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Very short copulations are enough for sperm transfer in *Littorina saxatilis*

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SPERM TRANSFER TIME IN *LITTORINA*

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ABSTRACT

Conflict over reproduction between females and males exists because of anisogamy and promiscuity. Together they generate differences in fitness optima between the sexes and result in antagonistic coevolution of female and male reproductive traits. Copulation duration is likely to be a compromise between male and female interests whose outcome depends on the intensity of sexual selection. The timing of sperm transfer during copulation is critical: For example, copulations may be interrupted before sperm is transferred as a consequence of female or male choice, or they may be prolonged to function as mate guarding. In the highly promiscuous intertidal snail *Littorina saxatilis*, copulations vary substantially in duration, from less than a minute to more than an hour, and it has been assumed that copulations of a few minutes do not result in any sperm being transferred. Here, we examined the timing of sperm transfer, a reproductive trait that is likely affected by sexual conflict. We performed time-controlled copulation trials using *L. saxatilis* males and virgin females, aiming to examine indirectly when the transfer of sperm starts. We observed the relationship between copulation duration and the proportion of developing embryos out of all eggs and embryos in the brood pouch. Developing embryos were observed in similar proportions in all treatments suggesting that sperm transfer begins rapidly (within 1 minute) in *L. saxatilis* and very short matings do not result in sperm shortage in the females. We discuss how the observed pattern can be influenced by predation risk, population density, and female status and receptivity.

INTRODUCTION

In sexually reproducing species, females and males share the benefits of reproductive success. However, while in strict, life-long monogamous species reproduction can be viewed as an alliance between the sexes, in other systems, such as polygynous and polyandrous species, the interests of males and females differ leading to reproductive conflict between the sexes (Parker, 1979). Copulation duration and number of matings are well-known examples of sexual conflict because long and numerous matings are generally observed to increase male fitness but to decrease female fitness (Chapman *et al.*, 2003).

Females are in general expected to invest much more energy per gamete than males (Janicke *et al.*, 2016; Trivers, 1972) and because of this asymmetry, females or their gametes can be considered as limiting resources. Male competition for such resources is inevitable and will select for traits or behaviors that increase male reproductive success (Bateman, 1948). Sexual conflict will then arise if those traits or behaviors reduce female fertility or survival (Chapman *et al.*, 1995; Wolfner, 1997).

Males have been shown to gain a fertility benefit by extending copulation duration and, thus, delaying the time when a female will remate with another male (Gilchrist & Partridge, 2000). Long copulations should be costly for both sexes (e.g., less time for feeding) (Daly, 1978) but they are expected to be more beneficial for males than they are for females (Edward , Stockley & Hosken, 2015; Simmons, 2001). For instance, in the common dung fly, males that copulated for longer transferred a larger quantity of ejaculate which was suggested to increase their reproductive success but not that of females who instead showed increased mortality during copulation and vigorous resistance to mating (Martin & Hosken, 2002). Another reason for males to copulate for longer is mate guarding, also exemplified by the common dung fly, which impedes other males from mating and fertilizing the guarded female. This benefits the male but may be costly to the guarded female, for example by preventing her from feeding properly (reviewed in Simmons 2001).

In addition to influencing the duration of mate guarding, population density is expected to influence sperm transfer and as a consequence, it may have an additional effect on copulation duration. In high-density populations, the theoretical prediction is that males should allocate sperm and seminal fluid with discrimination because ejaculates are costly to produce and represent a limit on how many successive females a male can

66 mate with and fertilize. There is strong agreement between theory and empirical
67 evidence that male investment per copulation is maximized when mating with high-
68 quality females (e.g., larger size) or with previously mated females, and when
69 competing with a low number of other males (delBarco-Trillo, 2011; Kelly & Jennions,
70 2011; Parker *et al.*, 1996; Parker & Pizzari, 2010; Simmons & Fitzpatrick, 2012; Wedell,
71 Gage & Parker, 2002).
72 The first step to understand how females and males interact with respect to copulation
73 duration is to measure fertilization success as a function of copulation duration.
74 Knowing when sperm transfer starts and ends is crucial for assessing how female and
75 male traits have coevolved. Here, we controlled copulation duration between males and
76 virgin females of the intertidal snail species *Littorina saxatilis*, a well-studied system for
77 adaptive divergence and reproductive isolation among populations inhabiting different
78 habitats. We tested whether copulation duration influences the proportion of eggs that
79 are fertilized, as a measure of sperm transfer. Eggs and developing embryos are carried
80 in the female's brood pouch in this species and so the result of sperm transfer can be
81 checked a few weeks after mating by dissecting the female. In littorinid gastropods,
82 sperm are transferred in a fluid and moved by cilia in a groove running along the male
83 penis (Reid, 1996). There is evidence in the sea hare *Aplysia parvula* that sperm
84 transferred in a fluid are very few at short copulation times (few minutes) and that their
85 number increases as copulation continues (Yusa, 1994). We expected a similar pattern
86 in *L. saxatilis* with short copulations being inadequate for sperm transfer whereas
87 longer copulations (ten minutes long or more) would be more likely to yield effective
88 transfer of sperm. However, we did not expect the relationship between copulation
89 duration and sperm transfer to be necessarily linear because in other studied
90 gastropods the correlation is either weak or absent (reviewed by Weggelaar,
91 Commandeur & Koene 2019).
92 Several ecotypes of *L. saxatilis* have been described and two in particular (so-called
93 'Crab' and 'Wave' forms) have been used as a model for studying the evolution of
94 reproductive isolation under a scenario with ongoing gene flow between locally adapted
95 populations (Johannesson *et al.*, 2010a). There is likely to be strong sexual conflict due
96 to high population density, risks associated with mating and opportunity for sperm
97 competition and/or cryptic female choice (Johannesson *et al.*, 2016; Johannesson *et al.*,
98 2010b). A wide range of copulation durations has been observed and this may be due to

slow sperm transfer (Hollander, Lindegarth & Johannesson, 2005; Perini *et al.*, 2020; Saur, 1990).

Here, we examined the timing of sperm transfer in *L. saxatilis* and performed time-controlled copulation trials using males and virgin females. Each female was mated only once and later dissected in order to count the number of developing embryos. The aim was to understand the relationship between copulation duration, sperm transfer and number of developing embryos. Knowing at which time males start transferring the sperm during copulation and whether longer copulations correspond to a larger number of offspring is needed for improving our understanding of the potential impact of sexual conflict on trait evolution in *L. saxatilis*.

MATERIAL AND METHODS

We performed one round of experiments in the autumn of 2016 and one in the summer of 2020 and for each experiment we followed the same protocol except that in 2020 we modified one treatment (see below).

We used a total of 38 virgin females (14 Wave in 2016, and 21 Crab and three Wave in 2020) that were sampled when immature (very small shell sizes, 2-4 mm long) from a rocky shore on the island of Saltö (58°52'17.0"N 11°07'04.1"E), west coast of Sweden, and reared in aquaria that were filled with sea water via a flow-through system. The aquaria were kept in a day-night cycle so that the virgin females could feed on microalgae that grew on the walls. After approximately ten months, we sampled adult snails of both ecotypes from the same locality and identified ~60 males by observing a fully developed penis. Females from the aquaria and males from the wild were measured (maximum shell length) and each female was matched with males of the same ecotype that were ~25% smaller. The probability of mating varies with the relative size of female and male, and the highest probability is reached for this size ratio (Perini*, et al.*, 2020). In each trial we used two males to increase further the chance that one male would start to mate with the virgin female.

Females and males adopt a characteristic mating position that can be clearly observed in the wild as well as in the lab. Typically, the male approaches the female and crawls on top of her shell until he stops at the front-right side of the female shell. At this specific mounting position, the male inserts the penis under the female shell and initiates

131 transfer of sperm. When exactly the penis is inserted is difficult to establish but a strong
132 correspondence has been found between male mounting position and copulation
133 attempt (Hollander*, et al.*, 2005).
134 We used unpublished data on copulation duration from an earlier experiment (Perini, *et*
135 *al*., 2020) to decide what we should consider as short, medium and long copulation
136 times (Fig. 1). Copulation trials were performed indoors under constant light and at
137 room temperature. At the start of each trial, the female and the two males (a trio) were
138 placed foot-down at the bottom of a transparent plastic sphere (80 mm in diameter)
139 one-third filled with sea water. In both the 2016 and 2020 experiments, each trio was
140 assigned to one of three treatments which corresponded to the time at which copulation
141 was artificially interrupted. In 2016, copulation was interrupted at either five, ten or 30
142 minutes after observing a pair to enter the characteristic mating position. In 2020,
143 copulation was instead interrupted at either one, five or 30 minutes. We replaced the 10
144 minute trial with a one minute treatment because we wanted to test the hypothesis that
145 very short copulations were insufficient for sperm to be transferred to the female. The
146 data from the two experiments were then combined by merging the 10 minute
147 treatment with the 30 minute treatment (10+) in order to increase the sample size for
148 the statistical analysis.
149 Copulations were interrupted at the predefined experimental times by separating the
150 mating pair. Copulations that lasted less than the pre-assigned time were recorded and
151 these females were assigned to a treatment group appropriate to the observed
152 copulation duration. Thereafter, the female was marked with a unique identifier and
153 placed in a new sea water aquarium without the male. The same aquarium was used for
154 all the treated females and also for virgin females that were not assigned to any of the
155 treatments and used as unmated controls.
156 If no copulation had been recorded throughout the length of the trial (two hours), the
157 same female was reused the next day and paired again with males with optimal relative
158 size. When available, new males were preferred, otherwise, the females were matched
159 with the same males as the previous day.
160 Mated and control females were dissected two to three weeks after the mating trials.
161 This time allowed the mated females to start using the sperm to fertilize eggs and for
162 embryogenesis of the first fertilized eggs to have proceeded to a developmental stage
163 that was easily distinguished from unfertilised eggs. Eggs and embryos of each female

were photographed using a Canon camera (model EOS 5D Mark iii) mounted on a Leica M80 microscope and counted using ImageJ (Schneider, Rasband & Eliceiri, 2012). Mis-developed embryos beyond egg stage were treated as fertilized eggs and included in the embryo count (Johannesson *et al.*, 2020). Embryos were classified as mis-developed if clumps of cells were spread throughout the egg capsule or they showed malformed shells (e.g., poorly coiled and dwarfed). Females with no eggs or developing embryos were discarded as they were likely immature and/or parasitized while females with at least one egg or one developing embryo were retained for the analysis.

We calculated the proportion of developing embryos for each female. We expected that females that had short copulations would have a limited sperm supply and so would show a reduced rate of fertilization in the eggs they produced over two to three weeks after the mating trials. Any such effect might be influenced by the total number of eggs and embryos carried by a female. In order to assess the relationship between copulation duration and fertilization success, we used a generalized linear model with error distribution following a beta-binomial function. We chose a beta-binomial distribution to account for over-dispersion in the response variable due to factors that may be important during fertilization (e.g., sperm storage) but that were not analyzed in this study. To test for a difference in proportion of developing embryos between the different treatments, we fitted a beta-binomial model using the R package "aod" (Lesnoff & Lancelot, 2012) in which the proportion of developing embryos was the response variable and copulation duration was the independent variable (categorical). The null model was beta-binomial with the same response variable but without the treatment effects and models were compared using the Akaike Information Criterion (AIC). Whether the different treatments had significantly different effects on the proportion of developing embryos was tested using the Tukey-Kramer method (Kramer, 1956; Tukey, 1949): the effects were considered significantly different if the absolute value of the difference of two treatment means was greater than or equal to the Honestly Significant Difference statistic (HSD). By adding the year when the two experiments were performed as a second independent variable, we were also able to test whether the relationship between the proportion of developing embryos and copulation durations differed between the two experiments. Finally, we included female size and total number of eggs and embryos as covariates to the beta-binomial model to

check whether these variables had a significant effect on the proportion of developing embryos.

RESULTS

We used a total of 38 virgin females but analyzed 33 mated females (Table 1), discarding five females that were likely immature or parasitized.

We examined the variation in proportion of developing embryos between treatments by fitting a beta-binomial model to account for dispersion of the response variable (dispersion parameter = 0.47, standard error = 0.06, p-value < 0.01). Including the treatment effects in the model explained significantly more variation in the response variable (treatment model AIC = 234.5, null model AIC = 251.3). The estimated coefficients of the treatments were significantly different from the control but treatments did not differ from one another (Table 2; Fig. 2). Short matings were as successful as long ones because similar proportions of developing embryos were found in all treatments and longer matings were not associated with a greater proportion of developing embryos. In all except one female in the ten-plus minute treatment, in addition to developing embryos, we also found eggs in which we could not detect development. There was no significant effect on the proportion of developing embryos due to the 2016 and 2020 experiment (estimate = -1.04, standard error = 4.23, p-value = 0.81), due to the female size (estimate = 0.02, standard error = 0.16, p-value = 0.93) nor due to the total number of eggs and embryos (estimate = 0.00, standard error = 0.00, p-value = 0.27).

Copulations that lasted less than the pre-assigned time (7 cases) ranged between three and 28 minutes duration and in all the females fertilization had occurred (proportion of developing embryos ranged between 0.2 and 0.9).

DISCUSSION

In species with internal fertilization, sperm have to be transferred into the female to fertilize the eggs. When, and for how long sperm transfer occurs is still uncertain for most species (Weggelaar*, et al.*, 2019). The number of sperm that are transferred to the female may be strongly correlated with copulation duration if a large quantity of sperm increases male and/or female reproductive success. This correlation between sperm

transfer and copulation duration may be complex and not necessarily linear, or absent, because the relationship is expected to depend on the interaction between female and male traits and their corresponding fitness optima (Edward *, et al.*, 2015; Perry & Rowe, 2015). Copulation duration may then be used for understanding whether the optima for sperm transfer are divergent (sexual conflict) or the same between the two sexes.

In this study, we have measured sperm transfer indirectly based on the relationship between the proportion of developing embryos and copulation duration in the highly promiscuous, internally-brooding snail *L. saxatilis*. We have shown that, surprisingly, very short copulations are sufficient for the sperm transport into the female to begin and that females involved in interrupted copulations of short, medium and long duration did not carry different proportions of developing embryos.

For species such as *L. saxatilis* in which males transfer sperm in a fluid via ciliary movements (Reid, 1996), very short copulations were not expected to be effective for transferring the sperm to the female (Hollander*, et al.*, 2005). However, experimental evidence for this assumption is not clear-cut, especially in other gastropods where the number of studies is limited to a few species (reviewed by Weggelaar, *et al.* 2019). For example, in the freshwater snail *Lymnaea stagnalis*, very few sperm were found after 10 to 25 minutes of copulation and most of the sperm were transferred near the end of copulation (Weggelaar*, et al.*, 2019). In *Littoraria cingulata* and *L. filosa,* Hollander *et al.* (2018) observed an increased probability of sperm transfer for longer copulation durations. In the opisthobranch sea hare *Aplysia parvula*, Yusa (1994) found that more sperm were transferred in longer copulations but that a few minutes were already sufficient for sperm transfer in a fluid. Hence, even though long copulations might be required for transferring a large amount of sperm, short copulations, as we observed in *L. saxatilis*, can be effective to transfer enough sperm to fertilize a batch of embryos. The experimental interruption of copulation itself does not appear to be the cause of rapid sperm transfer in our experiment with *L. saxatilis* because pairs that ended copulation before the pre-assigned time achieved similar transfer of sperm to the females (even after 3 minutes). This suggests that short copulations in nature can provide enough sperm for many of a female's eggs to be fertilized.

We cannot exclude the possibility that more sperm were transferred in longer matings. High sperm loading might be beneficial for males mainly to displace sperm from previous matings or to dilute their contribution (Parker & Pizzari, 2010). Previous

261 results on biased paternity towards large males in *L. saxatilis* would support this
262 possibility (Johannesson*, et al.*, 2016) suggesting that, like in many insects (Simmons,
263 2001) and a few aquatic gastropods (Anthes, Werminghausen & Lange, 2014; Oppliger
264 *et al.*, 2003; Xue, Zhang & Liu, 2014), sperm competition would select for large males
265 with a large/long penis that produce many sperm. Because here we have used virgins
266 and single matings, such a correlation between sperm transfer and copulation duration
267 may not be captured. All except one female showed eggs where we did not detect
268 development but we cannot be sure whether these were unfertilized or fertilized but
269 not sufficiently advanced embryos to be detectable as undergoing development at the
270 time when we dissected the females. The proportion of undeveloped embryos and eggs
271 in treated females matches well with the proportion of similarly early embryo stages
272 ("preveligers") (~20%) in wild-collected females (Johannesson*, et al.*, 2020) that are not
273 likely to be sperm-limited (Panova *et al.*, 2010). Such a similarity in proportions of
274 developed and undeveloped embryos would suggest that females that were interrupted
275 at any time during copulation (even after only one minute) in our experiment were
276 unlikely to be sperm-limited in the short term.
277 One hypothesis that could explain rapid sperm transfer in *L. saxatilis* is that of high
278 predation risk. There is empirical evidence in littorinid snails that when females and
279 males enter the mating position, the risk of being dislodged from the intertidal and/or
280 being eaten by crabs and fish increases compared to single individuals (Johannesson,
281 1986; Johannesson*, et al.*, 2010b; Kemppainen *et al.*, 2005; Koch, Lynch & Rochette,
282 2007). If this risk is high, then it may be beneficial for both sexes to transfer sperm
283 rapidly to assure fertilization at a lower cost of mating. This might explain why we
284 observed *L. saxatilis* developing embryos already in the one minute treatment. A similar
285 effect of predation was also found in fireflies, which usually copulate for hours or days.
286 In the species *Photinus collustrans*, where an increased predation risk was observed
287 compared to other fireflies, copulations lasted only a few minutes (Wing, 1988). If the
288 same was true for *L. saxatilis*, we would have expected copulation duration to reflect
289 such predation risk and thus, be on average a few minutes long, both in the lab and in
290 the field. What we see is, instead, an average mating time of 20 minutes and many
291 matings lasting up to one hour (Fig. 1). Hence, other factors are likely to influence
292 copulation duration in *L. saxatilis*.

In *L. saxatilis*, entering the mating position may not correspond exactly to the time when the penis is inserted under the female's shell. For this reason, the start of copulation may have been later than the time we recorded. At the same time, watching multiple trials, the observer might have missed the start of the mating by up to 30 seconds. The true duration of 'one minute' matings is, therefore, somewhat uncertain but this uncertainty is relatively low for the other treatments. Nevertheless, our general conclusion still holds: short matings in *L. saxatilis* (approximately one minute duration) are sufficient for sperm transfer to begin. This duration is shorter than what has been previously expected to be the required time for sperm of *L. saxatilis* to be transferred into the female and much shorter than the majority of observed matings (Fig. 1). Extended copulations do not necessarily mean that sperm transfer is delayed or that a larger quantity of sperm is transferred to the female. In many insects (Weggelaar*, et al.*, 2019) but also in some hermaphroditic land snails (Dillen, Jordaens & Backeljau, 2009), males have been found to increase their fertilization success by mate guarding the females after having transferred their sperm. This behavior is expected to be especially beneficial in low-density populations, whereas in high-density populations its benefits are lost. As the population density increases and, thus, both availability of females and intensity of male competition increases, males are instead expected to invest less in mate guarding as well as investing less in sperm quantity per mating (Parker, 1974). The prediction is that, compared to low-density populations, males in high-density populations should allocate time and energy into mate searching and consecutive inseminations, which should be especially beneficial when female receptivity is not time constrained (Parker, 1974). Hence, shorter copulations should be more cost-effective in high rather than in low-density populations and for mating systems with long rather than short sexual activity periods. In the populations sampled for this experiment, males and females of *L. saxatilis* live in high density and females are reproductively active year round and so the mate guarding hypothesis seems unlikely to explain why copulations last longer than what is required for initial sperm transfer.

We have shown that sperm transfer in *L. saxatilis* begins rapidly during copulation but it remains unclear whether the evolution of rapid sperm transfer is influenced by increased predation risk, high population density or year round female receptivity. The evidence that copulations in *L. saxatilis* are on average much longer than a few minutes strongly argues against any of these effects. We showed that enough sperm are

transferred in a short time to achieve fertilization as successfully after a few minutes as after ten or more minutes but we did not test for how long sperm transfer continues or whether the duration of transfer influences the total number of sperm transferred, and so male reproductive success, particularly when females are multiply mated. Once this information becomes available, we should be able to say more about sperm competition and the potential for sexual conflict over copulation duration.

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447 Table 1. Number of females (N) per ecotype, treatment and experiment (Year).

Ecotype	Treatment	Year	N
Crab	Control	2020	3
Crab	1	2020	4
Wave	1	2020	1
Crab	5	2020	3
Wave	5	2020	1
Crab	10+	2020	5
Wave	Control	2016	3
Wave	5	2016	4
Wave	10+	2016	2

448

449 Table 2. Summary of parameter estimates for the beta-binomial model and Tukey-
450 Kramer's Honestly Significant Difference (HSD). Back-transformed Maximum Likelihood
451 Estimate and 95% confidence intervals (95% CIs) for the control group, one minute
452 treatment (T1), five minute treatment (T5) and ten-plus minute treatment (T10+).

			Tukey-Kramer HSD		
Coefficient	**Estimate**	**95% CIs**	**Control**	**T1**	**T5**
Control	0.00[a]	0.00 to 0.46			
T1	0.91[b]	0.74 to 0.97	0.38		
T5	0.75[b]	0.56 to 0.88		0.58	
T10+	0.79[b]	0.57 to 0.91		0.58	0.49

453 Estimates followed by the same letter are not significantly different from each other
454 (Tukey–Kramer test, P>0.05).

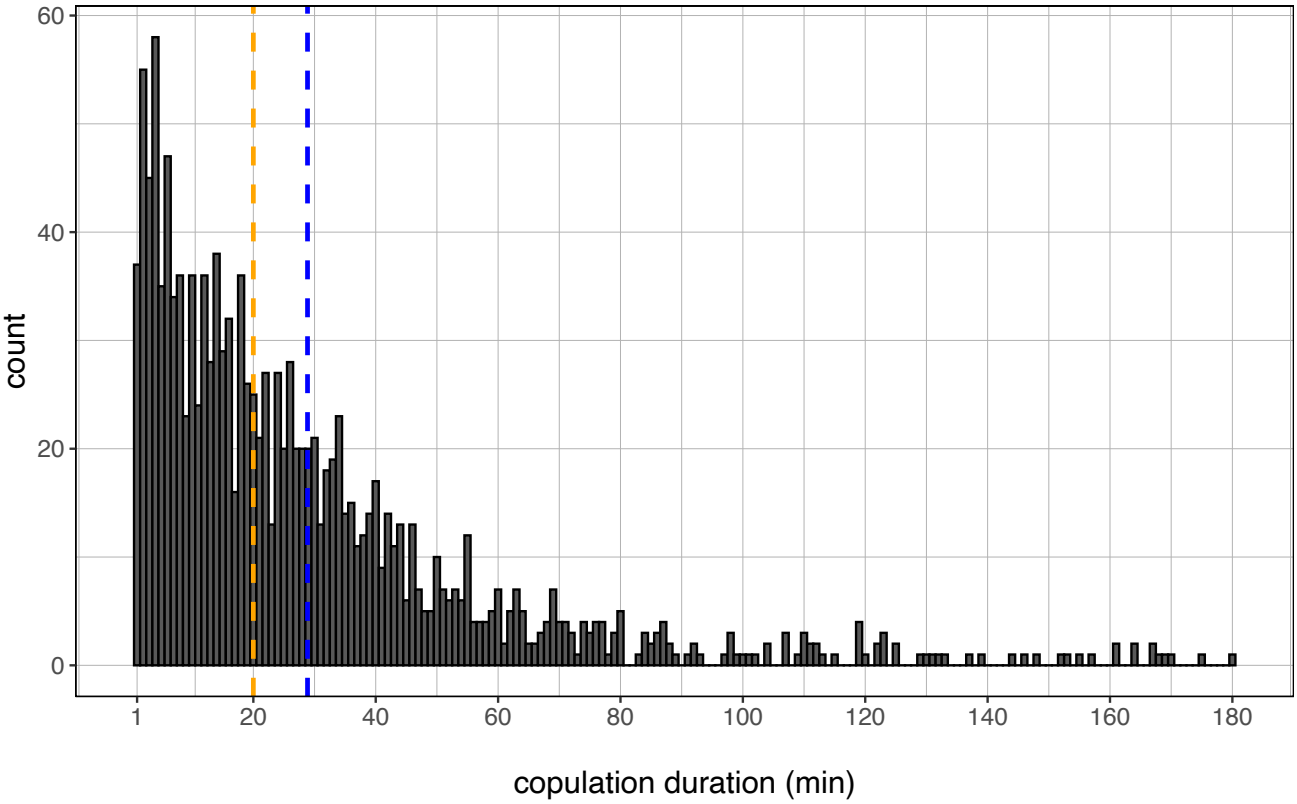

Figure 1. Distribution of copulation duration under laboratory conditions (unpublished data from the mating experiments described by Perini *et al.* 2020). Count (y axis) of how many matings occurred, with duration in one minute bins (x axis), with mean (blue dashed line) and median (orange dashed line) durations.

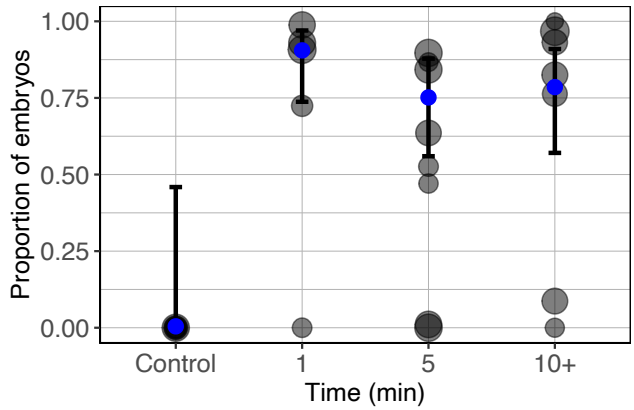

Figure 2. Proportion of developing embryos in the control and treatments. For each female (black points), the proportion (y axis) was calculated as the number of developing embryos divided by the total number of embryos (size of the black points ∝ natural logarithm of total number of embryos, range 1.1-6.1). For the control group and each time treatment (x axis), the fitted value (blue points) and 95% confidence intervals (black bars) were calculated using a beta-binomial model and back-transformed to the scale for proportions (0 to 1).