Podocotyle atomon (Trematoda: Digenea) impacts reproductive behaviour, survival and physiology in Gammarus zaddachi (Amphipoda)

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1 ***Podocotyle atomon* (Trematoda: Digenea) impacts reproductive**
2 **behaviour, survival and physiology in *Gammarus zaddachi* (Amphipoda)**
3 **on the UK coastline**

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13 Running title: Trematode infection and reproduction in *Gammarus zaddachi*

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18

19 **Abstract**

20 The Trematoda are a group of phylogenetically diverse metazoan parasites that
21 exhibit complex life cycles that often pass through invertebrate and vertebrate hosts.
22 Some trematodes influence their host's behaviour to benefit transmission. Their
23 parasitic influence may impact host population size by inhibiting an individual's
24 reproductive capacity.

25 We assessed the impact of infection by *Podocotyle atomon* on the reproductive
26 behaviour and fecundity of its amphipod intermediate host, *Gammarus zaddachi,*
27 using laboratory and field studies. Parasite prevalence was high in the field, with
28 males more likely to be infected (prevalence in males 64%, in females 39%). Males
29 also suffered a higher parasite burden than females. Infected females were less
30 active, but we found no evidence for a reduction in female reproductive success.
31 Infected females also had comparable pairing success to uninfected females. In
32 males, infection reduced survival and fecundity, with mortality being highest, and

33   sperm numbers lowest, in heavily infected individuals. Trematode parasites are

34   sometimes associated with altered host fecundity, but studies often lack the relevant

35   experimental data to explore the evolution of the trait. We discuss this among

36   information specific to the effect of *P. atomon* infection in *G. zaddachi.*

37

1. Introduction

39   The evolution of a host in response to parasitism can take several routes, including

40   (but not restricted to) the evolution of resistance (Dagan et al. 2017), parasite

41   avoidance behaviours (Behringer et al. 2018), and the evolution of sex characteristics

42   and physiological change (Hamilton & Zuk 1982; Howard & Lively 1994). There are a

43   wide variety of organisms that can induce host pathology and subsequently drive the

44   evolution of their host (viruses, bacteria, Microsporidia etc.). Some of the most

45   influential parasites for host evolution and adaption are trophically transmitted

46   metazoans, such as acanthocephalans, cestodes and trematodes (Reisinger &

47   Lodge 2016; Blasco-Costa & Poulin 2017).

48   The phylogenetically diverse Trematoda are obligate parasites that often exhibit

49   variable transmission methods and are found in freshwater, marine and terrestrial

50   habitats (Bojko et al. 2017; Galaktionov et al. 2018). The trophic transmission

51   methods used by trematodes are possible due to the parasites ability to manipulate

52   the behaviour and physiology of their host. Host manipulation by trematodes has

53   been linked with behavioural, developmental and pigmentation processes that

54   increase the susceptibility of the next host (Poulin 1995; Lefèvre et al. 2009; Cézilly

55   et al. 2010; Thomas et al. 2010).

56   Infections by trematodes often do not lead to rapid transmission, meaning that

57   infections can be present in populations for prolonged periods of time along with any

58   host manipulation effects (Etges & Gresso 1965). In the process of infection and

59   transmission, trematodes can influence several host traits that appear to be largely

60   unrelated (side-effects) to trematode transmission success. Such "side-effects"

61   include an altered fecundity in some cases, which can have consequences for the

62   host population size and associated niche (Kelly et al. 2001; Pai & Yan 2003). In

63   some circumstances and intermediate hosts, trematodes cause complete castration,

64   potentially due to resource siphoning from the host gonad (Minchella & Loverde

65   1981). The trematode *Cercaria batillariae* castrates its snail host *Batillaria cumingi*

66   and induces gigantism, diverting resources away from reproduction to the production

67   of parasite larvae (Miura et al. 2006). Host defences have also been observed,
68   whereby hosts may compensate for parasite-induced reduction in their reproduction
69   (Fredensborg & Poulin 2006; Minchella & Loverde 1981). The trematode
70   *Gynaecotyla adunca* induces increased crawling behaviour to facilitate its
71   transmission by predation in its amphipod host *Corophium volutator*; however, newly
72   infected males show fecundity compensation, increasing mating initiation and
73   possibly ejaculate size (McCurdy et al. 1999; McCurdy et al. 2000).

74   The Amphipoda, an order of crustaceans, have been used as a model species to
75   understand disease in many contexts (Bojko & Ovcharenko 2019), including work on
76   understanding parasitic Metazoa such as the Trematoda (Lagrue 2017; Gates et al.
77   2018; McPherson et al. 2018). *Podocotyle atomon* is a widely distributed species of
78   trematode common in Northern European seas. It uses the gastropod *Littorina* sp. as
79   a first intermediate host, an amphipod as a second intermediate host, and a marine
80   fish as the definitive host (Hunninen & Cable 1943; Kesting et al. 1996). This
81   trematode has been reported in a number of amphipod intermediate hosts, including
82   *Gammarus zaddachi* in the British Isles, and throughout several European marine
83   ecosystems (Zander et al. 2000; Álvarez et al. 2002; Kristmundsson & Helgason
84   2007; Markowski 2009).

85   *Gammarus zaddachi* is an intertidal species with a predicted lifespan of around one
86   year. They have two generations per year and breed iteratively, with direct
87   development of brooded embryos. The first broods are hatched in early spring when
88   food is abundant, with a series of 7-8 broods being produced throughout spring and
89   summer. The next generation then begins to breed in late autumn, producing 3-4
90   broods (Sutcliffe 1993). As in all *Gammarus* sp., brood size (number of eggs) is
91   positively correlated with female mass and in *G. zaddachi* there is little change in
92   investment (in terms of brood size vs. egg volume) over the year (Sutcliffe 1993).

93   Mating in *Gammarus* is restricted by the asynchronous and limited female receptivity
94   period, which has led to selection pressure for pre-copulatory guarding by males.
95   Males prefer to guard larger more fecund females, within the constraints of male-
96   male competition and their ability to carry the female in precopula (Hatcher & Dunn
97   1997). Females that are closer to their moult (when eggs are released), hence
98   minimising guarding costs, are also preferred (Adams et al. 1989; Poulin et al. 1994).

99   Infection by *P. atomon* includes cercarial penetration of the host cuticle, where the
100   parasite encysts within the body cavity of the *G. zaddachi* host (Gollasch & Zander

101 1995). The effects of trematode parasitism by *P. atomon* on the behaviour of *G.*
102 *zaddachi* remain understudied.

103 In this study we explore fecundity and parasitism in the estuarine amphipod *G.*
104 *zaddachi* infected by *P. atomon*. We assess the relationship between trematode
105 infection and host size, pairing success and female fecundity in the field. In
106 laboratory studies we quantify the effect of parasitism on the behaviour and survival
107 of the host, as well as assessing the impact of infection on male and female
108 fecundity. We also examine whether *G. zaddachi* show mate discrimination against
109 infected individuals, testing the predictions that males should avoid pairing with, and
110 allocate fewer sperm to, infected females.

111

112 **2.   Materials and Methods**

113 *2.1 Animal Collection and Husbandry*

114 Adult *G. zaddachi* were sampled from Budle Bay, Northumberland, U.K. (55°36'N,
115 1°45'W) (Fig. 1) (http://edina.ac.uk/digimap) (http://edina.ac.uk/digimap), using a fine
116 mesh net, in the spring and autumn of 2009 and 2010, and in spring 2011, to
117 coincide with *G. zaddachi* breeding seasons. A subset of animals (*n*=393) collected
118 in May 2009 were used immediately to evaluate parasite prevalence and burden, and
119 pairing success, in the field. For experimental studies, animals were kept in stock
120 tanks and experimental pots in aerated brackish water (salinity 6.5 ‰), at 14°C, 16h
121 light: 8h dark, corresponding to field conditions. Rotted sycamore leaves (*Acer*
122 *pseudoplatanus)* and algae (*Enteromorpha* spp.) were provided for food and shelter.
123 All dissections and records of sex, fecundity and parasitic status were carried out
124 after the animals were anaesthetized in carbonated water and performed under a
125 dissecting microscope (Leica SD-6). All weights were recorded "wet", after the
126 gammarid had been blotted with tissue paper to remove excess water. Wet weight is
127 preferable to length, as it minimizes damage and stress to the animals and is highly
128 correlated with length (Naylor and Adams, 1987). Gravid females were weighed
129 following removal of eggs for fecundity counts. Any animals remaining at the end of
130 the study were either maintained in the laboratory in stock tanks or returned to the
131 field.

132

133 *2.2 Parasite Detection and Identification*

134 Cysts were visible through the cuticle of infected *G. zaddachi*, and dissection
135 revealed the presence of a single trematode in each cyst (30 cysts dissected) (Fig.
136 2). Identification of the trematode encysted in *G. zaddachi* was based on genetic data
137 (DNA barcoding) and on morphological/geographical criteria. For molecular
138 identification, trematodes were dissected from four different *G. zaddachi* individuals
139 and preserved in 100% ethanol and held at -20°C, until DNA extraction. DNA was
140 extracted (using appropriate controls) as described by Sambrook et al. (1989) and
141 Ironside et al. (2003). Polymerase chain reaction (PCR) amplifications of partial
142 ribosomal genes (18S, 28S, 5.8S and ITS2) were then carried out with various
143 primers and Tc conditions using mgH$_2$O as a negative control reaction
144 (Supplementary data 1).

145 The PCR products were purified using a Qiaquick PCR purification kit (Qiagen Inc,
146 Sussex, UK) and sequenced at the University of Leeds Sequencing Service. These
147 sequences were subsequently entered into existing databases to search for
148 homologies with known trematode species. This analysis indicated that this species
149 belongs to the Opecoelidae (Digenea: Plagiorchiida: Opecoelata: Opecoelidae). A
150 concatenated 18S-28S alignment (2969 positions) was then constructed to determine
151 the phylogenetic position of the species, using data available from GenBank
152 (Supplementary data 2). This alignment included representative data for 29 species
153 within the Opecoelidae family as defined in Bray et al. (2016) and Martin et al. (2018
154 a-f). The outgroup comprised two taxa representative of the Brachycladiidae and
155 Acanthocolpidae, two sister families of the Opecoelidae (Cribb et al. 2003).
156 Sequence data were aligned using MAFFT, and concatenated and trimmed using
157 Geneious (Kearse et al. 2012). A Maximum Likelihood analysis was then run using
158 the Tamura-Nei model with gamma distribution with invariant sites (G+I) and 200
159 bootstrap replications using MEGA7 (Kumar et al. 2016).

160 In addition to the genetic information, morphological features described by Hunninen
161 and Cable (1943), including the metacercariae length, ratio of ventral and oral
162 suckers, and single continuous cytoplasm, were used to identify the parasite as
163 *Podocotyle atomon.* This parasite is known to infect *Gammarus* spp., including *G.*
164 *zaddachi.* The geographical location in which *G. zaddachi* were sampled (Markowski
165 2009), the size of the trematode, the dorsal position of the trematode within the
166 gammarid body cavity (Hunninen & Cable 1943), and the host-parasite relationship
167 observed (Kesting et al. 1996) were all in agreement with this species identification.

168

2.3 Parasite Prevalence and Burden

Parasite prevalence varied between specimens but we did not collect data for sufficient years to explore any seasonal effects. Trematode prevalence and burden in the field were evaluated for *G. zaddachi* sampled in May 2009. To evaluate the prevalence of the trematode, we recorded the presence of cysts for each individual (Fig. 2). These criteria was deemed to be more reliable and much more rapid to evaluate infection status than a molecular method we tested (i.e. PCR using Digenea specific primers and appropriate negative controls) (Supplementary data 3). Sex, infection status, number of visible cysts and weight were recorded for each individual (*n*=393).

179

2.4 Effect of Parasitism on Survival

To investigate the impact of parasitism on survival, *G. zaddachi* individuals [75 males (45 infected, 30 uninfected) and 120 females (37 infected, 83 uninfected)] were isolated for six weeks in the laboratory. These were isolated in individual pots, but otherwise maintained under the same conditions as the stock populations. Following inspection to determine their sex, weight, infection status and parasite burden, individuals were supplied with food and checked at weekly intervals for mortalities.

187

2.5 Effect of Parasitism on Reproductive Behaviour and Fecundity

2.5.1 Pairing success

To compare the pairing success of infected and uninfected *G. zaddachi* in the field, animals were sampled in May 2009 and immediately sorted into pairs (*n*=110 pairs, i.e. 220 individuals) and singles (*n*=173), before being transported to the lab where the sex, weight, pairing status and number of visible trematode cysts was recorded.

In the laboratory, we tested the hypothesis that males should discriminate against infected females in pairing decisions. Two size-matched females (1 infected and 1 uninfected) were placed in a 50ml pot (5cm diameter) together and left to settle for 5 minutes. A single uninfected male (*n*=25) was added and all contact and pairing attempts were recorded for 10 minutes, along with time of pairing and choice of female. All *G. zaddachi* individuals used in this experiment came from precopula

pairs, to ensure they were receptive to pairing, and males and females used in the same trial had not previously encountered each other. The size-matched females were marked for identification using white correction fluid (Tippex). Marking was alternated between the infected and uninfected female to control for any effects of marking; however, previous studies have shown no effect of this method of identification on pairing behaviour (Kelly et al. 2001).

We observed incidences of contact between the male and either the infected or uninfected female. For males we recorded pairing behaviors: assess (upon contact the male examined the female using his antennae) and pair (the male maneuvered the female into precopula positioning and commenced mate guarding). For females we recorded resistance behaviors: flee (upon contact the female swam quickly away from the male), and escape (following a pairing attempt by the male, the female flexed her body in resistance and escaped from his grasp). We also compared the pairing behavior of size-matched infected and uninfected males; the original male was removed following the trial, the females left to settle again, and then an infected male of a suitable size (n=25) added and again monitored for 10 minutes. For half of the experiments, the order of males was reversed, with an infected male being tested first. As a control, we also compared the general activity of infected and uninfected males (n=40; 20 infected, 20 uninfected) and females (n=37; 17 infected, 20 uninfected). Test individuals were placed in a 200ml capacity, 15cm diameter, clear plastic pot, which was filled with 60ml aerated brackish water, and marked with a diameter line across the center. A 5 min acclimatisation period was allowed between adding the individual to the pot and commencing the trial. For each trial, we counted the number of times the gammarid crossed the central line in five minutes.

2.5.2 Fecundity

To compare the number of sperm produced by infected and uninfected *G. zaddachi*, 32 infected and 30 uninfected males were isolated in individual pots for 6 weeks, to allow build-up of sperm stores (Dunn et al 2006; Lemaître et al. 2009). After this time sperm counts were recorded, using a modification of the methods in Lemaître et al. (2009). Males were anaesthetised in carbonated water and then dissected in crustacean Ringer solution (Van Harreveld 1936). One of the testes was removed and transferred to a 10µl droplet of distilled water on a cavity slide. The testis was ruptured with a fine hypodermic needle to release the sperm, which was then washed into an Eppendorf tube and made up to a volume of 1.5ml with distilled water. The

235 solution was briefly vortexed to prevent agglutination and ensure even mixing of the
236 sperm. Three 10µl samples were then pipetted onto a clean microscope slide and
237 allowed to dry. The sperm counts were conducted blind, at a magnification of x40,
238 using an Olympus BH-2 microscope. For each male, an estimate of the total sperm
239 number was calculated by multiplying the mean of the three replicate sperm counts
240 by the dilution factor.

241 The impact of infection status on female fecundity (*n*=183) was investigated in a
242 subset of *G. zaddachi* females collected in June (2009). Brooding females were
243 anaesthetized in carbonated water, and the embryos flushed from the brood pouch
244 using a syringe filled with brackish water. Weight, infection status, trematode number,
245 embryo number and embryo developmental stage were recorded for all females.

246

247 *2.5.3 Sperm allocation*

248 To test whether *G. zaddachi* males allocate fewer sperm to infected females, and to
249 compare the numbers of sperm allocated by infected versus uninfected males, 55
250 males (28 infected, 27 uninfected) were isolated in individual pots to allow
251 replenishment of sperm stores. After four weeks, receptive females (22 infected, 33
252 uninfected) were taken from pairs in the stock population and added to the males.
253 The individual pairs were allowed to mate. Successful mating was determined by the
254 presence of the female exuviae (as gammarids mate immediately following the
255 female molt) and presence of eggs in the brood pouch, followed by separation of the
256 pair. Within 24 hours of mating, all males were dissected and the number of
257 spermatozoa remaining in the seminal vesicle and testes counted as described
258 above.

259 To investigate whether female fecundity was affected by infection or by any ejaculate
260 tailoring by the males, the embryos were left for one week to develop and then
261 flushed from the brood pouch. The number of viable embryos, determined by
262 evidence of normal development (Weedall et al. 2006) upon visual inspection under a
263 dissecting microscope, was then counted.

264

265 *2.6 Data Analysis*

266 Data were analysed using statistical models constructed in R version 3.1.1 (R
267 Development Core Team 2014). All models were initially constructed as maximal

268 models, including all relevant terms and interactions. Models were compared using
269 P-values from the "dropterm" function (MASS package; Venables & Ripley 2002) to
270 determine whether terms significantly improved the fit of the model.

271

272 *2.6.1 Prevalence and burden*

273 The factors associated with infection status were assessed using a generalised linear
274 model (GLM), with binomial error distribution, including sex and weight as predictor
275 variables. Within infected individuals, we examined the variables influencing parasite
276 burden, using a GLM with Poisson error distribution, and again including sex and
277 weight as predictor variables.

278

279 *2.6.2 Survival*

280 To determine whether or not infection status influences mortality, the time to death
281 data were analysed using separate parametric survival models (PSMs) for males and
282 females, using the psm function within the rms package in R (Harrell Jr 2014a;
283 Harrell Jr 2014b). The impact of trematode burden on mortality was similarly
284 investigated for all infected females (n=37) and for a subset of 35 of the 45 infected
285 males, for which trematode number was recorded, using PSMs. Weight was included
286 in the models as it was found to have a significant impact on infection status and
287 parasite burden in the field data.

288

289 *2.6.3 Pairing success*

290 In the field, the impact of infection status on pairing status, and the impact of parasite
291 burden on pairing status in infected individuals, were analysed using separate GLMs,
292 with binomial error distribution, and with sex and weight included as predictor
293 variables. To analyse the impact of infection on male: female weight ratios in pairs,
294 the ratio was first calculated by dividing male weight by female weight, to determine
295 the relative sizes of paired individuals. The data were then normalised by log
296 transformation and tested using general linear models (LMs) of male or female
297 infection status as a predictor of pairing ratio. As individual weight was known to
298 affect infection status, male and female weight, respectively, were controlled for in
299 the models.

300 For the laboratory mate choice trials (where competing individuals were size-
301 matched), a contingency table Chi-squared test was used to assess differences in
302 mate selection between infected and uninfected males, with standard Chi-squared
303 tests used to test for discrimination against infected females. The likelihood of males
304 pairing during the 10 min trial period was assessed using Fisher's exact test, due to
305 low expected values. To investigate whether male infection status affected length of
306 time taken to pair, whilst accounting for the inherent right censoring of the data (due
307 to the fact that not all individuals paired), a PSM was used, also including number of
308 assessments by the male per second and number of incidences of resistance by the
309 females per second as predictor variables. The effects of both male and female
310 infection status on number of assessments by males and number of resistance
311 attempts by females were analysed using separate GLMs, with quasi-Poisson error
312 structure (as data could not be normalised and were over-dispersed), of either
313 assessments or resistances per second. To analyse the impact of infection status on
314 general *G. zaddachi* activity, the data were analysed using separate LMs for males
315 and females, each also including infection status as a predictor variable. The data for
316 females were first normalised by square root transformation.

317

318 *2.6.4 Fecundity analysis*

319 The impact of infection status on female fecundity in the field was analysed using a
320 GLM, with quasi-Poisson error distribution. Weight was also included in the model as
321 larger gammarid females are known to be more fecund (Hatcher & Dunn 1997). As
322 embryo loss occurs during embryo development in gammarids (Ford et al. 2003), we
323 also tested for an effect of embryo stage. Including stage as a factor in the model
324 was not significant ($p=0.97$) and so the term was removed from the minimal model.

325 The impact of infection on sperm numbers, and the impact of parasite burden on
326 sperm numbers in infected individuals, were analysed using separate LMs, each also
327 including male weight as a predictor variable. Similarly, sperm allocation to infected
328 vs. uninfected females was assessed by analysing remaining sperm number
329 following mating, with female infection status and female weight as predictor
330 variables; male weight and infection status were also controlled for by including them
331 as additive variables. All sperm count data were first normalised by square root
332 transformation. Numbers of viable embryos produced by uninfected and infected
333 females were also analysed using an LM, following square root transformation;
334 female weight and male sperm number were also included as predictor variables.

335

3. Results

3.1 Parasite Identification

18S, 28S and 5.8S/ITS2 sequences obtained from the trematode studied here are deposited respectively under the accession numbers HE983824, HE983825 and HE983826. BLAST analyses on these sequences shown closest similarity with trematodes belonging to the Podocotylinae subfamily (Martin et al. 2018a (Trematoda: Digena: Opecoelidae) (Supplementary data 4). Phylogenetics further confirms that the trematode used in this study belongs to this subfamily, strongly supported by a high (100%) bootstrap value (Supplementary data 5).

345

3.2 Parasite Prevalence and Burden

The *G. zaddachi* field sample collected in May 2009 consisted of 110 pairs, 112 single females and 61 single males. Of these, 64% of males and 39% of females were infected, with 50% individuals infected overall. For infection status, a significant interaction between weight and sex was found (GLM, with binomial error distribution: $LRT_1= 3.905$, *P*=0.048). Infection was more likely in larger individuals (Fig. 3a), an effect which was stronger in males than females. Within infected individuals, parasite burden (number of cysts) increased with weight (GLM, with Poisson error distribution: $LRT_1=39.401$, *P*<0.001); but did not differ between males and females ($LRT_1=1.934$, *P*=0.164; in infected males burden range = 9 (1-10 cysts), mean ± 1 S.E. = 2.43 ± 0.18; in infected females burden range = 5 (1-6 cysts), mean ± 1 S.E. = 1.59 ± 0.11; Fig. 3b).

358

3.3 Effect of Parasitism on Survival

The survival curves suggest that infection affects mortality in males, with a greater and steeper decline in infected individuals (Fig. 4). However, the field data revealed that larger (and hence older) males were more likely to be infected and had a higher average parasite burden. Controlling for weight, we found no significant effect of infection status on survival (PSM: $\chi^2_1=2.33$, *P*=0.127) and no significant effect of the interaction between infection status and weight (PSM: $\chi^2_1=0.03$, *P*=0.863); however, for the subset of males (*n*=35) for which parasite burden was recorded, survival was

367   significantly affected by the interaction between trematode number and weight (PSM:

368   χ^2_1=4.40, P=0.036), such that mortality is greatest when individuals have a high

369   trematode burden for their size. In females, which are smaller than males and may

370   generally hold a lower maximum parasite burden, we found no significant effect on of

371   either infection status (PSM: χ^2_1=1.52, P=0.217) or parasite burden (PSM: χ^2_1=2.32,

372   P=0.128) on survival, when controlling for individual weight.

373

374   *3.4 Effect of Parasitism on Reproductive Behaviour and Fecundity*

375   *3.4.1 Pairing success*

376   In the field, pairing status was influenced by weight and by sex, with a significant

377   interaction between the two (GLM with binomial error distribution: LRT$_1$=5.942,

378   P=0.015), such that likelihood of pairing increased with weight in males, but not in

379   females. In the field, 69% of infected males were paired, in comparison to 55% of

380   uninfected males, a discrepancy which was not found in females (44% infected

381   females and 49% uninfected females were paired). However, infected males are

382   generally larger and controlling for weight, infection itself was not found to

383   significantly affect pairing status (LRT$_1$=0.857, P=0.355). Additionally, for infected

384   individuals, parasite burden was not found to affect pairing status (GLM with binomial

385   error distribution: LRT$_1$=0.521, P=0.470).

386   The mean male: female weight ratio of animals in pairs (± 1 S.E.) was 2.58 ± 0.08,

387   N=110, which is similar to that reported by Adams and Greenwood (1987). This

388   weight ratio was not affected by either male infection status (LMs of log male: female

389   ratio: $F_{1,96}$=0.050, P=0.824) or female infection status ($F_{1,96}$=0.983, P=0.324).

390   In the laboratory trials, there was a trend for infected males to be less likely to pair

391   than uninfected males (Fisher's exact test: P=0.069, Fig. 5a). However, no significant

392   difference in mate choice was found between infected and uninfected males

393   (Contingency table Chi-squared test: χ^2_2=3.97, P=0.138); indicating that neither

394   infected (Chi-squared test: χ^2_1=0.22, P=0.637) nor uninfected (Chi-squared test:

395   χ^2_1=0.5, P=0.480) males discriminated between infected and uninfected females.

396   Infected males took significantly longer to pair than uninfected males (mean time to

397   pair ± 1 S.E. for uninfected males = 184s +/- 35s, N=25; for infected males = 275s +/-

398   50s, N=25; PSM: χ^2_1=6.36, P=0.012, Fig. 5b). However, we found no evidence of

399   discrimination against infected males or females, in terms of number of resistance-
400   attempts by females or number of assessments by males, respectively. Number of
401   female resistance attempts did not differ for infected and uninfected males (GLMs,
402   with quasi-Poisson error structure: $F_{1,98}=0.416$, $P=0.510$). Similarly, number of
403   assessments by males did not differ for infected and uninfected females $F_{1,98}=0.114$,
404    $P=0.738$). Additionally, number of assessments did not differ between infected and
405   uninfected males ($F_{1,98}=0.000$, $P=0.986$), and number of resistance attempts did not
406   differ between infected and uninfected females ($F_{1,98}=0.722$, $P=0.400$).

407   Parasitism affected the general activity of female, but not of male *G. zaddachi*. We
408   found infected female *G. zaddachi* to be 23% less active than uninfected ones (LMs
409   of sqrt line crosses: $F_{1,35}=6.225$, $P=0.017$; mean line crosses ± 1 S.E. for infected
410   females = 88.47 ± 7.14; for uninfected females = 114.0 ± 7.62). This difference was
411   not observed in males ($F_{1,38}=0.171$, $P=0.682$; mean line crosses ± 1 S.E. for males =
412   85.5 ± 4.31). General activity was not significantly affected by individual weight
413   (females: $F_{1,34}=1.020$, $P=0.320$; males: $F_{1,38}=0.849$, $P=0.363$).

414

415   *3.4.2 Female fecundity*

416   In the field, number of embryos increased with female mass (GLM, with quasi-
417   Poisson error distribution: $F_{1,92}=178.27$, $P<0.001$), but we found no effect of infection
418   on female fecundity ($F_{1,91}=0.630$, $P=0.429$). In the laboratory sperm allocation
419   experiment, the number of viable embryos (showing normal development after one
420   week) brooded by females was positively correlated with female weight only ($F_{1,52}=$
421   12.962, $P<0.001$), but not affected by female infection status ($F_{1,51}=0.047$, $P=0.829$)
422   or sperm allocated ($F_{1,51}=0.047$, $P=0.830$). We found no evidence for male
423   discrimination against infected females via prudent sperm allocation, with no
424   significant effect of female infection status on remaining sperm numbers post-
425   copulation (LM of sqrt sperm number: $F_{1,52}=0.537$, $P=0.467$). Male weight did not
426   have a significant effect on remaining sperm numbers ($F_{1,52}=0.190$, $P=0.665$).

427

428   *3.4.3 Male fecundity*

429   Infection status itself was not found to have a significant effect on total sperm
430   numbers (LM of sqrt sperm number: $F_{1,60}=0.006$, $P=0.939$). Across all males, weight
431   also had no effect on sperm numbers ($F_{1,60}=0.474$, $P=0.494$). However, within
432   infected individuals, there was a significant effect of trematode burden on sperm

433 numbers (LM of sqrt sperm number: $F_{1,29}$=4.994, P=0.033), when male weight was
434 controlled for (retained in model at P=0.052). The predicted graph from the model
435 (Fig. 6) indicates that sperm number increases with male weight, but decreases with
436 parasite burden, such that the individuals with the lowest sperm counts are those
437 with the highest trematode loads for their size.

438

439 **4. Discussion**

440 This study explores the effect of *P. atomon* (Trematoda) on the reproductive
441 behaviour, survival and physiology of its second intermediate host *G. zaddachi*
442 (Amphipoda). We identified the parasite using both morphological and genetic (18S,
443 28S, 5.8S and ITS2) information, providing partial genetic data to aid its identification
444 in further studies. The parasite prevalence within the population was skewed, with
445 larger males more likely to be infected and at risk of a greater parasite burden.
446 Female fecundity seems relatively unaffected by the presence of the parasite(s);
447 however, males with an excessive burden of trematodes reduced sperm production.
448 Our data suggest that *P. atomon* infection on the coastline is a driving force for
449 population control and can be the cause of mortality in larger males of the host
450 species.

451

452 *4.1* Podocotyle atomon *infection affecting host fecundity and physiology*

453 The overall prevalence of 50% observed in this study compares with 8 to 12%
454 reported in *G. zaddachi* by Kesting et al. (1996) and 11% reported in naturally
455 infected *Gammarus* by Hunninen & Cable (1943), although Hunninen & Cable noted
456 that 80 to 89% of *Gammarus* exposed to cercariae in the laboratory became infected.
457 The observed burden range of 1-10 cysts in infected individuals compares with a
458 range of 1-5 reported by Hunninen & Cable (1943) in natural infections, and as many
459 as 134 metacercariae removed from a single individual in experimental infections.
460 Trematode prevalence and burden increased with weight for both males and
461 females, which is likely to reflect increased opportunities for infection with age. Our
462 data suggest that older males, which grow larger and can accommodate more
463 parasites, as well as having had longer to accumulate infections, are at risk of
464 mortality from infection at high burden. However, the female's smaller size may
465 prevent them from acquiring a similar burden to the male. Females with such high

466 burdens may have a reduced lifespan, making them less likely to be collected from
467 the field. There is also a possibility that infection by *P. atomon* reduces growth,
468 suggesting that heavily infected smaller males may be older, and more likely to die,
469 than uninfected males of the same size; however, we think this interpretation is less
470 likely as infected males were, on average, larger than uninfected males. Previous
471 studies have found mixed results of trematode infection on mortality; for example,
472 *Microphallus papillorobustus* leads to increased mortality in its *Gammarus aequaticus*
473 host, although mortality is not affected in the alternative host *G. insensibilis* (Thomas
474 et al. 1995b).

475 By contrast, we found evidence for an impact of infection on general activity in
476 females, but not in males. Infected females were less active than uninfected females.
477 As a decrease in activity is unlikely to enhance parasite transmission, this is unlikely
478 to reflect parasite manipulation, but may be due to the metabolic costs of infection;
479 alternatively, a decrease in activity could cause females to be less able to escape
480 predation by specialised predators that may be definitive hosts. Reduced activity in
481 infected individuals has also been suggested as an explanation for lower respiration
482 rates seen in *G. insensibilis* infected by microphallid trematodes (Gates et al. 2018).
483 Gates et al. (2018) suggest that this reduction in activity may reflect the absence of
484 disturbance cues from potential definitive hosts in laboratory conditions, which could
485 equally apply to this study. Overall, we found that females were generally more active
486 than males, regardless of infection status, which may reflect differing behaviour and
487 foraging strategies; male activity decisions will be mostly driven by the search for
488 receptive females, whereas female activity will revolve around foraging. These
489 behavioural differences could relate to the likelihood of parasite contraction. This may
490 also explain why we observed no reduction in general activity in infected males; the
491 importance of obtaining mates may mean that infected males maintain investment in
492 searching for receptive females, at the cost of mortality.

493 Mate guarding is energetically costly, and guarding males are less able to feed
494 (Robinson and Doyle 1985); hence a tendency for infected males to show reduced
495 pairing success and guard smaller females, reflecting the metabolic burden imposed
496 by the parasite, might be expected. However, we found no effect of parasite infection
497 or burden on pairing success. Similarly, size-assortative pairing was not influenced
498 by infection, beyond the fact that infected individuals tend to be larger. This contrasts
499 with *M. papillorobustus* infection in *G. insensibilis*, where unpaired gammarids have
500 higher parasite burdens than paired individuals (Thomas et al. 1995a; Thomas et al.
501 1996), and infected males pair with smaller females than uninfected males of

502 comparable size (Thomas et al. 1995a). Again, our data suggest that infected males
503 maintain investment in reproductive effort, despite the decreased survival of heavily
504 infected males suggesting a metabolic cost of infection. Hence, individuals appear to
505 trade-off current and future reproductive success in response to infection.

506 We also found little evidence for prudent sperm allocation in response to female
507 infection status. This is expected as we found no effect of parasitism on female
508 embryo numbers; hence males are unlikely to experience strong selection to avoid
509 infected mates due to fecundity loss. Despite this, we did see a reduction in sperm
510 numbers in small males with relatively heavy parasite burdens. Similarly, in cestode
511 (*Cyathocephalus truncatus*) infected *Gammarus pulex,* sperm numbers are lower
512 than in uninfected individuals (Galipaud et al. 2011). The reduced investment in
513 sperm in heavily infected males may be due to direction of host resources away from
514 reproduction by the trematode; for example, tapeworm-infected male red flour
515 beetles show up to a 20% reduction in fertilisation success, possibly resulting from a
516 reduction in sperm numbers (Pai & Yan 2003). Alternatively, infected hosts may
517 increase investment in immune response at the expense of investment in
518 reproduction (Honkavaara et al. 2009; Mills et al. 2010).

519 In conclusion, despite high prevalence and burdens of infection observed in the field,
520 the overall impact of *P. atomon* on the reproductive behaviour and fecundity of its
521 secondary intermediate host (*G. zaddachi*) is lower than that reported for other
522 trematodes (Table 1). Despite the limited impact observed by this study, the high
523 prevalence of the parasite may mean that infections are having some small effect on
524 the continued evolution of *G. zaddachi.* We propose that a greater understanding of
525 this association may be accomplished by conducting continued sampling and
526 measuring of the host populations behavioural and physiological change over time
527 alongside continued laboratory experiments to explore the effects of parasitism
528 through several amphipod generations.

529

533

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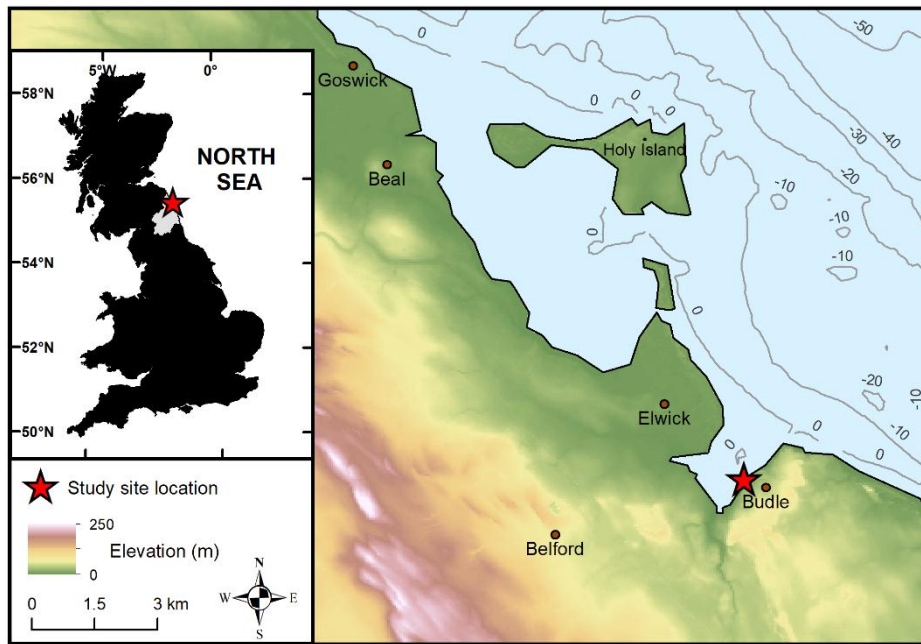
703 **Tables and Figures**

704

705

Figure 1: A map of the Northumberland Coast, north east England. The location of the study site, any adjacent major coastal settlements, hinterland topography and shoreface bathymetry are displayed at larger scale. The topography is derived from a 50m Digital Terrain Model (DTM) and the raster layers are extracted from the EDINA Ordinance Survey Digimap. Bathymetry are extracted from EDINA Marine Digimap. Geographic Information System (GIS) software (ArcGIS) was used to generate this figure. All layers are projected onto a WGS 84 coordinate system.

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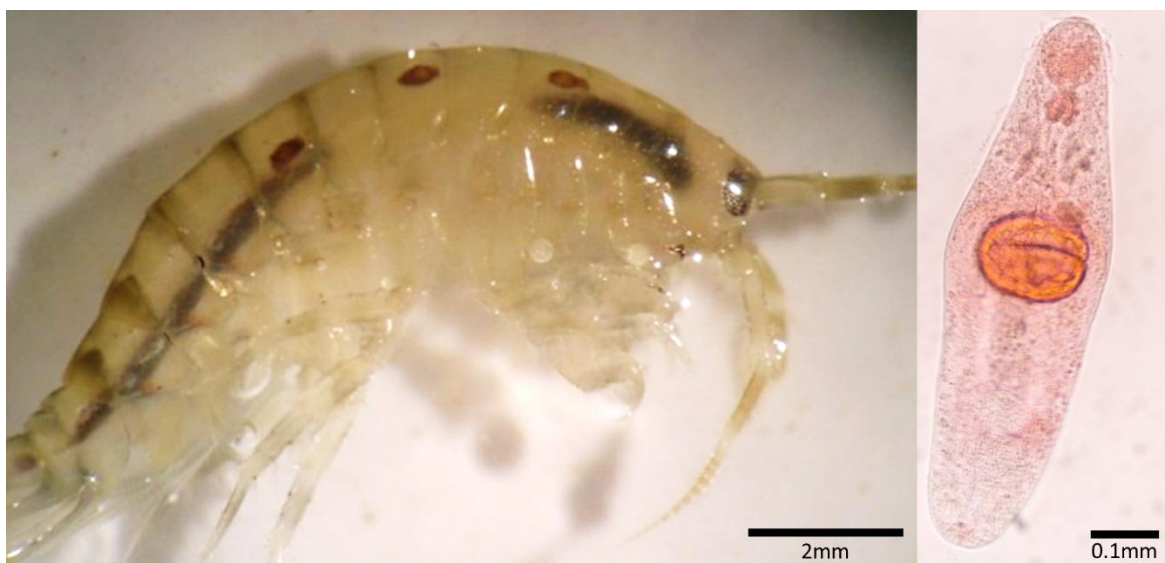
715 Figure 2: *Gammarus zaddachi* infected with three *Podocotyle atomon* and a high
716 magnification of the trematode parasite after dissection from the body cavity.
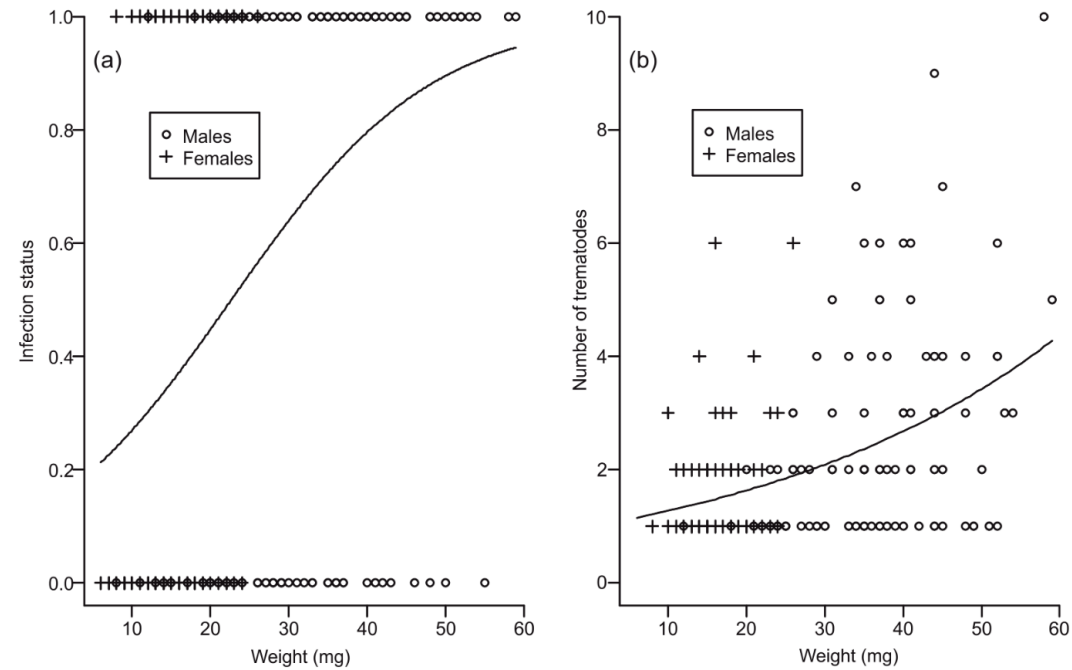
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719 Figure 3: (a) Weight distribution of *G. zaddachi* males and females, with a line
720 indicating the predicted probability of infection; (b) Trematode burden vs. weight for
721 infected males and females, with trend line predicted by the minimum adequate
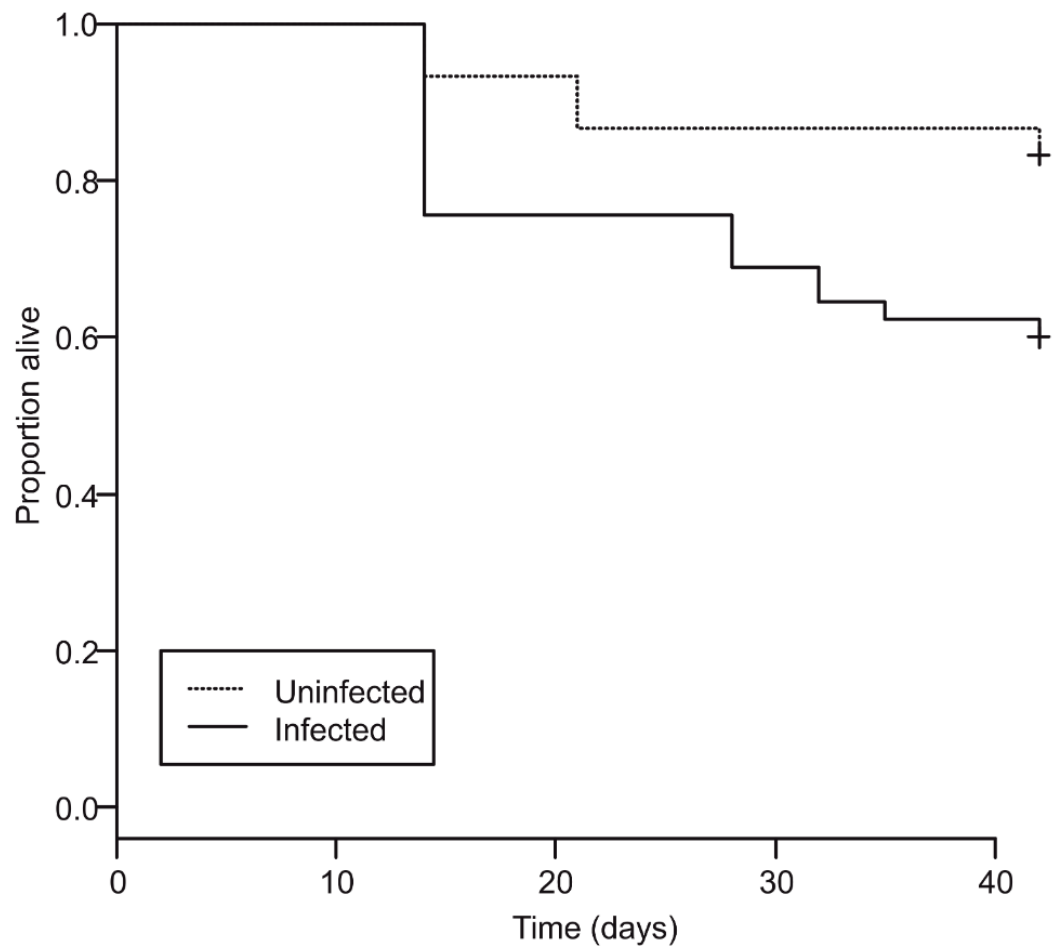722 model.

723

Figure 4: Survival curves for infected and uninfected *G. zaddachi* males over the 6-week period. The dashed line refers to healthy animals and the solid line refers to infected animals.
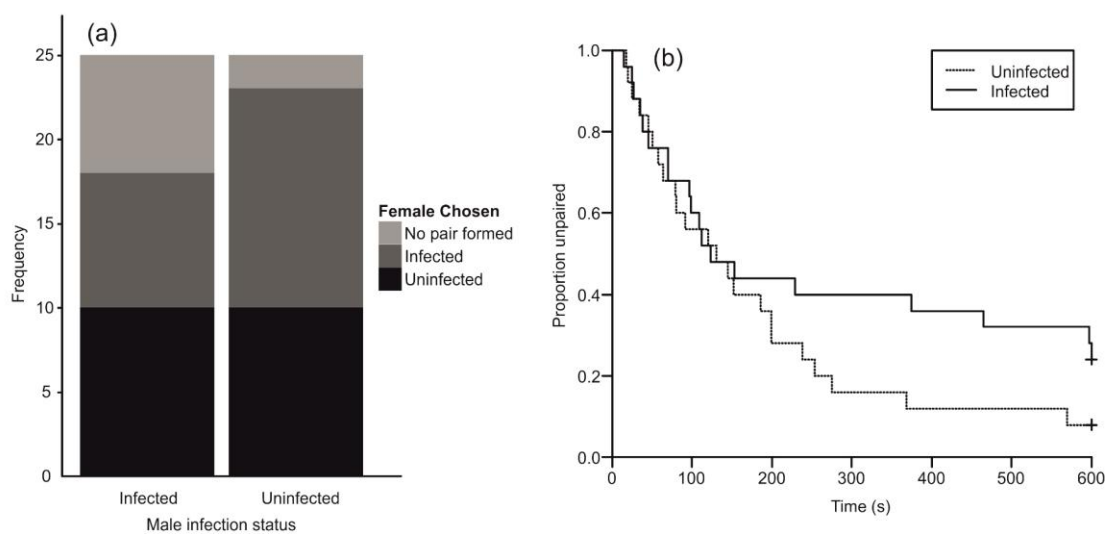
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729 Figure 5: (a) Frequency of *G. zaddachi* females selected by infected and uninfected
730 males in the mate choice trials; (b) Proportion of unpaired uninfected vs. infected *G.*
731 *zaddachi* males over the 10 min trial period. The dashed line refers to healthy
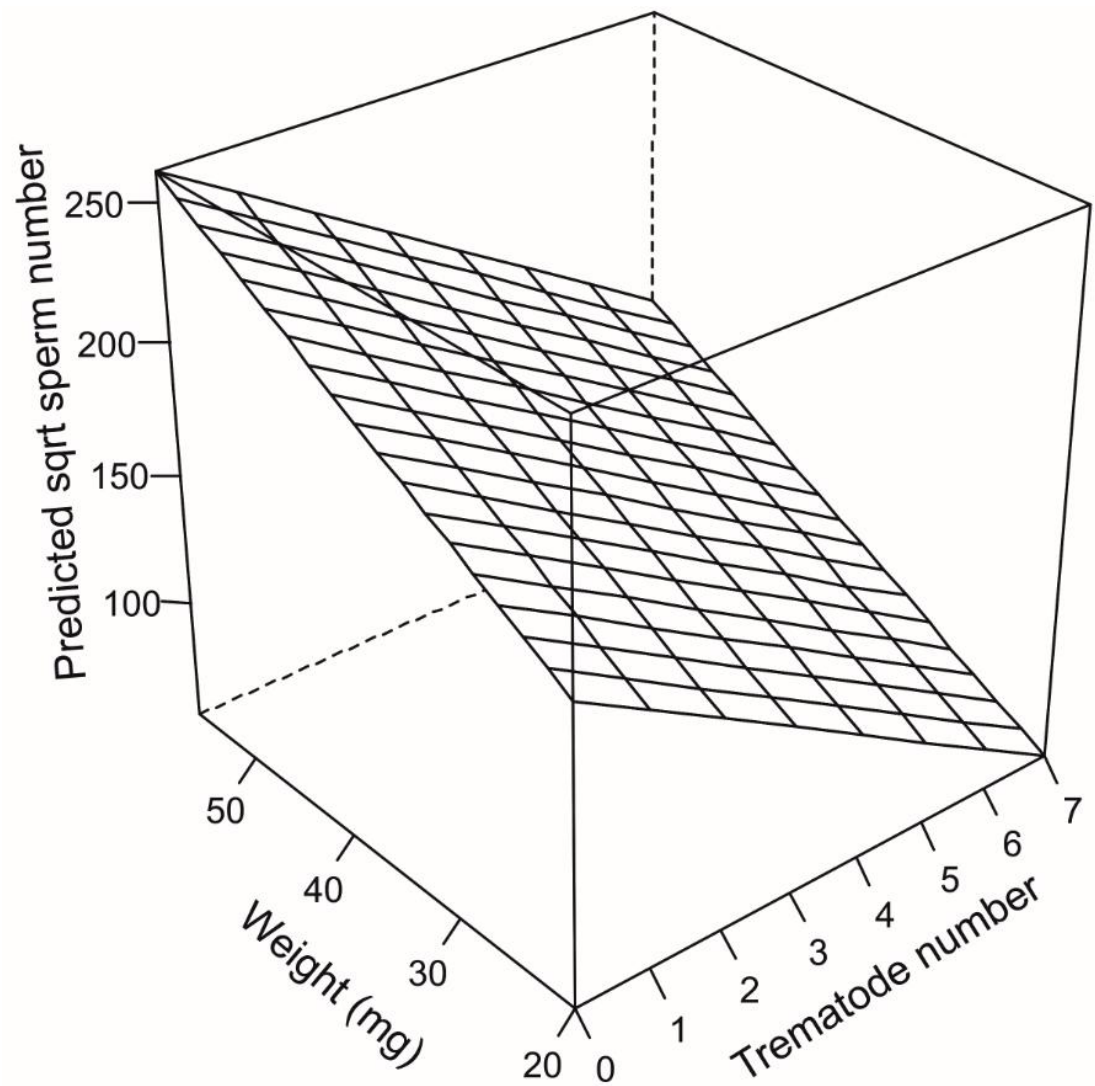732 animals and the solid line refers to infected animals.

733

734

735 Figure 6: Predicted relationship between sperm number, weight and parasite burden,
736 for infected *G. zaddachi* males.