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**Male *Gammarus roeseli* provide smaller ejaculates to females infected
with vertically transmitted microsporidian parasites**

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Abstract

The effects of parasites on the reproduction of their hosts are widespread, but studies investigating the effect of female parasitic status on sperm allocation in the males, a form of post-copulatory mate choice, remain scarce. Because males are often sperm-limited, strategic sperm investment, in which females of low reproductive value receive fewer sperm, is predicted to occur so as to maximise long-term male reproductive success. In this study based on pairs collected *in natura*, we investigated how *Gammarus roeseli* (Crustacea: Amphipoda) males allocated sperm when paired with females infected with the vertically-transmitted, sex ratio distorting, microsporidian parasites, *Nosema granulosis* or *Dictyocoela* sp. Since infected females had similar fecundity than uninfected ones, and offspring of females infected with *N. granulosis* showed a higher survival rate, we predicted equivalent or even larger sperm investment from males paired with infected females. Contrary to our predictions, males paired with infected females had lower sperm reserves prior to ejaculation and provided smaller size ejaculates compared to those paired with uninfected females. This pattern suggests either a strategic sperm investment as a function of the female parasitic status, or that males in good condition managed to pair with uninfected females with a higher probability than those in bad condition.

Keywords: mate choice, mate guarding, sperm investment, *Nosema granulosis*, *Dictyocoela*

43

44 The effects of parasites on the reproduction of their hosts are widespread and
45 diverse (Poulin 2007). In particular, the seminal paper by Hamilton & Zuk (1982) drew
46 attention to the fact that parasite infections could be a major evolutionary force
47 driving host's sexual selection and sexual behaviour. Among this broad area of
48 research, studies on effects of parasites on male sperm production and investment
49 are scarce. Most of them have investigated the direct effect of the parasitic status of
50 the male on its sperm production, with evidence of either negative effects (e.g. Yan &
51 Stevens 1995; Galipaud, Dechaume-Moncharmont, Oughadou & Bollache, 2011) or
52 positive effects (reflecting an increased investment to compensate negative effects of
53 parasitism on other life-history traits) (Figenschou et al. 2013; Haeussler, Schmera &
54 Baur, 2014). Only a few studies have investigated the effect of female parasitic status
55 on sperm allocation in the male (Edward & Chapman 2011). Due to intense male-
56 male competition and because males are sperm-limited, strategic sperm investment
57 is predicted to occur at each mating event to maximise the overall fertilisation
58 success (Wedell, Gage & Parker, 2002; Jarrige, Riemann, Goubault & Schmoll,
59 2015; but see Arundell, Wedell & Dunn, 2014). In particular, males may provide
60 females of low reproductive value with fewer sperm (Reinhold, Kurtz & Engqvist,
61 2002). Parasitic infections decrease host fitness, and are therefore a source of
62 variation in the quality of potential mates. Female parasitic status could therefore
63 promote strategic sperm allocation by males, with greater allocation of sperm to high
64 quality (uninfected) females, and prudent sperm allocation to females of low quality.
65 This is especially true in the eventuality of numerous potential future mates, because
66 these future mates could be of higher quality (Reinhold, Kurtz & Engqvist, 2002,
67 Edward & Chapman 2011).

When parasites are vertically transmitted (from mother to offspring via the eggs), they are under strong evolutionary pressure to distort the primary sex ratio of their hosts, through male killing or feminisation (Bandi, Dunn, Hurst & Rigaud 2001). By reversing genetic males into phenotypic females, feminising microbes increase their transmission efficiency by increasing the frequency of the transmitting sex (females). For instance, feminisation is induced by the bacterium *Wolbachia* (Bouchon, Rigaud & Juchault 1998; Kageyama, Nishimura, Hoshizaki & Ishikawa 2002) and by parasites from the eukaryotic phylum Microspora (Terry et al. 2004). Because they often result in female-biased populations, parasitic sex ratio distorters are a selective force in the evolution of mating behaviour (Charlat, Hurst & Mercot 2003) and mate choice (Jiggins, Hurst & Majerus 2000; Moreau, Bertin, Caubet & Rigaud 2001). In the crustacean isopod *Armadillidium vulgare*, not only males prefer to mate with uninfected (real) females vs. infected ones (genetic males reversed by the feminising bacterium *Wolbachia*) (Moreau, Bertin, Caubet & Rigaud 2001), but they also allocate smaller size ejaculates to infected females, resulting in a decrease in fertility (Rigaud & Moreau 2004). Because on average infected and uninfected females had the same fecundity, this lesser allocation to infected females has been attributed to the abnormal (incomplete) behaviour expressed by the reversed-females during courtship and copulation (Moreau & Rigaud 2001, Rigaud & Moreau 2004). The crustacean amphipod *Gammarus duebeni* has been found to be infected with sex ratio distorting parasites: the microsporidian parasites *Nosema granulosis* and *Dictyocoela duebenum* (Terry, Smith, & Dunn 1998; Ironside & Alexander 2015). Dunn et al. (2006) showed that males provide smaller size ejaculates to females infected with *N. granulosis*, relative to uninfected females. As infected females produce fewer eggs than uninfected ones and males are sperm-limited, the smaller

ejaculate size has been interpreted as a strategic sperm allocation; the males saving most of their sperm reserve for females of high quality. Assessment of female quality could be favoured in amphipods because of a long-lasting phase of precopulatory mate guarding involving close proximity with the female for up to three weeks (Galipaud, Bollache, Oughadou & Dechaume-Moncharmont 2015).

In most freshwater crustaceans, including *Gammarus roeseli*, reproduction is characterized by a precopulatory mate guarding phase during which males guard a potential mate by carrying a female beneath their ventral surface for several days before copulation. This behaviour is tightly linked to females' moulting cycle. While males are considered available for mating during most of their moult cycle (Sutcliffe 1992), females are only receptive to copulation shortly after moulting and just for a few hours. After copulation, they begin a new moulting cycle, which can last from several days up to several weeks depending on the species (Jormalainen 1998). *Gammarus roeseli* belong to the group of multivoltine iteroparous annuals (lifetime 12-24 months) and females moult six to eight times, thus potentially producing six to eight broods (Pöckl 1993).

Because females' moulting cycles, and hence receptivity to copulation, are asynchronous, the operational sex ratio is predicted to be strongly biased towards males. Precopulatory mate guarding behaviour is thought to have evolved as a male competitive strategy in response to the brief period of female receptivity (Parker 1974; Grafen & Ridley 1983). Males are in competition for access to females, in particular to 'high-quality' females. From a male perspective, the female's quality as a mate strongly depends on fecundity, and the male pairing decision to engage mate guarding is known to be negatively influenced by female parasitism status (Bollache et al. 2002).

Most populations of the amphipod *G. roeseli* are infected with microsporidian parasites in the rivers of Western Europe (Haine et al. 2004; Gismondi, Rigaud, Beisel & Cossu-Leguille 2012; Grabner et al. 2015). Three of these parasite species (*Nosema granulosis*, *Dictyocoela muelleri* and *Dictyocoela sp. (roeselum)*) have been shown to be vertically-transmitted, and associated with female-biased sex ratios (Haine et al. 2004; Haine, Motreuil & Rigaud 2007). In contrast with the parasites infecting *G. duebeni*, the microsporidia in *G. roeseli* are not associated with a decrease in fecundity (Haine et al. 2004) and the microsporidia of the genus *Dictyocoela* had only a slight impact on the host's physiology in absence of other stresses (Gismondi, Rigaud, Beisel & Cossu-Leguille 2012). *Nosema granulosis* even provides its hosts an advantage in survival relative to uninfected females (Haine, Motreuil & Rigaud 2007). Here, we tested the hypothesis of an effect of females' parasitic status on males' reproductive strategy. Male gammarids are sperm-limited, and long lasting precopulatory mate guarding leaves opportunities for an accurate assessment of female quality. We thus predicted that *G. roeseli* males allocate more sperm to females infected with *N. granulosis*, but show no difference in sperm allocation between uninfected females and females infected with parasites of the genus *Dictyocoela*.

Methods

Ethical note

This work followed the ABS/ASAB guidelines for the treatment of animals in behavioural research. Information about individuals' origin, and housing conditions are described below. Transport between sampling site and laboratory, housing

conditions, handling, as well as experimental monitoring, have been conducted in order to reduce stress and maximise animals' welfare. We complied with the French regulations for experiments on invertebrates.

Animal collection, maintenance and general procedures

Animals were collected in the river Ouches in Dijon (47°17'51.6"N; 5°02'33.3"E), using kick sampling method with a hand net. Gammarids exhibit a precopulatory mate guarding behaviour (they grasp females several days before egg laying) in order to ensure the reproduction of the future batch of eggs (Sutcliffe 1992). Immediately upon sampling, couples were isolated in individual plastic tubes, and were brought back to the laboratory. Each pair was then housed individually under a 12:12 light:dark cycle regime, at 15°C (± 1), in boxes (h=7cm; Ø=9cm) filled with water from the river mixed with tap water previously dechlorinated, UV-treated and oxygenated.

Couples were then randomly assigned to one of the two following experiments. The first experiment aimed at understanding the effect of female infection status on male ejaculate size. Fertilisation is semi-external in gammarids: after the female has laid eggs, the male deposits sperm in her ventral incubating 'pouch'. As water can flow in the pouch, it is impossible to collect male ejaculates. Sperm investment is therefore estimated by comparing sperm reserves in testes between two treatment groups; males before copulation (during mate guarding), and males after a copulation event (not holding the female anymore, the female having released eggs) (Dunn et al. 2006; Lemaître, Rigaud, Cornet & Bollache 2009). Males were anaesthetised using carbonised water and dissected to estimate their sperm reserves. In the first group (n = 64), males were dissected before insemination (within two days after their arrival in

the lab). In the second group (n = 61), sperm remaining in testes were counted after copulation, on the same day. Females were also anaesthetised and dissected. The eggs were flushed out from the incubating pouch and counted. To assess the female's infection status by microsporidia, the gonads were dissected and stored in pure ethanol until molecular analysis. Prior to dissection, the size of each animal was estimated by measuring the height of the fourth coxal plate, using a Nikon SMZ 1500 stereoscopic microscope and the Lucia G 4.81 software.

The second experiment aimed at assessing sperm replenishment kinetics after copulation. Sperm were therefore counted in males 2, 4, 8 and 12 days after the insemination (n = 21, 22, 20, 20, respectively). The second group of the first experiment served as reference for sperm reserves just after insemination (0 days after insemination, n = 64). At the end of the experiments, all non-dissected individuals were released back into the river they came from.

Sperm counting

Sperm was counted as described in Lemaître, Rigaud, Cornet & Bollache (2009). One testis per individual was isolated in a watch glass, in 1000 µL of demineralised water. After isolation, the gonad was dissected under a binocular microscope. The fragments of gonad were submitted to 10 s of ultra-wave treatment to separate the membranes from the sperm (Ultra-waves tank, Branson 2200 Branson Cleaning Equipment Company, Shelton, Co, U.S.A.) and homogenised. For each male, four 10 µL samples were placed on a slide and dried for 10 min at 37°C. All sperm in each drop were counted under an optic microscope Nikon Eclipse E600 (magnification x100). Statistical analyses were carried out using the sum of the number sperms in

the four drops, since the counting appeared repeatable between drops ($R = 0.964$, 95% CI = [0.953; 0.975]).

Infection status

Microsporidia detection and identification were performed using a PCR-RFLP method, following Haine et al. (2004). After DNA extraction from the female's gonads, a PCR test was conducted with the primers V1f and 530r, amplifying a fragment of the microsporidian 16S ribosomal gene only in case of parasite infection (Haine et al. 2004). The size of the amplification product allowed to discriminate between *Nosema granulosis* and *Dictyocoela* parasites, and the use of restriction enzymes *VspI* and *Bst1107I* (MBI fermentas) allowed to discriminate between the *D. muelleri* and the *D. sp. (roeselum)* sequences, respectively (Haine et al. 2004). *VspI* enzyme revealed only females infected with *Dictyocoela sp. (roeselum)* in this study, hereafter referred as "*Dictyocoela*". Males were not tested for the presence of parasites since prevalence in males in this population is close to zero (Haine et al. 2004).

Statistical analyses

Female fecundity was analysed with a general linear model including the following factors: female size, insemination status of males (before or after insemination), and infection status of females (uninfected, infected with *N. granulosis*, infected with *Dictyocoela*) and their two-order interactions. The repeatability of sperm count was estimated using 'rptR' packages (Stoffel, Nakagawa & Schielzeth 2017). Sperm reserves in males were analysed using a general linear model including the following factors: male size, insemination status (before or after insemination), and infection status of females (uninfected, infected with *N. granulosis*, infected with *Dictyocoela*)

and their two-order interactions. Sperm replenishment data were analysed using a general linear model including the following factors: male size, day after insemination (treated as categories, 0, 2, 4, 8 and 12 days after insemination) and their interaction. We reported Cohen's *d* with their bootstrapped 95% confidence intervals (Nakagawa & Cuthill 2007) as measures of effect size for the comparison between mean sperm reserves before and after copulation (Garamszegi 2009). We reported standardised slopes as a measure of effect size for the relationship between female body length and fecundity (Schielzeth 2010). Non-parametric post-hoc comparisons after Kruskal-Wallis' tests were performed using Conover's-test implemented in PMCMR packages (Pohlert 2014). The tests were performed using *R* 3.2.5 (R core team 2016) or JMP 10.0 (SAS institute).

Results

Animal size and female fecundity

A total of 108 pairs (*n* = 53 before ejaculation, and *n* = 55 after ejaculation) were measured. Thirty-seven females were infected with *N. granulosis*, 23 were infected with *Dictyocoela*, and 48 were uninfected (control). Body length did not differ as a function of the female's parasitic status in either males ($F_{2,105} = 0.43$, *p* = 0.65, supplementary Fig. S1a) or females ($F_{1,106} = 0.03$, *p* = 0.97, supplementary Fig. S1b). There was no difference in body length between the two groups (before and after ejaculation) in males ($F_{1,106} = 0.42$, *p* = 0.52, Cohen's *d* = -0.13, 95% CI = [-0.53; 0.22], supplementary Fig. S1c) or in females ($F_{1,106} = 0.18$, *p* = 0.68, Cohen's *d* = -0.08, 95% CI = [-0.48; 0.30], supplementary Fig. S1d). The mean body size was 3.00 mm (95%IC = [2.94; 3.06]) for males, and 2.70 mm (95% CI = [2.64;2.75]) for

females. There was a significant positive relationship between female size and fecundity ($F_{1,106} = 22.3$, $p < 10^{-5}$, standardised slope = 0.42, 95%CI = [0.24; 0.59], Fig.1), but there was no effect of the parasitic status of the female ($F_{2,104} = 0.18$, $p = 0.82$) or the insemination status of the male ($F_{2,104} = 0.0002$, $p = 0.99$) on female size.

Sperm reserves in males before and after insemination

There was no effect of male body size on sperm reserves before ($F_{1,51} = 1.89$, $p = 0.18$) or after ejaculation ($F_{1,53} = 0.68$, $p = 0.41$). Sperm reserves were significantly affected by the interaction between the insemination status and the parasitic status ($F_{2,104} = 3.32$, $p = 0.040$, Fig.2A). The sperm count was significantly larger for males in amplexus (i.e. before copulation) than for males after copulation in each treatment groups: *N. granulosis* infected females (Cohen's $d = 1.37$ with 95% CI = [0.92;1.95], $F_{1,35} = 17.14$, $p = 0.00021$), *Dictyocoela* infected females (Cohen's $d = 1.04$ with 95% CI = [0.39;1.84], $F_{1,21} = 6.07$, $p = 0.022$) and uninfected females (control) (Cohen's $d = 2.78$ with 95% CI = [2.029;4.13], $F_{1,46} = 92.078$, $p < 10^{-5}$). Sperm reserves before ejaculation were significantly different among males, depending on the parasitic status of the female (Kruskal-Wallis test $\chi^2 = 6.7824$, $df = 2$, $p = 0.034$). Males paired with a female infected with *N. granulosis* (Conover's post-hoc test, $p = 0.048$) or *Dictyocoela* ($p = 0.016$) had significantly lower sperm reserves than males from the control group. After ejaculation, sperm reserves were not significantly different among males, whether paired with infected females or uninfected females (Kruskal-Wallis test $\chi^2 = 1.77$, $df = 2$, p -value = 0.41). The comparison of sperm investment as a function of the parasitic status of females was based on the effect size (Cohen's d) of the average difference in sperm count before and after ejaculation. For each treatment, the value of the effect size was large (sensu Cohen 1988) but a direct

comparison of the 95% CIs (Cumming & Finch 2005, Krzywinski & Altman 2013) revealed a significantly larger sperm investment (twice larger) for the uninfected group than for the two infected groups (Fig. 2B).

We estimated the average sperm number in the ejaculate as the difference between the mean sperm reserves before and after ejaculation ($\pm$ 95% confidence interval based on 10 000 bootstraps): $n = 532$ (95% CI = [415; 639]) for the uninfected females, $n = 383$ (95% CI = [212; 565]) for females infected with *N. granulosis*, and $n = 234$ (95% CI = [49; 427]) for females infected with *Dictyocoela*. Because we counted the sperm number in 40 μ L among the 1000 μ L in which one testis was dissected, we can estimate the total ejaculate size as $((532 \times 1000)/40) \times 2 = 26600$ sperm for uninfected females (CI = [20750; 31950]), 19150 for females infected with *N. granulosis* (CI = [10600; 28250]), and 11700 sperm for females infected with *Dictyocoela* (CI = [2450; 21350]).

Sperm replenishment

Sperm reserves increased significantly in males with time spent since copulation ($F_{4,138} = 47.19$, $P < 10^{-5}$) but was not influenced by male size ($F_{1,137} = 2.36$, $P = 0.140$) nor by the interaction between male size and time since copulation ($F_{4,133} = 0.767$, $P = 0.548$). Post hoc comparisons showed no difference in sperm reserves between freshly-mated males and males two days after a copulation event (Fig. 3). Eight and 12 days after a copulation event, males had more sperm than other males, without any significant difference between them (Fig. 3).

Discussion

We observed a negative effect of females' infection status on males paired with them, in terms of both sperm reserves before ejaculation and ejaculate size. Indeed, males paired with infected females had significantly lower initial sperm reserves than males paired with uninfected females, whereas their final sperm reserves after insemination did not differ, reflecting a lower number of sperm provided during insemination. This could be explained by three non-exclusive hypotheses. First, based on our screening of sperm replenishment dynamics, males paired with infected females could be those that had less time to replenish their reserves between two copulations. Such a pattern could occur if infected females moulted more rapidly than uninfected ones, which would reduce amplexus duration before copulation. Alternatively, males paired with uninfected females could be in pair for longer prior collection. However, since the dynamics of sperm replenishment is quite rapid (around 8 days), this possible difference was not huge. If such a pairing pattern was true, this would mean that males engaging pairing with uninfected females would be those able to monopolize females earlier. These hypotheses remain to be tested. Second, when paired with infected females, males could strategically allocate less energy in the production of gametes. As first argued by Dewsbury (1982), a single male gamete may be cheap, but as males transfer large numbers of sperm, gamete production could be energetically expensive. Thus, males could modulate their allocation in sperm production according to female quality by allocating less energy in gamete production when paired with infected females (Reinhold, Kurtz & Engqvist, 2002). Third, males paired with infected females could differ in competitiveness or body condition from males paired with uninfected females, as suggested by the fact that unpaired males have generally lower sperm reserves than paired males (Lemaître, Rigaud, Cornet & Bollache 2009). Consistent with the major role of male-

317 male competition for access to receptive females in gammarids (Ward & Porter 1993,
318 Dick & Elwood 1996, Bollache & Cézilly, 2004), it is possible that those in the best
319 condition have a higher access to uninfected females. If males in poorer condition
320 were less competitive and less efficient in finding or monopolising uninfected
321 females, they might have to accept the second-choice females remaining in the
322 population. Furthermore, the decision to enter into precopula might not solely be
323 under male control, and females could resist male attempts to grasp them
324 (Jormalainen & Merilaita 1995, Jormalainen 1998). If uninfected females were more
325 able to resist attempts from males than infected ones, only the most competitive and
326 in best condition males would be able to pair with them.

327 Our results are consistent with previous observations in *G. roeseli* reporting that the
328 parasitic status of females does not appear to affect their fitness, neither in terms of
329 fecundity nor body length (Haine et al. 2004). This pattern is in contrast with the
330 situation in *G. duebeni*, where infection confers a fitness cost in terms of fecundity
331 (e.g. Dunn et al. 2006), and therefore, makes it difficult to invoke for *G. roeseli* the
332 adaptive strategic male sperm allocation based on differences in female fecundity
333 proposed in *G. duebeni* (Dunn et al. 2006). Differences in sperm reserves and
334 ejaculate size may therefore result from differences in cues used by males to identify
335 infected females. As precopulatory mate guarding is a long-lasting and intimate
336 interaction between the partners, we may propose that the male relies on behavioural
337 cues to assess whether its partner is a genuine female or a feminised male. *Nosema*
338 *granulosis* and *Dictyocoela* sp. have been shown to be sex ratio distorting parasites
339 in gammarids, reversing male hosts into phenotypic females (Terry et al. 2004;
340 Rodgers-Gray et al. 2004; Haine, Motreuil & Rigaud 2007; Ironside & Alexander
341 2015). A number of infected females are therefore genetic males reversed by

parasites. In terrestrial isopod crustaceans infected with the feminising *Wolbachia* bacteria, such 'false females' do not behave entirely as normal females during the copulation sequence, resulting in higher rates of copulation failure and, in case of successful copulations, lower sperm investment in infected females compared to uninfected ones (Moreau, Bertin, Caubet & Rigaud 2001, Rigaud & Moreau 2004). Since mate recognition in crustaceans is based on contact pheromones present on the cuticle (Caskey, Hasenstein & Bauer 2009; Zhang et al. 2011), differences in cuticular compounds between infected and uninfected females may explain the observed pattern. Investigating the behaviour and cuticle compounds of infected vs. uninfected *G. roeseli* females during mate guarding would therefore be helpful to further understand the sperm investment pattern observed in this study. Another explanation, which does not involve a directional preference for uninfected female, could be that infected females show less resistance to pairing attempts (Jormalainen & Merilaita 1995). Thus, only the most competitive males (with the largest reserves of sperm) could pair with more resistant uninfected females, the less competitive males only being able to pair with the less resistant infected females.

Gammarus roeseli males showed large sperm investment at each insemination event (between c.a. 50% and 75% of their sperm reserves constitute the ejaculate). Such a sperm depletion is consistent with the values reported in *Gammarus pulex* (Lemaître, Rigaud, Cornet & Bollache 2009), and confirms that the sperm investment is substantial in *Gammarus*. However, as also noted by Lemaître, Rigaud, Cornet & Bollache (2009), sperm replenishment was achieved within less than a dozen days. Initial sperm reserves in *G. roeseli* were double those in *G. pulex*: around 10 sperm/ μ L in *G. pulex* before copulation, and less than 2.5 sperm/ μ L after copulation, while in *G. roeseli* they were around 22 sperm/ μ L before copulation and more than 5

sperm/ μ L after copulation. As observed in terrestrial isopods (Moreau & Rigaud 2003), we may propose that these higher sperm reserves could be due to a selection pressure induced by the excess of females in populations of *G. roeseli* (Haine et al. 2004).

Future studies should carefully assess the influence of parasites on male mate choice and female behaviour in order to understand pairing processes leading to these mating patterns, and the link between male sperm reserves and female infection status.

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Caption for figure

Fig. 1. Female fecundity as a function of female body size (estimated from the height of the fourth coxal plate, in millimetres) and female parasitic status: uninfected females, females infected with *N. granulosis*, females infected with *Dictyocoela*. The regression line is given for each treatment group.

Fig. 2. (A) Mean sperm number ($\pm$ 95% confidence interval) as a function of the male status (solid circle: before copulation, open circle: after copulation) and the treatment group (the male is either paired with an uninfected female, a female infected with *N. granulosis*, or a female infected with *Dictyocoela*). (B) Effect size of the sperm investment (Cohen's *d*) with bootstrapped 95% CI as a function of the treatment group. Different letters indicate statistically significant differences based on comparison of the 95% CIs.

Figure 3. Mean sperm number ($\pm$ 95% confidence interval) as a function of the time since copulation. The first points correspond to the mean sperm number measured in the testes a few hours after copulation. Different letters indicate statistically significant differences (Tukey post-hoc comparisons).

554 **Fig. 1**

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Fig. 2

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Fig. 3

Supplementary figure

Fig S1. Boxplot of body length (estimated from the height of the fourth coxal plate, in millimetres) as a function of treatment group (parasitic status of the female) for males (a) and females (b), and as a function of pairing status (before or after copulation) for males (c) and females (d). For each group, the box figures the interval interquartile (IIQ), the dark line the median value, the solid circle the mean value, and the open circles the outliers.