1   Decoding the reproductive system of the olive fruit fly, *Bactrocera oleae*

2   M-E Gregoriou[1], M Reczko[2], K.T Tsoumani[1], K.D Mathiopoulos[1]

3   [1]Department of Biochemistry and Biotechnology, University of Thessaly,

4   Larissa, Greece

5   [2] Institute of Molecular Biology and Genetics, Biomedical Sciences Research

6   Centre "Alexander Fleming", Greece

7

8   Corresponding author: Kostas D. Mathiopoulos

9   Department of Biochemistry and Biotechnology, University of Thessaly, Larissa,

10   Greece 2410-565284, kmathiop@bio.uth.gr

11

12   <u>Abstract and keywords</u>

13   A great deal of behavioral and molecular interactions between male and

14   female insects takes place during insect reproduction. Here, we comprehensively

15   analyze the reproductive system of the olive fruit fly. Specifically, transcriptomic and

16   genomic analysis of the reproductive tissues from virgin and once mated insects were

17   performed. Genes encoding proteins implicated in immune response, mucins, antigen

18   5 proteins, proteases inhibitors and proteins with putative secretory activity were

19   identified. Comparison of the transcriptomes between virgin and mated insects

20   resulted in the identification of genes that are up- or down-regulated after mating. In

21   testes 106 genes were up-regulated and 344 genes were down-regulated, whereas in

22   male accessory glands with ejaculatory bulb 1,607 genes were up-regulated and 384

23   genes were down-regulated in mated male insects. Respectively, in mated females

24   1,705 genes were up-regulated and 120 genes were down-regulated in mated insects.

25   To get a deeper insight, the expression profiles of selected genes throughout sexual

26   maturation for the male tissues and throughout different time points after mating for

27   the female reproductive tissues were determined. Identification of genes that take part

28   in the mating procedure not only gives an insight in the biology of the insects, but it

29   could also help the identification of new target genes in order to disturb the

30   reproductive success of the olive fly and thus develop alternative pest control method.

31   Keywords: Tephritidae; post mating response; seminal fluid; accessory glands

32

1.   Introduction

34   In all arthropods, insects are the most divergent and abundant group, equipped
35   with high reproductive rates and numerous behavioral and physiological adaptations
36   that are critical to the maintenance of their populations. During insect reproduction,
37   sperm and seminal fluid that are produced in testes and male accessory glands
38   (MAGs) respectively are delivered to the female reproductive system [1, 2, 3].

39   Male seminal fluid proteins have been characterized mainly as proteases,
40   peptidases, serpins and protease inhibitors [4, 5, 6]. Although the functional classes of
41   these proteins are conserved across species, the genes that encode them rarely are.
42   Genes expressed in the accessory glands, as it was previously shown in Drosophila
43   species, exhibit rapid evolutionary change and gene expansion because of their critical
44   role in encoding products that underlie striking, fitness-related phenotypes [7]. Until
45   today, male seminal fluid proteins have been identified in different Drosophilidae [8, 9],
46   in the major disease vectors *Anopheles gambiae* [10] and *Aedes aegypti* [11], and in
47   Tephritid fruit flies like *Ceratitis capitata* [12, 13] and *Bactrocera cucurbitae* [14].

48   Female accessory glands also produce a secretory material that serves a
49   number of functions. It may act as a lubricant for egg passage, as a protective oothecal
50   cover, or as a glue to attach eggs to various substrates [15]. To date, female reproductive
51   genes have been comprehensively studied in very few insects like the sandfly
52   *Phlebotomus papatasi* [16], the house fly *Musca domestica* [17] and the Mediterranean
53   fruit fly *C. capitata* [18, 19].

54   After mating, the presence of sperm and seminal fluid in female insects
55   induces multiple physiological and behavioral changes such as repression of sexual
56   receptivity to further mating [20, 21, 22, 23], egg-laying stimulation, stimulation of immune
57   responses and reduced longevity [24, 25, 12, 26]. These post-mating responses have been
58   addressed in genome-wide studies in species such as *Drosophila melanogaster* [27, 28],
59   the honeybee queen *Apis mellifera* [29, 30, 31], *C. capitata* [32], *An. gambiae* [33, 34] and *Ae.*
60   *aegypti* [35]. Such studies have revealed that post-mating responses differ between
61   different insect species.

62   For the olive fruit fly, *Bactrocera oleae*, the major pest of olive cultivation, the

63   morphology and ultrastructure of the male accessory glands with ejaculatory bulb

64   tissues have been analyzed by Marchini et al [36]. The first molecular analysis of the

65   reproductive system was presented in 2014 by Sagri et al [37]. That study focused on the

66   identification of sex differentiation genes, i.e. differentially expressed genes either in

67   male testes or female accessory glands and spermathecae [37]. In order to go a step

68   further and understand the molecular processes that underlie reproduction of the olive

69   fly, one needs to identify genes that are over- or under-expressed during mating of the

70   flies. The usefulness of the knowledge of such genes and mechanisms in insect

71   control is obvious: if one could somehow impair the function of these genes, one

72   could also disturb the reproductive success of the olive fly and lead to population

73   control.

74   In this study, an extensive transcriptomic and genomic analysis of the

75   reproductive tissues from virgin and once-mated olive flies was performed.

76   Comparison of the different conditions resulted in the identification of differentially

77   expressed genes. To investigate their contribution in reproduction, we analyzed the

78   expression profiles of selected genes throughout the sexual maturation for the male

79   tissues and throughout different time points after mating for the female reproductive

80   tissues.

81

82   **2.   Results and Discussion**

83   The tissues selected for the transcriptomic analysis of the olive fly's

84   reproductive system were: 1. the male accessory glands from virgin (V_MALE) and

85   mated (M_MALE) male insects, 2. the testes from mated insects (M_TESTES) and 3.

86   the lower female reproductive tract, comprising of the spermathecae, the uterus and

87   the female accessory glands from virgin (V_FEMALE) and mated (M_FEMALE)

88   female insects.

89   In order to obtain tissues from sexually mature virgin flies, dissections were

90   performed on DAY-7 after eclosion. The sexual maturation of the insects was

91   determined by their ability to mate and give offspring. The olive fly laboratory strain

92   used for the experiments is sexually mature and can mate successfully at the selected

93   day. Dissection of the tissues from mated olive flies was performed 12 hours after

3

94   mating. Specifically, on DAY-7 after eclosion virgin male and female flies were
95   mixed and allowed to mate. A successful mating lasts for at least one hour [38]. When
96   the insects completed mating (i.e., they were voluntarily separated from each other),
97   they were kept in different cages for 12 hours before dissections. In *D. melanogaster*
98   the highest post-mating gene expression occurs after 6 hours [28] and in *C. capitata*
99   there is a general increase in the transcriptional activity only after three repeated
100 matings [39]. As there is no such evidence for the reproductive tissues of *B. oleae*, two
101 pieces of information guided our decision for the determination of the appropriate
102 time-point after mating to analyze. Firstly, male olive flies can remate at least 24
103 hours after a previous mating [40] and, secondly, oviposition of the mated females also
104 starts 24 hours after mating.

105

2.1. Transcriptome sequencing assembly

107 A single representative *de novo* assembly for *B. oleae* was generated from a
108 concatenation of the libraries obtained with the Illumina platform [41] using the Trinity
109 pipeline [42]. After assembly, transcript and unigene level expression values were
110 calculated using RSEM [43] for the four libraries (V_MALE, V_FEMALE, M_MALE,
111 M_FEMALE) sequenced using the Ion Proton. The average read length was 669.78bp
112 and the totally sequenced 203,690,146 bp gave 255,077 genes. From the libraries
113 V_MALE and M_MALE 11,452 transcripts were obtained whereas from the libraries
114 V_FEMALE and M_FEMALE 10,478 transcripts were obtained.

115

2.2. Before vs after mating differential expression

117 To identify the differentially expressed genes between virgin and mated
118 insects, the edgeR algorithm [44] with a stringent cutoff (q value < 0.05) was used.
119 Specifically, in the reproductive tissues from the mated female insects, 1,705 genes
120 were up-regulated and 120 genes were down-regulated. On the other hand, in male
121 accessory glands with ejaculatory bulb 1,607 genes were up-regulated and 384 genes
122 were down-regulated, whereas in testes 106 genes were up-regulated and 344 genes
123 were down-regulated. Less differentially expressed genes were detected in testes,
124 compared to the other two reproductive tissues, showing a limited transcriptional

4

125 activity. Indeed, in *D. melanogaster*, it was shown that spermatozoa are generally

126 metabolically quiescent and transcriptionally silent in adult insects [45, 46]. The entire

127 lists of all significantly (q<0.05) differentially expressed genes in all analyzed tissues

128 are given in supplementary Tables S1, S2, S3. Moreover, volcano plots were created

129 for the visualization of the distribution of the transcripts; the red dots indicate

130 differentially expressed genes between virgin and mated insects (Figure 1).

131   Functional annotation of the top100 over-expressed transcripts in the tissues of

132 the mated flies was performed based on the Gene Ontology (GO) categorization level

133 II using BLAST2GO tool [47]. The GO categorization is presented in Figure 2 and

134 involves biological process (BP) and molecular function (MF). The Gene Ontology

135 (GO) terms of the testes transcriptome, with the most abundant hits on the GO

136 functional annotation based on categorization level II, were similar to those obtained

137 from the *Bactrocera dorsalis* respective testes-transcriptome [48]. Regarding the

138 "molecular function" classification, the identified genes mostly fell in the main groups

139 "binding" and "catalytic activity". These groups were also identified as the most

140 abundant in similar studies in *B. dorsalis* 48 and *C. capitata* [32, 39], two closely related

141 insects to the olive fly, showing a conservation of the functions that alter during

142 mating in these insects. Regarding the male accessory glands and ejaculatory bulb

143 tissues, the GO analysis in response to biological processes and molecular function

144 showed enrichment of "metabolic processes" and "biological regulation" as in *C.*

145 *capitata* [32]. As sexually mature males are actively involved in pheromone response

146 and female courting, they show significant enrichment of these GO terms, indicating

147 the high energy investment required in mating. Finally, as it was extensively analyzed

148 in many Drosophila species, the female reproductive genes encode proteases, protease

149 inhibitors and genes related to immune response and energy metabolism [28, 27, 49, 50]. In

150 consistence with those data, homologous genes were also observed in the GO

151 annotation of the upregulated genes in the lower female reproductive tract of *B. oleae*.

152 This transcriptional activity of mated olive flies is characterized by rapid cell

153 proliferation and secretory activity, as supported by the categorization of the

154 transcripts in functional classes related to biological regulation, metabolic and cellular

155 processes. As it comes to olive fly, indeed the general transcriptomic profiles of the

156 analyzed tissues were similar to other dipteran reproductive systems such as *C.*

157 *capitata* [32, 39]. However, a more detailed analysis of the transcripts showed that there

5

158 is diversity in the mating response among species. Specifically, compared to *C.*
159 *capitata,* there were two distinct differences. Firstly, there was a profound increase in
160 the number of transcripts in once mated *B. oleae* insects. This rise in the number of
161 transcripts was detected only in three times mated *C. capitata* insects. Secondly, in *B.*
162 *oleae* there was a modification of the immunity response of the reproductive tissues
163 while in *C. capitata* there were not [32]. Similarly, comparison of *D. simulans* male
164 accessory gland proteins with their orthologues in its close relative *D. melanogaster*
165 demonstrated rapid divergence of many of these reproductive genes [51]. The
166 divergence of the reproductive genes is based on the important role that they play in
167 ensuring the successful mating and fertilization [52, 53].

168   For the validation of the differential expression of various genes observed after
169 the RNAseq analysis of reproductive tissues before and after mating, further
170 functional analysis was performed for 5 loci in testes and 6 loci in male accessory
171 glands and female accessory glands, respectively. These genes were selected based on
172 their known involvement in the reproductive system and their differential expression
173 between virgin and mated insects. In testes of mated flies, significant overexpression
174 was confirmed for the genes *c58283*, *c37552*, *hemolectin*, *mucin* and *cation*
175 *transporter* and downregulation for *scribbler* gene. qRT-PCR did not confirm the
176 expression profile of the genes *c15699* and *c52071* obtained from RNAseq, whereas
177 *c42528* showed very low expression (Figure 3). In male accessory glands of mated
178 flies, six reproductive loci were analyzed and their overexpression was confirmed
179 through qRT-PCR: *timeless*, *c52416*, *c57257*, *c52655*, *yellow-g* and *c53574* (Figure
180 4A). In female accessory glands, qRT-PCR results showed that *troponin C* had high
181 expression (18 fold) in virgin flies, in contrast to the RNAseq results that showed
182 overexpression in mated female flies. The *glutathione S-transferase epsilon class*
183 gene showed very low expression. Overexpression of the other four genes, *lingerer*,
184 *yolk protein-2, bestrophin-2* and *ornithine decarboxylase antizyme* observed in
185 RNAseq was also confirmed in qRT-PCR (Figure 4B).

186

187 *2.3. Genomic annotation of reproductive genes*

188   The 100 highly differentially expressed genes were annotated to the recently
189 sequenced genome of the olive fly (https://i5k.nal.usda.gov/Bactrocera_oleae) (Table

190   S2). In an attempt to annotate more seminal fluid proteins (SFPs), the genome

191   scaffolds were queried (tBLASTn, e-value $<10^{-10}$) using the amino acid sequences of

192   the 139 characterized *D. melanogaster* SFPs [8]. Only 43 of the Drosophila genes gave

193   significant hits in the olive fly genome. The homologous genes were grouped into 17

194   functional classes based on the categories defined for *D. melanogaster* [9] and *C.*

195   *capitata* [13] seminal fluid proteins (Table S4). The annotated genes obtained from this

196   procedure encode proteins that belong to the conserved functional classes such as

197   proteases and protease inhibitors, lipases, sperm-binding proteins and antioxidants [54].

198   Four sperm protein genes including the testes-specific protein *betaTub85D* and one

199   odorant binding receptor, *or82a*, were also annotated.

200

2.4. Transcriptional analysis of the differentially expressed genes

202   In order to get a closer look into the transcriptional changes of the

203   reproductive genes, a more detailed follow-up expression profile analysis was

204   performed for the male accessory glands with ejaculatory bulb tissues and the female

205   lower reproductive tract.

206   For the male accessory gland genes, we determined the expression profiles of

207   the selected genes from DAY-0 (first day of insect eclosion) to DAY-7 (sexually

208   mature insects). Presumably, a gene encoding a seminal fluid protein should be

209   expressed before mating, so that the protein will be present at the time of mating. In

210   agreement with this hypothesis, the highest expression of most genes was detected

211   before DAY-7 (Figure 5). The genes *timeless*, *c52416* and *c53574* showed highest

212   expression on DAY-0, while their expression dropped to lower levels and remained

213   stable until DAY-7. The genes *brunelleschi* and *yellow-g* showed their highest

214   expression on DAY-5 and gene *CG2254-like* on DAY-6.

215   *Timeless* expression increased after mating, indicating possibly a role in the

216   rhythmic cycle. *Timeless* along with *per* (*period*) regulate the circadian cycle of

217   insects. Knockout of *timeless* in male *D. melanogaster* showed a change in mating

218   time [55]. In *Spodoptera litorralis* it has been demonstrated that the sperm release

219   rhythm is controlled by an intrinsic circadian mechanism located in the reproductive

220   system [56]. *Brunelleschi* encodes a protein that belongs to the TRAPII complex which

221   is involved in vesicle trafficking in the secretory pathway [57]. As male accessory

222   glands are the secretory tissues of the reproductive system, *brunelleschi* is involved in

223   the maturation of accessory glands in order to produce the secretory proteins of the

224   seminal fluid. *Yellow-g* belongs to the MRJP/YELLOW family that includes the

225   major royal jelly proteins and the yellow proteins. The *yellow* gene family has been

226   associated with behavior [58, 59, 60, 61, 62, 63], pigmentation [64, 65], and sex-specific

227   reproductive maturation [62] in *D. melanogaster* and *A. mellifera*.

228   *CG2254-like* encodes a dehydrogenase that is localized in the lipid droplets,

229   organelles that store lipids and have a significant role in metabolism and membrane

230   synthesis [66]. The ejaculatory bulb is a muscle tissue and its contractions help to

231   transfer the seminal fluid to the female flies during mating. During mating, the tissue

232   has high energy demands and the presence of lipid droplets give them an alternative

233   source of energy. Moreover, these lipid organelles could serve as a source of substrate

234   for steroid hormone synthesis, such as ecdysteroid hormone that plays a significant

235   role in reproduction.

236   With regard to the female reproductive tract, the expression profile of the

237   selected genes was determined in DAY-7 old virgin females and at five time points

238   (0, 3, 6, 9, 12, 24 h) after mating (Figure 6). This was based on the hypothesis that if a

239   gene codes for a protein which is induced by mating, it should be expressed some

240   time after mating. The obtained expression profiles of the 6 genes were variable.

241   *Troponin C* showed limited expression after mating. *Ornithine decarboxylase*

242   *antizyme* showed an increasing expression with the highest expression at 24 hours

243   after mating, while *lingerer* (10-fold) and *bestrophin-2* (10-fold) showed highest

244   expression at 12 hours. *Yolk protein-2* showed 2-fold overexpression 9 hours after

245   mating and *glutathione S-transferase* showed highest expression immediately after

246   mating (0 Hours).

247   Troponin C protein plays a significant role in muscle contractions. In *Pieris*

248   *rapae*, the small cabbage white butterfly, it was identified as a component of the bursa

249   copulatrix female reproductive tissue that is responsible for the digestion of the

250   nutrient-rich spermatophore produced by the male accessory glands [67]. The

251   overexpression of this gene in the female reproductive tract of virgin flies indicates its

252   involvement in muscle contraction, probably aiding the digestion of the seminal fluid

253   proteins that are transferred to the female during mating.

8

254   An upregulation of *Ornithine decarboxylase antizyme* (ODC-AZ) was
255   observed in mated females. ODC-AZ binds and destabilizes the ornithine
256   decarboxylase (ODC), a key enzyme in polyamine synthesis [68]. Correlative changes
257   between hormone levels and polyamine metabolism were described in several insects.
258   For example, 20-hydroxyecdysone increases ODC activity in silk moth pupal tissues
259   [69] and juvenile hormone stimulates ODC activity during vitellogenesis in *D.*
260   *melanogaster* [70]. Ornithine decarboxylase antizyme is an inhibitor of ODC. Inhibition
261   of ODC activity causes impaired vitellogenesis in *Ae. aegypti* [71] and oviposition delay
262   in the silkmoth, *Hyalophora cecropia* [69]. The observed upregulation of their inhibitor
263   indicates that ODC-AZ is probably involved in the control of ODCs levels in mated
264   female olive flies.

265   *Lingerer* showed upregulation 12 hours after mating. Mutations of *lingerer* in
266   male *D. melanogaster* result in abnormal matings and the "stuck" phenotype where
267   males cannot be separated from females after the end of mating. It has been also
268   identified as a maternal gene expressed in *D. melanogaster* early embryos [72]. A
269   similar expression profile has been demonstrated for the *bestrophin 2* gene. In *D.*
270   *melanogaster* it encodes an oligomeric transmembrane protein that is thought to act as
271   chloride channel [73]. It facilitates the transportation of small molecules that are
272   transferred into the female flies as part of the seminal fluid during mating.

273   *Yolk protein-2* homologue in *D. melanogaster* is expressed almost exclusively
274   in females and it was associated with a female-sterile mutation [74, 75]. *Yolk protein-2*
275   gene encodes for a precursor of the major egg storage protein, the vitelline. There are
276   three main factors that regulate vitellogenesis in *D. melanogaster*: a brain factor, an
277   ovarian factor that stimulates fat body vitelline synthesis and a thoracic factor that is
278   involved in the uptake of the vitelline by the ovaries [76]. Moreover, vitellogenins have
279   also been implicated in the transportation of various molecules such as sugars, lipids
280   and hormones in insects [77].

281   Glutathione S-transferase epsilon class is a predicted intracellular or
282   membrane-bound protein [78]. Predicted intracellular proteins have been reported in the
283   reproductive system of *D. melanogaster* [4], *A. mellifera* [79] and *Ae. aegypti* [80]. For both
284   *A. mellifera* and *Ae. aegypti* these proteins are suggested to be secreted through

285   apocrine and holocrine secretion, non-standard secretion routes [81, 82]. Macro apocrine

286   secretion has been reported in the *B. oleae* male reproductive system [36].

287   The transcriptional profiles of the aforementioned genes identified in the

288   reproductive tissues of *B. oleae* showed changes associated with the production of the

289   proteins with relation to the sexual maturation of the males and the induction of

290   several post-mating responses for the females. However, the very complex

291   transcriptional profile of several of these genes necessitates further investigation. A

292   key focus for future studies is a more detailed functional analysis and characterization

293   of the processes that are involved in the reproduction of olive fly. Further

294   experiments, including transient silencing through RNA interference (RNAi) of the

295   aforementioned genes, would be helpful towards the clarification of the specific role

296   of the genes in the mating procedure or the post-mating response of the female

297   insects.

298

299   3. Conclusions

300   Undoubtedly, the reproduction system is one of the major systems of living

301   organisms, primarily responsible for any species' existence on earth. The present

302   study sheds light on various aspects of the reproduction system of a major agricultural

303   pest in an effort to understand its basic characteristics but also help in the

304   development of specific and more ecologically sound approaches to insect control.

305   During the last sixty years, agrochemical companies have been in a constant quest for

306   all the more specific, effective and environmentally friendly approaches to contain

307   populations of innocuous insects. Recently, RNA interference (RNAi) has been

308   explored as a strategy for pest control by administering insect-targeted double-

309   stranded RNA (dsRNA) to specifically block the expression of essential genes [83, 84]. In

310   that regard, inhibition of reproductive genes could lead to impairments in courtship

311   behavior, fertility, oviposition, reproductive physiology or direct death of the

312   developing embryo. Whatever the reason, the effect would be a dramatic drop in

313   population size of the targeted insect. Alternatively, one can envision a CRISPR-

314   based gene drive system in which a Cas9 (with its guide RNA) targets an essential

315   reproductive gene [85, 86]. The result would be a rapid substitution of the particular

316   reproductive gene in a population with an impaired copy (or the complete deletion of

317 the gene), thus damaging the reproductive ability of the pest and leading to its
318 population collapse.

319

320 **4. Methods**

321 *4.1 Ethics statement*

322 For these experiments no specific permissions are required. This study did not
323 interfere with any endangered or protected species as it was carried out on laboratory
324 reared olive flies.

325

326 *4.2 Fly culture and stock*

327 The laboratory strain of the olive fly is part of the original stock from the
328 Department of Biology, "Democritus" Nuclear Research Centre, Athens, Greece, and
329 has been reared in our laboratory for over 20 years. The flies are reared at 25°C with a
330 12h light/12h dark photoperiod and humidity 65% in laboratory cages with diameter
331 $30*30*30cm^3$ with wax cones inside for oviposition as described by Tzanakakis 1967
332 [87].

333

334 *4.3 Tissue collection*

335 To obtain the male adult flies used to generate the testes and accessory glands
336 libraries as well as the female adult flies for the lower reproductive tract libraries, a
337 standard laboratory rearing cage was set up with about 50 males and 50 females, one
338 day after eclosion. The insects were maintained separately, in order to obtain tissues
339 from two distinct groups:1. Virgin flies and 2. Mated flies. For the group of the virgin
340 insects, males and females were removed from the cages 7 days after eclosion. For the
341 group of the mated flies, we mixed virgin male and female flies to mate once. When
342 the mating procedure was completed, the insects were kept in different cages
343 separated for 12 hours before we perform dissections. The testes and MAGs with
344 ejaculatory bulb from the male insects and the lower reproductive tract from the
345 female insects were dissected in ddH_2O. The dissected material was immediately
346 immersed in the TRIzol Reagent (Ambion-Invitrogen). The RNA isolation was

11

347 performed based on the TRIzol Reagent following the manufacturer's instructions
348 with slight modifications. An additional DNA removal using the TURBO DNA-free
349 Kit (Ambion-Invitrogen), was performed, according to manufacturer's instructions.
350 RNA's integrity was assessed by 1% agarose gel electrophoresis. The purity of all
351 RNA samples was evaluated at Fleming Institute (Greece) with the use of (Agilent
352 2100 Bioanalyzer) and NanoDrop (2000).

353 For the validation of the differentially expressed genes, RNA was extracted
354 from three pools each one containing 10 pairs of the lower reproductive tract (FAGs/
355 spermathecae, uterus), 10 pairs of testes and 10 pairs of ejaculatory bulb/ MAGs
356 (three biological pool replicates) before and 12 hours after one mating of the
357 aforementioned laboratory strain. To determine the expression profiles of the selected
358 genes: 1. mRNA was extracted from 10 pairs of ejaculatory bulb/ MAGs from virgin
359 males at DAY-0 (first day of eclosion) until DAY-7 and 2. RNA was extracted from
360 three pools of 10 pairs of the lower reproductive tract from mated once females
361 immediately after mating (0 hours) and 3 hours, 6 hours, 9 hours, 12 hours and 24
362 hours after mating for the female tissues. The RNA isolation was performed as
363 described above.

364

365 *4.4 RNA isolation for library preparation and functional analysis*

366 mRNA transcripts from the samples were used to construct cDNA libraries for
367 sequencing analysis. The cDNA libraries of accessory glands and ejaculatory bulb
368 from virgin and mated male flies, the lower reproductive tract from virgin and mated
369 female flies and the gut tissue from virgin male flies were sequenced on the Ion
370 Proton^{TM} system for Next-Generation. Sequencing was performed at the Fleming
371 Institute (Greece) using the Ion Torrent™ Ion Chef™ automated platform. The cDNA
372 library obtained from the testes of mated male insect (M_TESTES) was sequenced by
373 Illumina Hi-Seq 2000 using the Illumina TruSeq RNA Sequencing protocol at the
374 Genome Quebec in Canada.

375

376 *4.5 Expression analyses of selected gene*

377   Following extraction, the RNA was treated with 1.0 unit of DNase I
378 (Invitrogen) according to manufacturer's instructions. The total amount of DNA-free
379 RNA obtained from each tissue was converted into cDNA using 300ng Random
380 hexamer primers (equimolar mix of N5A, N5G, N5C and N5T), 200units MMLV
381 Reverse Transcriptase (NEB), 5x Reaction buffer, 40mM dNTP mix and 40 units
382 RNAse Inhibitor (NEB) according to the manufacturer's instructions. Reverse
383 transcription was conducted at 42°C for 50 minutes and 70°C for 15 minutes. The
384 resulting cDNA was used in the followed qPCR reactions.

385   Specific primers to amplify genes identified on the transcriptomic analysis
386 were designed by Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast)
387 (Table S5). Normalized relative quantitation was used to analyze changes in
388 expression levels of the selected genes using a Real-time PCR approach. Expression
389 values were calculated relatively to the housekeeping genes. *Rpl19* were used as
390 reference in male reproductive tissues while *GAPDH* in FAGs/spermathecae [41].

391   The qRT-PCR conditions were: polymerase activation at 50 °C for 2 min,
392 DNA denaturation step at 95 °C for 4 min, followed by 50 cycles of denaturation at
393 95 °C for 10 s, annealing/ extension and plate read at 55 °C for 20 s and finally, a step
394 of melting curve analysis at a gradual increase of temperature over the range 55 °C to
395 95 °C. In this step, the detection of one gene-specific peak and the absence of primer
396 dimer peaks were guaranteed. Each reaction was in a total volume of 15 μl, containing
397 5 μl from a 1:10 dilution of the cDNA template, 2X SYBR Select Master Mix
398 (Applied Biosystem) and 300nM of each primer. The reactions were performed on a
399 Bio-Rad Real-time thermal cycler CFX96 (Bio-Rad, Hercules, CA, USA) and data
400 were analyzed through the CFX Manager™ software. All qRT-PCRs were performed
401 in triplicate (i.e., three technical replicates).

402

403 *4.6 Statistical tests*

404   One-way ANOVA followed by Dunnett's multiple comparisons test was
405 performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La
406 Jolla California USA, www.graphpad.com. Values were stated as mean ± standard
407 error and a p value of < 0.05 was considered statistically significant.

408

13

Competing interests

All the authors declare no competing interests.

Authors' contributions

Maria-Eleni Gregoriou: Conceptualization; Methodology; Validation; Formal analysis; Resources; Data curation; Validation; Investigation. **Martin Reczko**: Data curation; Software; Formal analysis. **Konstantina Tsoumani:** Validation; Resources; Investigation. **Kostas Mathiopoulos:** Conceptualization; Supervision; Project administration; Funding Acquisition. All authors participated in drafting the manuscript and read and approved the final document.

Acknowledgments

References

1. Tombes, A. S. & Roppel, R. M. Ultrastructure of the spermatheca of the granary weevil, *Sitophilus granarius* (L.) (Coleoptera : Curculionidae). International Journal of Insect Morphology and Embryology 1, 141–152 (1972).

2. Bailey, W. J. & Nuhardiyati, M. Copulation, the dynamics of sperm transfer and female refractoriness in the leafhopper *Balclutha incisa* (Hemiptera: Cicadellidae: Deltocephalinae). Physiological Entomology 30, 343–352 (2005).

3. Oppelt, A. & Heinze, J. Dynamics of sperm transfer in the ant *Leptothorax gredleri*. Naturwissenschaften 94, 781–786 (2007).

14

4. Walker, M. J. et al. Proteomic identification of *Drosophila melanogaster* male accessory gland proteins, including a pro-cathepsin and a soluble γ-glutamyl transpeptidase. Proteome Science 4, 1–10 (2006).

5. Laflamme, B.A & Wolfner M.F Identification and function of proteolysis regulators in seminal fluid. Molecular Reproductive Development, 80-101 (2013).

6. Sirot, L. K. et al. Towards a Semen Proteome of the Dengue Vector Mosquito: Protein Identification and Potential Functions. PLoS Neglected Tropical Diseases 5, e989 (2011).

7. Begun, D. J., Lindfors, H. A., Thompson, M. E. & Holloway, A. K. Recently evolved genes identified from *Drosophila yakuba* and *Drosophila erecta* accessory gland expressed sequence tags. Genetics 172, 1675–1681 (2006).

8. Findlay, G.D., Yi, X., Maccoss, M.J., Swanson, W.J. Proteomics reveals novel Drosophila seminal fluid proteins transferred at mating. PLoS Biololy; 6(7):e178 (2008).

9. Findlay, G. D., MacCoss, M. J. & Swanson, W. J. Proteomic discovery of previously unannotated, rapidly evolving seminal fluid genes in Drosophila. Genome Research 19, 886–895 (2009).

10. Dottorini, T. et al. A genome-wide analysis in *Anopheles gambiae* mosquitoes reveals 46 male accessory gland genes, possible modulators of female behavior. Proceedings of the National Academy of Sciences 104, 16215–16220 (2007).

11. Sirot, L. K. et al. Control of Female Feeding and Reproduction. Insect Biochemistry 38, 176–189 (2009).

12. Miyatake, T., Chapman, T. & Partridge, L. Mating-induced inhibition of remating in female mediterranean fruit flies *Ceratitis capitata*. Journal of Insect Physiology 45, 1021–1028 (1999).

13. Papanicolaou, A. et al. The whole genome sequence of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), reveals insights into the biology and adaptive evolution of a highly invasive pest species. Genome Biology 17, 1–31 (2016).

14. Kuba, H. & Itô, Y. Remating inhibition in the melon fly, *Bactrocera* (=Dacus) *cucurbitae* (Diptera: Tephritidae): Copulation with spermless males inhibits female remating. Journal of Ethology 11, 23–28 (1993).

15

15. Soltani-Mazouni, N. & Bordereau, C. Changes in the Cuticle , Ovaries and colleterial glands suring the pseudergate and neotenic molt in *Kalotermesfla vicollis* (Fabr.) (Isoptera, Kalotermitidae). In. J. Insect Morphol. & Embryol. 16, 221–235 (1987).

16. Rosetto, M. et al. A mammalian-like lipase gene is expressed in the female reproductive accessory glands of the sand fly *Phlebotomus papatasi* (Diptera, Psychodidae). Insect Molecular Biology 12, 501–508 (2003).

17. Wagner, R. M. et al. Isolation and identification of a novel peptide from the accessory sex gland of the female house fly, *Musca domestica*. Biochemical and Biophysical Research Communications 194, 1336–1343 (1993).

18. Marchini, D., Bernini, L. F., Marri, L., Giordano, P. C. & Dallai, R. The female reproductive accessory glands of the medfly *Ceratitis capitata*: Antibacterial activity of the secretion fluid. Insect Biochemistry 21, 597–605 (1991).

19. Marchini, D., Marri, L., Rosetto, M., Manetti, A. G. O. & Dallai, R. Presence of antibacterial peptides on the laid egg chorion of the medfly *Ceratitis capitata*. Biochemical and Biophysical Research Communications 240, 657–663 (1997).

20. Craig, G. B. & Hickey, W. A. Current status of the formal genetics of *Aedes aegypti*. Bulletin of the World Health Organization 36, 559–562 (1967).

21. Radhakrishnan, P. & Taylor, P. W. Seminal fluids mediate sexual inhibition and short copula duration in mated female Queensland fruit flies. Journal of Insect Physiology 53, 741–745 (2007).

22. Shutt, B., Stables, L., Aboagye-Antwi, F., Moran, J. & Tripet, F. Male accessory gland proteins induce female monogamy in anopheline mosquitoes. Medical and Veterinary Entomology 24, 91–94 (2010).

23. Abraham, S., Goane, L., Cladera, J. & Vera, M. T. Effects of male nutrition on sperm storage and remating behavior in wild and laboratory *Anastrepha fraterculus* (Diptera: Tephritidae) females. Journal of Insect Physiology 57, 1501–1509 (2011).

24. Chen, S. Biochemistry of Insect Male Accessory Glands. Annual review of entomology 29, 233–255 (1984).

25. Jang, E. B. Effects of Mating and Accessory-Gland Injections on Olfactory-Mediated Behavior in the Female Mediterranean Fruit-Fly, *Ceratitis capitata*. Journal of Insect Physiology 41, 705–710 (1995).

26. Barnes, A. I., Wigby, S., Boone, J. M., Partridge, L. & Chapman, T. Feeding, fecundity and lifespan in female *Drosophila melanogaster*. Proceedings of the Royal Society B: Biological Sciences 275, 1675–1683 (2008).

27. Lawniczak, M. K. & Begun, D. J. A genome-wide analysis of courting and mating responses in *Drosophila melanogaster* females. Genome 47, 900–910 (2004).

28. Mack, P. D., Kapelnikov, A., Heifetz, Y. & Bender, M. Mating-responsive genes in reproductive tissues of female *Drosophila melanogaster*. Proceedings of the National Academy of Sciences 103, 10358–10363 (2006).

29. Kocher, S. D., Richard, F. J., Tarpy, D. R. & Grozinger, C. M. Genomic analysis of post-mating changes in the honey bee queen (*Apis mellifera*). BMC Genomics 9, 1–15 (2008).

30. Kocher, S. D., Tarpytt, D. R. & Grozinger, C. M. The effects of mating and instrumental insemination on queen honey bee flight behaviour and gene expression. Insect Molecular Biology 19, 153–162 (2010).

31. Manfredini, F., Brown, M. J. F., Vergoz, V. & Oldroyd, B. P. RNA-sequencing elucidates the regulation of behavioural transitions associated with the mating process in honey bee queens. BMC Genomics 16, 1–13 (2015).

32. Gomulski, L. M. et al. Transcriptome profiling of sexual maturation and mating in the mediterranean fruit fly, *Ceratitis capitata*. PLoS ONE 7, (2012).

33. Rogers, D.W., Whitten, M.M., Thailayil, J., Soichot, J., Levashina, E.A., Catteruccia, F. Molecular and cellular components of the mating machinery in *Anopheles gambiae* females. Proceedings of the National Academy of Sciences of the United States of America 105: 19390–19395 (2008)

34. Gabrieli, P. et al. Sexual transfer of the steroid hormone 20E induces the postmating switch in *Anopheles gambiae*. Proceedings of the National Academy of Sciences 111, 16353–16358 (2014).

35. Alfonso-Parra, C. et al. Mating-Induced Transcriptome Changes in the Reproductive Tract of Female *Aedes aegypti*. PLoS Neglected Tropical Diseases 10, 1–24 (2016).

17

36. Marchini, D. & Del Bene, G. Comparative fine structural analysis of the male reproductive accessory glands in *Bactrocera oleae* and *Ceratitis capitata* (Diptera, Tephritidae). Italian Journal of Zoology 73, 15–25 (2006).

37. Sagri, E. et al. The molecular biology of the olive fly comes of age. BMC Genetics 15, (2014).

38. Zervas, G. A. & Economopoulos, A. P. Mating frequency in caged populations of wild and artificially reared (normal or γ-sterilized) olive fruit flies. Environ Entomol, 17-20 (1982).

39. Scolari, F. et al. Transcriptional Profiles of Mating-Responsive Genes from Testes and Male Accessory Glands of the Mediterranean Fruit Fly, *Ceratitis capitata*. PLoS ONE 7, (2012).

40. Tsiropoulos, G. J. & Tzanakakis, M. E. Mating frequency and inseminating capacity of rediation-sterilized and normal males of the olive fruit fly. Annals of the Entomological Society of America 63, 1007–1010 (1970).

41. Sagri, E. et al. Housekeeping in Tephritid insects: The best gene choice for expression analyses in the medfly and the olive fly. Scientific Reports 7, 1–9 (2017).

42. Haas, B.J., et al. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nature Protocols 8, 1494–1512 (2013).

43. Li, B. & Dewey, C. N. RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics 12, (2011).

44. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140 (2009).

45. Olivieri, G. & Olivieri, A. Autoradiographic study of nucleic acid synthesis during spermatogenesis in *Drosophila melanogaster*. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 2, 366–380 (1965).

46. Wasbrough, E. R. et al. The *Drosophila melanogaster* sperm proteome-II (DmSP-II). Journal of Proteomics 73, 2171–2185 (2010).

47. Götz, S. et al. High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Res, 36:3420-35 (2008)

48. Wei, D. et al. Transcriptome profiling of the testis reveals genes involved in spermatogenesis and marker discovery in the oriental fruit fly, *Bactrocera dorsalis*. Insect Molecular Biology 24, 41–57 (2015).

49. McGraw, L. A., Clark, A. G. & Wolfner, M. F. Post-mating gene expression profiles of female *Drosophila melanogaster* in response to time and to four male accessory gland proteins. Genetics 179, 1395–1408 (2008).

50. Prokupek, A. et al. An evolutionary expressed sequence tag analysis of Drosophila spermatheca genes. Evolution 62, 2936–2947 (2008).

51. Swanson, W. J. & Vacquier, V. D. The rapid evolution of reproductive proteins. Genetics 3, 137–144 (2002).

52. Braswell, W.E., Andrés, J.A., Maroja, L.S., Harrison, R.G., Howard, D.J., Swanson, W.J. Identification and comparative analysis of accessory gland proteins in Orthoptera. Genome 49: 1069–1080 (2006).

53. Tian, C.-B. et al. Comparative Transcriptome Analysis of three *Bactrocera dorsalis* (Diptera: Tephritidae) organs to identify functional genes in the male accessory glands and ejaculatory duct. Florida Entomologist 100, 42–51 (2017).

54. Mueller, J. L., Ripoll, D. R., Aquadro, C. F. & Wolfner, M. F. Comparative structural modeling and inference of conserved protein classes in Drosophila seminal fluid. Proceedings of the National Academy of Sciences 101, 13542–13547 (2004).

55. Beaver, L. M., Rush, B. L., Gvakharia, B. O. & Giebultowicz, J. M. Noncircadian regulation and function of clock genes *period* and *timeless* in oogenesis of *Drosophila melanogaster*. Journal of Biological Rhythms 18, 463–472 (2003).

56. Kotwica-Rolinska, J., Gvakharia, B. O., Kedzierska, U., Giebultowicz, J. M. & Bebas, P. Effects of period RNAi on V-ATPase expression and rhythmic pH changes in the vas deferens of *Spodoptera littoralis* (Lepidoptera: Noctuidae). Insect Biochemistry and Molecular Biology 43, 522–532 (2013).

57. Robinett, C. C., Giansanti, M. G., Gatti, M. & Fuller, M. T. TRAPPII is required for cleavage furrow ingression and localization of Rab11 in dividing male meiotic cells of Drosophila. Journal of Cell Science 122, 4526–4534 (2009).

19

58. Dow, M. A. The genetic basis of receptivity of yellow mutant *Drosophila melanogaster* females. Behavior Genetics 6, 141–143 (1976).

59. Wilson, R., Burnet, B., Eastwood, L. & Connolly, K. Behavioral pleiotropy of *yellow* gene in *Drosophila melanogaster*. Genetical Research 28, 75–88 (1976).

60. Burnet, B. & Wilson, R. Pattern mosaicism for behavior controlled by the yellow locus in *Drosophila melanogaster*. Genet Res 36, 235–247 (1980).

61. Drapeau, M. D., Radovic, A., Wittkopp, P. J. & Long, A. D. A gene necessary for normal male courtship, yellow, acts downstream of fruitless in the *Drosophila melanogaster* larval brain. Journal of Neurobiology 55, 53–72 (2003).

62. Drapeau, M. D., Cyran, S. A., Viering, M. M., Geyer, P. K. & Long, A. D. A cis-regulatory sequence within the yellow locus of *Drosophila melanogaster* required for normal male mating success. Genetics 172, 1009–1030 (2006).

63. Prud'homme, B. et al. Repeated morphological evolution through cis-regulatory changes in a pleiotropic gene. Nature 440, 1050–1053 (2006).

64. HAN, Q. et al. Identification of *Drosophila melanogaster* yellow-f and yellow-f2 proteins as dopachrome-conversion enzymes. Biochemical Journal 368, 333–340 (2002).

65. Wittkopp, P. J., True, J. R. & Carroll, S. B. Reciprocal functions of the Drosophila yellow and ebony proteins in the development and evolution of pigment patterns. Development (Cambridge, England) 129, 1849–1858 (2002).

66. Thiam, A. R., Farese, R. V. & Walther, T. C. The biophysics and cell biology of lipid droplets. Nature Reviews Molecular Cell Biology 14, 775–786 (2013).

67. Meslin, C. et al. Digestive organ in the female reproductive tract borrows genes from multiple organ systems to adopt critical functions. Molecular Biology and Evolution 32, 1567–1580 (2015).

68. Cayre, M. et al. Inhibition of polyamine biosynthesis alters oviposition behavior in female crickets. Behavioral Neuroscience 110, 1117–1125 (1996).

69. Wyatt, G.R., Rothaus, K., Lawler, D., Herbst, E.J. Ornithine decarboxylase and polyamines in silkmoth pupal tissues: effect of ecdysone and injury. Biochim. Biophys. Acta. 304, 482–494 (1973).

20

637   70. Birnbaum, M. J. & Gilbert, L. I. Juvenile hormone stimulation of ornithine
638   decarboxylase activity during vitellogenesis in *Drosophila melanogaster*. J
639   Comp Physiol B 160, 145–151 (1990).

640   71. Kogan, P. H. & Hagedorn, H. H. Polyamines, and effects from reducing their
641   synthesis during egg development in the yellow fever mosquito, *Aedes
642   aegypti*. J Insect Physiol 46, 1079–1095. (2000).

643   72. Kuniyoshi, H. et al. *lingerer*, a Drosophila gene involved in initiation and
644   termination of copulation, encodes a set of novel cytoplasmic proteins.
645   Genetics 162, 1775–1789 (2002).

646   73. Kaartinen, V. & Nagy, A. dbest1, a Drosophila homolog of human Bestrophin,
647   is not required for viability or photoreceptor integrity. Genesis 31, 130–136
648   (2001).

649   74. Williams, T. D. Mechanisms underlying the costs of egg production.
650   BioScience 55, 39–48 (2005).

651   75. Goldman, T. D. & Arbeitman, M. N. Genomic and functional studies of
652   Drosophila sex hierarchy regulated gene expression in adult head and nervous
653   system tissues. PLoS Genet 3, e216 (2007).

654   76. Handler, A. M. & Postlethwait, J. H. Endocrine control of vitellogenesis in
655   *Drosophila melanogaster*: Effects of the brain and corpus allatum. Journal of
656   Experimental Zoology 202, 389–401 (1977).

657   77. Sappington, T.W, Raikhel, A.S. Molecular characteristics of insect
658   vitellogenins and vitellogenin receptors. Insect Biochem Mol Biol 28: 277–
659   300 (1998).

660   78. Balogh, L. M. & Atkins, W. M. Interactions of glutathione transferases with 4-
661   hydroxynonenal. Drug Metabolism Reviews 43, 165–178 (2011).

662   79. Baer, B., Heazlewood, J. L., Taylor, N. L., Eubel, H. & Millar, A. H. The
663   seminal fluid proteome of the honeybee *Apis mellifera*. Proteomics 9, 2085–
664   2097 (2009).

665   80. Jones, J. C. & Wheeler, R. E. Studies on Spermathecal Filling in Aedes
666   Aegypti (Linnaeus). II. Experimental Authors: Jack Colvard Jones and
667   Ronald E . Wheeler. Biological Bulletin 129, 532–545 (1965).

668   81. Dapples, C. C., Foster, W. A. & Lea, A. O. Ultrastructure of the accessory
669   gland of the male mosquito, *Aedes aegypti* (L.) (Diptera: CUlicidae).

21

International Journal of Insect Morphology and Embryology 3, 279–291 (1974).

82. Ramalingam, S. Secretion in the male accessory-glands of *Aedes aegypti* (L.) (Diptera, Culicidae). Int J Insect Morphol Embryol 12: 87–96 (1983).

83. Borovsky, D. Insect peptide hormones and RNA-mediated interference (RNAi): promising technologies for future plant protection. Phytoparasitica 33, 109- 112 (2005).

84. Knorr, E. et al. Gene silencing in *Tribolium castaneum* as a tool for the targeted identification of candidate RNAi targets in crop pests. Scientific Reports 8, 1–15 (2018).

85. Esvelt, K. M., Smidler, A. L., Catteruccia, F. & Church, G. M. Concerning RNA-guided gene drives for the alteration of wild populations. eLife 3, 1–21 (2014).

86. Burt, A. Site-specific selfish genes as tools for the control and genetic engineering of natural populations. Proceedings of the Royal Society B: Biological Sciences 270, 921–928 (2003).

87. Tzanakakis, M., Economopoulos, A. P., Tsitsipis, J. The importance of conditions during the adult stage in evaluating an artificial food for larvae of *Dacus oleae* (Gmel.) (Diptera, Tephritidae). Z. Angew. Entomol. 59, 127–130 (1967).

Figure captions

Figure 1: Volcano plots represent the differentially expressed genes between virgin and mated flies in the three dissected tissues: A. testes, B. male accessory glands with ejaculatory bulb, C. lower female reproductive tract. The Y axis represents significance and the X axis represents logarithmic fold change. The red dots represent differentially expressed genes (p value < 0.05).

22

698   Figure 2: Functional annotation of the top 100 over expressed genes in *B. oleae*

699   reproductive tissues from mated insects showing top 20 hits of different category for

700   molecular function (MF) and biological process (BP).

701

702   Figure 3: Relative expression profiles of genes expressed in the reproductive tissue of

703   testes from virgin and mated male insects. Mean values ± standard error of triplicate

704   data from three biological replicates are shown. No statistical significance as

705   determined by t-test ($p < 0.05$).

706

707   Figure 4: Relative expression profiles of genes expressed in virgin and mated insects

708   from: A. male accessory glands with ejaculatory bulb and B. flower female

709   reproductive tract between virgin and mated female insects. Mean values ± standard

710   error of triplicate data from three biological replicates are shown. The * indicates

711   significantly different, as determined by t-test ($p < 0.05$).

712

713   Figure 5: Expression profiles in female reproductive tissues . Expression profiles of

714   the selected genes from the first day of eclosion (DAY-0) until DAY-7. The error bars

715   show the standard error of the mean between the three biological samples.

716

717   Figure 6: Expression profiles in female reproductive tissues. Expression profile of the

718   selected genes from the virgin flies and several time points after mating (0, 3, 6, 9, 12,

719   24 hours). The error bars show the standard error of the mean between the three

720   biological samples.
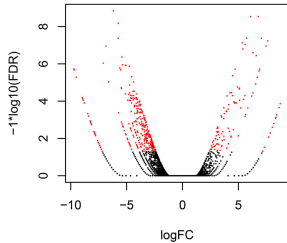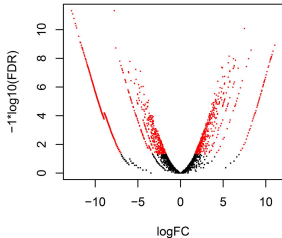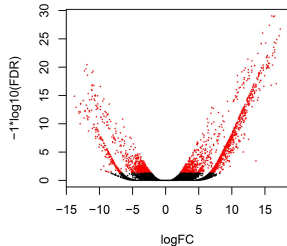
721

722   **Supplementary material**

23

723 Supplementary table S1: Genes differentially expressed in testes between virgin and

724 mated males. Transcript_id, p value and fold values (for all the transcripts) and

725 gene_id and annotation name (for the top100) are shown. Positive fold values indicate

726 upregulation while negative fold values indicate downregulation in mated flies

727 compared to virgin flies.

728 Supplementary table S2: Genes differentially expressed in male accessory glands and

729 ejaculatory bulb between mature virgin and mated males. Transcript_id, p value and

730 fold values (for all the transcripts) and gene_id and annotation name (for the top100)

731 are shown. Negative fold values indicate upregulation in mated flies while positive

732 fold values indicate downregulation compared to virgin flies.

733 Supplementary table S3: Genes differentially expressed in female lower reproductive

734 tract between mature virgin and mated males. Transcript_id, p value and fold values

735 (for all the transcripts) and gene_id and annotation name (for the top100) are shown.

736 Negative fold values indicate upregulation in mated flies while positive fold values

737 indicate downregulation compared to virgin flies.

738 Supplementary table S4: Annotated genes of *Bactrocera oleae* based on the homology

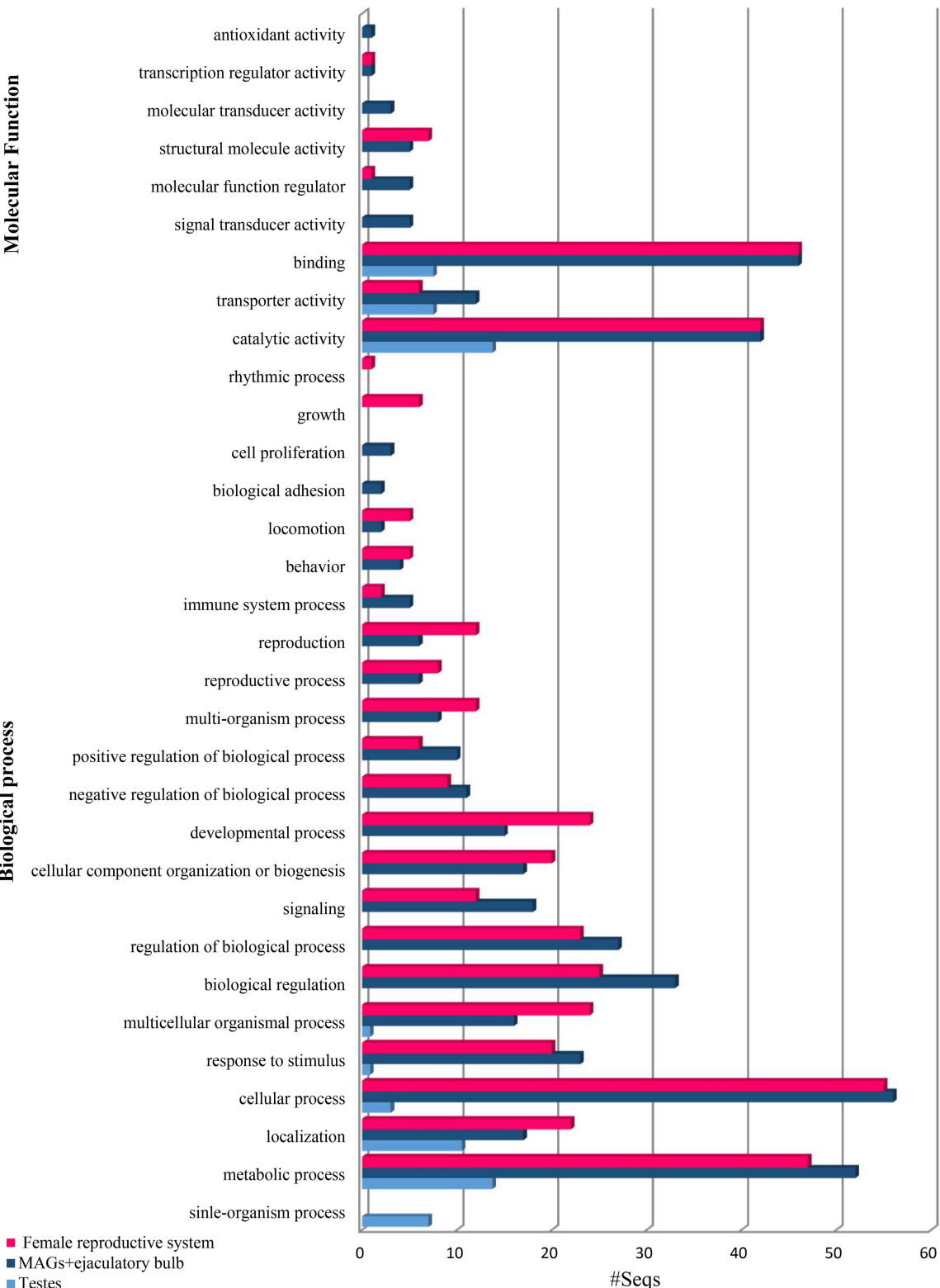739 of known seminal fluid proteins in *D. melanogaster* [54].

740 Supplementary table S5: Primers used for the qRT-PCR. The *RPL19* was used for the

741 normalization of the values obtained from qRT-PCR for male tissues and *GAPDH* for

742 the normalization of the values obtained from qRT-PCR for female tissues.

743

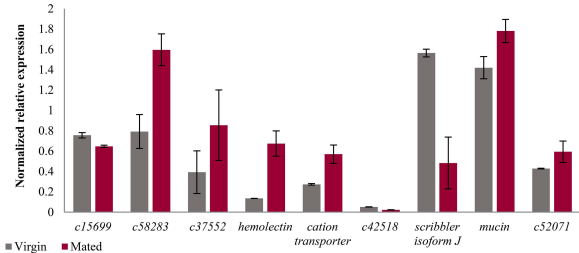744

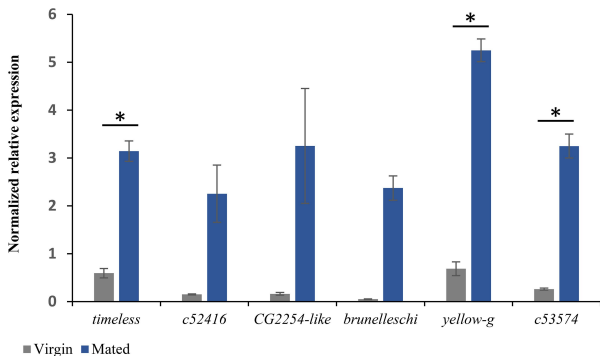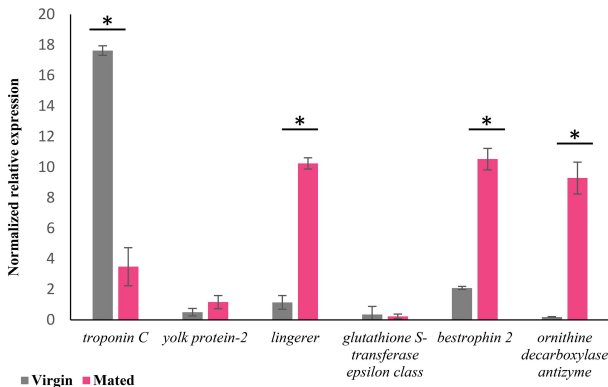**GO-term distribution**

Molecular Function

- antioxidant activity
- transcription regulator activity
- molecular transducer activity
- structural molecule activity
- molecular function regulator
- signal transducer activity
- binding
- transporter activity
- catalytic activity

Biological process

- rhythmic process
- growth
- cell proliferation
- biological adhesion
- locomotion
- behavior
- immune system process
- reproduction
- reproductive process
- multi-organism process
- positive regulation of biological process
- negative regulation of biological process
- developmental process
- cellular component organization or biogenesis
- signaling
- regulation of biological process
- biological regulation
- multicellular organismal process
- response to stimulus
- cellular process
- localization
- metabolic process
- sinle-organism process

■ Female reproductive system
■ MAGs+ejaculatory bulb
■ Testes

#Seqs

Testes

A Male accessory glands/ejaculatory bulb

Normalized relative expression

timeless · *c52416* · *CG2254-like* · *brunelleschi* · *yellow-g* · *c53574*

■ Virgin ■ Mated

B Female lower reproductive tract

Normalized relative expression

troponin C · *yolk protein-2* · *lingerer* · *glutathione S-transferase epsilon class* · *bestrophin 2* · *ornithine decarboxylase antizyme*

■ Virgin ■ Mated
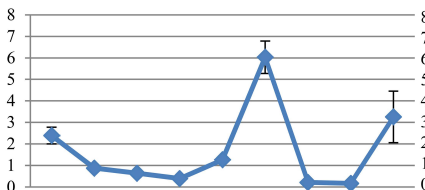
Normalized relative expression vs Days for six genes: *brunelleschi*, *timeless*, *c52416*, *yellow-g*, *c53574*, and *CG2254-like*. X-axis shows Days (0, 1, 2, 3, 4, 5, 6, 7, mated).

troponin C *bestrophin 2* *lingerer* *ornithine decarboxylase antizyme* *yolk protein-2* *glutathione S-transferase*

Normalized relative expression

Hours after mating