Sperm removal during copulation confirmed in the oldest extant damselfly, *Hemiphlebia mirabilis* (#9342)

First submission

Important notes

Editor and deadline

Giovanni Benelli / 22 Mar 2016

Files	1 Raw data file(s) Please visit the overview page to **download and review** the files not included in this review pdf.
Declarations	**Involves a field study on animals or plants.**

Review guidelines

Please in full read before you begin

How to review

When ready **submit your review online**. The review form is divided into 5 sections. Please consider these when composing your review:

1. **BASIC REPORTING**
2. **EXPERIMENTAL DESIGN**
3. **VALIDITY OF THE FINDINGS**
4. General comments
5. Confidential notes to the editor

You can also annotate this **pdf** and upload it as part of your review

To finish, enter your editorial recommendation (accept, revise or reject) and submit.

BASIC REPORTING

- ✓ Clear, unambiguous, professional English language used throughout.
- ✓ Intro & background to show context. Literature well referenced & relevant.
- ✓ Structure conforms to **PeerJ standard**, discipline norm, or improved for clarity.
- ✓ Figures are relevant, high quality, well labelled & described.
- ✓ Raw data supplied (See **PeerJ policy**).

EXPERIMENTAL DESIGN

- ✓ Original primary research within **Scope of the journal**.
- ✓ Research question well defined, relevant & meaningful. It is stated how research fills an identified knowledge gap.
- ✓ Rigorous investigation performed to a high technical & ethical standard.
- ✓ Methods described with sufficient detail & information to replicate.

VALIDITY OF THE FINDINGS

- ℹ Impact and novelty not assessed. Negative/inconclusive results accepted. *Meaningful* replication encouraged where rationale & benefit to literature is clearly stated.
- ✓ Data is robust, statistically sound, & controlled.
- ✓ Conclusion well stated, linked to original research question & limited to supporting results.
- ✓ Speculation is welcome, but should be identified as such.

The above is the editorial criteria summary. To view in full visit **https://peerj.com/about/editorial-criteria/**

Sperm removal during copulation confirmed in the oldest extant damselfly, *Hemiphlebia mirabilis*

Adolfo Cordero-Rivera

Postcopulatory sexual selection may favour mechanisms to reduce sperm competition, like physical sperm removal by males. To investigate the origin of sperm removal, I studied the reproductive behaviour and mechanisms of sperm competition in the only living member of the oldest damselfly family, *Hemiphlebia mirabilis*, one species that was considered extinct in the 1980s. This species displays scramble competition behaviour, whose males search for females with short flights and both sexes exhibit a conspicuous “abdominal flicking”. This behaviour is used by males during an elaborate precopulatory courtship, unique among the Odonata. Females use a similar display to reject male attempts to form tandem, but eventually signal receptivity by a particular body position. Males immobilise females during courtship using their legs, which, contrarily to other damselflies, never autotomize. Copulation is short (range 4.1-18.7 min), and has two stages. In the first stage, males remove part of the stored sperm, and inseminate during the second stage, at the end of mating. The examination of genitalia indicates that males have two horns covered by back-oriented spines, which match the size and form of female genitalia. The volume of sperm in females after copulation was 2.8 times larger than the volume stored in females whose copulation was interrupted at the end of stage I, indicative of a significant sperm removal. These results point out that sperm removal is an old character in the evolution of odonates, probably dating back to the Permian.

Sperm removal during copulation confirmed in the oldest extant damselfly, *Hemiphlebia mirabilis*

Adolfo Cordero-Rivera
Grupo ECOEVO, Universidade de Vigo, EUE Forestal, Campus Universitario
36005 Pontevedra, Spain

adolfo.cordero@uvigo.es

Running head: Reproductive behaviour of *Hemiphlebia mirabilis*

Word count: 4595

Abstract

Postcopulatory sexual selection may favour mechanisms to reduce sperm competition, like physical sperm removal by males. To investigate the origin of sperm removal, I studied the reproductive behaviour and mechanisms of sperm competition in the only living member of the oldest damselfly family, *Hemiphlebia mirabilis*, one species that was considered extinct in the 1980s. This species displays scramble competition behaviour, whose males search for females with short flights and both sexes exhibit a conspicuous "abdominal flicking". This behaviour is used by males during an elaborate precopulatory courtship, unique among the Odonata. Females use a similar display to reject male attempts to form tandem, but eventually signal receptivity by a particular body position. Males immobilise females during courtship using their legs, which, contrarily to other damselflies, never autotomize. Copulation is short (range 4.1-18.7 min), and has two stages. In the first stage, males remove part of the stored sperm, and inseminate during the second stage, at the end of mating. The examination of genitalia indicates that males have two horns covered by back-oriented spines, which match the size and form of female genitalia. The volume of sperm in females after copulation was 2.8 times larger than the volume stored in females whose copulation was interrupted at the end of stage I, indicative of a significant sperm removal. These results point out that sperm removal is an old character in the evolution of odonates, probably dating back to the Permian.

Keywords. Odonates, damselflies, postcopulatory sexual selection, sperm competition, Hemiphlebiidae

Introduction

The relevance of Sexual Selection in shaping reproductive behaviour is well established (Andersson, 1994), and in particular its effects on the evolution of primary (Leonard & Córdoba-Aguilar, 2010) and secondary sexual characters (Clutton-Brock, 2007). Our understanding of sexual selection processes is based on the conceptual framework that differentiates between pre-copulatory and post-copulatory forces and mechanisms (Eberhard, 1996), and the traditional differentiation of intra- and inter-sexual selection, which can be dated back to the original texts of Darwin (1871). The origin and maintenance of many bizarre behaviours is explained by sexual selection theory, even if such behaviours might not be favoured by natural selection. Males and females do not necessarily have the same interests, and conflicts are expected (Parker, 1979; Arnqvist, 2004), which is particularly evident in species whose females store sperm for long periods. Animal genitalia is therefore under intense sexual selection (Eberhard, 1985), and it may drive coevolutionary arms races between sexes (e.g. Sánchez & Cordero, 2014).

Several mechanisms explain the evolution of male reproductive strategies, both to increase paternity when in competition with other males, and to reduce the success of other males in the context of sperm competition (for a review, see Cordero Rivera & Córdoba Aguilar, 2016). Perhaps the most surprising adaptation is the ability to physically remove the sperm from rivals, stored inside the female, using the intromittent organ. In a ‘seminal’ (literally) paper, Waage (1979) demonstrated, for the first time, that male damselflies are able to use their genital ligula to trap sperm from previous mates, and remove it during copulation, so that paternity success of the last male is usually near 100% in the first clutch laid after copulation (e.g. Cordero & Miller, 1992). Since Waage’s discovery, the mechanism of sperm removal by males has been demonstrated in several animal groups, including Dermaptera (Kamimura, 2005), Orthoptera

(Ono, Siva-Jothy & Kato, 1989; von Helversen & von Helversen, 1991) and Coleoptera (Yokoi, 1990) among the insects, but also in Crustaceans (Galeotti et al., 2008) and Cephalopods (Wada et al., 2010). This is therefore a widespread phenomenon in animals, which has evolved several times, but we have little information on its origin in any animal group.

Although Odonates are well known model species for studies of sexual selection and evolutionary biology in general (Córdoba-Aguilar, 2008), most research is in fact confined to a few families (Cordero-Rivera and Córdoba-Aguilar 2010). This taxonomic bias might induce wrong interpretations about the generality of some behaviours. We do not have fossil information to track the evolution of significant behaviours, like the ability to remove sperm from previous mates when a male copulates with a female. An alternative is to study behaviour on a phylogenetic perspective. From this point of view, *Hemiphlebia mirabilis*, the only living member of the Hemiphlebiidae, given its basal position in the Zygoptera (Dumont, Vierstraete & Vanfleteren, 2010), is a priority taxon in this context. This family dates back to the late Jurassic at least (Lak et al., 2009), and *H. mirabilis* can been considered a "living-fossil", a survival from the Permian (Fraser, 1955).

The goals of this study were therefore two fold. First, I aimed at observing and describing the reproductive behaviour of this species, still unknown. Fraser (1955) published a description of the male intromittent organ, with two flagella or horns similar to that of other species known to use these structures to remove sperm (Córdoba-Aguilar & Cordero-Rivera, 2008). However, female internal organs remain unknown. Given that female structures are the "arena" where postcopulatory sexual selection takes place, their study is crucial. Therefore, my second goal was to study genital morphology and sperm competition mechanisms. If *H. mirabilis* males displace sperm, this would suggest that sperm displacement and the dual function of male genitalia (Waage, 1979) is a very old character in Odonates.

Methods

Most observations and experiments were done at Long Swamp, a large freshwater system in the Discovery Bay Coastal Park, near to Nelson (Victoria, Australia), between 17 November and 7 December 2013. At this place, population density of *H. mirabilis* was very high during the field work (Cordero-Rivera, 2015). A second population, inhabiting Ming Ming swamp in Grampians National Park, was visited for further observations.

Animals included in this study were individually marked. Having a marked population is a convenient fact for demographical and behavioural studies (Cordero-Rivera & Stoks, 2008), because individually marked specimens are best for focal observations. Marking was done with a permanent black ink pen (Faber-Castell Multimark 1525 S) on the external side of the right hind wing (see Figures 2 and 3).

Copulatory behaviour was rarely observed. For instance, I observed one mating on 19 and 25 November and two on 26 November. Therefore, I decided to try to increase inter-individual encounters by using a mosquito net as an outdoor insectary, where a set of marked specimens was introduced. The insectary was used to elicit mating behaviour on days 29, 30 November and 1 and 3 December, between 11 and 16-17 h. At the end of the observations, the insectary was removed and remaining individuals released. I observed 28 copulations in total, and 11 were interrupted at different times, to study sperm competition. The pair, or sometimes only the female, was immediately preserved in 70% ethanol for further analyses. One thermometer was situated on the shade of a shrub at about 1.5 m over water. Temperature was recorded approximately once every hour.

In the laboratory, females were dissected and the sperm storage organs extracted. Three females (two after copula and one interrupted) had no sperm in their bursa copulatrix. This fact might be due to failure in insemination (Sánchez-Guillén, Wellenreuther & Cordero-Rivera, 2012), and were excluded from sperm volume analyses. The sperm storage organs of a further female were damaged during dissection and could not be analysed. Final sample sizes were therefore seven females after copulation and four interrupted at the end of stage I. The volume of sperm was estimated following standard protocols (Cordero & Miller, 1992), using the software ImageJ (http://imagej.nih.gov/ij/) to measure the area of the sperm mass from microscope pictures. Male secondary genitalia was dissected and observed under Scanning Electron Microscope (SEM). Permits to collect odonates were issued by the Victorian Department of Environment and Primary Industries (permit number 10006907).

Mean values are presented with their standard error (SE) and sample size: mean±SE (N).

Results

Reproductive behaviour

Hemiphlebia mirabilis shows limited flying activity, and remains most of the time perched. Males are not territorial, and the mating system is scramble competition. The most conspicuous behaviour was abdominal flicking, which occurred continuously over the day, was performed by both sexes, and has been studied elsewhere (Cordero-Rivera, 2015).

Mating behaviour was very infrequent. Over the study period, I observed 28 copulations, but only eight of them outside the insectary. Mating behaviour reminds that of Coenagrionidae, but with several peculiarities. When a male detects a potential partner, he makes a fast flight, similar to a feeding flight, and grasps the female by the wings with his legs (Figure 1A). Occasionally, males try to get in tandem other males (even of other species), but this behaviour lasts only a few seconds. Some females curl their abdomen upwards when are immobilised by males, and try to dislodge the male, in a clear refusal behaviour (Figure 2), which sometimes was successful. In the insectary, these tandems which did not end in copula lasted 8.4±2.4 (4) minutes.

Receptive females remain motionless, and adopt a characteristic position with their abdomen curved downwards in the junction between the first and second abdominal segment, and upwards between third and fourth segment. The position recalls a capital Z (Figures 1B-F and 2C). After a variable time motionless, males start to make their characteristic abdominal flicking, in a clear courtship behaviour (see the sequence in Figure 1B-F, and also Video 1). Then, males curve their abdomen upwards and grasp the female's prothorax with their abdominal appendages (Figure 2C). The average time between female capture and tandem formation was 6.20±1.17 (19) minutes, with a range from 1.67 to 23.0 minutes. During this time, males use their abdominal flicking display to court the female, with motionless periods intercalated. After grasping the female with his anal appendages, the male widely opens its legs ~~when~~ releases female's wings (Video 1).

After tandem, males perform the sperm translocation, from segment 9th to 2nd (Figure 3), which lasted on average 3.5±0.24 (26) seconds (range 1-6 sec; Video 1). This behaviour always preceded copulation, contrarily to what was previously reported (Sant & New, 1988). Copulation starts immediately after sperm translocation. Copulatory movements are similar to Coenagrionidae, and had two clear phases, which correspond with stage I and II as described for *Enallagma cyathigerum* (Miller & Miller, 1981) (see Figure 4).

Copulation lasted 10.53±1.17 (14) minutes (range 4.1-18.7 min), and its duration is not significantly related to time of day (Figure 5) or air temperature (Figure 6) (multiple regression analysis with copula duration as the response variable and time of day and temperature as explanatory variables; time effect: -0.0053±0.0105, t_8=-0.51, p=0.624; temperature effect: 0.0008±0.0006, t_8=1.32, p=0.224). A similar analysis of the duration of courtship and precopulatory tandem also suggests that time of day and temperature have no effect (data not shown). Most of the variation in copulation duration is due to stage I, which lasts 9.96±1.30 (12) minutes. Stage II is of short duration and shows little variability in duration (1.08±0.11 (13) min). At the end of copulation, all males flew off immediately leaving the female alone, but usually perched nearby. Females either flew (N=5) or remained perched for a short time (N=4). One female could be observed for about 2 minutes after copula. She apparently did not expel sperm (Córdoba-Aguilar, 2006), but made conspicuous movements of the external genitalia.

I could not observe a single female laying eggs. It is clear that oviposition does not take place in tandem after mating, so it should be performed by females alone. On 25 November I collected 10 females apparently mature and put them in plastic containers with humid filter paper, which elicits oviposition behaviour in many zygopterans (Van Gossum, Sánchez-Guillén & Cordero-Rivera, 2003). They were retained for two hours on a shaded spot, and afterwards were released, but none laid eggs. A second attempt to obtain eggs was done with 22 females from the population at Ming Ming Swamp, which were fully mature judging by their enlarged abdomen. Females were maintained with humid filter paper from 12:15 to 15:30 h, but once more, no oviposition was observed. Five females were retained and maintained overnight with humid filter paper and a piece of vegetation, but yet again, no eggs were laid.

Anatomy of genitalia and sperm competition

Figure 7 shows the anatomy of male genitalia at SEM, and female genitalia at optical microscope. The aedeagus measures about 2 mm in length, and ends with a flexible head, culminated by two dorsal horns (Figure 7A, D). These are covered by backwards oriented spines (Figure 7F). The aedeagus is covered by small spines in both sides (Figure 7E), and by a group of large spines in the ventral median part (Figure 7C). Female genitalia shows two chitinized plates with embedded sensilla (Figure 7B), and a large *bursa copulatrix*, full of sperm in postcopula females. There is no spermatheca, although in some specimens the bursa seems divided into two parts, like the example in Figure 7B. The size and structure of male genitalia compared to female *bursa copulatrix*, as well as the presence of spines, both suggest that males can remove sperm during the stage I of copulation.

The volume of sperm in females after copulation was 2.8 times larger than the volume stored in females whose copulation was interrupted at the end of stage I (Figure 8; ANOVA, $F_{1,9}$=9.70, p=0.012), indicating a significant sperm removal.

Discussion

The reproductive behaviour of *H. mirabilis* is unique from several points of view. Both sexes perform abdominal flicking very frequently, particularly after flights, even in the absence of conspecifics (Cordero-Rivera, 2015). The results of this study indicate that this abdominal display, which is the most conspicuous behaviour of *H. mirabilis* (Sant & New, 1988), is also part of the courtship (Figure 1). There have been suggestions in the literature indicating that males use the curling of the abdomen to display to and attract females (Tillyard, 1913), and that females respond to male abdominal flicking by performing the same display (Davies, 1985). My observations nevertheless do not confirm this. Although to some extent both males and females show this behaviour when conspecifics also display, females can perform abdominal flicking even more actively than males when they are alone and undisturbed (up to 172 es in 10 minutes compared to 119 times in males; Cordero-Rivera, 2015). Courtship lasted a maximum of

23 min, which is a substantial amount of time, and is likely to be energetically expensive. This was an unexpected result, because small non-territorial damselflies do not have elaborate courtships, and suggests that precopulatory sexual selection might be intense in this species. The complexity of this precopulatory courtship is also relevant from a different point of view: this species is not only unique from its morphology and taxonomic position; it is also unique from the ethological point of view.

When zygopterans are handled for marking, it is not rare that some loose one or more legs, a fact that might reduce their survivorship (Cordero-Rivera, Egido Pérez & Andrés, 2002). In the case of *H. mirabilis* this never happened, and all individuals had six legs when first captured. Furthermore, I found that pulling legs out from adult *H. mirabilis* to sample tissues for DNA, required the use of scissors instead of forceps, because forcibly removing one leg could damage the animal, due to muscular tissues remaining attached to the leg. Given that legs are used to immobilise females during courtship (Figures 1 and 2), perhaps this explains why *H. mirabilis* males do not autotomize their legs. Legs are crucial for male courtship in *H. mirabilis*, another unusual characteristic.

A third peculiarity of *H. mirabilis* is that mating frequency is surprisingly low. Even if males and females were observed in large numbers, they seldom interacted. This low mating frequency, combined with the intense precopulatory sexual selection during courtship, and the fact that there is no postcopulatory association between males and females, which is typical when polyandry is common (Alcock, 1994), suggest that females mate very few times during their lifetime. This is further indicated by the finding that one female (out of 5) interrupted at the end of stage I of copulation had no sperm from previous matings, which suggests that she might be mating for the first time.

Even if mating frequency is low, it is unlikely that female *H. mirabilis* is monogamous. The anatomy of genitalia is typical of sperm removers, and the measurement of sperm volumes confirmed this possibility. If the images in Figures 7A and 7B are compared, it is evident that the size and morphology of the ligula matches the size and morphology of the female genitalia. Note that images in Figure 7 are oriented with the dorsal part above, but during copulation, the abdomen of the female contacts with the male upside-down, like in Figure 4. This means that the foldable head of the ligula is orientated during copulation in a way that favours the introduction of its horns into the bursa, facilitating sperm capture and removal. In all Zygopteran species so far studied, stage I is used to remove sperm from previous matings and stage II to inseminate (Córdoba-Aguilar, Uhía & Cordero-Rivera, 2003). My results are compatible with this scenario also for *H. mirabilis*. Therefore, sperm competition has been a relevant force in the evolution of reproductive behaviour in this species, and given its phylogenetic situation, in the odonates as a group, ~~It~~ may date back to the Permian. A study of the evolution of genitalia in a phylogenetic context is certainly a priority (e.g. Rudoy & Ribera, 2016). It is interesting to note that two (out of nine) females dissected after a complete copulation did not have sperm in their bursa. This suggests that some matings might be unsuccessful at insemination, and therefore females may need to mate more than once to be able to reproduce. This possibility needs further study.

I could not observe oviposition, neither elicit egg-laying on humid filter paper and plant tissue. Oviposition has apparently never been observed in this species (Sant & New, 1988). The dense vegetation of Long Swamp was too thick to allow detailed behavioural observations of these small damselflies. If females lay eggs at the base of the reeds, this is unlikely to be detected. Even mating pairs were very difficult to notice among the vegetation. Furthermore, individuals in copula were never seen flying (a further peculiarity of this species), which also ~~difficult~~

detection. Focal observation of 79 females in this population (during 10 min each) allowed witnessing three of them mating, but none laying eggs (Cordero-Rivera, 2015). One possibility is that oviposition takes place during the night. Nevertheless, this seems unlikely because animals showed no activity at low temperatures, and five females were maintained over night with humid filter paper, and did not lay eggs. Further detailed observations at localities where the vegetation is less dense (like Ming Ming swamp) might be fruitful to detect oviposition. It surely is endophytic, given the structure of the ovipositor (Sant & New, 1988).

To conclude, this study offers the first description of reproductive behaviour of a key taxon in the evolution of the Odonata, considered the sister group to all Lestoidea (Dumont, Vierstraete & Vanfleteren, 2010), and suggests that sperm removal is a very old adaptation of male odonates in the arena of sexual selection. Some mysteries remain: "*Hemiphlebia mirabilis* will always be an enigma" (Fraser, 1955).

Acknowledgements

I acknowledge the help of many colleagues, which shared with me their experiences and information about the fascinating *H. mirabilis* during my sabbatical leave in Nov-Dec 2013, with a license from the University of Vigo. Many thanks to Di Crowther, Ian Endersby, Gerry Quinn, John Hawking, Reiner Ritchter, and Richard Rowe. Funding was provided by a grant from the Spanish Ministry of Science and Innovation, which included FEDER funds (CGL2011-22629).

References

Alcock J. 1994. Postinsemination associations between males and females in insects: The mate guarding hypothesis. *Annual Review of Entomology* 39:1–21.

Andersson M. 1994. *Sexual selection*. Princeton: Princeton University Press.

Arnqvist G. 2004. Sexual conflict and sexual selection: lost in the chase. *Evolution* 58:1383–1388.

Clutton-Brock T. 2007. Sexual selection in males and females. *Science* 318:1882–1885.

Cordero A., Miller PL. 1992. Sperm transfer, displacement and precedence in Ischnura graellsii (Odonata: Coenagrionidae). *Behavioral Ecology and Sociobiology* 30:261–267.

Cordero Rivera A., Córdoba Aguilar A. 2016. Selección postcópula: competencia espermática y elección femenina críptica. In: Carranza J ed. *Etología adaptativa: el comportamiento como producto de la selección natural*. UCOpress y Publicaciones de la Universidad de Extremadura, 471–497.

Cordero-Rivera A. 2015. Demographics and adult activity of Hemiphlebia mirabilis: a short-lived species with a huge population size (Odonata: Hemiphlebiidae). *Insect Conservation and Diversity*:in press. DOI: 10.1111/icad.12147.

Cordero-Rivera A., Córdoba-Aguilar A. 2010. Selective forces propelling genitalic evolution in Odonata. In: Leonard J, Córdoba-Aguilar A eds. *The evolution of primary sexual characters in animals*. Oxford: Oxford University Press, 332–352.

Cordero-Rivera A., Egido Pérez FJ., Andrés JA. 2002. The effect of handling damage, mobility, body size, and fluctuating asymmetry on lifetime mating success of Ischnura graellsii (Rambur) (Zygoptera: Coenagrionidae). *Odonatologica* 31:117–128.

Cordero-Rivera A., Stoks R. 2008. Mark-recapture studies and demography. In: Córdoba-Aguilar A ed. *Dragonflies: model organisms for ecological and evolutionary studies*. Oxford: Oxford University Press, 7–20.

Córdoba-Aguilar A. 2006. Sperm ejection as a possible cryptic female choice mechanism in

Odonata (Insecta). *Physiological Entomology* 31:146–153.

Córdoba-Aguilar A. 2008. *Dragonflies and damselflies. Model organisms for ecological and evolutionary research*. Oxford: Oxford University Press.

Córdoba-Aguilar A., Cordero-Rivera A. 2008. Cryptic female choice and sexual conflict. In: Córdoba-Aguilar A ed. *Dragonflies: model organisms for ecological and evolutionary studies*. Oxford: Oxford University Press, 189–202.

Córdoba-Aguilar A., Uhía E., Cordero-Rivera A. 2003. Sperm competition in Odonata (Insecta): the evolution of female sperm storage and rivals' sperm displacement. *Journal of Zoology* 261:381–398.

Darwin C. 1871. *The descent of man and selection in relation to sex*. London: John Murray.

Davies DAL. 1985. Hemiphlebia mirabilis Selys: some notes on distribution and conservation status (Zygoptera: Hemiphlebiidae). *Odonatologica* 14:331–339.

Dumont HJ., Vierstraete A., Vanfleteren JR. 2010. A molecular phylogeny of the Odonata (Insecta). *Systematic Entomology* 35:6–18.

Eberhard WG. 1985. *Sexual selection and animal genitalia*. Cambridge, Massachusetts: Harvard University Press.

Eberhard WG. 1996. *Female control: sexual selection by cryptic female choice*. Princeton: Princeton University Press.

Fraser FC. 1955. A study of Hemiphlebia mirabilis Selys (Odonata), a survival from the Permian. *Entomologists Monthly Magazine* 91:110–113.

Galeotti P., Rubolini D., Pupin F., Sacchi R., Fasola M. 2008. Sperm removal and ejaculate size correlate with chelae asymmetry in a freshwater crayfish species. *Behavioral Ecology and Sociobiology* 62:1739–1745. DOI: 10.1007/s00265-008-0602-8.

Van Gossum H., Sánchez-Guillén RA., Cordero-Rivera A. 2003. Observations on rearing damselflies under laboratory conditions. *Animal Biology* 53:37–45.

von Helversen D., von Helversen O. 1991. Pre-mating sperm removal in the bushcricket Metaplastes ornatus Ramme 1931 (Othoptera, Tettigonoidea, Phaneropteridae). *Behavioral Ecology and Sociobiology* 28:391–396.

Kamimura Y. 2005. Last-male paternity of Euborellia plebeja, an earwig with elongated genitalia and sperm-removal behavior. *Journal of Ethology* 23:35–41. DOI: 10.1007/s10164-004-0125-3.

Lak M., Fleck G., Azar D., Engel Fls MS., Kaddumi HF., Neraudeau D., Tafforeau P., Nel A., Engel MS., Kaddumi HF., Neraudeau D., Tafforeau P., Nel A. 2009. Phase contrast X-ray synchrotron microtomography and the oldest damselflies in amber (Odonata: Zygoptera: Hemiphlebiidae). *Zoological Journal of the Linnean Society* 156:913–923. DOI: 10.1111/j.1096-3642.2008.00497.x.

Leonard J., Córdoba-Aguilar A. 2010. *The evolution of primary sexual characters in animals*. Oxford: Oxford University Press.

Miller PL., Miller CA. 1981. Field observations on copulatory behaviour in Zygoptera, with an examination of the structure and activity of male genitalia. *Odonatologica* 10:201–218.

Ono T., Siva-Jothy MT., Kato A. 1989. Removal and subsequent ingestion of rivals' semen during copulation in a tree cricket. *Physiological Entomology* 14:195–202.

Parker GA. 1979. Sexual selection and sexual conflict. In: Blum MS, Blum NA eds. *Sexual* *selection and reproductive competition in insects*. New York: Academic Press, 123–166.

Rudoy A., Ribera I. 2016. The macroevolution of size and complexity in insect male genitalia. *PeerJ* In press.

Sánchez V., Cordero C. 2014. Sexual coevolution of spermatophore envelopes and female genital traits in butterflies: Evidence of male coercion? *PeerJ* 2:e247. DOI: 10.7717/peerj.247.

Sánchez-Guillén RA., Wellenreuther M., Cordero-Rivera A. 2012. Strong asymmetry in the relative strengths of prezygotic and postzygotic barriers between two damselfly sister species. *Evolution* 66:690–707.

Sant GJ., New TR. 1988. *The biology and conservation of Hemiphlebia mirabilis Selys (Odonata, Hemiphlebiidae) in southern Victoria*. Melbourne: Department of Conservation Forests and Lands.

Tillyard RJ. 1913. On some new and rare Australian Agrionidae (Odonata). *Proceedings of the Linnean Society of New South Wales* 37:404–479.

Waage JK. 1979. Dual function of the damselfly penis: sperm removal and transfer. *Science* 203:916–918.

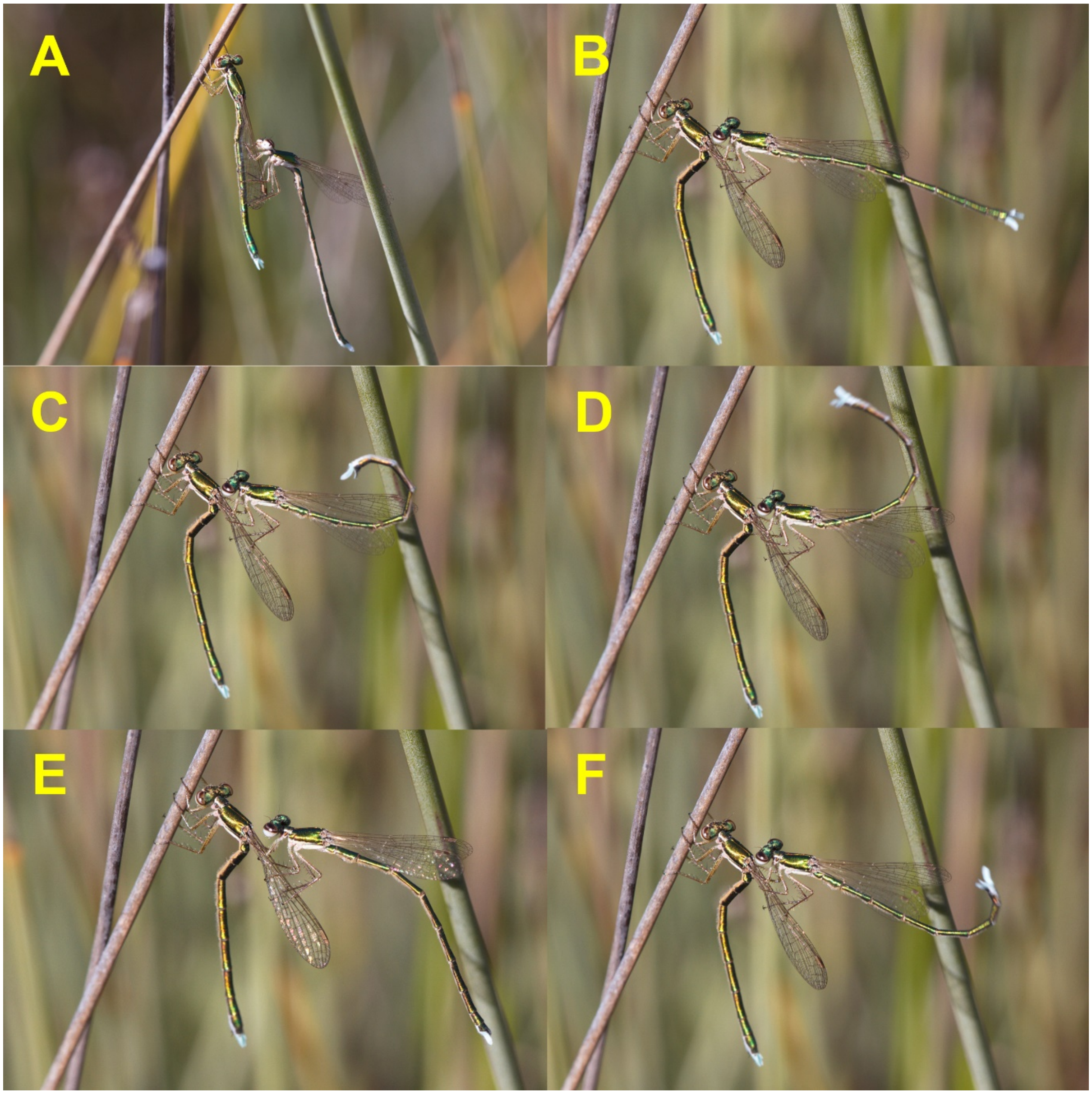
Wada T., Takegaki T., Mori T., Natsukari Y. 2010. Sperm removal, ejaculation and their behavioural interaction in male cuttlefish in response to female mating history. *Animal Behaviour* 79:613–619. DOI: 10.1016/j.anbehav.2009.12.004.

Yokoi N. 1990. The sperm removal behavior of the Yellow Spotted Longicorn Beetle Psacothea hilaris (Coleoptera: Cerambycidae). *Applied Entomology and Zoology* 25:383–388.

Supplementary material

Video 1. A summary of reproductive behaviour of *H. mirabilis*.

Available at: https://youtu.be/ROfXhv_bg00

354 

355 Figure 1. Courtship behaviour of male *H. mirabilis*. Males use the "flicking" display characteristic of this species. Note the "Z" position of female abdomen, which signals receptivity. Males repeatedly curl their abdomen up and downwards during this display. See also Video 1.

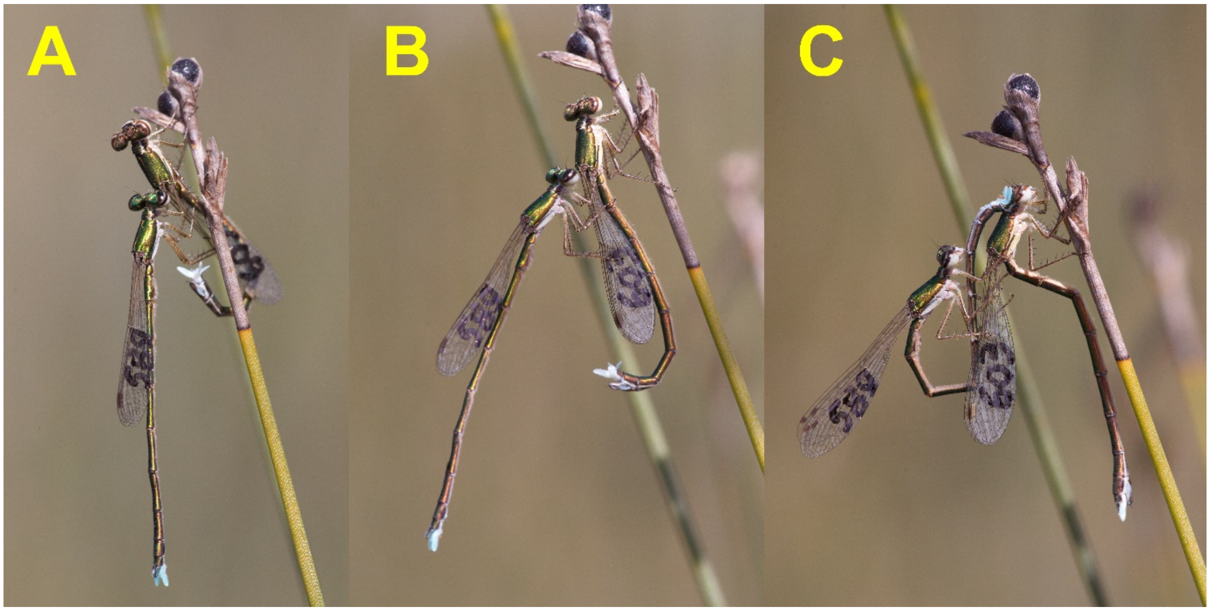


Figure 2. Female refusal behaviour, using the abdomen to try to dislodge the male (A and B), and the start of precopulatory tandem (C), once female shows signs of receptivity. Female acceptance of copulation is indicated by the “Z” position of her abdomen.

Figure 3. The intra-male sperm translocation behaviour. All matings observed (N=28) were preceded by this behaviour.

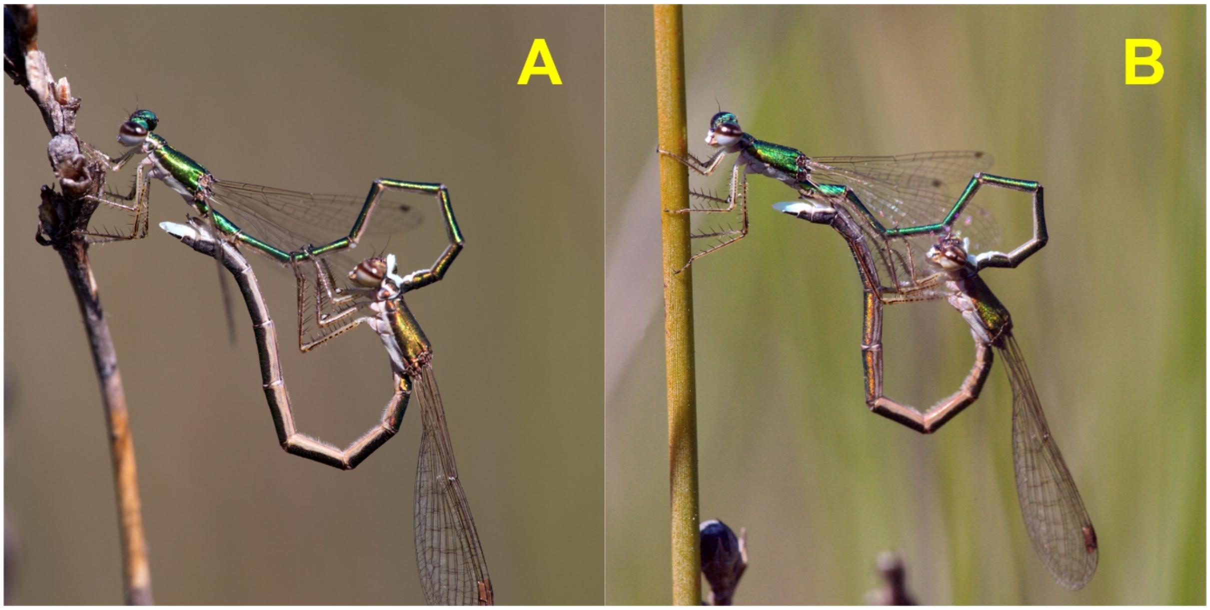


Figure 4. Copulatory phases in *H. mirabilis* match the description of stage I (A) and stage II (B) of Miller and Miller (1981). Stage I is involved in rivals' sperm removal and insemination takes place during stage II.

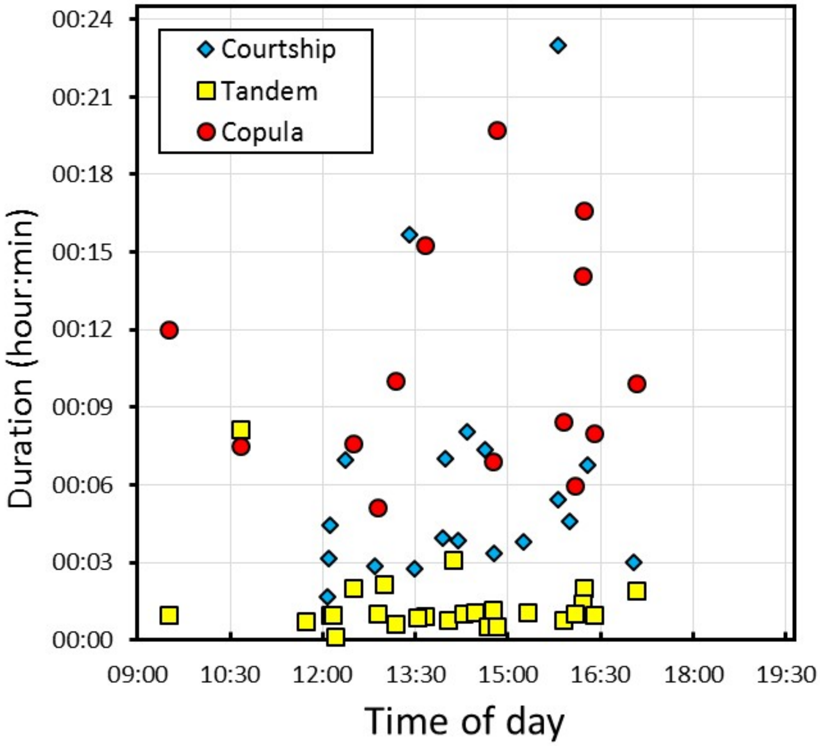


Figure 5. The relationship between time of day and duration of reproductive behaviours of *H. mirabilis*. Courtship refers to the time between female capture and tandem formation. Tandem indicates the time the pair remained in tandem before copulation. Finally, copulation refers to the time between the start and the end of the copulatory wheel.

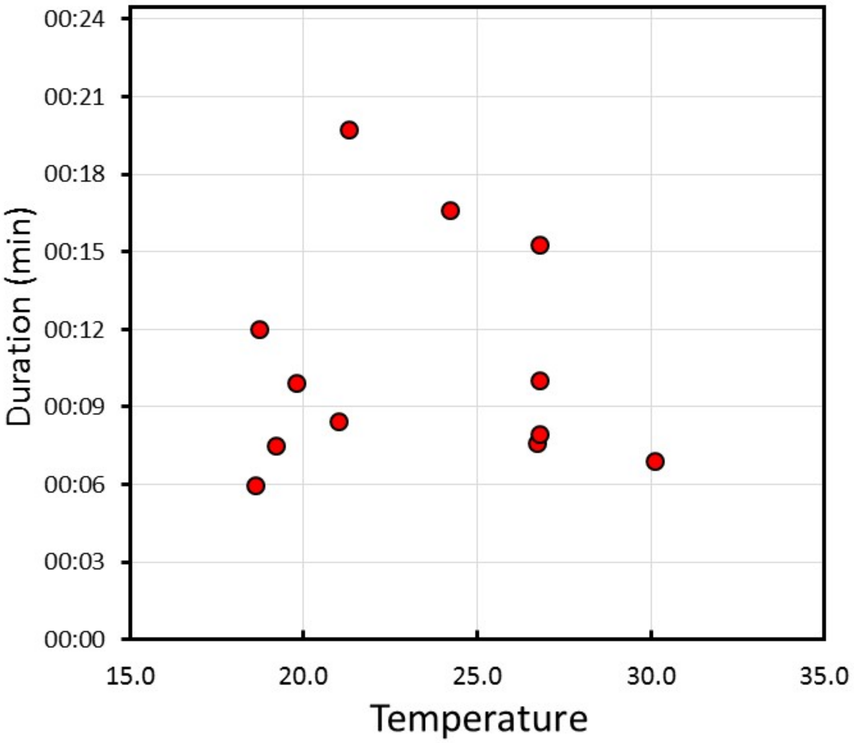


Figure 6. The relationship between air temperature and copulation duration in *H. mirabilis.*

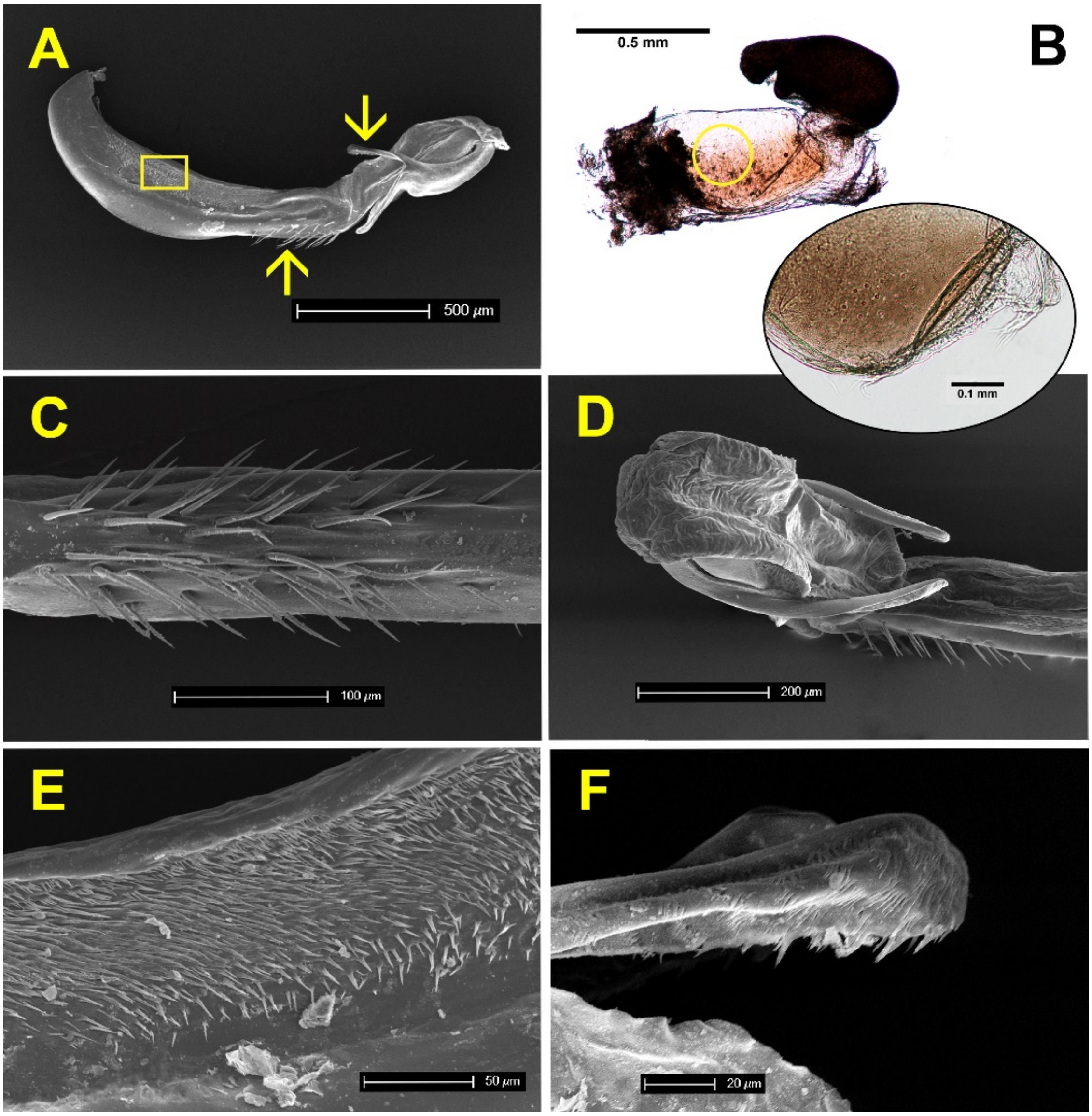


Figure 7. Male and female genitalia of *H. mirabilis*. (A) Lateral view of aedeagus and (B) female vagina and bursa copulatrix, both at the same scale, and orientated with the dorsal part above. The insert in (B) shows a detail of the genital valves of the female, with mechanical sensilla, situated in the area indicated by the circle. Further details of male aedeagus are shown. (C) Ventral view of the spines, in the region indicated by the upwards arrow in (A). (D) Dorso-lateral view of the head of the aedeagus. (E) Detail of the spines of the aedeagus in the region indicated by the rectangle in (A). (F) Lateral view of the aedeagus horns, with backward-directed spines,

whose position is indicated by the downward arrow in (A). The image in (B) has been edited to remove dust.

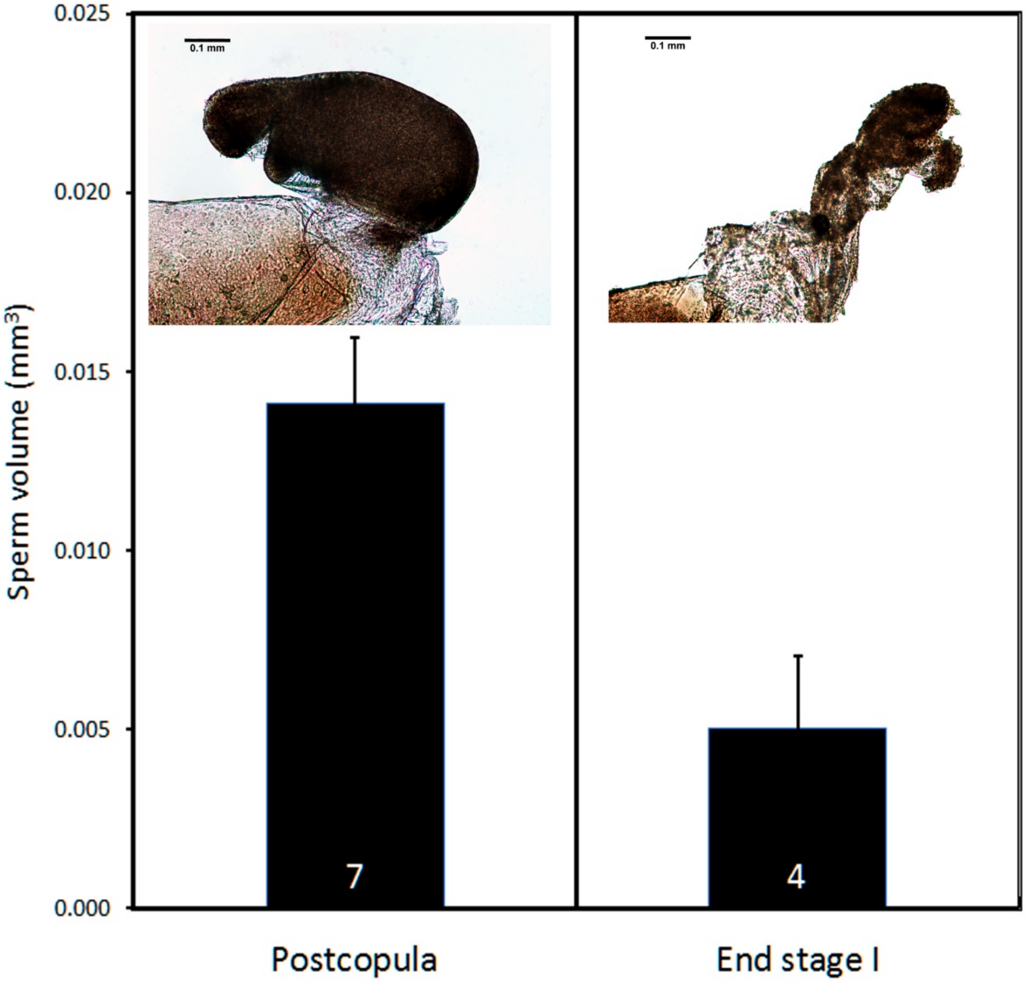


Figure 8. The volume of sperm (mean+SE) in females after a complete copulation and interrupted at the end of stage I, before insemination. Numbers are sample size. The pictures show examples of the bursa copulatrix full of sperm after copulation (left) and partially emptied at the end of stage I (right). Scale bar in the images 0.1 mm. Images edited to remove dust.