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1 **Crayfish plague affects juvenile survival and adult behaviour of invasive signal crayfish**

2

3 Running title: Crayfish plague negatively affects signal crayfish

4

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13 Abstract

14 The spread of invasive, non-native species is a key threat to biodiversity. Parasites can play a
15 significant role by influencing their invasive host's survival or behaviour, which can
16 subsequently alter invasion dynamics. The North American signal crayfish (*Pacifastacus*
17 *leniusculus*) is a known carrier of *Aphanomyces astaci*, an oomycete pathogen that is the
18 causative agent of crayfish plague and fatal to European crayfish species, whereas North
19 American species are considered to be largely resistant. There is some evidence, however, that
20 North American species, can also succumb to crayfish plague, though how *A. astaci* affects
21 such 'reservoir hosts' is rarely considered. Here, we tested the impact of *A. astaci* infection on
22 signal crayfish, by assessing juvenile survival and adult behaviour following exposure to *A.*
23 *astaci* zoospores. Juvenile signal crayfish suffered high mortality 4-weeks post-hatching, but
24 not as older juveniles. Furthermore, adult signal crayfish with high infection levels displayed
25 altered behaviours, being less likely to leave the water, explore terrestrial areas and exhibit
26 escape responses. Overall, we reveal that *A. astaci* infection affects signal crayfish to a much
27 greater extent than previously considered, which may not only have direct consequences for
28 invasions, but could substantially affect commercially harvested signal crayfish stocks
29 worldwide.

30

31 Keywords: crayfish plague; *Aphanomyces astaci*; behaviour; invasive species; signal crayfish;
32 reservoir hosts

33

34

35 Key findings

- 36 • Juvenile signal crayfish suffered mortality after exposure to *A. astaci*
- 37 • Adult signal crayfish exhibited altered behavioural responses following exposure
- 38 • Susceptibility to *A. astaci* could affect signal crayfish population management

39 1. Introduction

40 Parasites have a significant impact on communities and ecosystems by directly affecting host
41 fitness, with subsequent impacts on population dynamics and overall biodiversity (Hudson et
42 al., 2006; Tompkins et al., 2011; Cable et al., 2017). Despite this, parasites are a fundamental
43 component of healthy ecosystems with wide reaching impacts, from influencing the cycle of
44 biogeochemical nutrients to regulating host density and functional traits (Hatcher et al., 2014;
45 Preston et al., 2016). Parasites can also influence their host's behaviour, which can in turn alter
46 the outcome of competitive interactions, reproductive behaviour and dispersal ability (Bakker
47 et al., 1997; Macnab and Barber, 2012; Barber et al., 2017). During invasions by non-native
48 species to new areas, parasites can play a key role facilitating or hindering the successful spread
49 of invaders, while potentially having catastrophic effects on other related native species
50 (Vilcinskas, 2015).

51 Crayfish are freshwater crustaceans that are commercially harvested in many countries,
52 but can also reach high densities and exert a significant impact on ecosystems, with several
53 species having become widespread, damaging invaders (Holdich et al., 2014; James et al.,
54 2014; Ercoli et al. 2015). For example, in Great Britain, the North American signal crayfish
55 (*Pacifastacus leniusculus*) has become the most common crayfish species, having largely
56 replaced the native white clawed crayfish (*Austropotamobius pallipes*, see Holdich et al., 2014;
57 James et al., 2014). Crayfish are hosts to many parasites and symbionts, including viruses,
58 bacteria, fungi and helminths that can cause chronic, long-term infections (Longshaw et al.,
59 2012; Kozubíková-Balcarová et al., 2013). One such parasite, the oomycete *Aphanomyces*
60 *astaci*, the causative agent of crayfish plague, is a key threat to crayfish biodiversity worldwide
61 (Svoboda et al., 2017), having eradicated many populations of native European crayfish
62 (Filipová et al., 2013; Kozubíková-Balcarová et al., 2014) and recent evidence suggests it may
63 have also caused a decline in commercially harvested North American crayfish stocks (Edsman

64 et al., 2015; Jussila et al., 2015). This obligate parasitic oomycete penetrates host tissues
65 (Söderhäll et al., 1978) and produces motile reproductive zoospores (Cerenius and Söderhäll,
66 1984), which can reach high densities (up to several hundred zoospores per litre) during a
67 crayfish plague outbreak (Strand et al., 2014). An infected individual can release about 2700
68 zoospores per week (Strand et al., 2012), and this number can be much higher when the crayfish
69 is dying or moulting (Makkonen et al., 2013; Svoboda et al., 2013).

70 Generally, North American crayfish species which have co-evolved with *Aphanomyces*
71 *astaci*, are considered to be chronic but largely asymptomatic carriers. They combat *A. astaci*
72 through consistent production of prophenoloxidase, which activates a melanisation cascade
73 resulting in melanisation of hyphae that prevents their invasion into host soft tissues (Cerenius
74 et al., 1988). Most native European crayfish, on the other hand, apparently only produce
75 prophenoloxidase only in response to infection, which is too slow to effectively melanise the
76 hyphae that then spread into host tissues leading to paralysis and death (Cerenius et al., 2003).
77 The Australian yabby (*Cherax destructor*) also suffers high mortality as a result of crayfish
78 plague, though this species shows some resistance to less virulent strains and survives longer
79 when exposed to highly virulent strains compared to highly susceptible species (Mrugała et al.,
80 2016). In infected European crayfish, severe behavioural changes before death include a lack
81 of coordination and paralysis (Gruber et al., 2014), though to what extent carrier crayfish
82 exhibit behavioural changes is largely unknown and this could play a vital role during new
83 invasions and in commercial crayfish farms. Highly infected crayfish, for example, might be
84 less likely to disperse, which would alter invasion success and introduction to new habitats.

85 Few studies have directly assessed the effect of the *A. astaci* on North American
86 species, although there is some evidence that they can succumb to the disease and display
87 altered behaviour if also stressed by other factors (Cerenius et al., 1988; Aydin et al., 2014;
88 Edsman et al., 2015). Co-infection of *A. astaci* and *Fusarium* spp., for example, results in

89 Eroded Swimmeret Syndrome (ESS) in signal crayfish, which causes females to carry fewer
90 eggs (Edsman et al., 2015). Mortality of adult signal crayfish has also been observed in
91 experimental settings, though only when crayfish were exposed to very high zoospore numbers
92 (Aydin et al., 2014). Furthermore, vertical transmission of *A. astaci* (from adults to eggs) has
93 been reported (Makkonen et al., 2010), and little is known on how *A. astaci* might affect
94 juvenile North American crayfish.

95 Here, we addressed two key issues regarding the effects of *A. astaci* on signal crayfish.
96 First, we tested the hypothesis that juvenile signal crayfish would suffer high mortality upon
97 infection by *A. astaci* zoospores, as it has previously been suggested that juvenile crayfish may
98 be more susceptible to infection compared to adults (Mrugała et al., 2016). Additionally, we
99 assessed the effect of *A. astaci* on adult signal crayfish, hypothesising that even if adults may
100 not suffer significant mortality, behavioural changes would be apparent.

101

102 2. Materials and Methods

103 2.1 Signal crayfish trapping

104 All adult signal crayfish were collected in February and March 2017 using cylindrical traps
105 ('Trappy Traps', Collins Nets Ltd., Dorset, UK) baited with cat food and checked daily
106 (trapping licence: NT/CW081-B-797/3888/02). The crayfish were collected from a population
107 displaying negligible levels of infection (maximum agent level A1) when assessed in 2014
108 (Derw Farm pond, Powys, Wales, SO 13891 37557; James et al., 2017). A small subset of
109 individuals (n = 3) re-tested by qPCR (see 2.2) before the experiments began in May 2017 all
110 revealed low levels of infection by *A. astaci*, although elevated compared to 2014 (agent level
111 A2/A3). After removal from traps, crayfish were transferred to individual containers with 500
112 mL of pond water and transported to the Cardiff University Aquarium (holding licence: W C
113 ILFA 002), where they were maintained individually in 20 L aquaria containing a plant pot

114 refuge, gravel and air supply delivered via an airstone. The crayfish were held at $13\pm 1^\circ\text{C}$ under
115 a 12 h light: 12 h dark lighting regime and fed a mixture of frozen peas and *Tubifex* bloodworm
116 (Shirley Aquatics, Solihull, West Midlands, U.K.) once every 2 days. A 50% water change was
117 conducted 1 h after feeding to maintain water quality and remove excess food. Crayfish were
118 acclimatised to the laboratory for at least 4 weeks before the experiments began. Four females
119 were carrying eggs, and upon hatching, the offspring were mixed, moved to 120 L communal
120 aquaria and used in the juvenile infection experiment. Only male crayfish were used in the
121 adult behavioural tests; since a relatively low number of females ($n = 6$) were caught and
122 therefore it was not possible to test an equal number of males and females in this experiment.

123

124 2.2 *Aphanomyces astaci* culture and quantification

125 Crayfish in the present study were exposed to a Group B strain (Pec14) of *Aphanomyces astaci*
126 provided by Charles University in Prague. This strain was isolated from dead *Astacus astacus*
127 from a crayfish plague outbreak in the Černý Brook, Czech Republic (Kozubíková-Balcarová
128 et al., 2014) and demonstrated similarly high virulence towards European *A. astacus* (see
129 Becking et al., 2015) as the strains from Group B (PsI) used in other experimental studies
130 (Makkonen et al., 2012; Jussila et al., 2013). The culture was maintained in Petri dishes of
131 RGY agar (Alderman, 1982; Becking et al., 2015; Mrugała et al., 2016) and zoospores were
132 produced according to the methodology of Cerenius et al. (1988). Briefly, 2-4 agar culture
133 plugs ($\sim 2\text{ mm}^2$) were cut from an RGY culture and placed in flasks containing 200 mL of liquid
134 RGY-medium. Multiple replicates were done each time in order to produce a sufficient number
135 of zoospores. These cultures were allowed to grow at 16°C for 2-4 days on a shaker. Once
136 sufficient mycelial growth had occurred, the cultures were washed to induce sporulation and
137 transferred to separate flasks (containing 500 mL of distilled water). The washing was repeated
138 in distilled water 3-4 times over ~ 8 h. Then, the cultures were incubated at $13\pm 1^\circ\text{C}$ for 24-36

139 h until motile zoospores were produced. The number of zoospores was quantified using a
140 haemocytometer.

141 **Following** both experiments, crayfish were euthanized by freezing at -20°C for 1 h. For
142 juveniles, the whole crayfish was lysed (TissueLyser, Qiagen) and DNA extracted using a
143 Qiagen DNeasy extraction kit (Qiagen). For adult crayfish, a section of tail-fan and soft-
144 abdominal tissue was removed by dissection, **lysed (TissueLyser, Qiagen)**, and both tissues
145 were pooled (~ 20 mg total), and the DNA extracted using the same kits. Infection intensity
146 was estimated based on the number of PCR-forming units (PFU) determined by qPCR using
147 TaqMan MGB probes and expressed using the semi-quantitative levels A0-A7 for adults (as
148 described by Vrålstad et al., 2009); with slight modification of the protocol as in Svoboda et
149 al. (2014). For juveniles, infection intensity was expressed as number of PCR forming units
150 because a direct comparison cannot be made here between juvenile (whole body) and adult
151 (sample body) infection levels.

152

153 *2.3 Juvenile crayfish infection*

154 Here, we monitored the survival of juvenile signal crayfish that hatched in the laboratory when
155 exposed either to **A. astaci** at 1, 10 or 100 zoospores mL⁻¹ or to a sham treatment (control). All
156 crayfish used in this experiment hatched within a 3-day period in May 2017. The infection was
157 conducted twice in separate experiments, the first time approximately 4 weeks after the crayfish
158 hatched (n = 25 crayfish per zoospore treatment) and the second time after 10 weeks with
159 different crayfish (n = 17 individuals per zoospore treatment). When the experiment began,
160 crayfish were housed individually in 1 L pots containing distilled water with a gravel substrate
161 for 48 h. After this acclimatisation period, the pots were spiked with 1, 10 or 100 zoospores
162 mL⁻¹ (the control treatment was given a 20% water change). After a 24 h infection period, 80%
163 of the water in all pots was changed. The crayfish were fed crushed algae wafers and frozen

164 *Tubifex* bloodworm (Shirley Aquatics, Solihull, West Midlands, U.K.) once every 2 days. A
165 50% water change was conducted 1 h after feeding to maintain water quality. For 14 days, we
166 recorded crayfish deaths and any moults daily. Crayfish and moulted carapaces were stored in
167 ethanol at -20°C until DNA was extracted.

168

169 2.4 Adult crayfish behaviour

170 Male crayfish behaviour was tested in an arena (Fig. 1) consisting of a tank (L 100 cm x H 53
171 cm x W 48 cm) with access to a terrestrial area (L 120 cm x H 20 cm x W 20 cm). At the start
172 of the experiment, crayfish were divided into two groups: those destined for ‘high infection’
173 and those to be kept at ‘low infection’ levels. Those destined for the ‘high-infection’ group (n
174 = 15, mean carapace length 52.2 mm, sd = 4.44) were individually exposed to a dose of 1000
175 zoospores mL⁻¹ in 500 mL of water for 24 h. Simultaneously, the ‘low-infection’ crayfish (n =
176 17, mean carapace length 53.1 mm, sd = 4.66) were sham-infected by adding the same amount
177 of distilled water instead of spore-containing water. After the 24 h period, all crayfish were
178 returned to their individual tanks, where they were held for 1 week before their behaviour was
179 assessed. Individual crayfish were placed into the behavioural arena (Fig. 1) and left to
180 acclimatise overnight. Then, at 09:00 h the next day, their behaviour was recorded using an
181 infrared CCTV camera (Sentient Pro HDA DVR 8 Channel CCTV, Maplin, Rotherham, UK)
182 for 24 h (09:00 – 21:00 light and 21:00 – 09:00 dark). During video analysis, the time spent
183 engaged in each of the following four behaviours was recorded for each crayfish: actively
184 walking in water, in a refuge, stationary out of the refuge and moving out of water.

185 Following this, each crayfish was moved to an aquarium (W 30 cm x L 61 cm x D 37
186 cm) with covered sides and allowed to settle for 30 min before their response to being gently
187 touched on the rostrum for 10 s was tested. Crayfish typically reacted by raising their chelae
188 (an aggressive, threatening response) and/or retreating using a characteristic ‘tail-flip’

189 response. If a crayfish retreated, the glass rod was immediately moved again to touch the
190 rostrum. This test was repeated three times with 5 min intervals. Whether the crayfish reacted
191 with a ‘tail-flip’ and/or raised its chelae to attack was recorded. These responses were recorded
192 since behavioural changes that affect a crayfish’s ability to retreat or interact with conspecifics
193 may have subsequent effects on competitive ability, resource acquisition, and ultimately,
194 survival.

195 Following behavioural tests, crayfish were euthanized and *A. astaci* infection levels
196 were quantified as described in section 2.2.

197

198 2.5 Statistical analysis

199 All statistical analyses were performed in R version 3.5.1 (R Core Team, 2018). For the
200 juvenile crayfish experiment, Kaplan-Meier survival analyses were performed using the
201 ‘survival’ package in R (Therneau, 2018) with separate models run for both time points (4- and
202 10-weeks post-hatching). Both models included spore concentration and carapace length as
203 independent variables. Model selection was based on AIC. It was not possible to statistically
204 assess the effect of moulting on mortality as an insufficient number of moulting events were
205 recorded.

206 For the adult crayfish, the time spent moving (active), in shelter, stationary (outside of
207 a shelter) or out of the water was quantified over 24 h for each individual. Generalised Additive
208 Models for Location, Scale and Shape (GAMLSS) models (Stasinopoulos et al., 2008) with
209 appropriate distributions (see Table 1) were used to determine whether ‘treatment’ (i.e. high or
210 low infection) or carapace length (mm) influenced the proportion of time crayfish spent
211 moving, in shelter, out of water or stationary. In the GAMLSS with beta inflated or beta zero
212 inflated distributions, the μ parameter refers to the average amount of time spent engaging in a
213 particular behaviour, whilst v relates to the likelihood of a behaviour not occurring

214 (Stasinopoulos et al., 2008). To assess the response of crayfish to a touch stimulus, threatening
215 or tail flip escape responses were scored separately. The crayfish were tested three times, and
216 it was noted whether they retreated by tail flipping and/or threatened by raising the chelae at
217 least once during the three tests. These data were analysed in binomial models (i.e., threat/no
218 threat, tail flip/no tail flip), using GAMLSS. Treatment group and carapace length were
219 included as independent variables.

220

221 3. Results

222

223 3.1 Juvenile crayfish infection

224 At 4-weeks old, zoospore concentration significantly affected survival of juvenile signal
225 crayfish ($z = 5.971$, $p < 0.001$), with almost all crayfish dying in both the 10 and 100 zoospore
226 mL^{-1} treatments after the 14-day experimental period (Fig. 2). Around half of the crayfish died
227 in the 1 zoospore mL^{-1} treatment, whilst 92% of control treatment crayfish survived. Carapace
228 length also had a significant effect on the survival of these crayfish, with larger individuals
229 surviving longer ($z = -4.387$, $p < 0.001$). In contrast, survival of crayfish exposed to the same
230 infection doses at 10 weeks of age was not significantly affected by the zoospore treatment; all
231 crayfish in the control and 1 zoospore mL^{-1} treatments survived, whilst 88% and 82% of those
232 in the 10 and 100 zoospore mL^{-1} treatments survived. All juvenile crayfish that were tested
233 (Fig. 2) were previously infected (as they were descended from infected females), although
234 those that were exposed to zoospores exhibited significantly elevated infection levels (subset
235 tested for *A. astaci* infection using qPCR; Kruskal-Wallis $X^2 = 9.7534$, $df = 3$, $p = 0.021$; Fig.
236 2).

237

238 3.2 Adult crayfish behaviour

239

240 All crayfish in the 'high-infection' group displayed agent levels A4-A6 (median number of
241 PFU = 23,050; n[A4] = 3; n[A5] = 13; n[A6] = 1), whilst all crayfish from the 'low-infection'
242 group remained at very low (n[A2] = 9) to low (n[A3] = 6) infection levels (median number of
243 PFU = 43). As such, for all analyses, crayfish behaviour was compared in terms of high and
244 low infection.

245

246 Adults exposed to *A. astaci* zoospores (high-infection: 1000 zoospores mL⁻¹) were significantly
247 less likely to leave the water and spent on average 1.3% (range: 0 - 3.8%) of the 24 h period in
248 the terrestrial arena compared to those in the low infection-group (sham-infected), which spent
249 3.5% (range: 0.3 - 9.2%) out of water (GAMLSS, ν , LRT = 5.671, $p = 0.017$). In terms of the
250 other behaviours, there was no significant difference between crayfish from both the high and
251 low-infection groups, which spent 31.8 (standard deviation $\pm 9.1\%$) of the time active, 47.2 \pm
252 25% stationary outside of a shelter and 18.6 $\pm 26\%$ in a shelter (Table 1; Appendix Table 2).

253

254 Crayfish from the high-infection group were also significantly less likely to mount a tail-flip
255 response to tactile stimulation (GAMLSS, μ , LRT = 4.036, $p = 0.045$), where 35% of those in
256 the high-infection group initiated a tail-flip response at least once compared to 75% of those
257 from the low-infection group. Overall, though there was no significant difference between the
258 two treatment groups, larger crayfish were more likely to display a threat response (GAMLSS,
259 μ , LRT = 4.758, $p = 0.029$), spend less time in a shelter (GAMLSS, ν , LRT = 5.514, $p = 0.019$)
260 and more time stationary outside of a shelter (GAMLSS, ν , LRT = 5.730, $p = 0.017$) compared
261 to smaller crayfish.

262

263 **4. Discussion**

264 Here, we show that *A. astaci* can cause almost total mortality in juvenile signal crayfish at
265 ecologically relevant zoospore densities (Strand et al., 2012, 2014), though larger, older
266 individuals were less affected. Additionally, we show that a high *A. astaci* burden affects the
267 behaviour of adult crayfish, making them almost half as likely to spend time on land and to
268 escape from tactile stimulation compared to less infected individuals. The low infection levels
269 of our control crayfish did not differ from those frequently observed in *P. leniusculus*
270 populations across Europe (Kozubíková et al., 2011; Filipová et al., 2013; Tilmans et al., 2014)
271 and in Japan (Mrugała et al., 2017); although slightly higher infection levels (A2-A5) were
272 reported in the UK (James et al., 2017). Thus, the high infection group in our study represents
273 the outbreak of a highly virulent strain. Whilst signal crayfish are a highly successful invasive
274 species in Europe that continue to spread (Peay et al., 2010; Holdich et al., 2014; James et al.,
275 2014), the negative impacts of crayfish plague reported here, especially in terms of juvenile
276 mortality, could have consequences for commercially harvested stocks by reducing recruitment
277 and possibly resulting in population crashes. This also supports previous studies which have
278 shown that commercially harvested signal crayfish populations can decline when *A. astaci* is
279 present (Jussila et al., 2016). Furthermore, these results add to growing evidence that *A. astaci*
280 could play a more significant role in regulating invasive signal crayfish population dynamics
281 than previously considered, which could play a role in determining invasion success (Jussila et
282 al., 2015).

283 In North American crayfish species, *A. astaci* can grow within the carapace, though
284 constant host melanisation of new hyphae prevents spore penetration to soft tissues (Unestam
285 & Weiss, 1970; Nyhlén & Unestam, 1975; Cerenius et al., 2003). In the present study, juvenile
286 signal crayfish suffered extensive dose-dependent mortality when exposed to *A. astaci*
287 zoospores around 4-weeks post-hatching. Slightly older (and therefore larger) crayfish,
288 however, avoided this cost. Many juvenile crayfish studied here probably became infected

289 rapidly after hatching, having acquired an infection from their mothers via horizontal
290 transmission. Older and larger crayfish possibly have a better-developed immune response,
291 capable of efficiently melanising hyphae. It has been suggested that the immune response of
292 juvenile crayfish to *A. astaci* infection is generally reduced compared to adults (Mrugała et al.,
293 2016), which seems to be the case in the present study. In other invertebrates too, younger
294 individuals exhibit lower immune responses, for instance, snails showing greater susceptibility
295 to schistosome parasites (Dikkeboom et al., 1985). It has also been hypothesised, however, that
296 juvenile crayfish could be less affected due to their higher moulting frequency compared to
297 adults (Reynolds, 2002), allowing them to shed the growing hyphae and lower their *A. astaci*
298 burden. Further research comparing the immunological capacity of juvenile and adult crayfish
299 is required to confirm this. By the 10-week time-point, particularly susceptible individuals may
300 have already succumbed to infection and therefore those used in the current experiment would
301 have been more resistant to the pathogen. This appears unlikely though, since high levels of
302 mortality were not observed in the communal holding tanks. Ecologically, the finding that
303 relatively young crayfish hatchlings are highly susceptible to high doses of zoospores could
304 have significant implications for signal crayfish recruitment and survival, especially in lentic
305 environments, where zoospores are less likely to be washed away from the maternal crayfish.

306 Adult crayfish suffering from higher *A. astaci* infection levels during the present study
307 exhibited a reduced tendency to leave the water. Although crayfish spend little time out of
308 water in general, this finding suggests that populations of invasive signal crayfish with high
309 burdens of *A. astaci* could be less likely to disperse overland to reach new aquatic habitats, a
310 behavioural trait that can contribute to the spread of invasive crayfish (Grey and Jackson, 2012;
311 Holdich et al., 2014; Puky, 2014; Ramalho and Anastácio, 2014). Other invertebrates are less
312 active when infected by parasites, potentially to avoid the associated fitness costs of dispersal.
313 Flat back mud crabs (*Eurypanopeus depressus*) infected with rhizocephalans, for example,

314 spend more time in shelter and are less active than uninfected crabs (Belgrad and Griffen,
315 2015), whilst sponge-dwelling snapping shrimp (*Synalpheus elizabethae*) infected by bopyrid
316 isopods show 50% lower activity levels compared to uninfected individuals (McGrew and
317 Hultgren, 2011). In other invertebrates, many studies have shown that parasites can influence
318 dispersal, though these studies focus on direct host manipulation, which does not seem to be
319 the case here as there is no evidence of *A. astaci* actively manipulating the host. In terms of
320 native European crayfish management, a lower tendency of infected individuals to disperse
321 overland might be beneficial, by reducing the transmission of *A. astaci* to new waterbodies.

322 Highly infected crayfish were also less likely to respond to tactile stimulation by
323 retreating in a characteristic ‘tail-flip’ manner. This reduced ability to escape could lead to
324 increased predation of highly infected crayfish. *A. astaci* zoospores largely penetrate soft
325 abdominal tissue (Vrålstad et al., 2009), and it is possible that the reduced escape response is
326 directly due to the general pathological effects of the parasite (Unestam & Weiss, 1970). Other
327 parasites, such as *Thelohania contejeani*, also penetrate crayfish tissues, parasitising the
328 muscles and reducing the ability of crayfish to predate and feed (Haddaway et al., 2012). It is
329 also possible that highly infected crayfish exhibit a reduced tendency to move on land to reduce
330 the risk of predation. In the same way, crustaceans become less active and tend to stay in a
331 refuge when moulting, during which they are vulnerable to predators and largely unable to
332 escape (Thomas, 1965; Cromarty et al., 2000).

333 The crayfish used in the current study were from a population previously considered to
334 be below the detection limit (n = 30 tested by James et al. (2017) exhibited A0-A1 levels).
335 However, given that infection levels A2-A3 were found both among crayfish tested before the
336 experiment began, as well as among those in the group not exposed to zoospores, it is evident
337 that this population has either become infected since 2014, that a previously very low
338 prevalence of *A. astaci* has since increased, or that crayfish present with A2-A3 infection levels

339 in 2014 were just not trapped by James et al. (2017). Signal crayfish in Europe are generally
340 associated with the Group B strain of *A. astaci* (see Huang, Cerenius and Söderhäll, 1994;
341 Grandjean et al., 2014), which has also been found infecting another Welsh population,
342 approximately 45 miles away from the population studied here (James et al., 2017). Although
343 not confirmed, the crayfish used in the present study were most likely initially infected with a
344 Group B strain and subsequently exposed to another strain from the same group. It is also
345 possible that the tested crayfish were locally adapted to their original *A. astaci* infection
346 (Gruber et al., 2014; Jussila et al., 2015), and the observed behavioural effects resulted from
347 the exposure to the new *A. astaci* strain. As observed by Jussila et al. (2013), even assumed
348 identical *A. astaci* strains may differently affect their crayfish hosts; therefore, the experimental
349 crayfish in the present study likely dealt with multiple infections of closely related *A. astaci*
350 strains. Further research is required, to explicitly compare the behaviour and survival of
351 infected and uninfected signal crayfish, as well as investigate the effects of different *A. astaci*
352 strains (both in single and multiple infections) on the behaviour and survival of infected
353 crayfish.

354 In summary, we have shown that high levels of *A. astaci* cause severe mortality in
355 young juveniles and affect the behaviour of adult signal crayfish. Mounting evidence suggests
356 that signal crayfish may succumb to *A. astaci* more often than previously considered, which
357 could be having an impact on commercially harvested populations (Aydin et al., 2014; Edsman
358 et al., 2015). The crayfish exposed to zoospores in the present study displayed relatively high
359 plague agent levels of A4-A6 (A7 being the highest level of infection; Vrålstad et al., 2009). A
360 longer period of infection, or higher infection dose, may induce further behavioural responses
361 beyond those reported here, and in some cases even cause mortality as observed by Aydin et
362 al. (2014), where signal crayfish were exposed to 10,000 zoospores mL⁻¹. Female crayfish
363 suffering from ESS carry far fewer fertilised eggs than uninfected females (Edsman et al.,

2015) which, coupled with the high juvenile mortality documented in the present study, could drastically reduce juvenile recruitment and result in population crashes. Similarly, crayfish plague could also have implications for the further spread of signal crayfish by affecting population dynamics, though this species has already successfully colonised large parts of Europe (Holdich et al., 2014) and so the ecological impact may be negligible. Anecdotally, it was assumed that most North American crayfish are infected with *A. astaci*, though molecular methods have demonstrated that it is less prevalent than once thought. In France, for example, just over half of the signal crayfish populations tested were found to be positive for crayfish plague (Filipová et al., 2013), and in the UK the prevalence was 56.5% (James et al., 2017). It is possible, therefore, that the population dynamics of uninfected invasive populations may be affected when infected individuals are translocated and introduced.

375

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380

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562

563

564 Fig. 1 – Experimental arena used to assess crayfish behaviour. The tank was filled with water
565 up to 3 cm below the level of the terrestrial area. The base of the arena, ramp (incline 30°) and
566 bridge were coated in 1-2 cm of pea gravel.

567

568 Fig. 2 – Survival (a, b) and infection levels (c, d) of juvenile signal crayfish infected with *A.*
569 *astaci* for two weeks. Infection treatments were sham-infection, 1, 10 and 100 zoospores mL⁻¹;
570 a & c) 4 weeks after hatching; b & d) 10 weeks after hatching. Note in b) sham-infection
571 treatment is identical to infection treatment 1 (grey / dashed grey). A subset of juvenile crayfish
572 from each treatment was tested using qPCR, c) sham-infection, 0 zoospores ml⁻¹ [n = 5], 1 [n
573 = 6], 10 [n = 5], 100 [n = 4]; d) 0 [n = 3], 1 [n = 3], 10 [n = 5], 100 [n = 6]. See Appendix Table
574 1 for absolute values.

575

576 Table 1 – Results of GAMLSS (**Generalised Additive Models for Location, Scale and Shape**)
 577 statistical analyses and mean proportion of time crayfish spent engaged in different behaviours
 578 over 24 h. Significant results ($p < 0.05$) are highlighted in bold. BE = beta, BEZI = beta zero-
 579 inflated, BEINF = beta inflated, BI = binomial, SD = standard deviation. * denotes proportion
 580 of crayfish, not the mean.
 581

Response variable	Infection treatment group	Mean (%)	Range (%)	GAMLSS Parameter	Family	Variable	DF	LRT	P-value
Proportion of time active	Low	31	21-49	μ	BE	Treatment	1, 28	0.016	0.898
	High	32	18-48			CL		0.024	0.876
Proportion of time out of water	Low	4	0-9	μ	BEZI	Treatment	1, 25	2.095	0.147
	High	1	0-4			CL		0.075	0.784
				ν		Treatment		5.671	0.017
						CL		0.125	0.724
Proportion of time in shelter	Low	21	0-55	μ	BEINF	Treatment	1, 24	0.046	0.830
	High	17	0-75			CL		2.478	0.116
				ν		Treatment		0.043	0.835
						CL		5.514	0.019
Proportion of time stationary	Low	44	11-76	μ	BEINF	Treatment	1, 26	0.383	0.536
	High	50	0-76			CL		5.730	0.017
Proportion of crayfish that tail flipped	Low	75%	na	μ	BI	Treatment	1, 26	4.036	0.045

	High	35%	na			CL	1.607	0.205
Proportion of crayfish that exhibited threat behaviour	Low	67%	na	μ	BI	Treatment	0.177	0.674
	High	65%	na			CL	4.758	0.029

582

583