A eDNA-qPCR combination assay to non-invasively detect the presence of the parasite *Schistocephalus solidus* inside its intermediate host, the threespine stickleback.

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ABSTRACT

To study parasite-host interactions, it is of major importance to detect the presence of the parasite during its development in the host. The *Schistocephalus solidus*-threespine stickleback pair has been used extensively to study host phenotypic alterations caused by a parasite with a complex life cycle. This cestode worm is localized inside the abdominal cavity of its intermediate fish host, which limits its detection to the time when it has grown substantially or to multiple infections. Here, we present a non-invasive species-specific quantitative real time PCR approach based on the detection of environmental DNA from the worm (eDNA), sampled directly in the abdominal cavity of the threespine stickleback. Using this approach on two fish populations (n=151), 98% of fish were correctly assigned to their *S. solidus* infection status. Among the 35 sticklebacks that harboured single or multiple infections, only 3 were not detected as infected. Furthermore, non-infected sticklebacks were never incorrectly identified as infected. There was a significant correlation between eDNA concentration and the total mass of parasites. We also assessed ventilation rates as a rapid and complimentary mean to detect infection (n=20). Ventilation rates were repeatable over 3 measurements, did not vary significantly between non-infected and infected fish, were not significantly correlated to the parasite index, and thus could not reliably be used to predict infection. Our eDNA approach could be used in this parasite-host system alone or in combination with morphological measurements to detect *S. solidus*.

Key Words: *Schistocephalus solidus*, threespine stickleback, parasite, detection, quantitative real-time PCR, environmental DNA

INTRODUCTION

Internal parasites with complex life cycles often have multiple effects on their hosts (Marcogliese and Cone 1997). Studying these parasite-hosts interactions requires to be able to track the parasite during its development in the hosts. One of the model systems in the study of phenotypic alterations of the intermediate host infected by a parasite with a complex life cycle is the interaction between *Schistocephalus solidus* and its obligatory intermediate host, the threespine stickleback (*Gasterosteus aculeatus*, (Barber 2013)). *S. solidus* is a freshwater flatworm with a complex life cycle that has to infect three distinct hosts in order to survive and reproduce: a copepod, the threespine stickleback, and a fish-eating bird. The threespine stickleback is the specific intermediate host of *S. solidus* and can be infected by one or several worms after consumption of a *S. solidus*-infected copepod. Within 24 hours after oral uptake, the worm penetrates the intestine wall of the fish and eventually migrates into the abdominal cavity where it establishes as a small plerocercoid (Hammerschmidt and Kurtz 2007). During the first twelve weeks, *S. solidus* shows a sigmoid growth curve in the stickleback abdominal cavity (Barber and Svensson 2003). When the worm has gained a sufficient mass, it becomes infective and can reproduce in the intestinal system of its final host, a fish-eating bird (Tierney and Crompton 1992). Previous studies demonstrated that *S. solidus* infection has major impacts on the stickleback energy demands, physiology and immunity. Furthermore, effects of the macro parasite on its fish host include behavioural perturbations that result in risky behaviours, such as loss of anti-predatory responses and of schooling tendency (reviewed in (Barber and Scharsack 2010)).

The whole life cycle of this parasite can be reproduced in the laboratory (Barber and Scharsack 2010), which makes it highly amenable to manipulation. However, the success rate of infection in the laboratory when experimental single infections are used is around 15-20% ((Grécias et al. 2016); (Weber et al. 2017)). Thus, a high number of individuals must be included and assayed in a study to obtain an acceptable final sample size of infected ones. Furthermore, variation in infection rates for sticklebacks caught in the wild is high (Weber et al. 2017), which means that wild individuals considered as uninfected controls may turn out to be infected when studying wild sticklebacks. A simple detection method would allow selecting, or avoiding, infected individuals depending on the needs of a specific project. This would allow minimizing the number of individuals used in an experimental procedure, an important aspect for the refinement of animal use protocols. However, despite the fact that the *S. solidus*-threespine stickleback system is a very useful model in behaviour, ecology, evolution, immunology and parasitology (Barber 2013), there is currently a lack of a simple, reliable and non-invasive method to systematically determine if a threespine stickleback is infected by *S. solidus*.

Available methods to detect *S. solidus* are based on morphological criteria. Indeed, sticklebacks parasitized by *S. solidus* display modifications in the shape of their head and exhibit a swollen and distended belly. These morphological modifications are exacerbated with an increase in the parasitic load (Dingemanse et al. 2009). The principal morphometric technique that is thereby currently used to determine if a stickleback is infected by *S. solidus* relies on a visual analysis of the level of the

79 abdominal distension of the fish. This allows to predict the infection status (infected or
80 not) and the parasite index (PI, the proportion of infected fish weight that is contributed
81 by parasite tissue) (Barber 1997). However, this visual approach is less reliable when
82 the stickleback is infected by one worm or by a few small worms, which do not induce
83 clear morphological changes. Furthermore, some confounding factors can impact the
84 level of the abdominal distension. During the breading season, gravid females exhibit a
85 distended belly that is very similar to the one of fish infected by *S. solidus,* and a large
86 stomach or liver can also influence the abdominal distension (Barber 1997). X-rays are
87 another morphometric technique that can be used in parasitology to determine the
88 infection status of a host, but several problems are associated with their application,
89 such as the difficulty to distinguish between the parasite and the surrounding fish tissue
90 (Shahidi et al. 1997) or the high financial cost that limits their application to some
91 laboratories. In this context, it is necessary to develop a method that will not be based
92 on morphological criteria.

93

94 Our objective was to design a low cost and non-lethal method to detect *S. solidus* in the
95 abdominal cavity of its threespine stickleback host. To do so, we used two
96 complementary approaches: detection of environmental DNA (eDNA) and measurement
97 of a physiological trait. Our first hypothesis was that *S. solidus* produces cell-free DNA in
98 the abdominal cavity of the fish, and that this eDNA could be identified by a PCR
99 approach to correctly detect *S. solidus* infection in sticklebacks (Bass et al. 2015). eDNA
100 produced by parasites is a common target in epidemiology to detect infection using a
101 PCR approach in organic samples like serum, feces ((Pontes et al. 2002); (Xu et al.

102 2017)) or plasma (Imwong et al. 2015). eDNA is also used to detect invasive species in
103 aquatic environments (Takahara et al. 2013). We thus tested a non-invasive quantitative
104 real-time PCR method (qPCR), based on eDNA extraction from fluids of the abdominal
105 cavity of the stickleback. We used qPCR for its sensitivity and higher capacity of
106 detecting false positive compared to PCR products detected on an electrophoresis gel
107 (Bass et al. 2015). We designed primers that amplify a gene sequence from the *S.*
108 *solidus* genome but that also do not amplify DNA from the specific host, the threespine
109 stickleback. To do so, we chose a nuclear gene (GEEE01010589.1 TSA:
110 Schistocephalus solidus ssol_TR119934_c5_g1_i2 transcribed RNA sequence) that
111 does not have known homologous sequences in other species (Hébert et al. 2016),
112 allowing to specifically detect *S. solidus*. We tested if we could assign a fish to a given
113 infection status. Since eDNA has been previously used to make quantitative predictions
114 about the biomass of organism detected (Lacoursiere-Roussel et al. 2016), we also
115 tested if the eDNA concentration we measured covaried with the total mass of the
116 parasite(s) present within the abdominal cavity of an individual.

117

118 Our second hypothesis was that infected individuals would show a significantly higher
119 ventilation rate, which could be used to distinguish between non-infected and infected
120 fish. As mentioned previously, the energy demands imposed by the presence of *S.*
121 *solidus* have been well documented and affect growth (Barber and Svensson 2003) and
122 reproductive output (Heins and Baker 2003). For example, oxygen consumption is
123 higher in infected sticklebacks compared to uninfected ones for a given swimming speed
124 (Lester 1971) and for fish of similar mass (Meakins and Walkey 1975). Infected fish shift

125 to surface respiration at a higher dissolved oxygen level than uninfected ones, being
126 only rivalled by females harbouring mature eggs, which are also energy demanding
127 (Meakins and Walkey 1975). We thus tested the use of the ventilation rate of healthy
128 and infected sticklebacks as a rapid and non-invasive indicator of *S. solidus* infection,
129 using a previously published protocol to measure ventilation in threespine stickleback (Di
130 Poi et al. 2016).

131

132 **MATERIALS AND METHODS**

133 **Populations of interest**

134 This study was conducted in two populations of threespine sticklebacks. The first
135 population was Lac Témiscouata, Québec (47°40'33"N 68°50'15"O, freshwater
136 environment), where sticklebacks are known to be infected by *S. solidus* and to show
137 typical behaviour alterations (Grecias et al. 2017). The second population was
138 composed of anadromous fish from a salt marsh at Isle-Verte, Québec (48°1'0"N
139 69°26'59"O; salinity 22-26 ppt (Poulin and FitzGerald 1989)), for which infection by *S.*
140 *solidus* is not expected as the worm does not tolerate high levels of salinity (Macat et al.
141 2015).

142

143 **Fish sampling and rearing**

144 In Lac Témiscouata, adults were caught in July 2016 and September 2017, and
145 juveniles in August 2016, using a seine and minnow traps. Juvenile sticklebacks from
146 the anadromous Isle-Verte salt marsh population were sampled in tide pools in July

147 2016 using hand nets. Sticklebacks were brought to the “Laboratoire Regional des
148 Sciences Aquatiques” at Université Laval. Adults and juveniles from each population
149 were kept in separate 80 L-tanks under a Light:Dark photoperiod and a temperature of
150 15°C reflecting the conditions in their natural environments (Québec, Canada). For
151 anadromous fish, salinity was maintained at 28 ppt. Fish were fed daily with brine
152 shrimps. All sticklebacks were adults at the start of the experiment. They were isolated
153 in 2 L-tanks and were assigned an identification number. 151 sticklebacks were used: 96
154 from Lac Témiscouata and 55 from Isle-Verte.

155

156 **Sampling eDNA from the host abdominal cavity**

157 We sampled the internal fluids of the fish within the intra-peritoneal cavity to obtain
158 eDNA. The fish of interest was taken out of its tank and placed on a sponge. The needle
159 of a syringe filled with 100 µL of PBS (Phosphate Buffered Saline, pH 7.4 Life
160 Technologies) was inserted into its abdominal cavity and PBS was injected. Then,
161 without removing the needle from the abdominal cavity, the plunger of the needle was
162 pulled back until the syringe filled with the PBS that was just injected (up to 100 µL).
163 After the needle was removed, the fish was directly put back in its tank. Between 10 and
164 100 µL of PBS was obtained and directly added to a tube of 700 µL of Longmire lysis
165 preservation buffer (Longmire et al. 1997). This protocol was done twice for each fish on
166 two consecutive days. On the second day, the PBS used to wash the abdominal cavity
167 was added to the same Longmire tube as the first sample (i.e. up to 200 µL in the 700
168 µL Longmire tube). Each day, the needle was inserted into a different face of the fish.
169 Abdominal cavity fluid samples were placed at 4°C between the first and the second

170 day, and during the night following the second day. Samples were kept at -20°C before
171 DNA extraction. Fish were fed with brine shrimps after the sampling of the abdominal
172 cavity.

173

174 After these two samplings, fish were euthanized with an overdose of MS-222 (75 mg/L
175 mg/kg) followed by exsanguination and dissected to determine if they were infected by
176 *S. solidus*. Fish sex, size and mass, and *S. solidus* mass and number in each fish were
177 noted. The parasite index was calculated using the formula (total weight (mg) of
178 parasites / Total weight (mg) of host plus parasites) x 100. A value of 50 indicates a
179 situation where the total weight of parasites is equal to the net weight of the stickleback
180 (Arme and Owen 1967).

181

182 The protocol was approved by the Comité de Protection des Animaux de l'Université
183 Laval (CPAUL 2014069-3).

184

185 **DNA extraction from samples**

186 DNA extraction from the abdominal cavity samples was performed according to an
187 environmental DNA method developed by Lacoursière-Roussel et al. (Lacoursière-
188 Roussel et al. under revision), with small modifications to the protocol. To summarize, 30
189 µL of proteinase K (4 mg/mL) (VWR) was directly added to a Longmire buffer sample
190 and the tube was incubated at 55°C for the night. The sample was transferred into a
191 new 2 mL tube and 950 µL of phenol chloroform isoamyl alcohol (Invitrogen) was added.

192 After shaking and centrifugation, supernatant was transferred into a new tube and 950
193 µL of chloroform (VWR) was added. After shaking and centrifugation, a maximum of 750
194 µL of the supernatant was put into a new tube. 750 µL of ice cold isopropanol (Fisher
195 Scientific) and 375 µL of room temperature 5M sodium chloride NaCl were added to the
196 tube, which was placed at -20°C for the night. After centrifugation, all liquid was
197 removed from the tube and 1500 µL of 70% ethanol was added. After centrifugation,
198 ethanol was removed and the tube was air-dried. The DNA pellet was resuspended in
199 80 µL water and the tube was placed at 55°C for 10 minutes. The tube was placed at
200 4°C for the night and then stored at -20°C.

201

202 **Design of primers specific to the *Schistocephalus solidus* genome**

203 A pair of primers that amplifies a sequence from a nuclear gene (GEEE01010589.1
204 TSA: Schistocephalus solidus ssol_TR119934_c5_g1_i2 transcribed RNA sequence)
205 from the *S. solidus* genome was designed. The gene sequence was obtained from the
206 de novo transcriptome of *S. solidus* (sequence available on NCBI:
207 https://www.ncbi.nlm.nih.gov/nuccore/GEEE01010589.1?report=fasta) and does not
208 have known homologues in other species (Hébert et al. 2016). This nuclear gene, whose
209 function is unknown, is highly expressed during early developmental stages of the worm
210 when it is not able to reproduce (Hebert et al. 2017). We targeted the DNA sequence of
211 this gene with the forward for-CGGATTGTCTTCTCGTTGTA and reverse rev-
212 GGACAACCACTGTCCACTAA primers, designed with Primer3 (Untergasser et al.
213 2012). The primers generated a 200 bp amplicon. We verified with the Basic Local

214 Alignment Search Tool (nucleotide BLAST) on NCBI that the 200 bp amplicon did not
215 align with other sequences except for *S. solidus*.

216

217 **PCR assays to determine specificity of amplification**

218 Polymerase Chain Reaction (PCR) assays were conducted on genomic DNA samples to
219 confirm that the designed primers amplified only *S. solidus* DNA and not threespine
220 stickleback DNA. DNA was extracted from *S. solidus* and stickleback samples (Lac
221 Témiscouata) kept at -20°C in ethanol 90%. Tissue samples of 0.3 cm^2 (*S. solidus* body,
222 stickleback tail) were used for the extraction. Extraction was performed using a salt
223 method (Aljanabi and Martinez 1997). PCR reaction was performed in a total volume of
224 24.3 µL (13.75 µL of nuclease free water, 1 µL of 10mM dNTPs, 0.25 µL of TAQ DNA
225 polymerase (5U/µL Bio Basic), 2.5 µL of 10x TAQ buffer with $MgCL_2$, 5 µL of DNA (*S.*
226 *solidus* or stickleback) and 1.8 µL of primer mix (4.56 µM of each primer)). Two negative
227 controls were done (no primers, no DNA). The PCR program was realized with a
228 Mastercycler (Eppendorf) under these conditions: a denaturation step of 2 min at 95°C,
229 an annealing and elongation step of 40 cycles including 20 sec at 95°C, 40 sec at 56°C
230 and 60 sec at 72°C, and a final step of 10 min at 72°C. PCR products were visualized on
231 a 2% agarose gel using SYBR safe staining (Invitrogen).

232

233 **qPCR assays**

234 eDNA extracted from the abdominal cavity samples of fish was amplified with a real-time
235 quantitative polymerase chain reaction (qPCR) using the designed primers specific to *S.*

236 *solidus*. Each 96-well plate included eDNA samples extracted from the body cavity and a
237 standard curve obtained from a pool of *S. solidus* genomic DNA (3 individuals) diluted at
238 different concentrations. The concentrations of the 7-point standard curve ranged from
239 0.9 ng/µL to 0.01 ng/µL with a dilution factor of 2. qPCR reaction was performed in a
240 qPCR mix including 12.5 µL of PowerUp SYBR Green Master mix (Life Technologies)
241 and 1 µL of primer mix (4.56 µM of each primer). For the standard curve, genomic DNA
242 samples, 6.5 µL of RNAse free water and 5 µL of DNA were added to the qPCR mix. For
243 the eDNA samples, no water was used and 11.5 µL of solution containing the
244 resuspended eDNA was added to the qPCR mix (for a total qPCR mix volume of 25 µL
245 in each case). Two negative controls (no primers, no DNA) were done in each plate.
246 Each reaction was done in triplicate. qPCR was performed with a 7500 Real-Time PCR
247 system (Life Technologies). The amplification was realized according to the conditions
248 described by (Lacoursière-Roussel et al. 2016): 2 min at 50 °C, 10 min at 95 °C,
249 followed by 70 cycles of 15 sec at 95 °C and 60 sec at 56 °C. A melt curve protocol was
250 then performed with temperature going from 60 celsius to 95 celsius to detect primer
251 dimers, check specificity and obtain a Tm value for that sample, which can be compared
252 to the standard curve of Schistocephalus DNA.

253

254 **Ventilation rate measurements**

255 A subset of individuals used in the *S. solidus* eDNA detection experiment was used for
256 this analysis (captured in Lac Témiscouata in July 2016, n=20). Ventilation
257 measurements were repeated three days in a row with individuals sampled in the same
258 order each day. For each individual fish, a 250 mL beaker was filled with 50 mL of clean

259 water from the supply used to fill experimental 2 L-tanks. The individual was filmed from
260 the side (JVC Everio camera) to be able to detect opercular movement. At then end of
261 the three days of measurements, these fish were euthanized and their infection status
262 determined. Dissection showed that 13 individuals were infected and 7 were parasite-
263 free. All the infected fish were also detected as infected by the eDNA approach (see
264 below). Films were subsequently analyzed at 0.46 X speed by an observer (NAH) blind
265 to treatment using the open source VLC Media Player (Videolan). The first 30 seconds
266 of the movie where the fish was clearly visible were used to measure ventilation rates. If
267 the individual moved in a position where it was not visible during the first-30-second
268 sampling period, the ventilation measurement was made for the usable seconds within
269 that period and reported back to 30 seconds. The observer repeated all measurements
270 for the first day of sampling to assess accuracy. Measures done twice on the same fish
271 on the same video were highly correlated (Spearman r = 0.99) with most samples
272 having the exact same number of ventilations when measured twice, with a maximum
273 difference of 3 ventilation movements and an average difference of 0.7. A single
274 measurement per video was thus used. A ventilation rate was calculated by dividing the
275 number of ventilation measured in 30 seconds by 30. This measurement was used for
276 further statistical analyses. The total number of ventilation measured during a 30 second
277 interval was used to estimate the number of ventilation over 1 minute for graphical
278 purposes and for comparison to previous studies.

279

280 **Analyses**

281 Following qPCR amplification, the presence or the absence of *S. solidus* eDNA in the
282 body cavity samples was determined by comparing the Ct values (amplification curves)
283 and the Tm values (melt curves) of the samples with the ones of the standard curves. A
284 Ct ("cycle threshold") is the cycle at which a sample reaches a preset threshold of
285 fluorescence, which is the proxy for the quantity of eDNA. A sample with a low Ct means
286 that there were more templates in the original sample and that it thus reached the
287 threshold sooner in the 70 PCR cycles. A body cavity sample showing at least one of its
288 Ct values (among the triplicates) above 0 in combination with the associated Tm
289 value(s) close (+/- 2) to the mean Tm of the standard curve (79.64) was considered
290 positive for *S. solidus* eDNA. On the contrary, if a Ct value above 0 was associated with
291 a Tm value more distant to the mean Tm of the standard curve, then this value was
292 considered negative for *S. solidus* eDNA and the result of primer dimer formation.
293 Finally, if the body cavity sample had only "undetermined" Ct values, it was considered
294 negative for *S. solidus e*DNA, irrespectively of Tm values. For each fish, qPCR results
295 were analyzed with regard to its infection status (parasitized or not by *S. solidus* upon
296 dissection). A true positive was obtained when there was concordance between the
297 infection status and the qPCR results. A false negative was defined when the fish was
298 infected by *S. solidus*, but no traces of *S. solidus* eDNA were found using qPCR. A false
299 positive was associated with fish not infected by *S. solidus* but for which *S. solidus*
300 eDNA was inferred using qPCR results. All individual results are found in supplementary
301 data.

302

303 Statistical analysis was done in R version 3.3.3 (R Core Team 2017) using R Studio
304 (Version 1.0.136) and the ggplot2 package (Wickham 2009). To determine if our method
305 allows a quantitative estimate of parasite mass, we tested if the eDNA concentration
306 obtained for infected fish (using the Ct value) negatively correlated with their total
307 parasite mass, using a non-parametric Spearman correlation (a small Ct value should be
308 associated with a larger parasite mass and vice-versa if the eDNA method is
309 quantitative). We used the lowest Ct measured for a given fish if more than one triplicate
310 amplified DNA. Repeatability of ventilation rates over the three days was calculated
311 following (Lessells and Boag 1987). We compared ventilation rates between infected
312 and non-infected individuals using a t-test, as they followed a normal distribution (based
313 on a Shapiro-wilk test). We tested if ventilation rates covaried positively with the parasite
314 load (parasite index) using a non-parametric correlation (Spearman correlation).

315

316 **RESULTS**

317 **Primers specificity**

318 PCR tests performed with primers that target a sequence from the *S. solidus* nuclear
319 gene NCBI ID GEEE01010589.1 (TSA: Schistocephalus solidus
320 ssol_TR119934_c5_g1_i2 transcribed RNA sequence) confirm the amplification of *S.*
321 *solidus* genomic DNA, and no amplification of threespine stickleback genomic DNA. The
322 PCR product was sequenced using Sanger ABI3730xl (Plateforme de séquençage et de
323 génotypage des génomes, Centre Hospital Universitaire du Québec) to confirm the
324 amplification of the 200 pb amplicon in the *S. solidus* genome. Controls (no template, no
325 primers) were negative as expected.

326

327 **Detection of *S. solidus* DNA in the abdominal cavity of infected fish with qPCR**

328 qPCR was performed in combination with a E-DNA extraction protocol to try to detect *S.*
329 *solidus* presence in the abdominal cavity of sticklebacks. Standard curves realized with
330 a pool of *S. solidus* genomic DNA samples diluted at different concentrations had mean
331 Ct values ranging from 29.65 (highest concentration) to 36.86 (lowest concentration) and
332 a mean Tm value of 79.64. All the controls were negative as expected. The earliest Ct
333 value we obtained for abdominal cavity E-DNA samples with our protocol and amount of
334 starting material varied between 34.4 and 49.6 for positively identified individuals. Using
335 this approach, 148 fish out of 151 were correctly assigned to their infection status
336 (infected or not by *S. solidus*, 98 % of true positive) (Table 1). The 3 remaining fish were
337 false negative. We did not obtain any false positive with our approach.

338

339 In the Témiscouata population, 35 fish out of 96 were infected by *S. solidus.* Single and
340 multiple infections (up to 6 worms) were reported upon dissection, with a median of 1.5
341 worm, illustrating the predominance of single or double infections. Note that one fish
342 harboured 12 worms, but we did not obtain reliable mass for the worms and as such is
343 included in the dataset only to calculate the infection status assignation rate. Among the
344 infected sticklebacks, 18 exhibited a single worm upon dissection and no external visual
345 clues suggested the presence of *S. solidus* in the fish body cavity (Figure 1). For all
346 infected fish, the total parasite mass was comprised between 130 mg and 2356 mg and
347 the parasite index was always below 50 (between 4.9 and 40.4), meaning that the total
348 weight of parasites was always below the net weight of the host fish (Table 2). Thirty-two
349 out of the 35 infected fish were successfully detected as infected using qPCR. For the 3
350 remaining infected fish, no trace of *S. solidus* eDNA was detected with our protocol
351 (1.98% of samples tested, low false negative rate) (Table 1). The 3 fish had a total
352 parasite mass that span almost the whole range found in the study (168 mg and 274 mg
353 for the single infections and 1644 mg for the double infection, from the second smallest
354 to the third largest). Their parasite index (respectively 12.5, 6.5 and 25.3) was above the
355 minimal values from infected fish that were correctly detected (Table 2). They had no
356 eDNA detected in any of the triplicates (Ct "undetermined"). As predicted, none of the
357 fish from the Isle-Verte anadromous population were infected by *S. solidus* (n=55). We
358 never detected *S. solidus* DNA in the samples of the uninfected fish from this population,
359 nor from uninfected fish from Lac Temiscouata (0 false positive) (Table 1). There was a
360 significant correlation between Ct values (which are inversely correlated to the amount

361 of *S. solidus* eDNA) and the total parasite mass found within a fish (one-sided Spearman
362 correlation, rho= -0.41, p = 0.01, figure 2).

363

364 **Variations of ventilation rates between uninfected and infected sticklebacks**

365 We measured the ventilation rates of 20 fish, 3 times over 3 consecutive days, and then
366 determined that 13 of these were infected. The infected individuals harboured from 1 to
367 5 worms with a total mass ranging between 618 mg and 2356 mg (parasite index
368 between 13.2 and 40.4). One infected individual died after the first ventilation
369 measurement and we could use the videos for only the 2 first days for one non-infected
370 fish. We had 3 ventilation measurements for all other individuals. Repeatability of
371 ventilation across 3 days for a given fish was 0.41 (Lessells and Boag 1987). As
372 ventilation rates were repeatable over this time scale, we used ventilation rates
373 measured on the first day in subsequent analysis, to reflect how this method would be
374 used to quickly detect infection. Ventilation rates estimated over 30 seconds and
375 reported in number of opercular movement per minute ranged between 99 and 146
376 opercular beats/min in un-infected fish and between 112 and 160 in *S. solidus* infected
377 ones (figure 3). The average number of ventilation per minute was 122 for uninfected
378 fish and 134 for infected fish. We found that ventilation rates did not vary significantly
379 between infected and non-infected individuals (two-sided t-test, t = -1.57, p = 0.15, n=20,
380 figure 3). Ventilation was positively correlated to the parasite index but this covariation
381 was not significant (one-sided Spearman correlation, rho = 0.35, p = 0.07, n=20). There
382 was a substantial overlap in ventilation rate between the two groups, which prevented
383 the use of this trait to discriminate between *S. solidus* infected and healthy individuals.

384

385 **DISCUSSION**

386 In parasite-host system studies, being able to detect the presence of a parasite during
387 its development in the host is of major importance to better understand the interactions
388 between the two organisms. Here, we demonstrate the power of a method based on
389 detecting eDNA from the parasite *S. solidus* directly sampled from the abdominal cavity
390 of its specific intermediate host, the threespine stickleback.

391

392 Using our eDNA-qPCR combination method in two populations of threespine
393 sticklebacks, 148 fish out of 151 were correctly assigned to their infection status
394 (infected or not), including 32 fish parasitized by *S. solidus* (with single and multiple
395 infections). For 3 sticklebacks only (1.98% of samples tested), *S. solidus* eDNA was not
396 detected despite the fact that these fish were infected (low false negative rate). We
397 never assigned an "infected" status to an uninfected fish (0 false positive). The amount
398 of S. solidus eDNA was correlated to the total mass of parasites found within a fish. The
399 ventilation rate was repeatable when measured over 3 days, but had very low
400 discriminatory power.

401

402 Environmental DNA has recently emerged as a promising tool to detect aquatic species
403 that are sometimes at very low densities in past and present heterogeneous
404 environments (Bass et al. 2015). Detecting eDNA therefore finds application in ecology
405 and conservation ((Rees et al. 2014); (Thomsen and Willerslev 2015)). eDNA is also

406 combined with PCR approaches to detect pathogens in water (trematode *Ribeiroia*
407 *ondatrae*, pathogen of amphibians: (Huver et al. 2015); liver fluke: (Hashizume et al.
408 2017)). The originality of our method to detect an internal parasite lies in the fact that the
409 eDNA extraction was made from the internal fluids of the fish abdominal cavity instead of
410 water samples, as *S. solidus* is free to move in the abdominal cavity of its host,
411 potentially releasing cell-free DNA. We detected *S. solidus* eDNA in almost all infected
412 fish (98% of true positive). We found only 3 false negatives out of 35 infected
413 sticklebacks (1.98% of tested samples). In a preliminary test, we first tried to amplify *S.*
414 *solidus* eDNA from body cavity samples or from water samples by standard PCR and to
415 visualize the amplification products on an electrophoresis gel. For the body cavity
416 samples, the PCR approach was less sensitive compared to the qPCR method, with a
417 much higher false negative rate that made this simpler method unusable. For the water
418 samples from tanks of infected fish, *S. solidus* DNA was never detected by PCR
419 (although we could detect a stickleback positive control). We propose that
420 concentrations of *S. solidus* DNA are too low or absent to be detected in the excretions
421 of the fish.

422

423 All sticklebacks from the anadromous population (n=55) and 61 fish from the
424 Témiscouata population were free of infection by *S. solidus*. Our method did not yield
425 false positive, i.e. non-infected fish that would be detected as infected with our qPCR
426 results. False positive could have resulted from a lack of specificity of the primers used.
427 Thus, the absence of false positive for 116 samples confirms the specificity of the
428 method. A false positive could also have resulted from a signal of an exposed

429 stickleback, i.e. a fish that was exposed to a *S. solidus*-infected copepod, but for which
430 infection did not succeed and the worm did not develop in its abdominal cavity. The
431 absence of false positive in our data suggests that our method does not allow to detect
432 exposed sticklebacks. However, all the fish used were of wild origin, which prevents us
433 to have a record of exposure over the life of an individual fish such that we do not know
434 if any of our non-infected fish were exposed to this parasite. Testing this possibility of
435 laboratory-exposed fish could add to the usefulness of the present detection method.

436

437 The 3 false negatives (infected sticklebacks from the Témiscouata population for which
438 *S. solidus* DNA was not detected using qPCR) had a total parasite mass and a parasite
439 index above the minimal values from infected fish that were correctly detected. This
440 observation indicates that these false negatives are not due to a lack of sensitivity of the
441 method at low parasitic weight. We rather propose that the critical step of our method is
442 during the sampling of the internal fluids of the fish intraperitoneal cavity. As mentioned
443 previously, *S. solidus* is free in the abdominal cavity of its host. When the PBS washing
444 is done, it is possible that the worm may be too far from the needle (e.g. between two
445 organs of the stickleback) to allow the capture of its DNA. To overcome this issue, we
446 performed body cavity sampling on two consecutive days, and each time the needle was
447 inserted into a different face of the fish. This approach was quite reliable and probably
448 resulted in our low false negative rate. However, we suggest that the eDNA sampling
449 protocol could be extended over three days in order to maximize *S. solidus* DNA
450 capture. The correlation we found between the levels of *S. solidus* amplified eDNA (Ct
451 values) and the parasitic load suggest that in the future this approach could be used in a

452 quantitative way to estimate not only the presence but the total mass of parasites within
453 a host. The correlation is non linear and there is large variation in total parasite mass for
454 a given Ct value and further refinement would be needed. Therefore, our method in its
455 current state gives a very reliable presence/absence response and its use for
456 quantitative assessment looks promising for the future.

457

458 Ventilation rates of un-infected fish were in the same range as found in previous studies
459 of freshwater sticklebacks (Bell et al. 2010, Di Poi et al. 2016). The ventilation rate was
460 repeatable when measured over 3 days, which suggest that a single measurement gives
461 sufficient information on ventilation requirements of each individual at this life stage and
462 that there are significant inter-individual differences in physiology. However, ventilation
463 rates did not differ between infected and non-infected sticklebacks, although the lowest
464 and highest rates were found in un-infected and infected fish respectively and there was
465 a trend for ventilation rates to covary positively with the parasite index. Therefore, the
466 discriminatory power of this simple measurement was low and could only be used as a
467 complementary indicator of infection by *S. solidus* in its fish host.

468

469 We expect the eDNA method presented here to find applications beyond the *S. solidus*-
470 threespine stickleback system. Indeed, a lot of fish species, including fish of economic
471 importance, have parasites found in their abdominal cavity (Hoffman 1999). To apply our
472 method to these systems, the main challenge would be to design highly specific primers

473 for the parasite of interest (Taylor et al. 2013). Our low cost method could be a simple
474 alternative to morphological-based methods already used in these systems.

475

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482 lab first suggested to us to sample the stickleback abdominal cavity fluids to study the
483 *Schistocephalus* secretome, which led to this eDNA detection protocol.

484

485 **Authors' contributions**

486 C.S.B. designed the study with input from N.A.-H. C.S.B. performed the eDNA-qPCR
487 experiments and analyzed the data. N.A.-H. performed the ventilation rate
488 measurements and did the statistical analyses. C.S.B. and N.A-H. wrote the manuscript.

489

490 **Ethics**

491 This study was done in compliance with Animal Care and Use Program and was
492 approved by the Comité de Protection des Animaux de l'Université Laval (CPAUL
493 2014069-3).

Data availability

The complete dataset is available as a supplementary data file.

Competing interests

The authors declare no competing or financial interests.

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606

607

608 **Table 1. Fish distribution according to their infection status at dissection (infected**
609 **or uninfected by *S. solidus*) and their qPCR results (*S. solidus* eDNA detected or**
610 **not).** Numbers of fish from the Témiscouata population are indicated in normal font and
611 the ones from the anadromous Isle-Verte population are indicated in italic font.

612

	Status at dissection: infected	**Status at dissection: uninfected**
qPCR= detected as infected (*S. solidus* eDNA)	32; *0*	0; *0*
qPCR= detected as uninfected (no *S. solidus* eDNA)	3; *0*	61; *55*

613

614

615 **Table 2. Infection by *S. solidus* in the Témiscouata population.** Minimum, maximum
616 and median values for number of parasites, total parasite mass and parasite index.
617 Infected fish ID T12 was not included in calculations of min, max, median (no specific
618 data available other than known presence of worms).

number of infected fish	number of parasites per fish			total parasite mass (mg)			parasite index (PI, %)		
	min	max	median	min	max	median	min	max	median
35	1	6	1.5	130	2356	495	4.9	40.4	16.6

619

620

621 **Figures**

622 **Figure 1. External morphology of threespine sticklebacks from Lac Témiscouata**
623 **that were infected by *S. solidus* (single or multiple infections) or not. A.** Non-
624 infected fish. **B.** Infected fish with 1 *S. solidus* worm (parasite mass 618.2 mg; PI 13.2).
625 **C.** Infected fish with 5 *S. solidus* worms (total parasite mass 2356 mg; PI 39.3). A and B
626 do not exhibit abdominal distension while abdominal distension is clearly visible in C.
627 Fish B and C were both detected as infected using the E-DNA method.

628

629 **Figure 2. eDNA concentrations covary with total parasite mass**. Low Ct values,
630 which represent high levels of eDNA, are associated with a high parasite mass
631 (Spearman correlation, rho = -0.41, p = 0.01, n= 31).

632

633 **Figure 3. Ventilation rates do not differ significantly between un-infected and**
634 **infected threespine sticklebacks** (two-sided t-test, t = -1.57, p = 0.15, n=20).
635 Ventilation per minute ranged between 99 and 146 opercular movement per minute in
636 un-infected fish (n=7) and between 112 and 160 in *S. solidus* infected fish (n=13). The
637 lower and upper hinges correspond to the 25th and 75th percentiles and the vertical
638 bars extend to the largest value no further than 1.5 times the interquartile range.

639

640

641

Figure 1

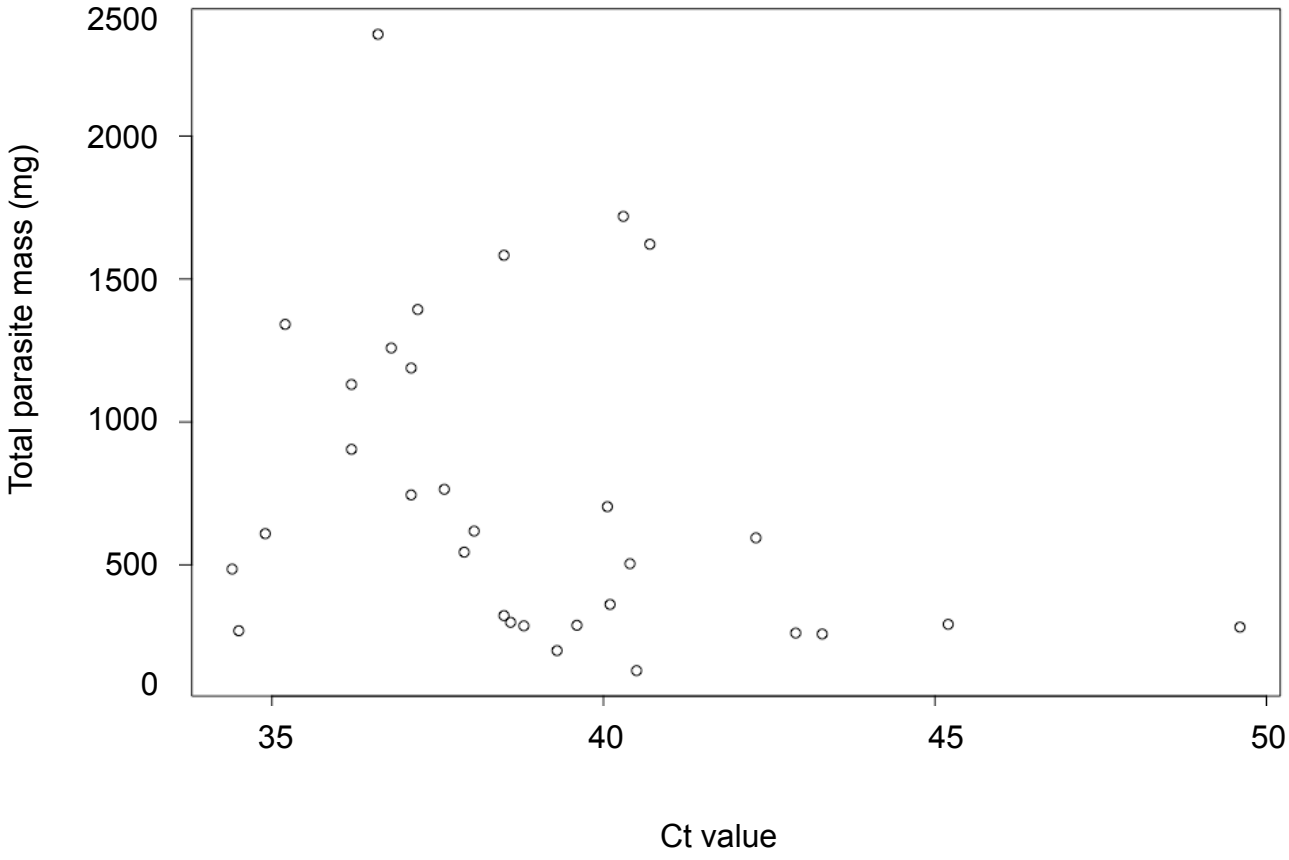
2500
2000
1500
1000
500
0
Total parasite mass (mg)
35
40
45
50
Ct value

Figure 2

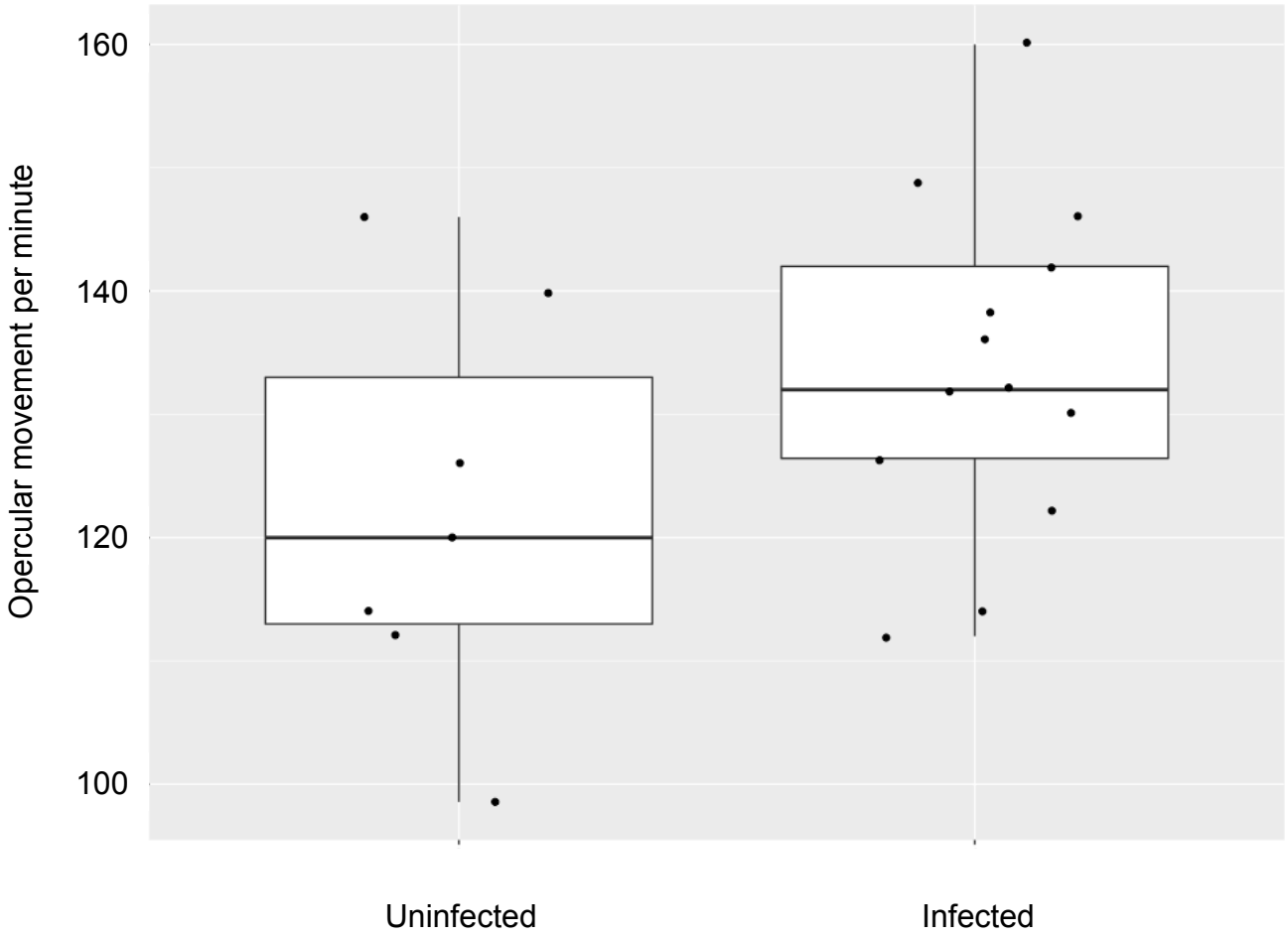
160
140
120
100
Opercular movement per minute
Uninfected
Infected

Figure 3