

1            **Temporal and genetic variation in female aggression after mating**

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23 **Keywords**

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25 *melanogaster*

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30 **Abstract**

31 Aggression between individuals of the same sex is almost ubiquitous across the animal  
32 kingdom. Winners of intrasexual contests often garner considerable fitness benefits, through  
33 greater access to mates, food, or social dominance. In females, aggression is often tightly  
34 linked to reproduction, with females displaying increases in aggressive behavior when mated,  
35 gestating or lactating, or when protecting dependent offspring. In the fruit fly, *Drosophila*  
36 *melanogaster*, females spend twice as long fighting over food after mating as when they are  
37 virgins. However, it is unknown when this increase in aggression begins or whether it is  
38 consistent across genotypes. Here we show that aggression in females increases between 2  
39 to 4 hours after mating and remains elevated for at least a week after a single mating. In  
40 addition, this increase in aggression 24 hours after mating is consistent across three diverse  
41 genotypes, suggesting this may be a universal response to mating in the species. We also  
42 report here the first use of automated tracking and classification software to study female  
43 aggression in *Drosophila* and assess its accuracy for this behavior. Dissecting the genetic  
44 diversity and temporal patterns of female aggression assists us in better understanding its  
45 generality and adaptive function, and will facilitate the identification of its underlying  
46 mechanisms.

47

48

## 49 **Introduction**

50 Aggression towards conspecifics is prevalent throughout the animal kingdom. Winners in  
51 aggressive encounters often benefit from increased access to resources or mates, better  
52 positions in social hierarchies, and ultimately, higher reproductive success (Holekamp et al.  
53 1996; Stockley and Bro-Jørgensen 2011; Clutton-Brock and Huchard 2013; Stockley and  
54 Campbell 2013). Male aggression has been shown to be at least partly heritable in flies, mice,  
55 rats, and chickens, suggesting a genetic basis (5–8). Although we know a substantial amount  
56 about the genetic basis of male aggression, we know little about it in the context of female  
57 aggression. As males and females show striking differences in the frequency, form, and  
58 intensity of aggression they display, it seems likely that there are also significant sex  
59 differences in the genetic architecture underlying intrasexual aggression. As genetic variation  
60 forms the pool of variation on which selection can act, determining how much genotypes  
61 differ is a key question for understanding how female aggression has evolved.

62

63 Females display a large degree of plasticity in their aggressive behavior across time, adding  
64 another important source of variation - temporal variation. Females typically fight over  
65 resources associated with offspring production or survival, and there is often a tight temporal  
66 association between female aggression and reproduction (2,9,10). Classifying these temporal  
67 associations between aggression and reproduction in females can help us understand female  
68 aggression in a number of ways. First, investigating what stages of reproduction lead to an  
69 increase (or decrease) in aggression can help us to establish the adaptive value of aggression  
70 for females. As aggression is expected to have costs, in the form of energy expenditure, injury,  
71 and opportunity costs, females should only elevate their aggression when the benefits  
72 outweigh the costs. If these costs and benefits shift over a female's reproductive cycle, we

73 expect to see plastic expression of aggression with the most elevated levels of aggression  
74 displayed when aggression is most beneficial for females. Second, dissecting the temporal  
75 associations between aggression and reproduction can assist us in pinpointing potential  
76 mechanisms that regulate female aggression and whether these differ from those regulating  
77 male aggression (11). By tracking the timing of aggression and any behavioral or physiological  
78 correlates, we are able to identify putative mechanisms for further testing.

79

80 *Drosophila melanogaster* is a model organism for the study of male aggression and sexual  
81 selection (12), though fewer studies have examined female aggression (out of 65 empirical  
82 studies found on a Web of Science search using the keywords ‘*Drosophila melanogaster*’ and  
83 ‘aggress\*’, 61 measured male aggression and 8 measured female aggression – search  
84 conducted 30.4.19). Females in this species fight over food, particularly valuable protein-rich  
85 resources, such as live yeast (13–15). Mated females will fight for twice as long over access to  
86 food as virgin females, and this change is stimulated by the transfer of sperm and seminal  
87 fluid proteins during mating (16). Mating also dramatically alters other aspects of female  
88 behavior and physiology - mated females show increased rates of ovulation and egg laying,  
89 reduced receptivity to mating attempts, changed feeding patterns and preferences, altered  
90 immune responses, and even changes in sleep patterns (17). However, there is substantial  
91 variation amongst populations and genotypes in the strength and speed of these post-mating  
92 responses (PMRs). For example, females from the commonly used w1118 lab strain eject  
93 mating plugs almost twice as fast as females from a Canton S background (18). Males from  
94 different populations also differ in their ability to stimulate female PMRs (19), which suggests  
95 differences in male ejaculate composition or co-evolution between males and females that  
96 alters the strength of PMRs in different populations. The variation amongst different

97 populations and lab strains in PMRs suggest that mating-induced female aggression may vary  
98 in magnitude (or existence) between genotypes; such variation thus constitutes a key area  
99 for further research in the field of female aggression.

100

101 We first test here whether there was an effect of male or female genotype on female  
102 aggression in three commonly used lab strains (w1118, Canton S, and Dahomey), both before  
103 and after mating. We predicted that there would be differences between the genotypes in  
104 virgins' levels of aggression due to underlying genetic differences. We also predicted that  
105 differences amongst genotypes in males' ability to stimulate PMRs and differential female  
106 PMRs would lead to differences between genotypes in how much female aggression increases  
107 after mating.

108

109 Second, we tested the timing of mating-induced female aggression to identify putative  
110 mechanisms regulating it. We have shown previously that the transfer of sperm is necessary  
111 for females to increase aggression 24 hours after mating (16), which suggests that the timing  
112 of sperm transfer and sperm storage may be important mediators of the induction of female  
113 aggression. We had two predictions as to when female aggression should begin to increase:

- 114 1. Aggression should increase immediately after mating due to the stimulation of mating  
115 per se or the transfer of male ejaculate components (similar to the post-mating  
116 receptivity effect) OR;
- 117 2. Aggression should only increase a few hours after mating, when oviposition rates  
118 begin to increase, suggesting a direct link between female aggression and egg laying  
119 (20,21).

120

121 Finally, we evaluated how well existing automated tracking and classification software is able  
122 to track and score female aggression using our current set-up. Studying behaviors such as  
123 aggression can be time-consuming as individuals often spend a relatively small fraction of  
124 time engaging in aggressive encounters. The invention of software to track animal locomotion  
125 and behavior has dramatically improved our ability to study broad-scale and individual-level  
126 behaviors (22). Machine-learning techniques can now recognize specific suites of behaviors  
127 automatically, while automated tracking and classification software remove the need to  
128 manually score behaviors – empowering us to conduct high-throughput behavioral analyses.  
129 The use of such software to study male aggression in *Drosophila melanogaster* has led to the  
130 discovery of sex-specific genes, hormones, and neuronal pathways responsible for male  
131 aggression in flies and other animals (12,23). However, to our knowledge, no studies have yet  
132 used automated tracking and classification software to study female aggression in *D.*  
133 *melanogaster* or other insects. Given that modules of fruit fly aggression (e.g. lunging) are  
134 discrete and distinct from one another, machine learning-based methods of study may also  
135 be particularly useful for behavioral analyses in the fruit fly (14). Developing a system to  
136 accurately record, track, and score female aggression is therefore a key methodological  
137 advancement which will facilitate further study of female aggression.

138

## 139 **Materials and Methods**

### 140 *Fly stocks and culture*

141 To test whether an increase in female aggression after mating is common across different  
142 genotypes, we used three common laboratory genotypes: Dahomey, Canton-S, and w1118.  
143 We chose these three genotypes as they are widely used as a genetic background for  
144 introgressing different mutations into in a variety of laboratory studies. Flies from the

145 Dahomey stock population were collected from Dahomey, Benin in the 1970s and have been  
146 kept in large, outbred population cages in the laboratory ever since (24). The Canton S stock  
147 was received from the Heisenberg lab at the University of Würzburg, while w1118 came from  
148 the Wilson lab at the University of Oxford. Both Canton S and w1118 stocks were kept in  
149 smaller populations, but raised in large population cages for multiple generations prior to this  
150 experiment. Eggs were collected from each population cage and larvae from all genotypes  
151 were raised at standardized larval density (25). Flies were collected within 8 hours of eclosion  
152 to ensure virginity, and the sexes were housed separately. Flies were raised and maintained  
153 as adults on standard fly food medium (for 1L of fly food, main ingredients followed this ratio:  
154 20g molasses, 14.6g yeast, 6.8g agar, 5.6mL propionic/phosphoric acid) without live yeast.  
155 Males were kept in vials in groups of 10, while females were kept in individual vials from the  
156 time of eclosion to the time they were used in a contest (i.e. five days). All flies were kept and  
157 experiments conducted at 25°C on a 12:12 dark: light cycle.

158

### 159 *Experimental design*

#### 160 **Genotype experiment**

161 In addition to testing whether females differ in aggression due to genotype, we also tested  
162 whether males from different genotypes differed in how much aggression they stimulated in  
163 females after mating. In all previous studies, females were mated to males from the same  
164 genetic background as them – i.e. Canton-S females were mated to Canton-S males, while  
165 Dahomey females were mated to Dahomey males (14,16). As males from different genotypes  
166 may differ in their ejaculate composition or ability to stimulate female aggression, we also  
167 incorporated male genotype into our experimental design. To investigate whether any  
168 differences in genotype were due to male or female genotype, or an interaction between the

169 two, we used a fully factorial design. Males from all genotypes were mated to females of all  
170 genotypes (sample sizes available in Supplementary Table 1). The genotype experiment was  
171 conducted in two blocks.

172

### 173 **Timing experiment**

174 To test when female aggression increases after mating, we tested females at 6 time points  
175 after mating:

- 176 • 1 hour post-mating
- 177 • 2 hours post-mating
- 178 • 4 hours post-mating
- 179 • 8 hours post-mating
- 180 • 24 hours post-mating
- 181 • 1 week (168 hours) post-mating

182

183 For this experiment, we used only Dahomey females, as the results from our first experiment  
184 suggested there were no differences between genotypes in mating-induced aggression. In the  
185 ‘mated’ treatments, females were mated to standard Dahomey males. Each mated female  
186 was paired with a mated female from the same treatment that had finished mating within 30  
187 minutes of each other, so that they were as closely matched as possible for end of mating  
188 time. For each pair of mated females, a pair of randomly selected virgin females was chosen  
189 and fought at the same time to get an equivalent control for each time point (sample sizes in  
190 Supplementary Table 2).

191

192 *Behavioral experiments*

193 In the genotype experiment, we painted females 3 days post-eclosion (one day prior to  
194 mating, two days prior to fighting). Females were painted with either a red or yellow dot of  
195 acrylic paint on their thorax to facilitate individual identification. Females were not painted  
196 in the timing experiment.

197

198 4 days post-eclosion, females in the ‘mated’ treatments of both experiments were placed into  
199 a vial with a single male and observed. Once a single mating occurred, they were separated  
200 from these males and put into a fresh vial containing regular fly food media. Females that did  
201 not mate within 5 hours were discarded. We recorded the latency to mating and the duration  
202 of each mating for all pairs.

203

204 In the genotype experiment, females remained in their vials for 24 hours after mating. These  
205 vials were subsequently frozen and we counted the number of eggs each female had laid. In  
206 the genotype experiment, females were used in contests 24 hours after mating (5 days post-  
207 eclosion). For the two hours directly before being used in a contest, females were kept in a  
208 vial with damp cotton wool but no food to increase the chance that we would see aggressive  
209 behavior (13).

210

211 In the timing experiment, the amount of time females spent in starvation vials prior to being  
212 used in contests differed depending on their treatment. Females from the ‘1 hour’ and ‘2  
213 hour’ treatments were placed immediately into starvation vials upon completion of mating.  
214 Females from the other treatments were placed into starvation vials 2 hours before being  
215 placed in the contest arena (i.e. for females in the ‘4 hour’ treatment, they remained in vials  
216 with access to food for 2 hours and then were in starvation vials for 2 hours).

217

218 Flies were then aspirated from these vials into a contest arena containing an Eppendorf tube  
219 cap filled with regular fly food (diameter 2 mm) and a ~2- $\mu$ l drop of yeast paste, providing a  
220 limited resource to fight over. Females were allowed 5 minutes to acclimatise to the arena  
221 and were then filmed for 30 minutes using Toshiba Camileo X400 video cameras.

222

223 In the genotype experiment, females were removed from the contest arena after being  
224 recorded and placed into new vials with fly food (again containing no live yeast) and left to  
225 lay eggs. 24 hours later, these females were removed and the vials frozen to count the  
226 number of eggs females laid in the 24 hours after the contest. We then measured wing area  
227 of females used in contests as a proxy of body size (26).

228

### 229 *Manual behavioral scoring*

230 Videos were scored blind to treatment. Only headbutts were recorded as a proxy for female  
231 aggression, as these have previously been shown to be the most common high intensity form  
232 of aggression engaged in by females (14,15). We recorded the number of headbutting  
233 encounters and duration of each encounter. An encounter began when one female  
234 headbutted the other and ended when the flies separated or stopped interacting (NB: an  
235 encounter may include multiple headbutts from one or both individuals). We then used total  
236 encounter duration as the primary response variable, as we have previously shown that it  
237 encompasses variation in both the number and duration of encounters to give a good overall  
238 indicator of the amount of aggression shown by a pair (16). Using headbutts also enabled us  
239 to have a direct comparison with the automated behavioral data, where headbutting is  
240 detectable, but not lower intensity behaviors, such as fencing.

241

242 *Automated tracking and analysis*

243 To track the females, we used the Caltech fly tracker (27). The program records the location  
244 and trajectory of individual flies, as well as producing data on other parameters, such as  
245 velocity, distance to other individuals, and location within an arena. These parameters and  
246 tracking data were then transferred to the program JAABA to use machine learning to  
247 automatically classify headbutting behavior in our videos (28). We first calibrated the JAABA  
248 machine learning algorithm by manually annotating several video frames in a subset of videos  
249 to specify which behaviors are of interest – in our case labelling frames that contained  
250 instances of headbutting and a sample of those frames that did not contain headbutting. The  
251 program then conducted an iterative process of machine learning to identify further cases of  
252 that behavior seen in the videos by using the annotated frames as a reference point. These  
253 predictions were then checked by the manual trainer, correcting and refining the program’s  
254 predictions. Once further refinements to the algorithm generated no improvement in the  
255 program’s ability to classify aggression (as measured using JAABA’s ground-truthing function),  
256 we used this classifier across all videos in both the timing and genotypes experiment.

257

258 We could use the automated tracking and classification analysis software on 189 of the 227  
259 videos that we were able to score manually in the timing experiment. In the genotype  
260 experiment, we were able to use tracking and classification software on 272/332 videos. The  
261 tracking software failed to successfully track the flies in the remaining videos in both  
262 experiments (potentially due to issues related to lighting and contrast levels).

263

264 *Statistical analysis*

265 We performed all statistical analysis in R (version 3.3.2) (29). In the timing experiment, for  
266 analyses involving contest duration we used generalized linear models with negative binomial  
267 distributions (using the function `glm.nb` from the ‘MASS’ package - Venables & Ripley, 2002),  
268 as these models best fit our data, and our data met the majority of the assumptions for such  
269 models. To compare the results of manual and automated scoring, we used a linear model  
270 with the manual scores as the response variable, with automated scores and mating status  
271 (and their interaction) as the explanatory variables.

272

273 For the genotype experiment, we used GLMs with Gamma distributions to analyze contest  
274 duration data, as these models fit our data better than linear models or GLMS with negative  
275 binomial distributions. We fitted two models:

276 a. To investigate whether males differed in their ability to stimulate female aggression  
277 (and whether there was an interaction between male and female genotype), we fit  
278 the following model on a dataset that contained only mated females:

279 
$$\text{Contest duration} \sim \text{Male genotype} * \text{Female genotype}$$

280 b. To test whether female genotypes differed in the magnitude of their response to  
281 mating, we pooled all male mating treatments together (i.e. for Dah females, the  
282 ‘mated’ treatment consisted of females mated to Dah males, Canton-S males, and  
283 w1118 males). We then fit the following model to a dataset containing all females:

284 
$$\text{Contest duration} \sim \text{Mating status} * \text{Female genotype}$$

285 In addition, we investigated the effect of female genotype on wing area, and the number of  
286 eggs a female laid in the 24 hours before-, and after being used in a contest. For the wing area  
287 analysis, we used a linear model with wing area as the response and female genotype as the  
288 explanatory variable. For the egg count analyses, we fit a GLM with a quasipoisson

289 distribution as the data was count data that was overdispersed and therefore did not fit a  
290 Poisson distribution. As body size has been shown to be linked to fecundity in *D.*  
291 *melanogaster*, we included it in the model as follows:

292 Egg count (either pre- or post-contest) ~ Mating status \* Female genotype \* Wing area

293

## 294 **Results**

### 295 *Genotype experiment*

#### 296 Males from different genotypes do not differ in their ability to stimulate female aggression

297 Males of different genotypes did not differ in the aggression they stimulated in mated females  
298 ( $Dev_{2, 178} = 0.12$ ,  $P = 0.87$ ; Supplementary Figure 1). Female genotype was marginally non-  
299 significant ( $Dev_{2, 176} = 2.26$ ,  $P = 0.071$ ), with a trend for w1118 females to fight for less time  
300 than Canton-S or Dahomey females. There was no significant interaction between male and  
301 female genotype ( $Dev_{4, 172} = 2.62$ ,  $0.19$ ), which suggests that males do not stimulate a  
302 different amount of aggression in females of their own genotype compared with those from  
303 a different genotype.

304

#### 305 Mating-induced female aggression is consistent across different genotypes

306 Mated females fought for longer than virgin females across all female genotypes (GLM with  
307 Gamma distribution:  $Dev_{1, 270} = 6.78$ ,  $P = 0.0002$ ; Fig. 1). There was also a significant effect of  
308 genotype, with w1118 females fighting for less time than either Canton-S or Dahomey  
309 females when both mated and virgin ( $Dev_{2, 268} = 4.95$ ,  $P = 0.006$ ). There was no significant  
310 interaction between mating status and female genotype, suggesting that the genotypes  
311 showed similar increases in aggression in response to mating ( $Dev_{2, 266} = 1.37$ ,  $P = 0.247$ ).

312

313

314 **Figure 1: Mated females fought for longer than virgin females in all genotypes and w1118 females**  
315 **fought for less time than Canton-S and Dahomey females**

316 Blue points represent mated females while yellow points represent virgin females in each genotype.  
317 Each point represents the contest duration for one pair of females. The black bars represent the  
318 treatment means  $\pm$  1 standard error. Sample sizes are recorded in Supplementary Table 1.

319

320 *Timing experiment*

321 Aggression increases 2-4 hours after mating

322 There was a significant interaction between mating status and number of hours after mating  
323 for contest duration (GLM with negative binomial distribution:  $Dev_{5, 213} = 25.23$ ,  $P = 0.0001$ ;  
324 Figure 2.a). There was no significant difference between mated and virgin females 1 or 2 hours  
325 after mating, but there were significant differences at 4 hrs, 8 hrs, 24 hrs, and a week after  
326 mating (GLMs conducted on each time point separately: 1 hr:  $Dev_{1,31} = 0.66$ ,  $P = 0.42$ , 2 hrs:  
327  $Dev_{1,32} = 0.11$ ,  $P = 0.74$ , 4 hrs:  $Dev_{1,43} = 24.81$ ,  $P < 0.001$ , 8 hrs:  $Dev_{1,24} = 6.36$ ,  $P = 0.01$ , 24 hrs:  
328  $Dev_{1,41} = 24.33$ ,  $P < 0.001$ , Week:  $Dev_{1,44} = 14.32$ ,  $P < 0.001$ ). There was a significant main  
329 effect of mating, whereby mated females fought for longer than virgin females ( $Dev_{1, 225} =$   
330  $52.877$ ,  $P < 0.0001$ ). There was no significant effect of number of hours after mating or of  
331 block (Hours:  $Dev_{5, 220} = 8.496$ ,  $P = 0.14$ ; Block:  $Dev_{2, 218} = 3.217$ ,  $P = 0.2$ ).

332

333 **Figure 2: Mated females start to fight for longer than virgins around four hours after mating**

334 a. Manual scoring of headbutt duration

335 b. Automated tracking and classification of headbutt duration.

336 Blue points represent mated females, while yellow points represent virgin females. Each point  
337 represents the contest duration for one pair of females. The black bars represent the treatment means  
338  $\pm 1$  standard error.

339

340 How effective is using automated tracking and machine learning software for studying female  
341 aggression?

342 We tested whether there was a correlation between manual observations and tracking  
343 observations of headbutts, as well as whether this effect differed by mating status. We found  
344 a significant positive correlation between manual and tracking data (LM:  $F_{1, 185} = 14.05$ ,  $P =$   
345  $0.0002$ , Adjusted  $R^2 = 0.21$ ; Fig. 3), a significant effect of mating status for manual data (as  
346 found previously:  $F_{1, 185} = 31.52$ ,  $P < 0.0001$ ), and a significant interaction between mating  
347 status and tracking data ( $F_{1, 185} = 6.04$ ,  $P = 0.015$ ). As can be seen in Figure 3, the correlation  
348 between the manual and tracking data was significantly positive for mated females (linear  
349 regression equation:  $Y = 12.54 + 1.25x$ ,  $P = 0.0002$ ), while the slope for virgin females was not  
350 significantly different from 0 ( $Y = 9.56 - 0.0005x$ ,  $P = 0.99$ ).

351

352

353 **Figure 3: Manual and tracking data are positively correlated for mated females but not for virgin**  
354 **females**

355 Blue points represent mated females from all treatments, while yellow points represent virgin females  
356 from all treatments. Each point represents the contest duration for one pair of females. The lines  
357 indicate the linear model fit between the manual scoring and tracking data for mated (blue) and virgin  
358 (yellow) females separately. The highlighted areas around the line indicate the 95% confidence  
359 interval.

360

361 **Discussion**

362 *Genotypes*

363 We found that mating stimulated female aggression in all three genotypes to a similar degree  
364 and that males from different genotypes did not differ in their ability to stimulate aggression.  
365 Our results suggest that mating-induced female aggression is common to multiple genotypes  
366 of *Drosophila melanogaster* and is present in similar magnitudes.

367

368 Genetic variation underlying female aggression is not well-studied across taxa. While sex-  
369 specific genetic architectures have been investigated for a number of life history and  
370 morphological traits (31,32), there have been fewer studies on behavioral traits (33).  
371 Behavioral traits are often highly plastic and depend on an individual's social and physical  
372 environment, which may suggest a relatively small genetic component responsible for  
373 variation in female aggression. Shorter et al (2015) tested male aggression in 200 inbred lines  
374 of *Drosophila melanogaster* to detect genetic variation underlying aggression. They found a  
375 20-fold difference between the most and least aggressive lines, showing a strong effect of  
376 genotype on male aggressive behavior. Although this study found high levels of broad-sense  
377 heritability ( $H^2 = 0.69 \pm 0.07$ ), they found very low narrow-sense heritability ( $h^2 = 0.00$ ), which  
378 suggests a complicated set of gene interactions determining male aggressive phenotypes  
379 (23).

380

381 Male aggression in *Drosophila melanogaster* has been shown to be regulated by a sex-specific  
382 neural pathway, which suggests that there may be very different genetic architectures for  
383 male and female aggression in this species (34). We found that there was a genotype effect  
384 on female aggression (for both mated and virgin females), whereby w1118 females fought for

385 less time than Canton-S or Dahomey females. These results could suggest genotypic  
386 differences underlying aggression, but it is also possible that it due to a phenotypic  
387 manifestation of the white-eye mutation in w1118 females. Flies with a white-eye mutation  
388 are essentially blind, which detrimentally affects their locomotion and courtship behavior  
389 during photophase (35,36). w1118 females fighting for less time is consistent with a specific  
390 effect of the white-eyed mutation, rather than relying on broader polygenic differences  
391 between genotypes. We also found that w1118 males were much slower to mate than either  
392 Canton-S or Dahomey males and mated for less time (Supplementary Information and  
393 Supplementary Figures 2 & 3), as expected given their reduced ability to locate and court  
394 females (37). Our results suggest that there could be underlying genetic differences related  
395 to female aggression, but there do not appear to be dramatic differences between our female  
396 genotypes that alter either their base level of aggression or their aggressive response to  
397 mating.

398

399 Females from different populations can vary significantly in their response to mating and male  
400 ejaculates (19), while males can also differ greatly in their ability to stimulate post-mating  
401 responses based on their condition and previous social environment (38–40). However, we  
402 did not find any effect of male genotype or its interaction with female genotype on inducing  
403 female aggression or for either of our egg production measures (Supplementary Information  
404 and Supplementary Figures 4 & 5). Our results suggest that males from these three genotypes  
405 may not differ significantly in their ejaculate, or at least in those components that influence  
406 female aggression and egg laying in the 48 hours after mating, or that the post-mating female  
407 aggression response is robust to variation in ejaculate composition. There is previous  
408 evidence, however, that shows that female aggression can vary in magnitude based on

409 qualities of the male ejaculate. Females raised at different larval densities showed different  
410 levels of increase in aggression after mating (41). Females raised at higher larval density  
411 showed a greater increase in aggression after mating, which is probably due to their small  
412 body size meaning that the ejaculate they receive from males is larger in proportion to their  
413 mass (40).

414

#### 415 *Timing*

416 We found that female aggression in *Drosophila melanogaster* increased in mated females  
417 between two and four hours after mating, and remained elevated for at least a week after a  
418 single mating. There did not appear to be an increase over time in the level of aggression after  
419 mating – once females displayed an increase in aggression (2-3.5-fold higher than virgins), the  
420 difference between mated and virgins seemed to remain consistent for at least a week. This  
421 consistency, combined with the fact that males of different genotypes did not stimulate  
422 different levels of aggression, suggests a potential ‘switch’-like mechanism for female  
423 aggression – i.e. there is no gradual build-up of aggressive behavior, but instead once  
424 aggression has been turned on, it remains on at the same level. Ovipositor extrusion has been  
425 suggested to be such a behavior – it is only present in mated females, not in virgin females  
426 (42). Other behavioral and physiological effects show more of a gradual build-up effect,  
427 turning certain pathways present in virgins up (or down) in mated females, such as sex  
428 peptide increasing oogenesis and reducing receptivity in mated females (43). It is possible  
429 that intermediate levels of aggression may produce few benefits but still display the same  
430 costs associated with expressing higher levels of aggression, suggesting an ‘on/off’ switch may  
431 be a more beneficial way to regulate aggression.

432

433 One possible interpretation of the switch-like nature of female aggression is that increased  
434 aggression after mating is an adaptive response by females. It may be beneficial for females  
435 to only upregulate their aggression after mating as only then is it necessary for them to  
436 compete over access to resources such as food or oviposition sites. As virgins, their rate of  
437 egg production is low, as is their need for protein-rich foods, which suggests that the benefits  
438 to competing over such resources are limited. Potentially, when females deplete their sperm  
439 stores we may expect a return to virgin-like aggression, although this remains unknown. As  
440 yet, we have shown no direct costs to aggression in females, but it seems likely that there are  
441 at least some energetic costs to engaging in aggressive encounters with other females in other  
442 taxa (44). Taken together, these suggest a low cost-benefit ratio for virgin females for  
443 engaging in aggression, but this may shift for mated females, leading to increased aggression.  
444 Females across taxa are quite plastic in their expression of aggression, with some authors  
445 suggesting that females should be even more plastic than males due to the potentially higher  
446 costs from engaging in aggressive encounters (3). Overall, there seems to be clear evidence  
447 that females can adjust their levels of aggression in response to their environment or  
448 reproductive status, and our study may represent another instance of females altering their  
449 aggression in such an adaptive fashion.

450

#### 451 Breakdown of timing in mated females

452 To identify the putative mechanisms regulating female aggression after mating, it is useful to  
453 consider the timing of other post-mating responses (Fig. 4). Matings generally take around  
454 15-20 minutes in *Drosophila melanogaster*, with sperm transfer taking 1-8 minutes and the  
455 remaining duration taken up with the transfer of Sfps (45). The timing of sperm transfer and  
456 sperm storage may be important mediators of the induction of female aggression as the

457 transfer of sperm is necessary for females to increase aggression 24 hours after mating (16).  
458 Sperm storage begins around 25 minutes after the start of mating, with females storing up to  
459 400 sperm in their seminal receptacle by 1 hour after the start of mating (21). Females then  
460 use these sperm to fertilize their eggs over the next 5-7 days, depleting their sperm store  
461 (21,46). Female aggression may be triggered by females reaching their maximum sperm  
462 storage capacity, which appears to occur before the onset of increased aggression in mated  
463 females (Fig. 4). Female aggression does not appear to follow the same pattern as sperm  
464 number - sperm stores usually diminish over a period of time (dependent on female fecundity  
465 and number of sperm stored initially - (21,46)), whereas female aggression remains at an  
466 elevated level from 4 hours to a week after mating. This could suggest that the 'off' part of  
467 the female aggression switch is not determined by the number of sperm in storage. However,  
468 we did not test the number of sperm females had at different time points, so cannot be sure  
469 that our females showed the same pattern of sperm depletion as other females. Our females  
470 were kept without live yeast, which results in fewer eggs being laid and therefore fewer sperm  
471 being used, so they may have retained high numbers of sperm.

472

473

474 **Figure 4: Schematic of timing of various processes in mated females for the first 24 hours after**  
475 **mating**

476 A. The top portion of the figure represents aggression in mated females over the first 24 hours after  
477 mating as contest duration in seconds. This matches Figure 1 in this paper.

478 B. The middle part of the figure demonstrates the patterns of sperm storage (in the seminal receptacle  
479 only), egg laying (oviposition), and ovulation in mated females. These lines represent general patterns

480 and are not to scale with each other, but merely to give an indication of when and how these processes  
481 change over time. Sources: sperm storage (21), ovulation (20), egg laying (20).

482 C. The bottom portion of the figure demonstrates the timing of the presence of various seminal fluid  
483 proteins in different parts of the female – e.g. the top line represents when ovulin has been detected  
484 in the female hemolymph after mating. Sources: ovulin in hemolymph (47,48), ovulin in ovaries (20),  
485 mating plug and sperm ejection (18,49), sex peptide bound to sperm (50), sex peptide in hemolymph  
486 (51).

487

488 Other than sperm, seminal fluid proteins may also be involved in inducing post-mating  
489 aggression in females. ‘Sex peptide’ (SP) is at least partially responsible for the increase in  
490 aggression after mating and acts at multiple timescales, from a few hours after mating to a  
491 week after mating (50,52,53). These timescales line up with our findings in this study – that  
492 female aggression increases 2-4 hours after mating and remains elevated for at least a week.

493

494 The increase in female aggression 2-4 hours after mating also appears to occur in conjunction  
495 with the stimulation of ovulation (1.5 hours after mating starts) and potentially also with the  
496 beginning of egg laying (~3 hours after the start of mating) (20,54). We have previously shown  
497 that the production of eggs is not necessary for mating-induced female aggression and our  
498 results from both the timing and genotype experiments lend support to this idea (16,41).

499

#### 500 *The use of automated tracking and classification software*

501 Interestingly, there was a significant difference in how closely matched the automated and  
502 tracking data were between mated and virgin pairs. For mated females, videos that had  
503 higher scores from manual scoring also generally had higher scores when tracked. The fitted

504 regression line for mated females had a slope of 1.25, suggesting that the tracking data may  
505 show lower values for dyads with less fighting than manual scoring and higher values for  
506 dyads with more fighting than manual scoring. Although there was a lot of variability in the  
507 tracking-manual data relationship, it seems tracking may give a relatively accurate indication  
508 of aggression for mated females. In contrast, the manual and tracking scores showed very  
509 little concordance for virgin females (slope of fitted line = 0.0005, Fig. 3). There were generally  
510 fewer virgin pairs with higher scores (as virgin females fought less overall), but we would still  
511 expect to see a positive correlation between manual and tracking data over a narrower range  
512 of values.

513

514 This difference in the ability of the tracking system to detect aggression in mated and virgin  
515 females may indicate that there are not only quantitative differences between the two, but  
516 also qualitative differences. In a detailed ethographic comparison of female aggression, (14)  
517 found only small differences in fighting behavior between mated and virgin females. They  
518 found that mated females performed more behavioral transitions than virgins between  
519 different types of aggressive behaviors – e.g. from different types of fencing to headbutting  
520 to wing threats. However, they did not report any differences in the nature of individual  
521 behaviors, such as differences in how fast females performed headbutts, which our study  
522 potentially provides some evidence for.

523

524 We report the particular issues and challenges we faced using automated tracking and  
525 classification software for female aggression in the Supplementary Information as we hope  
526 this will assist others when designing their own setups. If tracking and classification software  
527 could be made to work for the relatively subtle behaviors of female aggression, it would

528 dramatically reduce the amount of time required for each experiment. Currently, manually  
529 scoring videos can take weeks or months of valuable researcher time and restricts the number  
530 of treatments and replicates that can be used in any study on female aggression.

531

## 532 **Conclusions**

533 Increasing our understanding of variation in female aggression is crucial for our  
534 comprehension of its development, function, and evolutionary consequences. Given the  
535 relationship between aggression and fitness is likely to differ substantially between males and  
536 females, it is vital to consider female-specific morphology, physiology, and behavior to fully  
537 understand the evolution of female aggression across species. Many proximate mechanisms  
538 of male aggression have been shown to be conserved across taxa - the neurotransmitter  
539 serotonin is found to play a role in both invertebrate and vertebrate male aggression,  
540 suggesting one of its very early functions may have been to regulate male aggression (55,56).  
541 It is possible that there are similar mechanisms that regulate female aggression that have yet  
542 to be properly examined – candidates include juvenile hormone in insects, and testosterone  
543 in vertebrates (11,57). Studying the temporal and genetic variation of female aggression  
544 allows us to identify putative proximate mechanisms and further test their role in the  
545 development and evolution of female aggression.

546

## 547 **Supporting Information**

548 Supporting Information (including Supporting Figures 1-7)

549

550

551

552 **Data availability**

553 Data for all experiments conducted for this paper will be made publicly available on the  
554 Oxford University Research Archive (doi to be inserted here).

555

556 **Author contributions**

557 E.B. and S.W. conceived the project. E.B. designed the experiments, with S.W. providing  
558 advice. E.R.B, A.E-C, E.B. performed the behavioural experiments. E.R.B and A.E-C. collected  
559 the egg count and wing size data for the genotype experiment, while E.B. scored the  
560 behavioural data for the timing experiment. E.B. trained the automated tracking and analysis  
561 software, and analysed the data for both experiments. E.B. wrote the manuscript. S.W., E.R.B.  
562 and A.E-C. discussed the results and contributed to the manuscript.

563

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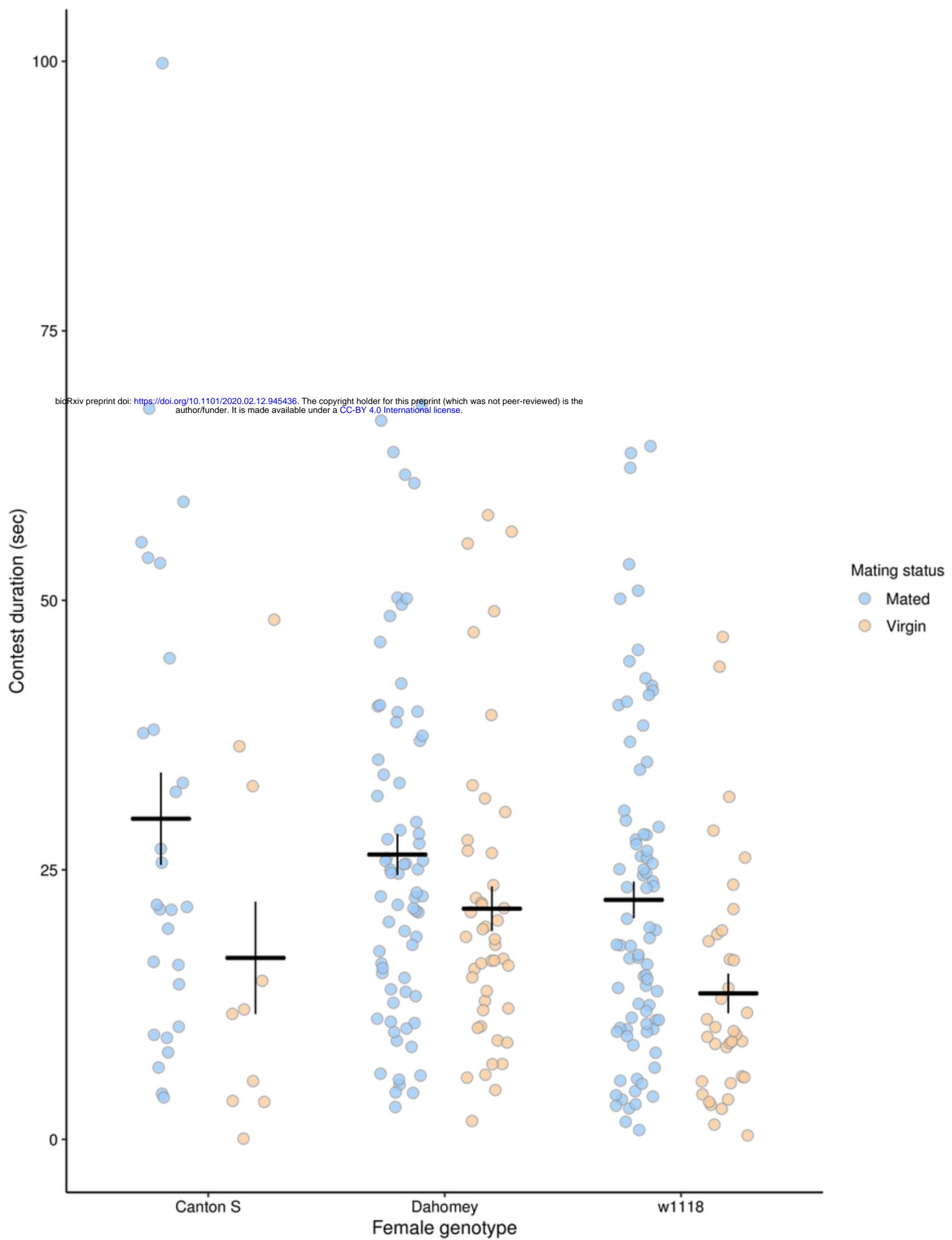


Figure 1

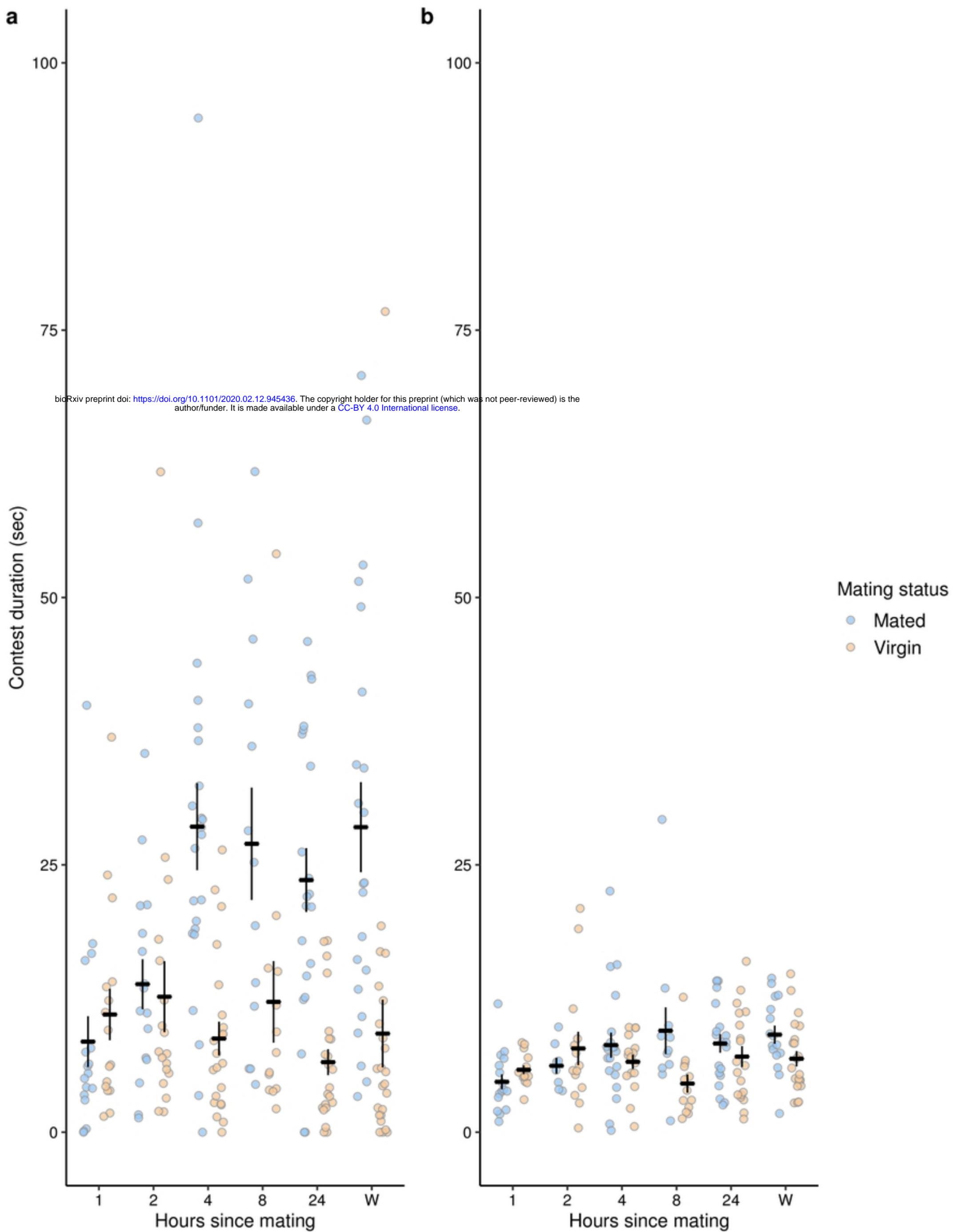


Figure 2

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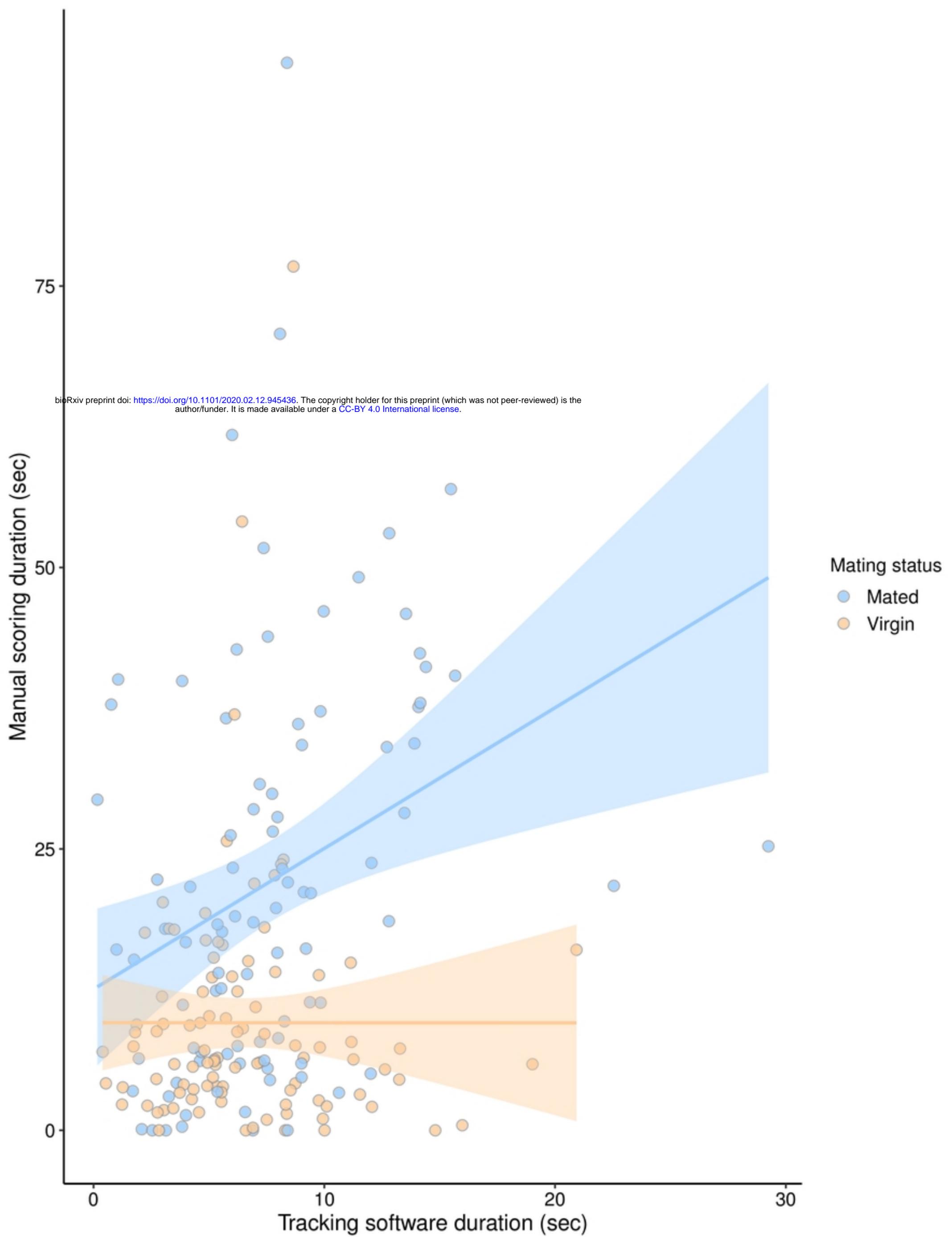


Figure 3

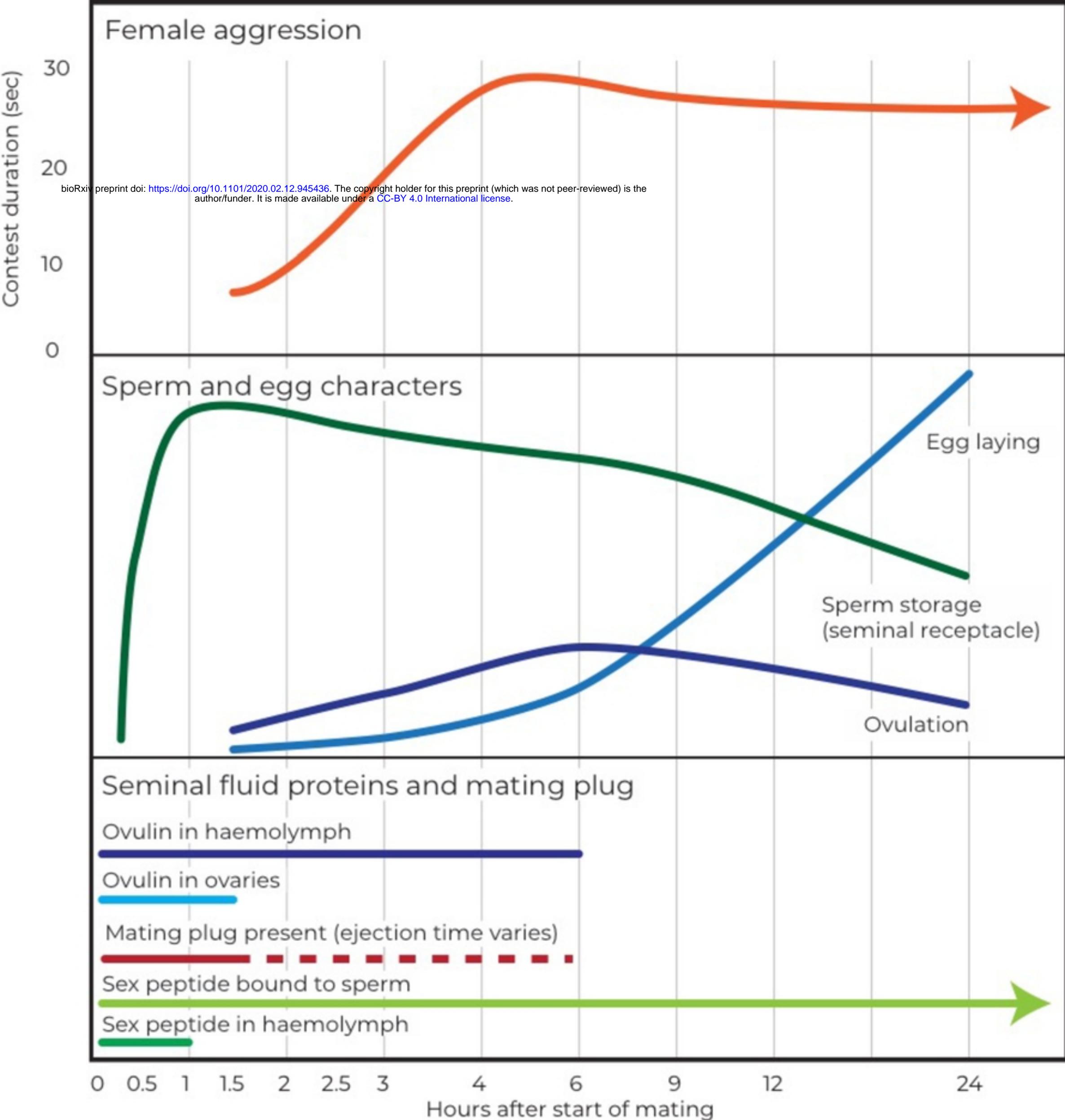


Figure 4