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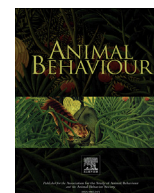
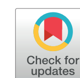


Male *Gammarus roeseli* provide smaller ejaculates to females infected with vertically transmitted microsporidian parasites



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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 males, a form of postcopulatory 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 to maximize 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 to 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 a lower sperm count before insemination and provided smaller ejaculates than those paired with uninfected females. This pattern suggests either a strategic sperm investment as a function of the female's parasitic status, or that males in good condition had a higher probability of pairing with uninfected females than those in poor condition.

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

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 feminization (Bandi, Dunn, Hurst, & Rigaud, 2001). By reversing genetic males into phenotypic females, feminizing microbes

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increase their transmission efficiency by increasing the frequency of the transmitting sex (females). For instance, feminization 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 do males prefer to mate with uninfected (real) females than infected ones (genetic males reversed by the feminizing bacterium *Wolbachia*; Moreau et al., 2001), but they also allocate smaller ejaculates to infected females, resulting in a decrease in fertility (Rigaud & Moreau, 2004). Because on average infected and uninfected females have 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, 2003; 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* (Ironside & Alexander, 2015; Terry, Smith, & Dunn, 1998). Dunn, Andrews, Ingrey, Riley, and Wedell (2006) showed that males provide smaller 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 has been interpreted as a strategic sperm allocation: the males save most of their sperm for females of high quality. Assessment of female quality could be favoured in amphipods because of a long phase of precopulatory mate guarding involving close proximity with the female for up to 3 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 strongly biased towards males provided there is an equal population sex ratio. Precopulatory mate-guarding behaviour is thought to have evolved as a male competitive strategy in response to the brief period of female receptivity (Grafen & Ridley, 1983; Parker, 1974). Males are in competition for access to females, in particular to 'high-quality' females. From a male perspective, the female's quality strongly depends on fecundity, and the male's decision to engage in mate guarding is known to be negatively influenced by the female's parasitic status (Bollache, Rigaud, & Cézilly, 2002).

Most populations of the amphipod *G. roeseli* are infected with microsporidian parasites in the rivers of Western Europe (Gismondi, Rigaud, Beisel, & Cossu-Leguille, 2012; Grabner et al., 2015; Haine et al., 2004). Three of these parasite species (*N. granulosis*, *Dictyocoela muelleri* and *Dictyocoela* sp. *(roeselum)*) have been shown to be vertically transmitted, and associated with female-biased sex ratios (Haine, Motreuil, & Rigaud, 2007; Haine et al., 2004). 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 the absence of other stressors (Gismondi et al., 2012). *Nosema granulosis* even increases its hosts' survival relative to uninfected females (Haine et al., 2007). Here, we tested the hypothesis that females' parasitic status affects males' reproductive strategy. Male gammarids are sperm limited, and prolonged precopulatory mate guarding allows males to assess female quality accurately. We thus predicted that *G. roeseli* males would 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 ASAB/ABS 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 and experimental monitoring were conducted to reduce stress and maximize the animals' welfare. We complied with the French regulations for experiments on invertebrates.

Animal Collection, Maintenance and General Procedures

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

Pairs 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. Fertilization 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. We therefore estimated sperm investment indirectly by comparing the average sperm reserve (i.e. the total number of sperm in the testes) of two treatment groups: males before copulation (during mate guarding) and males after copulation (no longer holding the female, which has released eggs; Dunn et al., 2006; Lemaître, Rigaud, Cornet, & Bollache, 2009). Males were anaesthetized using carbonized water and dissected to estimate their sperm reserves. In the first group ($N = 64$), males were dissected before insemination (within 2 days after their arrival in the laboratory). In the second group ($N = 61$), sperm remaining in testes were counted after insemination, on the same day. Females were also anaesthetized and dissected. The eggs were flushed out from the incubating pouch and counted. To assess whether the female was infected with 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 (http://www.lucia.cz/).

The second experiment aimed at assessing sperm replenishment kinetics after insemination. Sperm were therefore counted in males 2, 4, 8 and 12 days after 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 from where they came.

Sperm Counting

The sperm reserve of each male was estimated as described in Lemaître et al. (2009). One testis per individual was isolated in a watch glass, in 1000 μl of demineralized water. After isolation, the gonad was dissected under a binocular microscope. The fragments of gonad were submitted to 10 s of ultrasonic treatment to separate the membranes from the sperm (Branson 2200 Ultrasonic Cleaner, Branson Cleaning Equipment Co., Shelton, CT, U.S.A.) and homogenized. 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 optical microscope Nikon Eclipse E600 (magnification ×100). Statistical analyses were carried out using the sum of the number of sperm in the four drops, since the counting appeared repeatable between drops ($R = 0.964$, 95% confidence interval, 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 if there was a parasite infection (Haine et al., 2004). The size of the amplification product allowed us to discriminate between *N. granulosis* and *Dictyocoela* parasites, and the use of restriction enzymes VspI and Bst1107I (Fermentas, ThermoFisher Scientific, Waltham, MA, U.S.A.) allowed us to discriminate between the *D. muelleri* and the *D.* sp. *(roeselum)* sequences, respectively (Haine et al., 2004). The VspI enzyme revealed only females infected with *Dictyocoela* sp. (*roeselum*) in this study, hereafter referred to 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), infection status of females (uninfected, infected with *N. granulosis*, infected with *Dictyocoela*) and their two-way interactions. The repeatability of the 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), infection status of females (uninfected, infected with *N. granulosis*, infected with *Dictyocoela*) and their two-way 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 report Cohen's d with their bootstrapped 95% CIs (Nakagawa & Cuthill, 2007) as measures of effect size for the change in average sperm number in the testes before and after insemination (Garamszegi et al., 2009). For a given treatment group, this value of effect size was used as a measure of the average sperm investment. We report standardized slopes as a measure of effect size for the relationship between female body length and fecundity (Schielzeth, 2010). Nonparametric 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, Cary, NC, U.S.A.).

RESULTS

Animal Size and Female Fecundity

A total of 108 pairs ($N = 53$ before insemination and $N = 55$ after insemination) 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 ($F_{2,105} = 0.43$, $P = 0.65$; Appendix Fig. A1a) or females ($F_{1,106} = 0.03$, $P = 0.97$; Appendix Fig. A1b). There was no difference in body length between the two groups (before and after insemination) in males ($F_{1,106} = 0.42$, $P = 0.52$, Cohen's $d = -0.13$, 95% CI = [−0.53; 0.22]; Appendix Fig. A1c) or in females ($F_{1,106} = 0.18$, $P = 0.68$, Cohen's $d = -0.08$, 95% CI = [−0.48; 0.30]; Appendix Fig. A1d). The mean body size was 3.00 mm (95% CI = [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 ($F_{1,106} = 22.3$, $P < 0.001$, standardized slope = 0.42, 95% CI = [0.24; 0.59]; Fig. 1), but there was no effect of the parasitic status of the female

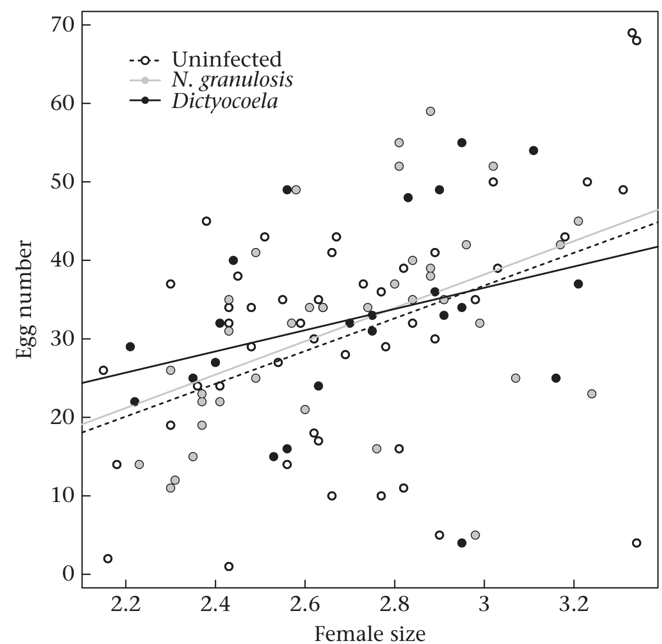

Figure 1. Female fecundity as a function of female body size (estimated from the height of the fourth coxal plate, in mm) and female parasitic status: uninfected females, females infected with *N. granulosis*, females infected with *Dictyocoela*. The regression line is given for each treatment group.

($F_{2,104} = 0.18$, $P = 0.82$) 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 insemination ($F_{1,53} = 0.68$, $P = 0.41$). Sperm reserves were significantly affected by the interaction between the insemination status and the parasitic status ($F_{2,104} = 3.32$, $P = 0.04$; Fig. 2a). The sperm count was significantly larger for males before than after insemination in each treatment group: *N. granulosis*-infected females (Cohen's $d = 1.37$ with 95% CI = [0.92;1.95], $F_{1,35} = 17.14$, $P < 0.001$), *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], $F_{1,46} = 92.078$, $P < 0.001$). Sperm reserves before insemination differed significantly between males, depending on the parasitic status of the female (Kruskal–Wallis test: $X^2{}_2 = 6.7824$, $P = 0.034$). 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 insemination, sperm reserves were not significantly different between males, whether paired with infected or uninfected females (Kruskal–Wallis test: $X^2{}_2 = 1.77$, $P = 0.41$). 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 insemination. 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 as large) 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 insemination (±95% CI 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 μl from the 1000 μl in which one testis was dissected, we can estimate the total ejaculate size as $((532 \times 1000)/40) \times 2 = 26\,600$ sperm for uninfected females (CI = [20 750; 31 950]), 19 150 for females infected with *N. granulosis* (CI = [10 600; 28 250]) and 11 700 sperm for females infected with *Dictyocoela* (CI = [2450; 21 350]).

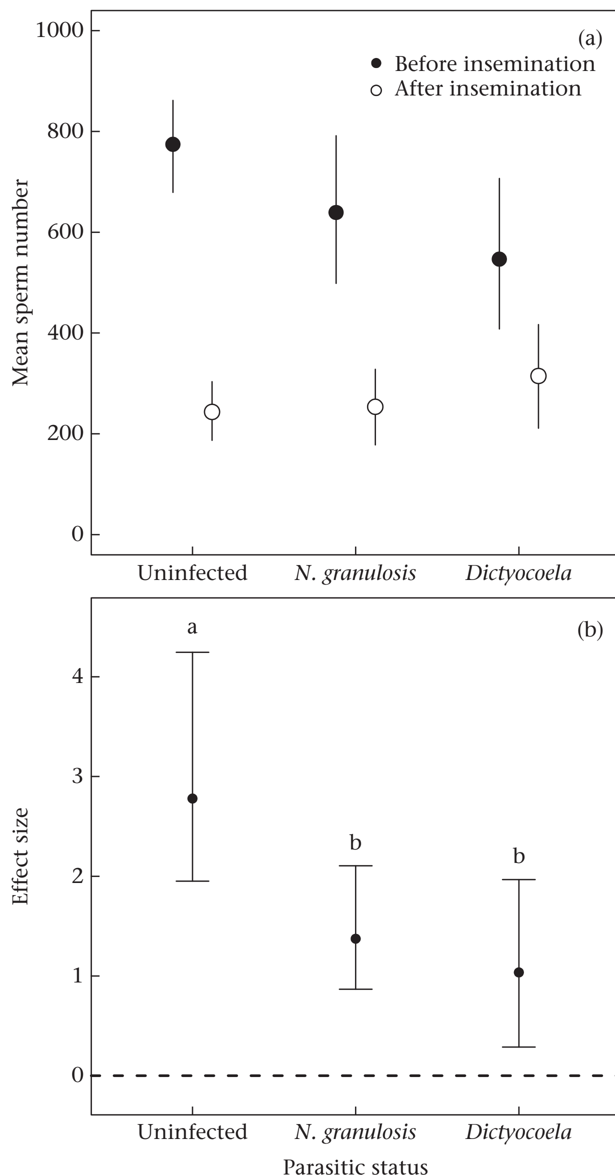

Figure 2. (a) Mean sperm number (± 95% CI) as a function of the male's insemination status (before or after insemination) and the treatment group (the male was paired with an uninfected female, a female infected with *N. granulosis* or a female infected with *Dictyocoela*). (b) Effect size of the sperm 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.

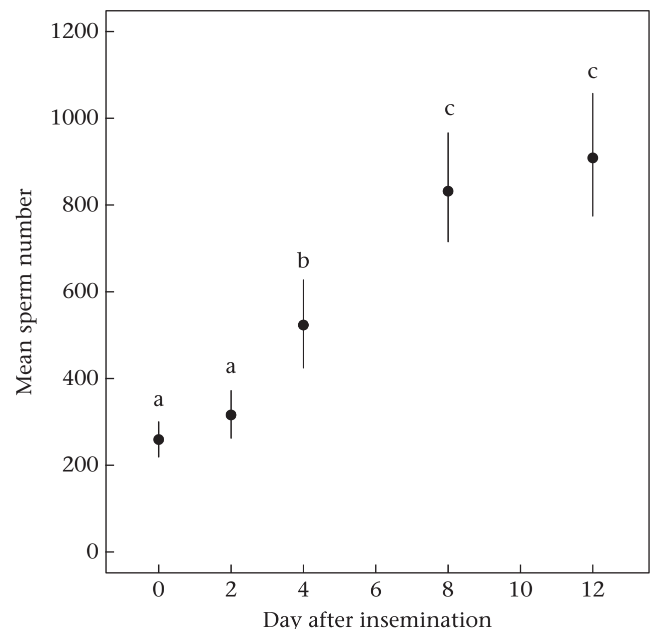

Figure 3. Mean sperm number (±95% CI) as a function of the time since insemination. The first point corresponds to the mean sperm number measured in the testes a few hours after insemination. Different letters indicate statistically significant differences (Tukey post hoc comparisons).

Sperm Replenishment

Sperm reserves increased significantly in males with time spent since insemination ($F_{4,138} = 47.19$, $P < 0.001$) 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 insemination ($F_{4,133} = 0.767$, $P = 0.548$). Post hoc comparisons showed no difference in sperm reserves between freshly mated males and males 2 days after insemination (Fig. 3). Eight and 12 days after insemination, males had more sperm than more recently mated males, without any significant difference between them (Fig. 3).

DISCUSSION

We observed a negative effect of females' infection status on both the number of sperm in the testes before insemination and the average ejaculate size in the males they were paired with. Indeed, males paired with infected females had a significantly lower initial sperm count than males paired with uninfected females, whereas their final sperm count after insemination did not differ, reflecting fewer sperm provided during insemination. This could be explained by three nonexclusive 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. This hypothesis remains to be tested. Second, when paired with infected females, males could strategically allocate less energy to 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 sperm production according to female quality by allocating less energy to gamete production when paired with infected females (Reinhold et al., 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 generally have a lower sperm count in the testes than paired males (Lemaître et al., 2009). Consistent with the major role of male–male competition for access to receptive females in gammarids (Bollache & Cézilly, 2004; Dick & Elwood, 1996; Ward & Porter, 1993), it is possible that those in the best condition have higher access to uninfected females. If males in poorer condition were less competitive and less efficient in finding or monopolizing uninfected females, they might have to accept the second-choice females remaining in the population.

Our results are consistent with previous observations in *G. roeseli* reporting that the parasitic status of females does not appear to affect their fitness, in terms of either fecundity or body length (Haine et al., 2004). This pattern contrasts with the situation in *G. duebeni*, where infection confers a fitness cost in terms of fecundity (e.g. Dunn et al., 2006) and, therefore, makes it difficult to propose that *G. roeseli* shows the same adaptive, strategic male sperm allocation based on differences in female fecundity proposed for *G. duebeni* (Dunn et al., 2006). Differences in sperm count in the testes and ejaculate size may therefore result from differences in cues used by males to identify infected females. As precopulatory mate guarding is a long-lasting and intimate interaction between the partners, we may propose that the male relies on behavioural cues to assess whether its partner is a genuine female or a feminized male. *Nosema granulosis* and *Dictyocoela* sp. have been shown to be sex ratio-distorting parasites in gammarids, reversing male hosts into phenotypic females (Haine et al., 2007; Ironside & Alexander, 2015; Rodgers-Gray, Smith, Ashcroft, Isaac, & Dunn, 2004; Terry et al., 2004). Some infected females are therefore genetic males reversed by parasites. In terrestrial isopod crustaceans infected with the feminizing *Wolbachia* bacteria, such 'false females' do not behave entirely as normal females during the copulation sequence, resulting in higher rates of copulation failure and, for successful copulations, lower sperm investment in infected females than uninfected ones (Moreau et al., 2001; Rigaud & Moreau, 2004). Since mate recognition in crustaceans is based on contact pheromones present on the cuticle (Caskey, Hasenstein, & Bauer, 2009; Zhang, Terschak, Harley, Lin, & Hardege, 2011), differences in cuticular compounds between infected and uninfected females may explain the observed pattern. Investigating the behaviour and cuticle compounds of infected versus 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 females, could be that infected females show less resistance to pairing attempts (Jormalainen & Merilaita, 1995). Thus, only the most competitive males (with the largest sperm count in the testes) 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 ca. 50% and 75% of their initial sperm count constitute the ejaculate). This sperm depletion is consistent with the values reported in *Gammarus pulex* (Lemaître et al., 2009), and confirms that the sperm investment is substantial in *Gammarus*. However, as also noted by Lemaître et al. (2009), sperm were replenished within 12 days. Initial sperm counts in *G. roeseli* were double those in *G. pulex*: around 10 sperm/μl in *G. pulex* before insemination and fewer than 2.5 sperm/μl after insemination, while in *G. roeseli* they were around 22 sperm/μl before insemination and more than 5 sperm/μl after insemination. As observed in terrestrial isopods (Moreau & Rigaud, 2003), we may propose that these higher sperm counts 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 to understand pairing processes leading to these mating patterns, and the link between male sperm count and female infection status.

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Appendix

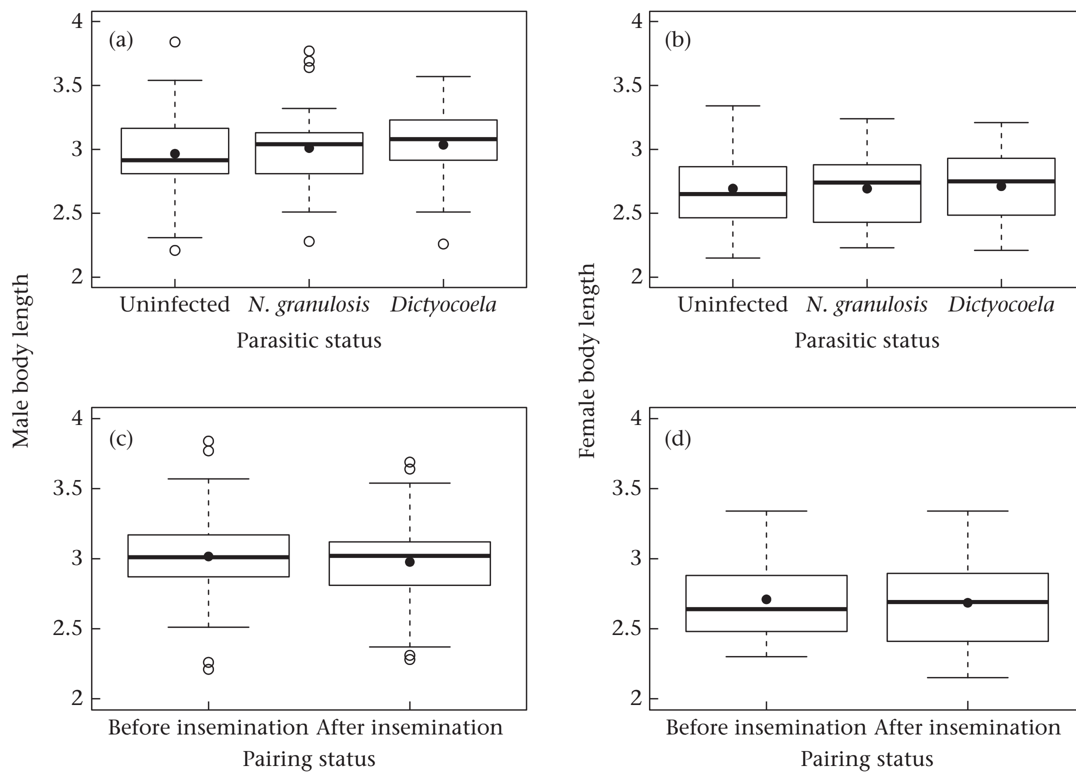

Figure A1. Box plots of body length (estimated from the height of the fourth coxal plate, in mm) as a function of treatment group (parasitic status of the female) for (a) males and (b) females and as a function of status (before or after insemination) for (c) males and (d) females. The box plots show the median (dark line), mean (solid circle) and 25th and 75th percentiles; the whiskers indicate the values within 1.5 times the interquartile range and the open circles are outliers.