Temporal and genetic variation in female aggression after mating

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Abstract

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

Introduction

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

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

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

Materials and Methods

Fly stocks and culture

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

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

Experimental design

Genotype experiment

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

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

Timing experiment

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

- 1 hour post-mating
- 2 hours post-mating
- 4 hours post-mating
- 8 hours post-mating
- 24 hours post-mating
- 1 week (168 hours) post-mating

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

Behavioral experiments

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

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

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

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

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

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

Manual behavioral scoring

Videos were scored blind to treatment. Only headbutts were recorded as a proxy for female aggression, as these have previously been shown to be the most common high intensity form of aggression engaged in by females (14,15). We recorded the number of headbutting encounters and duration of each encounter. An encounter began when one female headbutted the other and ended when the flies separated or stopped interacting (NB: an encounter may include multiple headbutts from one or both individuals). We then used total encounter duration as the primary response variable, as we have previously shown that it encompasses variation in both the number and duration of encounters to give a good overall indicator of the amount of aggression shown by a pair (16). Using headbutts also enabled us to have a direct comparison with the automated behavioral data, where headbutting is 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*

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

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

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

    Contest duration ~ Male genotype * Female genotype

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

    Contest duration ~ Mating status * Female genotype

In addition, we investigated the effect of female genotype on wing area, and the number of eggs a female laid in the 24 hours before-, and after being used in a contest. For the wing area analysis, we used a linear model with wing area as the response and female genotype as the 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

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

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

Timing experiment

Aggression increases 2-4 hours after mating

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

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

a. Manual scoring of headbutt duration

b. Automated tracking and classification of headbutt duration.

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

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

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

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

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

Discussion

Genotypes

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

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

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

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

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

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

Breakdown of timing in mated females

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

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

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

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

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

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

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

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

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

The use of automated tracking and classification software

Interestingly, there was a significant difference in how closely matched the automated and tracking data were between mated and virgin pairs. For mated females, videos that had 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

564 **References**

565 1. Holekamp KE, Smale L, Szykman M. Rank and reproduction in the female spotted
566 hyaena. Journal of Reproduction and Fertility. 1996;108(2):229–37.

567 2. Stockley P, Bro-Jørgensen J. Female competition and its evolutionary consequences in
568 mammals. Biological reviews of the Cambridge Philosophical Society. 2011
569 May;86(2):341–66.

570 3. Clutton-Brock T, Huchard E. Social competition and its consequences in female
571 mammals. Journal of Zoology. 2013 Mar;289(3):151–71.

572 4. Stockley P, Campbell A. Female competition and aggression: interdisciplinary
573 perspectives. Philosophical Transactions of the Royal Society B: Biological Sciences.
574 2013 Oct;368(1631):20130073.

575 5. Buitenhuis AJ, Kjaer JB. Long term selection for reduced or increased pecking
576 behaviour in laying hens. World's Poultry Science Journal. 2008 Dec;64(4):477–87.

577 6. Dierick HA, Greenspan RJ. Molecular analysis of flies selected for aggressive behavior.
578 Nature genetics. 2006;38(9):1023–31.

7. Heyne HO, Lautenschläger S, Nelson R, Besnier F, Rotival M, Cagan A, et al. Genetic Influences on Brain Gene Expression in Rats Selected for Tameness and Aggression. Genetics. 2014 Nov 1;198(3):1277–90.

8. Sluyter F, Bult A, Lynch CB, van Oortmerssen GA, Koolhaas JM. A comparison between house mouse lines selected for attack latency or nest-building: Evidence for a genetic basis of alternative behavioral strategies. Behav Genet. 1995 May 1;25(3):247–52.

9. Murphy TG, Hernandez-Mucino D, Osorio-Beristain M, Montgomerie R, Omland KE. Carotenoid-based status signaling by females in the tropical streak-backed oriole. Behavioral Ecology. 2009 Jul;20(5):1000–6.

10. Papadopoulos NT, Carey JR, Liedo P, Müller H-G, Sentürk D. Virgin females compete for mates in the male lekking species Ceratitis capitata. Physiological Entomology. 2009 Sep;34(3):238–45.

11. Rosvall KA. Proximate perspectives on the evolution of female aggression: good for the gander, good for the goose? Philosophical Transactions of the Royal Society B: Biological Sciences. 2013;368(1631):20130083–20130083.

12. Chen S, Lee AY, Bowens NM, Huber R, Kravitz EA. Fighting fruit flies: a model system for the study of aggression. Proceedings of the National Academy of Sciences of the United States of America. 2002 Apr;99(8):5664–8.

13. Edwards AC, Rollmann SM, Morgan TJ, Mackay TFC. Quantitative genomics of aggressive behavior in Drosophila melanogaster. PLoS genetics. 2006 Sep;2(9):e154–e154.

14. Nilsen SP, Chan Y-B, Huber R, Kravitz EA. Gender-selective patterns of aggressive behavior in Drosophila melanogaster. Proceedings of the National Academy of Sciences of the United States of America. 2004 Aug;101(33):12342–7.

15. Ueda A, Kidokoro Y. Aggressive behaviours of female Drosophila melanogaster are influenced by their social experience and food resources. Physiological entomology [Internet]. 2002; Available from: http://onlinelibrary.wiley.com/doi/10.1046/j.1365-3032.2002.00262.x/full

16. Bath E, Bowden S, Peters C, Reddy A, Tobias JA, Easton-Calabria E, et al. Sperm and sex peptide stimulate aggression in female Drosophila. Nature Ecology & Evolution. 2017 May 15;1(6):0154.

17. Kubli E, Bopp D. Sexual behavior: How sex peptide flips the postmating switch of female flies. Current Biology. 2012;22(13):R520–2.

18. Lee K-M, Daubnerová I, Isaac RE, Zhang C, Choi S, Chung J, et al. A Neuronal Pathway that Controls Sperm Ejection and Storage in Female Drosophila. Current Biology. 2015;1–8.

19. Wensing KU, Fricke C. Divergence in sex peptide-mediated female post-mating responses in Drosophila melanogaster. Proceedings of the Royal Society B: Biological Sciences. 2018 Sep 12;285(1886):20181563.

20. Heifetz Y, Lung O, Frongillo EA, Wolfner MF. The Drosophila seminal fluid protein Acp26Aa stimulates release of oocytes by the ovary. Current Biology. 2000 Jan 15;10(2):99–102.

21. Bloch Qazi MC. An early role for the Drosophila melanogaster male seminal protein Acp36DE in female sperm storage. Journal of Experimental Biology. 2003 Oct 1;206(19):3521–8.

22. Dell AI, Bender JA, Branson K, Couzin ID, de Polavieja GG, Noldus LPJJ, et al. Automated image-based tracking and its application in ecology. Trends in Ecology & Evolution. 2014 Jul;29(7):417–28.

23. Shorter J, Couch C, Huang W, Carbone MA, Peiffer J, Anholt RRH, et al. Genetic architecture of natural variation in Drosophila melanogaster aggressive behavior. PNAS. 2015 Jul 7;112(27):E3555–63.

24. Partridge L, Farquhar M. Lifetime mating success of male fruitflies (Drosophila melanogaster) is related to their size. Animal Behaviour. 1983 Aug;31(3):871–7.

25. Clancy DJ, Kennington WJ. A simple method to achieve consistent larval density in bottle cultures. Drosophila Information Service. 2001;84:168–9.

26. Gilchrist A, Partridge L. A comparison of the genetic basis of wing size divergence in three parallel body size clines of Drosophila melanogaster. Genetics. 1999 Dec;153(4):1775–87.

27. Eyjolfsdottir E, Branson S, Burgos-Artizzu XP, Hoopfer ED, Schor J, Anderson DJ, et al. Detecting Social Actions of Fruit Flies. In: Fleet D, Pajdla T, Schiele B, Tuytelaars T, editors. Computer Vision – ECCV 2014. Springer International Publishing; 2014. p. 772–87. (Lecture Notes in Computer Science).

28. Kabra M, Robie AA, Rivera-Alba M, Branson S, Branson K. JAABA: interactive machine learning for automatic annotation of animal behavior. Nature Methods. 2012 Dec 2;10:64.

29. R Core Team. R: A Language and Environment for Statistical Computing [Internet]. Vienna, Austria: R Foundation for Statistical Computing; 2012. Available from: http://www.r-project.org/

30. Venables WN, Ripley BD. Modern Applied Statistics with S [Internet]. New York: Springer; 2002. Available from: http://dx.doi.org/10.1007/978-0-387-21706-2

31. Hallsson LR, Björklund M. Sex-specific genetic variances in life-history and morphological traits of the seed beetle Callosobruchus maculatus. Ecology and Evolution. 2012;2(1):128–38.

652 32. Jensen H, Sæther B-E, Ringsby TH, Tufto J, Griffith SC, Ellegren H. Sexual variation in
653 heritability and genetic correlations of morphological traits in house sparrow (Passer
654 domesticus). Journal of Evolutionary Biology. 2003;16(6):1296–307.

655 33. Chang HC, Dingemanse NJ. Sex-dependent expression of behavioural genetic
656 architectures and the evolution of sexual dimorphism. Proceedings of the Royal Society
657 B: Biological Sciences. 2017 Oct 11;284(1864):20171658.

658 34. Vrontou E, Nilsen SP, Demir E, Kravitz EA, Dickson BJ. fruitless regulates aggression and
659 dominance in Drosophila. Nature neuroscience. 2006 Dec;9(12):1469–71.

660 35. Krstic D, Boll W, Noll M. Influence of the White Locus on the Courtship Behavior of
661 Drosophila Males. PLOS ONE. 2013 Oct 30;8(10):e77904.

662 36. Liu L, Davis RL, Roman G. Exploratory Activity in Drosophila Requires the kurtz
663 Nonvisual Arrestin. Genetics. 2007 Mar 1;175(3):1197–212.

664 37. Reed SC, Reed EW. Natural selection in laboratory populations of *Drosophila* . II.
665 Competition between a white-eye gene and its wild type allele. Evolution. 1950
666 Mar;4(1):34–42.

667 38. Bretman A, Westmancoat JD, Chapman T. Male control of mating duration following
668 exposure to rivals in fruitflies. Journal of Insect Physiology. 2013 Aug;59(8):824–7.

669 39. Bretman A, Fricke C, Hetherington P, Stone R, Chapman T. Exposure to rivals and
670 plastic responses to sperm competition in Drosophila melanogaster. Behavioral
671 Ecology. 2010 Jan;21(2):317–21.

672 40. Wigby S, Perry JC, Kim Y-H, Sirot LK. Developmental environment mediates male
673 seminal protein investment in Drosophila melanogaster. Functional Ecology.
674 2016;30(3):410–9.

675 41. Bath E, Morimoto J, Wigby S. The developmental environment modulates mating-
676 induced aggression and fighting success in adult female Drosophila. Functional Ecology.
677 2018;32(11):2542–52.

678 42. Rezával C, Pavlou HJ, Dornan AJ, Chan Y-B, Kravitz EA, Goodwin SF. Neural circuitry
679 underlying Drosophila female postmating behavioral responses. Current biology : CB.
680 2012 Jul;22(13):1155–65.

681 43. Billeter J-C, Wolfner MF. Chemical Cues that Guide Female Reproduction in Drosophila
682 melanogaster. J Chem Ecol. 2018 Sep 1;44(9):750–69.

683 44. Seebacher F, Ward AJW, Wilson RS. Increased aggression during pregnancy comes at a
684 higher metabolic cost. The Journal of experimental biology. 2013 Mar;216(Pt 5):771–6.

685 45. Mattei AL, Riccio ML, Avila FW, Wolfner MF. Integrated 3D view of postmating
686 responses by the Drosophila melanogaster female reproductive tract, obtained by
687 micro-computed tomography scanning. PNAS. 2015 Jul 7;112(27):8475–80.

688 46. Avila FW, Ram KR, Qazi MCB, Wolfner MF. Sex Peptide Is Required for the Efficient
689 Release of Stored Sperm in Mated Drosophila Females. Genetics. 2010 Oct
690 1;186(2):595–600.

691 47. Herndon LA, Wolfner MF. A Drosophila seminal fluid protein, Acp26Aa, stimulates egg
692 laying in females for 1 day after mating. Proceedings of the National Academy of
693 Sciences. 1995;92(22):10114–10118.

694 48. Monsma SA, Harada HA, Wolfner MF. Synthesis of two Drosophila male accessory
695 gland proteins and their fate after transfer to the female during mating.
696 Developmental Biology. 1990 Dec 1;142(2):465–75.

697 49. Avila FW, Cohen AB, Ameerudeen FS, Duneau D, Suresh S, Mattei AL, et al. Retention
698 of Ejaculate by Drosophila melanogaster Females Requires the Male-Derived Mating
699 Plug Protein PEBme. Genetics. 2015 Aug 1;200(4):1171–9.

700 50. Peng J, Chen S, Büsser S, Liu H, Honegger T, Kubli E. Gradual Release of Sperm Bound
701 Sex-Peptide Controls Female Postmating Behavior in Drosophila. Current Biology. 2005
702 Feb;15(3):207–13.

703 51. Pilpel N, Nezer I, Applebaum SW, Heifetz Y. Mating-increases trypsin in female
704 Drosophila hemolymph. Insect Biochemistry and Molecular Biology. 2008;38(3):320–
705 30.

706 52. Liu H, Kubli E. Sex-peptide is the molecular basis of the sperm effect in Drosophila
707 melanogaster. Proc Natl Acad Sci USA [Internet]. 2008;100. Available from:
708 http://dx.doi.org/10.1073/pnas.1631700100

709 53. Haussmann I, Hemani Y, Wijesekera T, Dauwalder B, Soller M. Multiple pathways
710 mediate the sex-peptide-regulated switch in female Drosophila reproductive
711 behaviours. Proceedings of the Royal Society B: Biological Sciences.
712 2013;280(October):20131938–20131938.

713 54. Rubinstein C, Wolfner M. Drosophila seminal protein ovulin mediates ovulation
714 through female octopamine neuronal signaling. Proceedings of the National Academy
715 of Sciences [Internet]. 2013;110(43). Available from:
716 http://www.pnas.org/content/110/43/17420.short

717 55. Dierick HA, Greenspan RJ. Serotonin and neuropeptide F have opposite modulatory
718 effects on fly aggression. Nature genetics. 2007;39(5):678–82.

719 56. Johnson O, Becnel J, Nichols CD. Serotonin 5-HT(2) and 5-HT(1A)-like receptors
720 differentially modulate aggressive behaviors in Drosophila melanogaster.
721 Neuroscience. 2009 Feb;158(4):1292–300.

722 57. Tibbetts EA, Crocker KC. The challenge hypothesis across taxa: Social modulation of
723 hormone titres in vertebrates and insects. Animal Behaviour. 2014;92:281–90.

724

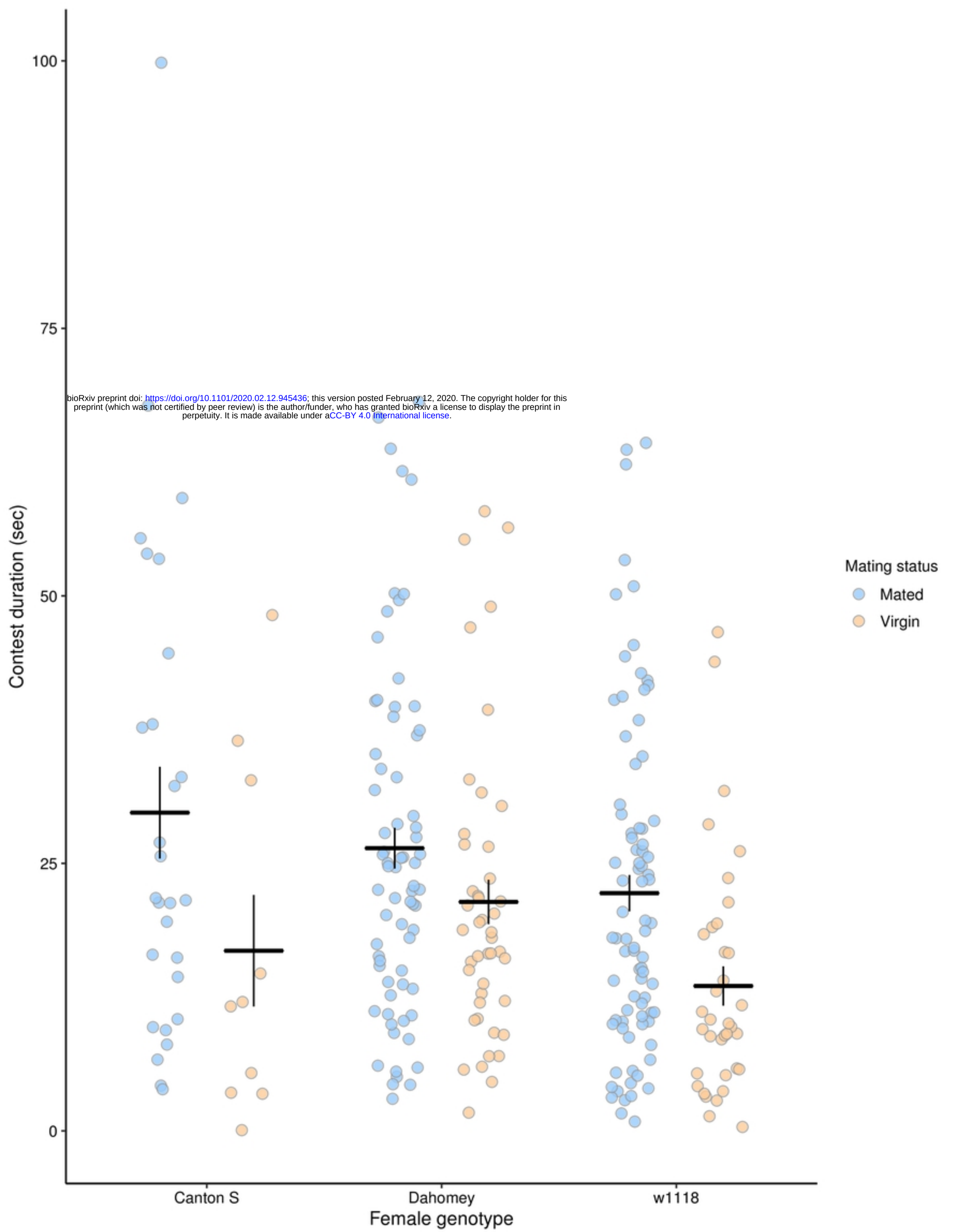

Figure 1

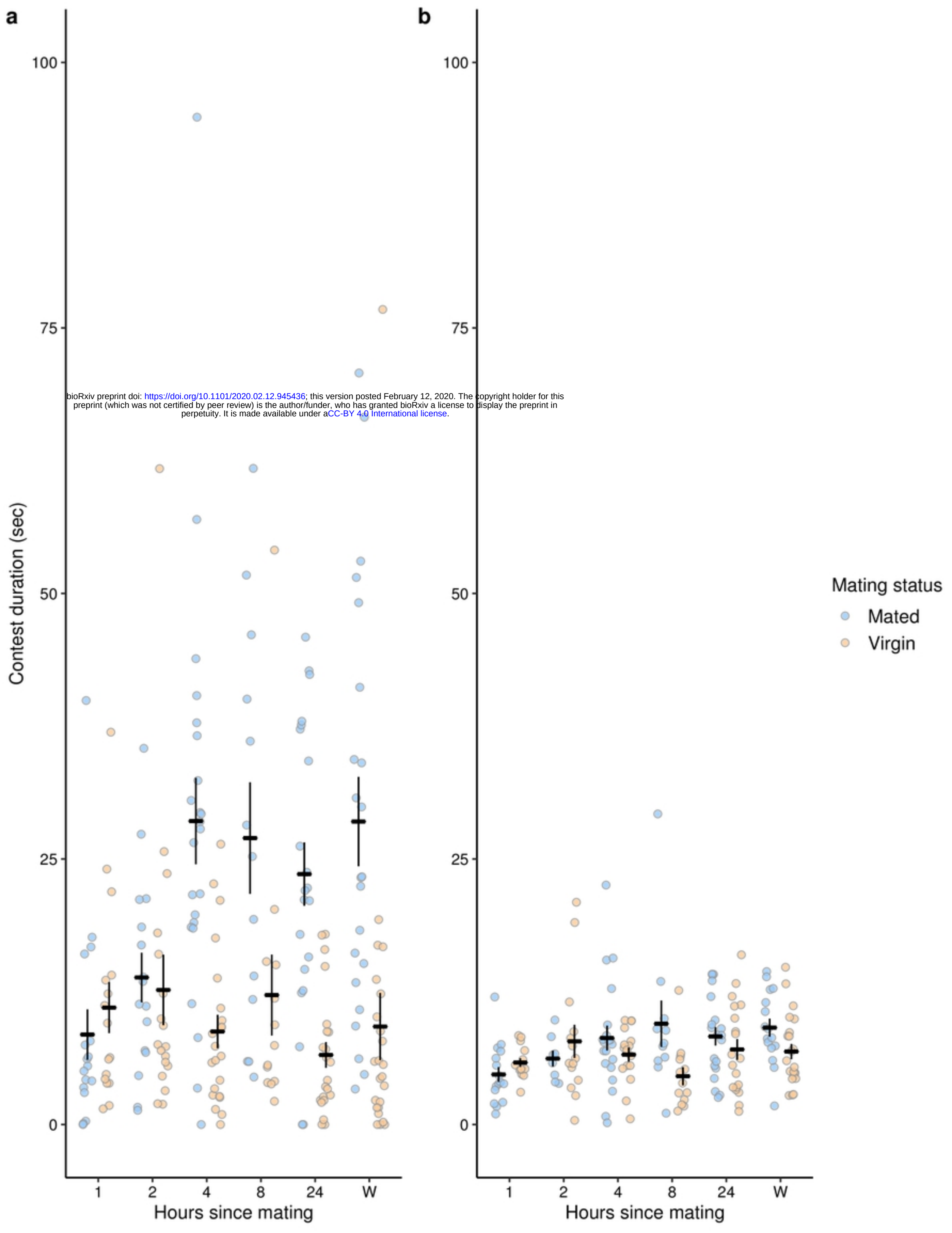

Figure 2

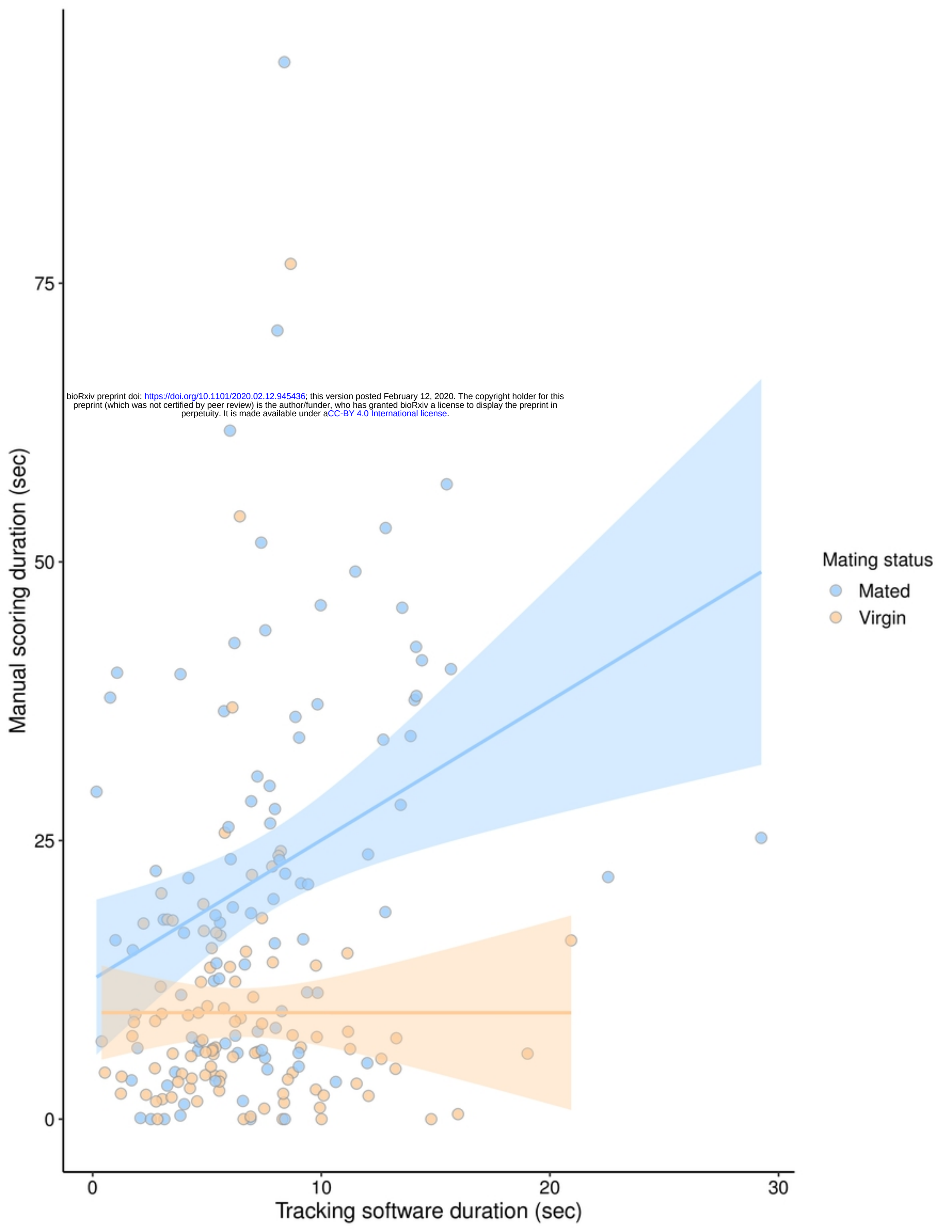

Figure 3

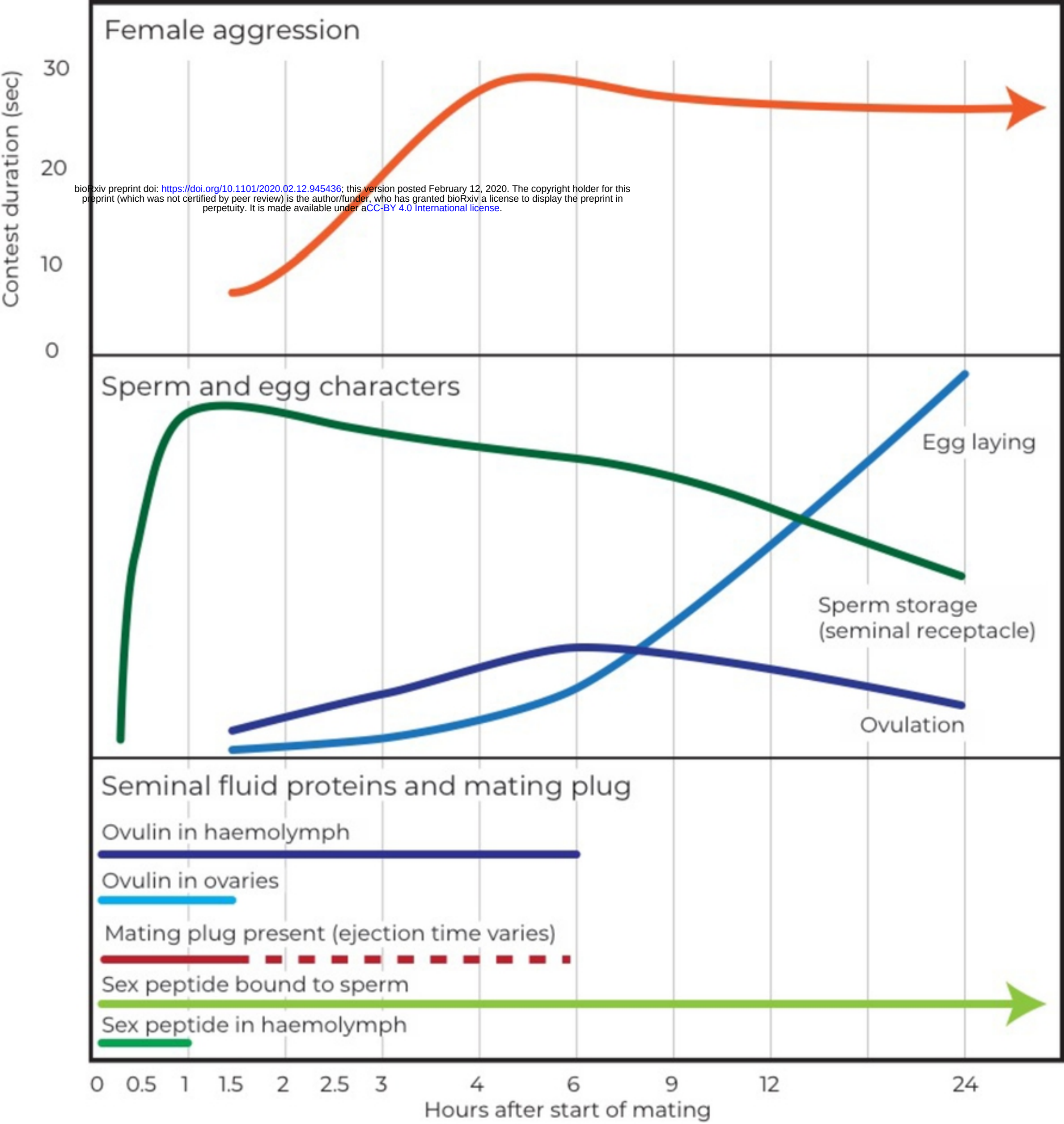

Figure 4