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

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

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Abstract

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

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

sperm numbers lowest, in heavily infected individuals. Trematode parasites are sometimes associated with altered host fecundity, but studies often lack the relevant experimental data to explore the evolution of the trait. We discuss this among information specific to the effect of *P. atomon* infection in *G. zaddachi.*

1. Introduction

The evolution of a host in response to parasitism can take several routes, including (but not restricted to) the evolution of resistance (Dagan et al. 2017), parasite avoidance behaviours (Behringer et al. 2018), and the evolution of sex characteristics and physiological change (Hamilton & Zuk 1982; Howard & Lively 1994). There are a wide variety of organisms that can induce host pathology and subsequently drive the evolution of their host (viruses, bacteria, Microsporidia etc.). Some of the most influential parasites for host evolution and adaption are trophically transmitted metazoans, such as acanthocephalans, cestodes and trematodes (Reisinger & Lodge 2016; Blasco-Costa & Poulin 2017).

The phylogenetically diverse Trematoda are obligate parasites that often exhibit variable transmission methods and are found in freshwater, marine and terrestrial habitats (Bojko et al. 2017; Galaktionov et al. 2018). The trophic transmission methods used by trematodes are possible due to the parasites ability to manipulate the behaviour and physiology of their host. Host manipulation by trematodes has been linked with behavioural, developmental and pigmentation processes that increase the susceptibility of the next host (Poulin 1995; Lefèvre et al. 2009; Cézilly et al. 2010; Thomas et al. 2010).

Infections by trematodes often do not lead to rapid transmission, meaning that infections can be present in populations for prolonged periods of time along with any host manipulation effects (Etges & Gresso 1965). In the process of infection and transmission, trematodes can influence several host traits that appear to be largely unrelated (side-effects) to trematode transmission success. Such “side-effects” include an altered fecundity in some cases, which can have consequences for the host population size and associated niche (Kelly et al. 2001; Pai & Yan 2003). In some circumstances and intermediate hosts, trematodes cause complete castration, potentially due to resource siphoning from the host gonad (Minchella & Loverde 1981). The trematode *Cercaria batillariae* castrates its snail host *Batillaria cumingi* and induces gigantism, diverting resources away from reproduction to the production

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

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

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

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

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

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

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

2. Materials and Methods

2.1 Animal Collection and Husbandry

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

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_2O 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.

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).

2.4 Effect of Parasitisrn 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.

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

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

2.6.1 Prevalence and burden

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

2.6.2 Survival

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

2.6.3 Pairing success

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

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

2.6.4 Fecundity analysis

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

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

335

336 **3. Results**

337 *3.1 Parasite Identification*

338 18S, 28S and 5.8S/ITS2 sequences obtained from the trematode studied here are
339 deposited respectively under the accession numbers HE983824, HE983825 and
340 HE983826. BLAST analyses on these sequences shown closest similarity with
341 trematodes belonging to the Podocotylinae subfamily (Martin et al. 2018a
342 (Trematoda: Digena: Opecoelidae) (Supplementary data 4). Phylogenetics further
343 confirms that the trematode used in this study belongs to this subfamily, strongly
344 supported by a high (100%) bootstrap value (Supplementary data 5).

345

346 *3.2 Parasite Prevalence and Burden*

347 The *G. zaddachi* field sample collected in May 2009 consisted of 110 pairs, 112
348 single females and 61 single males. Of these, 64% of males and 39% of females
349 were infected, with 50% individuals infected overall. For infection status, a significant
350 interaction between weight and sex was found (GLM, with binomial error distribution:
351 LRT_1= 3.905, *P*=0.048). Infection was more likely in larger individuals (Fig. 3a), an
352 effect which was stronger in males than females. Within infected individuals, parasite
353 burden (number of cysts) increased with weight (GLM, with Poisson error distribution:
354 LRT_1=39.401, *P*<0.001); but did not differ between males and females (LRT_1=1.934,
355 *P*=0.164; in infected males burden range = 9 (1-10 cysts), mean ± 1 S.E. = 2.43 ±
356 0.18; in infected females burden range = 5 (1-6 cysts), mean ± 1 S.E. = 1.59 ± 0.11;
357 Fig. 3b).

358

359 *3.3 Effect of Parasitism on Survival*

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

significantly affected by the interaction between trematode number and weight (PSM: χ^2_1=4.40, *P*=0.036), such that mortality is greatest when individuals have a high trematode burden for their size. In females, which are smaller than males and may generally hold a lower maximum parasite burden, we found no significant effect on of either infection status (PSM: χ^2_1=1.52, *P*=0.217) or parasite burden (PSM: χ^2_1=2.32, *P*=0.128) on survival, when controlling for individual weight.

3.4 Effect of Parasitism on Reproductive Behaviour and Fecundity

3.4.1 Pairing success

In the field, pairing status was influenced by weight and by sex, with a significant interaction between the two (GLM with binomial error distribution: LRT_1=5.942, *P*=0.015), such that likelihood of pairing increased with weight in males, but not in females. In the field, 69% of infected males were paired, in comparison to 55% of uninfected males, a discrepancy which was not found in females (44% infected females and 49% uninfected females were paired). However, infected males are generally larger and controlling for weight, infection itself was not found to significantly affect pairing status (LRT_1=0.857, *P*=0.355). Additionally, for infected individuals, parasite burden was not found to affect pairing status (GLM with binomial error distribution: LRT_1=0.521, *P*=0.470).

The mean male: female weight ratio of animals in pairs (± 1 S.E.) was 2.58 $\pm$ 0.08, *N*=110, which is similar to that reported by Adams and Greenwood (1987). This weight ratio was not affected by either male infection status (LMs of log male: female ratio: $F_{1,96}$=0.050, *P*=0.824) or female infection status ($F_{1,96}$=0.983, *P*=0.324).

In the laboratory trials, there was a trend for infected males to be less likely to pair than uninfected males (Fisher's exact test: *P*=0.069, Fig. 5a). However, no significant difference in mate choice was found between infected and uninfected males (Contingency table Chi-squared test: χ^2_2=3.97, *P*=0.138); indicating that neither infected (Chi-squared test: χ^2_1=0.22, *P*=0.637) nor uninfected (Chi-squared test: χ^2_1=0.5, *P*=0.480) males discriminated between infected and uninfected females.

Infected males took significantly longer to pair than uninfected males (mean time to pair ± 1 S.E. for uninfected males = 184s +/- 35s, *N*=25; for infected males = 275s +/- 50s, *N*=25; PSM: χ^2_1=6.36, *P*=0.012, Fig. 5b). However, we found no evidence of

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

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

3.4.2 Female fecundity

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

3.4.3 Male fecundity

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

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

4. Discussion

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

4.1 Podocotyle atomon *infection affecting host fecundity and physiology*

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

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

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

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

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

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

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

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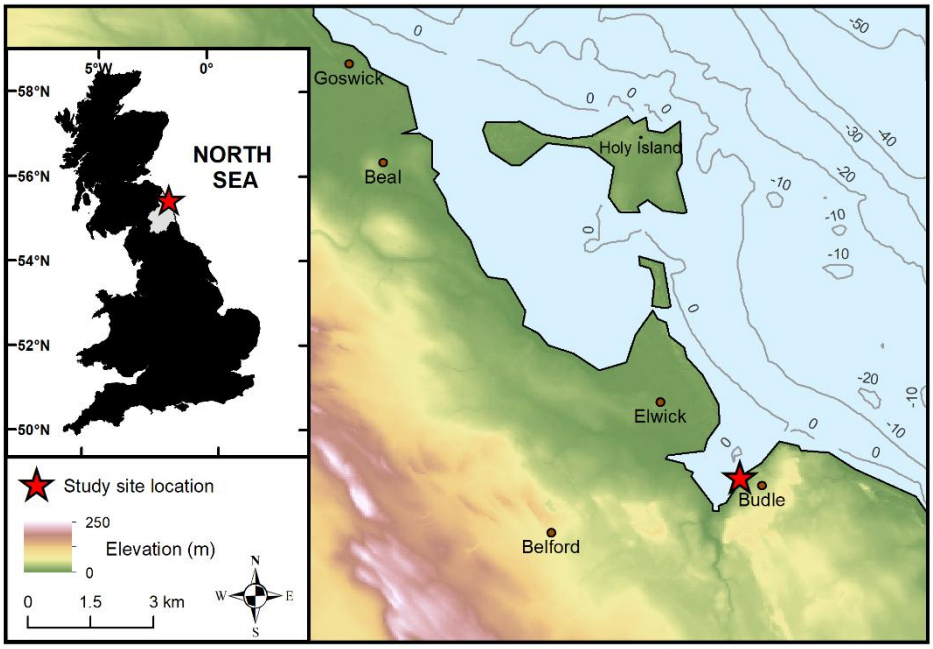
702

703 **Tables and Figures**

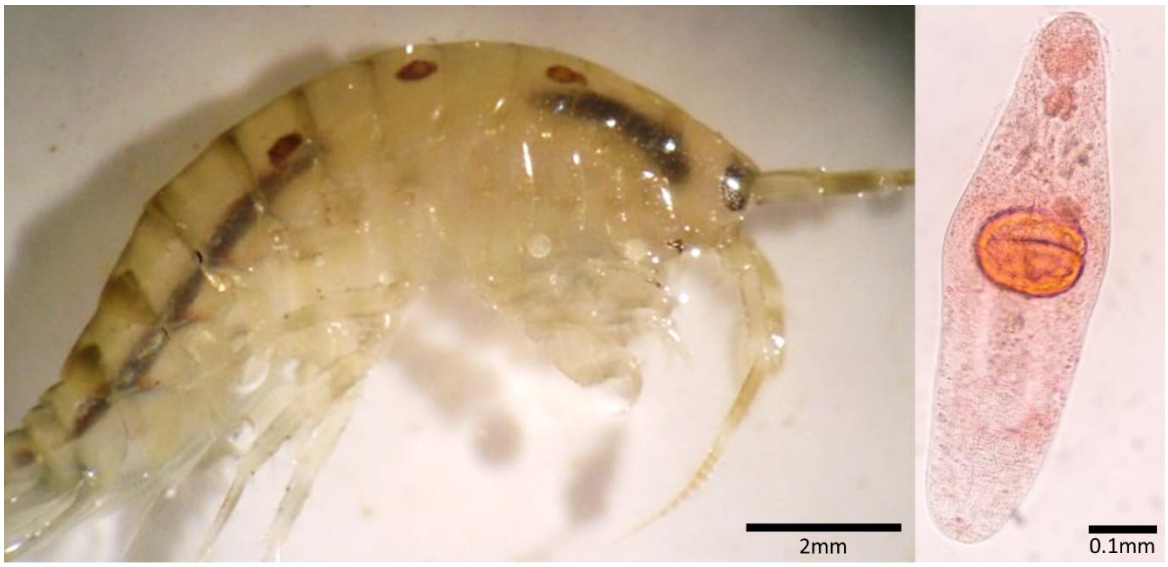
704



705

706 Figure 1: A map of the Northumberland Coast, north east England. The location of
707 the study site, any adjacent major coastal settlements, hinterland topography and
708 shoreface bathymetry are displayed at larger scale. The topography is derived from a
709 50m Digital Terrain Model (DTM) and the raster layers are extracted from the EDINA
710 Ordinance Survey Digimap. Bathymetry are extracted from EDINA Marine Digimap.
711 Geographic Information System (GIS) software (ArcGIS) was used to generate this
712 figure. All layers are projected onto a WGS 84 coordinate system.

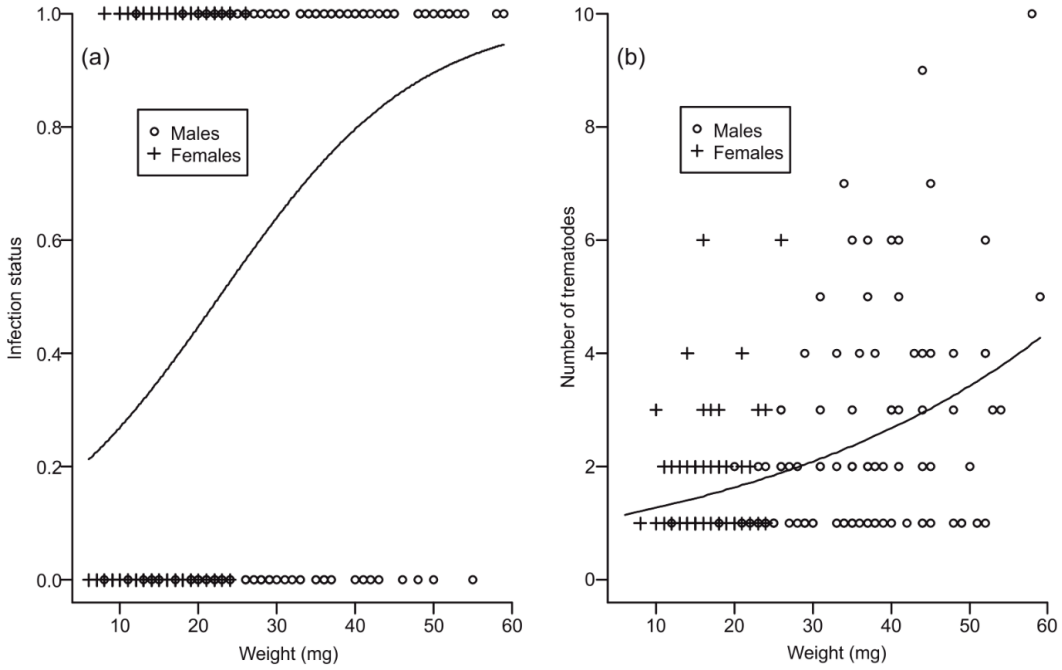
713



714

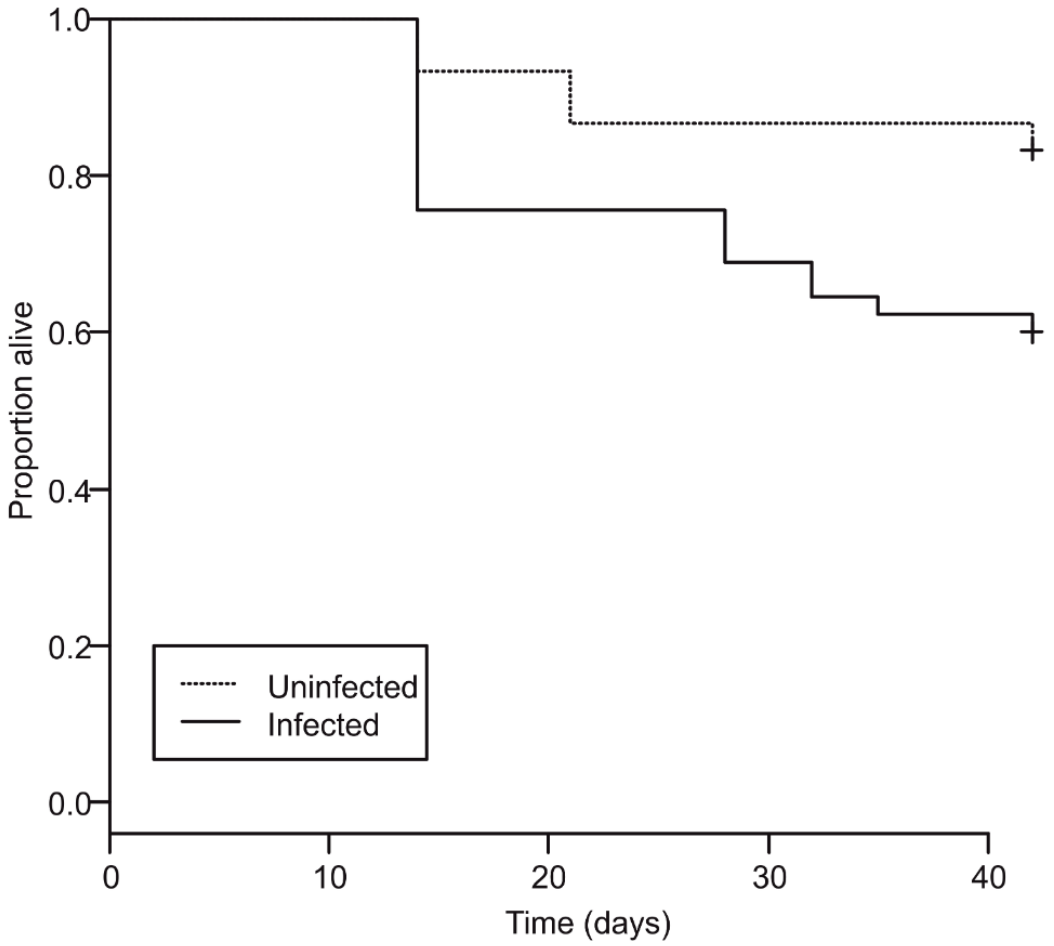
715 Figure 2: *Gammarus zaddachi* infected with three *Podocotyle atomon* and a high
716 magnification of the trematode parasite after dissection from the body cavity.

717



718

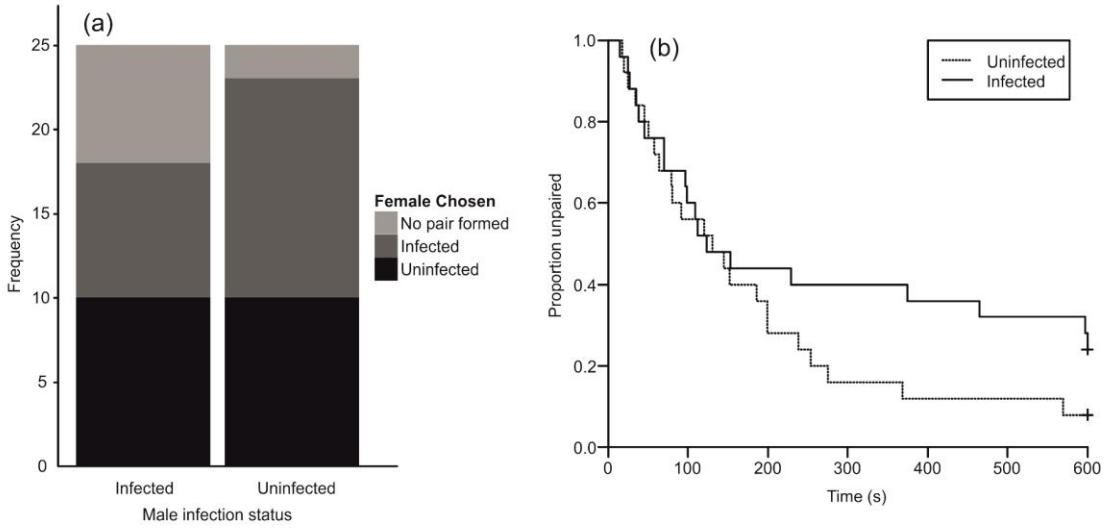
719 Figure 3: (a) Weight distribution of *G. zaddachi* males and females, with a line
720 indicating the predicted probability of infection; (b) Trematode burden vs. weight for
721 infected males and females, with trend line predicted by the minimum adequate
722 model.



723

724 Figure 4: Survival curves for infected and uninfected *G. zaddachi* males over the 6-
725 week period. The dashed line refers to healthy animals and the solid line refers to
726 infected animals.

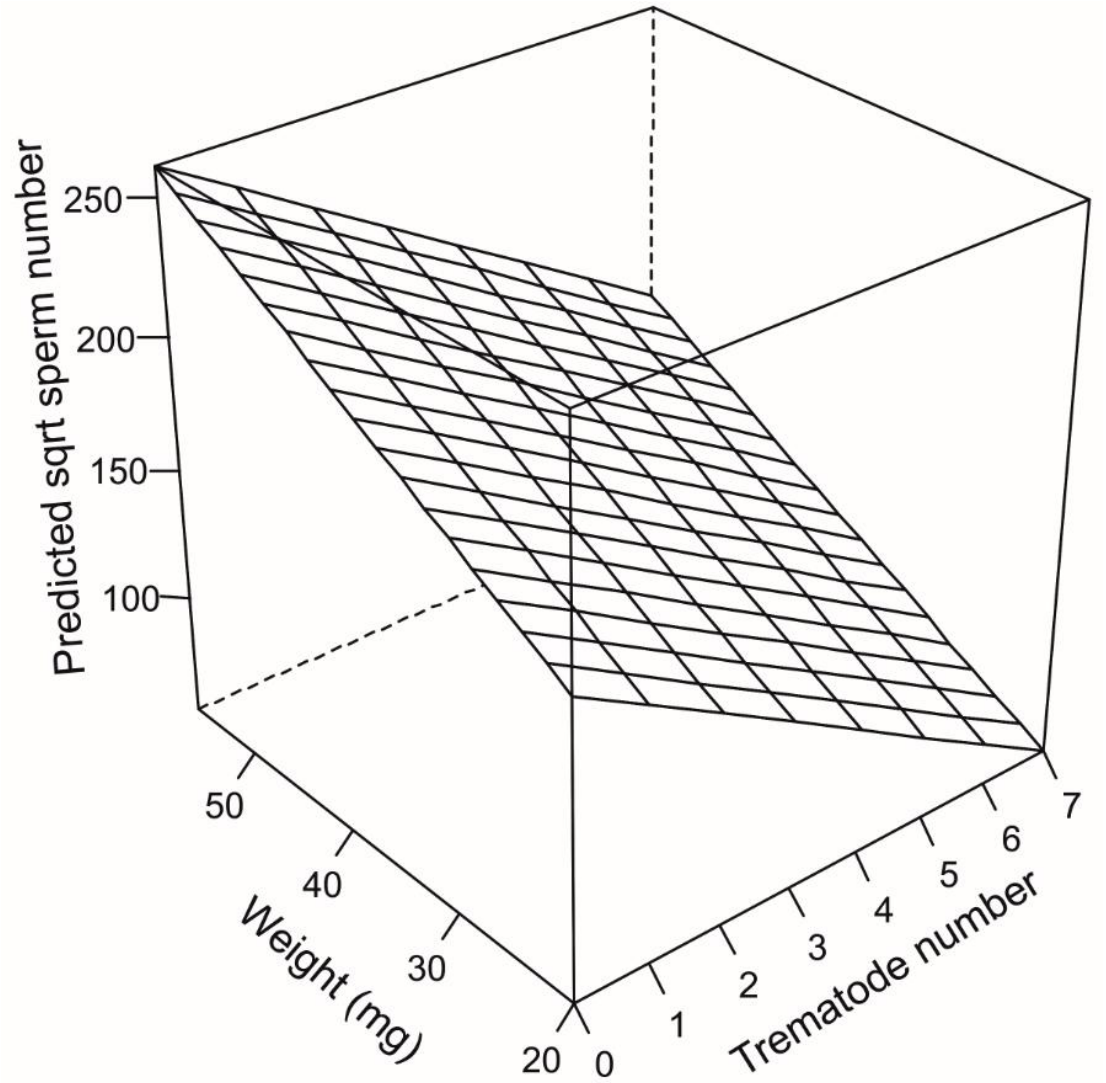
727



728

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.