A novel role for *Eip74EF* in male reproduction in promoting sperm elongation and male fecundity

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Running Head: *Eip74EF* promotes sperm length

Abbreviations:

Eip74EF - Ecdysone induced protein 74EF

GFP - green fluorescent protein

NCBI - National Center for Biotechnology Information

PAML - Phylogenetic Analysis by Maximum Likelihood

PBS - phosphate buffered saline

REML - restricted maximum likelihood

SR - seminal receptacle

ABSTRACT

Spermatozoa are the most morphologically variable cell type, yet little is known about genes controlling natural variation in sperm shape. *Drosophila* fruit flies have evolved the longest sperm known, which are evolving under postcopulatory sexual selection, driven by sperm competition and cryptic female choice. Long sperm outcompete short sperm but primarily when females have long seminal receptacles (SRs), the primary sperm storage organ. Thus, selection on sperm length is mediated by SR length, and the two traits are coevolving across the *Drosophila* lineage, driven by a genetic correlation and fitness advantage of long sperm and long SR genotypes in both males and females. *Ecdysone induced protein 74EF* (*Eip74EF*) is rapidly evolving under positive selection in *Drosophila*, and it is expressed during post-meiotic stages of spermatogenesis, when spermatid elongation occurs. Partial knockdown of *Eip74EF* leads to shorter sperm but does not affect SR length, suggesting that *Eip74EF* is involved in promoting spermatid elongation but is not a genetic driver of male-female coevolution. We also found that *Eip74EF* knockdown has opposing effects on fecundity in males and females, with an increase in fecundity for males but a decrease in females, consistent with its documented role in oocyte maturation. It is possible that knockdown males produce more sperm that are also shorter, which would explain the increase in fecundity, but this hypothesis remains to be tested. Our results document a novel function of *Eip74EF* in spermatogenesis and demonstrates that this gene influences both male and female reproductive success.

Keywords: *Eip74EF*, fecundity, fitness, seminal receptacle, sperm competition

1. INTRODUCTION

Spermatozoa are arguably the most variable cell type among metazoans, exhibiting dramatic variation in size and shape to encompass all possible permutations of presence or absence of a head, midpiece, or flagellum (Pitnick, Hosken, & Birkhead, 2009). With all this morphological variation, it is not surprising that sperm can evolve rapidly on short time scales (Pitnick, Miller, Schneider, & Markow, 2003; Manier & Palumbi, 2008; Lüpold, Westneat, & Birkhead, 2011; Stewart, Wang, & Montgomerie, 2016), with morphological differences emerging even over the course of invasive expansion into new environments (Kahrl & Cox, 2017). Sperm morphology can influence swimming speed (Blengini, Sergio, Gabriela, Giojalas, & Margarita, 2014; Lüpold, Calhim, Immler, & Birkhead, 2009; Firman & Simmons, 2010; Fitzpatrick, Montgomerie, Desjardins, Stiver, Kolm, & Balshine, 2009) and fertilization success (Bennison, Hemmings, Slate, & Birkhead, 2015), and its rapid evolution has been credited to postcopulatory sexual selection via sperm competition (Parker, 1970; Snook, 2005; Pitnick et al., 2009; Godwin, Vasudeva, Michalczyk, Martin, Lumley, Chapman, & Gage, 2017) and cryptic female choice (Eberhard 1986; Firman, Gasparini, Manier, & Pizzari, 2017).

Within the *Drosophila* lineage, sperm length exhibits among the greatest range of phenotypic variation within a clade, spanning over two orders of magnitude from 320 μm in *D. persimilis* to 58,290 μm in *D. bifurca* (Pitnick, Markow, & Spicer, 1995). Moreover, in the obscura group, males produce heteromorphic sperm, meaning that they come in two (Snook, 1997) or three (Alpern, Asselin, & Moehring, 2019) different sizes within an ejaculate. Sperm length evolution in *Drosophila* is mediated by both length of the female's primary sperm storage organ, the seminal receptacle or SR (Miller & Pitnick, 2002; Lüpold, Reil, Manier, Zeender, Belote, & Pitnick, *in review*), a genetic correlation with SR length (Lüpold, Manier, Puniamoorthy, Schoff,

73 Starmer, Buckley Luepold, Belote, & Pitnick, 2016), and moderate fitness advantage of a long
74 sperm genotype in both males and females (Zajitchek, Zajitschek, Josway, Al Shabeeb, Weiner,
75 & Manier, *in press*). As a result of this tight interaction between sperm length and SR length, the
76 two traits are coevolving both across species (Pitnick, Markow, & Spicer, 1999) and among
77 populations undergoing incipient speciation (Pitnick et al., 2003).

78 Little is known about the genetic basis of natural variation in sperm length, but we expect
79 genes controlling this trait to be rapidly evolving. In a screen for *Drosophila* genes showing
80 signatures of rapid adaptive evolution under positive selection, we identified *Ecdysone-induced*
81 *protein 74EF* (*Eip74EF*). This gene also has increased expression during post-meiotic stages of
82 spermatogenesis (https://mnlab.uchicago.edu/sppress/; Vibranovski, Lopes, Karr, & Long,
83 2009), when spermatid elongation occurs. *Eip74EF* is best understood in its roles in activating
84 programmed cell death of salivary glands during metamorphosis (Jiang, Lamblin, Steller, &
85 Thummel, 2000; Lee & Baehrecke, 2001) and promoting oocyte maturation (**Kozlova** &
86 Thummel, 2000). While it is expressed in testis (Vibranovsky et al., 2009), it has no known
87 function in spermatogenesis, and neither isoform Eip74A nor Eip74B is expressed in the sperm
88 proteome (Dorus, Busby, Gerike, Shabanowitz, Hunt, & Karr, 2006).

89 Here, we explored the role of *Eip74EF* in sperm length. Because sperm length and SR
90 length are genetically correlated (Lüpold et al., 2016) and coevolving (Pitnick et al., 1999; 2003),
91 we also measured SR length in *Eip74EF* knockdown females to look for pleiotropic effects on
92 both sperm length and SR length. We found that knockdown of *Eip74EF* resulted in shorter
93 sperm, so we also examined mating behavior and sperm competitive success in knockdown
94 males and fecundity in both males and females.

95

96

97 2. MATERIALS AND METHODS

98

99 **2.1 Stocks and crosses**

100 *Eip74EF* has only one major protein isoform expressed in adults, E74A (Liu, Landreh, Cao, Abe,
101 Hendriks, Kennerdell, Zhu, Wang, & Bonini, 2012). For this study, we used a hypomorphic
102 mutant of *Eip74EF* obtained from the Bloomington Drosophila Stock Center (stock #12619) that
103 has partial reduction of expression of E74A while retaining viability (Liu et al., 2012). The
104 mutant allele ($E74A^{BG01805}$) contains a *p*(*GT1*) transposon insertion in the sixth intron, within a
105 w^{1118} background. Experimental animals were reared from breeding vials containing 10 virgin
106 females and 20 virgin males that were transferred to new sugar-yeast-agar food vials daily for
107 four days, after which adults were discarded. Control animals were reared from breeding vials
108 containing 10 $E74A^{BG01805}$ females and 20 w^{1118} males. Progeny were collected within 8-12 hours
109 of eclosion using light CO_2 anesthesia into single-sex vials at densities of 10 females or 20 males
110 per vial. For both experimental and control treatments, progeny from two replicate sets of
111 breeding vials out of four potential replicates per treatment were phenotyped for all
112 experiments.

113 For reproductive success experiments, competitor males derived from a Canton-S stock
114 expressing a 3xP3-GFP construct in eye. These Canton-S Protamine-GFP lines were generated
115 using the piggybac germline transformation system (Handler and Harrell, 1999). The GFP-
116 tagged Protamine-B construct was cut out of the *P*-element plasmid construct pW8/ProtB-GFP1
117 (Manier, Belote, Berben, Novikov, Stuart, & Pitnick, 2010) with *Kpn*I and *Bam*HI and subcloned
118 into the pBS/2xAsc vector, a modified pBlueScript-KS+ plasmid (Stratagene, La Jolla, CA) in
119 which two *Asc*I sites, flanking the multiple cloning site, had been created by site-directed
120 mutagenesis. The insert was then cut out with *Asc*I and ligated into the single *Asc*I site of the

121 piggybac vector p3xP3/EGFPaf (Horn & Wimmer, 2000) to yield p3xP3-EGFP/ProtB-GFP. This
122 DNA was mixed with the helper plasmid pBΔSac (Handler & Harrell, 1999) and injected into
123 preblastoderm embyos from a Canton-S wild-type strain (generously provided by Mariana
124 Wolfner, Cornell University). Transformants were identified by green fluorescent pseudopupils
125 and ocelli. Two tagged lines were generated: *Canton-S pBac{3xP3-EGFP, ProtB-EGFP}27* and
126 *Canton-S pBac{3xP3-EGFP, ProtB-EGFP}16B* with the transgenes inserted on chromosome 2 and
127 chromosome 3, respectively. Although this stock (hereafter "Canton-S") contained a transgene,
128 the fluorescent eye and sperm markers were not specifically utilized in this series of
129 experiments.
130
131 **2.2 Sperm measurements**
132 To obtain sperm length measurements, seminal vesicles from 5-day-old ether-anesthetized
133 males were dissected into a large droplet of 1X phosphate-buffered saline (PBS) on a gelatin-
134 coated glass slide, using fine forceps. The seminal vesicle was ruptured and dragged several
135 times through the droplet to release and spread motile sperm. The slide was then dried down at
136 55 °C in a drying oven, fixed in 3:1 methanol:acetic acid, stained with DAPI (Molecular Probes),
137 and mounted under a coverslip in 80% glycerol in PBS and sealed with nail polish. We then
138 visualized sperm using darkfield microscopy at 200X and sperm heads using fluorescence
139 microscopy at 400X on a Nikon Ni-U microscope and captured images with a Zyla Andor
140 monochrome camera. Images were measured using the segmented line tool in ImageJ software
141 (https://imagej.nih.gov/ij/). Up to five sperm and sperm heads were measured for each of up
142 to 8 males across two replicates for both the mutant and control treatments.
143
144 **2.3 SR length**

145 Females were aged 5 days and then frozen in their food vials until dissection. Females were
146 then thawed, and thorax length was measured as a proxy for body size using a ruler reticle in a
147 Nikon SMZ-745 stereoscope at maximum magnification. The lower reproductive tract was
148 dissected with fine forceps into a droplet of 1X PBS, and the SR was carefully unraveled using a
149 fine insect pin. The sample was then mounted under a coverslip with clay on the corners to
150 allow compression of the sample into a single plane without overcompression. SRs were
151 immediately visualized at 100X or 200X under phase contrast microscopy, and images were
152 captured using a Canon EOS Rebel T3i camera. For each of two replicates for mutant and
153 control treatments, 10 females were measured.
154
155 **2.4 Fitness**
156 We evaluated the fitness consequences of *Eip74EF* knockdown by quantifying male and female
157 fecundity and male competitive fertilization success in . To measure fecundity, we housed 20
158 males or females per replicate individually with two Canton-S females or males, respectively,
159 for seven days, transferring triads (one focal individual and two mates) into new food vials
160 every two days, without anesthesia. We counted adult progeny that eclosed from all food vials.
161 This approach to counting progeny to assess fecundity does not account for effects of *Eip74EF*
162 on egg-to-adult viability, only on the total number of adult progeny that survived to
163 successfully eclose.
164 We assessed paternity success of mutant and control males by mating a w^{1118} female first
165 with a Canton-S male and allowing her to remate with a mutant or control male. All matings
166 occurred in individual vials and were observed directly, recording copulation duration and day
167 of remating. We scored paternity using eye color, since females, mutant males, and control
168 males carried a recessive white-eye mutation, while Canton-S competitor males had a dominant

169 wild type eye color. Paternity was expressed as P2, or proportion of offspring sired by the
170 second male (here, mutant or control males).
171
172 **2.5 Molecular evolution**
173 We estimated the rate of positive selection on *Eip74EF* as a measure of adaptive molecular
174 divergence using DNA sequences from 22 *Drosophila* species downloaded from NCBI. The
175 codeml program in PAML v4.6 (Yang, 1997; 2007) compares a null model of neutral divergence
176 (M8) with a model of adaptive divergence under positive selection (M8a), based on the ratio
177 dN/dS, which compares the number of non-synonymous substitutions (dN) with the number of
178 synonymous substitutions (dS). $dN/dS = \omega$, and generally $\omega < 1$ points to purifying selection, ω
179 $= 1$ indicates neutrality, and $\omega > 1$ suggests positive selection. However, ω is rarely greater than
180 1, since multiple sites across multiple lineages are unlikely to be under positive selection for an
181 extended period. Thus, ω estimates are conservative indicators of positive selection on codons.
182 The critical values for this test are 2.71 at 5% and 5.41 at 1%, with a null distribution of point
183 mass 0 and χ^2_1 (Self & Liang, 1987).
184
185 **2.6 Statistical analysis**
186 All statistical analyses were performed in R v.3.5.3 (R Core Team, 2013). Total sperm length and
187 sperm head length were analyzed using lmer within library lme4, with knockdown treatment as
188 a fixed effect, male identity nested within replicate as a random effect, and researcher identity
189 as a random effect, with restricted maximum likelihood (REML) parameter estimation. SR
190 length was found to be negatively correlated with body size (thorax length) (Pearson's product-
191 moment correlation, $t_{42} = -2.514$; $P = 0.0159$), so thorax length was included as a random effect in
192 a linear mixed model, with knockdown treatment and replicate as fixed effects, and thorax

193 length as a random effect. SR lengths were all measured by the same individual. Fecundity data
194 were analyzed using lmer with knockdown treatment as a fixed effect and replicate as a random
195 effect, with maximum likelihood parameter estimation, because there was only one random
196 effect. For all analyses implemented in lmer, P-values were estimated using a Type II Wald chi-
197 square test with Anova in the car library. Here, we report knockdown treatment effect sizes as
198 the *t*-value from the mixed model regression, along with the χ^2 and p-value from the Wald's
199 test.

200 From the sperm competition experiment, we also obtained remating rate (proportion of
201 females that remated), copulation duration, and day of remating. Remating rate was analyzed
202 using a chi-square test in two different ways: with replicates as categories (N = 4) and with
203 knockdown treatments as categories (N = 2), both yielding the same result. Copulation duration
204 was analyzed using lmer with knockdown treatment as a fixed effect and replicate as a random
205 effect, with maximum likelihood parameter estimation. We report results as for other lmer
206 analyses described above. For paternity success, we used logistic regression with a logit link
207 function and binomial error distribution (after ensuring no overdispersion in the data),
208 implemented using glm.

209

210

211 3. RESULTS

212

213 *Eip74EF* is rapidly evolving under positive selection, with an M8 model estimate of -20882.5,
214 M8a of -20875.6, and a χ^2 of 13.80, well above the 1% critical threshold of 5.41. Knockdown of
215 *Eip74EF* resulted in shorter sperm (t = -5.341, χ^2_1 = 28.53, P < 0.0001; Figure 1a) but had no effect
216 on sperm head length (t = -1.48, χ^2_1 = 2.191, P = 0.139; Figure 1b) nor female SR length (t = 1.132,

217 χ^2_1 = 1.281, P = 0.258; Figure 1c). Disruption of *Eip74EF* led to an increase in male fecundity (t =
218 3.529, χ^2_1 = 12.45, P = 0.0004; Figure 2a) but a decrease in female fecundity (t = -10.17, χ^2_1 =
219 103.43, P < 0.0001; Figure 2b). In other words, female fecundity was adversely affected while
220 male fecundity was positively affected. In the sperm competition experiment, remating rate
221 (replicates as groups: χ^2_3 = 3.795, P = 0.284; treatment as groups: χ^2_1 = 0.976, P = 0.323), day of
222 remating (t = 0.336, χ^2_1 = 0.113, P = 0.737), copulation duration (t = 0.109, χ^2_1 = 0.012, P = 0.913),
223 and paternity success (P_2; z = 0.163, P = 0.871) were all unaffected by *Eip74EF* knockdown.
224
225
226 4. DISCUSSION
227
228 We found that *Eip74EF* is evolving rapidly under positive selection, a surprising result given
229 that its roles in molting and metamorphosis, highly conserved processes, would suggest that it
230 should be under strong purifying selection. However, we identified a novel role for *Eip74EF*
231 during sperm morphogenesis, in the positive regulation of sperm length, a rapidly evolving
232 trait. *Eip74EF* is best understood as part of the ecdysone signaling pathway, with spikes in
233 expression associated with larval molts (Bodai, Zsindely, Gáspár, Kristó, Komonyi, & Boros,
234 2012) and puparium formation (Fletcher & Thummel, 1995). However, *Eip74EF* has also been
235 implicated in a wide variety of other developmental processes, including oogenesis (Kozlova &
236 Thummel, 2000), autophagic programmed cell death in salivary glands during metamorphosis
237 (Lee & Baehrecke, 2001), ageing-related processes in the brain (Liu et al., 2012), and silk
238 production in the silk moth *Bombyx mori* (Zhang, Cheng, Jin, Guo, Wu, Liu, Xu, Sun, Li, He, &
239 Xia, 2017). The ecdysone signaling pathway more generally influences spermatogenic cyst
240 differentiation through antagonistic interactions with EGF signaling (Qian, Dominado, Zoller,

241 Ng, Kudyba, Siddall, Hime, & Schulz, 2014), but it is unknown if *Eip74EF* is specifically
242 involved in this process.

243 *Eip74EF* is an E26 transformation specific (ETS) domain transcription factor, with little
244 known about the downstream genes that it regulates. We do know that Eip74EF is not present
245 in the sperm proteome (Dorus et al., 2006) and is therefore not directly involved in sperm
246 structure, motility, or physiology, but it may regulate expression of the sperm proteome. There
247 are two primary Eip74EF isoforms (A and B) whose expression is controlled by alternate
248 promoters and transcription start sites. However, both isoforms have overlapping functions in
249 larval development and metamorphosis (Bodai et al., 2012).

250 On the face of it, it is curious that knockdown of *Eip74EF* has opposing consequences in
251 males and females, with increased fecundity in males but decreased fecundity in females. The
252 result in females is more easily explained by the role that *Eip74EF* plays in oogenesis; without it,
253 oocyte maturation arrests at stage 9 (Kozlova & Thummel, 2000). In males, *Eip74EF* promotes
254 longer sperm but seemingly at a cost to male fecundity. There are a number of events between
255 mating and oviposition during which fecundity could ultimately be affected, including sperm
256 transfer, sperm storage, sperm retention in storage, ovulation, sperm release from storage,
257 fertilization, oviposition, and offspring viability to adulthood. It is possible that there is a trade-
258 off in *Eip74EF* mutants between producing longer sperm and producing more sperm, such that
259 because mutants produce shorter sperm, they are now able to produce more sperm. It is also
260 possible that more sperm are able to enter storage if they are shorter. *Eip74EF* is
261 transcriptionally active in male accessory glands (Flybase, modENCODE), but Eip74EF protein
262 was not identified in a recent proteomic screen as expressed in male accessory gland, much less
263 transferred to females (Sepil, Hopkins, Dean, Thézénas, Charles, Konietzny, Fischer, Kessler, &
264 Wigby, 2019).

265 Despite having shorter sperm, *Eip74EF* mutant males were not less competitive during
266 sperm competition. Previous work found that the long-sperm advantage only occurs when
267 mating with females with long SRs. We did not measure the sperm length of females used in the
268 paternity study, nor did we measure sperm length in the standard male competitors. It is
269 possible that the combinations of male sperm and female SR phenotypes were not conducive to
270 observing a fitness effect of *Eip74EF* under sperm competition. More controlled experiments in
271 which knockdown males competed against males of known sperm length within females of
272 known SR length would more clearly demonstrate a role for *Eip74EF* in sperm competition.
273 However, given the extensive work on the role of sperm length in sperm competition (Miller
274 and Pitnick, 2002; Lüpold, Manier, Berben, Smith, Daley, Buckley, Belote, & Pitnick, 2012; 2016;
275 *in review*; Manier, Belote, Starmer, Berben, Lüpold, Ala-Honkola, Collins, & Pitnick, 2013), we
276 can safely conclude that any genetic mechanism that promotes sperm length while leaving
277 fertility unaffected can be considered to be a "sperm competition gene".

278 What is driving the rapid evolution of *Eip74EF*? On an evolutionary level, it is selection
279 on sperm length, mediated by length of the SR. However, most reproductive proteins that are
280 rapidly evolving are ones that interact directly with female-derived molecules, such as gamete-
281 recognition proteins that mediate fertilization and seminal fluid proteins that modify and are
282 modified by molecules in the female reproductive tract (Wilburn & Swanson, 2016). Here, we
283 demonstrate rapid evolution of a protein that has no known direct interactions with female-
284 derived molecules, nor does it affect length of the SR, which does directly interact with sperm
285 length. Dorus et al. (2006) found that many sperm proteome proteins are well-conserved,
286 suggesting that structural proteins that form the building blocks of spermatozoa are evolving
287 slowly, while the molecular builders that regulate sperm morphogenesis evolve more rapidly.
288 Thus, the molecular secret to rapid evolution and the vast morphological diversity of sperm lies

289 not in its final structure but in its development, harkening back to the old adage of evo-devo:
290 we cannot understand the evolution of morphological diversity until we understand the
291 evolution of development.

292

293

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FIGURE CAPTIONS

FIGURE 1 $E74A^{BG01805}$ males had significantly shorter sperm ($P < 0.0001$) (a) with no difference in sperm head length ($P = 0.139$) (b), and $E74A^{BG01805}$ females had no change in SR length ($P = 0.258$) (c) relative to controls.

FIGURE 2 $E74A^{BG01805}$ males had higher fecundity (P = 0.0004) (a), while $E74A^{BG01805}$ females had lower fecundity (P < 0.0001) (b).

FIGURE 3 The $E74A^{BG01805}$ mutation had no effect on a male's ability to influence female remating rate ($P = 0.323$) (a), female latency to remate in days ($P = 0.737$) (b), copulation duration in minutes ($P = 0.913$) (c), and P_2 ($P = 0.871$) (d).

Figure 1

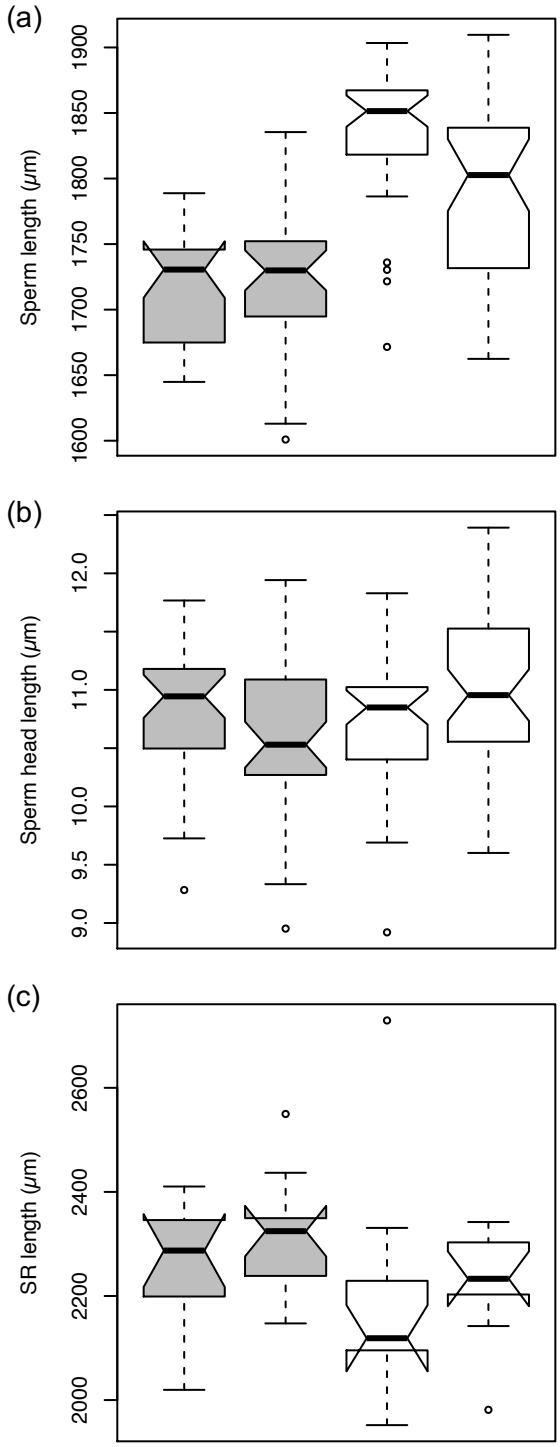
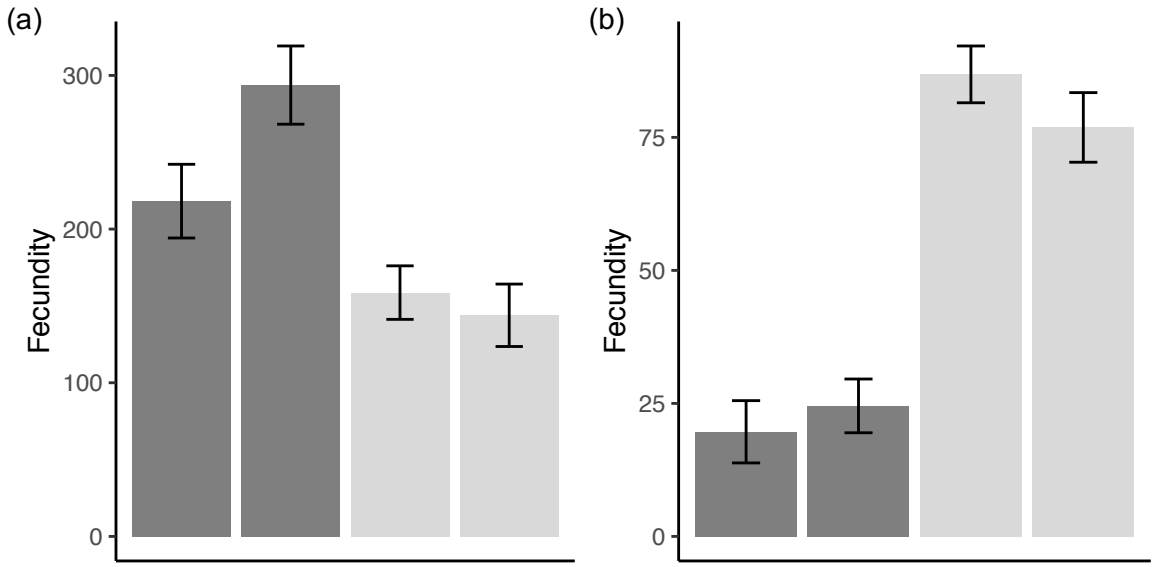
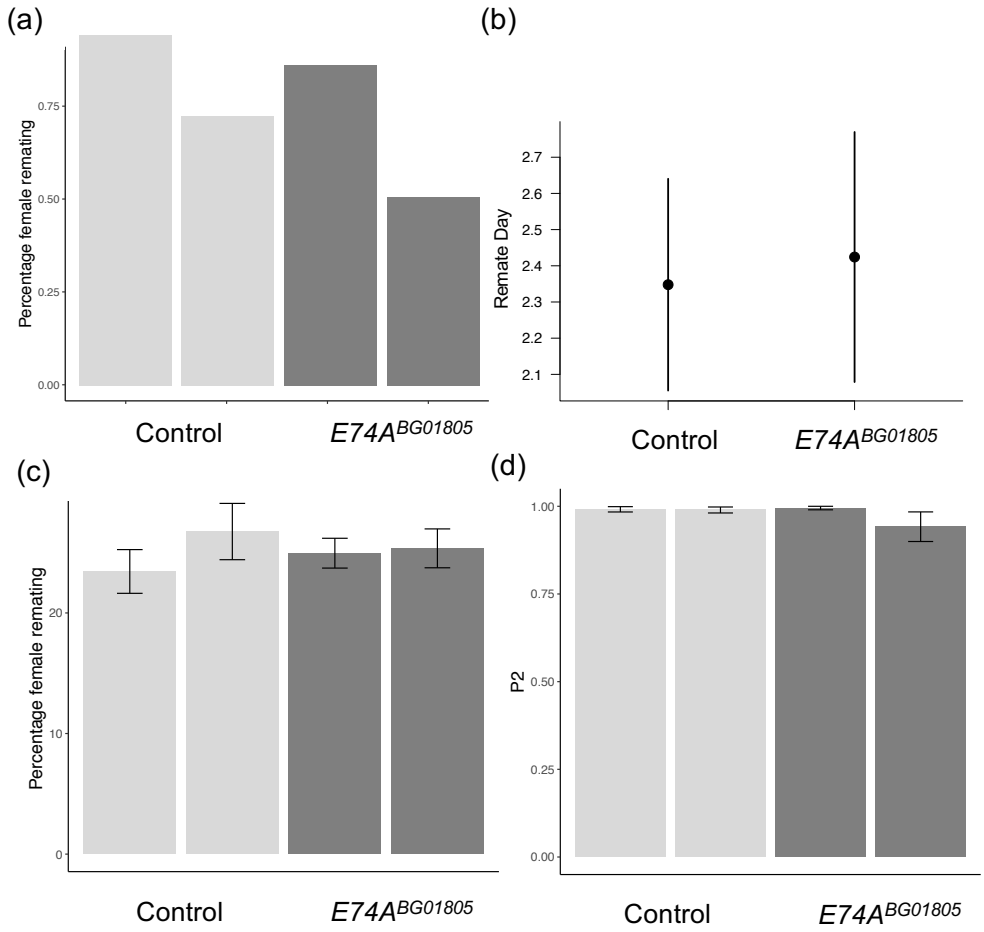

(a)
Sperm length (μm)
1600
1650
1700
1750
1800
1850
1900
(b)
Sperm head length (μm)
9.0
9.5
10.0
11.0
12.0
(c)
SR length (μm)
2000
2200
2400
2600

Figure 2



452 Figure 3



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