Variation in intraspecific sperm translocation behaviour in a damselfly and its consequences on sperm viability

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Abstract

Sperm quality and viability affect both male and female fitness. Most dragonfly and damselfly males translocate sperm from the testis to the seminal vesicle before each copulation, a behaviour known as intra-male sperm translocation (ST). However, some published observations indicate that odonate males can occasionally skip ST prior to copulation. Our aim was to determine the circumstances under which males skip ST and how this might affect sperm viability. We allowed males of the damselfly *Ischnura graellsii* to perform ST (interrupting the copulation at this stage) and we studied ST behaviour during subsequent copulation. Males were randomly assigned to four treatments, which consisted of allowing the experimental male to copulate again 15 minutes, 24 hours, 48 hours or 72 hours after his last ST. Fertility of females mated with the experimental males was analysed as a proxy to sperm viability. All males used the sperm that they translocated previously, when the second mating took place 15 min after the manipulation, while the proportion of males that repeated ST increased steadily, from 6.7% after one day to 57.1% after three days. Both treatment (time elapsed since last ST) and the interaction between treatment and ST (yes/no), had a significant effect on fertility, which decreased only in males that did not perform ST immediately before copulation. Additional experiments with damselflies of the genus *Calopteryx* showed also that males do not repeat ST when the time until next copulation is less than one day. Our results suggest that sperm quality decays over time in odonates, and that males can choose whether to keep and reuse the sperm in the seminal vesicle or to discard it. We conclude that the special anatomical disposition of odonate males might open selective pressures to maximize sperm viability and/or repeated intra-male ST behaviour.

Keywords: behavioural flexibility, *Calopteryx,* fertility, *Ischnura*, sperm quality, Odonata

Introduction

The ecological and evolutionary consequences that sperm viability and quality have on sexual selection and sexual conflict have been the subject of intense research over the last decade (Mautz et al. 2013). For instance, sperm viability is driven by post-copulatory sexual selection (*i.e*., viability of the sperm stored in the seminal vesicle is higher in polygamous males than in their monogamous relatives; Hunter & Birkhead 2002). In agreement with this observation, in the cricket *Teleogryllus oceanicus,* male-male interactions and female mating history also have an effect on the viability of the sperm inseminated by males (García-González & Simmons 2005; Thomas & Simmons 2009), which suggests that sperm viability plays an important role in sperm competition.

Sperm viability has a heritable component, and it is genetically and phenotypically correlated with other reproductive traits (Simmons & Roberts 2005). In guppies *(Poecilia reticulata)* sperm viability is positively correlated with male carotenoid levels, showing that selection for sperm viability or quality may also represent a trade-off in the evolution of primary and secondary male sexual traits (Locatello et al. 2006). However, in the cockroach *Nauphoeta cinerea*, sperm viability and testis size appear to be negatively correlated (Moore et al. 2004).

Odonates (dragonflies and damselflies) have evolved an unusual sexual anatomy, where the primary male genitalia have been reduced to vestigial scales at the end of the abdomen, and secondary genitalia have developed on the second abdominal segment (Cordero-Rivera & Córdoba-Aguilar 2010). The insemination of the female is a two-step process: first, the male must translocate sperm from the testis (opening at the tip of the abdomen) to the seminal vesicle on the second abdominal segment. This process is termed the intra-male sperm-translocation behaviour (hereafter ST), and it is commonly performed when the mating pair is already making contact (*i.e.* after the male grasps the female by the thorax or the head; Rivas-Torres et al. 2019). Secondly, the female bends her abdomen so that her genitalia contacts the secondary male genitalia, leading to the typical mating “wheel” position. Following ST, the sperm is transferred to the female, but only at the end of copulation (Cordero-Rivera & Córdoba-Aguilar 2010).

However, in several damselfly species, such as *Neurobasis chinensis* (Kumar & Prasad 1977) and *Ischnura graellsii* (Cordero 1989), males may sometimes mate without having recently been seen to perform ST. In the case of *Ischnura*, the absence of ST was easily explained because these males had stored sperm from previous ST followed by unsuccessful copulation, therefore suggesting that this sperm had not been inseminated and was still present in the seminal vesicle. These observations imply that males can skip some steps of the behavioural sequence with a focal female, although the specifics of how males may adjust ST behaviour to particular circumstances and how this influences the viability of the unused sperm stored in the seminal vesicle are unknown.

Here, we studied the ST behaviour in an experimental setting, using *Ischnura* and *Calopteryx* damselflies, with the aim to test how long the sperm stored in the seminal vesicle is viable and therefore used by males if they have the opportunity to mate again. Our prediction is that sperm viability in the secondary seminal vesicle regulates male behaviour so that males should not repeat ST if the sperm is viable, but they should discard sperm and repeat ST once the sperm reduces in viability after the first ST event. Therefore, as the time elapsed between ST and copulation increases, we predict that female fertility would decrease, and most males would therefore repeat ST after more than one day of interval. To test this hypothesis, we used a population of *Ischnura graellsii* maintained under laboratory conditions, where the history of each individual was followed in detail. Furthermore we re-analyzed previous data on hand-pairing experiments between *Calopteryx splendens* and *C. haemorrhoidalis,* where some matings were interrupted after ST and prior to insemination (Cordero-Rivera 2017), to test whether these males repeated ST when given a second chance of mating.

Materials and methods

Experimental design with I. graellsii

Newly emerged adults of *I. graellsii* were collected in March-April 2016-2018, at three ponds in the province of Pontevedra (Galicia, NW Spain) and transported to the laboratory in net bug containers. Once in the laboratory, individual body

length was measured to the nearest 0.1 mm, and damselflies were individually marked, separated by sex, and kept in insectaries of 50 × 50 × 50 cm. Insectaries were maintained at room temperature and humidity, with natural light and damselflies were fed with adult *Drosophila ad libitum* (Van Gossum et al. 2003).

After individuals reached maturity (7-9 days for females and 6-8 days for males; Van Gossum et al. 2003), females were introduced into an insectary with several mature males. Whenever a male grasped a female in tandem, he was allowed to translocate sperm from the 9th to the 2nd abdominal segment, and then the tandem was interrupted (experimental males) or allowed to mate (control males). Afterwards, experimental males were randomly assigned to one of five treatments, which consisted of allowing the male to copulate with a second female after: (i) 15 minutes (*N*=10); (ii) one day (*N*=15); (iii) two days (*N*=21); (iv) three days (*N*=21) and (v) control (*N*=8), that we let copulate without interruptions.

Study of the viability of sperm, in relation to the elapsed time from ST in I. graellsii

Every day, all mated females (*N*=75) from the previous experiment were placed in individual oviposition containers with moist filter paper as an oviposition substrate and left for 2-3 days. Eggs were incubated in dechlorinated tap water for 30 days after oviposition, at room temperature. The number of eggs laid was counted, and all hatched and unhatched eggs (showing a visible embryo) were considered as fertile, in order to account for possible problems in hatching due to the artificial substrate used for oviposition. Unhatched eggs (*i.e.* eggs without a visible embryo), were considered sterile (Fincke 1984b). Some females died before laying enough eggs, and therefore the sample size of this experiment was reduced to *N*=57.

The proportion of fertile eggs was analysed with a Generalized Linear Model (GLM), because the response variable [fertile/sterile] was binomial; using the total number of eggs as binomial totals. We compared the fertility of females mated with males that performed ST before copulation (including in this group

also control males) with the fertility of females mated with males that did not repeat ST. Treatment (time since last ST; numerical) and ST behaviour (yes/no, categorical) and their interaction, were included as predictor variables in the analysis, to detect if fertility decayed over time. Overdispersion was detected and estimates of parameters and standard errors were corrected using the appropriate option in Genstat 19th software (VSN International 2017)

Experiments with Calopteryx

A second experiment was conducted with *C. haemorrhoidalis* and *C. splendens*, from a field population in Central Italy, using the "hand-pairing" technique (Oppenheimer & Waage 1987). This experiment was designed to test postcopulatory genital coevolution, and it included interruption of some matings after ST but before insemination (total *N*=131; see Cordero-Rivera 2017 for further details). Here we re-examined these data to see if males repeated ST when given the opportunity to re-mate (the time elapsed between matings was never more than 2 h).

Results

Repetition of ST

Figure 1 shows the proportion of *I. graellsii* mated males that performed ST before copulation, as a function of experimental treatment. All males that had their first mating attempt interrupted 15 minutes after their first ST, used sperm stored from this first ST when they were allowed to mate again. In contrast, when the time between the first ST and the second tandem exceeded one day, the proportion of males performing ST increased proportionately to the time elapsed: 6.7% after one day, 33.3% after two days and 57.1% after three days.

The experiment with *C. haemorrhoidalis and C. splendens* (Cordero-Rivera 2017), has also shown that males do not repeat ST when the time elapsed between ST and the next copulation is less than one day: in 21 out of 137 copulations observed, *Calopteryx* males did not perform ST. From these 21 males, 20 had performed ST prior to an interrupted copulation a few minutes

earlier, while the remaining male had performed a compete copulation, but was not followed in detail, and it is possible that he had sperm translocated for another unsuccessful copulation.

Viability of sperm in relation to the time elapsed since ST in I. graellsii

Egg fertility was used as a proxy to analyse sperm viability. The number of fertile eggs (*N*=57 females) was analysed with a GLM with binomial errors and logit link in Genstat 19th software (VSN International 2017). The analyses showed that treatment (time) had a significant effect on fertility (deviance ratio: 4.28, *P*=0.009; Figure 2). We found that fertility decreased with time since the first ST (estimate: -0.420±0.155; t_{51}=-2.71, *P*=0.009), but only in males that did not perform ST immediately before copulation (the interaction Treatment*ST was marginally significant, t_{51}=1.80, *P*=0.077; Figure 2).

Discussion

Our predictions stated that males should not repeat ST if the sperm is viable, but they should discard sperm and repeat ST once the sperm loses viability after the first ST event. On the other hand, as the time elapsed between ST and copulation increases, we also predicted that female fertility would decrease.

Our results indicate that males of *Ischnura graellsii* are able to detect that their sperm vesicle is full, and choose whether to re-use this sperm, depending on the time elapsed since ST. The proportion of males repeating ST increased with the number of days since their last ST and in parallel, egg fertility decreased if males did not repeat sperm translocation after 2-3 days (see Figure 2). Additional experiments with *Calopteryx* showed a similar trend, with most of the males not repeating the translocation of sperm whenever the time elapsed between ST and the next copulation was less than 24 h.

Our observations suggest that males' sperm vesicles were presumably full. Altogether, the evidence from *I. graellsii* and *Calopteryx* suggest that odonates (at least damselflies) are able to keep sperm viable and alive inside the secondary sperm vesicle, which works as a storage organ, although our data

also suggest that the sperm is not kept alive for long (but see Miller, 1995). Several questions arise from these observations, which we discuss in detail below.

The first question would be how the males are able to keep the sperm viable and alive inside the sperm vesicle after ST. Most research, both in vertebrates and invertebrates, has focused on the viability of sperm once it has been transferred to the female (Aumuller & Riva 1992; Manaskova et al. 2002; Hartmann & Loher 1996; Chapman 2001). Ejaculate composition varies between species but generally speaking it comprises both cells (sperm, parasperm and inmunity cells) and molecules (seminal proteins, hormones, antimicrobial peptides, salts and sugars, fats and defensive compounds) (Perry et al. 2013). Some of these molecules (*i.e.* the non-seminal component of the ejaculate) play an important role in keeping the sperm alive and protecting it from damage inside the female reproductive tract. For example, the seminal fluid of some insects is composed of sugars such as glucose, glycogen or trehalase, which nourish the sperm (Gillot 1996; Poiani 2006); and several proteins in the ejaculate of *Drosophila* help to protect sperm cells from damage (Chapman 2001). We expect certain components of the odonate ejaculate to play a role keeping sperm viable, not only within the female tract, but also inside the male seminal vesicle. Exploring the seminal fluid composition in odonates would be a promising research field. For example, it is known that in the dragonfly *Orthetrum coerulescens* males retain some sperm in the vesicle after a copulation (Miller 1990) employing sperm aggregation (*i.e*. spermatodesms) to increase its longevity (Siva-Jothy 1997), but the composition of these sperm aggregations is yet unknown.

The second question would be how do *I. graellsii* males detect that the sperm stored in the seminal vesicle is still viable. This could be achieved by a neurological mechanism based on the time elapsed since the last ST. More complex physiological mechanisms are known in mammals, where some proteins decrease when the viability of sperm diminishes (Bebas et al. 2008). However again, this has not been investigated in odonates. An alternative possibility is that males detect the depletion of the seminal vesicle using some type of mechanical or chemical sensilla, so that they repeat ST whenever they

detect the depletion of the sperm vesicle. It is known that the spoon-like head of the genital ligula in some odonates is covered with small conical protuberances resembling chemical sensilla (Andrés & Cordero-Rivera 2000), therefore the existence of similar structures inside the seminal vesicle is plausible.

Finally, it is unknown what happens with the old sperm whenever the ST is repeated. It has been suggested that odonate males belonging to the suborder Anisoptera can expel their sperm in the absence of a female, by muscular compression of the vesicle. Ejection of sperm could act in this case as a mechanism similar to masturbation, which in humans and other non-human primates is used as a strategy to increase sperm fitness (Zimmerman et al. 1965; Thomsen 2000). On the other hand, males of Zygoptera such as *Enallagma cyathigerum* cannot actively empty their sperm vesicle but they can keep sperm alive in their seminal vesicle for up to 10 days (Miller 1995). Given that the cost of producing the seminal fluid to keep this sperm alive is high (Reinhardt et al. 2011), perhaps the translocated fresh sperm displaces the old sperm in the seminal vesicle, and this fact can explain why egg fertility did not decrease in the case of *I. graellsii* males that repeated ST (Figure 2). Last, the process could be similar to that observed in marine snails, where the old sperm is reabsorbed or phagocyted and then digested in the seminal vesicle (Buckland-Nicks & Fu-Shiang 1976).

There are some reports in the literature that indicate that occasionally ST does not precede copulation in odonates (Fraser & Herman 1993; Kano & Kita 1996). Our experiments indicate that these cases might be explained by males re-using sperm that was translocated up to 24 hours earlier. Only two species of odonates are known to routinely repeat ST in a single mating sequence (Fincke 1984a; Cordero-Rivera et al. 1995; Rivas-Torres et al. 2019). Nothing is known about fertility levels and seminal fluid composition in these species, and given their special behaviour, we suggest that future work should analyse how egg fertility relates to the time since ST or the number of ST events, and how seminal fluid composition varies according to different environmental factors such as male density, female status, season or temperature.

Conclusion

The sperm translocation behaviour of odonates is of special interest given that this is the only insect order with indirect ST (Cordero-Rivera & Córdoba-Aguilar 2010). To our knowledge, our study is the first investigating the role of time since ST on female fertility in odonates. Given the relevance of sperm quality in sexual selection, the social environment (*i.e.* sex-ratio or male-male competition) might be a significant selective force acting on seminal fluid proteins with a maintenance function (Fitzpatrick & Lüpold 2014).

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Figure 1: The proportion of males of *Ischnura graellsii* that repeated (YES, grey) or not (NO, blue) sperm translocation (ST), as a function of the time elapsed since the experimental interruption of their mating (after having translocated sperm to the vesicle).

Figure 2: The variation of fertility among females of *Ischnura graellsii* whose males did ST immediately before copulation (black circles) or used previously transferred sperm (red squares), as a function of the time between the first sperm translocation (ST) event and copulation (control, 15 min, 1, 2 and 3 days). The lines represent predicted responses from a GLM including Treatment (time), ST (yes/no) and their interaction.

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Figure

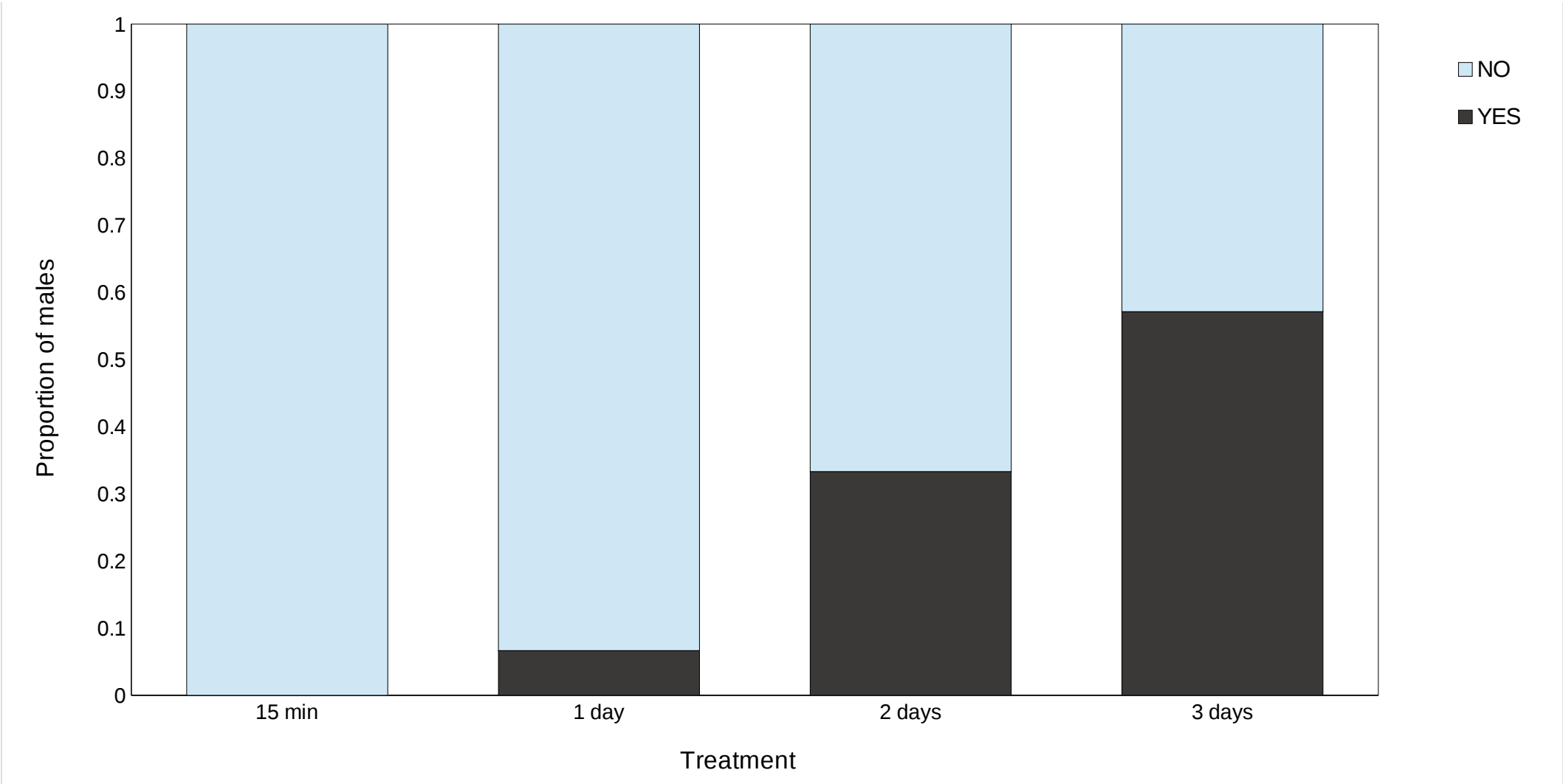
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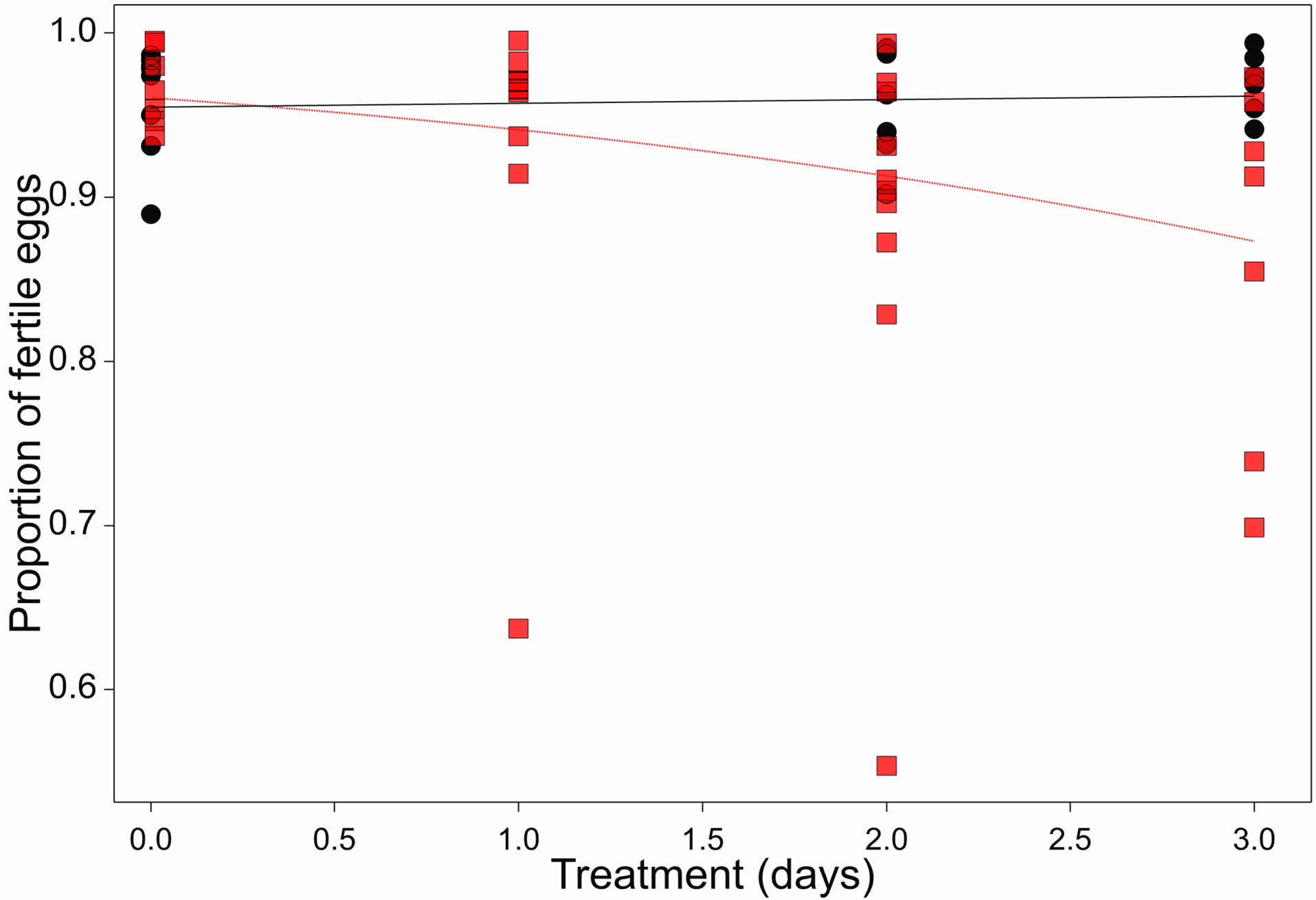
Figure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