteome and significantly altered by dehydration. C. Total mass before eclosion, D. post-
958 eclosion total mass, E. mass change after eclosion, and total egg production in females when control
959 (non-dehydrated) and dehydrated larvae were allowed to complete development. Analysis of variance
960 was utilized to examine statistical differences with the use of R statistic packages. Bars above indicate
961 significance at $P < 0.05$.

962

963 **Figure 11: Impact of larval dehydration stress on male fertility.** A. Expression profiles of male-
964 associated genes with expression differences after larval dehydration (D, dehydration, C, control, and
965 CD, cryoprotective dehydration, MAG, male accessory gland). B. Mass of males used in mating
966 experiments from dehydrated or control larvae. C. Female fertility (control or dehydrated) following
967 copulation with dehydrated or control males. Analysis of variance was utilized to examine statistical
968 differences with the use of R statistic packages. Bars above indicate significance at $P < 0.05$ unless
969 otherwise noted.

970

971 **Figure 12: Accessory gland gel is critical for larval development.** A. Amino acid composition and
972 putative phosphate and glycosylation sites of gel proteins based on sequence information. Relative
973 amounts are based on comparison levels between columns. B. Survival of developing larvae with (black)
974 and without (gray) gel presence at larval ecdysis. Open circles are the average and filled circles are each
975 replicate. C. Larvae length after 20 days with and without gel at larval ecdysis. Bar indicates significance
976 at $P < 0.05$. Analysis of variance was utilized to examine statistical differences with the use of R statistic
977 packages. Bars above or beside indicate significance at $P < 0.05$.

978

979 **Figure 13: Role of accessory gland gel in relation to thermal buffering of eggs.** A. Thermal profile within
980 (orange) and outside (blue) gel-egg mixture under field conditions, B. Egg viability following exposure to
981 20°C for three hours. Eggs without gel (constant) were held at 4°C for the duration of the trial. Maximum
982 temperature change during 24 hour period. D. Rate of temperature change (minimum to maximum).
983 Analysis of variance was utilized to examine statistical differences with the use of R statistic packages. *
984 above indicates significance at $P < 0.05$ unless otherwise noted.

985

986 **Figure 14: Population growth is impacted by dehydration and thermal stress in developing larvae.** A.
987 Population growth following altered egg production due to dehydration exposure as larvae in males,
988 females, and both sexes combined compared to control (no dehydration of larvae). B. Growth based on
989 the presence or absence of the gel under favorable conditions. C. Impact of thermal stress on egg
990 viability with and without accessory gland gel.

991

992 **Figure 15: Summary of Antarctic midge reproduction.** Larval development (four stages) is condensed
993 into a single representation or all stages. Adults live approximately two-three weeks. Egg development
994 occurs over thirty days. Impact of specific conditions are highlighted based on experimental evidence
995 from this study.

996

997 **Supplemental table 1.** RNA-seq results for complete *Belgica antarctica* gene set. Expression values are
998 in transcripts per million. RNA-seq datasets are available under the following NCBI Bioprojects
999 PRJNA174315 and PRJNA576639.

1000

1001 **Supplemental table 2**- BLAST results for female accessory gland specific *de novo* transcriptome against
1002 bacterial sequences from the NCBI nr database.

1003

1004 **Supplemental table 3.** RNA-seq results for female-enriched *Belgica antarctica* gene set. Expression
1005 values are in transcripts per million. RNA-seq datasets are available under the following NCBI Bioprojects
1006 PRJNA174315 and PRJNA576639.

1007

1008 **Supplemental table 4.** RNA-seq results for male-enriched *Belgica antarctica* gene set. Expression values
1009 are in transcripts per million. RNA-seq datasets are available under the following NCBI Bioprojects
1010 PRJNA174315 and PRJNA576639.

1011

1012 **Supplemental table 5.** RNA-seq results for larvae-enriched *Belgica antarctica* gene set. Expression
1013 values are in transcripts per million. RNA-seq datasets are available under the following NCBI Bioprojects
1014 PRJNA174315 and PRJNA576639.

1015

1016 **Supplemental table 6.** RNA-seq results for female accessory gland-enriched *Belgica antarctica* gene set.
1017 Expression values are in transcripts per million. RNA-seq datasets are available under the following NCBI
1018 Bioprojects PRJNA174315 and PRJNA576639.

1019

1020 **Supplemental table 7.** RNA-seq results for male accessory gland-enriched *Belgica antarctica* gene set.
1021 Expression values are in transcripts per million. RNA-seq datasets are available under the following NCBI
1022 Bioprojects PRJNA174315 and PRJNA576639.

1023

1024 **Supplemental table 8.** WGCNA module results for female-enriched *Belgica antarctica* gene set.
1025 Expression values are in transcripts per million. RNA-seq datasets are available under the following NCBI
1026 Bioproject PRJNA174315 and PRJNA576639.

1027

1028 **Supplemental table 9.** WGCNA module results for male-enriched *Belgica antarctica* gene set. Expression
1029 values are in transcripts per million. RNA-seq datasets are available under the following NCBI Bioprojects
1030 PRJNA174315 and PRJNA576639.

1031

1032 **Supplemental table 10.** WGCNA module results for larvae-enriched *Belgica antarctica* gene set.
1033 Expression values are in transcripts per million. RNA-seq datasets are available under the following NCBI
1034 Bioproject PRJNA174315 and PRJNA576639.

1035

1036 **Supplemental table 11.** WGCNA module results for female accessory gland-enriched *Belgica antarctica*
1037 gene set. Expression values are in transcripts per million. RNA-seq datasets are available under the
1038 following NCBI Bioproject PRJNA174315 and PRJNA576639.

1039

1040 **Supplemental table 12.** WGCNA module results for male accessory gland-enriched *Belgica antarctica*
1041 gene set. Expression values are in transcripts per million. RNA-seq datasets are available under the
1042 following NCBI Bioproject PRJNA174315 and PRJNA576639.

1043

1044 **Supplemental table 13.** Transcript levels for gel-derived proteins from the *Belgica antarctica* gene set.
1045 Expression values are in transcripts per million. RNA-seq datasets are available under the following NCBI
1046 Bioproject PRJNA174315 and PRJNA576639. Relative abundance of protein amounts were based on the
1047 number of protein fragments that match a specific genes.

1048

1049 **Supplemental table 14.** Transcript levels for transcription factors from the *Belgica antarctica* gene set.
1050 Expression values are in transcript per million. RNA-seq datasets are available under the following NCBI
1051 Bioproject PRJNA174315 and PRJNA576639.

1052

1053 **Supplemental table 15**- Quantitative PCR primers used for the validation of RNA-seq result.

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1488 34086.

1489

1490

1491

Figure 1

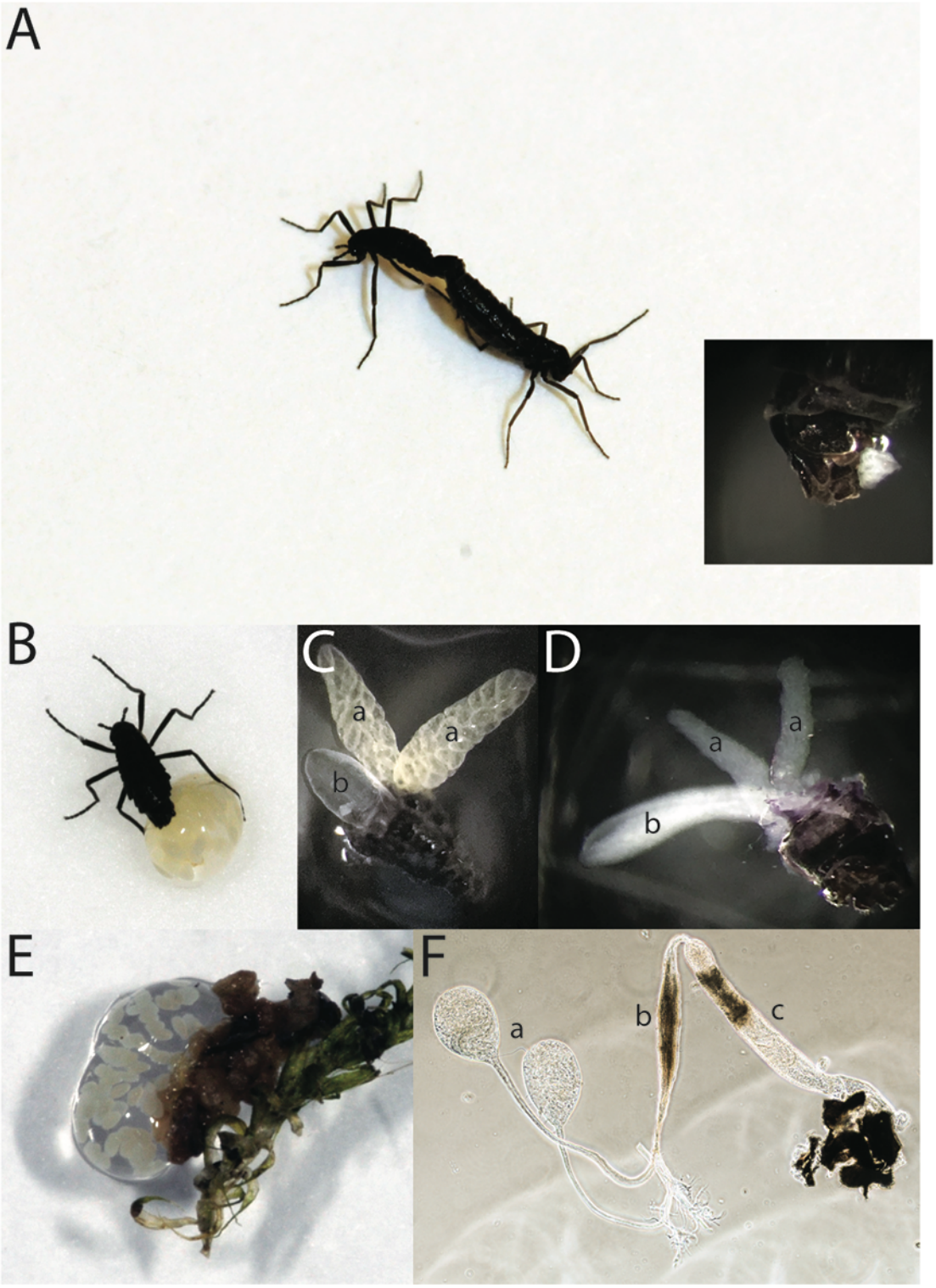


Figure 2

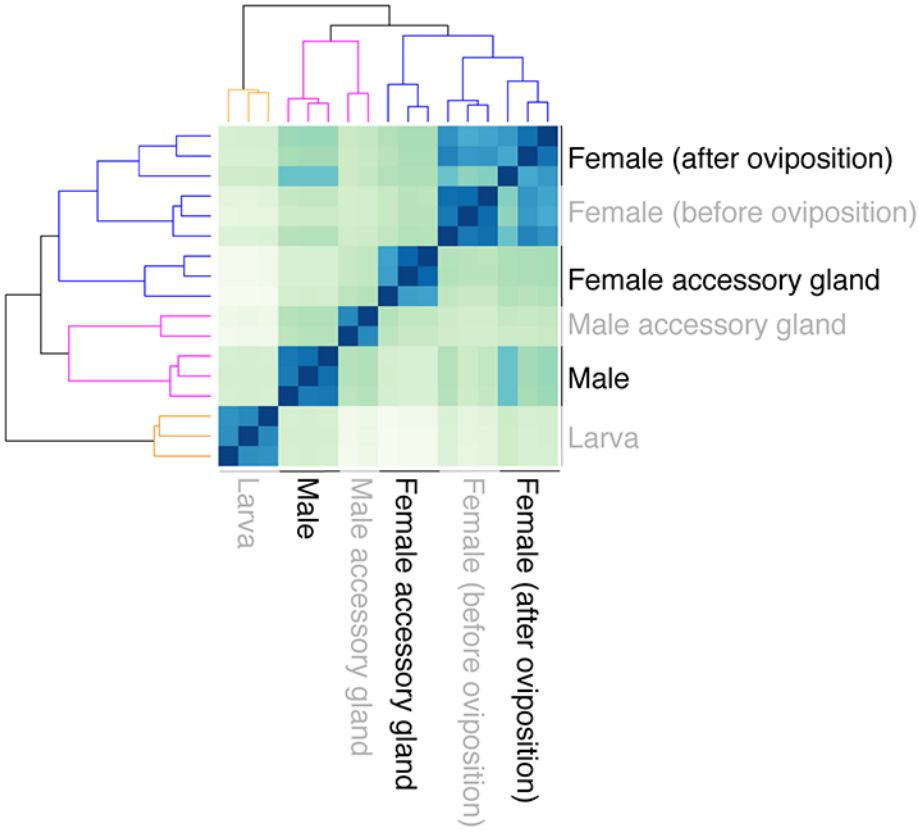
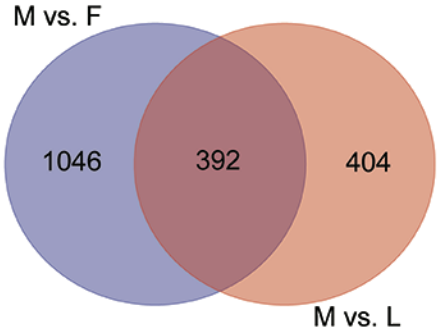
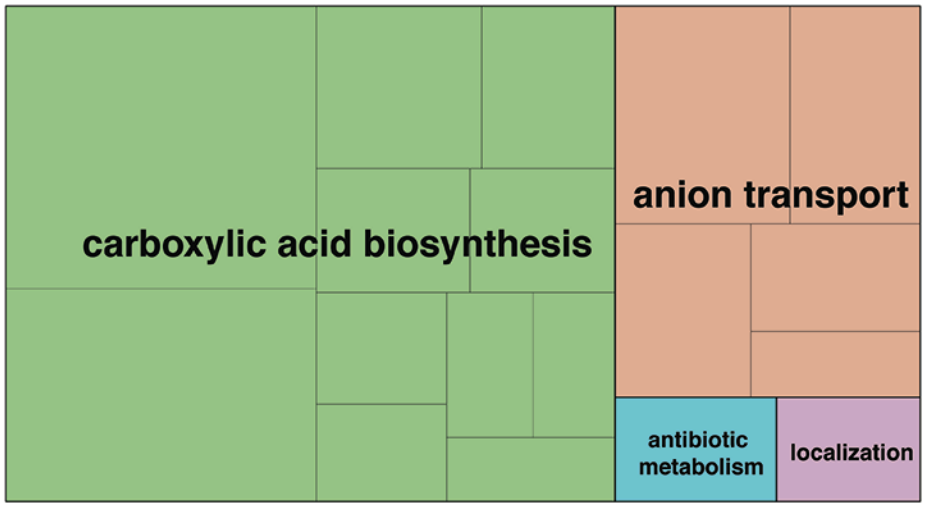

Female (after oviposition)
Female (before oviposition)
Female accessory gland
Male accessory gland
Male
Larva
Larva
Male
Male accessory gland
Female accessory gland
Female (before oviposition)
Female (after oviposition)

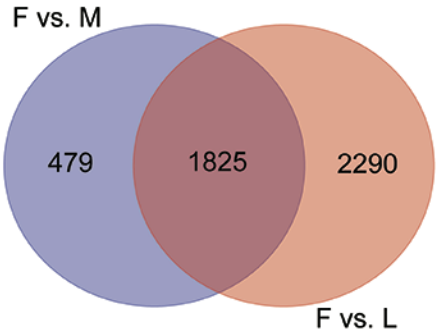
Figure 3

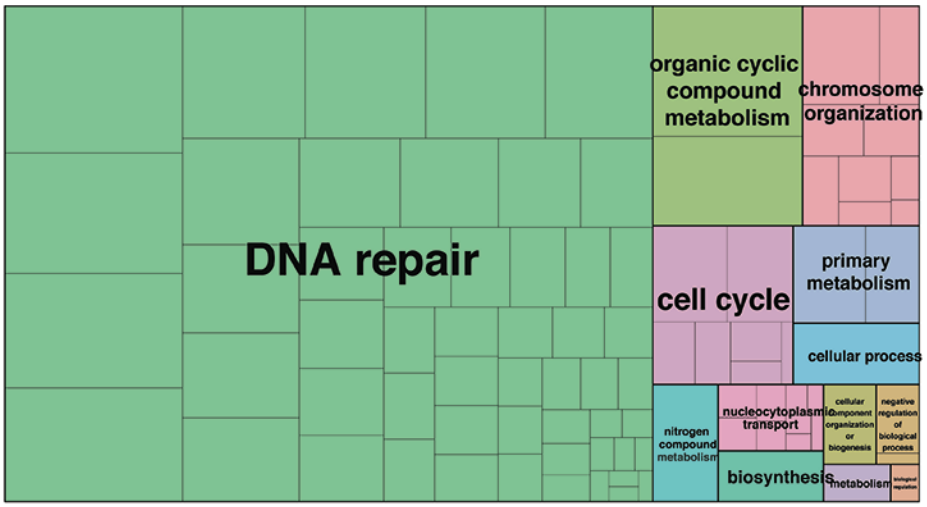
A.


M vs. F
1046
392
404
M vs. L

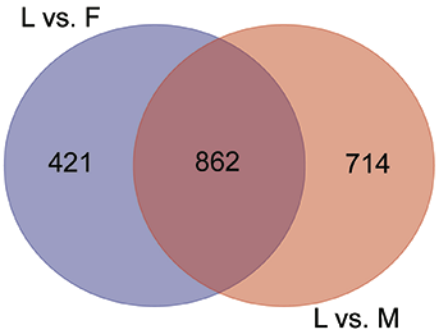

carboxylic acid biosynthesis
anion transport
antibiotic metabolism
localization

B.


F vs. M
479
1825
2290
F vs. L


DNA repair
organic cyclic compound metabolism
chromosome organization
cell cycle
primary metabolism
cellular process
nitrogen compound metabolism
nucleocytoplasmic transport
cellular component organization or biogenesis
negative regulation of biological process
biosynthesis
metabolism

C.


L vs. F
421
862
714
L vs. M

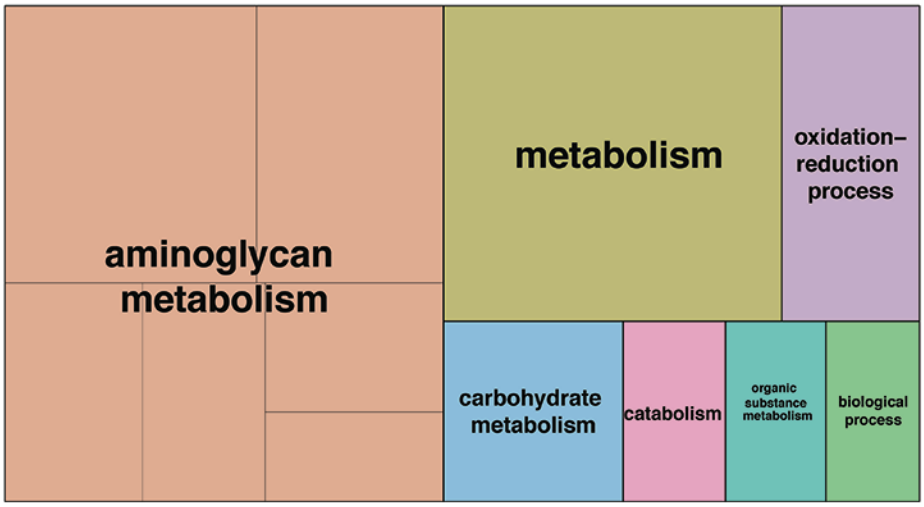
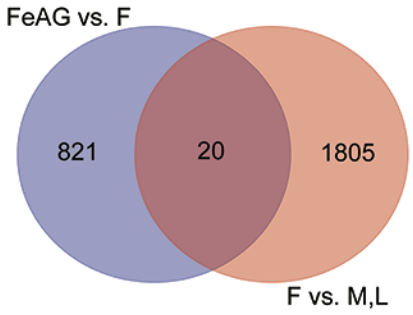
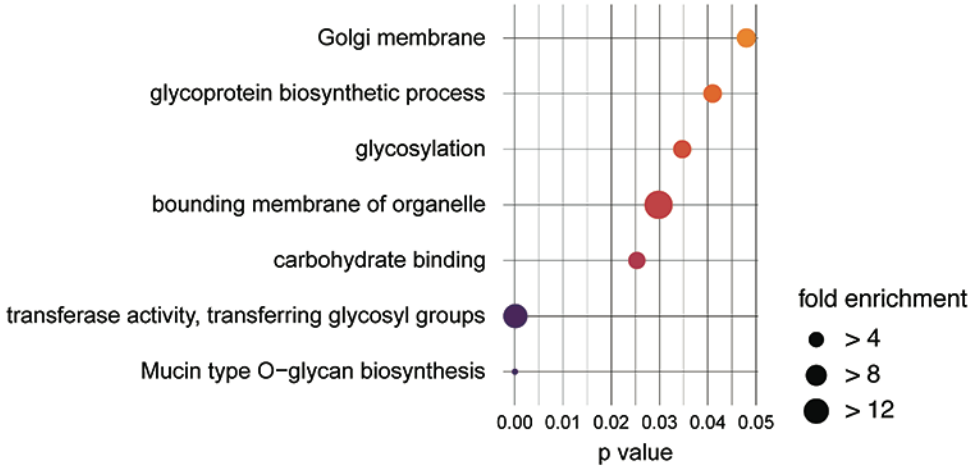

aminoglycan metabolism
metabolism
oxidation–reduction process
carbohydrate metabolism
catabolism
organic substance metabolism
biological process

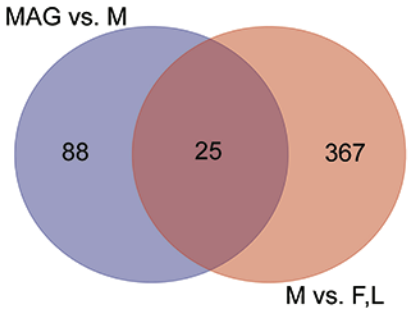
Figure 4

A.


FeAG vs. F
821
20
1805
F vs. M,L


Golgi membrane
glycoprotein biosynthetic process
glycosylation
bounding membrane of organelle
carbohydrate binding
transferase activity, transferring glycosyl groups
Mucin type O−glycan biosynthesis
0.00 0.01 0.02 0.03 0.04 0.05
p value
fold enrichment
> 4
> 8
> 12

B.


MAG vs. M
88
25
367
M vs. F,L

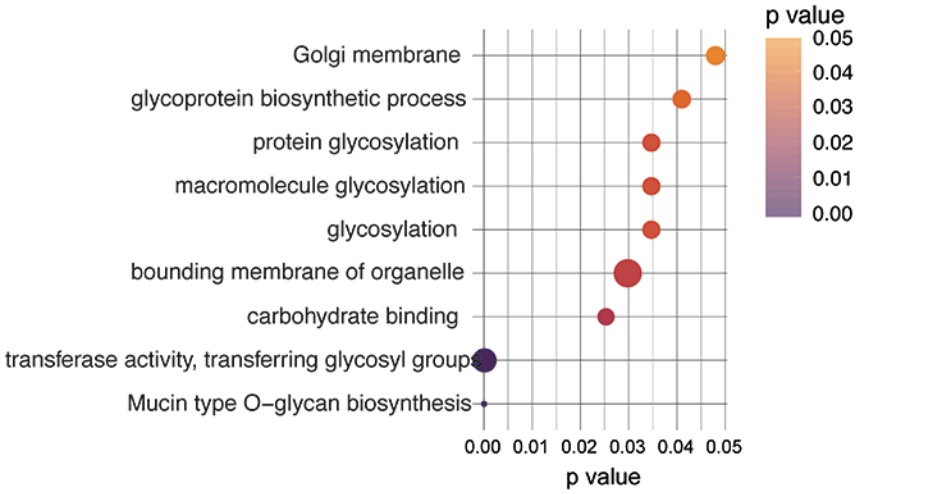
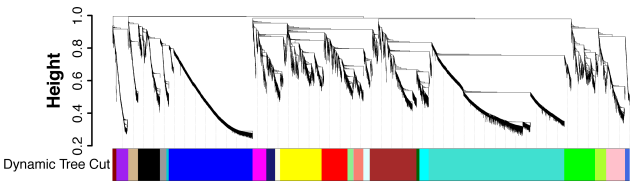
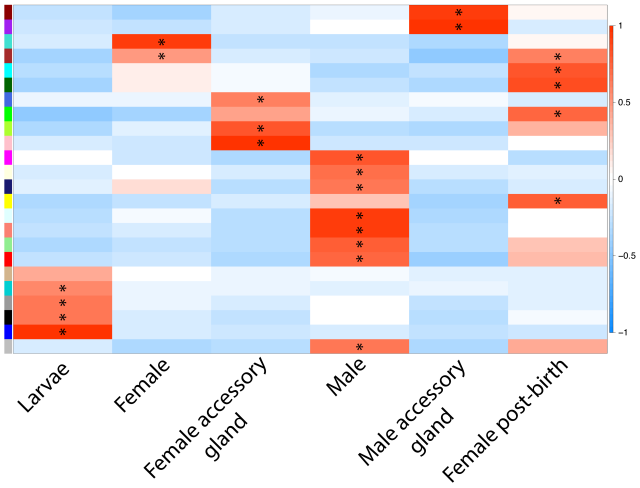

Golgi membrane
glycoprotein biosynthetic process
protein glycosylation
macromolecule glycosylation
glycosylation
bounding membrane of organelle
carbohydrate binding
transferase activity, transferring glycosyl groups
Mucin type O−glycan biosynthesis
0.00 0.01 0.02 0.03 0.04 0.05
p value
p value
0.05
0.04
0.03
0.02
0.01
0.00

Figure 5

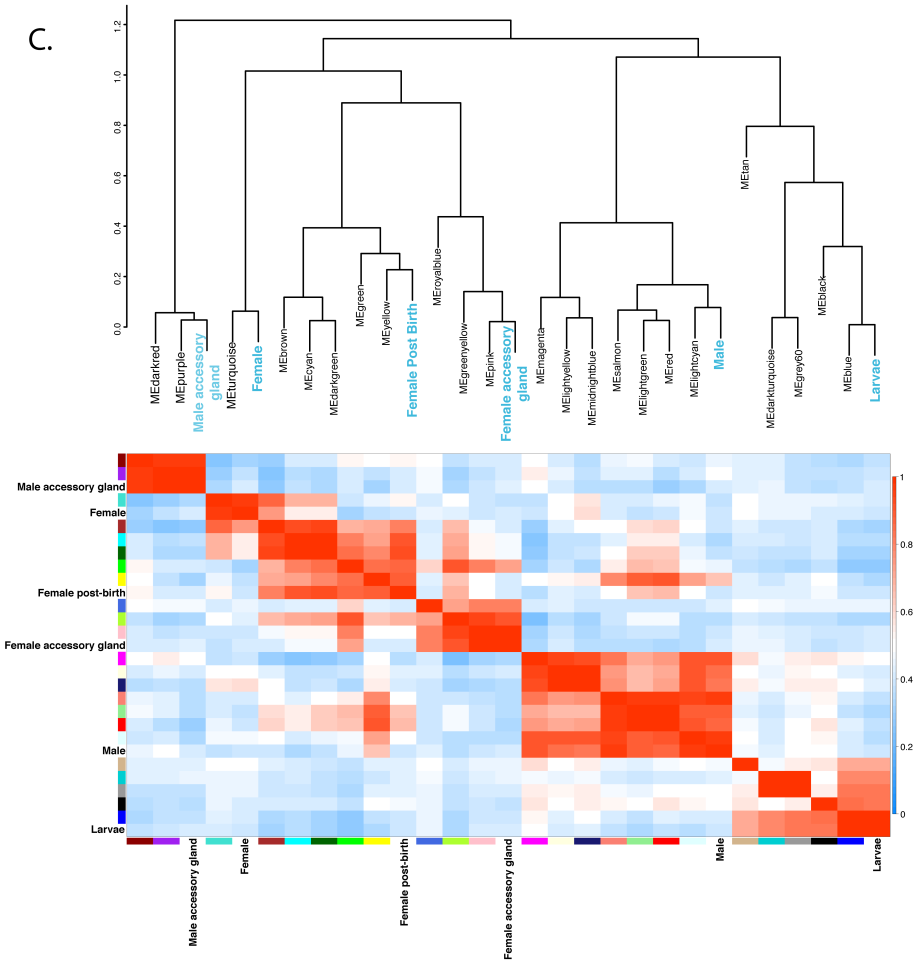
A.



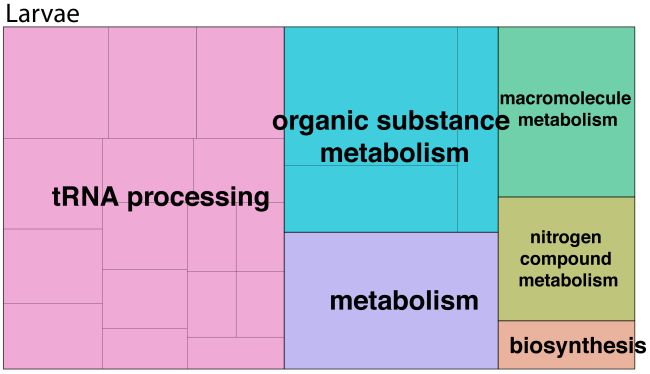
B.



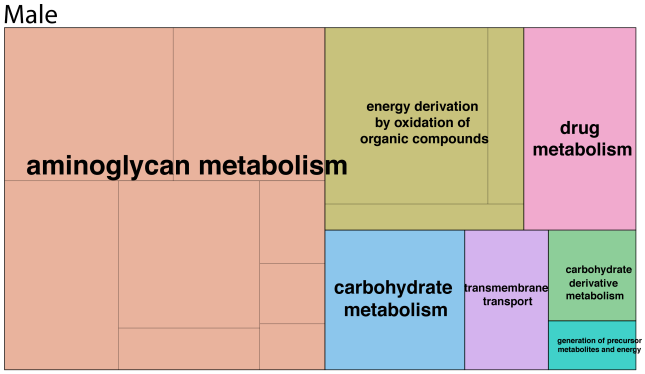
C.



D.
Larvae



E.
Male



F.
Female

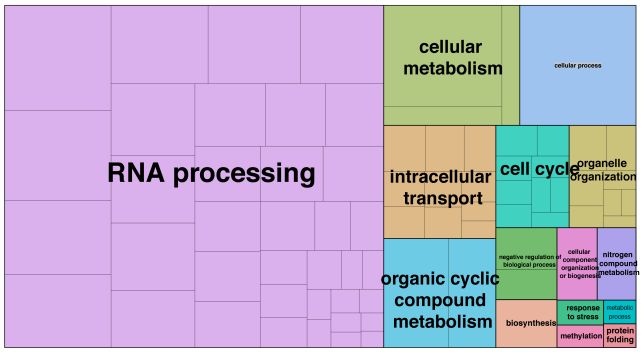


Figure 6

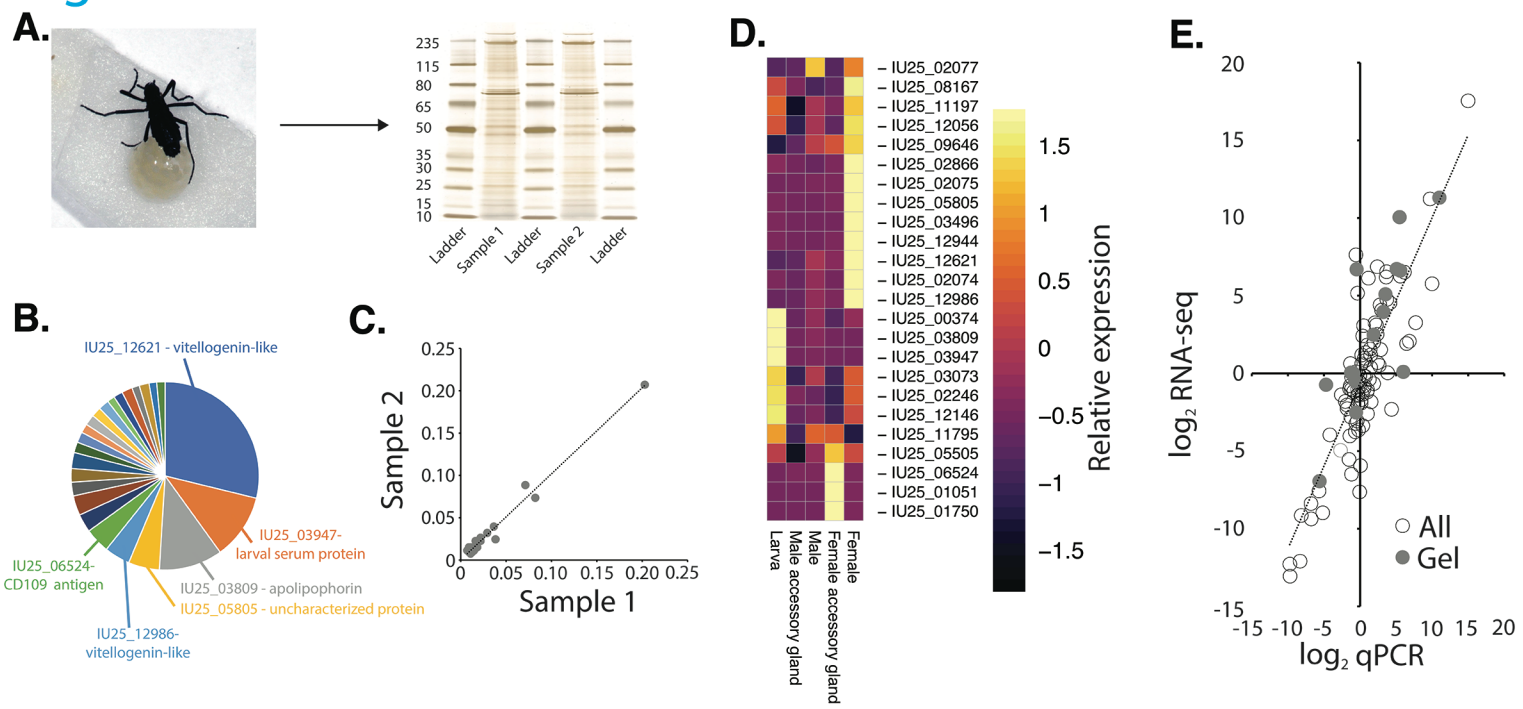

A.
235
115
80
65
50
35
30
25
15
10
Ladder
Sample 1
Ladder
Sample 2
Ladder
B.
IU25_12621 - vitellogenin-like
IU25_03947-
larval serum protein
IU25_06524-
CD109 antigen
IU25_03809 - apolipophorin
IU25_05805 - uncharacterized protein
IU25_12986-
vitellogenin-like
C.
Sample 2
Sample 1
0.25
0.20
0.15
0.10
0.05
0
0.05
0.10
0.15
0.20
0.25
D.
IU25_02077
IU25_08167
IU25_11197
IU25_12056
IU25_09646
IU25_02866
IU25_02075
IU25_05805
IU25_03496
IU25_12944
IU25_12621
IU25_02074
IU25_12986
IU25_00374
IU25_03809
IU25_03947
IU25_03073
IU25_02246
IU25_12146
IU25_11795
IU25_05505
IU25_06524
IU25_01051
IU25_01750
Larva
Male accessory gland
Male
Female accessory gland
Female
1.5
1
0.5
0
−0.5
−1
−1.5
Relative expression
E.
log2 RNA-seq
log2 qPCR
20
15
10
5
0
-5
-10
-15
-15
-10
-5
0
5
10
15
20
All
Gel

Figure 7

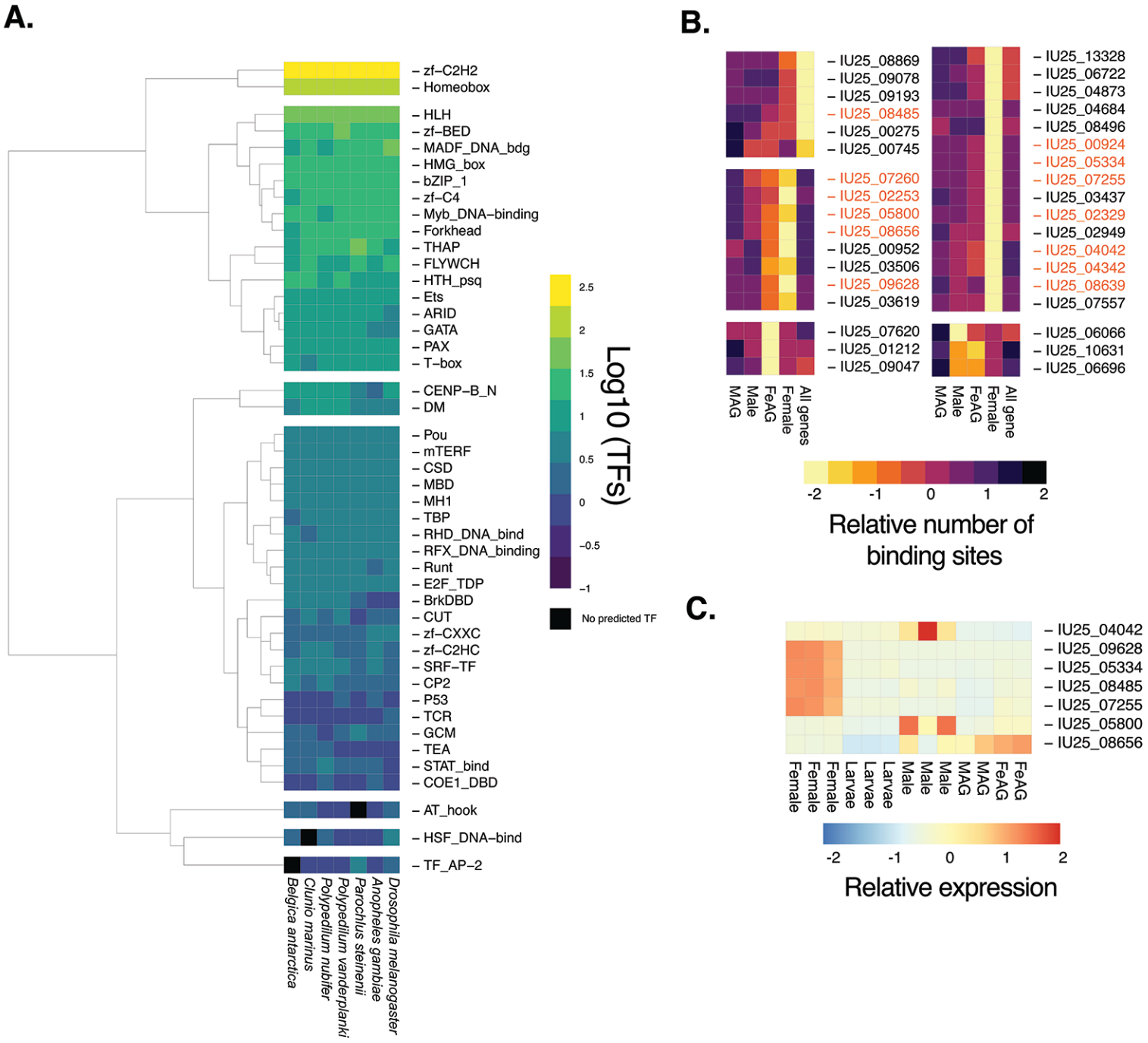

A.
zf–C2H2
Homeobox
HLH
zf–BED
MADF_DNA_bdg
HMG_box
bZIP_1
zf–C4
Myb_DNA–binding
Forkhead
THAP
FLYWCH
HTH_psq
Ets
ARID
GATA
PAX
T–box
CENP–B_N
DM
Pou
mTERF
CSD
MBD
MH1
TBP
RHD_DNA_bind
RFX_DNA_binding
Runt
E2F_TDP
BrkDBD
CUT
zf–CXXC
zf–C2HC
SRF–TF
CP2
P53
TCR
GCM
TEA
STAT_bind
COE1_DBD
AT_hook
HSF_DNA–bind
TF_AP–2
Belgica antarctica
Clunio marinus
Polypedilum nubifer
Polypedilum vanderplanki
Parochlus steinenii
Anopheles gambiae
Drosophila melanogaster
Log10 (TFs)
2.5
2
1.5
1
0.5
0
−0.5
−1
No predicted TF
B.
IU25_08869
IU25_09078
IU25_09193
IU25_08485
IU25_00275
IU25_00745
IU25_07260
IU25_02253
IU25_05800
IU25_08656
IU25_00952
IU25_03506
IU25_09628
IU25_03619
IU25_07620
IU25_01212
IU25_09047
IU25_13328
IU25_06722
IU25_04873
IU25_04684
IU25_08496
IU25_00924
IU25_05334
IU25_07255
IU25_03437
IU25_02329
IU25_02949
IU25_04042
IU25_04342
IU25_08639
IU25_07557
IU25_06066
IU25_10631
IU25_06696
MAG
Male
FeAG
Female
All genes
All gene
-2
-1
0
1
2
Relative number of binding sites
C.
IU25_04042
IU25_09628
IU25_05334
IU25_08485
IU25_07255
IU25_05800
IU25_08656
Female
Female
Female
Larvae
Larvae
Larvae
Male
Male
Male
MAG
MAG
FeAG
FeAG
-2
-1
0
1
2
Relative expression

Figure 8

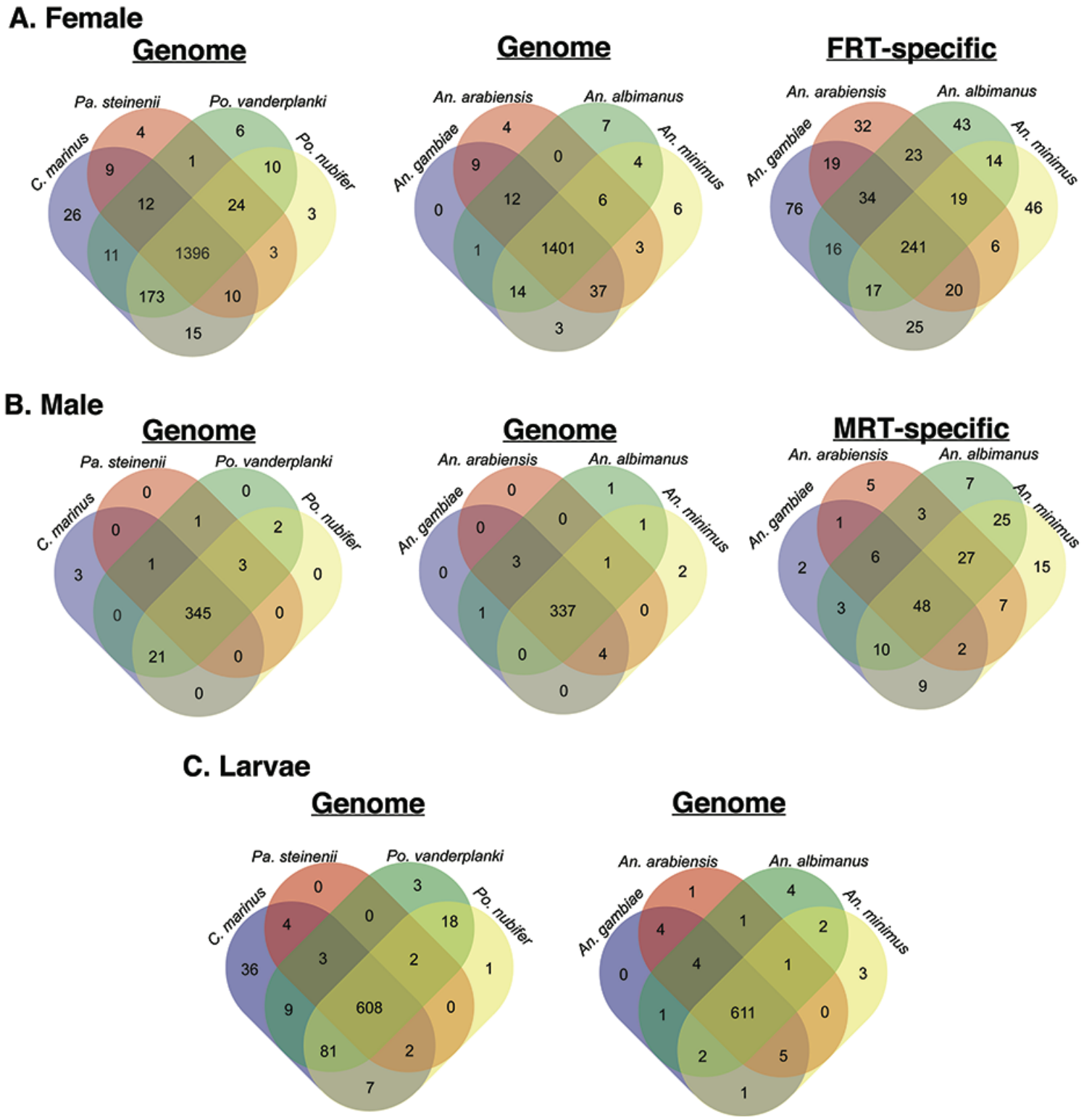
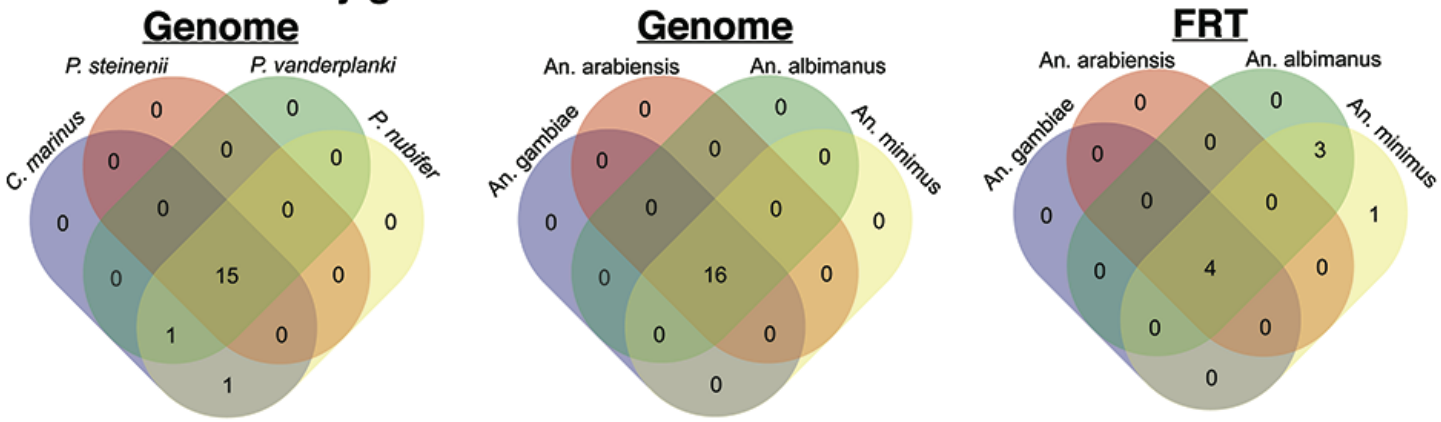
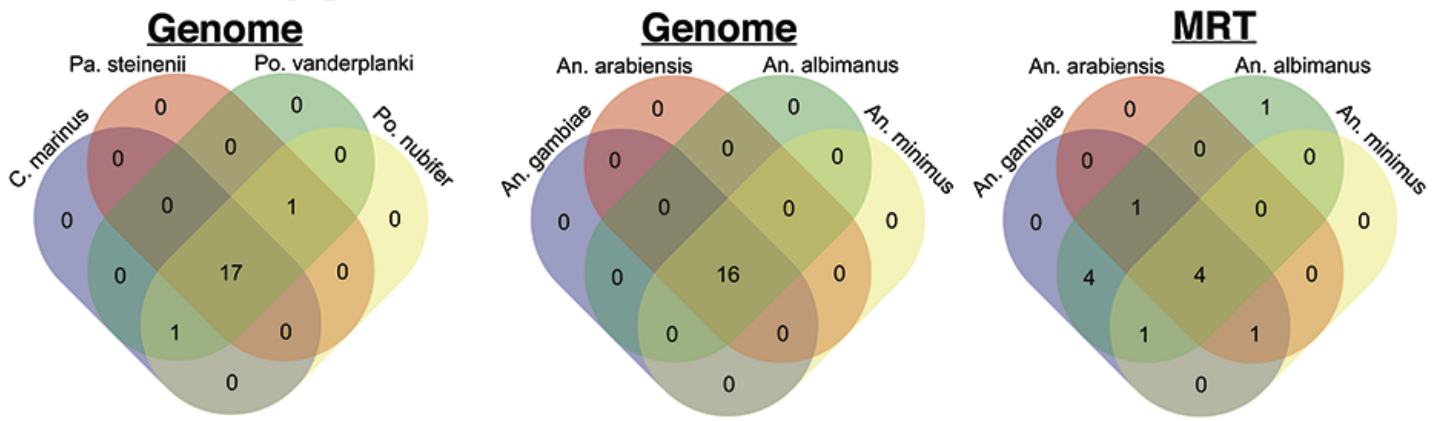

A. Female
Genome
Pa. steinenii
Po. vanderplanki
C. marinus
Po. nubifer
4
6
9
1
10
26
12
24
3
11
1396
3
173
10
15
Genome
An. arabiensis
An. albimanus
An. gambiae
An. minimus
4
7
9
0
4
0
12
6
6
1
1401
3
14
37
3
FRT-specific
An. arabiensis
An. albimanus
An. gambiae
An. minimus
32
43
19
23
14
76
34
19
46
16
241
6
17
20
25
B. Male
Genome
Pa. steinenii
Po. vanderplanki
C. marinus
Po. nubifer
0
0
0
1
2
3
1
3
0
0
345
0
21
0
0
Genome
An. arabiensis
An. albimanus
An. gambiae
An. minimus
0
1
0
0
1
0
3
1
2
1
337
0
0
4
0
MRT-specific
An. arabiensis
An. albimanus
An. gambiae
An. minimus
5
7
1
3
25
2
6
27
15
3
48
7
10
2
9
C. Larvae
Genome
Pa. steinenii
Po. vanderplanki
C. marinus
Po. nubifer
0
3
4
0
18
36
3
2
1
9
608
0
81
2
7
Genome
An. arabiensis
An. albimanus
An. gambiae
An. minimus
1
4
4
1
2
0
4
1
3
1
611
0
2
5
1

Figure 9

A. Female accessory gland



B. Male accessory glands



C. Male accessory glands enriched genes of mosquitoes

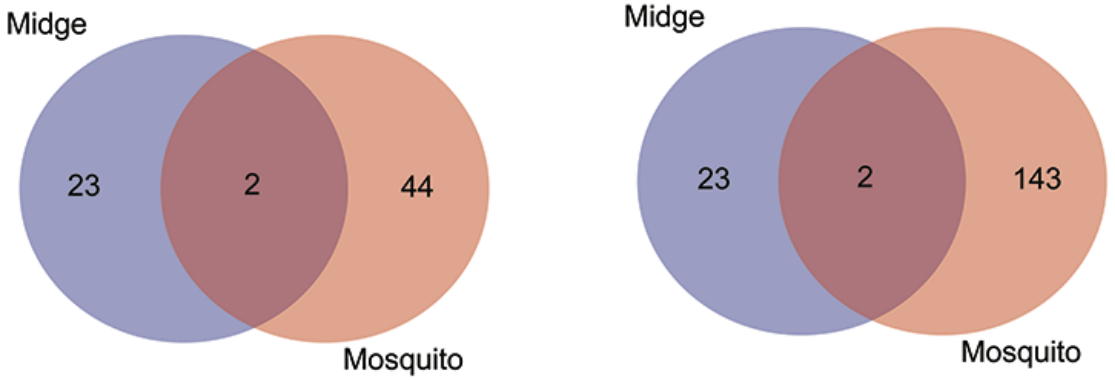


Figure 10

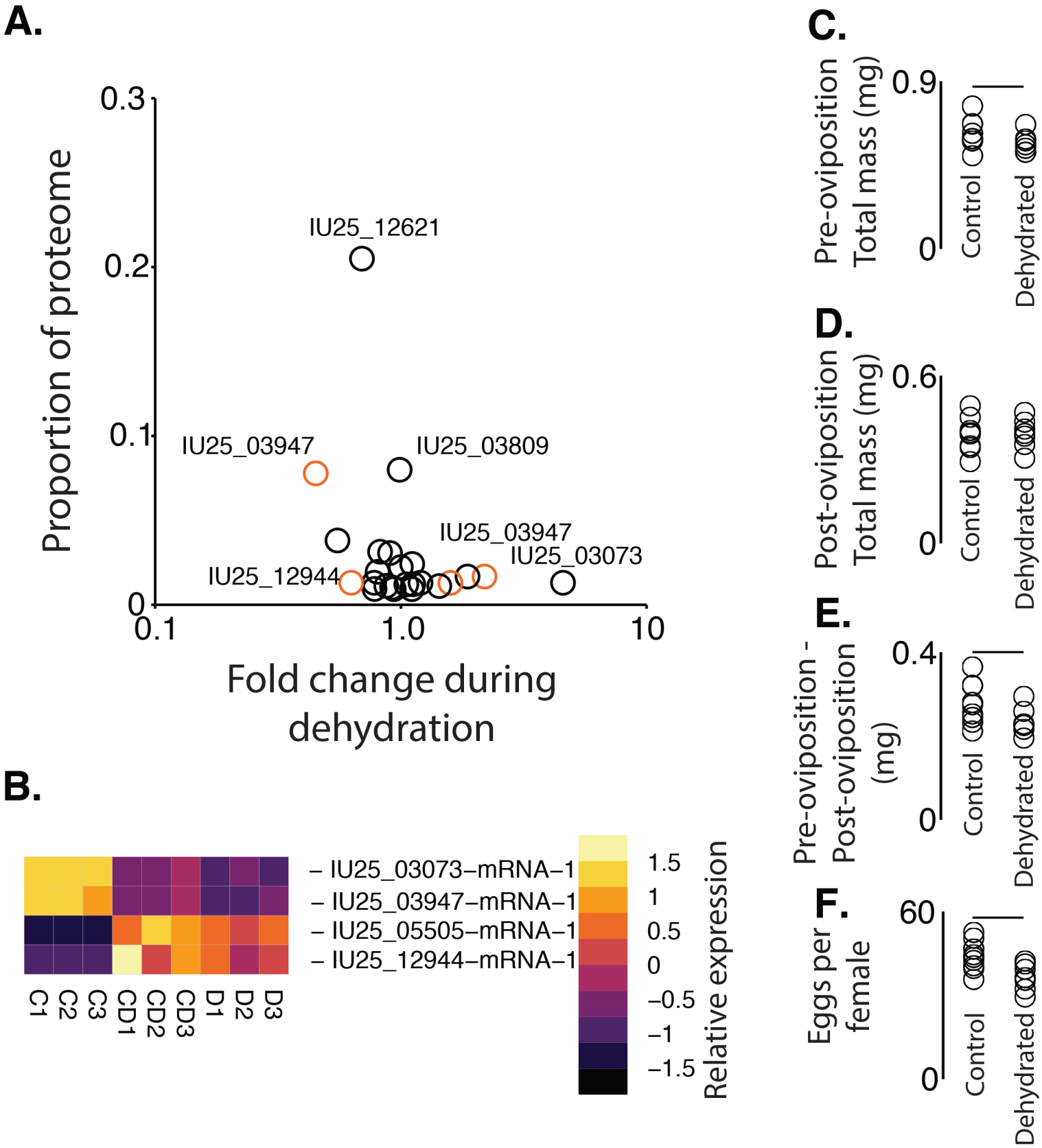
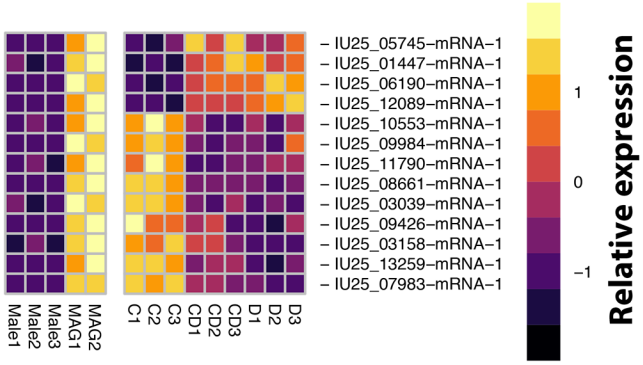
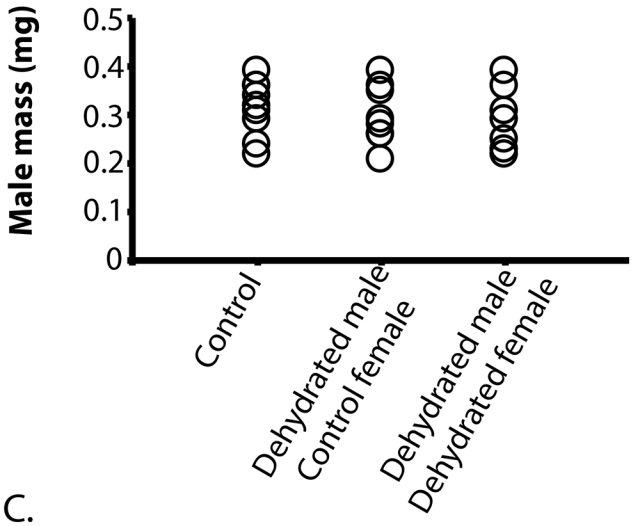

A.
Proportion of proteome
0.3
0.2
0.1
0
0.1
1.0
10
Fold change during dehydration
IU25_12621
IU25_03947
IU25_03809
IU25_03947
IU25_03073
IU25_12944
B.
– IU25_03073–mRNA–1
– IU25_03947–mRNA–1
– IU25_05505–mRNA–1
– IU25_12944–mRNA–1
C1
C2
C3
CD1
CD2
CD3
D1
D2
D3
1.5
1
0.5
0
–0.5
–1
–1.5
Relative expression
C.
Pre-oviposition Total mass (mg)
0.9
0
Control
Dehydrated
D.
Post-oviposition Total mass (mg)
0.6
0
Control
Dehydrated
E.
Pre-oviposition - Post-oviposition (mg)
0.4
0
Control
Dehydrated
F.
Eggs per female
60
0
Control
Dehydrated

Figure 11

A.



B.



C.

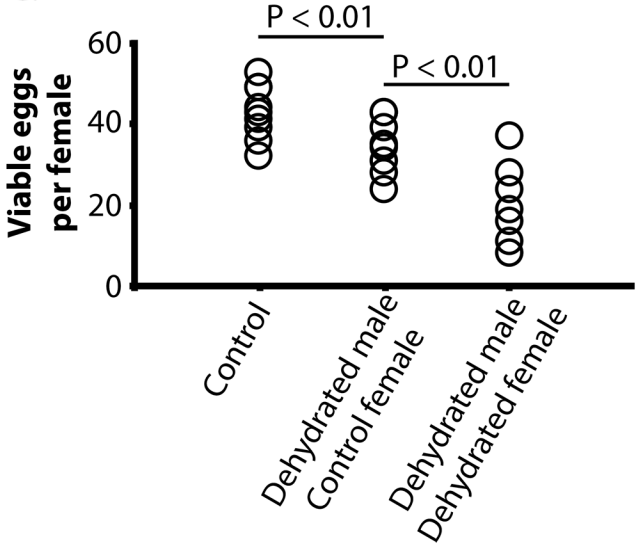
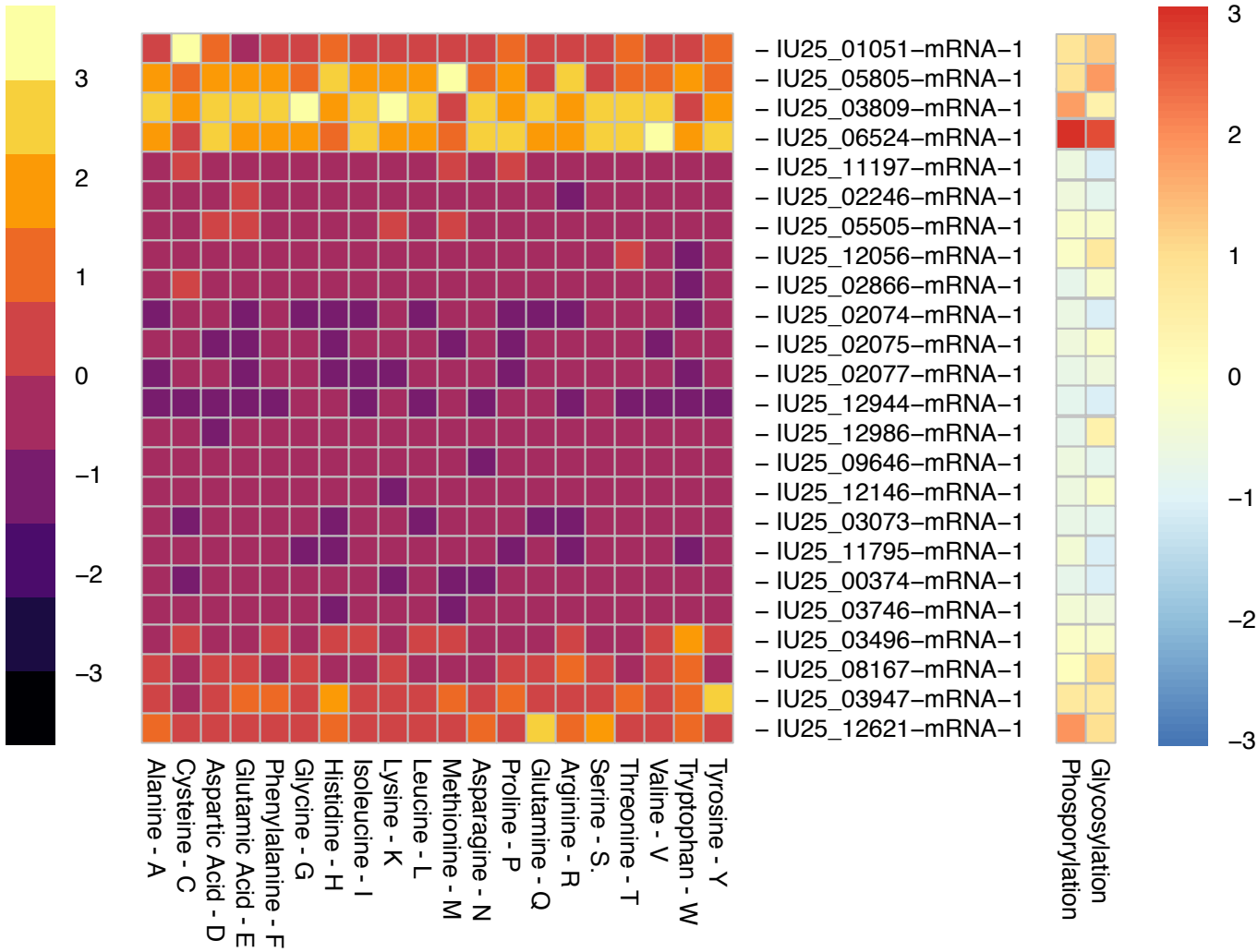
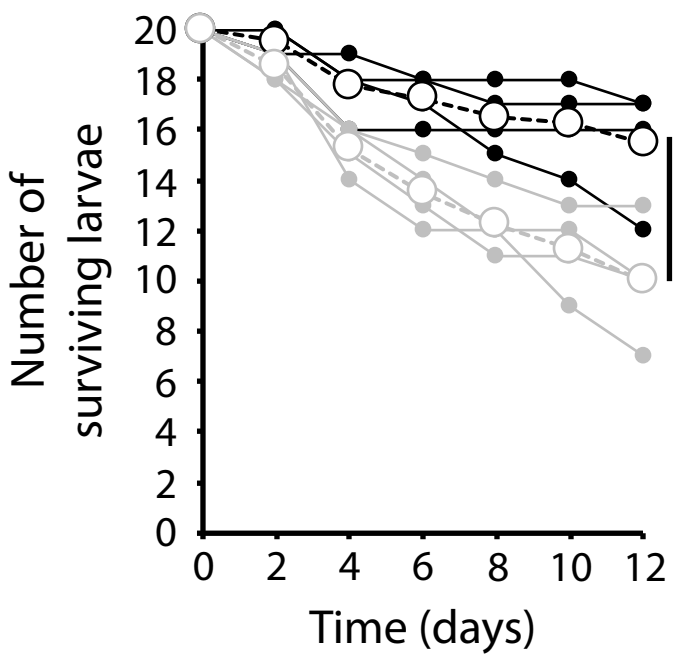


Figure 12

A.



B.



C.

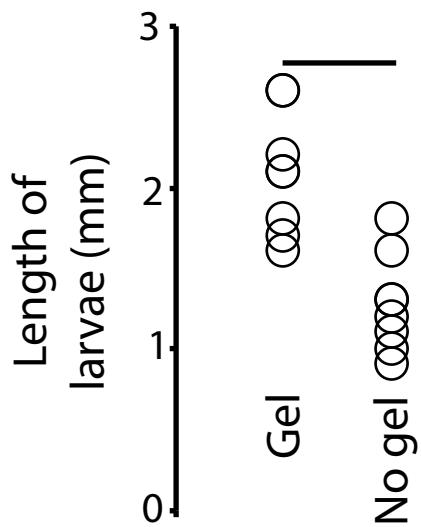


Figure 13

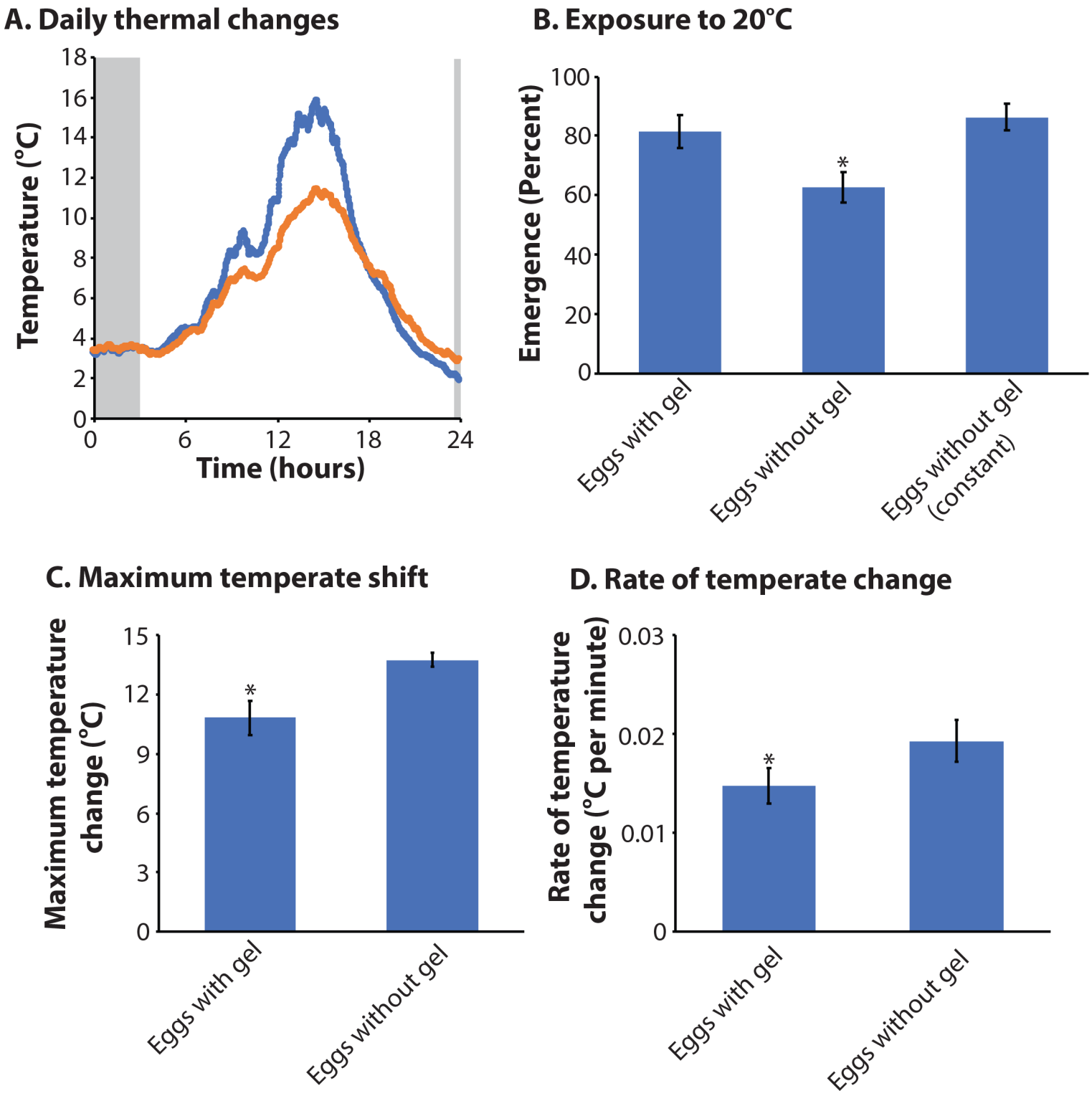
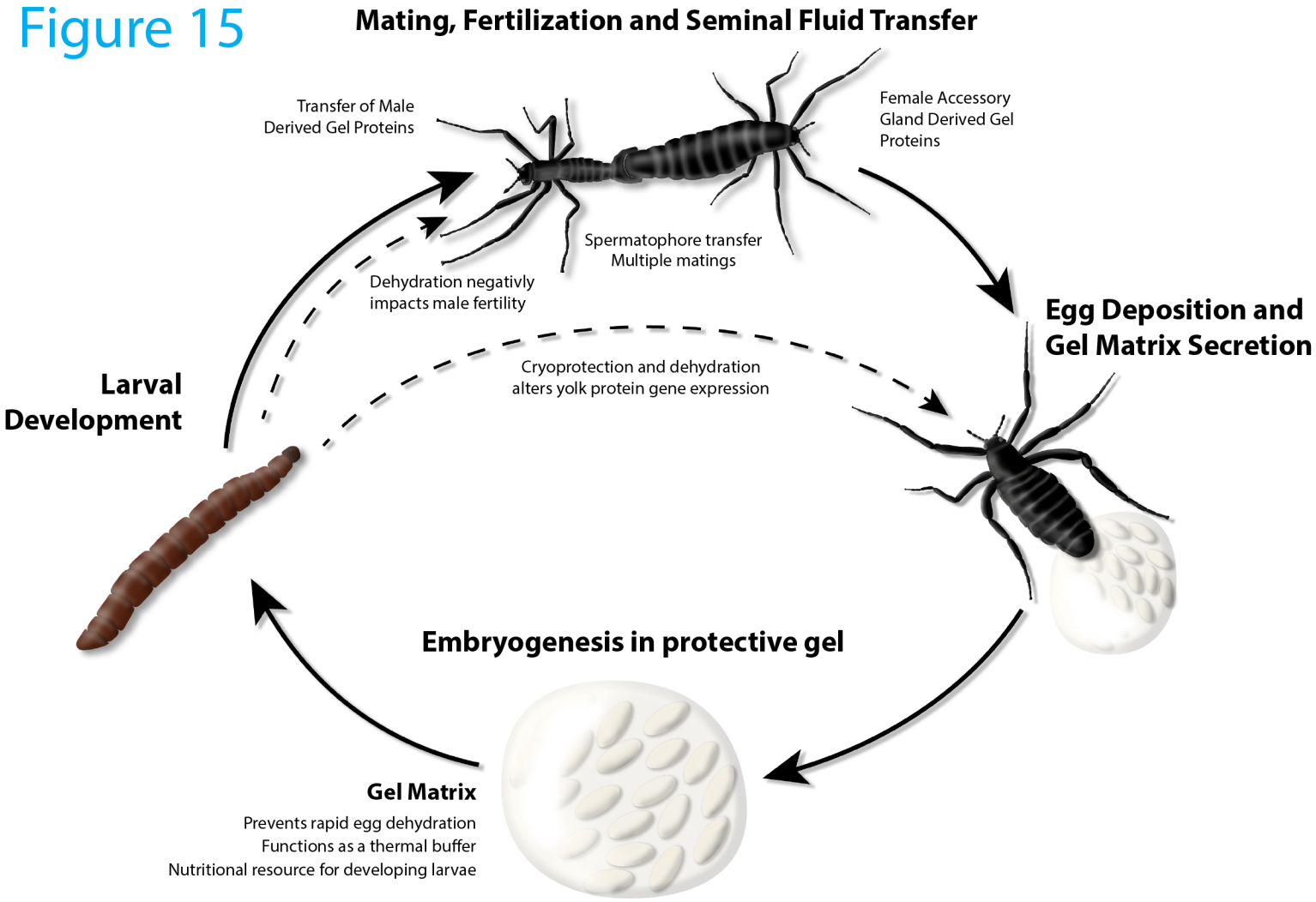

A. Daily thermal changes
Temperature (°C)
18
16
14
12
10
8
6
4
2
0
0
6
12
18
24
Time (hours)
B. Exposure to 20°C
Emergence (Percent)
100
80
60
40
20
0
*
Eggs with gel
Eggs without gel
Eggs without gel (constant)
C. Maximum temperate shift
Maximum temperature change (°C)
15
12
9
6
3
0
*
Eggs with gel
Eggs without gel
D. Rate of temperate change
Rate of temperature change (°C per minute)
0.03
0.02
0.01
0
*
Eggs with gel
Eggs without gel

Figure 12

A
Population growth (λ)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Male
Female
Male and Female
Dehydration treatment
B
Population growth (λ)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
No gel
Gel treatment
C
Population growth (λ)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Thermal stress
Thermal treatment

Figure 15


Mating, Fertilization and Seminal Fluid Transfer
Transfer of Male Derived Gel Proteins
Female Accessory Gland Derived Gel Proteins
Spermatophore transfer
Multiple matings
Dehydration negativly impacts male fertility
Egg Deposition and Gel Matrix Secretion
Cryoprotection and dehydration alters yolk protein gene expression
Larval Development
Embryogenesis in protective gel
Gel Matrix
Prevents rapid egg dehydration
Functions as a thermal buffer
Nutritional resource for developing larvae