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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 (Lagroe 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.

2.2 Parasite Detection and Identification

Cysts were visible through the cuticle of infected *G. zaddachi*, and dissection revealed the presence of a single trematode in each cyst (30 cysts dissected) (Fig. 2). Identification of the trematode encysted in *G. zaddachi* was based on genetic data (DNA barcoding) and on morphological/geographical criteria. For molecular identification, trematodes were dissected from four different *G. zaddachi* individuals and preserved in 100% ethanol and held at -20°C, until DNA extraction. DNA was extracted (using appropriate controls) as described by Sambrook et al. (1989) and Ironside et al. (2003). Polymerase chain reaction (PCR) amplifications of partial ribosomal genes (18S, 28S, 5.8S and ITS2) were then carried out with various primers and Tc conditions using mgH₂O as a negative control reaction (Supplementary data 1).

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

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

168

169 2.3 Parasite Prevalence and Burden

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

179

180 2.4 Effect of Parasitism on Survival

181 To investigate the impact of parasitism on survival, *G. zaddachi* individuals [75 males
182 (45 infected, 30 uninfected) and 120 females (37 infected, 83 uninfected)] were
183 isolated for six weeks in the laboratory. These were isolated in individual pots, but
184 otherwise maintained under the same conditions as the stock populations. Following
185 inspection to determine their sex, weight, infection status and parasite burden,
186 individuals were supplied with food and checked at weekly intervals for mortalities.

187

188 2.5 Effect of Parasitism on Reproductive Behaviour and Fecundity

189 2.5.1 Pairing success

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

194 In the laboratory, we tested the hypothesis that males should discriminate against
195 infected females in pairing decisions. Two size-matched females (1 infected and 1
196 uninfected) were placed in a 50ml pot (5cm diameter) together and left to settle for 5
197 minutes. A single uninfected male ($n=25$) was added and all contact and pairing
198 attempts were recorded for 10 minutes, along with time of pairing and choice of
199 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

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

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

2.5.3 Sperm allocation

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

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

2.6 Data Analysis

Data were analysed using statistical models constructed in R version 3.1.1 (R 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 Podocotylineae 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 \pm 1 S.E. = $2.43 \pm$
356 0.18 ; in infected females burden range = 5 (1-6 cysts), mean \pm 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: $\chi^2_1 = 2.33$, $P = 0.127$) and no significant effect of the
365 interaction between infection status and weight (PSM: $\chi^2_1 = 0.03$, $P = 0.863$); 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: $\chi^2_1=4.40$, $P=0.036$), 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: $\chi^2_1=1.52$, $P=0.217$) or parasite burden (PSM: $\chi^2_1=2.32$, $P=0.128$) 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 ± 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: $\chi^2_2=3.97$, $P=0.138$); indicating that neither infected (Chi-squared test: $\chi^2_1=0.22$, $P=0.637$) nor uninfected (Chi-squared test: $\chi^2_1=0.5$, $P=0.480$) 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 \pm 35s, $N=25$; for infected males = 275s \pm 50s, $N=25$; PSM: $\chi^2_1=6.36$, $P=0.012$, 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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703 **Tables and Figures**

704

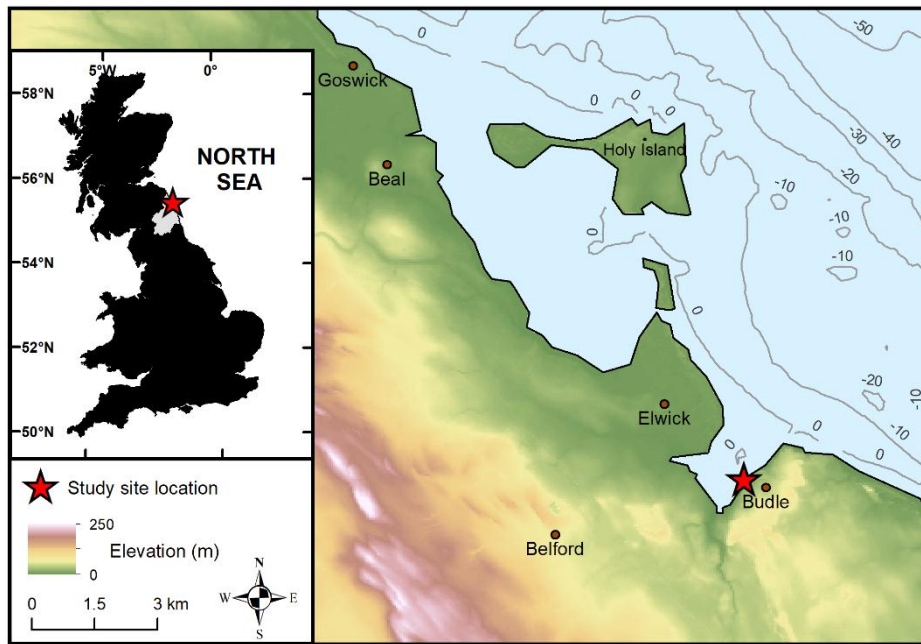


Figure 1: A map of the Northumberland Coast, north east England. The location of the study site, any adjacent major coastal settlements, hinterland topography and shoreface bathymetry are displayed at larger scale. The topography is derived from a 50m Digital Terrain Model (DTM) and the raster layers are extracted from the EDINA Ordnance 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.

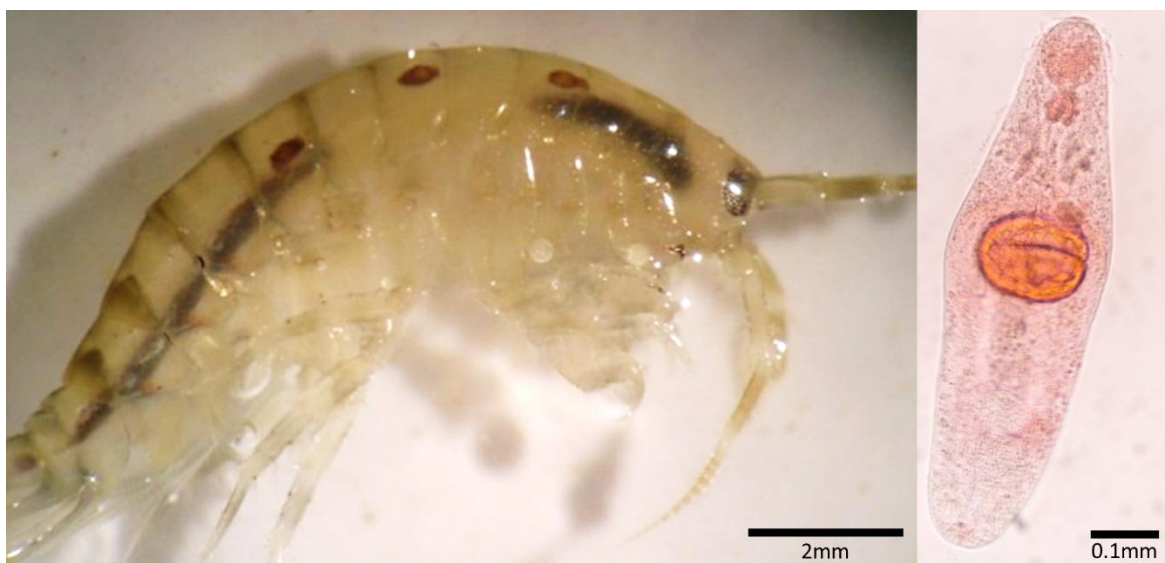


Figure 2: *Gammarus zaddachi* infected with three *Podocotyle atomon* and a high magnification of the trematode parasite after dissection from the body cavity.

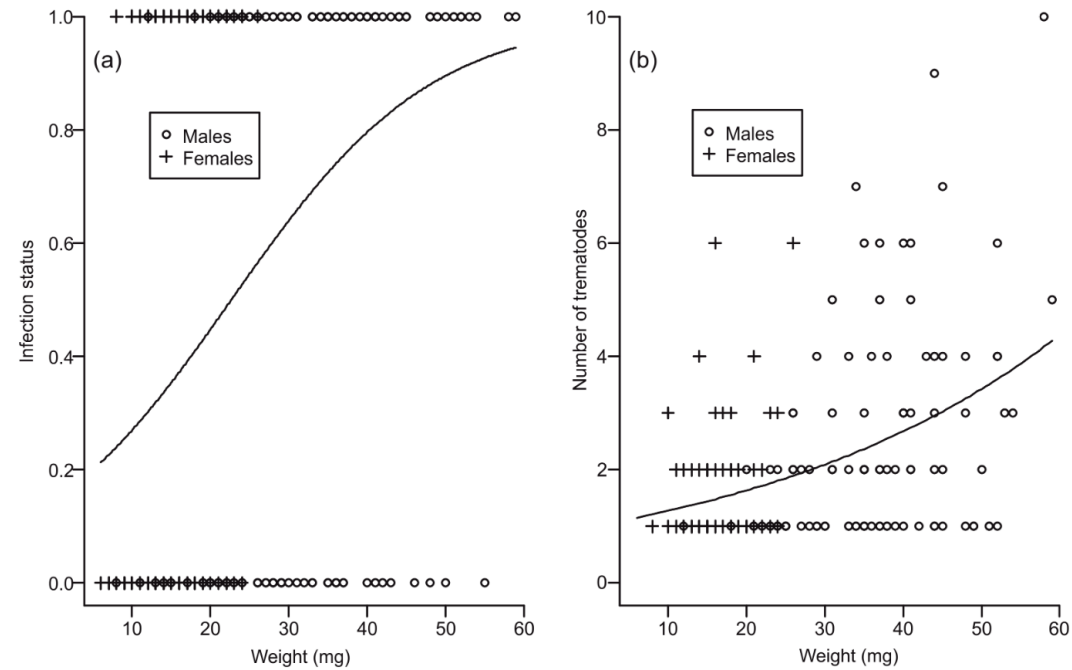
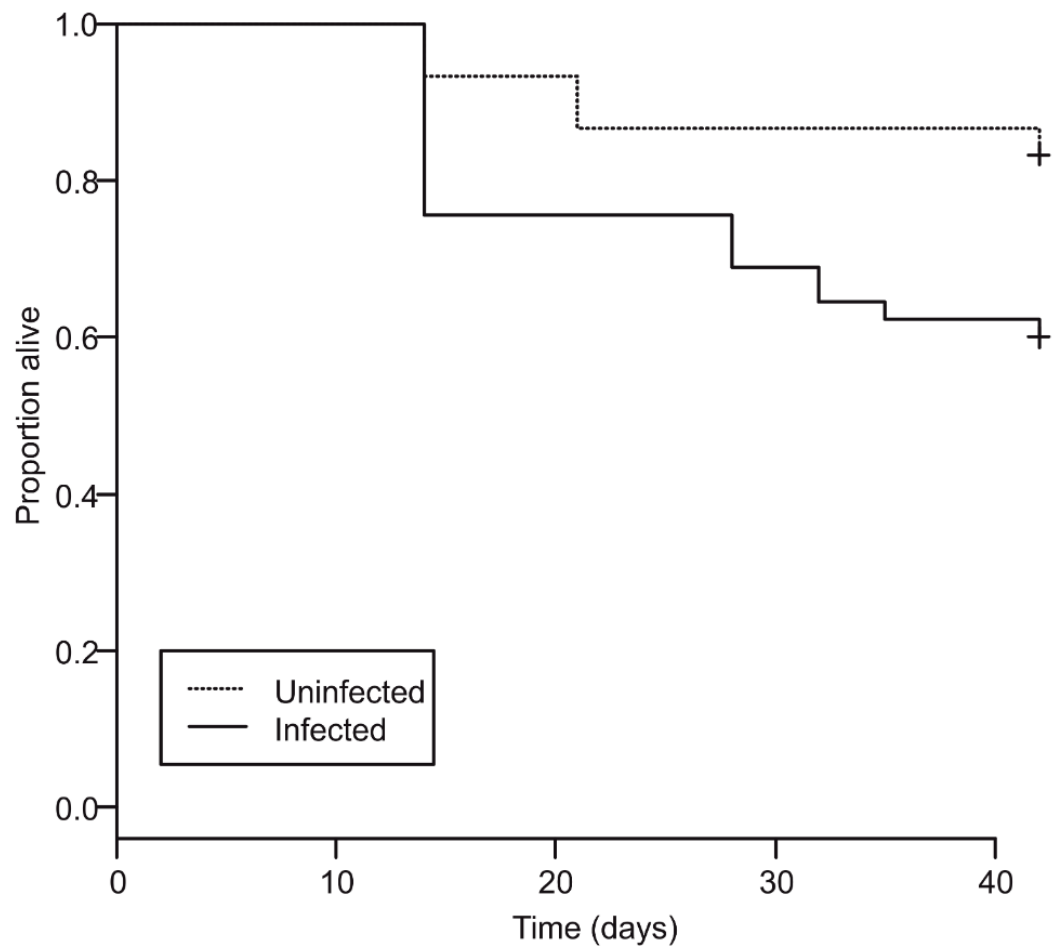


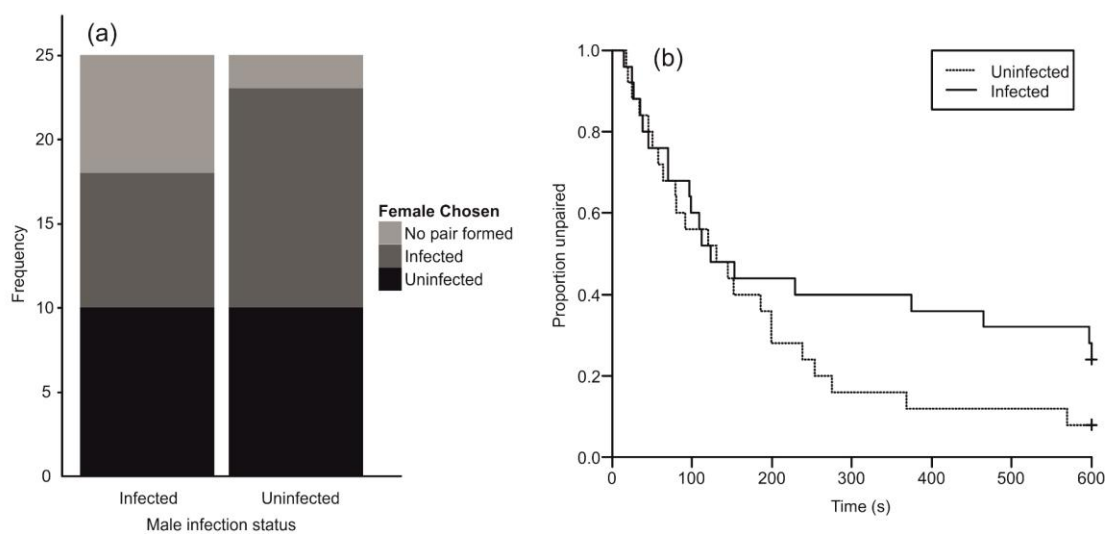
Figure 3: (a) Weight distribution of *G. zaddachi* males and females, with a line indicating the predicted probability of infection; (b) Trematode burden vs. weight for infected males and females, with trend line predicted by the minimum adequate model.



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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.

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Figure 5: (a) Frequency of *G. zaddachi* females selected by infected and uninfected males in the mate choice trials; (b) Proportion of unpaired uninfected vs. infected *G. zaddachi* males over the 10 min trial period. The dashed line refers to healthy animals and the solid line refers to infected animals.

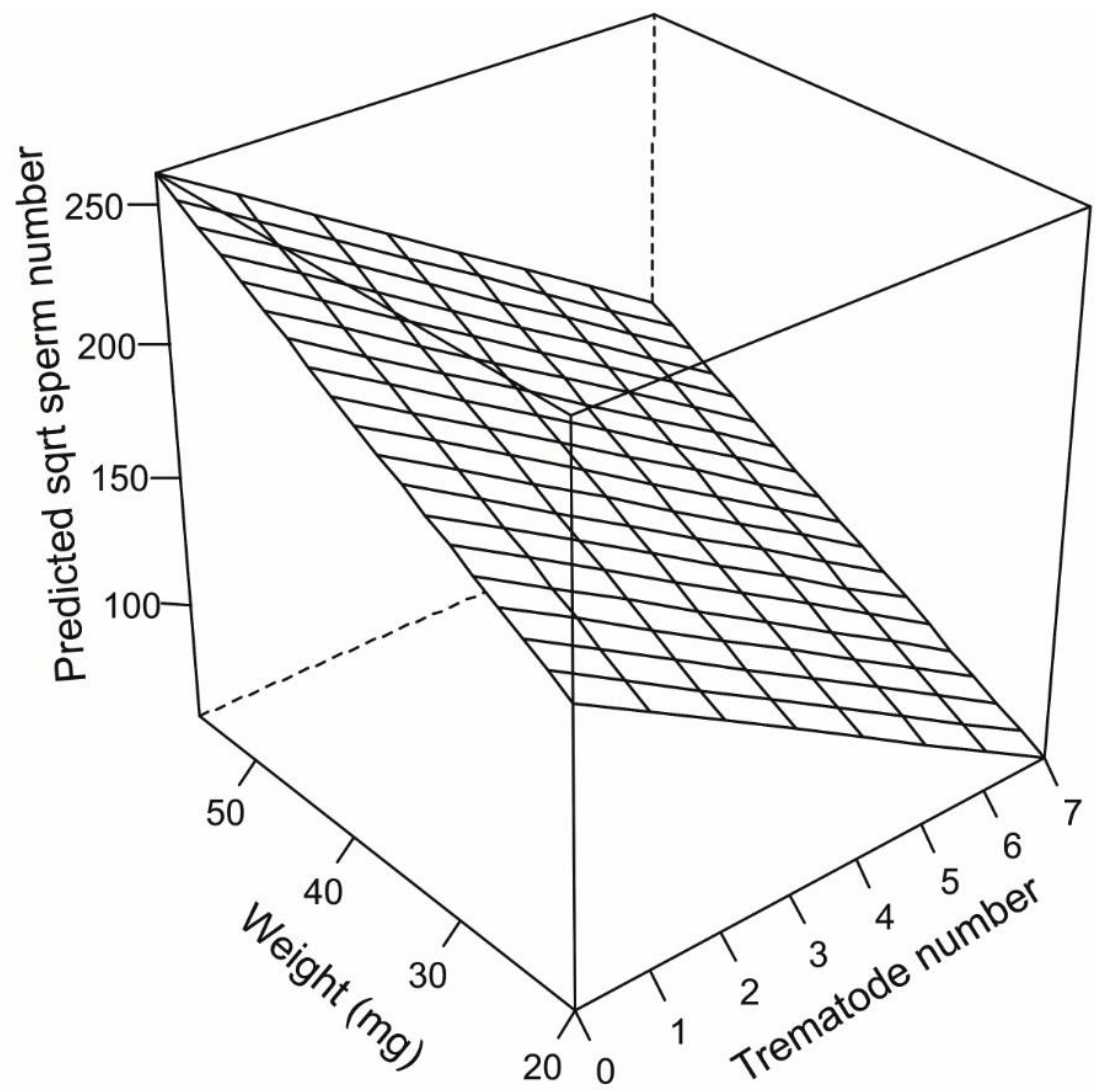


Figure 6: Predicted relationship between sperm number, weight and parasite burden, for infected *G. zaddachi* males.