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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.

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7   Adolfo Cordero-Rivera

8   Grupo ECOEVO, Universidade de Vigo, EUE Forestal, Campus Universitario

9   36005 Pontevedra, Spain

10

11   adolfo.cordero@uvigo.es

12

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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

58   (Ono, Siva-Jothy & Kato, 1989; von Helversen & von Helversen, 1991) and Coleoptera (Yokoi,

59   1990) among the insects, but also in Crustaceans (Galeotti et al., 2008) and Cephalopods (Wada

60   et al., 2010). This is therefore a widespread phenomenon in animals, which has evolved several

61   times, but we have little information on its origin in any animal group.

62   Although Odonates are well known model species for studies of sexual selection and

63   evolutionary biology in general (Córdoba-Aguilar, 2008), most research is in fact confined to a

64   few families (Cordero-Rivera and Córdoba-Aguilar 2010). This taxonomic bias might induce

65   wrong interpretations about the generality of some behaviours. We do not have fossil

66   information to track the evolution of significant behaviours, like the ability to remove sperm

67   from previous mates when a male copulates with a female. An alternative is to study behaviour

68   on a phylogenetic perspective. From this point of view, *Hemiphlebia mirabilis*, the only living

69   member of the Hemiphlebiidae, given its basal position in the Zygoptera (Dumont, Vierstraete &

70   Vanfleteren, 2010), is a priority taxon in this context. This family dates back to the late Jurassic

71   at least (Lak et al., 2009), and *H. mirabilis* can been considered a "living-fossil", a survival from

72   the Permian (Fraser, 1955).

73   The goals of this study were therefore two fold. First, I aimed at observing and describing the

74   reproductive behaviour of this species, still unknown. Fraser (1955) published a description of

75   the male intromittent organ, with two flagella or horns similar to that of other species known to

76   use these structures to remove sperm (Córdoba-Aguilar & Cordero-Rivera, 2008). However,

77   female internal organs remain unknown. Given that female structures are the "arena" where

78   postcopulatory sexual selection takes place, their study is crucial. Therefore, my second goal was

79   to study genital morphology and sperm competition mechanisms. If *H. mirabilis* males displace

80   sperm, this would suggest that sperm displacement and the dual function of male genitalia

81   (Waage, 1979) is a very old character in Odonates.

82   **Methods**

83   Most observations and experiments were done at Long Swamp, a large freshwater system in the

84   Discovery Bay Coastal Park, near to Nelson (Victoria, Australia), between 17 November and 7

85   December 2013. At this place, population density of *H. mirabilis* was very high during the field

86   work (Cordero-Rivera, 2015). A second population, inhabiting Ming Ming swamp in Grampians

87   National Park, was visited for further observations.

88   Animals included in this study were individually marked. Having a marked population is a

89   convenient fact for demographical and behavioural studies (Cordero-Rivera & Stoks, 2008),

90   because individually marked specimens are best for focal observations. Marking was done with a

91   permanent black ink pen (Faber-Castell Multimark 1525 S) on the external side of the right hind

92   wing (see Figures 2 and 3).

93   Copulatory behaviour was rarely observed. For instance, I observed one mating on 19 and 25

94   November and two on 26 November. Therefore, I decided to try to increase inter-individual

95   encounters by using a mosquito net as an outdoor insectary, where a set of marked specimens

96   was introduced. The insectary was used to elicit mating behaviour on days 29, 30 November and

97   1 and 3 December, between 11 and 16-17 h. At the end of the observations, the insectary was

98   removed and remaining individuals released. I observed 28 copulations in total, and 11 were

99   interrupted at different times, to study sperm competition. The pair, or sometimes only the

100   female, was immediately preserved in 70% ethanol for further analyses. One thermometer was

101   situated on the shade of a shrub at about 1.5 m over water. Temperature was recorded

102   approximately once every hour.

103   In the laboratory, females were dissected and the sperm storage organs extracted. Three females

104   (two after copula and one interrupted) had no sperm in their bursa copulatrix. This fact might be

105   due to failure in insemination (Sánchez-Guillén, Wellenreuther & Cordero-Rivera, 2012), and

106   were excluded from sperm volume analyses. The sperm storage organs of a further female were

107   damaged during dissection and could not be analysed. Final sample sizes were therefore seven

108   females after copulation and four interrupted at the end of stage I. The volume of sperm was

109   estimated following standard protocols (Cordero & Miller, 1992), using the software ImageJ

110   (http://imagej.nih.gov/ij/) to measure the area of the sperm mass from microscope pictures. Male

111   secondary genitalia was dissected and observed under Scanning Electron Microscope (SEM).

112   Permits to collect odonates were issued by the Victorian Department of Environment and

113   Primary Industries (permit number 10006907).

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

115   **Results**

116   Reproductive behaviour

117   *Hemiphlebia mirabilis* shows limited flying activity, and remains most of the time perched.

118   Males are not territorial, and the mating system is scramble competition. The most conspicuous

119   behaviour was abdominal flicking, which occurred continuously over the day, was performed by

120   both sexes, and has been studied elsewhere (Cordero-Rivera, 2015).

121   Mating behaviour was very infrequent. Over the study period, I observed 28 copulations, but

122   only eight of them outside the insectary. Mating behaviour reminds that of Coenagrionidae, but

123   with several peculiarities. When a male detects a potential partner, he makes a fast flight, similar

124   to a feeding flight, and grasps the female by the wings with his legs (Figure 1A). Occasionally,

125   males try to get in tandem other males (even of other species), but this behaviour lasts only a few

126   seconds. Some females curl their abdomen upwards when are immobilised by males, and try to

127   dislodge the male, in a clear refusal behaviour (Figure 2), which sometimes was successful. In

128   the insectary, these tandems which did not end in copula lasted 8.4±2.4 (4) minutes.

129   Receptive females remain motionless, and adopt a characteristic position with their abdomen

130   curved downwards in the junction between the first and second abdominal segment, and upwards

131   between third and fourth segment. The position recalls a capital Z (Figures 1B-F and 2C). After a

132   variable time motionless, males start to make their characteristic abdominal flicking, in a clear

133   courtship behaviour (see the sequence in Figure 1B-F, and also Video 1). Then, males curve their

134   abdomen upwards and grasp the female's prothorax with their abdominal appendages (Figure

135   2C). The average time between female capture and tandem formation was 6.20±1.17 (19)

136   minutes, with a range from 1.67 to 23.0 minutes. During this time, males use their abdominal

137   flicking display to court the female, with motionless periods intercalated. After grasping the

138   female with his anal appendages, the male widely opens its legs when releases female's wings

139   (Video 1).

140   After tandem, males perform the sperm translocation, from segment 9th to 2nd (Figure 3), which

141   lasted on average 3.5±0.24 (26) seconds (range 1-6 sec; Video 1). This behaviour always

142   preceded copulation, contrarily to what was previously reported (Sant & New, 1988). Copulation

143   starts immediately after sperm translocation. Copulatory movements are similar to

144   Coenagrionidae, and had two clear phases, which correspond with stage I and II as described for

145   *Enallagma cyathigerum* (Miller & Miller, 1981) (see Figure 4).

146   Copulation lasted 10.53±1.17 (14) minutes (range 4.1-18.7 min), and its duration is not

147   significantly related to time of day (Figure 5) or air temperature (Figure 6) (multiple regression

148 analysis with copula duration as the response variable and time of day and temperature as

149 explanatory variables; time effect: -0.0053 ± 0.0105, $t_8=-0.51$, $p=0.624$; temperature effect:

150 0.0008 ± 0.0006, $t_8=1.32$, $p=0.224$). A similar analysis of the duration of courtship and

151 precopulatory tandem also suggests that time of day and temperature have no effect (data not

152 shown). Most of the variation in copulation duration is due to stage I, which lasts 9.96 ± 1.30 (12)

153 minutes. Stage II is of short duration and shows little variability in duration (1.08 ± 0.11 (13)

154 min). At the end of copulation, all males flew off immediately leaving the female alone, but

155 usually perched nearby. Females either flew (N=5) or remained perched for a short time (N=4).

156 One female could be observed for about 2 minutes after copula. She apparently did not expel

157 sperm (Córdoba-Aguilar, 2006), but made conspicuous movements of the external genitalia.

158 I could not observe a single female laying eggs. It is clear that oviposition does not take place in

159 tandem after mating, so it should be performed by females alone. On 25 November I collected 10

160 females apparently mature and put them in plastic containers with humid filter paper, which

161 elicits oviposition behaviour in many zygopterans (Van Gossum, Sánchez-Guillén & Cordero-

162 Rivera, 2003). They were retained for two hours on a shaded spot, and afterwards were released,

163 but none laid eggs. A second attempt to obtain eggs was done with 22 females from the

164 population at Ming Ming Swamp, which were fully mature judging by their enlarged abdomen.

165 Females were maintained with humid filter paper from 12:15 to 15:30 h, but once more, no

166 oviposition was observed. Five females were retained and maintained overnight with humid filter

167 paper and a piece of vegetation, but yet again, no eggs were laid.

168 Anatomy of genitalia and sperm competition

169 Figure 7 shows the anatomy of male genitalia at SEM, and female genitalia at optical

170 microscope. The aedeagus measures about 2 mm in length, and ends with a flexible head,

171   culminated by two dorsal horns (Figure 7A, D). These are covered by backwards oriented spines

172   (Figure 7F). The aedeagus is covered by small spines in both sides (Figure 7E), and by a group

173   of large spines in the ventral median part (Figure 7C). Female genitalia shows two chitinized

174   plates with embedded sensilla (Figure 7B), and a large *bursa copulatrix*, full of sperm in

175   postcopula females. There is no spermatheca, although in some specimens the bursa seems

176   divided into two parts, like the example in Figure 7B. The size and structure of male genitalia

177   compared to female *bursa copulatrix*, as well as the presence of spines, both suggest that males

178   can remove sperm during the stage I of copulation.

179   The volume of sperm in females after copulation was 2.8 times larger than the volume stored in

180   females whose copulation was interrupted at the end of stage I (Figure 8; ANOVA, $F_{1,9}$=9.70,

181   p=0.012), indicating a significant sperm removal.

182   **Discussion**

183   The reproductive behaviour of *H. mirabilis* is unique from several points of view. Both sexes

184   perform abdominal flicking very frequently, particularly after flights, even in the absence of

185   conspecifics (Cordero-Rivera, 2015). The results of this study indicate that this abdominal

186   display, which is the most conspicuous behaviour of *H. mirabilis* (Sant & New, 1988), is also

187   part of the courtship (Figure 1). There have been suggestions in the literature indicating that

188   males use the curling of the abdomen to display to and attract females (Tillyard, 1913), and that

189   females respond to male abdominal flicking by performing the same display (Davies, 1985). My

190   observations nevertheless do not confirm this. Although to some extent both males and females

191   show this behaviour when conspecifics also display, females can perform abdominal flicking

192   even more actively than males when they are alone and undisturbed (up to 172 times in 10

193   minutes compared to 119 times in males; Cordero-Rivera, 2015). Courtship lasted a maximum of

194   23 min, which is a substantial amount of time, and is likely to be energetically expensive. This

195   was an unexpected result, because small non-territorial damselflies do not have elaborate

196   courtships, and suggests that precopulatory sexual selection might be intense in this species. The

197   complexity of this precopulatory courtship is also relevant from a different point of view: this

198   species is not only unique from its morphology and taxonomic position; it is also unique from the

199   ethological point of view.

200   When zygopterans are handled for marking, it is not rare that some loose one or more legs, a fact

201   that might reduce their survivorship (Cordero-Rivera, Egido Pérez & Andrés, 2002). In the case

202   of *H. mirabilis* this never happened, and all individuals had six legs when first captured.

203   Furthermore, I found that pulling legs out from adult *H. mirabilis* to sample tissues for DNA,

204   required the use of scissors instead of forceps, because forcibly removing one leg could damage

205   the animal, due to muscular tissues remaining attached to the leg. Given that legs are used to

206   immobilise females during courtship (Figures 1 and 2), perhaps this explains why *H. mirabilis*

207   males do not autotomize their legs. Legs are crucial for male courtship in *H. mirabilis*, another

208   unusual characteristic.

209   A third peculiarity of *H. mirabilis* is that mating frequency is surprisingly low. Even if males and

210   females were observed in large numbers, they seldom interacted. This low mating frequency,

211   combined with the intense precopulatory sexual selection during courtship, and the fact that there

212   is no postcopulatory association between males and females, which is typical when polyandry is

213   common (Alcock, 1994), suggest that females mate very few times during their lifetime. This is

214   further indicated by the finding that one female (out of 5) interrupted at the end of stage I of

215   copulation had no sperm from previous matings, which suggests that she might be mating for the

216   first time.

217   Even if mating frequency is low, it is unlikely that female *H. mirabilis* is monogamous. The

218   anatomy of genitalia is typical of sperm removers, and the measurement of sperm volumes

219   confirmed this possibility. If the images in Figures 7A and 7B are compared, it is evident that the

220   size and morphology of the ligula matches the size and morphology of the female genitalia. Note

221   that images in Figure 7 are oriented with the dorsal part above, but during copulation, the

222   abdomen of the female contacts with the male upside-down, like in Figure 4. This means that the

223   foldable head of the ligula is orientated during copulation in a way that favours the introduction

224   of its horns into the bursa, facilitating sperm capture and removal. In all Zygopteran species so

225   far studied, stage I is used to remove sperm from previous matings and stage II to inseminate

226   (Córdoba-Aguilar, Uhía & Cordero-Rivera, 2003). My results are compatible with this scenario

227   also for *H. mirabilis*. Therefore, sperm competition has been a relevant force in the evolution of

228   reproductive behaviour in this species, and given its phylogenetic situation, in the odonates as a

229   group. It may date back to the Permian. A study of the evolution of genitalia in a phylogenetic

230   context is certainly a priority (e.g. Rudoy & Ribera, 2016). It is interesting to note that two (out

231   of nine) females dissected after a complete copulation did not have sperm in their bursa. This

232   suggests that some matings might be unsuccessful at insemination, and therefore females may

233   need to mate more than once to be able to reproduce. This possibility needs further study.

234   I could not observe oviposition, neither elicit egg-laying on humid filter paper and plant tissue.

235   Oviposition has apparently never been observed in this species (Sant & New, 1988). The dense

236   vegetation of Long Swamp was too thick to allow detailed behavioural observations of these

237   small damselflies. If females lay eggs at the base of the reeds, this is unlikely to be detected.

238   Even mating pairs were very difficult to notice among the vegetation. Furthermore, individuals

239   in copula were never seen flying (a further peculiarity of this species), which also difficult

240   detection. Focal observation of 79 females in this population (during 10 min each) allowed

241   witnessing three of them mating, but none laying eggs (Cordero-Rivera, 2015). One possibility is

242   that oviposition takes place during the night. Nevertheless, this seems unlikely because animals

243   showed no activity at low temperatures, and five females were maintained over night with humid

244   filter paper, and did not lay eggs. Further detailed observations at localities where the vegetation

245   is less dense (like Ming Ming swamp) might be fruitful to detect oviposition. It surely is

246   endophytic, given the structure of the ovipositor (Sant & New, 1988).

247   To conclude, this study offers the first description of reproductive behaviour of a key taxon in

248   the evolution of the Odonata, considered the sister group to all Lestoidea (Dumont, Vierstraete &

249   Vanfleteren, 2010), and suggests that sperm removal is a very old adaptation of male odonates in

250   the arena of sexual selection. Some mysteries remain: "*Hemiphlebia mirabilis* will always be an

251   enigma" (Fraser, 1955).

252   **Acknowledgements**

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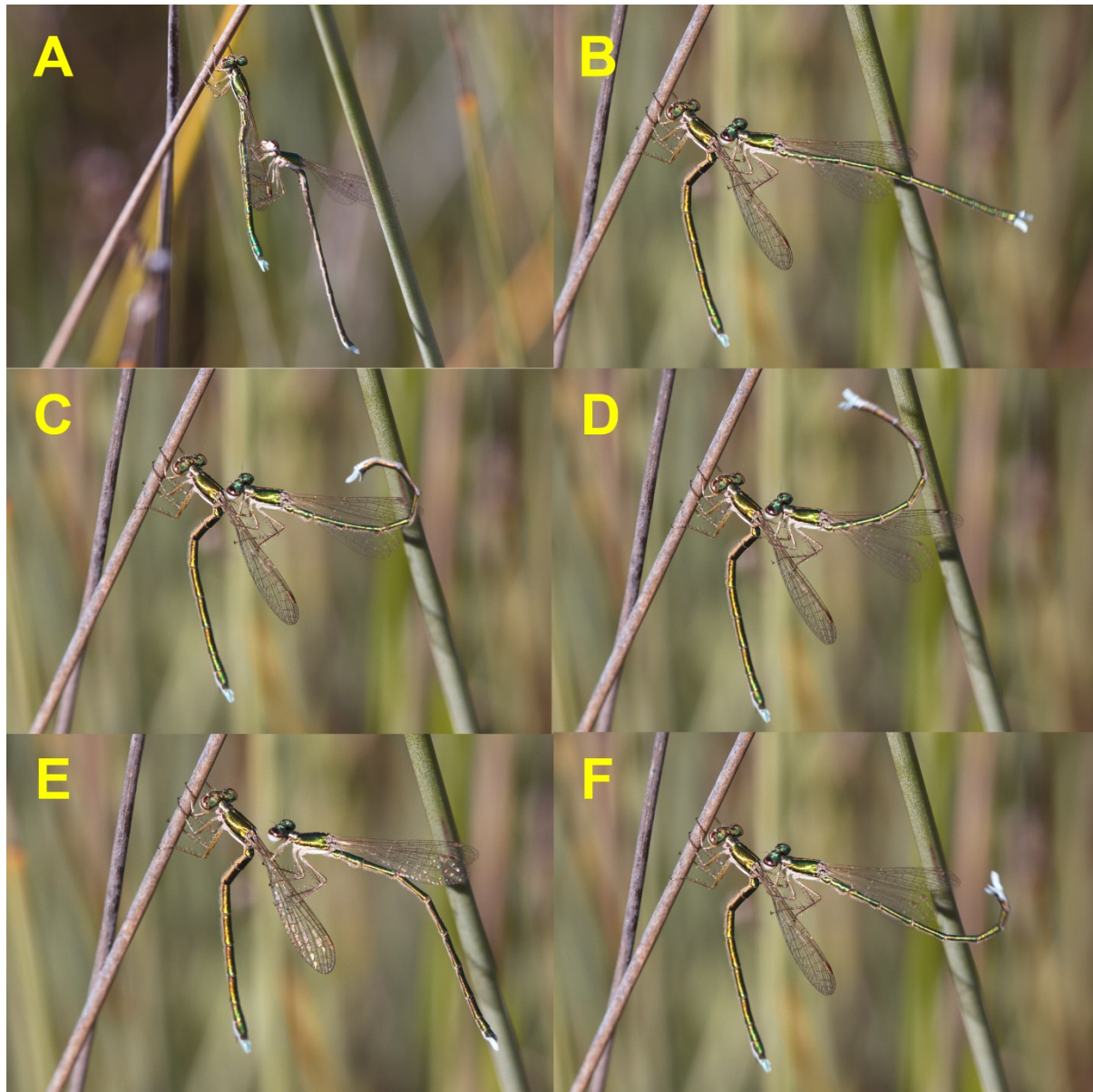347   hilaris (Coleoptera: Cerambycidae). *Applied Entomology and Zoology* 25:383–388.

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351 **Supplementary material**

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

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

354

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.

359

360 Figure 2. Female refusal behaviour, using the abdomen to try to dislodge the male (A and B), and

361 the start of precopulatory tandem (C), once female shows signs of receptivity. Female acceptance

362 of copulation is indicated by the "Z" position of her abdomen.

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

366 preceded by this behaviour.

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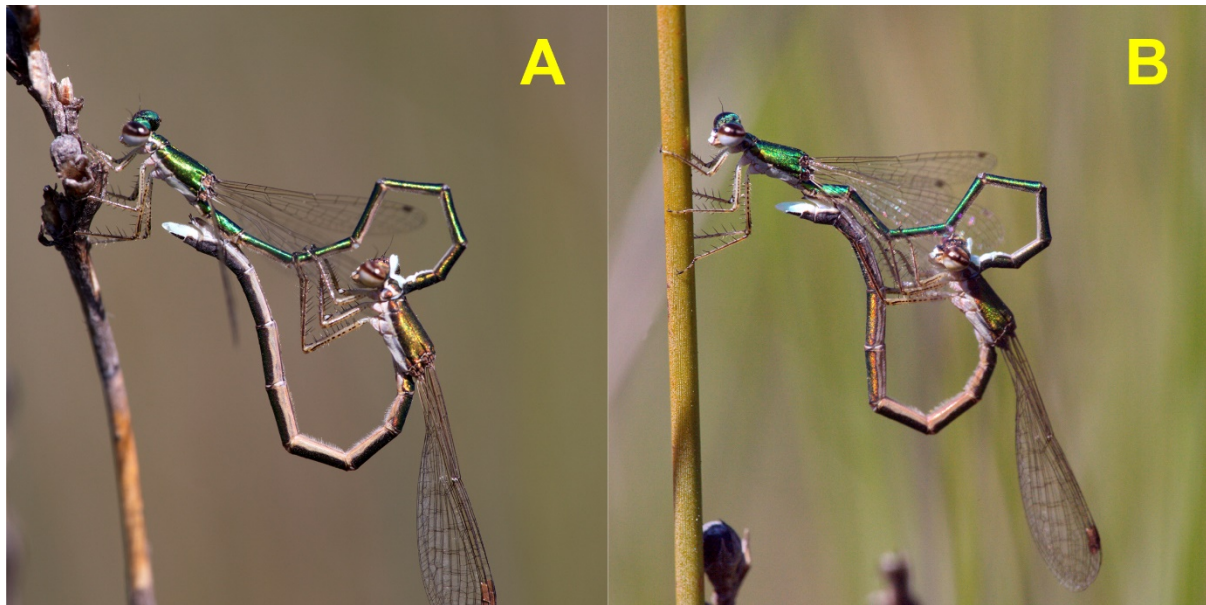
368
369   Figure 4. Copulatory phases in *H. mirabilis* match the description of stage I (A) and stage II (B)

370   of Miller and Miller (1981). Stage I is involved in rivals' sperm removal and insemination takes

371   place during stage II.

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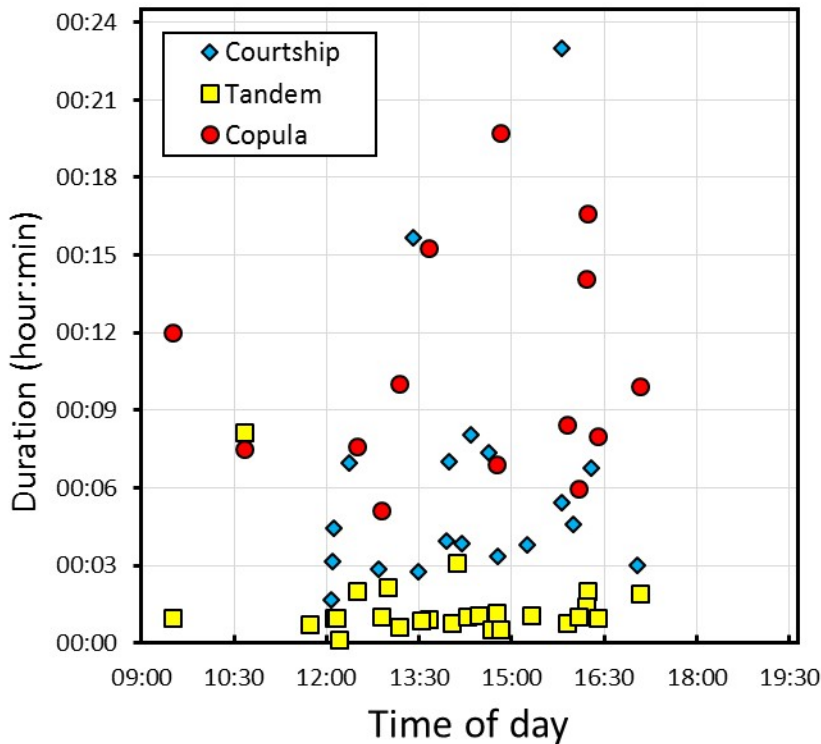
373 _____

374 Figure 5. The relationship between time of day and duration of reproductive behaviours of *H.*

375 *mirabilis*. Courtship refers to the time between female capture and tandem formation. Tandem

376 indicates the time the pair remained in tandem before copulation. Finally, copulation refers to the

377 time between the start and the end of the copulatory wheel.
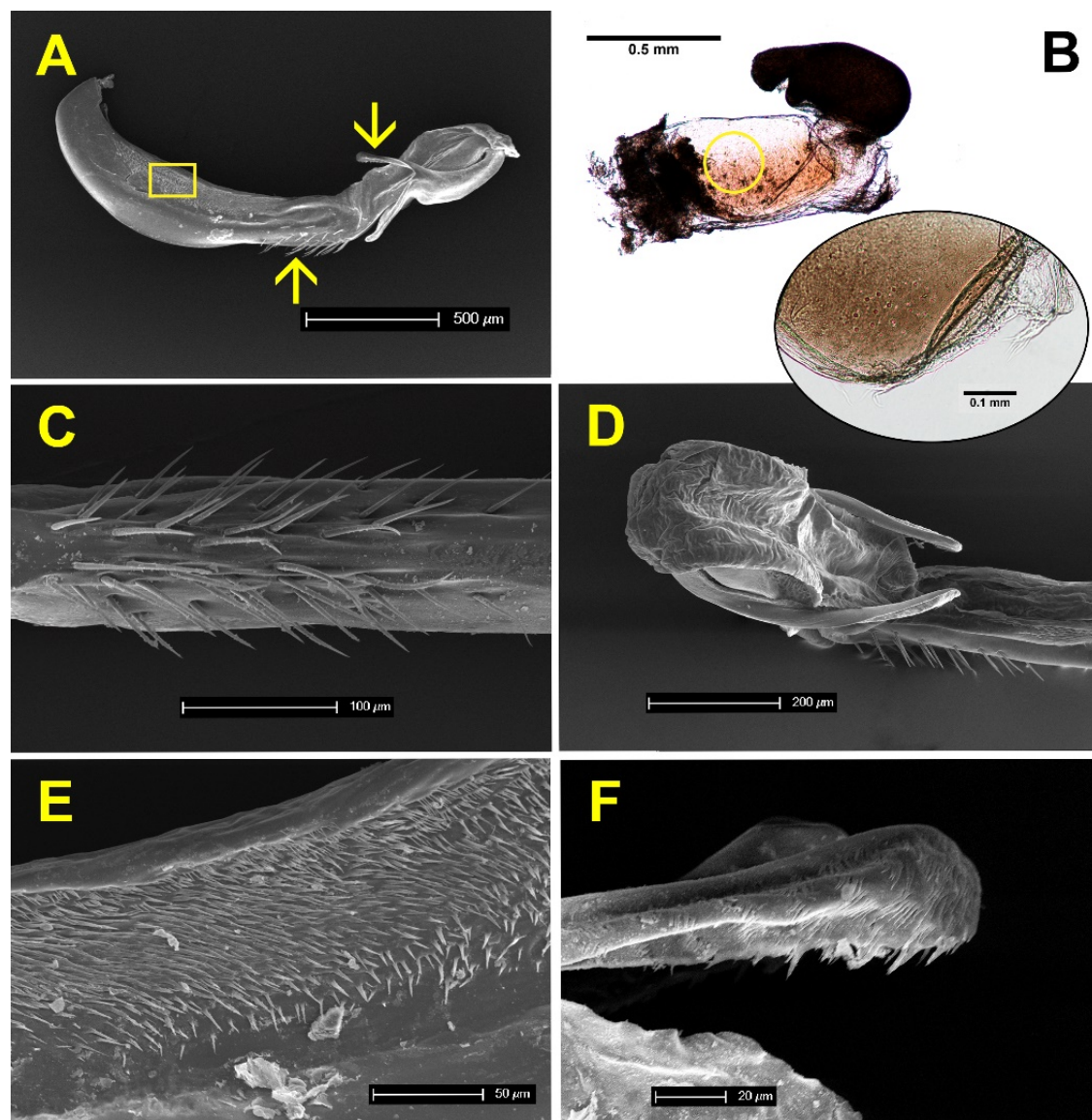
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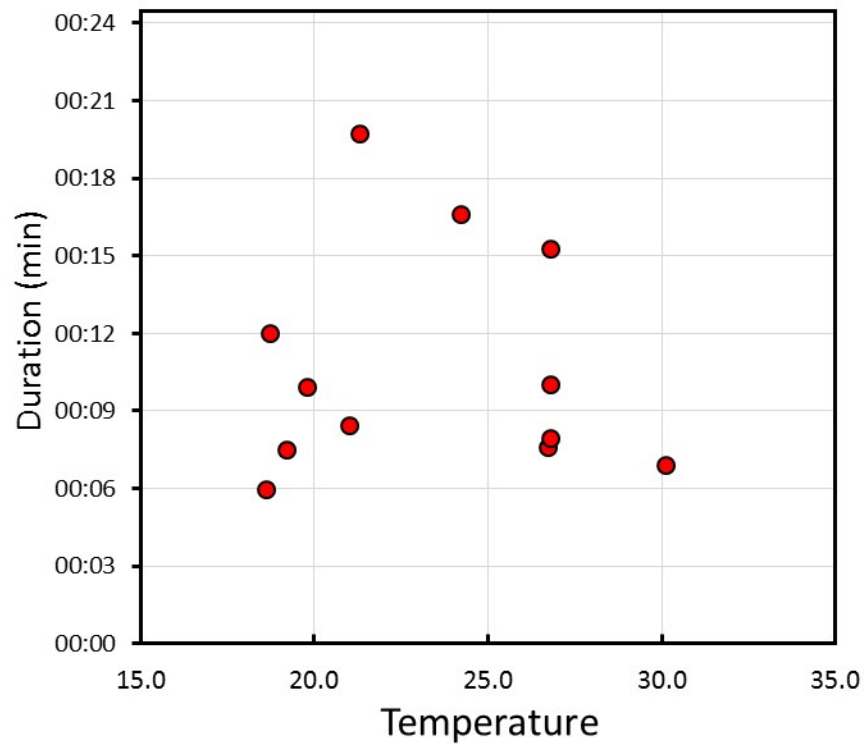
379

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

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382

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,

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

391   remove dust.
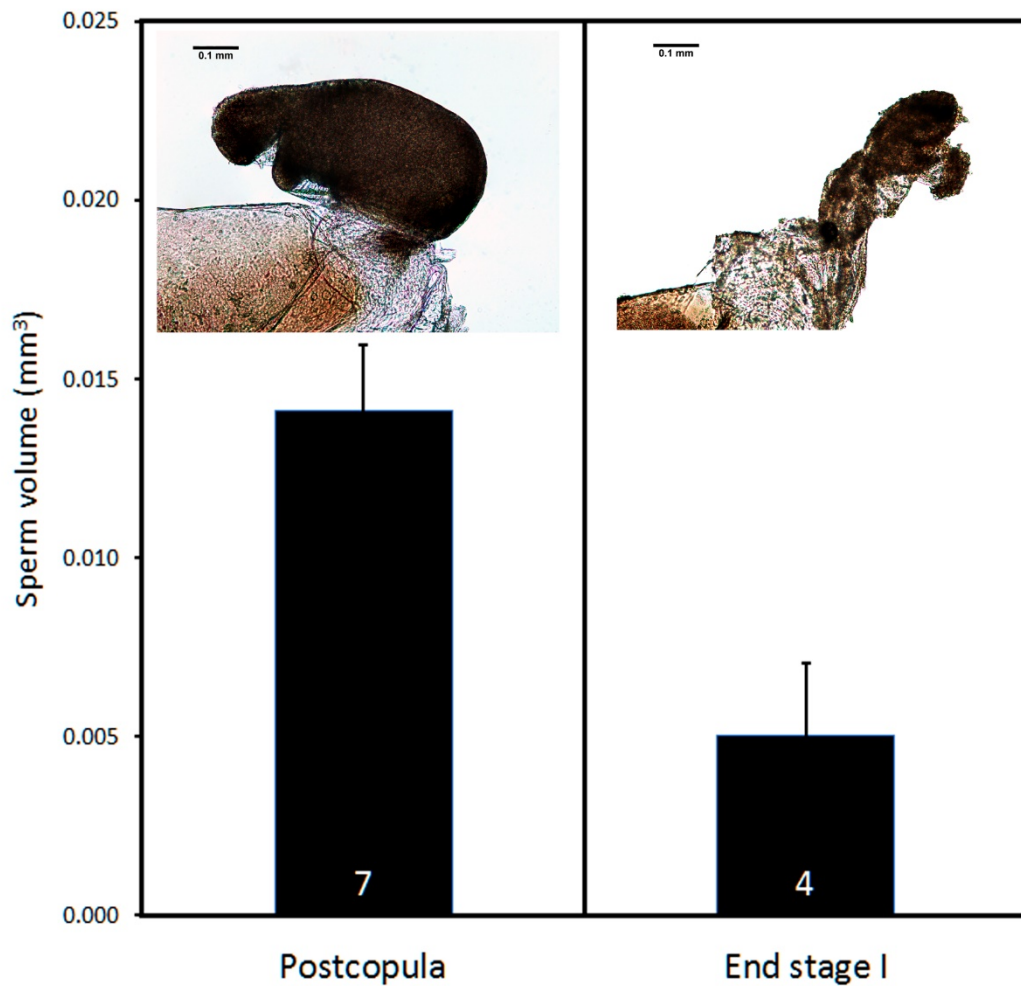
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393   Figure 8. The volume of sperm (mean+SE) in females after a complete copulation and

394   interrupted at the end of stage I, before insemination. Numbers are sample size. The pictures

395   show examples of the bursa copulatrix full of sperm after copulation (left) and partially emptied

396   at the end of stage I (right). Scale bar in the images 0.1 mm. Images edited to remove dust.