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

Running title: a eDNA method to detect an internal parasite

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## ABSTRACT

Detecting the presence of a parasite within its host is crucial to the study of host-parasite interactions. The *Schistocephalus solidus* - threespine stickleback pair has been studied extensively to investigate host phenotypic alterations associated with a parasite with a complex life cycle. This cestode is localized inside the stickleback's abdominal cavity and can be visually detected only once it passes a mass threshold. We present a non-invasive quantitative PCR approach based on detection of environmental DNA from the worm (eDNA), sampled in the fish abdominal cavity. Using this approach on two fish populations (n=151), 98% of fish were correctly assigned to their *S. solidus* infection status. There was a significant correlation between eDNA concentration and total parasitic mass. We also assessed ventilation rates as a complimentary mean to detect infection. Our eDNA detection method gives a reliable presence/absence response and its future use for quantitative assessment is promising.

## 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 track the parasite during its development in the hosts. One of the model systems for investigating phenotypic alterations of the intermediate host infected by a parasite with a complex life cycle is the *Schistocephalus solidus*-threespine stickleback pair (Barber, 2013). *S. solidus* is a freshwater flatworm that has to infect three distinct hosts to survive and reproduce: a copepod, the threespine stickleback, and a fish-eating bird. The threespine stickleback *Gasterosteus aculeatus* 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 small worm penetrates the intestine wall of the fish and migrates into the abdominal cavity (Hammerschmidt and Kurtz, 2007) where it shows a sigmoid growth curve during the first twelve weeks (Barber and Svensson, 2003). When the worm has gained a sufficient mass, it can reproduce in its avian final host (Tierney and Crompton, 1992). Previous studies demonstrated that *S. solidus* infection has major impacts on the stickleback energy demands, physiology, immunity and behaviour (Barber and Scharsack, 2010).

The entire life cycle of this parasite can be reproduced in the laboratory (Barber and Scharsack, 2010). However, the success rate 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

53 caught in the wild is high (Weber et al., 2017), which means that wild individuals
54 considered as uninfected controls may turn out to be infected. A simple, reliable and
55 non-invasive detection method would allow selecting, or avoiding, infected individuals
56 depending on the needs of a specific project. This would allow minimizing the number of
57 individuals used in an experimental procedure, an important aspect for the refinement of
58 animal use protocols.

59

60 Available methods to detect sticklebacks parasitized by *S. solidus* relies on
61 morphological modifications that are exacerbated with an increase in parasitic load
62 (Dingemanse et al., 2009). A visual analysis of the level of the abdominal distension of
63 the fish allows to predict the infection status and the parasite index (PI, the proportion of
64 infected fish weight that is contributed by parasite tissue) (Barber, 1997). However, this
65 visual approach is less reliable when the stickleback is infected by a single worm or by a
66 few small worms, which do not induce clear morphological changes. Furthermore, some
67 confounding factors can impact the level of abdominal distension, including eggs and
68 large organs (Barber, 1997). In this context, it is necessary to develop a method that will
69 not be based on morphological criteria.

70

71 Our objective was to design a non-lethal method to detect *S. solidus* in the abdominal
72 cavity of its threespine stickleback host. We used two complementary approaches:
73 detection of environmental DNA (eDNA) and measurement of a physiological trait. Our
74 first hypothesis was that *S. solidus* produces cell-free DNA in the fish abdominal cavity,

and that this eDNA could be identified by a PCR approach to correctly detect *S. solidus* infection in sticklebacks (Bass et al., 2015). eDNA produced by parasites is a common target in epidemiology to detect infection using a PCR approach in organic samples like serum, feces (Pontes et al., 2002; Xu et al., 2017) or plasma (Imwong et al., 2015). eDNA is also used to detect invasive species in aquatic environments (Takahara et al., 2013). We thus tested a non-invasive quantitative real-time PCR method (qPCR), based on eDNA extraction from fluids of the stickleback abdominal cavity. We designed primers that amplify a gene sequence from the *S. solidus* genome but not stickleback DNA. We tested if we could assign a fish to a given infection status. Since eDNA has been previously used to make quantitative predictions about the biomass of organism detected (Lacoursiere-Roussel et al., 2016), we also tested if eDNA concentration covaried with the total mass of the parasite(s) present within the abdominal cavity of an individual.

Our second hypothesis was that infected fish would show a significantly higher ventilation rate compared to uninfected ones, which could be used to discriminate them. It has been shown that oxygen consumption is higher in infected sticklebacks compared to uninfected ones for a given swimming speed (Lester, 1971) and for fish of similar mass (Meakins and Walkey, 1975). We thus tested the use of the ventilation rate as a rapid and non-invasive indicator of *S. solidus* infection, using a previously published protocol to measure ventilation in stickleback (Di Poi et al., 2016).

## MATERIALS AND METHODS

### Populations of interest

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

### Fish sampling and rearing

Individuals were caught using a seine and minnow traps in Lac Témiscouata as adults in July 2016 and September 2017, and as juveniles in August 2016, Juvenile sticklebacks from the anadromous Isle-Verte salt marsh population were sampled in tide pools in July 2016 using hand nets. Sticklebacks were brought to the “Laboratoire Regional des Sciences Aquatiques” at Université Laval. Adults and juveniles from each population were kept in separate 80 L-tanks under a Light:Dark photoperiod and a temperature of 15°C, reflecting the conditions in their natural environments (Québec, Canada). For anadromous fish, salinity was maintained at 28 ppt. Fish were fed daily with brine shrimps. All sticklebacks were adults at the start of the experiment. They were isolated in 2 L-tanks and were assigned an identification number. 151 sticklebacks were used: 96 from Lac Témiscouata and 55 from Isle-Verte.

120

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

122 We sampled the internal fluids of the fish within the intra-peritoneal cavity to obtain
123 eDNA. The fish of interest was taken out of its tank and placed on a sponge. The needle
124 of a syringe filled with 100 µL of PBS (Phosphate Buffered Saline, pH 7.4 Life
125 Technologies) was inserted into its abdominal cavity and PBS was injected. Then,
126 without removing the needle from the abdominal cavity, the plunger of the needle was
127 pulled back until the syringe filled with the PBS that was just injected (up to 100 µL).
128 After the needle was removed, the fish was directly put back in its tank. Between 10 and
129 100 µL of PBS was obtained and directly added to a tube of 700 µL of Longmire lysis
130 preservation buffer (Longmire et al., 1997). This protocol was done twice for each fish on
131 two consecutive days. On the second day, the PBS used to wash the abdominal cavity
132 was added to the same Longmire tube as the first sample (i.e. up to 200 µL in the 700
133 µL Longmire tube). Each day, the needle was inserted into a different face of the fish.
134 Abdominal cavity fluid samples were placed at 4°C between the first and the second
135 day, and during the night following the second day. Samples were kept at -20°C before
136 DNA extraction. Fish were fed with brine shrimps after the sampling of the abdominal
137 cavity.

138

139 After these two samplings, fish were euthanized with an overdose of MS-222 (75 mg/L
140 mg/kg) followed by exsanguination and dissected to determine if they were infected by
141 *S. solidus*. Fish sex, size and mass, and *S. solidus* mass and number in each fish were

142 noted. The parasite index was calculated using the formula (total weight (mg) of
143 parasites / Total weight (mg) of host plus parasites) x 100. A value of 50 indicates a
144 situation where the total weight of parasites is equal to the net weight of the stickleback
145 (Arme and Owen, 1967).

146

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

149

150 **DNA extraction from samples**

151 DNA extraction from the abdominal cavity samples was performed according to an
152 environmental DNA method previously developed (Lacoursière-Roussel et al., under
153 revision), with small modifications to the protocol. To summarize, 30 µL of proteinase K
154 (4 mg/mL) (VWR) was directly added to a Longmire buffer sample and the tube was
155 incubated at 55°C for the night. The sample was transferred into a new 2 mL tube and
156 950 µL of phenol chloroform isoamyl alcohol (Invitrogen) was added. After shaking and
157 centrifugation, supernatant was transferred into a new tube and 950 µL of chloroform
158 (VWR) was added. After shaking and centrifugation, a maximum of 750 µL of the
159 supernatant was put into a new tube. 750 µL of ice cold isopropanol (Fisher Scientific)
160 and 375 µL of room temperature 5M sodium chloride NaCl were added to the tube,
161 which was placed at -20°C for the night. After centrifugation, all liquid was removed from
162 the tube and 1500 µL of 70% ethanol was added. After centrifugation, ethanol was
163 removed and the tube was air-dried. The DNA pellet was resuspended in 80 µL water

164 and the tube was placed at 55°C for 10 minutes. The tube was placed at 4°C for the
165 night and then stored at -20°C.

166

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

168 A pair of primers that amplifies a sequence from a nuclear gene (GEEE01010589.1
169 TSA: Schistocephalus solidus ssol_TR119934_c5_g1_i2 transcribed RNA sequence)
170 from the *S. solidus* genome was designed. The gene sequence was obtained from the
171 de novo transcriptome of *S. solidus* (sequence available on NCBI:
172 https://www.ncbi.nlm.nih.gov/nuccore/GEEE01010589.1?report=fasta) and does not
173 have known homologues in other species (Hébert et al., 2016). The only available
174 information on the function of this gene is that it is highly expressed during early
175 developmental stages of the worm when it is not able to reproduce (Hebert et al., 2017).
176 We targeted the DNA sequence of this gene with the forward for-
177 CGGATTGTCTTCTCGTTGTA and reverse rev-GGACAACCACTGTCCACTAA primers,
178 designed with Primer3 (Untergasser et al., 2012). The primers generated a 200 bp
179 amplicon. We verified with the Basic Local Alignment Search Tool (nucleotide BLAST)
180 on NCBI that the 200 bp amplicon did not align with other sequences except for *S.*
181 *solidus*.

182

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

184 Polymerase Chain Reaction (PCR) assays were conducted on genomic DNA samples to
185 confirm that the designed primers amplified only *S. solidus* DNA and not threespine

186 stickleback DNA. DNA was extracted from *S. solidus* and stickleback samples (Lac
187 Témiscouata) kept at -20°C in ethanol 90%. Tissue samples of 0.3 $cm^2$ (*S. solidus* body,
188 stickleback tail) were used for the extraction. Extraction was performed using a salt
189 method (Aljanabi and Martinez, 1997). PCR reaction was performed in a total volume of
190 24.3 µL (13.75 µL of nuclease free water, 1 µL of 10mM dNTPs, 0.25 µL of TAQ DNA
191 polymerase (5U/µL Bio Basic), 2.5 µL of 10x TAQ buffer with $MgCL_2$, 5 µL of DNA (*S.*
192 *solidus* or stickleback) and 1.8 µL of primer mix (4.56 µM of each primer)). Two negative
193 controls were done (no primers, no DNA). The PCR program was realized with a
194 Mastercycler (Eppendorf) under these conditions: a denaturation step of 2 min at 95°C,
195 an annealing and elongation step of 40 cycles including 20 sec at 95°C, 40 sec at 56°C
196 and 60 sec at 72°C, and a final step of 10 min at 72°C. PCR products were visualized on
197 a 2% agarose gel using SYBR safe staining (Invitrogen).

198

199 **qPCR assays**

200 eDNA extracted from the abdominal cavity samples of fish was amplified with a real-time
201 quantitative polymerase chain reaction (qPCR) using the designed primers specific to *S.*
202 *solidus*. Each 96-well plate included eDNA samples extracted from the body cavity and a
203 standard curve obtained from a pool of *S. solidus* genomic DNA (3 individuals) diluted at
204 different concentrations. The concentrations of the 7-point standard curve ranged from
205 0.9 ng/µL to 0.01 ng/µL with a dilution factor of 2. qPCR reaction was performed in a
206 qPCR mix including 12.5 µL of PowerUp SYBR Green Master mix (Life Technologies)
207 and 1 µL of primer mix (4.56 µM of each primer). For the standard curve, genomic DNA
208 samples, 6.5 µL of RNAse free water and 5 µL of DNA were added to the qPCR mix. For

209 the eDNA samples, no water was used and 11.5 µL of solution containing the
210 resuspended eDNA was added to the qPCR mix (for a total qPCR mix volume of 25 µL
211 in each case). Two negative controls (no primers, no DNA) were done in each plate.
212 Each reaction was done in triplicate. qPCR was performed with a 7500 Real-Time PCR
213 system (Life Technologies). The amplification was realized according to the conditions
214 described by (Lacoursière-Roussel et al., 2016): 2 min at 50 °C, 10 min at 95 °C,
215 followed by 70 cycles of 15 sec at 95 °C and 60 sec at 56 °C. A melt curve protocol was
216 then performed with temperature going from 60 celsius to 95 celsius to detect primer
217 dimers, check specificity by determining that a single amplicon was produced, and
218 obtain a Tm value for that sample, which can be compared to the standard curve of
219 *Schistocephalus* DNA.

220

221 **Ventilation rate measurements**

222 A subset of individuals used in the *S. solidus* eDNA detection experiment was used to
223 quantify ventilation rates (captured in Lac Témiscouata in July 2016, n=20). Ventilation
224 measurements were repeated three days in a row with individuals sampled in the same
225 order each day. For each individual fish, a 250 mL beaker was filled with 50 mL of clean
226 water from the supply used to fill experimental 2 L-tanks. The individual was filmed from
227 the side (JVC Everio camera) to be able to detect opercular movement. At the end of the
228 three days of measurements, these fish were euthanized and their infection status
229 determined. Dissection showed that 13 individuals were infected and 7 were parasite-
230 free. All the infected fish were also detected as infected by the eDNA approach (see
231 below). Films were subsequently analyzed at 0.46 X speed by an observer (NAH) blind

232 to treatment using the open source VLC Media Player (Videolan). The first 30 seconds
233 of the movie where the fish was clearly visible were used to measure ventilation rates. If
234 the individual moved in a position where it was not visible during the first-30-second
235 sampling period, the ventilation measurement was made for the usable seconds within
236 that period and reported back to 30 seconds. The observer repeated all measurements
237 for the first day of sampling to assess accuracy. Measures done twice on the same fish
238 on the same video were highly correlated (Spearman r = 0.99) with most samples
239 having the exact same number of ventilations when measured twice, with a maximum
240 difference of 3 ventilation movements and an average difference of 0.7. A single
241 measurement per video was thus used. A ventilation rate was calculated by dividing the
242 number of ventilation measured in 30 seconds by 30. This measurement was used for
243 further statistical analyses. The total number of ventilation measured during a 30 second
244 interval was used to estimate the number of ventilation over 1 minute for graphical
245 purposes and for comparison to previous studies.

246

247 **Analyses**

248 Following qPCR amplification, the presence or the absence of *S. solidus* eDNA in the
249 body cavity samples was determined by comparing the Ct values (amplification curves)
250 and the Tm values (melt curves) of the samples with the ones of the standard curves. A
251 Ct ("cycle threshold") is the cycle at which a sample reaches a preset threshold of
252 fluorescence, which is the proxy for the quantity of eDNA. A sample with a low Ct means
253 that there were more templates in the original sample and that it thus reached the
254 threshold sooner in the 70 PCR cycles. A body cavity sample showing at least one of its

255 Ct values (among the triplicates) above 0 in combination with the associated Tm
256 value(s) close (+/- 2) to the mean Tm of the standard curve (79.64) was considered
257 positive for *S. solidus* eDNA. On the contrary, if a Ct value above 0 was associated with
258 a Tm value more distant to the mean Tm of the standard curve, then this value was
259 considered negative for *S. solidus* eDNA and the result of primer dimer formation.
260 Finally, if the body cavity sample had only "undetermined" Ct values, it was considered
261 negative for *S. solidus e*DNA, irrespectively of Tm values. For each fish, qPCR results
262 were analyzed with regard to its infection status (parasitized or not by *S. solidus* upon
263 dissection). A true positive was obtained when there was concordance between the
264 infection status and the qPCR results. A false negative was defined when the fish was
265 infected by *S. solidus*, but no traces of *S. solidus* eDNA were found using qPCR. A false
266 positive was associated with fish not infected by *S. solidus* but for which *S. solidus*
267 eDNA was inferred using qPCR results. All individual results are found in supplementary
268 data.

269

270 Statistical analysis was done in R version 3.3.3 (R Core Team, 2017) using R Studio
271 (Version 1.0.136) and the ggplot2 package (Wickham, 2009). To determine if our
272 method allows a quantitative estimate of parasite mass, we tested if the eDNA
273 concentration obtained for infected fish (using the Ct value) negatively correlated with
274 their total parasite mass, using a non-parametric Spearman correlation (a small Ct value
275 should be associated with a larger parasite mass and vice-versa if the eDNA method is
276 quantitative). We used the lowest Ct measured for a given fish if more than one triplicate
277 amplified DNA. Repeatability of ventilation rates over the three days was calculated

278 following a previously developed method (Lessells and Boag, 1987). We compared
279 ventilation rates between infected and non-infected individuals using a t-test, as they
280 followed a normal distribution (based on a Shapiro-wilk test). We tested if ventilation
281 rates covaried positively with the parasite load (parasite index) using a non-parametric
282 correlation (Spearman correlation).

283

284 **RESULTS AND DISCUSSION**

285 In parasite-host system studies, being able to detect a parasite during its development in
286 the host is important to better understand their interactions. Here, we demonstrate the
287 power of a method based on detecting eDNA from an internal parasite directly sampled
288 from the abdominal cavity of its specific intermediate host.

289

290 **Primers specificity**

291 PCR tests performed with primers that target a sequence from the *S. solidus* nuclear
292 gene NCBI ID GEEE01010589.1 confirmed the amplification of *S. solidus* genomic DNA,
293 and not of threespine stickleback genomic DNA. The PCR product was sequenced using
294 Sanger ABI3730xl (Centre Hospital Universitaire du Québec) to confirm the amplification
295 of the 200 pb amplicon in the *S. solidus* genome. Controls (no template, no primers)
296 were negative as expected.

297

298 **Detection of *S. solidus* eDNA in the abdominal cavity of infected fish**

299 qPCR was performed in combination with a eDNA extraction protocol to try to detect *S.*
300 *solidus* presence in the stickleback abdominal cavity. Standard curves realized with a
301 pool of *S. solidus* genomic DNA samples diluted at different concentrations had mean Ct
302 values ranging from 29.65 (highest concentration) to 36.86 (lowest concentration) and a
303 mean Tm value of 79.64. All the controls were negative. The earliest Ct value we
304 obtained for abdominal cavity eDNA samples with our protocol and amount of starting
305 material varied between 34.4 and 49.6 for positively identified individuals. Using this
306 approach on two fish populations, 148 fish out of 151 were correctly assigned to their
307 infection status (infected or not by *S. solidus*, 98 % of true positive).

308

309 In the Témiscouata population, 35 fish out of 96 were infected by *S. solidus.* Single and
310 multiple infections (up to 6 worms) were reported upon dissection, with a median of 1.5
311 worm, illustrating the predominance of single or double infections. Note that one fish
312 harboured 12 worms, but we did not obtain reliable mass for the worms and as such is
313 included in the dataset only to calculate the infection status assignation rate. Among the
314 infected sticklebacks, 18 exhibited a single worm upon dissection and no external visual
315 clues suggested the presence of *S. solidus* in the fish body cavity (Figure 1). For all
316 infected fish, the total parasite mass was comprised between 130 mg and 2356 mg
317 (median of 495 mg) and the parasite index was always below 50 (between 4.9 and 40.4,
318 median = 16,6%), meaning that the total weight of parasites was always below the net
319 weight of the fish host. 32 out of the 35 infected fish were successfully detected as
320 infected using qPCR. For the 3 remaining infected fish, no trace of *S. solidus* eDNA was
321 detected with our protocol (1.98% of samples tested, low false negative rate). They had

322 no eDNA detected in any of the triplicates (Ct “undetermined”). The 3 fish had a total
323 parasite mass that span almost the whole range found in the study (168 mg and 274 mg
324 for the single infections and 1644 mg for the double infection, from the second smallest
325 to the third largest). Their parasite index (respectively 12.5, 6.5 and 25.3) was above the
326 minimal values from infected fish that were correctly detected. These observations
327 indicate that these false negatives are not due to a lack of sensitivity of the method at
328 low parasitic weight. We rather propose that the critical step of our method is during the
329 sampling of the internal fluids of the fish intraperitoneal cavity. *S. solidus* is free in the
330 abdominal cavity of its host. It is possible that the PBS injection was done too far from
331 the worm to allow the sampling of its DNA. To overcome this issue, we performed body
332 cavity sampling on two consecutive days, and each time the needle was inserted into a
333 different face of the fish. This approach was quite reliable and probably resulted in our
334 low false negative rate. However, we suggest that the eDNA sampling protocol could be
335 extended over three days in order to maximize *S. solidus* DNA capture.

336

337 None of the fish from the anadromous population were infected by *S. solidus* (n=55). We
338 never detected *S. solidus* DNA in the samples of the uninfected fish from this population,
339 nor from the 61 uninfected fish from Lac Témiscouata (0 false positive). False positive
340 could have resulted from a lack of specificity of the primers used. The absence of false
341 positive for 116 samples confirms the specificity of the method. A false positive could
342 also have resulted from a signal of an exposed stickleback, i.e. a fish that was exposed
343 to a *S. solidus*-infected copepod, but for which infection did not succeed and the worm
344 did not develop in its abdominal cavity. The absence of false positive suggests that our

345 method does not allow to detect sticklebacks that have been exposed but that did not
346 develop an infection. However, all the fish used were of wild origin, which prevents us to
347 have a record of exposure over the life of an individual fish. Testing this possibility with
348 laboratory-exposed fish could add to the usefulness of the present detection method.

349

350 We found a significant correlation between Ct values (which are inversely correlated to
351 the amount of *S. solidus* eDNA) and the total parasite mass found within a fish (one-
352 sided Spearman correlation, rho= -0.41, p = 0.01, Figure 2). This result suggests that in
353 the future this approach could be used in a quantitative way to estimate not only the
354 presence but the total mass of parasites within a host. The correlation is non-linear and
355 there is large variation in total parasite mass for a given Ct value and thus further
356 refinement would be needed. Therefore, our method in its current state gives a very
357 reliable presence/absence response and its use for quantitative assessment looks
358 promising for the future.

359

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

361 We measured the ventilation rates of 20 fish, 3 times over 3 consecutive days, and then
362 determined that 13 of these were infected. The infected individuals harboured from 1 to
363 5 worms with a total mass ranging between 618 mg and 2356 mg (parasite index
364 between 13.2 and 40.4). One infected individual died after the first ventilation
365 measurement and we could use the videos for only the 2 first days for one of the non-
366 infected fish. We had 3 ventilation measurements for all other individuals. Repeatability

367 of ventilation across 3 days for a given fish was 0.41 (Lessells and Boag, 1987). As
368 ventilation rates were repeatable over this time scale, we used ventilation rates
369 measured on the first day in subsequent analysis, to reflect how this method would be
370 used to quickly detect infection. Ventilation rates estimated over 30 seconds and
371 reported in number of opercular movement per minute ranged between 99 and 146
372 opercular beats/min in uninfected fish and between 112 and 160 in *S. solidus* infected
373 ones (Figure 3). The average number of ventilation per minute was 122 for uninfected
374 fish and 134 for infected fish. Ventilation rates of uninfected fish were in the same range
375 as found in previous studies of freshwater sticklebacks (Bell et al., 2010; Di Poi et al.,
376 2016). We found that ventilation rates did not differ significantly between non-infected
377 and infected individuals (two-sided t-test, $t = -1.57$, $p = 0.15$, $n = 20$, Figure 3).
378 Ventilation was positively correlated to the parasite index, but this covariation was not
379 significant (one-sided Spearman correlation, rho = 0.35, $p = 0.07$, $n=20$). There was a
380 substantial overlap in ventilation rate between the two groups, which prevented the use
381 of this trait to discriminate between *S. solidus* infected and healthy individuals.
382 Therefore, the power of this simple measurement is low and can only be used as a
383 complementary indicator of infection by *S. solidus* in its host.

384

385 We expect the eDNA method presented here to find applications beyond the *S. solidus*-
386 threespine stickleback system. Indeed, several fish species have parasites found in their
387 abdominal cavity (Hoffman, 1999). To apply our method to these systems, the main
388 challenge would be to design highly specific primers for the parasite of interest (Taylor et

al., 2013). Our low cost method could be a simple alternative to morphological-based methods already used in these systems.

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## Competing interests

The authors declare no competing or financial interests.

## Author contributions

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

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412 **Ethics**

413 This study was approved by the Comité de Protection des Animaux de l'Université Laval
414 (CPAUL 2014069-3).

415

416 **Data availability**

417 The complete dataset is available as a supplementary data file.

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515 **Figures**

516 **Figure 1. External morphology of threespine sticklebacks from Lac Témiscouata**
517 **that were infected by *S. solidus* (single or multiple infections) or not. A.** Non-
518 infected fish. **B.** Infected fish with 1 *S. solidus* worm (parasite mass 618.2 mg; PI 13.2).
519 **C.** Infected fish with 5 *S. solidus* worms (total parasite mass 2356 mg; PI 39.3). A and B
520 do not exhibit abdominal distension while it is clearly visible in C. Fish B and C were
521 both detected as infected using the eDNA method.

522

523 **Figure 2. eDNA concentration covaries with total parasite mass**. Low Ct values,
524 which represent high levels of eDNA, are associated with a high parasite mass
525 (Spearman correlation, rho = -0.41, p = 0.01, n= 31).

526

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

533

Figure 1

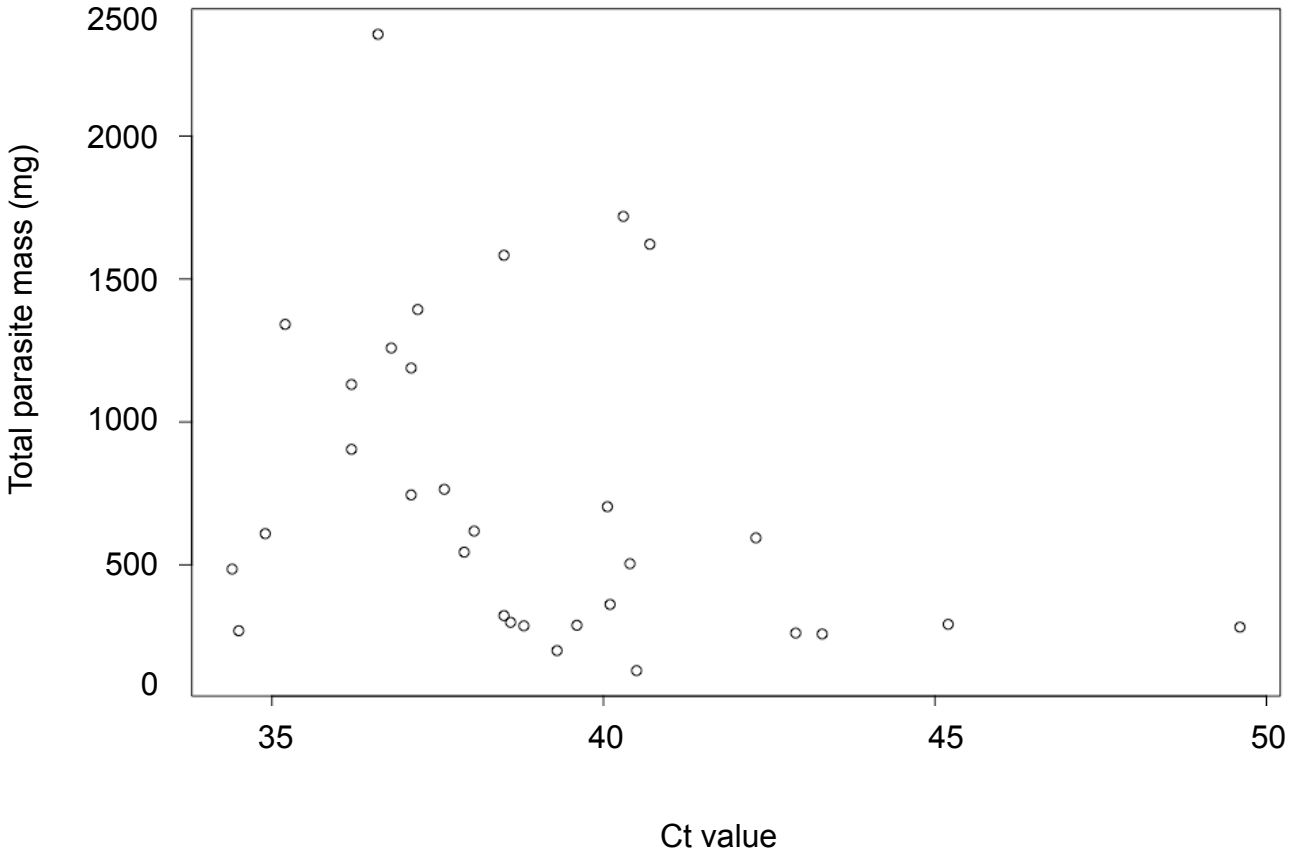
2500
2000
1500
1000
500
0
Total parasite mass (mg)
35
40
45
50
Ct value

Figure 2

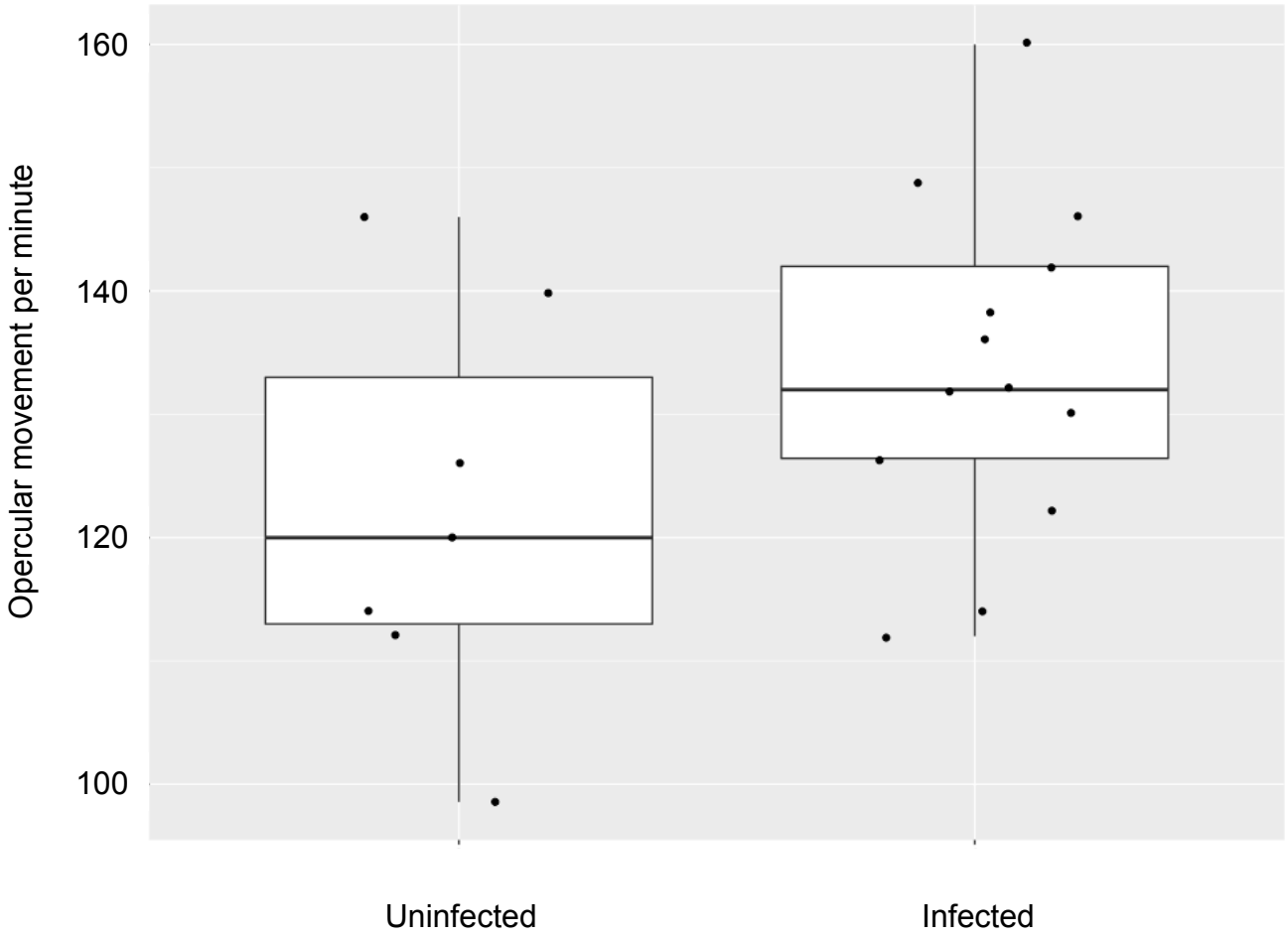
160
140
120
100
Opercular movement per minute
Uninfected
Infected

Figure 3