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1 *Saprolegnia parasitica* zoospore activity and host survival indicates isolate variation in host  
2 preference

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## 8 **Abstract**

9 The ubiquitous freshwater pathogen *Saprolegnia parasitica* has long been considered a true  
10 generalist, capable of infecting a wide range of fish species. It remains unclear, however,  
11 whether different isolates of this pathogen, obtained from distinct geographic locations and  
12 host species, display differences in host preference. To assess this, the current study examined  
13 the induced zoospore encystment responses of four *S. parasitica* isolates towards the skin of  
14 four fish species. While three of the isolates displayed ‘specialist’ responses, one appeared to  
15 be more of a ‘generalist’. *In vivo* challenge infections involving salmon and sea trout with the  
16 ‘generalist’ (salmon isolate EA001) and a ‘specialist’ (sea trout isolate EA016) pathogen,  
17 however, did not support the *in vitro* findings, with no apparent host preference reflected in  
18 infection outcomes. Survival of sea trout and salmon though was significantly different  
19 following a challenge infection with the sea trout (EA016) isolate. These results indicate that  
20 while *S. parasitica* isolates can be considered true generalists, they may target hosts to which  
21 they have been more frequently exposed (potential local adaptation). Understanding host  
22 preference of this pathogen could aid our understanding of infection epidemics and help with  
23 the development of fish management procedures.

24

25 **Keywords:** host preference; specialist; generalist; local adaptation; oomycete.

26

27 Declarations of interest: none.

28

## 29 **Introduction**

30 Host specificity is an important parasite trait; providing an accurate depiction of a parasite's  
31 ecological niche (Poulin and Mouillot 2003). It is determined by the number of host species  
32 that a parasite can successfully invade and the taxonomic relationship between these host  
33 species. While some parasites might trade off their virulence (the severity of infection) against  
34 transmissibility (ability to spread infection from host to host) with optimum parasite fitness  
35 striking a balance between the production of transmission stages and damage to the host, for  
36 others the interaction is more complex (Acevdeo et al. 2019). This is partly driven by host  
37 range; a given parasite species may infect a wide range of phylogenetically distinct host taxa,  
38 with parasite fitness varying from host to host. Thus, the composition of the host population  
39 presents a selective pressure that contributes to evolution of parasite generalism or specialism  
40 (Futuyma and Moreno 1988). Parasites with low host specificity are considered generalists,  
41 capable of switching between distantly related host species, moreover they tend to exhibit a  
42 similar level of virulence across their broad host range (Poulin and Mouillot 2003; Leggett et  
43 al. 2013). More specialised parasites may possess a high specificity for certain host species or  
44 taxonomic group for which they exhibit an optimal level of virulence.

45 Fungal and fungal-like parasites are thought to possess the broadest host range of any  
46 parasite group (Fisher et al. 2012). Perhaps the most notorious example being the aquatic

47 chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) that infects over 700 amphibian species  
48 (Olson et al. 2013) and is also capable of infecting fish (Liew et al. 2017). Furthermore, several  
49 fish pathogenic oomycetes are considered true generalists able to infect several different host  
50 families (Gozlan et al. 2014). Members of the *Saprolegnia* genus are particularly destructive;  
51 *Saprolegnia diclina* is a virulent pathogen of fish eggs (Kitancharoen et al. 1997; Fregeneda-  
52 Grandes et al. 2007; van den Berg et al. 2013) and *S. ferax* is believed to be partly responsible  
53 for declines in amphibian populations (Kiesecker et al. 2001; Pounds 2001). *S. parasitica* is  
54 arguably the most important animal pathogenic oomycete with a reported 1 in 10 of all farmed  
55 raised salmon succumbing to saprolegniasis and frequent associations with declining natural  
56 wild fish populations (van West 2006). Previously, *S. parasitica* was considered opportunistic,  
57 only able to infect fish hosts as a secondary pathogen. Several salmonid studies, however, have  
58 highlighted that certain isolates of *S. parasitica* are primary invaders and highly virulent (Neish  
59 1977; Willoughby and Pickering 1977). Despite this, marked differences in virulence have  
60 been observed between *S. parasitica* isolates (Yuasa and Hatai 1995) and the host range of  
61 individual isolates remains unexplored.

62 *S. parasitica* produces free-swimming zoospores during the infective stage of its life  
63 cycle. These zoospores are unicellular, single nucleated cells that are able to swim freely via  
64 two flagella; one tinselated and one whiplash flagellum (Burr and Beakes 1994). They are  
65 responsible for the first essential step in establishing an infection, namely locating and  
66 attaching to a host. Upon achieving this, these infectious propagules encyst, germinate and  
67 sprout mycelial hyphae which then penetrate the host tissues (Willoughby et al. 1983; Diéguez-  
68 Uribeondo et al. 1994). If isolates of *S. parasitica* are specialised for particular hosts, this could  
69 be reflected in their induced zoospore encystment responses towards different fish species.

70 The current study combines *in vitro* induced zoospore encystment data with targeted *in*  
71 *vivo* challenge experiments to uncover the extent of specialism/generalism within this species.

72 The *in vitro* investigations assessed the host preference of four *S. parasitica* isolates that are  
73 both geographically distinct and originally isolated from different host species. The *in vivo*  
74 studies aimed to determine whether the *in vitro* findings were reflected in challenge infection  
75 outcomes. Moreover, infections involving different populations of the same species were  
76 included to determine whether *S. parasitica* host preference exists at a population level.

77 We hypothesise that while the isolates investigated may not be highly specialised to a  
78 single host species, they do display preferences towards a limited number of host species. A  
79 phylogenetic analysis of the four *S. parasitica* isolates based on nuclear ribosomal internal  
80 transcribed spacer (nrITS) sequence data has also been included to examine their position  
81 within the *Saprolegnia* taxonomic system proposed by Sandoval-Sierra et al. (2014). This data  
82 could potentially expand our understanding of *S. parasitica* infections and inform future  
83 aquaculture practices.

## 84 **Materials and Methods**

### 85 *Host origin and maintenance*

86 Atlantic salmon (*Salmo salar*), sea trout (*Salmo trutta*), common carp (*Cyprinus carpio*) and  
87 three-spined stickleback (*Gasterosteus aculeatus*) (n=6 per species) were net caught from  
88 hatcheries or the wild and delivered to our aquarium facilities at Cardiff University for use in  
89 the induced encystment assays (see Table 1). Moreover, further net-caught Atlantic salmon and  
90 sea trout (n=60 per species) were obtained for use in the experimental challenge infections (see  
91 Table 1). Prior to experimental procedures, fish were maintained in 90 L tanks at a density of  
92 1 fish L<sup>-1</sup>. Both prior to and during experimental procedures, fish were exposed to a water  
93 temperature of 12±0.5°C, oxygen saturation of >91%, 12 h light: 12 h dark cycle and fed trout  
94 pellets daily unless otherwise stated.

95 *Saprolegnia culture and zoospore production*

96 Four *Saprolegnia parasitica* isolates were obtained directly from four naturally infected fish  
97 hosts collected during routine sampling by the Environment Agency (see Table 2). On the  
98 riverbank, a small mycelial clump (approx. 4-5 cm<sup>2</sup>) was extracted from the affected tissue of  
99 a live, recently caught fish using forceps and placed immediately onto a potato dextrose agar  
100 (PDA, 39g L<sup>-1</sup>) plate. The plate was sealed using parafilm and sent to our facilities at Cardiff.  
101 Cultures were sub-cultured monthly onto fresh PDA plates according to Stewart et al. (2017).  
102 For zoospore production, petri dishes containing ~40 ml glucose-yeast broth (Glucose 10g L<sup>-1</sup>,  
103 Yeast Extract 2.5g L<sup>-1</sup>) were inoculated with three 5 mm diameter plugs of healthy white  
104 mycelia from the stock culture. The *S. parasitica* mycelia were left to grow for 72 h at 20°C,  
105 then washed with dechlorinated water in order to remove excess glucose-yeast broth. To induce  
106 zoospore production, the mycelia were placed in a 50/50 mixture of dechlorinated water and  
107 aquarium water at 10°C for 72 h. The resulting zoospore suspension was concentrated via  
108 centrifugation at 4600 rpm for 10 min at room temperature. Zoospores in the concentrated  
109 suspension were enumerated using a haemocytometer.

110 *DNA extraction and sequencing of internal transcribed spacer (ITS) rDNA*

111 DNA was extracted using the following modified protocol outlined by Vilgalys and Hester  
112 (1990). Briefly, ~0.3g of mycelia from each of the respective *S. parasitica* isolates was ground  
113 under liquid nitrogen and suspended in 500 µl of 2X (w/v) CTAB extraction buffer (100 mM  
114 Tris, 20 mM Na<sub>2</sub>EDTA, 1.4M NaCl, pH 8.0). The samples were then subject to a freeze-thaw  
115 step in which they were placed at -80°C for 10 min and subsequently incubated at 65°C for 30  
116 min. Samples were extracted twice using equal volumes of chloroform-isoamyl alcohol (24:1);  
117 DNA was precipitated by adding 2 volumes of isopropyl alcohol and placing the samples at -  
118 20°C for 24 h. The resulting genomic DNA pellets were washed once with 70% EtOH, dried

119 under a laminar flow hood and re-suspended in 50 µl of TE buffer (10 mM Tris, 1 mM EDTA,  
120 pH 8.0).

121 PCR amplification of the ITS region was performed using the universal fungal primers  
122 of White et al. (1990): 5'-GGAAGTAAAAGTCGTAACAAGG-3' (ITS 5-Forward) and 5'-  
123 TCCTCCGCTTATTGATATGC-3' (ITS 4-Reverse). The PCR reaction mix consisted of  
124 15 µl of Taq PCR Master Mix (Qiagen), 1.5 µl of each forward and reverse primer, 20-50 ng  
125 genomic DNA and nuclease-free water to give a total reaction volume of 30 µl. The PCR  
126 protocol was as follows: initial denaturation at 94°C for 5 min, 5 amplification cycles of:  
127 denaturation at 94°C for 30s, annealing at 58°C for 30s and extension at 72°C for 1 min;  
128 subsequently a further 33 cycles were included where the annealing temperature was changed  
129 to 48°C. A final extension at 72°C for 10 min concluded the PCR. In order to check the PCR  
130 products were the correct size (approx. 700 bp), they were run on a 1% agarose gel and  
131 visualised using UV-transillumination. PCR products were then sequenced via Sanger  
132 sequencing and a NCBI BLAST search for related sequences was used to confirm each sample  
133 as *S. parasitica* (>98% sequence identity to *S. parasitica*).

#### 134 *Phylogenetic analysis*

135 ITS sequence data for the four *S. parasitica* isolates (see Supplementary materials) investigated  
136 here were included in a phylogenetic analysis alongside *Saprolegnia* spp. sequences from  
137 GenBank that have been previously designated into phylogenetic clusters by Sandoval-Sierra  
138 et al. (2014) and an isolate of *Aphanomyces astaci* served as an outgroup (Supplementary Table  
139 S1). The Molecular Evolutionary Genetics Analysis (MEGA) software v10.0.2 was used to  
140 first align the ITS sequences using the ClustalW algorithm with default settings, and  
141 subsequently construct a phylogenetic tree using the Maximum Likelihood method based on  
142 the Jukes-Cantor model. Relative branch support of the tree was estimated using a bootstrap

143 analysis with 1000 replicates, all other settings were set to default. The tree was converted into  
144 Newick format and imported into FigTree v1.4.4 to produce a circular phylogenetic tree.

#### 145 *In vitro induced zoospore encystment assays*

146 To obtain skin samples for the induced zoospore encystment assays, Atlantic salmon, sea trout,  
147 common carp and three-spined stickleback (n=6 per species; details in Table 1) were euthanised  
148 with an overdose of MS-222 and pithed to destroy the brain (Home Office Schedule 1 method).  
149 Skin was subsequently removed from each fish and samples from each respective fish species  
150 were pooled together and homogenised in phosphate buffered saline (PBS 1X) at a  
151 concentration of 0.1g skin ml<sup>-1</sup>. The homogenised solution was centrifuged at 1000 rpm for 5  
152 min. The resulting pellet was discarded and the supernatant was aliquoted and stored at -20°C  
153 until required.

154 A modified capillary root model (Halsall 1976) was used to assess the induced zoospore  
155 encystment responses of the four *S. parasitica* isolates listed in Table 2. The assay was  
156 performed in a plastic petri dish (48 mm base diameter x 12 mm depth) containing 5 ml of  
157 zoospore suspension prepared as described previously and adjusted to a concentration of ~300  
158 zoospores ml<sup>-1</sup>. A cell scraper was used to ensure no spores were encysted on the sides or base  
159 of the petri dishes and that the spores were evenly dispersed throughout the suspension. Two  
160 micro-capillary tubes (Drummond Scientific, 2.9 cm length, 20 µl volume) were introduced  
161 into the petri dish, containing either the fish skin solution ('test' solution) or PBS (1X 'control'  
162 solution) and positioned at specific distances apart using forceps (see Fig. 1A). The test and  
163 control tubes were left in the zoospore solution for 10 min, then removed and the contents of  
164 each expelled into an Eppendorf tube. These tubes were vortexed for 45 s, causing any  
165 zoospores to encyst. The number of encysted zoospores in each tube was then counted using a  
166 haemocytometer.



167 To account for potential differences between batches of zoospores, the assays were  
168 conducted in the following manner; for each *S. parasitica* isolate two batches of zoospores  
169 were produced. Per batch, 10 replicates of the assay were performed against each fish species.  
170 Hence, across the two batches of spores per isolate, a total of 20 replicates were achieved  
171 against each fish species. To control for potential side bias, the position of the test and control  
172 tubes was alternated. Room lighting (constant overhead LED lighting) and temperature  
173 ( $20\pm 1^{\circ}\text{C}$ ) were kept constant throughout the assays. The induced zoospore encystment  
174 responses were expressed by a ratio that was calculated for each assay replicate using the  
175 following equation:

$$\text{Induced zoospore encystment ratio (IZER)} = \frac{\text{Mean no. of zoospores in 'test' tube}}{\text{Mean no. of zoospores in 'control' tubes}}$$

178 The assay was subsequently modified to assess the induced zoospore encystment  
179 responses of the salmon (EA001) and sea trout (EA016) *S. parasitica* isolates (see Table 2)  
180 when presented with a direct choice between the skins of two fish species. Micro-capillary  
181 tubes containing salmon skin solution ('salmon test') and sea trout skin solution ('sea trout  
182 test') were used in the same assay alongside a PBS control (Fig. 1B). All other experimental  
183 conditions were consistent with those described previously. The induced zoospore encystment  
184 responses of these isolates towards salmon and sea trout were calculated using the induced  
185 zoospore encystment ratio (IZER) equation above.

#### 186 In vivo *Saprolegnia parasitica* challenge infections

187 Challenge infections were conducted to assess whether the induced zoospore encystment ratios  
188 (IZERs) obtained for the salmon (EA001) and sea trout (EA016) *S. parasitica* isolates were  
189 reflected in infection outcomes. The experimental procedure described here was performed  
190 separately for these isolates in order to avoid cross contamination. Zoospore suspensions were

191 prepared as described above and a concentration of  $\sim 3 \times 10^5 \text{ L}^{-1}$  used for all of the experimental  
192 infections. Juvenile salmon and sea trout ( $n=60$  per species, see Table 1) were subjected to an  
193 adjusted ‘ami-momi’ technique (Hatai and Hoshiai, 1994) in which they were individually  
194 shaken in a net for 30 s to introduce abrasions to the fish body and remove protective mucus.  
195 Of these fish,  $n=50$  per species were assigned to the ‘treatment’ condition and placed into glass  
196 aquaria (31 W x 61 D x 31 H cm) separated by species, containing a well-oxygenated zoospore  
197 suspension at a density of 1 fish  $\text{L}^{-1}$  for 24 h without food. Following zoospore exposure, fish  
198 were transferred into individual transparent plastic 1 L containers of dechlorinated water and  
199 daily feeding was resumed. The remaining fish acted as the ‘controls’ ( $n=10$  per species) and  
200 were handled in exactly the same manner without exposure to zoospores, before transfer to  
201 individual containers. Water in both the treatment and control containers was changed every  
202 24 h. Fish were checked hourly for signs of saprolegniasis over the duration of the experimental  
203 period of 168 h. Fish were categorised as either symptomatic (mild and cleared or severe) or  
204 asymptomatic according to the severity of their symptoms (Table 3). Any fish displaying severe  
205 signs of infection were euthanised via overdose with MS-222 and pithing.

206 This experiment was subsequently repeated using two different stocks of juvenile  
207 salmon from the River Tyne: one from the North Tyne and another from the South Tyne. While  
208 there is no available data to confirm whether these were genetically distinct populations of fish,  
209 the literature on within-river genetic structure of salmon suggests that river tributaries generally  
210 contain distinct populations (Vähä et al. 2007). Hence these will be referred to as different  
211 populations from here onwards. The fish were kept separated according to population and all  
212 other experimental procedures were the same as those described previously.

213 *Animal Ethics*

214 All procedures and protocols followed ARRIVE guidelines, were approved by the Cardiff  
215 University Animal Ethics Committee and conducted under UK Home Office license PPL  
216 30/3424.

217 *Statistical Analyses*

218 Analyses were conducted using R statistical software (version 3.5.1, R Core Team 2018) with  
219 the significance threshold  $P < 0.05$  used for all models. Non-significant terms were removed  
220 during model refinement based on Analysis of Variance (Crawley 2007) while model  
221 robustness was assessed using residual plots (Pinheiro and Bates 2000).

222 A Generalised Linear Model (GLM) fitted with a Gaussian error family and identity  
223 link function was used to examine the induced zoospore encystment of four *S. parasitica*  
224 isolates towards the skin of four fish species. Induced zoospore encystment ratio (IZER) was  
225 the dependent term in the model, fixed terms included: *S. parasitica* isolate (EA001, EA016,  
226 EA012, CF006), fish skin (salmon, sea trout, common carp, three-spined stickleback - herein  
227 referred to as stickleback) and the interaction between these two terms. Zoospore batch (1, 2)  
228 was included as a fixed factor in the original model but was removed during model refinement  
229 due to non-significance ( $P > 0.05$ ). *Post-hoc* analysis (lsmeans package; Lenth 2016) was  
230 conducted to compare the IZERS of each *S. parasitica* isolate.

231 A second GLM fitted with a Gaussian error family and identity link function was used  
232 to examine the induced zoospore encystment of the salmon (EA001) and sea trout (EA016)  
233 isolates when presented with salmon and sea trout skin in a single assay. The dependent and  
234 independent terms were the same as those outlined in the previous model and again, zoospore  
235 batch (1, 2) was included in the original model but removed due to non-significance ( $P > 0.05$ ).

236 Comparisons between the IZERs of the two *S. parasitica* isolates were assessed using *post-hoc*  
237 analysis (lsmeans package; Lenth 2016).

238 Proportional odds logistic regression (POLR) models (MASS package; Venables and  
239 Ripley 2002) were used to examine the infection outcomes of the *S. parasitica* experimental  
240 challenge infections. Infection outcome of each fish (asymptomatic, mild and cleared, severe)  
241 was the dependent term in the models, fixed effects included; *S. parasitica* isolate (salmon  
242 isolate EA001, sea trout isolate EA016), and fish species/population (model 1 – salmon, sea  
243 trout; model 2 – North Tyne, South Tyne salmon). Fish standard length (mm), initially included  
244 in the models, was non-significant ( $P>0.05$ ) and therefore was consequently removed.

245 Kaplan-Meier survival plots (survival package; Therneau 2015) were generated for  
246 challenge infections involving the sea trout isolate (EA016) only, log-rank tests were used to  
247 compare the survival plots. Survival analysis was not possible for the salmon isolate (EA001)  
248 as no mortalities occurred during the challenge infections with this isolate.

## 249 **Results**

### 250 *Phylogenetic analysis*

251 All four isolates of *S. parasitica* used in the current study fell within Cluster 3 of the taxonomic  
252 system proposed by Sandoval-Sierra et al. (2014) (Supplementary Fig. S1)**Error! Reference**  
253 **source not found.** Although, identical by ITS, subsequent genome resequencing revealed  
254 significant genetic differences between isolates (Matthews 2020).

### 255 *In vitro induced zoospore encystment assays*

256 Induced zoospore encystment ratios (IZERs) displayed by the *S. parasitica* isolates (EA001,  
257 EA016, EA012, CF006) were dependent on fish skin (salmon, sea trout, common carp,  
258 stickleback) (GLM;  $df=9$ ,  $P<0.0001$ ), but there was no clear trend in preference. The salmon

259 isolate (EA001) showed a significant preference for salmonid and carp over stickleback (Fig.  
260 2A). The sea trout isolate (EA016) IZERs were significantly higher for sea trout and common  
261 carp compared to salmon or stickleback skin (Fig. 2B). The common carp (EA012) isolate  
262 displayed a preference for common carp and salmon over sea trout and stickleback skin (Fig.  
263 2C). Lastly, the IZERs of the stickleback isolate (CF006) were significantly higher for salmon  
264 compared to sea trout or stickleback skin (Fig. 2D). Mean zoospore numbers in the 'test' and  
265 'control' tubes for each isolate are presented in Table 4. In light of these results, the salmon  
266 EA001 and the sea trout EA016 isolates were selected for further *in vitro* induced zoospore  
267 encystment testing and *in vivo* challenge experiments (EA001 showing no preference for sea  
268 trout or salmon, compared to EA016 preferring sea trout skin).

269         When simultaneously presented with salmon and sea trout skin, zoospores of the  
270 salmon isolate (EA001) and the sea trout isolate (EA016) displayed consistently generalist and  
271 specialist induced zoospore encystment responses, respectively. For EA001, there were no  
272 significant differences in IZER for salmon and sea trout skin ( $P>0.05$ ) (Fig. 3). Conversely, we  
273 confirmed the preference of EA016 for sea trout compared to salmon skin ( $P<0.0001$ ) (Fig. 3).  
274 Mean zoospore numbers in 'salmon test', 'sea trout test' and 'control' tubes for each isolate are  
275 presented in Table 5.

#### 276 *In vivo Saprolegnia parasitica challenge infections*

277 Infection outcomes (asymptomatic, mild and cleared, severe) from the *S. parasitica* challenge  
278 infections revealed no evidence of isolate-specific host preference. Neither fish species  
279 (salmon, sea trout; Figs. 4A and B) nor fish population (North, South Tyne salmon; Figs. 4C  
280 and D) significantly affected fish infection outcomes for either parasite isolate (salmon isolate  
281 EA001, sea trout isolate EA016; GLMs;  $df=1$ ,  $P>0.05$ ). Infection outcomes were, however,  
282 significantly different between the two *S. parasitica* isolates (GLMs;  $df=1$ ,  $P<0.0001$ ); EA001

283 established infections in only 34% and 24% of salmon and sea trout respectively (Fig. 4A) in  
284 addition to 20% and 24% of North and South Tyne salmon (Fig. 4C). Conversely, EA016  
285 successfully infected 100% of challenged fish (Figs. 4B and D).

286 Indications of host-preference were observed from the survival analysis; while there  
287 was no overall significant difference in the survival of salmon and sea trout challenged with  
288 the sea trout isolate (EA016) (log-rank test;  $P>0.05$ ), there was a significant difference during  
289 the initial 48 h of the infection (Fig. 5A). At 24 h, sea trout survival was reduced to 84%,  
290 whereas none of the salmon died during this period (log-rank test;  $P<0.05$ ; Fig. 5A). At 48 h,  
291 60% of the sea trout were alive compared to 96% of the salmon (log-rank test;  $P<0.0001$ ; Fig  
292 5A). There was no significant difference in survival for North and South Tyne salmon  
293 challenged with the sea trout isolate (EA016) (log-rank test;  $P>0.05$ ; Fig. 5B).

## 294 **Discussion**

295 The current study is the first to investigate the host preference of *S. parasitica* at an isolate  
296 level. The *in vitro* induced zoospore encystment assays indicate that the salmon isolate  
297 (EA001) is a generalist, exhibiting a similar level of preference for three fish species, whereas  
298 the sea trout (EA016), carp (EA012) and stickleback (CF006) isolates are more specialised,  
299 showing a higher preference for one or two fish species. These *in vitro* results, however, were  
300 not reflected in the *in vivo* challenge infections; no differences between salmon and sea trout  
301 infection outcomes were observed within the salmon isolate (EA001) or sea trout isolate  
302 (EA016) challenges. Infection outcomes between salmon populations (North and South Tyne)  
303 were also not significantly different for either isolate, suggesting host preference does not occur  
304 at a population level. Despite this, survival analysis revealed a significantly higher number of  
305 sea trout mortalities compared to salmon within 48 h of the sea trout isolate (EA016) challenge  
306 infection, which could be a potential indication of host preference/adaptation.

307           The phylogenetic analysis presented here offered no separation between the four  
308 isolates as they all possessed an identical ITS sequence and fell within Cluster 3 of the  
309 taxonomic system proposed by Sandoval-Sierra et al. (2014) alongside other isolates classified  
310 as *S. parasitica*. Despite the four *S. parasitica* isolates sharing the same ITS sequence – the  
311 DNA barcode traditionally used for *Saprolegnia* species identification – isolates were  
312 genetically distinct (confirmed via whole genome resequencing see Matthews 2020) and  
313 exhibited considerable differences in host preference and virulence, suggesting this region is  
314 not suitable for isolate discrimination.

315           Interestingly, all isolates examined here yielded a low induced zoospore encystment  
316 response to sticklebacks. Moreover, even the more ‘specialist’ isolates demonstrated a similar  
317 level of induced zoospore encystment towards fish from different families; the sea trout isolate  
318 (EA016), for instance, displayed a comparable level of preference for sea trout and carp  
319 (Family Salmonidae and Cyprinidae, respectively). Hence, reaffirming the postulation that  
320 *Saprolegnia* spp. are able to target a wide range of phylogenetically distant species. A potential  
321 criticism of the induced zoospore encystment assay is that homogenisation of the fish skin in  
322 solution would have destroyed its structure and biological components. However, El-Feki *et*  
323 *al.* (2003) employed the same methodology in their chemotaxis assay and found that fish skin  
324 induced the highest chemotactic response compared to other fish tissue extracts including  
325 mucus, blood, and gills.

326           The drastically different levels of virulence displayed by the salmon (EA001) and sea  
327 trout (EA016) isolates in the challenge infections may be a reflection of different time in  
328 culture; EA001 was isolated 510 days before EA016. Maintaining pathogens (fungi, bacteria  
329 and viruses) on/in artificial culture media for extended periods can cause an attenuation of  
330 virulence (Druelle et al. 2008; Almaguer-Chávez et al. 2011; Ansari and Butt 2011). Passage  
331 through a susceptible fish host and subsequent re-isolation can restore virulence in *S. parasitica*

332 cultures (Songe et al. 2014), there are, however contamination risks associated with re-  
333 culturing which could confound results.

334         Disparity between sea trout and salmon survival during the initial 48 h of the sea trout  
335 isolate (EA016) challenge infection could reflect the different induced zoospore encystment  
336 responses displayed by this isolate; if its zoospores are less attracted to salmon skin, as our  
337 induced zoospore encystment results would suggest, it may have taken longer to locate the  
338 salmon in comparison to the sea trout. It may also reflect subtle differences in the early stages  
339 of host-pathogen interaction. The initial attachment of *S. parasitica* zoospores to host cells is  
340 purportedly achieved via cell-binding proteins such as lectins (Jiang et al. 2013). In salmonids,  
341 this triggers a strong inflammatory response via the induction of proinflammatory cytokines  
342 and antimicrobial peptides (AMPs), in particular interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6 and tumour  
343 necrosis factor- $\alpha$  (TNF- $\alpha$ ) (de Bruijn et al. 2012; Belmonte et al. 2014). Effector proteins and/or  
344 proteases then suppresses several constituents of the adaptive immune system by  
345 downregulating T-helper cell cytokines, antigen presentation machinery and immunoglobulins  
346 (Jiang et al. 2013; Belmonte et al. 2014). Subsequently host cells are attacked by a multitude  
347 of virulence factors in the form of proteases, lipases and lysing enzymes (Jiang et al. 2013). It  
348 may be that the salmon immune response was able to suppress the initial EA016 invasion,  
349 however immune defences were eventually overcome and salmon survival plummeted from 96  
350 to 60% between 48 and 60 h. Sequencing of the *S. parasitica* genome revealed the arsenal of  
351 virulence proteins employed by this pathogen are rapidly evolving due to co-evolution with the  
352 host (Jiang et al. 2013). Hence, isolates of *S. parasitica* could potentially target host species to  
353 which they have been more frequently exposed and consequently adapted to (Williams 1966;  
354 Kawecky and Ebert 2004; Savolainen et al. 2013). The sea trout isolate (EA016) examined here  
355 may be better adapted to sea trout, which is reflected in its induced zoospore encystment



356 responses and ability to initiate rapid virulence and host mortality during the challenge  
357 experiment.

358 Overall, this study indicates that *S. parasitica* is a generalist with isolate variation in  
359 host preference. Uncovering the host preference of the key isolates within the UK could aid  
360 our understanding of disease outbreaks in the wild and fish management practises within  
361 aquaculture. For example, if the sea trout isolate (EA016) investigated here was present in a  
362 salmon aquaculture facility, knowledge of the 48 h lag in salmon mortality would prompt the  
363 application of treatments to boost immune function and potentially reduce mortalities.  
364 Furthermore, we demonstrate that the ITS region does not offer *S. parasitica* isolate separation  
365 and is not indicative of isolate severity.

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503 **Tables and Figures**

504

505 Table 1. Origin of fish used for induced zoospore encystment assays and challenge infections.

506

Experiment	Fish Species	Life stage	Mean weight (g) + range	Mean standard length (mm) + range	Source	Date of arrival at Cardiff University
Induced zoospore encystment assays	Atlantic salmon ( <i>Salmo salar</i> )	Juvenile	17.4 (14.8-20.4)	111.0 (104.6-117.8)	Kielder Salmon Hatchery, Hexham, Northumberland	June 2016
	Sea trout ( <i>Salmo trutta</i> )	Juvenile	9.2 (7.8-10.2)	92.3 (88.5-96.2)		
	Common carp ( <i>Cyprinus carpio</i> )	Juvenile	41.41 (23.5-63.5)	123.9 (103.8-138.2)	DC Freshwater Fish, Brookwood, Surrey	
	Three-spined stickleback ( <i>Gasterosteus aculeatus</i> )	Adult	1.7 (0.6-2.8)	48.0 (43.9-57.2)	Roath Brook, Cardiff	
Challenge infection 1	Atlantic salmon	Juvenile	1.3 (0.7- 2)	40.1 (34.9-48.2)	Kielder Salmon Hatchery, Hexham, Northumberland	July 2017
	Sea trout	Juvenile	1.2 (0.6-2.1)	41.4 (32.9-48.1)		
Challenge infection 2	North Tyne Atlantic salmon	Juvenile	2.7 (1 - 4.8)	50.8 (36.9-64.9)		
	South Tyne Atlantic salmon	Juvenile	2.2 (1-4.1)	49.1 (36.8-61.6)		

507



508 Table 2. Origin of *Saprolegnia parasitica* isolates used for the *in vitro* induced zoospore  
 509 encystment assays. \*Denotes isolates used in subsequent induced zoospore encystment assays  
 510 and challenge infections.

511

Isolate	Host species	River/waterbody	Date isolated
EA001*	Atlantic Salmon ( <i>Salmo salar</i> )	River Esk, Yorkshire, England. (54°26'59.1"N, 0°48'12.42"W)	10/01/2015
EA016*	Sea trout ( <i>Salmo trutta</i> )	River Dart, Devon, England. (50°27'36.432"N, 3°41'42.144"W)	03/06/2016
EA012	Common carp ( <i>Cyprinus carpio</i> )	Lake near Romsey, Hampshire, England. (50°59'40.6"N, 1°34'46.7"W)	22/03/2016
CF006	Three-spined stickleback ( <i>Gasterosteus aculeatus</i> )	Roath Brook, Cardiff, Wales. (51°29'54.1572"N, 3°9'54.2484"W)	15/07/2016

512

513 Table 3. Fish in the *Saprolegnia parasitica* experimental infections were categorised as either  
 514 asymptomatic, symptomatic (mild and cleared) or symptomatic (severe) of saprolegniasis  
 515 according to these symptoms.

Asymptomatic	Symptomatic	
	Mild and cleared infection	Severe infection
<ul style="list-style-type: none"> <li>No signs of saprolegniasis</li> </ul>	<ul style="list-style-type: none"> <li>Small tufts of mycelial growth on the body which were no longer present upon conclusion of the experiment</li> </ul>	<ul style="list-style-type: none"> <li>Extensive mycelial body coverage</li> <li>Lethargy</li> <li>Respiratory distress</li> <li>Loss of equilibrium</li> </ul>

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517

518 Table 4. Mean zoospore numbers of the salmon (EA001), sea trout (EA016), common carp  
 519 (EA012) and three-spine stickleback (CF006) isolates in the ‘test’ and ‘control’ tubes of the *in*  
 520 *vitro* induced zoospore encystment assays

521

Isolate	Fish skin	Mean no. of zoospores in ‘test’ tubes	Mean no. of zoospores in ‘control’ tubes
EA001	Atlantic Salmon ( <i>Salmo salar</i> )	5.25	0.5
	Sea trout ( <i>Salmo trutta</i> )	5	0.35
	Common carp ( <i>Cyprinus carpio</i> )	3.65	0.35
	Three-spined stickleback ( <i>Gasterosteus aculeatus</i> )	0.85	0.35
EA016	Salmon	2.6	0.45
	Sea trout	5.6	0.25
	Common carp	5.45	0.3
	Three-spined stickleback	1.7	0.2
EA012	Salmon	2.55	0.2
	Sea trout	1.6	0.25
	Common carp	2.7	0.2
	Three-spined stickleback	0.85	0.35
CF006	Salmon	3.05	0.2
	Sea trout	1.25	0.15
	Common carp	1.95	0.2
	Three-spined stickleback	2.5	0.45

522

523

524 Table 5. Mean zoospore numbers of the salmon (EA001) and sea trout (EA016) isolates in the  
 525 ‘salmon test’, ‘sea trout test’ and ‘control’ tubes of the *in vitro* induced zoospore encystment  
 526 assays

Isolate	Mean no. of zoospores in ‘salmon test’ tubes	Mean no. of zoospores in ‘sea trout test’ tubes	Mean no. of zoospores in ‘control’ tubes
EA001	3.15	3.1	0.25
EA016	3.15	8.3	0.3

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533 Fig. 1. Induced zoospore encystment assays - experimental set-up. (A) Assay used to assess  
534 the induced zoospore encystment of four *Saprolegnia parasitica* isolates: EA001 (salmon),  
535 EA016 (sea trout), EA012 (common carp) and CF006 (three-spined stickleback) against a fish  
536 skin extract (from salmon, sea trout, common carp or three-spined stickleback) and PBS  
537 control. (B) Modified assay used to compare the induced zoospore encystment of isolates  
538 EA001 (salmon) and EA016 (sea trout) when simultaneously exposed to salmon and sea trout  
539 skin extracts alongside a PBS control.

540

541 Fig. 2. Induced zoospore encystment ratios of four *Saprolegnia parasitica* isolates: (A) Salmon  
542 isolate (EA001), (B) Sea trout isolate (EA016), (C) Common carp isolate (EA012) and (D)  
543 Three-spined stickleback isolate (CF006) against skin extracts from four fish species (salmon,  
544 sea trout, common carp and three-spined stickleback). Statistical significance displayed;  
545  $P < 0.05$  (\*),  $< 0.01$  (\*\*),  $< 0.001$  (\*\*\*). Dots above and below the box and whisker plots signify  
546 outliers.

547

548 Fig. 3. Induced zoospore encystment ratios of the *Saprolegnia parasitica* zoospore isolates;  
549 salmon isolate (EA001) and sea trout isolate (EA016) when presented with a direct choice  
550 between salmon and sea trout skin extracts. Statistical significance displayed;  $P < 0.0001$  (\*\*\*).

551

552 Fig. 4. Percentage of salmon and sea trout, and North and South Tyne salmon that were  
553 asymptomatic, mild and cleared and severely infected with saprolegniasis when challenged  
554 with the *Saprolegnia parasitica* salmon isolate (EA001; (A) and (C) respectively), and the sea  
555 trout isolate (EA016; (B) and (D) respectively).

556

557 Fig. 5. Survival plots showing percent survival (+95% confidence intervals) of (A) Salmon  
558 and sea trout, and (B) North and South Tyne salmon challenged with *Saprolegnia parasitica*  
559 sea trout isolate (EA016). Control fish (both species and populations) not challenged with *S.*  
560 *parasitica* displayed 100% survival (dashed line).

561