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

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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. Mounting 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 mounting is critical. For example, mountings 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*, mountings vary substantially in duration, from less than a minute to more than an hour, and it has been assumed that mountings 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 mounting trials using *L. saxatilis* males and virgin females, aiming to examine indirectly when the transfer of sperm starts. We observed the relationship between mounting 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 (i.e. 1, 5 and 10 or more min at which mountings were artificially interrupted), suggesting that sperm transfer begins rapidly (within 1 min) 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). Mounting 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 (Trivers, 1972; Janicke *et al.*, 2016) 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 behaviours that increase male reproductive success (Bateman, 1948). Sexual conflict will then arise if those traits or behaviours reduce female fertility or survival (Chapman *et al.*, 1995; Wolfner, 1997).

Males have been shown to gain a fertility benefit by extending mounting duration and, thus, delaying the time when a female will remate with another male (Gilchrist & Partridge, 2000). Long mountings 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 (Simmons, 2001; Edward , Stockley & Hosken, 2015). 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 mounting 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 by 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 mounting 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 mate with and fertilize (reviewed by Parker & Pizzari, 2010). There is strong agreement between theory and empirical evidence that male investment per mounting is maximized when mating with high-quality females (e.g. larger female size is often used as proxy) or with previously mated females, and when competing with a low number of other males (Parker *et al.*, 1996; Wedell, Gage & Parker, 2002; delBarco-Trillo, 2011; Kelly & Jennions, 2011; Simmons & Fitzpatrick, 2012).

The first step to understanding how females and males interact with respect to mounting duration is to measure fertilization success as a function of mounting duration. Knowing when sperm transfer starts and ends is crucial for assessing how female and male traits have coevolved. Here, we controlled mounting duration between nonvirgin males (hereafter simply males) and virgin females of the intertidal snail species *Littorina saxatilis*, a well-studied system for studying adaptive divergence and reproductive isolation among populations inhabiting different habitats. We explored the relationship between mounting duration and the proportion of eggs that are fertilized, as a measure of sperm transfer. Eggs and developing embryos are carried in the female's brood pouch in this species and so the result of sperm transfer can be checked a few weeks after mating by dissecting the female. In littorinid gastropods, sperm are transferred in a fluid and moved by cilia in a groove running along the male penis (Reid, 1996). There is evidence in the sea hare *Aplysia parvula* that only a few sperm are transferred in a fluid over short mounting times (few minutes) and that their number increases as mounting continues (Yusa, 1994). We expected a similar pattern in *L. saxatilis* with short mountings being inadequate for sperm transfer, whereas longer mountings (10 min or longer) would be more likely to yield effective transfer of sperm. However, we did not expect the relationship between mounting duration and sperm transfer to be necessarily linear because in other studied gastropods the correlation is either weak or absent (reviewed by Weggelaar, Commandeur & Koene, 2019).

Several ecotypes of *L. saxatilis* have been described and two in particular (so-called 'crab' and 'wave' forms) have been used as a model for studying the evolution of reproductive isolation under a scenario with ongoing gene flow between locally adapted populations (Johannesson *et al.*, 2010). There is likely to be strong sexual conflict due to high population density, risks associated with mating and opportunity for sperm competition and/or cryptic female choice (Johannesson *et al.*, 2010b, 2016). Mating is strongly dependent on size and the highest probability of mating was estimated in pairs where the male was *c.* 25% smaller than the female (i.e. optimum size ratio) (Perini *et al.*, 2020). A wide range of mounting durations has been observed and this may be indicative of slow sperm transfer (Saur, 1990; Hollander, Lindegarth & Johannesson, 2005; Perini *et al.*, 2020). Females have been found to store sperm for more than a year (Johannesson *et al.*, 2016).

Here, we examined the timing of sperm transfer in *L. saxatilis* indirectly by performing time-controlled mounting 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 mounting duration, sperm transfer and number of developing embryos. Knowing at which time males start transferring the sperm during mounting and whether longer mountings 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. 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 of *Littorina saxatilis* (14 wave in 2016, and 21 crab and 3 wave in 2020). These 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 *c.* 10 months, we sampled adult snails of both ecotypes from the same locality and identified *c.* 60 males by observing a fully developed penis. These males were kept at 4 °C in individual tubes, like the virgin females, before the start of the experiment and also when they were not used. In this way, both virgin females and wild males experienced the same laboratory conditions immediately prior to mating. Females from the aquaria and males from the wild were measured [(mean maximum shell length (mm) ± SD: crab females = 9.8 ± 1.8; crab males = 10.1 ± 1.9; wave females = 7.8 ± 0.6; wave males = 4.5 ± 1.0)] and each female was matched with two males of the same ecotype, which were *c.* 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 transfer of sperm. When exactly the penis is inserted is difficult to establish but a strong correspondence has been found between male mounting position and mounting attempt (Hollander *et al.*, 2005).

We used unpublished data on mounting duration from an earlier experiment (Perini *et al.*, 2020) to decide what we should consider as short, medium and long mounting times (Fig. 1; selecting values close to the minimum, mode and mean of the distribution). The experiment by Perini *et al.* (2020) had similar conditions to those used here (see below) except for using one male and one female of the same or of different ecotypes per trial. Ecotype did not influence mounting duration. Mounting trials in the experiment reported here were performed indoors under constant light and at room temperature. At the start of each trial, the female and two males (a trio) were placed foot-down at the bottom of a transparent plastic sphere (80 mm in diameter) one-third filled with sea water. In both the 2016 and 2020 experiments, each trio was assigned to one of three treatments, each of which corresponded to the time at which mounting was artificially interrupted. In 2016, mounting was interrupted at either 5, 10 or 30 min after observing a pair to enter the characteristic mating position. In 2020, mounting was instead interrupted at either 1, 5 or 30 min. We replaced the 10 min trial with a 1 min treatment because we wanted to test the hypothesis that very short mountings were insufficient for sperm to be transferred to the female. The data from the two experiments were then combined by merging the 10 min treatment with the 30 min treatment (hereafter referred to as the 10+ min treatment) in order to increase the sample size for the statistical analysis.

Mountings were interrupted at the predefined experimental times by separating the mating pair. Mountings that lasted less than the pre-assigned time were recorded and these females were assigned to a treatment group appropriate to the observed

mounting duration. Thereafter, the female was marked with a unique identifier and placed in a new sea water aquarium without the male. The same aquarium was used for all the treated females and also for virgin females that were not assigned to any of the treatments and used as unmated controls. These controls were included in the experiment in order to check for the possibility of embryonic development without fertilization and to ensure that females were virgin, despite our precautions.
If no mounting had been recorded throughout the length of the trial (2 h), the same female was reused the next day and paired again with males with optimal relative size. When available, new males were preferred, otherwise, the females were matched with the same males as the previous day.

Mated and control females were dissected 2 to 3 weeks after the mating trials. This time allowed the mated females to start using the sperm to fertilize eggs and for embryogenesis of the first fertilized eggs to have proceeded to a developmental stage that was easily distinguished from unfertilized eggs. Females have been found to carry up to 1,011 embryos (mean + SD = 130 + 123; data for 500 wild females from Johannesson *et al.*, 2020). 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 v. 1.53a (Schneider, Rasband & Eliceiri, 2012). Misdeveloped embryos beyond egg stage were treated as fertilized eggs and included in the embryo count (Johannesson *et al.*, 2020). Embryos were classified as misdeveloped if clumps of cells were spread throughout the egg capsule or they showed malformed shells (e.g. poorly coiled and dwarfed). Mated females with no eggs and no 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 mountings would have a limited sperm supply and so would show a reduced rate of fertilization in the eggs they produced over 2 to 3 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 mounting 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 overdispersion in the response variable due to factors that may be important during fertilization (e.g. sperm storage) but that were not analysed 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 v. 1.3.1 (Lesnoff & Lancelot, 2012), in which the proportion of developing embryos was the response variable and mounting duration was the independent (categorical) variable. The null model was beta-binomial with the same response variable but without the treatment effects; 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 (Tukey, 1949; Kramer, 1956): 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 mounting durations differed between the two experiments. Finally, we included ecotype, 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 analysed 33 mated females (Table 1), discarding 5 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, SE = 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. The number of developing embryos ranged between 0–285 in the 1 min treatment, 0–226 in the 5 min treatment and 0–418 in the 10+ min treatment. In all, except one female in the 10+ min treatment, in addition to developing embryos, we also found eggs in which we could not detect development. The number of eggs/undeveloped embryos ranged between 2–29 in the 1 min treatment, 2–258 in the 5 min treatment and 0–126 in the 10+ min treatment. There was no significant effect on the proportion of developing embryos due to the difference in ecotype (estimate = -0.68, SE = 0.70, *P* = 0.34), due to the 2016 and 2020 experiment (estimate = -1.29, SE = 0.72, *P* = 0.09), due to the female size (estimate = 0.02, SE = 0.16, *P* = 0.93) nor due to the total number of eggs and embryos (estimate = 0.00, SE = 0.00, *P* = 0.27).

Mountings that lasted less than the pre-assigned time (7 cases) ranged between 3 and 28 min in duration, and we found that fertilization had occurred in all the females (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 mounting duration if a large quantity of sperm increases male and/or female reproductive success. This correlation between sperm transfer and mounting duration may be complex and may not be necessarily linear, or may be absent; this is 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). Mounting 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 mounting duration in the highly promiscuous, internally-brooding snail *Littorina saxatilis*. We have shown that, surprisingly, very short mountings are sufficient for the sperm transport into the female to begin and that females involved in interrupted mountings 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 mountings 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 clearcut, 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 min of mounting and most of the sperm were transferred near the end of mounting (Weggelaar *et al.*, 2019). In *Littoraria cingulata* and *L. filosa,* Hollander *et al.* (2018) observed an increased probability of sperm transfer for longer mounting durations. In the opisthobranch sea hare *Aplysia parvula*, Yusa (1994) found that more sperm were transferred in longer mountings but that a few minutes were already sufficient for sperm transfer in a fluid. Hence, even though long mountings might be required for transferring a large amount of sperm, short mountings, as we observed in *L. saxatilis*, can be effective to transfer enough sperm to fertilize a batch of eggs. The experimental interruption of mounting itself does not appear to be the cause of rapid sperm transfer in our experiment with *L. saxatilis* because pairs that ended mounting before the pre-assigned time achieved similar transfer of sperm to the females (even after 3 min). This suggests that short mountings 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. Transferring more sperm may be advantageous in some circumstances and this might help to explain long mounting durations. High sperm loading might be beneficial for males mainly to displace sperm from previous matings or to dilute their contribution (Parker & Pizzari, 2010). Previous results on biased paternity towards large males in *L. saxatilis* would support this possibility (Johannesson *et al.*, 2016) suggesting that, like in many insects (Simmons, 2001) and a few aquatic gastropods (Oppliger *et al.*, 2003; Anthes, Werminghausen & Lange, 2014; Xue, Zhang & Liu, 2014), sperm competition would select for large males with a large/long penis that produces many sperm. Nevertheless, in *L. saxatilis*, only 18% of the variation in male reproductive output was explained by male size, implying that fertilization is also influenced by other factors that are involved before, during and after mounting (Johannesson *et al.*, 2016; Johannesson *et al.*, 2020; Perini *et al.*, 2020). Because here we have used virgin females and single matings, such effects may not be captured.

All except one female showed eggs where we did not detect development, but we cannot be sure whether these were unfertilized or fertilized but not sufficiently advanced embryos to be detectable as undergoing development at the time when we dissected the females. The proportion of undeveloped embryos and eggs in treated females matches well with the proportion of similarly early embryo stages in wild-collected females (*c.* 20% were “preveligers”; Johannesson *et al.*, 2020) that are not likely to be sperm-limited (Panova *et al.*, 2010). Such a similarity in proportions of developed and undeveloped embryos would suggest that females that were interrupted at any time during mounting (even after only 1 min) in our experiment were unlikely to be sperm-limited in the short term.

One hypothesis that could explain rapid sperm transfer in *L. saxatilis* is that of high predation risk. There is empirical evidence in littorinid snails that when females and males enter the mating position, the risk of being dislodged from the intertidal and/or being eaten by crabs and fish increases compared to single individuals (Johannesson, 1986; Kemppainen *et al.*, 2005; Koch , Lynch & Rochette, 2007; Johannesson *et al.*, 2010b). If this risk is high, then it may be beneficial for both sexes to transfer sperm rapidly to assure fertilization at a lower cost of mating. This might explain why we observed *L. saxatilis* developing embryos already in the 1min treatment. A similar effect of predation was also found in fireflies, which usually copulate for hours or days. In the species *Photinus collustrans*, where an increased predation risk was observed compared to other fireflies, mountings lasted only a few minutes (Wing, 1988). If the same was true for *L. saxatilis*, we would have expected mounting duration to reflect such predation risk and thus, be on average a few minutes long, both in the lab and in the field. What we see is, instead, an average mating time of 30 min and many matings lasting longer than 10 min and up to 1 h (Fig. 1). Hence, other factors are likely to influence mounting duration in *L. saxatilis*. For instance, in littorinid snails, seminal fluid proteins are also being transferred during mounting (Buckland-Nicks *et al.*, 1999), but we still do not know the importance of this transfer for explaining the observed variation in mounting duration of *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 sperm transfer must have been later than the time we recorded and copulation duration must have been shorter than the observed mounting duration. At the same time, watching multiple trials, the observer might have missed the start of the mating by up to 30 s. The true duration of ‘1 min’ 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* (*c.* 1 min 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 mountings 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 behaviour 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 mountings 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 mountings last longer than what is required for initial sperm transfer. The mate guarding hypothesis was also unlikely to explain the paternity patterns in a closely related species, *L. obtusata*, where extended mating position (up to 2.5 h without genital contact) was observed but in the absence of a clear effect on reproductive success (Paterson, Partridge & Buckland-Nicks, 2001).

We have shown that sperm transfer in *L. saxatilis* begins rapidly during mounting, 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 mountings 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 10 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 mounting duration.

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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

Table 2. Summary of parameter estimates for the beta-binomial model and Tukey–Kramer's HSD.

			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

Back-transformed maximum likelihood estimates and 95% confidence intervals (95% CIs) of the proportion of developing embryos for the control group, 1 min treatment (T1), 5 min treatment (T5) and 10+ min treatment (T10+). Tukey–Kramer's HSD is the minimal difference between two treatment means for considering them significantly different. Estimates followed by the same letter are not significantly different from each other (Tukey–Kramer test, $P > 0.05$).

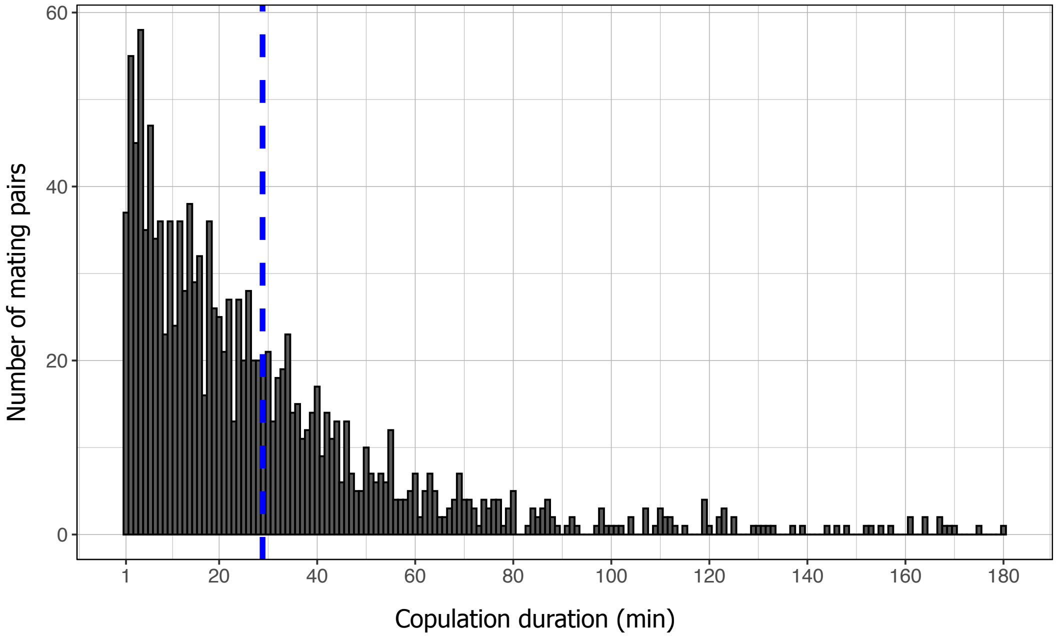

Figure 1. Distribution of mounting duration under laboratory conditions [summed over all matings in Perini*, et al.* (2020), regardless of ecotype]. Count (*y*-axis) of how many matings occurred, with duration in 1 min bins (*x*-axis), with mean (blue dashed line) duration indicated.

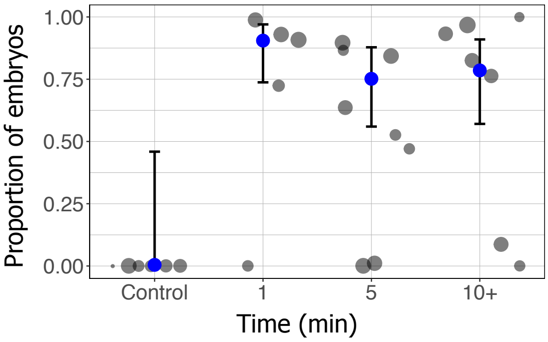

Figure 2. Proportion of developing embryos in the control and treatments. For each female (black jittered points), the proportion (*y*-axis) was calculated as the number of developing embryos divided by the total number of embryos (size of the black points ∝ natural logarithm of total number of embryos, 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).