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4 **Insect Male Reproductive Glands and Their Products**

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23 bulb, evolution, longevity, ovulation, post-mating responses, remating receptivity, seminal

24 fluid, seminal proteins, sexual conflict, sexual selection, SFPs, sperm, sperm storage, sperm

25 competition

26 **Abstract:**

27 The seminal fluid of insects demonstrates complexity at several levels. At the molecular level,
28 it includes numerous biochemical classes, with protein sequences varying across taxa. At the
29 functional level, seminal components induce diverse changes in female behavior, physiology,
30 and anatomy. At the structural level, seminal components can be packaged into higher-order
31 structures or contribute to multi-layered ejaculate conformations. Seminal components are
32 produced by reproductive glands whose structure and origins vary among insect species. The
33 biology of these glands and their products provide insights into the fundamentals of fertility,
34 reproductive health, and the evolutionary forces that shape reproduction.

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44 **Introduction**

45 The female insect is changed by mating. Behaviorally, physiologically, even anatomically, the
46 mated female differs dramatically from her virgin self. In many cases, these changes are
47 induced by proteins and other molecules carried in the male's ejaculate. Exactly how the post-
48 mating phenotype differs from the pre-mating condition varies across taxa, being honed by
49 selective forces that must strike a balance between the male-female cooperation necessary to
50 achieve a successful fertilization, and the broader, often divergent, evolutionary interests of the
51 sexes.

52

53 The male reproductive glands, particularly the accessory glands, are tasked with the production
54 of the non-sperm component of the ejaculate (the seminal fluid) in insects. Divergent in
55 number, structure, and developmental origin, they are functional analogues of mammalian
56 glands such as the prostate and seminal vesicles. In this chapter, we begin by outlining the
57 complexity of insect seminal fluid in terms of both its organization and its molecular
58 composition. Following this, we briefly summarize the general functions of the secretions of
59 male insect reproductive glands, which induce profound changes in female behavior,
60 physiology, and anatomy. Much of our understanding of the activities of seminal fluid
61 components comes from the fruit fly *Drosophila melanogaster*. Therefore, we describe two
62 paradigmatic examples of its seminal fluid proteins, sex peptide (SP) and ovulin, before
63 outlining what is known about another seminal fluid component, the male-transferred hormone
64 20-hydroxyecdysone, in the malaria vector mosquito *Anopheles gambiae*. We then review the
65 considerable variation in the ultrastructure of reproductive glands across insects, including a
66 detailed overview of *D. melanogaster* accessory glands and their two distinct secretory cell
67 types, the main and secondary cells. Finally, we discuss forces that are thought to have
68 influenced the evolution of accessory glands and their products. This evolutionary framework

69 helps to explain some of the more surprising functions of accessory gland products, such as
70 those that cause female harm.

71

72 **1) The complexity of insect seminal fluid**

73 The ejaculate is composed of sperm and seminal fluid, the latter encompassing a rich diversity
74 of lipids, proteins, carbohydrates, nucleic acids, water, hormones, mucus, vitamins, vesicles,
75 microbes and, in some species, glandular cells (Avila et al., 2011; Gillott, 2003; Hopkins et al.,
76 2017; Perry et al., 2013; Poiani, 2006). In many insects, the protein component of the ejaculate,
77 derived largely from the male accessory glands, is particularly diverse. To date, 121 accessory
78 gland-expressed genes have been identified in the malaria vector *An. gambiae* (Baldini et al.,
79 2012; Dottorini et al., 2007; Rogers et al., 2009), while nearly 100 seminal fluid proteins (SFPs)
80 are transferred to females during mating in another mosquito, the yellow fever vector *Aedes*
81 *aegypti* (Sirot et al., 2011). In *D. melanogaster*, over 200 SFPs are known. These SFPs
82 encompass a range of functions from antioxidants and lectins to proteases and protease
83 inhibitors (Avila et al., 2011, 2015; Findlay et al., 2008; Gillott, 2003; McGraw et al., 2015;
84 Ram & Wolfner, 2007b); proteases are particularly diverse in both insect and mammalian
85 seminal fluid (Laflamme & Wolfner, 2013; Mueller et al., 2004). In *Drosophila* (and likely in
86 other insects), SFPs' amino acid diversity is further supplemented by a variety of post-
87 translational modifications, such as glycosylation, that may further influence their functions
88 (Gligorov et al., 2013).

89

90 Insect ejaculates are generally of two types (Zizzari et al., 2014). First, sperm may be free-
91 swimming within a seminal fluid medium, as in *D. melanogaster*. Alternatively, the ejaculate
92 may be transferred as a spermatophore, as in butterflies and springtails, where both sperm and
93 non-sperm components are encased within a proteinaceous capsule (Meslin et al., 2017; Zizzari

94 et al., 2014). Spermatophores may adopt complex, multi-layered conformations as in the rove
95 beetle *Aleochara cutula*. In this species, the male secretes a ‘tube-like structure’ into the female
96 sperm storage organ, which serves to guide the elongation of a secondary tube that will
97 eventually inflate and burst to release sperm (reviewed in Meslin et al., 2017).

98

99 **2) General functions of male insect reproductive gland products**

100 More than just a vehicle for sperm, seminal fluid components have been implicated in the
101 induction of diverse female post-mating phenotypes (Avila et al., 2011; Gillott, 2003; Hopkins
102 et al., 2017; McGraw et al., 2015; Perry et al., 2013; Poiani, 2006). SFPs have been shown to
103 be particularly important in driving these changes, at least in *D. melanogaster*. Roles of SFPs
104 in females can be broadly categorized into those affecting behavior, physiology, or anatomy.
105 Some SFPs also play an important role within the male, by processing other seminal proteins
106 as they pass through the male’s reproductive tract *en route* to the female (LaFlamme et al.,
107 2012, 2014; Laflamme & Wolfner, 2013). Others promote activities of the sperm within the
108 female reproductive tract, such as the successful release of sperm from storage (Avila et al.,
109 2010).

110

111 **a) Modulation of female behavior**

112 After mating, females of many insect species show reduced receptivity to re-mating. This
113 change may be short-term, e.g. just a few days in the Mediterranean fruit fly *Ceratitis*
114 *capitata* (Miyatake et al., 1999), or permanent, as suggested by some studies of the dengue
115 vector mosquito *Ae. aegypti* (Craig, 1967). In *D. melanogaster* females, a ~2-week reduction
116 in receptivity post-mating, accompanied by a suite of specific rejection behaviors, is
117 controlled by the SFP sex peptide (SP; see section Sex Peptide of *Drosophila melanogaster*)
118 (Bussell et al., 2014; Connolly & Cook, 1973).

119

120 Injection of accessory glands, or their extracts, into virgin females can elicit some of the
121 same phenomena as mating (Garcia-Bellido, 1964). Many of these effects can also be
122 induced by injecting purified SP into the circulation of virgin females, indicating that SP
123 can also access targets from the hemolymph (Chen et al., 1988). Similarly, in *Ae. aegypti*,
124 injection of accessory gland extracts into the thorax of virgin females or implantation of
125 whole accessory glands prevents re-mating (Craig, 1967; Helinski et al., 2012). In *An.*
126 *gambiae*, injection of accessory gland extracts into virgin females makes them less likely to
127 remate (Shutt et al., 2010). Furthermore, *An. gambiae* females mated to spermless males
128 (with fully functional accessory glands) exhibit typical changes in post-mating behavior,
129 such as the induction of blood-feeding and mating refractoriness, indicating that sperm are
130 not needed for these responses (Thailayil et al., 2011).

131

132 Mating often also causes a change in female feeding behavior. Nutritional geometry
133 experiments in house crickets (*Gryllus bimaculatus*) have demonstrated a shift in dietary
134 preference towards protein-rich food sources following mating (Tsukamoto et al., 2014). In
135 *D. melanogaster*, SP elevates female appetite and leads females to favor sodium- and
136 protein-rich food sources (Ribeiro & Dickson, 2010; Vargas et al., 2010; Walker et al.,
137 2015).

138

139 **b) Modulation of female physiology**

140 In the mated female, seminal molecules affect egg production and ovulation. Consumption
141 of a spermatophore in the two-spot ladybird (*Adalia bipunctata*) is associated with an
142 increase in oviposition rate. This effect is independent of female diet, suggesting that it is
143 driven by molecules from the spermatophore rather than through broader nutritional effects

144 (Perry & Rowe, 2008). Injection of male accessory gland extracts into virgin female cotton
145 bollworms (*Helicoverpa armigera*) is also associated with earlier egg maturation and
146 oviposition (Jin & Gong, 2001). In *Aedes* species, increased egg-production is induced by
147 accessory gland extracts (Klowden & Chambers, 1991). In *D. melanogaster* the analogous
148 effect has been shown to be due to the activity of specific SFPs, such as SP and ovulin,
149 which act through the female's nervous or neuromuscular systems (see section Modulation
150 of female anatomy, below). An increase in egg-laying has been shown to be a mating-
151 induced effect that is independent of sperm in *An. gambiae* (Thailayil et al., 2011), again
152 suggesting a role for seminal fluid components.

153

154 Transfer of SP to *D. melanogaster* females induces an increase in juvenile hormone
155 synthesis (Carvalho et al., 2006; Moshitzky et al., 1996). This in turn is associated with
156 diminished female sex pheromone production (Bontonou et al., 2015), vitellogenic oocyte
157 progression (Soller et al., 1999), and reduced resistance to systemic bacterial infection
158 (Schwenke & Lazzaro, 2017). The latter is suggested to reflect trade-offs between
159 investment in reproduction and self-maintenance (Schwenke & Lazzaro, 2017). Conversely,
160 SP activates expression of genes encoding antimicrobial (AMP) peptides, via the Toll and
161 Imd pathways, perhaps to protect against sexually transmitted microorganisms (Peng et al.,
162 2005). SP also influences gut physiology and growth, leading to the production of more
163 concentrated excreta (Apger-McGlaughon & Wolfner, 2013; Cognigni et al., 2011;
164 Lemaitre & Miguel-Aliaga, 2013).

165

166 **c) Modulation of female anatomy**

167 The SFP ovulin (see section Ovulin of *Drosophila melanogaster*) induces visible physical
168 changes in the conformation of the *D. melanogaster* female reproductive tract (Mattei et al.,

169 2015). By stimulating octopaminergic signalling, ovulin relaxes the musculature
170 surrounding the oviduct (Rubinstein & Wolfner, 2013). This relaxation leads to the
171 uncurling of a tightly coiled loop in the upper oviduct within 90-minutes of mating, allowing
172 for transit of an oocyte (Mattei et al., 2015). Another *Drosophila* SFP, Acp36DE, relaxes a
173 constriction in the upper uterus, facilitating the passage of sperm into storage (Avila &
174 Wolfner, 2009; Mattei et al., 2015).

175

176 d) **Supporting the activities of sperm**

177 Across insects, ejaculate components can form novel structures within the female
178 reproductive tract (Meslin et al., 2017; Parker, 1970). These ‘mating plugs’ provide a
179 physical barrier to the entry of sperm from rival males, and have been implicated in reducing
180 female receptivity to remating (Avila et al., 2011; Lung & Wolfner, 2001).

181

182 In some butterfly species, the mating plug includes an external component that covers the
183 female copulatory opening. In contrast, in some other insects, including *D. melanogaster*,
184 the mating plug sits within the posterior uterus at the distal end of the reproductive tract
185 (Lung & Wolfner, 2001). The *D. melanogaster* mating plug is bipartite, combining a
186 posterior portion composed predominantly of ejaculatory bulb derived proteins, such as
187 PEBme (a protein with some similarity to homopolymer-forming proteins in spider-silk),
188 PEBII, and PEBIII, with an anterior section composed of accessory gland-derived proteins
189 such as Acp36DE (Avila et al., 2011, 2015; Bertram et al., 1996b; Bretman et al., 2010;
190 Lung & Wolfner, 2001). While the posterior component forms within the first 5 min of
191 mating, the anterior section is formed by around 20 min after the start of mating.

192

193 Mating plugs also function in promoting sperm storage and retention. The seminal fluid of
194 *An. gambiae* coagulates to form a mating plug in females and is required for the initial entry
195 of sperm into the female storage organs (Giglioli & Mason, 1966; Rogers et al., 2009). The
196 *An. gambiae* accessory gland-derived SFP transglutaminase cross-links the SFP Plug to
197 form the mating plug, which is slowly degraded in the female reproductive tract over the
198 course of 1-2 days (Giglioli & Mason, 1966).

199

200 Individual SFPs have been identified that regulate the storage of sperm in females. For
201 example, *D. melanogaster* Acp36DE is required for sperm accumulation into the storage
202 organs by ‘corralling’ sperm and by altering uterine conformation, as noted in section
203 Modulation of female anatomy (Avila & Wolfner, 2017; Bertram et al., 1996; Neubaum &
204 Wolfner, 1999) Once in storage, the SFP Acp29Ab, a C-type lectin, is required for the
205 retention of sperm (Wong et al., 2008), while the SFPs SP, seminase, CG1652, CG1656,
206 CG9997, and CG17575 are required for efficient release of sperm from storage (Avila et al.,
207 2010; Ram & Wolfner, 2007a). In the hymenopteran species *Apis mellifera* and *Atta*
208 *colombica*, fertility may be promoted by the viability enhancing effect of accessory gland
209 secretions on sperm (Avila et al., 2011; Susanne P.A. den Boer, Baer, et al., 2009; Susanne
210 P.A. den Boer, Boomsma, et al., 2009). However, in polyandrous lineages, this promotion
211 of viability is restricted to self-sperm and actively decreases the viability of rival sperm (S.
212 P A den Boer et al., 2010).

213

214 **3) Seminal fluid proteins in focus**

215 **a). Sex Peptide of *Drosophila melanogaster***

216 Transplantation of accessory glands and injection of their extracts into virgin females each
217 initially implicated their products in driving post-mating reduction in female sexual receptivity

218 and elevation of egg-laying (Bodnaryk, 1978; Garcia-Bellido, 1964; Kummer, 1960; Leahy,
219 1966). These effects were traced to a 36-amino acid ‘sex peptide’ (SP) by fractionation
220 experiments and injection of purified sex peptide into the female abdominal cavity (Chen et
221 al., 1988), as well as by ectopic expression of SP in transgenic virgin females (Aigaki et al.,
222 1991), and phenotypic characterization of the effects of RNAi-knockdowns (Chapman et al.,
223 2003) or SP-knockouts (Liu & Kubli, 2003). The name ‘sex peptide’ derives from early
224 chromatographic studies by Fox, who noted the presence of male specific peptides in the male
225 body fluid (Bodnaryk, 1978; Fox et al., 1959).

226

227 SP is secreted by ‘main cells’ (Gligorov et al., 2013; Kalb et al., 1993) into the accessory gland
228 lumen, where it may reach quantities of 3.1pMoles (Kubli, 1992). During mating, mature males
229 transfer between 30% and 50% of the gland’s SP (Kubli, 1992), and are seemingly able to
230 exercise some control over the quantity that they transfer (see section Phenotypic plasticity).
231 SP alters the transcriptome of the mated female, significantly changing the level of RNAs
232 relating to immunity, egg development, behavior, early embryogenesis, nutrient sensing, and
233 phototransduction (Gioti et al., 2012). In females, SP also induces many of the behavioral,
234 physiological, and anatomical effects noted in section 2, as well as endocrine changes through
235 activation of juvenile hormone synthesis (Moshitzky et al., 1996) (Figure 1).

236

237 SP acts through a G-protein coupled receptor (sex peptide receptor; SPR) primarily in neurons
238 that innervate the female reproductive tract to induce the post-mating change in receptivity
239 (Hasemeyer et al., 2009; Yang et al., 2009; Yapici et al., 2008). The involvement of additional
240 receptors has also been suggested (Hausmann et al., 2013). While SP’s precise neural targets
241 are not yet known, the ultimate target of its signalling pathway is likely the brain. Targets in

242 the brain may also contribute to the mechanism by which SP reduces daytime ‘siesta’ sleep
243 (Isaac et al., 2010) and elevates aggression towards other females (Bath et al., 2017).
244

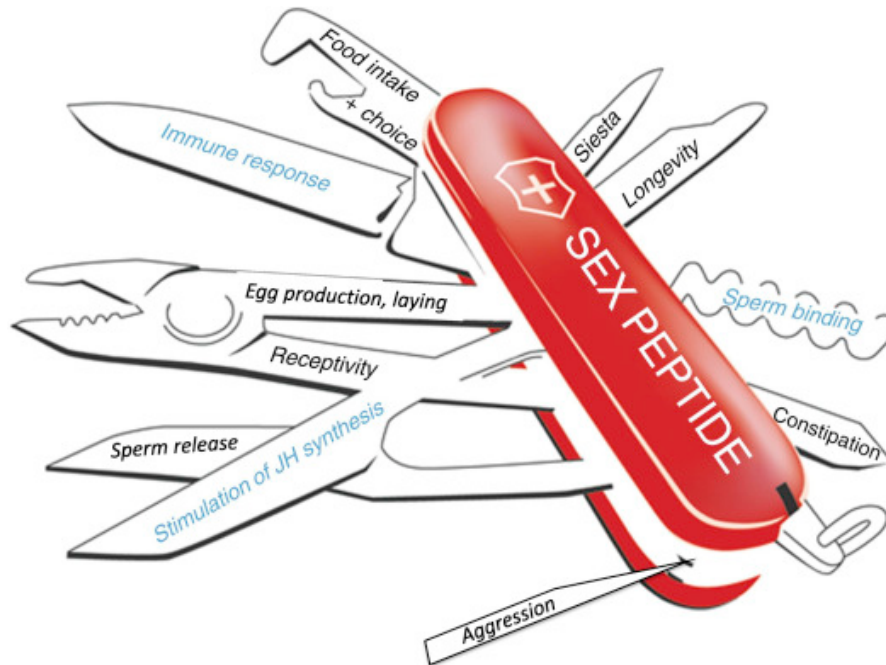


Figure 1. The many effects induced by the seminal sex peptide in mated female *Drosophila melanogaster*.

Modified from Kubli, E., Bopp, D., 2012. *Curr. Biol.* 13, R520–R522.

245

246

247 While SP is able to induce physiological change in females on its own, its effects are extended
248 in the presence of sperm (David, 1963; Manning, 1962, 1967). Studies in the 1960s noted that
249 the female post-mating response could be separated into a short- and long-term component.
250 The former occurs within the first 48 hours of mating and is independent of sperm, while the
251 latter can extend beyond 10 days post-mating but requires sperm (the ‘sperm effect’) (Manning,
252 1962, 1967). It has since been shown that SP binds to both the head and tail of sperm via the
253 N-terminus region of the peptide (Peng et al., 2005), a process that depends upon the action of
254 an interdependent network of SFPs (Findlay et al., 2014; Ram & Wolfner, 2007a, 2009; Sitnik

255 et al., 2016). SP's C-terminus, which induces much of the female post-mating response
256 (Schmidt et al., 1993), is gradually released from the tails of stored sperm by cleavage of SP at
257 a trypsin site by an unknown protease (Peng et al., 2005). Stimulation of juvenile hormone
258 synthesis, which is achieved by the peptide's N-terminus, is thought to be carried out by free
259 SP in the ejaculate, prior to sperm-binding (Liu & Kubli, 2003; Moshitzky et al., 1996).

260

261 **b). Ovulin of *Drosophila melanogaster***

262 Ovulin, a 264-amino acid prohormone-like seminal protein (Monsma & Wolfner, 1988), is one
263 of the most rapidly-evolving proteins in *Drosophila* (Aguade et al., 1992). Found in both
264 secretory cell types in the male's accessory gland (Monsma et al., 1990), ovulin is transferred
265 to females during mating, where it acts to increase ovulation rate (Herndon & Wolfner, 1995).
266 In particular, it induces the ovulation of mature oocytes that have accumulated in the unmated
267 female, clearing the way for the increased oogenesis triggered by SP (Chapman et al., 2001).

268

269 During mating, some ovulin enters the mated female's circulation (Monsma et al., 1990); the
270 remainder stays within her reproductive tract, localizing primarily to the upper oviduct and the
271 base of the ovaries (Heifetz et al., 2000). In the female reproductive tract, ovulin is cleaved by
272 the seminal metalloprotease Semp1 (LaFlamme et al., 2012). During ejaculation, Semp1 is
273 activated by the serine protease SFP seminase as it transits through the male reproductive tract
274 (LaFlamme et al., 2014). Semp1 then cleaves ovulin only after it is transferred to the female
275 (LaFlamme et al., 2012) (Figure 2). Full-length ovulin and two of its cleavage products
276 stimulate ovulation (Heifetz et al., 2005).

277

278 Ovulin increases octopaminergic signaling in the female's reproductive tract, although its site
279 of action is unknown (Rubinstein & Wolfner, 2013). As noted in section Modulation of female

280 anatomy, this leads to relaxation of the oviduct musculature, changing oviduct conformation,
 281 thus facilitating ovulation (Mattei et al., 2015). Although ovulin is only detectable in the mated
 282 female for a few hours after mating, its action increases the number of boutons made by
 283 octopaminergic neurons on the oviduct musculature (Rubinstein & Wolfner, 2013). Thus,
 284 ovulin's effects can in theory persist long after it has disappeared. However, its effects are only
 285 detectable on the first day post-mating; after that, other factors, such as SP activity (Chapman
 286 et al., 2003; Liu & Kubli, 2003), account for the increased egg production.
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 288

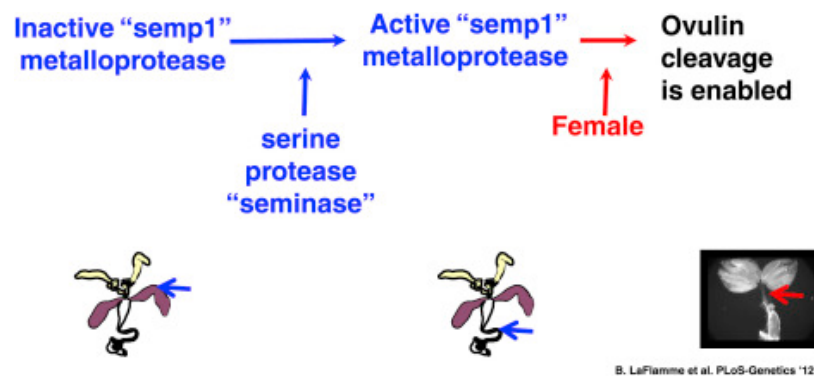


Figure 2. The proteolytic pathway that cleaves ovulin. The seminal serine protease *seminase* cleaves the inactive precursor form of the seminal metalloprotease *Semp1* as the two move through the male during ejaculation. This cleavage activates *Semp1*, but the latter only cleaves its target, ovulin, once the proteins have entered the female. Arrows on the diagrams or photographs below the pathway show where the cleavages occur.

289

290 c). 20-hydroxyecdysone of *Anopheles gambiae*

291 Although SFPs are the major mediator of female post-mating phenotypes in *Drosophila* and
 292 most insects that have been studied, in *An. gambiae* the steroid hormone 20-hydroxyecdysone
 293 (20E) acts as a major effector of post-mating changes (Gabrieli et al., 2014). 20E is a common

294 insect ecdysteroid that regulates molting in juvenile stages (Yamanaka et al., 2013) and
295 multiple processes in adults, including controlling lifespan (Tricoire et al., 2009), learning
296 (Geddes et al., 2013), sleep and social interactions (Ishimoto & Kitamoto, 2011).

297

298 *An. gambiae* males produce 20E in their accessory glands and pass it to the female reproductive
299 tract during mating (Pondeville et al., 2008). Sexual transfer of 20E sets off a series of events
300 that ends with the inhibition of female remating (Gabrieli et al., 2014) and the induction of
301 oogenesis and oviposition (Baldini et al., 2013; Gabrieli et al., 2014). Furthermore, the mating-
302 induced increase in oogenesis simultaneously decreases the efficiency of the *An. gambiae*
303 immune system to kill the malaria-causing *Plasmodium* parasites (Rono et al., 2010).

304

305 Within the female *An. gambiae* reproductive tract, degradation of the mating plug occurs over
306 a similar timeframe to the reduction in 20E level (Baldini et al., 2013). Given that sexual
307 transfer of 20E stimulates oviposition (Gabrieli et al., 2014), that large titers of 20E are
308 transferred to females within the mating plug (Baldini et al., 2013), and the timing of the plug's
309 degradation within the female, it may be that the plug acts as a store of 20E, gradually releasing
310 the hormone following its transfer to females.

311

312 Interestingly, mating plug formation and 20E transfer may have coevolved in anopheline
313 mosquitoes. Species that transfer high levels of 20E during mating produce a fully coagulated
314 mating plug, and these species tend to be found in global regions where malaria transmission
315 is high. Conversely, the species that have been studied from areas with low malaria
316 transmission rates neither form a plug nor transfer 20E to females during mating (Mitchell et
317 al., 2015). This has led to the suggestion that the divergent sexual transfer of 20E has influenced
318 the ability of anopheline species to transmit malaria.

319

320 **4) The diversity of insect reproductive glands**

321 A typical insect male reproductive system consists of an ejaculatory duct, with an ejaculatory
322 bulb at its base, and testes and accessory glands branching off from its proximal end. Products
323 of these tissues meet during passage through the ejaculatory duct *en route* to the female.

324

325 Across insects, the accessory glands are the most studied contributor to the ejaculate. There is
326 great morphological diversity among these glands (Figure 3), ranging from *Drosophila*'s two
327 lobes to the house cricket's (*Acheta domesticus*) tangled mass of several hundred tubules
328 (Chen, 1984). Even within a single family, such as *Diptera*, there is considerable
329 morphological diversity of accessory glands (Throckmorton, 1962).

330

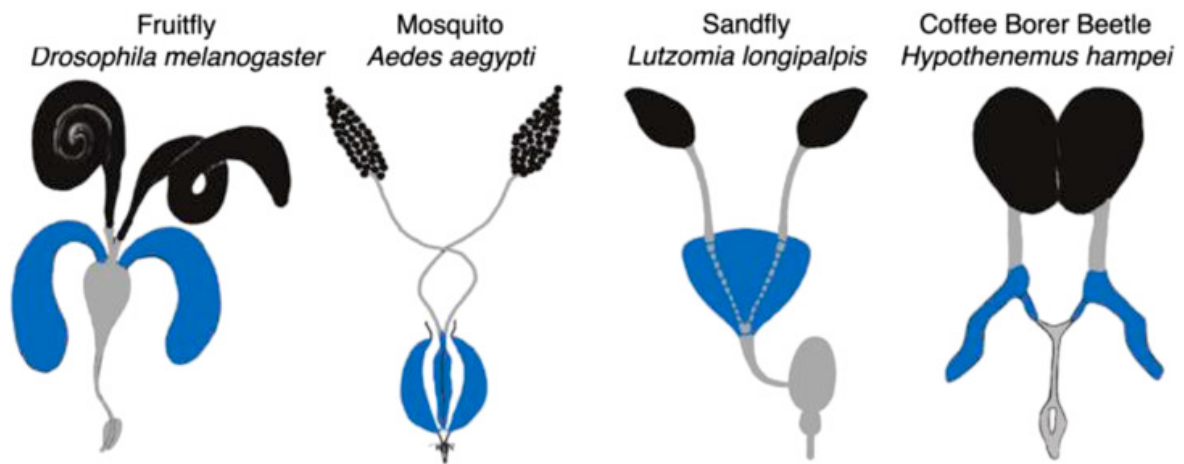


Figure 3. Diagram of the male reproductive tracts of several insects, with reproductive glands shown in blue. Within-tract views are drawn to-scale. As noted in the text, male reproductive glands' developmental origins differ among insects so the blue tissues, although analogous in function, are not necessarily homologous.

Drawing by S. Suarez is reprinted from McGraw, L.A., Suarez, S.S., Wolfner, M.F., *et al.*, 2015. *Bioessays* 37, 142–147 with permission.

331

332 Species vary in accessory gland number. For example, male mealworm beetles (*Tenebrio*
333 *molitor*) contain a pair of tubular accessory glands as well as a separate pair of bean-shaped
334 accessory glands, whereas males of *Drosophila* species and the Colorado potato beetle
335 (*Leptinotarsa decemlineata*) each have just a single pair of accessory glands (PChen, 1984).
336 This morphological diversity is paralleled in the developmental origins of insect accessory
337 glands, which may be ectodermal, mesodermal, or both (Leopold, 1976). Indeed, in the
338 Queensland fruit fly (*Bactrocera tryoni*), males have a pair of highly-innervated, sac-shaped
339 mesoderm-derived glands as well as three or four pairs of spongy, distally bifurcated
340 ectodermic glands (Radhakrishnan et al., 2009).

341

342 The cellular complexity of accessory glands is also variable. For example, the bean-shaped
343 glands of *T. molitor* contain seven distinct secretory cell types, whereas the accessory glands
344 of *Drosophila* and *Leptinotarsa decemlineata* contain two and one visibly different cell type
345 respectively (Chen, 1984). The accessory glands of *Ae. aegypti* have anterior and posterior
346 portions that differ in cell-type and density, as well as in the proteins - and potentially other
347 secretory materials - they produce and contain (Alfonso-Parra et al., 2014; Dapples et al.,
348 1974). Transplantation experiments have demonstrated that the anterior accessory gland cells
349 contain the active molecule(s) that elicit the post-mating female phenotypes of inhibited re-
350 mating and increased egg-laying (Ramalingam & Craig, 1978). Less is known about the
351 posterior cells of the *Ae. aegypti* accessory gland, but they have been suggested to secrete a
352 mucus-like substance that binds granules secreted from the anterior cells (Ramalingam, 1983).

353

354 Different reproductive glands, as well as different cell types in these glands, work
355 interdependently to produce the seminal fluid. For example, the *D. melanogaster* mating plug
356 includes contributions from at least the ejaculatory bulb and accessory glands (Avila et al.,
357 2015; Bretman et al., 2010; O Lung & Wolfner, 2001). In another case, spermatophores in
358 beetles, crickets, and moths are composed of layers derived from the secretions of different
359 reproductive glands (Grimnes et al., 1986; Meslin et al., 2017). Among species within the
360 suborder *Ensifera*, the ‘rough glands’ produce the larger spermatophylax component of the
361 spermatophore, while the ‘smooth glands’ produce the ampulla, which bears the sperm (Lewis
362 & South, 2012). In the cabbage white butterfly (*Pieris rapae*), both the accessory glands and
363 the distal section of the mating duct contribute to the soft inner matrix of the spermatophore,
364 whereas the proximal region contributes most to the tough outer envelope (Meslin et al., 2017).

365

366 **5) The accessory glands of *Drosophila melanogaster***

367 Each lobe of the *D. melanogaster* male accessory gland is an epithelium that consists of a single
368 layer of ~1000 binucleate secretory cells, encased by a muscular sheath (Figure 4) (Bairati,
369 1968). The secretory cells belong to two morphologically and biochemically distinct types,
370 whose development is differentially specified by the action of Hox, or other homeodomain,
371 proteins (Bertram et al., 1992; Federer & Chen, 1982; Gligorov et al., 2013; Kalb et al., 1993;
372 Monsma et al., 1990). Small, flattened, and hexagonal in shape, the ‘main cells’ account for
373 96% of the secretory cells. Main cells produce SP (DiBenedetto et al., 1990), and several other
374 SFPs including the proteases Semp1, seminase, and CG9997, as well as other proteins
375 important for the binding of the sex peptide to sperm (DiBenedetto et al., 1990; Findlay et al.,
376 2014; Gligorov et al., 2013; Ram & Wolfner, 2009). At the distal tips of the accessory glands,
377 ~40 secondary cells protrude into the lumen (Bertram et al., 1992). These large, spherical cells

378 contain numerous vacuoles as well as secretory granules and filamentous bodies that resemble
 379 structures found among sperm in the female storage organs (Bairati, 1968; Monsma et al.,
 380 1990; Perotti, 1971). Secretion granule-like structures have also been observed in the accessory
 381 gland lumens of a number of insects, including species of leafhoppers (Zhang et al., 2016),
 382 bees (Cruz-Landim & Dallacqua, 2005), and mosquitoes (Chen, 1984).
 383

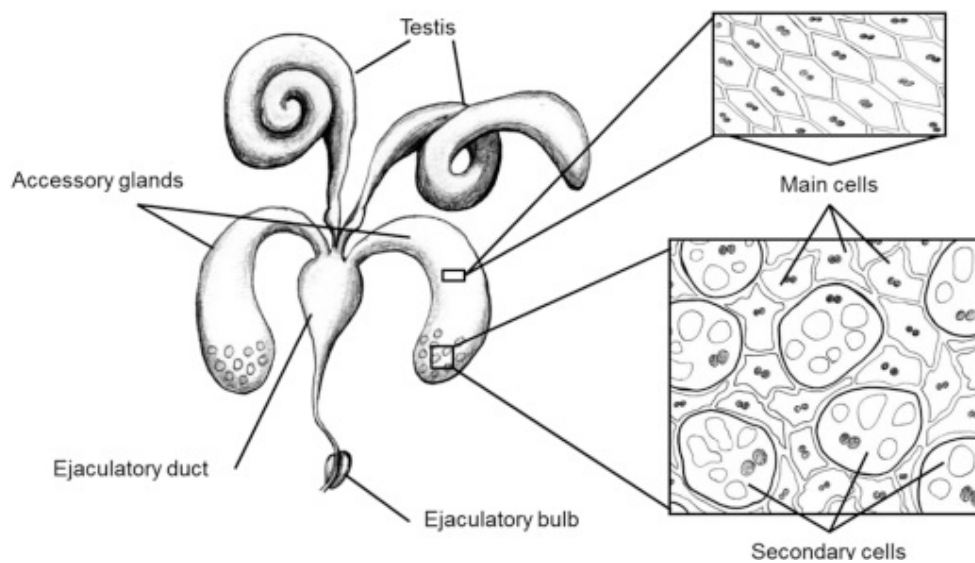


Figure 4. The *Drosophila melanogaster* male reproductive tract. This drawing, approximately to scale, shows the male reproductive glands and testes. Insets show the position and appearance of the two secretory cell types of the male's accessory glands; nuclei in these binucleate cells are shaded.

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384 Secondary cells produce a cell-type specific set of SFPs including some of those required for
 385 the sperm-binding and storage of SP (Gligorov et al., 2013; Minami et al., 2012; Sitnik et al.,
 386 2016). Unlike main cells, secondary cells continue to grow throughout the life of the adult fly
 387 and, in older multiply-mated males, can delaminate from the apical surface of the epithelium,
 388 migrate towards the proximal end of the gland, and eventually can be transferred to females in

389 the ejaculate (Leiblich et al., 2012). These processes are under the control of bone
390 morphogenetic protein (BMP) signalling and seem to be accelerated by age (as well as mating
391 frequency)(Leiblich et al., 2012). The age-dependent growth and delamination has led to the
392 suggestion that the *Drosophila* accessory gland, and its secondary cells in particular, can serve
393 as a model for the human prostate (Wilson et al., 2017).

394

395 BMP-signalling in the secondary cells also drives the secretion of small, ~30-100nm diameter,
396 extracellular vesicles (exosomes) and secretory organelles termed dense core granules
397 (Corrigan et al., 2014; Leiblich et al., 2012; Redhai et al., 2016). Each cell contains ~10 dense
398 core granules, of which approximately 4 are lost during mating and replenished within ~24
399 hours (Redhai et al., 2016). Blockage of BMP-mediated secretion in secondary cells impairs
400 the male's ability to decrease remating by his mate, but does not affect his ability to stimulate
401 her egg production (Corrigan et al., 2014; Leiblich et al., 2012). At least part of these effects
402 have been suggested to depend on secondary cell exosomes, which have been reported to fuse
403 with sperm once inside the female and to associate with female reproductive tract tissues
404 (Corrigan et al., 2014). Such fusion could potentially introduce RNAs, proteins, or other
405 molecules to the sperm or female tissues, thereby influencing the female's post-mating
406 response. This model is based on studies in mammals, where the contents of exosomes secreted
407 by malignant cells have been reported to prime nearby tissues for metastatic invasion (Hoshino
408 et al., 2015; Rak, 2015), and where exosomes from male reproductive tissues have been shown
409 to fuse with sperm and affect sperm activation, capacitation, and motility (Aalberts et al.,
410 2013). These 'epididymosomes' and 'prostasomes' can deliver regulatory RNA species
411 (including tRNA fragments, Y RNAs and microRNAs), as well as growth factors and cytokines
412 (Aalberts et al., 2013; Colombo et al., 2014; Vojtech et al., 2014), in combinations sensitive to

413 the male's rearing environment and have been suggested to transmit information
414 transgenerationally (Q. Chen et al., 2016; Qi Chen et al., 2016; Sharma et al., 2016).

415

416 **6) Evolution of insect reproductive glands and their products**

417 Several aspects of reproductive gland biology are commonly found among insects: the
418 existence of accessory glands, along with other types of reproductive gland; the biochemical
419 classes of the molecules that these glands produce; and the role of these products in inducing
420 important post-mating responses such as increased egg laying, ovulation, immune gene
421 expression, and feeding (Avila et al., 2011; Gillott, 2003; Hopkins et al., 2017; Mueller et al.,
422 2004; Poiani, 2006). Although many of the effects induced by seminal secretions appear to
423 benefit both sexes in terms of their regulated fertility, some actions of seminal proteins can be
424 deleterious to the female, decreasing her longevity or manipulating her behavior in ways that
425 do not seem to serve her interests. To paraphrase Dobzhansky (Dobzhansky, 1973), in order to
426 fully appreciate the biology of insect reproductive glands, we must understand the forces that
427 have guided their evolution. These forces include:

428

429 **a). Sexual selection**

430 Due to their importance for reproductive success, male reproductive processes have been
431 subject to strong and divergent selection. This concept of 'selection in relation to sex' was
432 first laid out by Darwin 12 years after the publication of his theory of evolution by natural
433 selection. 'Sickened' by the peacock's exaggerated plumage, Darwin sought to understand
434 why such elaborate and seemingly deleterious traits can be maintained in populations. His
435 solution came through focusing not on an individual's struggle for survival, but rather on its
436 struggle for matings (Darwin, 1871). Sexual selection theory is built on these conceptual
437 foundations, positing that evolutionary change can be driven by (a) competition between

438 individuals of one sex for access to individuals of the other (intrasexual selection), and (b)
439 preferences held by members of one sex for certain features in the other (intersexual
440 selection).

441

442 **b). Post-copulatory sexual selection: sperm competition**

443 In 1970, Geoff Parker recognized that if a female mates multiply, as occurs in many insect
444 species, a male's reproductive success depends on more than just his ability to compete for
445 a mate (Parker, 1970). In such polyandrous mating systems, the opportunity for sexual
446 selection continues after ejaculation through 'sperm competition', the competition between
447 sperm from rival males for access to oocytes. In recent years, the development of *Drosophila*
448 lines expressing green- or red-fluorescent proteins in sperm heads has facilitated the
449 visualization of this process in these insects (Figure 5).

450

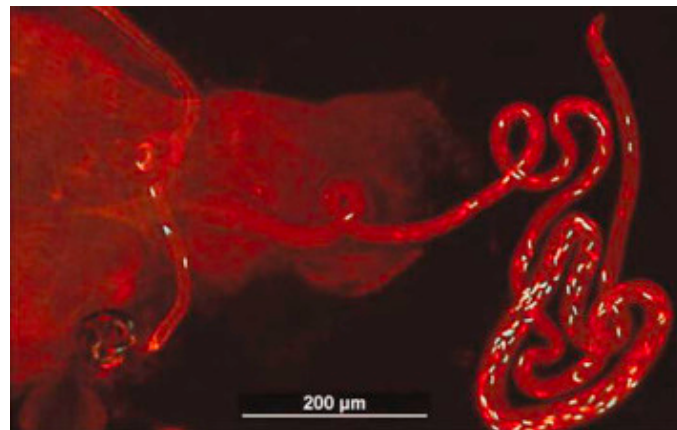


Figure 5. Visualizing sperm competition in *Drosophila*. Sperm from two males are seen within the seminal receptacle of a doubly-mated female. The heads of the sperm from the first male are green (due to protamine-GFP); from the second male are red (protamine-RFP).

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451

452 Many traits promote the competitiveness of a male's ejaculate: changes to sperm
453 morphology to promote faster swimming (even including the formation of motility-boosting
454 aggregations) (Fisher & Hoekstra, 2010; Higginson & Pitnick, 2011), specialized genital
455 morphologies that can physically displace a rival's sperm (Gallup et al., 2003; Waage, 1979,
456 1986), or the physical guarding of a female from rival males (Mazzi et al., 2009). Products
457 of the male's reproductive glands also contribute to these phenomena by affecting sperm
458 storage and viability in females, potentially disabling the sperm of a rival (S. P A den Boer
459 et al., 2010), and by a chemical form of mate-guarding: rendering a female unreceptive to
460 remating (Tracey Chapman et al., 2003; Liu & Kubli, 2003) or stopping her production of
461 'calling' pheromones, thus preventing other males from being attracted to her (Fan et al.,
462 1999; Kingan et al., 1993; Nagalakshmi et al., 2007; Raina et al., 1994).

463

464 **c). Post-copulatory sexual selection: cryptic female choice**

465 The female, however, does not present a passive environment in which sperm competition
466 plays out. Instead, she provides a partisan space in which the outcome of competition
467 between rival males can be influenced in accordance with her own distinct evolutionary
468 interests (Birkhead & Pizzari, 2002; W. G. Eberhard, 2009; William G. Eberhard, 1996;
469 Firman et al., 2017; Thornhill, 1983). This process, therefore, represents the post-copulatory
470 analogue of inter-sexual selection and is named cryptic female choice (William G. Eberhard,
471 1996; Firman et al., 2017; Thornhill, 1983). Males are expected to evolve mechanisms in
472 response to female choice processes to boost the probability that their sperm will be

473 preferentially retained for use in fertilizations. The roles of seminal fluid components in
474 modulating female insect behaviors (such as sexual receptivity and sperm ejection (Lee et
475 al., 2015; Lupold et al., 2013)), reproductive tract conformation, physiology, and immunity
476 (Firman et al., 2017) were likely shaped by their acting within a dynamic female
477 environment.

478

479 **d). Sexual conflict**

480 Selection for traits that promote the success of a male in sperm competition can result in
481 traits that cause collateral damage to females (Parker, 2006; Tatarnic et al., 2014). This is
482 thought to apply in the case of *Drosophila* SP, which limits female remating and thus sperm
483 competition risk, but has toxic effects that shorten the female's lifespan (Wigby & Chapman,
484 2005). Additional SFPs, such as the *Drosophila* protease inhibitor Acp62F, have also been
485 shown to be toxic to females (Lung et al., 2002; Wolfner, 2002).

486

487 Conflict can even arise between the interests of males and females long after mating.
488 Individuals have a finite quantity of resources to allocate to reproduction (Curio, 1983;
489 Parker et al., 2002; Stearns, 1992; Trivers, 1972). How a female should best partition these
490 resources across successive breeding attempts depends on factors including mate quality,
491 reproductive experience, and age (Curio, 1983; Horváthová et al., 2012; Sheldon, 2000).
492 For example, her best strategy may be to withhold some resources in a given mating to
493 allocate to her own immunocompetence, foraging ability, and/or somatic maintenance, thus
494 boosting the probability that she survives to mate with a subsequent (possibly more fit) male
495 (Adkins-Regan & Tomaszycki, 2007; Krakauer & Johnstone, 1995). However, the optimal
496 outcome for her mate is that she should invest at a high level in their brood, increasing the

497 number or viability of his offspring. Thus, it is often not possible to simultaneously
498 maximize the fitness of both male and female, giving rise to evolutionary tension.

499

500 These conflicts can lock males and females into co-evolutionary arms races, each
501 responding over evolutionary time to the other's adaptations (Holland & Rice, 1999;
502 Holland & Rice, 1998; Hosken et al., 2001; Parker, 2006; Rice, 1996; Wigby & Chapman,
503 2004). For example, if males transfer seminal fluid components that lead to females over-
504 investing in a current reproductive effort, selection for a counter-response could lead to
505 reduced sensitivity of females to male molecules or mechanisms to neutralize the effects of
506 those molecules. This, in turn, could drive renewed selection on males for new or stronger
507 molecules to produce the male-beneficial effect. More complicated three-way evolutionary
508 arms races arise when selection frees females from male control over their mating rate. This
509 may intensify the degree of sperm competition, selecting for seminal fluid components in
510 males that promote their sperm competitive success, but harm females in the process. These
511 co-evolutionary processes are thought to contribute to rapid evolution in the primary
512 sequence of a significant fraction of SFPs (Aguade et al., 1992; Findlay et al., 2009; Haerty
513 et al., 2007; Swanson et al., 2001; Swanson & Vacquier, 2002). The arms races may also
514 help explain the diversity of seminal fluid products, evolving in response to female counter-
515 evolution (Sirot et al., 2017).

516

517 The conflicts described occur in the context of a process, reproduction, that also requires
518 cooperation between the sexes: coordination is required between male and female cells and
519 molecules, for egg-sperm binding, or for the processing of certain SFPs in *D. melanogaster*
520 by molecules from both sexes (Avila & Wolfner, 2017; Aydin et al., 2016; Bianchi et al.,
521 2014; Findlay et al., 2014; Inoue et al., 2005; Laflamme & Wolfner, 2013; Ravi Ram et al.,

522 2006; Yapici et al., 2008). The extent to which conflict should manifest in reproductive
523 processes will depend upon the degree of asymmetry in the fitness interests of interacting
524 parties. In species with lifetime monogamy (where that monogamy is not enforced by one
525 sex against the evolutionary interests of the other (Hosken et al., 2009)), male and female
526 fitness interests will align closely. Moreover, conflict traits may not evolve even when male
527 and female fitness interests diverge: the extent of the costs and benefits associated with
528 antagonistic traits, as well as the opportunity for such traits to evolve (e.g. presence of
529 sufficient genetic variation), can constrain the manifestation of conflict (Chapman, 2006).

530

531 **e). Phenotypic plasticity**

532 Depletion of sperm and SFPs over successive matings constrains male fitness. In *D.*
533 *melanogaster*, for example, SFP replenishment after mating can take ~3 days (Sirot et al.,
534 2009). Therefore, selection should favor the male's allocating these products in line with the
535 reproductive context in which he finds himself. Consistent with this hypothesis, males have
536 evolved to strategically allocate sperm and SFPs to females in response to factors such as
537 female mating status, sexual novelty, and quality (Lüpold et al., 2011; Pizzari et al., 2003;
538 Price et al., 2012; Sirot et al., 2011; Wedell et al., 2002; Wigby et al., 2009). In *D.*
539 *melanogaster*, males transfer less ovulin when mating with recently mated females (Sirot,
540 Wolfner, et al., 2011), but more sex peptide and ovulin when mating in the presence of rival
541 males (Wigby et al., 2009).

542

543 **Concluding remarks**

544 Beyond the sperm and egg, a diversity of seminal fluid molecules are essential for the
545 reproductive success of insects, as in many other animals. Seminal fluid components regulate
546 processes that move sperm through the female reproductive tract and into storage, modify the

547 reproductive tract to facilitate egg production and transit, and influence feeding and digestion
548 to assist in high levels of egg production. Effects of seminal fluid components on females may
549 be beneficial to both members of the mating pair, such as by coupling increased egg production
550 to mating. Alternatively, they may primarily benefit the male, as is likely the case in their
551 induction of female refractoriness to remating. Seminal fluid molecules are produced in male
552 reproductive glands, which themselves vary across insects in number, structure, and cellular
553 constitution. Ejaculate molecules fall into biochemically-conserved classes, but their sequences
554 have come under strong selection to boost reproductive success under sperm competition
555 situations or to co-evolve with reproductive processes in females. Nevertheless, the overlap in
556 molecular and organellar types in the seminal fluid of insects and other taxa makes it
557 increasingly apparent that the study of insect accessory glands provides significant and broad
558 insights into mechanisms of fertility, has implications for our understanding of human male
559 reproductive gland health and disease, and offers powerful opportunities for targeted control
560 of insect disease vectors and pests.

561

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