Animal Behaviour 156 (2019) 51–55

Contents lists available at ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav

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

Anais Rivas-Torres [a, *], M. Olalla Lorenzo-Caballa [a], Rosa Ana Sánchez-Guillén [b], Adolfo Cordero-Rivera [a]

[a] ECOEVO Lab, Universidade de Vigo, Escola de Enxeñaría Forestal, Pontevedra, Spain
[b] Biología Evolutiva, Instituto de Ecología A.C., Xalapa, Mexico

ARTICLE INFO

Article history:
Received 9 April 2019
Initial acceptance 17 May 2019
Final acceptance 8 July 2019

MS. number: 19-00260R

Keywords:
behavioural flexibility
Calopteryx
fertility
Ischnura
Odonata
sperm quality

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 intramale 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 min or 1–3 days after his last ST. Fertility of females mated with the experimental males was analysed as a proxy for 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 over time. 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 did not repeat ST when the time to the next copulation was less than a 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 increase selective pressures to maximize sperm viability and/or repeated intramale ST behaviour.

The ecological and evolutionary consequences of sperm viability and quality for sexual selection and sexual conflict have been the subject of intense research over the last decade (Mautz, Møller, & Jennions, 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 than monogamous males; Hunter & Birkhead, 2002). In agreement with this observation, in the cricket *Teleogryllus oceanicus*, male–male interactions and female mating history also affect 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, Rasotto, Evans, & Pilastro, 2006). However, in the cockroach *Nauphoeta cinerea*, sperm viability and testis size appear to be negatively correlated (Moore, Harris, Montrose, Levin, & Moore, 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

* Correspondence: A. Rivas-Torres, ECOEVO Lab, Universidade de Vigo, Escola de Enxeñaría Forestal, Campus Universitario A Xunqueira s/n, 36005, Pontevedra, Galiza, Spain.
E-mail address: arivasto@gmail.com (A. Rivas-Torres).

https://doi.org/10.1016/j.anbehav.2019.08.001
0003-3472/

the intramale 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, Outomuro, Lorenzo-Carballa, & Cordero-Rivera, 2019). Second, the female bends her abdomen so that her genitalia touch 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 have been seen to mate apparently without performing ST. In the case of *Ischnura*, the absence of ST was easily explained because these males had stored sperm from a 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 of testing 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 was 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 has lost viability after the first ST event. Therefore, as the time elapsed between ST and copulation increases, we predicted that female fertility would decrease, and most males would therefore repeat ST after an interval of more than 1 day. To test this hypothesis, we used a population of *I. graellsii* maintained under laboratory conditions, where the history of each individual was followed in detail. We also reanalysed previous data on hand-pairing experiments between *Calopteryx splendens* and *Calopteryx haemorrhoidalis*, in which 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.

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, northwest 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 $50 \times 50 \times 50$ cm. Insectaries were maintained at room temperature and humidity, with natural light and damselflies were fed with adult *Drosophila* ad libitum (Van Gossum, Sánchez, & Cordero-Rivera, 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 ninth to the second 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 15 min ($N = 10$), 1 day ($N = 15$), 2 days ($N = 21$) or 3 days ($N = 21$) or to copulate without interruptions as a control ($N = 8$).

Sperm Viability in Relation to Time from ST

Egg fertility was used as a proxy to analyse sperm viability. 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; we used the total number of eggs as binomial totals. We compared the fertility of females mated with males that performed ST before copulation (including 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 whether fertility decayed over time. Overdispersion was detected and estimates of parameters and standard errors were corrected using the appropriate option in Genstat 19th edition 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 whether males repeated ST when given the opportunity to remate (the time elapsed between matings was never more than 2 h).

Ethical Note

All the animals were collected in the field with a permit issued by the Regional Government of Galicia (permit number EB-089/2017). They were kept in the laboratory for behavioural purposes only and until they died naturally.

RESULTS

Repetition of ST

Fig. 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 min 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 1 day, the proportion of males performing ST increased proportionately to the time elapsed: 0.07 after 1 day, 0.03 after 2 days and 0.57 after 3 days.

The experiment with *C. haemorrhoidalis and C. splendens* (Cordero-Rivera, 2017) also showed that males do not repeat ST when the time elapsed between ST and the next copulation is less than 1 day: in 21 of 137 copulations observed, *Calopteryx* males did not perform ST. Of these 21 males, 20 had performed ST prior to an

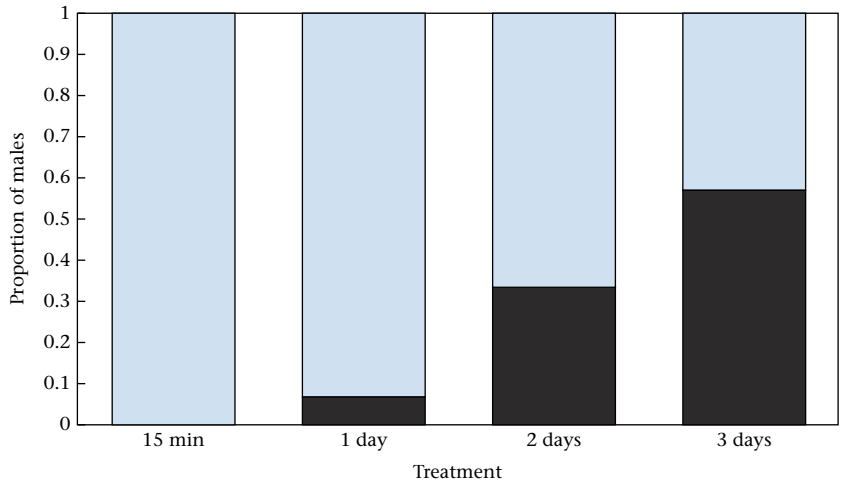

Figure 1. The proportion of males of *Ischnura graellsii* that repeated (grey) or did not repeat (blue) sperm translocation, as a function of the time elapsed since the experimental interruption of their mating (after having translocated sperm to the vesicle).

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.

Sperm Viability in Relation to Time from ST

The GLM showed that treatment (time) had a significant effect on fertility (deviance ratio: 4.28, $P = 0.009$; Fig. 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 for males that did not perform ST immediately before copulation (the interaction Treatment*ST was marginally significant: $t_{51} = 1.80$, $P = 0.077$; Fig. 2).

DISCUSSION

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

Our results indicate that male *I. graellsii* are able to detect that their sperm vesicle is full, and choose whether to reuse 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 Fig. 2). Additional experiments with *Calopteryx* showed a similar trend, with most of the males not

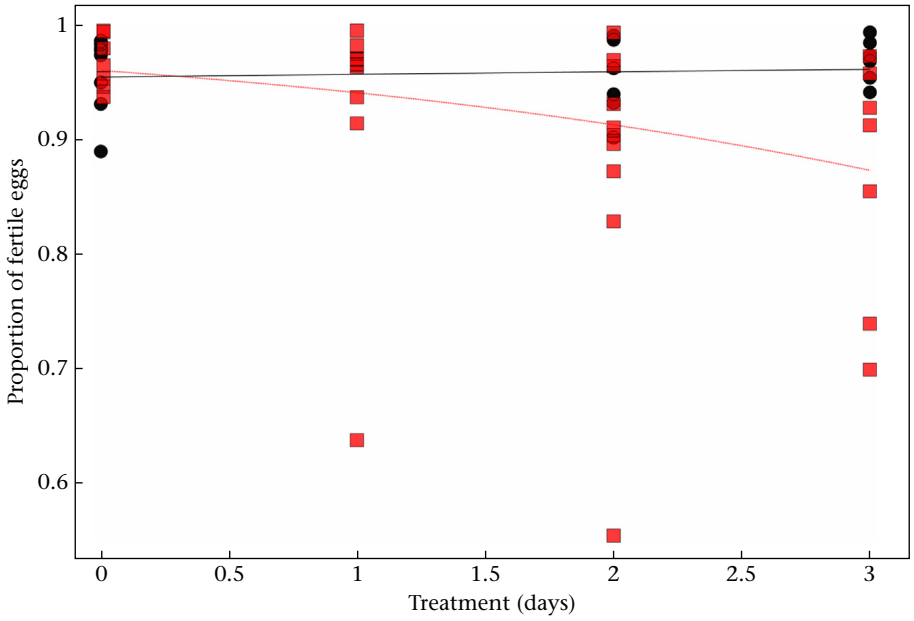

Figure 2. The variation in fertility among females of *Ischnura graellsii* that had mated with males that performed sperm translocation (ST) immediately before copulation (black circles) or used previously transferred sperm (red squares), as a function of the time between the first ST event and copulation (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.

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 results 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 can males 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; Chapman, 2001; Hartmann & Loher, 1996; Manaskova, Ryslava, Ticha, & Jonakova, 2002). Ejaculate composition varies between species but it generally comprises both cells (sperm, parasperm and immunity cells) and molecules (seminal proteins, hormones, antimicrobial peptides, salts and sugars, fats and defensive compounds; Perry, Sirot, & Wigby, 2013). Some of these molecules (i.e. the nonseminal 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, in which some proteins decrease when the viability of sperm diminishes (Bebas, Kotwica, Joachimiak, & Giebultowicz, 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 to 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 in this case act as a mechanism similar to masturbation, which in humans and other nonhuman primates is used as a strategy to increase sperm fitness (Thomsen, 2000; Zimmerman, Maude, & Moldawer, 1965). 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, Naylor, & Siva-Jothy, 2011), perhaps the translocated fresh sperm displaces the old sperm in the seminal vesicle, which would explain why egg fertility did not decrease for *I. graellsii* males that repeated ST (Fig. 2). Last, the process could be similar to that observed in marine snails, in which 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 reusing sperm that was translocated up to 24 h earlier. Only two species of odonates are known to routinely repeat ST in a single mating sequence (Cordero, Santolamazza-Carbone, & Utzeri, 1995; Fincke, 1984a; 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 ST 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 to investigate 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).

Acknowledgments

Professor Michael Jennions, an anonymous referee and the Editor provided comments that helped us improve our manuscript. Funding was provided by a grant from the Spanish Ministry of Economy and Competitiveness, including FEDER funds (CGL2014-53140-P). A.R.T. was supported by an FPI grant (BES-2015-071965).

References

Andrés, J. A., & Cordero-Rivera, A. (2000). Copulation duration and fertilization success in a damselfly: An example of cryptic female choice? *Animal Behaviour, 59*, 695–703.

Aumuller, G., & Riva, A. (1992). Morphology and functions of the human seminal vesicle. *Andrologia, 24*, 183–196.

Bebas, P., Kotwica, J., Joachimiak, E., & Giebultowicz, J. M. (2008). Yolk protein is expressed in the insect testis and interacts with sperm. *BMC Developmental Biology, 8*, 64.

Buckland-Nicks, J. A., & Fu-Shiang, C. (1976). Spermatogenesis of a marine snail, *Littorina sitkana*. *Cell and Tissue Research, 170*, 455–475.

Chapman, T. (2001). Seminal fluid-mediated fitness traits in *Drosophila*. *Heredity, 87*, 511–521.

Cordero, A. (1989). Reproductive behaviour of *Ischnura graellsii* (Rambur) (Zygoptera: Coenagrionidae). *Odonatologica, 18*, 237–244.

Cordero-Rivera, A. (2017). Sexual conflict and the evolution of genitalia: Male damselflies remove more sperm when mating with a heterospecific female. *Scientific Reports, 7*, 7844.

Cordero-Rivera, A., & Córdoba-Aguilar, A. (2010). Selective forces propelling genitalic evolution in Odonata. In J. Leonard, & A. Cordoba-Aguilar (Eds.), *The evolution of primary sexual characters in animals* (pp. 332–352). Oxford, U.K.: Oxford University Press.

Cordero, A., Santolamazza-Carbone, S., & Utzeri, C. (1995). Male disturbance, repeated insemination and sperm competition in the damselfly *Coenagrion scitulum* (Zygoptera: Coenagrionidae). *Animal Behaviour, 49*, 437–449.

Fincke, O. M. (1984a). Giant damselflies in a tropical forest: Reproductive biology of *Megaloprepus coerulatus* with notes on Mecistogaster (Zygoptera: Pseudostigmatidae). *Advances in Odonatology, 2*, 13–27.

Fincke, O. M. (1984b). Sperm competition in the damselfly *Enallagma hageni* Walsh (Odonata: Coenagrionidae): Benefits of multiple mating to males and females. *Behavioral Ecology and Sociobiology, 14*, 235–240.

Fitzpatrick, J. L., & Lüpold, S. (2014). Sexual selection and the evolution of sperm quality. *Molecular Human Reproduction, 20*, 1180–1189.
Fraser, A. M., & Herman, T. B. (1993). Territorial and reproductive behavior a sympatric species complex of the neotropical damselfly *Cora* Selys (Zygoptera, Polythoridae). *Odonatologica, 22*, 411–429.
García-González, F., & Simmons, L. W. (2005). Sperm viability matters in insect sperm competition. *Current Biology, 15*, 271–275.
Gillot, C. (1996). Male insect accessory glands: Functions and control of secretory activity. *Invertebrate Reproduction and Development, 30*, 1–3.
Hartmann, R., & Loher, W. (1996). Control mechanisms of the behavior 'secondary defense' in the grasshopper *Gomphocerus rufus* L. (Gomphocerinae: Orthoptera). *Journal of Comparative Physiology A: Neuroethology, Sensory Neural and Behavioral Physiology, 178*, 329–336.
Hunter, F. M., & Birkhead, T. R. (2002). Sperm viability and sperm competition in insects. *Current Biology, 12*, 121–123.
Kano, K., & Kita, H. (1996). Solitary sperm translocation of male of *Euphaea yayeyamana* Matsumura & Oguma in Oguma. *Gekkan-Mushi, 303*, 36–37.
Kumar, A., & Prasad, M. (1977). Reproductive behaviour in *Neurobasis chinensis* chinensis (Linnaeus) (Zygoptera: Calopterygidae). *Odonatologica, 6*, 163–171.
Locatello, L., Rasotto, M. B., Evans, P., & Pilastro, A. (2006). Colourful male guppies produce faster and more viable sperm. *Journal of Evolutionary Biology, 19*, 1595–1602.
Manaskova, P., Ryslava, H., Ticha, M., & Jonakova, V. (2002). Characterization of proteins from boar prostate. *American Journal of Reproductive Immunology, 48*, 283–290.
Mautz, B. S., Møller, A. P., & Jennions, M. D. (2013). Do male secondary sexual characters signal ejaculate quality? A meta-analysis. *Biological Reviews, 88*, 669–682.
Miller, P. L. (1990). Mechanisms of sperm removal and sperm transfer in *Orthetrum coerulescens* (Fabricius) (Odonata: Libellulidae). *Physiological Entomology, 15*, 199–209.
Miller, P. L. (1995). *Dragonflies*. Slough, U.K: Richmond.
Moore, P. J., Harris, W. E., Montrose, V. T., Levin, D., & Moore, A. J. (2004). Constraints on evolution and postcopulatory sexual selection: Trade-offs among ejaculate characteristics. *Evolution, 58*, 1773–1780.
Oppenheimer, S. D., & Waage, J. K. (1987). Hand-pairing: A new technique for obtaining copulations within and between *Calopteryx* species (Zygoptera: Calopterygidae). *Odonatologica, 16*, 291–296.
Perry, J. C., Sirot, L., & Wigby, S. (2013). The seminal symphony: How to compose an ejaculate. *Trends in Ecology & Evolution, 28*, 414–422.
Poiani, A. (2006). Complexity of seminal fluid: A review. *Behavioral Ecology and Sociobiology, 60*, 289–310.
Reinhardt, K., Naylor, R., & Siva-Jothy, M. T. (2011). Male mating rate is constrained by seminal fluid availability in bedbugs, *Cimex lectularius*. *PLoS One, 6*, e22082.
Rivas-Torres, A., Outomuro, D., Lorenzo-Carballa, M. O., & Cordero-Rivera, A. (2019). The evolution and diversity of intra-male sperm translocation in Odonata: A unique behaviour in animals. *Behavioral Ecology and Sociobiology, 73*, 54.
Simmons, L. W., & Roberts, B. (2005). Bacterial immunity traded for sperm viability in male crickets. *Science, 309*, 2031.
Siva-Jothy, M. T. (1997). Odonate ejaculate structure and mating systems. *Odonatologica, 26*, 415–437.
Thomas, M. L., & Simmons, L. W. (2009). Male dominance influences pheromone expression, ejaculate quality, and fertilization success in the Australian field cricket, *Teleogryllus oceanicus*. *Behavioral Ecology, 20*, 118–1124.
Thomsen, R. (2000). *Sperm Competition and the Function of Masturbation in Japanese Macaques (*Macaca fuscata*)* (Ph.D. thesis). Munich, Germany: Ludwig-Maximilians-Universitat Munchen.
Van Gossum, H., Sánchez, R., & Cordero-Rivera, A. (2003). Observations on rearing damselflies under laboratory conditions. *Animal Biology, 53*, 37–45.
VSN International. (2017). *Genstat for Windows* (19th ed.). Hemel Hempstead, U.K.: VSN International.
Zimmerman, S. J., Maude, M. B., & Moldawer, M. (1965). Frequent ejaculation and total sperm count, motility, and form in humans. *Fertility and Sterility, 16*, 342–345.