# A putative novel role for *Eip74EF* in male reproduction in promoting sperm elongation at the cost of 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

## RESEARCH HIGHLIGHTS

*Eip74EF* promotes oocyte maturation in *Drosophila*. We found evidence that it also promotes sperm elongation in males, but at a cost to male fecundity. Mutant males have shorter sperm but have higher reproductive success, while females have reduced fecundity.

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 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 a long seminal receptacle (SR), 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 expressed during post-meiotic stages of spermatogenesis, when spermatid elongation occurs, and we found that it is rapidly evolving under positive selection in *Drosophila*. Hypomorphic knockout of the *E74A* isoform leads to shorter sperm but does not affect SR length, suggesting that E74A may be involved in promoting spermatid elongation but is not a genetic driver of male-female coevolution. We also found that *E74A* knockout 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. Our results suggest a novel function of *Eip74EF* in spermatogenesis and demonstrates that this gene influences both male and female reproductive success. We speculate on possible roles for E74A in spermatogenesis and male reproductive success.

Keywords: ecdysone signaling, E74A, fitness, sperm length, spermatogenesis, testis

53 1. INTRODUCTION

54

55 Spermatozoa are arguably the most morphologically variable cell type among metazoans,
56 exhibiting dramatic diversity in size and shape to encompass all possible permutations of
57 presence or absence of a head, midpiece, or flagellum (Pitnick et al., 2009). With this level of
58 morphological variation, it is not surprising that sperm can evolve rapidly on short time scales
59 (Pitnick et al., 2003; Manier and Palumbi, 2008; Lüpold et al., 2011; Stewart et al., 2016), with
60 morphological differences emerging even over the course of invasive expansion into new
61 environments (Kahrl and Cox, 2017). Sperm morphology can influence swimming speed
62 (Fitzpatrick et al., 2009; Lüpold et al., 2009; Firman and Simmons, 2010; Blengini et al., 2014) and
63 fertilization success (Bennison et al., 2014), and its rapid evolution has been credited to
64 postcopulatory sexual selection via sperm competition (Parker, 1970; Snook, 2005; Pitnick et al.,
65 2009; Godwin et al., 2017) and cryptic female choice (Eberhard, William G., 1986; Firman et al.,
66 2017).

67 Within the *Drosophila* lineage, sperm length exhibits among the greatest range of
68 phenotypic variation within a clade, spanning over two orders of magnitude from 320 μm in *D.*
69 *persimilis* to 58,290 μm in *D. bifurca* (Pitnick et al., 1995). Moreover, in the obscura group, males
70 produce heteromorphic sperm, meaning that they come in two (Snook, 1997) or three (Alpern et
71 al., 2019) different sizes within an ejaculate. Sperm length evolution in *Drosophila* is mediated by
72 both length of the female's primary sperm storage organ, the seminal receptacle or SR (Miller
73 and Pitnick, 2002), a genetic correlation with SR length (Lüpold et al., 2016), and moderate
74 fitness advantage of a long sperm genotype in both males and females (Zajitschek et al., 2019).
75 As a result of this tight interaction between sperm length and SR length, the two traits are

76 coevolving both across species (Pitnick et al., 1999) and among populations undergoing
77 incipient speciation (Pitnick et al., 2003).

78 Little is known about the genes that control natural variation in sperm length, but we
79 expect them to be rapidly evolving. In a screen for *Drosophila* genes showing signatures of rapid
80 adaptive evolution under positive selection, we identified *Ecdysone-induced protein 74EF*
81 (*Eip74EF*). This gene also has increased expression during post-meiotic stages of
82 spermatogenesis (Vibranovski et al., 2009), when spermatid elongation occurs, though it has no
83 known function in spermatogenesis. Neither isoform E74A nor E74B is expressed in the sperm
84 proteome (Dorus et al., 2006), suggesting that *Eip74EF* is more likely to be involved in
85 regulation of spermatogenesis rather than encoding a structural component of mature sperm.
86 *Eip74EF* is an E26 transformation specific (ETS) domain transcription factor, best understood in
87 its role as an "early response" gene in the ecdysone signaling pathway. Ecdysone regulates
88 many stages of development, including embryogenesis, larval molting, pupation, and
89 metamorphosis (Baehrecke, 1996; Yamanaka et al., 2013) and is also important in early ovary
90 development and virtually all stages of oogenesis (Swevers, 2019). The isoform E74A is
91 specifically required for maintenance and proliferation of germline stem cells during oogenesis
92 (Ables and Drummond-Barbosa, 2010). In spermatogenesis, the ecdysone pathway is critical for
93 the maintenance and proliferation of germline and cyst stem cells (Li et al., 2014; Qian et al.,
94 2014), but it has no known role at later stages.

95 Here, we explored the role of E74A in sperm length. Because sperm length and SR length
96 are genetically correlated (Lüpold et al., 2016) and coevolving (Pitnick et al., 1999, 2003), we also
97 measured SR length in E74A hypomorphic mutant females to look for pleiotropic effects on
98 both sperm length and SR length. We found that E74A mutant males had shorter sperm, so we

99 also examined mating behavior and sperm competitive success in mutant males and fecundity
100 in both males and females.
101
102
103 2. MATERIALS AND METHODS
104
105 **2.1 Molecular evolution**
106 We estimated the rate of positive selection on *Eip74EF* as a measure of adaptive molecular
107 divergence using DNA sequences from 22 *Drosophila* species downloaded from NCBI. The
108 codeml program in PAML v4.6 (Yang, 1997, 2007) compares a null model of neutral divergence
109 (M8a) with a model of adaptive divergence under positive selection (M8), based on the ratio
110 $dN/dS$, which compares the rate of non-synonymous substitutions ($dN$) with the rate of
111 synonymous substitutions ($dS$). $dN/dS = \omega$, and generally $\omega < 1$ points to purifying selection, $\omega$
112 $= 1$ indicates neutrality, and $\omega > 1$ suggests positive selection. However, $\omega$ is rarely greater than
113 1, since multiple sites across multiple lineages are unlikely to be under positive selection for an
114 extended period. Thus, $\omega$ estimates are conservative indicators of positive selection on codons.
115 The critical values for this test are 2.71 at 5% and 5.41 at 1%, with a null distribution of point
116 mass 0 and $\chi^2_1$ (Self and Liang, 1987).
117
118 **2.2 Stocks and crosses**
119 *Eip74EF* has only one major protein isoform expressed in adults, E74A (Liu et al., 2012). For this
120 study, we used a hypomorphic mutant of *Eip74EF* obtained from the Bloomington Drosophila
121 Stock Center (stock #12619) that has partial reduction of expression of E74A while retaining
122 viability (Liu et al., 2012). The mutant allele ($E74A^{BG01805}$) contains a *p*(*GT1*) transposon insertion

123 in the sixth intron, within a $w^{1118}$ background. Experimental animals were reared from breeding
124 vials containing 10 virgin females and 20 virgin males that were transferred to new sugar-yeast-
125 agar food vials daily for four days, after which adults were discarded. Control animals were
126 reared from breeding vials containing 10 $E74A^{BG01805}$ females and 20 $w^{1118}$ males. Progeny were
127 collected within 8-12 hours of eclosion using light $CO_2$ anesthesia into single-sex vials at
128 densities of 10 females or 20 males per vial. For both experimental and control treatments,
129 progeny from two replicate sets of breeding vials out of four potential replicates per treatment
130 were phenotyped for all experiments.

131 For reproductive success experiments, competitor males were derived from a Canton-S
132 stock expressing a 3xP3-GFP construct in eye. These Canton-S Protamine-GFP lines were
133 generated using the piggybac germline transformation system (Handler and Harrell, 1999). The
134 GFP-tagged Protamine-B construct was cut out of the *P*-element plasmid construct pW8/ProtB-
135 GFP1 (Manier et al., 2010) with *Kpn*I and *Bam*HI and subcloned into the pBS/2xAsc vector, a
136 modified pBlueScript-KS+ plasmid (Stratagene, La Jolla, CA) in which two *Asc*I sites, flanking
137 the multiple cloning site, had been created by site-directed mutagenesis. The insert was then cut
138 out with *Asc*I and ligated into the single *Asc*I site of the piggybac vector p3xP3/EGFPaf (Horn
139 and Wimmer, 2000) to yield p3xP3-EGFP/ProtB-GFP. This DNA was mixed with the helper
140 plasmid pBΔSac (Handler and Harrell, 1999) and injected into preblastoderm embyos from a
141 Canton-S wild-type strain (generously provided by Mariana Wolfner, Cornell University).
142 Transformants were identified by green fluorescent pseudopupils and ocelli. Two tagged lines
143 were generated: *Canton-S pBac{3xP3-EGFP, ProtB-EGFP}27* and *Canton-S pBac{3xP3-EGFP,*
144 *ProtB-EGFP}16B* with the transgenes inserted on chromosome 2 and chromosome 3,
145 respectively. Although this stock (hereafter "Canton-S") contained a transgene, the fluorescent
146 eye and sperm markers were not specifically utilized in this series of experiments.

147

148 **2.3 Sperm measurements**

149 To obtain sperm length measurements, seminal vesicles from 5-day-old ether-anesthetized
150 males were dissected into a large droplet of 1X phosphate-buffered saline (PBS) on a gelatin-
151 coated glass slide, using fine forceps. The seminal vesicle was ruptured and dragged several
152 times through the droplet to release and spread motile sperm. The slide was then dried down at
153 55 °C in a drying oven, fixed in 3:1 methanol:acetic acid, stained with DAPI (Molecular Probes),
154 and mounted under a coverslip in 80% glycerol in PBS and sealed with nail polish. We then
155 visualized sperm using darkfield microscopy at 200X and sperm heads using fluorescence
156 microscopy at 400X on a Nikon Ni-U microscope and captured images with a Zyla Andor
157 monochrome camera. Images were measured using the segmented line tool in ImageJ software
158 (https://imagej.nih.gov/ij/). Up to five sperm and sperm heads were measured for each of up
159 to 8 males across two replicates for both the mutant and control treatments. Sperm
160 measurements that were abnormally short for a given male were assumed to be due to breakage
161 and were excluded.

162

163 **2.4 SR length**

164 Females were aged 5 days and then frozen in their food vials until dissection. Females were
165 then thawed, and thorax length was measured as a proxy for body size using a ruler reticle in a
166 Nikon SMZ-745 stereoscope at maximum magnification. The lower reproductive tract was
167 dissected with fine forceps into a droplet of 1X PBS, and the SR was carefully unraveled using a
168 fine insect pin. The sample was then mounted under a coverslip with clay on the corners to
169 allow compression of the sample into a single plane without overcompression. SRs were
170 immediately visualized at 100X or 200X under phase contrast microscopy, and images were

171 captured using a Canon EOS Rebel T3i camera. For each of two replicates for mutant and
172 control treatments, 10 females were measured using the segmented line tool in ImageJ.
173
174 **2.5 Fitness**
175 We evaluated the fitness consequences of *Eip74EF* knockdown by quantifying male and female
176 fecundity and male competitive fertilization success. To measure fecundity, we housed 20 males
177 or females per replicate individually with two Canton-S females or males, respectively, for
178 seven days, transferring triads (one focal individual and two mates) into new food vials every
179 two days, without anesthesia. We counted adult progeny that eclosed from all food vials. This
180 approach to counting progeny to assess fecundity does not account for effects of *Eip74EF* on
181 egg-to-adult viability, only on the total number of adult progeny that survived to successfully
182 eclose.
183 We assessed paternity success of mutant and control males by mating a $w^{1118}$ female first
184 with a Canton-S male (day 0) and giving her a four-hour opportunity to remate with a mutant
185 or control male over the following 4 days (days 1-4). All matings occurred in individual vials
186 and were observed directly, recording copulation duration and day of remating. We scored
187 paternity using eye color, since females, mutant males, and control males carried a recessive
188 white-eye mutation, while Canton-S competitor males had a dominant wild type eye color.
189 Paternity was expressed as $P_2$, or proportion of offspring sired by the second male (here, mutant
190 or control males).
191
192 **2.6 Statistical analysis**
193 All statistical analyses were performed in R v.3.5.3 (Team, 2018). Total sperm length and sperm
194 head length were analyzed using lmer within library lme4, with knockdown treatment as a

195 fixed effect, male identity nested within replicate as a random effect, and researcher identity as
196 a random effect, with restricted maximum likelihood (REML) parameter estimation. SR length
197 was found to be negatively correlated with body size (thorax length) (Pearson's product-
198 moment correlation, $t_{42}$ = -2.514; $P$ = 0.0159), so thorax length was included as a random effect in
199 a linear mixed model, with knockdown treatment and replicate as fixed effects, and thorax
200 length as a random effect. SR lengths were all measured by the same individual. Fecundity data
201 were analyzed using lmer with knockdown treatment as a fixed effect and replicate as a random
202 effect, with maximum likelihood parameter estimation, because there was only one random
203 effect. For all analyses implemented in lmer, P-values were estimated using a Type II Wald chi-
204 square test with Anova in the car library. Here, we report knockdown treatment effect sizes as
205 the *t*-value from the mixed model regression, along with the $\chi^2$ and p-value from the Wald's
206 test.

207 From the sperm competition experiment, we also obtained remating rate (proportion of
208 females that remated), copulation duration, and day of remating. Remating rate was analyzed
209 using a chi-square test in two different ways: with replicates as categories (N = 4) and with
210 knockdown treatments as categories (N = 2), both yielding the same result. Copulation duration
211 was analyzed using lmer with knockdown treatment as a fixed effect and replicate as a random
212 effect, with maximum likelihood parameter estimation. We report results as for other lmer
213 analyses described above. For paternity success, we used logistic regression with a logit link
214 function and binomial error distribution (after ensuring no overdispersion in the data),
215 implemented using glm.
216
217
218 3. RESULTS

219

220 *Eip74EF* is rapidly evolving under positive selection, with an M8a model estimate of -20882.5,
221 M8 of -20875.6, and a $\chi^2$ of 13.80, well above the 1% critical threshold of 5.41. Knockdown of
222 *Eip74EF* resulted in shorter sperm ($t$ = -5.341, $\chi^2_1$ = 28.53, $P$ < 0.0001; Figure 1a) but had no effect
223 on sperm head length ($t$ = -1.48, $\chi^2_1$ = 2.191, $P$ = 0.139; Figure 1b) nor female SR length ($t$ = 1.132,
224 $\chi^2_1$ = 1.281, $P$ = 0.258; Figure 1c). Disruption of *E74A* led to an increase in male fecundity ($t$ =
225 3.529, $\chi^2_1$ = 12.45, $P$ = 0.0004; Figure 2a) but a decrease in female fecundity ($t$ = -10.17, $\chi^2_1$ =
226 103.43, $P$ < 0.0001; Figure 2b). In other words, female fecundity was adversely affected while
227 male fecundity was positively affected. In the sperm competition experiment, female remating
228 rate (replicates as groups: $\chi^2_3$ = 3.143, $P$ = 0.370; treatment as groups: $\chi^2_1$ = 0.762, $P$ = 0.383;
229 Figure 3a), day of remating ($t$ = 0.336, $\chi^2_1$ = 0.113, $P$ = 0.737; Figure 3b), copulation duration ($t$ =
230 0.109, $\chi^2_1$ = 0.012, $P$ = 0.913; Figure 3c), and paternity success ($P_2$; $z$ = 0.163, $P$ = 0.871; Figure 3d)
231 were all unaffected by E74A knockout.

232

233

234 4. DISCUSSION

235

236 We found that *Eip74EF* is evolving rapidly under positive selection, a surprising result given
237 that its roles in molting and metamorphosis, which are both highly conserved processes, would
238 suggest that it should be under strong purifying selection. However, we found evidence that
239 E74A plays a novel role during sperm morphogenesis, in the positive regulation of sperm
240 length, a rapidly evolving trait. The decrease in sperm length in *E74A* mutants is attributable
241 primarily to change in sperm flagellum length, since head length was unaffected. At the same
242 time, mutant males had increased fecundity. It should be noted that we have only included one

243 genetic experiment here, and our sperm length results may be due to other genetic factors
244 linked to *Eip74EF* rather than this gene itself. More experimentation is needed to further
245 confirm the effect of E74A on sperm length including, but not limited to, examining sperm
246 length in a knock-in mutant with expression under control of a heat-shock promoter (Fletcher et
247 al., 1997).

248 We know little about E74A's role in testis. Within the ecdysone pathway more generally,
249 it is one of several genes that are activated when the steroid hormone 20-hydroxyecdysone
250 (ecdysone) binds to a heteromeric receptor in the nuclear envelope comprised of ecdysone
251 receptor (EcR) and ultraspiracle (USP) (Yamanaka et al., 2013). EcR is also a transcription factor
252 that directly initiates expression of a number of early response genes, including *Eip74EF*
253 (Uyehara and McKay, 2019). In the male germline, EcR is required for growth and proliferation
254 of both germline stem cells and cyst stem cells, which co-differentiate throughout
255 spermatogenesis (Li et al., 2014; Qian et al., 2014). Further indirect evidence for E74A function in
256 spermatogenesis comes from defects found in *belle* (*bel*) mutants. *Bel* is a DEAD box RNA
257 helicase that regulates translation of *E74A* transcripts in salivary glands during metamorphosis
258 (Ihry et al., 2012). Although it is unknown if *bel* similarly regulates *E74A* translation in testis,
259 hypomorphic mutants of *bel* have spermatogenic defects in chromosome segregation and
260 cytokinesis at the round spermatid stage, approximately when spermatid elongation begins
261 (Johnstone et al. 2005).

262 We can only speculate about E74A's possible function during spermatogenesis. We do
263 know that it is a transcription factor that regulates downstream genes in the ecdysone signaling
264 pathway. Ecdysone signaling generally tends to promote the maintenance of developmental
265 stages or to trigger a switch between stages (Gancz and Gilboa, 2013; Li et al., 2014; Yatsenko
266 and Shcherbata, 2018; Leiblich et al., 2019; Swevers, 2019), with parallel functions in

267 larval/pupal development and gametogenesis (Ables et al., 2016). Because germline
268 development is regulated largely by non-autonomous paracrine signaling from supporting
269 somatic cells (Spradling et al., 2011; Zoller and Schulz, 2012; Li et al., 2014; Qian et al., 2014;
270 Ables and Drummond-Barbosa, 2017), E74A is likely to be expressed in somatic cyst cells.
271 Indeed, ecdysone signaling is detectable in nurse cells and follicle cells throughout oogenesis
272 (Carney and Bender, 2000) and regulates multiple stages from development of the ovarian niche
273 (Gancz and Gilboa, 2013; Yatsenko and Shcherbata, 2018) to ovulation (Knapp and Sun, 2017;
274 Swevers, 2019). In the ovary, ecdysone signaling is orchestrated on a fine spatio-temporal scale
275 throughout oogenesis (Swevers 2019). In spermatogenesis, E74A is also likely to be tightly
276 regulated at very specific timepoints, especially since expression in the testis overall is very low
277 (Brown et al., 2014; Li et al., 2014). Less is known about the role of ecdysone signaling in
278 morphogenesis, though it is required for morphogenesis and function of Malpighian tubules
279 (Gautam et al., 2015). Perhaps most relevant to flagellar elongation, an early study on *Drosophila*
280 Kc cells in culture found that application of ecdysone induced elongation and motility in cells
281 mediated by the formation of filopodia through tubulin assembly (Berger et al., 1980). This
282 result suggests that ecdysone could also mediate flagellar assembly during spermatogenesis.

283 On the face of it, it is curious that knockdown of *Eip74EF* has opposing consequences in
284 males and females, with increased fecundity in males but decreased fecundity in females. The
285 result in females is more easily explained by the role that *Eip74EF* plays in oogenesis; without it,
286 germline stem cells arrest development and degenerate. In males, *Eip74EF* seems to promote
287 longer sperm but at a cost to male fecundity. There are a number of events between mating and
288 oviposition during which fecundity could ultimately be affected, including sperm transfer,
289 sperm storage, sperm retention in storage, ovulation, sperm release from storage, fertilization,
290 oviposition, and offspring viability to adulthood. It is possible that there is a trade-off in

291 *Eip74EF* mutants between producing longer sperm and producing more sperm, such that
292 because mutants produce shorter sperm, they are now able to produce more sperm (Pitnick and
293 Markow, 1994; Immler et al., 2011). This hypothesis is unlikely to explain the whole story,
294 however, since fecundity in mutant males increased by approximately 50%, while sperm length
295 only decreased by around 5%. Further experiments should examine the rate of spermatogenesis
296 in testes of E74A mutant males.

297 It is also possible that fewer mutant sperm are able to enter storage. Decreased storage
298 may be a function of sperm storage organs being able to store more shorter sperm. An
299 alternative and non-mutually exclusive hypothesis is that E74A regulates seminal fluid
300 composition and/or transfer. Seminal fluid proteins produced in the accessory glands can have
301 profound effects on many aspects of female reproduction, behavior, and physiology (Avila et al.
302 2011), including sperm storage after mating (Neubaum & Wolfner 1999; Bloch Qazi & Wolfner
303 2003; Avila & Wolfner 2009). Accessory gland development and function require ecdysone
304 receptor (EcR) (Herndon et a. 1997; Sharma et al. 2017; Leiblich et al 2019), which directly
305 upregulates E74A in salivary glands and wing disc (Burtis et al., 1990; Thummel et al., 1990;
306 Uyehara and McKay, 2019). It is therefore possible that E74A is also involved in accessory gland
307 function and production of seminal fluid proteins independent of a possible role in
308 morphogenesis during spermatogenesis.

309 Despite having shorter sperm, *E74A* hypomorphic mutants were not less competitive
310 during sperm competition. Previous work found that the long-sperm advantage only occurs
311 when mating with females with long SRs. We did not measure the SR length of females used in
312 the paternity study, nor did we measure sperm length in the standard male competitors. It is
313 possible that the combinations of male sperm and female SR phenotypes were not conducive to
314 observing a fitness effect of E74A under sperm competition. More controlled experiments in

315 which knockout males competed against males of known sperm length within females of
316 known SR length would more clearly demonstrate a role for E74A in sperm competition.

317 What is driving the rapid evolution of *Eip74EF*? On an evolutionary level, it may reflect
318 selection on sperm length, mediated by length of the SR (Miller and Pitnick, 2002) and a genetic
319 correlation between the two traits (Lüpold et al., 2016). However, most reproductive proteins
320 that are rapidly evolving are ones that interact directly with female-derived molecules, such as
321 gamete-recognition proteins that mediate fertilization and seminal fluid proteins that modify
322 and are modified by molecules in the female reproductive tract (Wilburn and Swanson, 2016).
323 Here, we demonstrate rapid evolution of a protein that has no known direct interactions with
324 female-derived molecules, nor does it seem to affect length of the SR, which does directly
325 interact with sperm length. Dorus et al. (2006) found that many sperm proteome proteins are
326 well-conserved, suggesting that structural proteins that form the building blocks of
327 spermatozoa are evolving slowly, while the molecular builders that regulate sperm
328 morphogenesis evolve more rapidly. Thus, the molecular secret to rapid evolution and the vast
329 morphological diversity of sperm may lie not in its final structure but in its development,
330 harkening back to the old adage of evo-devo: we cannot understand the evolution of
331 morphological diversity until we understand the evolution of development.

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334 DATA AVAILABILITY STATEMENT

335

336 The data that support the findings of this study are openly available in Dryad at
337 https://doi.org/10.5061/dryad.k0p2ngf4k.

338

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499

500

501 FIGURE CAPTIONS

502

503 **FIGURE 1** *E74A*$^{BG01805}$ males had significantly shorter sperm ($P < 0.0001$) (a) with no difference

504 in sperm head length ($P = 0.139$) (b), and *E74A*$^{BG01805}$ females had no change in SR length ($P =$

505 $0.258$) (c) relative to controls. Two replicates are shown per treatment, with the mutant in dark

506 gray and control in light gray.

507

508 **FIGURE 2** *E74A*$^{BG01805}$ males had higher fecundity (P = 0.0004) (a), while *E74A*$^{BG01805}$ females had
509 lower fecundity (P < 0.0001) (b). Two replicates are shown per treatment, with the mutant in
510 dark gray and control in light gray.
511
512 **FIGURE 3** The *E74A*$^{BG01805}$ mutation had no effect on a male's ability to influence female
513 remating rate (*P* = 0.323) (a), female latency to remate in days (*P* = 0.737), illustrated as effect
514 sizes of the statistical model (b), copulation duration in minutes (*P* = 0.913) (c), and $P_2$ (*P* =
515 0.871) (d). Two replicates are shown per treatment, with the mutant in dark gray and control in
516 light gray.

Fig. 1

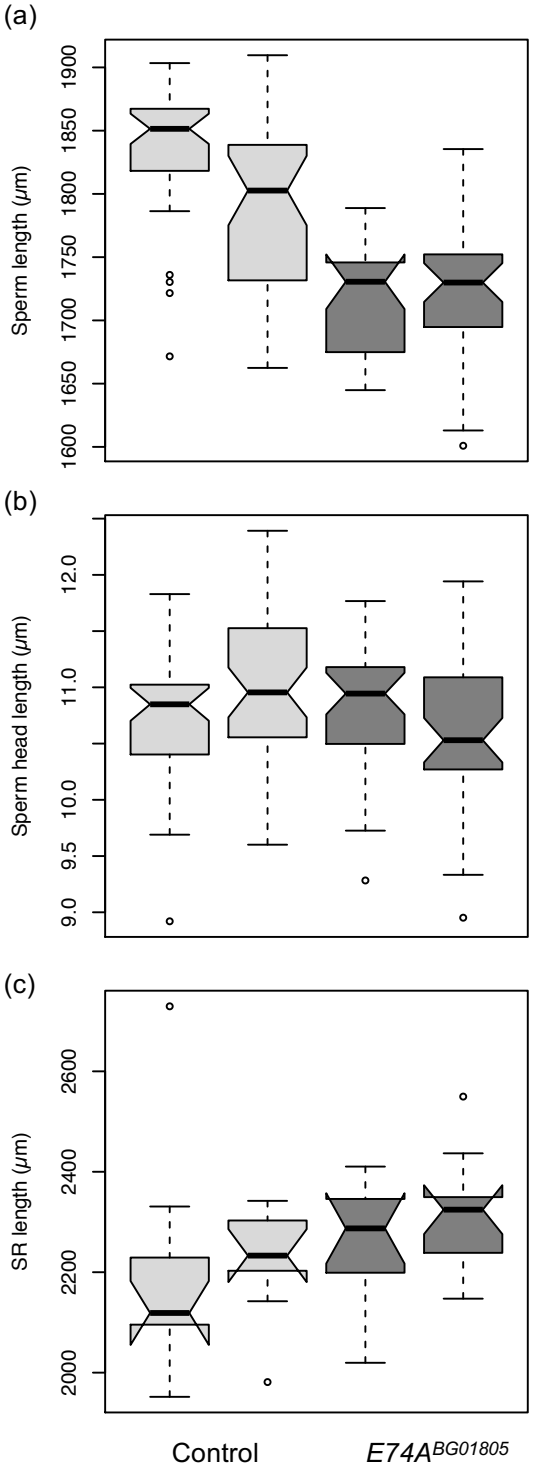

(a)
Sperm length (μm)
1600
1650
1700
1750
1800
1850
1900
(b)
Sperm head length (μm)
9.0
9.5
10.0
11.0
12.0
(c)
SR length (μm)
2000
2200
2400
2600
Control
E74A^BG01805

Fig. 2

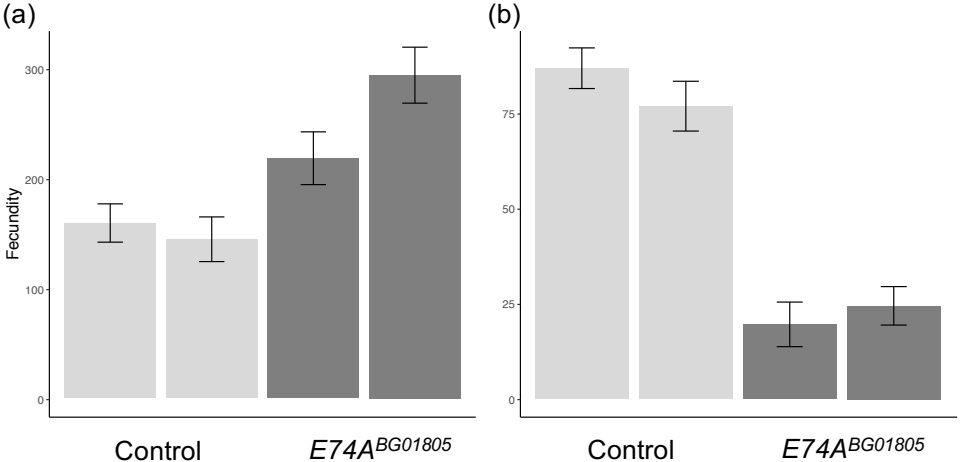
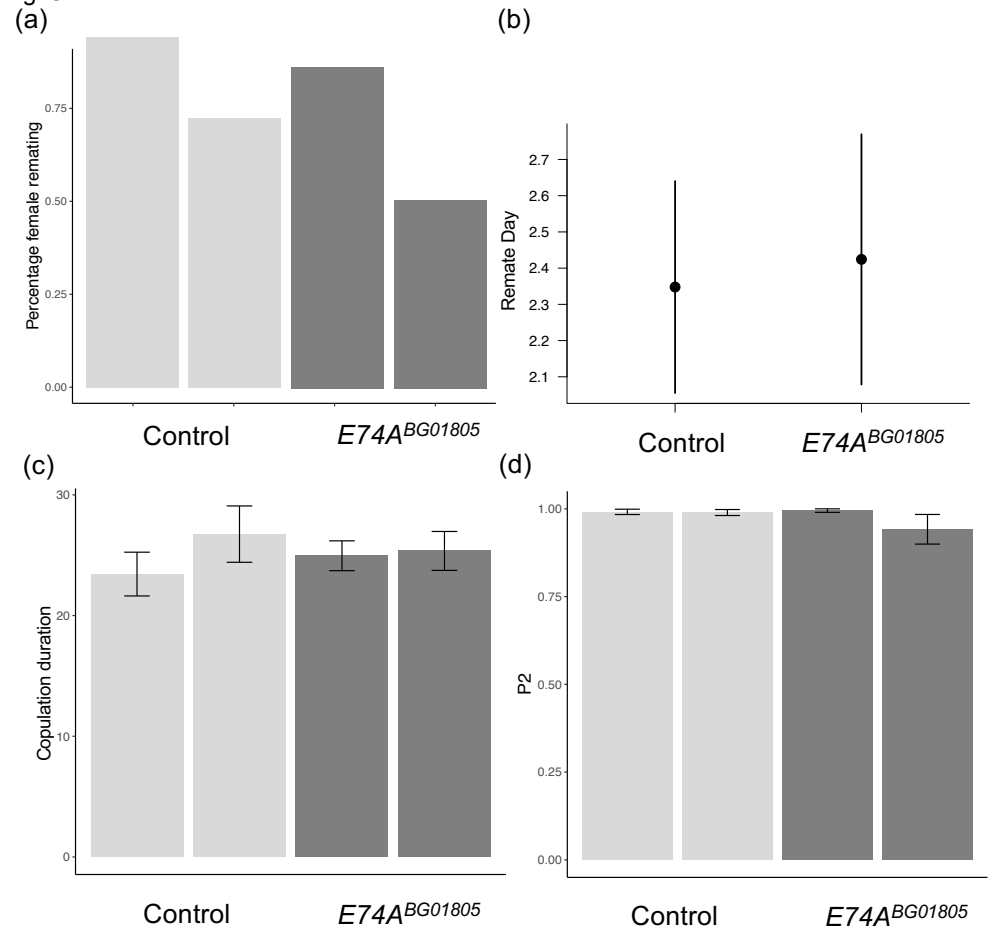

(a)
Fecundity
300
200
100
0
Control
E74A^BG01805
(b)
75
50
25
0
Control
E74A^BG01805

Fig. 3


(a)
Percentage female remating
0.00
0.25
0.50
0.75
Control
E74A^BG01805
(b)
Remate Day
2.1
2.2
2.3
2.4
2.5
2.6
2.7
Control
E74A^BG01805
(c)
Copulation duration
0
10
20
30
Control
E74A^BG01805
(d)
P2
0.00
0.25
0.50
0.75
1.00
Control
E74A^BG01805