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METHODS AND TECHNIQUES

An eDNA-qPCR assay to detect the presence of the parasite *Schistocephalus solidus* inside its threespine stickleback host

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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-lethal quantitative PCR (qPCR) 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 rate as a complementary mean to detect infection. Our eDNA detection method gives a reliable presence/absence response and its future use for quantitative assessment of infection is promising.

KEY WORDS: Cestode, *Gasterosteus aculeatus*, Parasitic load, 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–host interactions requires tracking the parasite during its development in the host. One model system 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). *Schistocephalus solidus* Müller 1776 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* Linnaeus 1758 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 h of 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 12 weeks (Barber and Svensson, 2003). When the worm has gained 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's energy demands, physiology, immunity and behaviour (Barber and Scharsack, 2010).

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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 caught in the wild is high (Weber et al., 2017), which means that wild individuals considered to be uninfected controls may turn out to be infected. A simple, reliable and non-lethal detection method would allow selection, or avoidance, of infected individuals, depending on the needs of a specific project. This would allow the number of individuals used in an experimental procedure to be minimized, an important aspect for the refinement of animal-use protocols.

Available methods to detect sticklebacks parasitized by *S. solidus* rely on morphological modifications that are exacerbated by an increase in parasitic load (Dingemanse et al., 2009). A visual analysis of the level of the abdominal distension of the fish allows prediction of the infection status and the parasite index (PI, the proportion of infected fish mass that is contributed by parasite tissue) (Barber, 1997). However, this visual approach is less reliable when the stickleback is infected by a single small worm or by a few small worms. Furthermore, some confounding factors can impact the level of abdominal distension, including eggs and large organs (Barber, 1997). In this context, it is necessary to develop a method that will not be based on morphological criteria.

Our objective was to design a non-lethal method to detect *S. solidus* in the abdominal cavity of its threespine stickleback host. We used two complementary approaches: detection of environmental DNA (eDNA) and measurement of a physiological trait. Our first hypothesis was that *S. solidus* produces cell-free DNA or cell-bound 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, faeces or plasma (Imwong et al., 2015; Pontes et al., 2002; Xu et al., 2017). eDNA is also used to detect invasive species in aquatic environments (Takahara et al., 2013). We thus tested a non-lethal quantitative real-time PCR method (qPCR), based on eDNA extraction from fluids of the stickleback abdominal cavity, using primers designed to amplify a gene sequence from the *S. solidus* genome but not stickleback DNA. We tested whether we could assign a fish to a given infection status based on the qPCR results. As eDNA has been previously used to make quantitative predictions about the biomass of organism detected (Lacoursière-Roussel et al., 2016), we also tested whether the eDNA concentration co-varied 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 with uninfected ones, which could be used to discriminate between them. It has been

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shown that oxygen consumption is higher in infected sticklebacks than in 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 ventilation rate as a rapid and non-lethal indicator of *S. solidus* infection, using a previously published protocol to measure ventilation in sticklebacks (Di Poi et al., 2016).

MATERIALS AND METHODS

Populations of interest

This study was conducted on two populations of threespine sticklebacks. The first population was at Lac Témiscouata, QC, Canada (47°40′33″N, 68°50′15″W, freshwater environment), where sticklebacks are known to be infected by *S. solidus* and to show typical behaviour alterations (Grecias et al., 2018). The second population was composed of anadromous fish from a salt marsh at Isle-Verte, QC, Canada (48°1′0″N, 69°26′59″W; salinity 22–26 ppt; Poulin and FitzGerald, 1989), for which infection by *S. solidus* is not expected, as the worm does not tolerate high salinity (Mačát 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 (QC, 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. In total, 151 sticklebacks were used: 96 from Lac Témiscouata and 55 from Isle-Verte.

Sampling eDNA from the host abdominal cavity

We sampled the internal fluid of the fish within the intra-peritoneal cavity to obtain eDNA. The fish of interest was taken out of its tank and placed on a sponge. The needle of a syringe (U-100 BD micro-fine IV insulin syringes with single-unit graduations, 28 gauge, 1 ml volume, 12.7 mm needle length) filled with 100 µl of phosphate-buffered saline (PBS, pH 7.4; Life Technologies, Carlsbad, CA, USA) was inserted into its abdominal cavity and PBS was injected. Then, without removing the needle from the abdominal cavity, the plunger of the needle was pulled back until the syringe filled with the PBS that had just been injected (up to 100 µl). After the needle was removed, the fish was put directly back into its tank. Between 10 and 100 µl of PBS was obtained and directly added to a tube of 700 µl of Longmire lysis preservation buffer (Longmire et al., 1997). This protocol was done twice for each fish on 2 consecutive days. On the second day, the PBS used to wash the abdominal cavity was added to the same Longmire tube as the first sample (i.e. up to 200 µl in the 700 µl Longmire tube). The needle was inserted into a different side of the fish on each sampling day. During eDNA sampling, fish were not anaesthetized as manipulation took less than 1 min. Abdominal cavity fluid samples were placed at 4°C between the first and the second day, and during the night following the second day. Samples were kept at −20°C before DNA extraction. Fish were fed with brine shrimps after sampling of the abdominal cavity.

After these two samplings, fish were killed with an overdose of MS-222 (75 mg l^{-1}) followed by exsanguination and were dissected to determine whether they were infected by *S. solidus*. Fish sex, size and mass, and *S. solidus* mass and number in each fish were noted. Fish were weighed to the nearest 0.01 g and parasites to the nearest 0.1 mg. The PI was calculated using the formula [total mass of parasites (mg)/total mass of host plus parasites (mg)]×100. A value of 50 indicates a situation where the total mass of parasites is equal to the net mass of the stickleback (Arme and Owen, 1967).

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

DNA extraction from samples

DNA extraction from the abdominal cavity samples was performed according to an eDNA method previously developed (Lacoursière-Roussel et al., 2018), with small modifications to the protocol. To summarize, 30 µl of proteinase K (4 mg ml^{-1}; VWR, Radnor, PA, USA) was directly added to a Longmire buffer sample and the tube was incubated at 55°C overnight. The sample was transferred into a new 2 ml tube and 950 µl of phenol:chloroform: isoamyl alcohol (Invitrogen, Carlsbad, CA, USA) was added. After shaking and centrifugation, the supernatant was transferred into a new tube and 950 µl of chloroform (VWR) was added. After shaking and centrifugation, a maximum of 750 µl of the supernatant was put into a new tube; 750 µl of ice-cold isopropanol (Fisher Scientific, Hampton, NH, USA) and 375 µl of room-temperature 5 mol l^{-1} NaCl were added to the tube, which was placed at −20°C overnight. After centrifugation, all liquid was removed from the tube and 1500 µl of 70% ethanol was added. After centrifugation, the ethanol was removed and the tube was air-dried. The DNA pellet was resuspended in 80 µl water and the tube was placed at 55°C for 10 min, then at 4°C overnight, and stored at −20°C.

Design of primers specific to the *Schistocephalus solidus* genome

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

PCR assays to determine specificity of amplification

PCR assays were conducted on genomic DNA samples to confirm that the designed primers amplified only *S. solidus* DNA and not threespine stickleback DNA. DNA was extracted from *S. solidus* and stickleback samples (Lac Témiscouata) kept at −20°C in 90% ethanol. Tissue samples of 0.3 cm^2 (*S. solidus* body, stickleback tail) were used for the extraction, which was performed using a salt method (Aljanabi and Martinez, 1997). The PCR reaction was performed in a total volume of 24.3 µl [13.75 µl of nuclease-free water, 1 µl of 10 mmol l^{-1} dNTPs, 0.25 µl of Taq DNA polymerase (5 U $µl^{-1}$ Bio Basic, Markham, ON, Canada), 2.5 µl of 10× Taq buffer with $MgCl_2$, 5 µl of DNA (*S. solidus* or stickleback) and 1.8 µl

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of primer mix (4.56 µmol l^{-1} of each primer)]. Two negative controls were performed (no primers, no DNA). The PCR program was realized with a Mastercycler (Eppendorf) under these conditions: a denaturation step of 2 min at 95°C, an annealing and elongation step of 40 cycles including 20 s at 95°C, 40 s at 56°C and 60 s at 72°C, and a final step of 10 min at 72°C. PCR products were visualized on a 2% agarose gel using SYBR safe staining (Invitrogen).

qPCR assays

eDNA extracted from the abdominal cavity samples of fish was amplified by qPCR using the designed primers specific to *S. solidus*. Each 96-well plate included eDNA samples extracted from the fish body cavity and a standard curve obtained from a pool of *S. solidus* genomic DNA (3 individuals) diluted at different concentrations. The concentrations of the 7-point standard curve ranged from 0.9 to 0.01 ng μl^{-1} with a dilution factor of 2. The qPCR reaction was performed in a qPCR mix including 12.5 µl of PowerUp SYBR Green Master mix (Life Technologies) and 1 µl of primer mix (4.56 µmol l^{-1} of each primer). For the genomic DNA samples, 6.5 µl of RNAse-free water and 5 µl of DNA were added to the qPCR mix. For the eDNA samples, no water was used and 11.5 µl of solution containing the resuspended eDNA was added to the qPCR mix (for a total qPCR mix volume of 25 µl in each case). Two negative controls (no primers, no DNA) were included in each plate. Each reaction was done in triplicate. qPCR was performed with a 7500 Real-Time PCR system (Life Technologies). The amplification was realized according to the conditions described by Lacoursière-Roussel et al. (2016): 2 min at 50°C, 10 min at 95°C, followed by 70 cycles of 15 s at 95°C and 60 s at 56°C. Using such a large number of amplification cycles is not standard when detecting mRNA levels, but has been found to be useful for detecting the presence of eDNA when the appropriate negative controls are used (Lacoursière-Roussel et al., 2016). A melt curve protocol was then performed, with temperature going from 60 to 95°C. This melt curve was used to detect primer dimers, to check specificity by determining that a single amplicon was produced, and to obtain a melting temperature (T_m) value for that sample, which can be compared with the standard curve of *Schistocephalus* DNA.

Ventilation rate measurements

A subset of individuals used in the *S. solidus* eDNA detection experiment was used to quantify ventilation rates (captured in Lac Témiscouata in July 2016, n=20, randomly chosen). Ventilation measurements were repeated 3 days in a row, with individuals sampled in the same order each day. For each individual fish, a 250 ml beaker was filled with 50 ml of clean water from the supply used to fill experimental 2 l tanks. The individual was filmed from the side (JVC Everio camera) to enable detection of opercular movements. At the end of the 3 days of measurements, these fish were killed and their infection status was determined. Dissection showed that 13 individuals were infected and 7 were parasite free. All the infected fish were also detected as infected by the eDNA approach (see below). Films were subsequently analysed at 0.46× speed by an observer (N.A.H.) blind to the treatment using the open source VLC Media Player (Videolan). The first 30 s of the movie where the fish was clearly visible were used to measure ventilation rates. If the individual moved into a position where it was not visible during the first 30 s sampling period, the ventilation measurement was made for the usable seconds within that period and reported back to 30 s. The observer repeated all measurements for the first day of sampling to assess accuracy. Measurements done twice on the same fish in the same video were highly correlated (Spearman's ρ=0.99) with most samples having the exact same number of ventilations when measured twice, with a maximum difference of 3 ventilation movements and an average difference of 0.7. A single measurement per video was thus used. A ventilation rate was calculated by dividing the number of ventilation movements measured in 30 s by 30. This measurement was used for further statistical analysis. The total number of ventilation movements measured during a 30 s interval was used to estimate the number of ventilations over 1 min for graphical purposes and for comparison with previous studies.

Analyses

Following qPCR amplification, the presence or absence of *S. solidus* eDNA in the body cavity samples was determined by comparing the cycle threshold (Ct) values (amplification curves) and the T_m values (melt curves) of the samples with the ones of the standard curves. The Ct is the cycle at which a sample reaches a preset threshold of fluorescence, which is a proxy for the quantity of eDNA. A sample with a low Ct means that there was more template in the original sample and that it thus reached the threshold sooner in the 70 PCR cycles. A body cavity sample showing at least one of its Ct values (among the triplicates) above 0 in combination with an associated T_m value(s) close (±2°C) to the mean T_m of the standard curve (79.64°C) was considered positive for *S. solidus* eDNA. In contrast, if a Ct value above 0 was associated with a T_m value more distant from the mean T_m of the standard curve, then this value was considered negative for *S. solidus* eDNA and the result of primer-dimer formation. Finally, if the body cavity sample had only 'undetermined' Ct values, it was considered negative for *S. solidus* eDNA, irrespective of the T_m value. For each fish, qPCR results were analysed with regard to its infection status (parasitized or not by *S. solidus* upon dissection). A true positive was obtained when there was concordance between the infection status and the qPCR results. A false negative was defined when the fish was infected by *S. solidus* but no traces of *S. solidus* eDNA were found using qPCR. A false positive was associated with fish not infected by *S. solidus* but for which *S. solidus* eDNA was inferred using qPCR results. All individual results are found in Dataset 1.

Statistical analysis was done in R version 3.3.3 (http://www.R-project.org/) using R Studio (version 1.0.136) and the ggplot2 package (Wickham, 2009). To determine whether our method allows a quantitative estimate of parasite mass, we tested whether the eDNA concentration obtained for infected fish (using the Ct value) negatively correlated with their total parasite mass, using a non-parametric Spearman correlation (a small Ct value should be associated with a larger parasite mass and vice versa if the eDNA method is quantitative). We used the lowest Ct measured for a given fish if more than one triplicate amplified DNA. Repeatability of ventilation rates over the 3 days was calculated following a previously developed method (Lessells and Boag, 1987). We compared ventilation rates between infected and non-infected individuals using a t-test, as they followed a normal distribution (based on a Shapiro–Wilk test). We tested whether ventilation rates co-varied positively with the parasite load (PI) using a non-parametric correlation (Spearman correlation).

RESULTS AND DISCUSSION

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

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

Primer specificity

PCR tests performed with primers that target a sequence from the *S. solidus* nuclear gene (NCBI ID GEEE01010589.1) confirmed the amplification of *S. solidus* genomic DNA, and not of threespine stickleback genomic DNA. The PCR product was sequenced using Sanger ABI3730xl (Centre hospitalier universitaire de Québec - Université Laval) to confirm the amplification of the 200 bp amplicon in the *S. solidus* genome. Controls (no template, no primers) were negative, as expected.

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

qPCR was performed in combination with an eDNA extraction protocol to detect *S. solidus* presence in the stickleback abdominal cavity. Standard curves made with a pool of *S. solidus* genomic DNA samples diluted at different concentrations had mean Ct values ranging from 29.65 (highest concentration, 0.9 ng μl^{-1}) to 36.86 (lowest concentration, 0.01 ng μl^{-1}) and a mean T_m value of 79.64°C. All the controls were negative. The earliest Ct value we obtained for abdominal cavity eDNA samples with our protocol and amount of starting material varied between 34.4 and 49.6 for positively identified individuals. Using this approach on two fish populations, 148 fish out of 151 were correctly assigned to their infection status (infected or not by *S. solidus*, 98% of true positive).

In the Lac Témiscouata population, 35 fish out of 96 were infected by *S. solidus*. Single and multiple infections (up to 6 worms) were reported upon dissection, with a median of 1.5 worms, illustrating the predominance of single or double infections. Note that one fish harboured 12 worms, but we did not obtain reliable mass for the worms because that individual died before euthanasia and dissections were performed, and as such is included in the dataset only for calculation of infection status. Among the infected sticklebacks, 18 exhibited a single worm upon dissection without external visual clues that would suggest the presence of *S. solidus* in the fish body cavity (Fig. 1). For all infected fish, the total parasite mass comprised between 130 and 2356 mg (median of 495 mg) and the PI was always below 50% (between 4.9% and 40.4%, median 16.6%), meaning that the total mass of parasites was always below the net mass of the fish host. Thirty-two out of the 35 infected fish were successfully detected as infected using qPCR. For the 3 remaining infected fish, no trace of *S. solidus* eDNA was detected with our protocol (1.98% of samples tested, low false-negative rate). No eDNA was detected for these fish in any of the triplicates (Ct 'undetermined'). These 3 fish had a total parasite mass that spanned almost the whole range found in the study (168 and 274 mg for the single infections and 1644 mg for the double infection, from the second smallest to the third largest worm). Their PI (respectively 12.5%, 6.5% and 25.3%) was above the minimal values from infected fish that were correctly detected. These observations indicate that these false negatives are not due to a lack of sensitivity of the method at low parasitic mass. We instead propose that the critical step of our method is during the sampling of the internal fluid from the fish intraperitoneal cavity. *Schistocephalus solidus* is free in the abdominal cavity of its host. It is possible that the PBS injection was done too far from the worm to allow sampling of its DNA. To overcome this issue, we performed body cavity sampling on 2 consecutive days, and each time the needle was inserted into a different side of the fish. This approach was quite reliable and probably resulted in our low false-negative rate. The eDNA sampling protocol may be extended over 3 days in order to maximize *S. solidus* DNA capture, with the caveat that this would expose the fish to more stress. Another aspect that greatly influences eDNA detection is storage method and time (Hinlo et al., 2017). Following eDNA sampling from the fish body cavity, we suggest performing DNA extraction directly after refrigeration at 4°C, without freezing.

None of the fish from the anadromous population were infected by *S. solidus* (n=55). We never detected *S. solidus* DNA in the samples of the uninfected fish from this population, or from the 61 uninfected fish from Lac Témiscouata (0 false positive). False positives could have resulted from a lack of specificity of the primers used. The absence of false positive for 116 samples confirms the specificity of the method. False positives could also have resulted from a signal from an exposed stickleback, i.e. a fish that was exposed to a *S. solidus*-infected copepod, but for which infection did not succeed and the worm did not develop in its abdominal cavity. The absence of false positives suggests that our method does not allow detection of sticklebacks that have been exposed but that do not develop an infection. However, all the fish used were of wild origin, which means we have no record of exposure over the lifetime of an individual fish. Testing this possibility with laboratory-exposed fish could add to the usefulness of the present detection method.

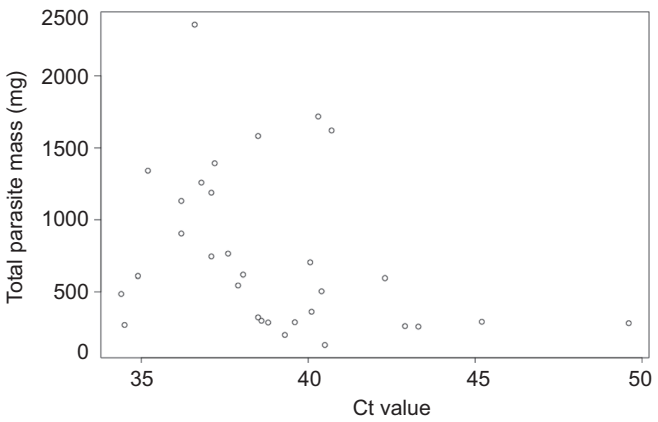

Fig. 2. eDNA concentration co-varies with total parasite mass. Low cycle threshold (Ct) values, which represent high levels of eDNA, are associated with a high parasite mass (Spearman correlation, ρ=−0.41, P=0.01, n=31).

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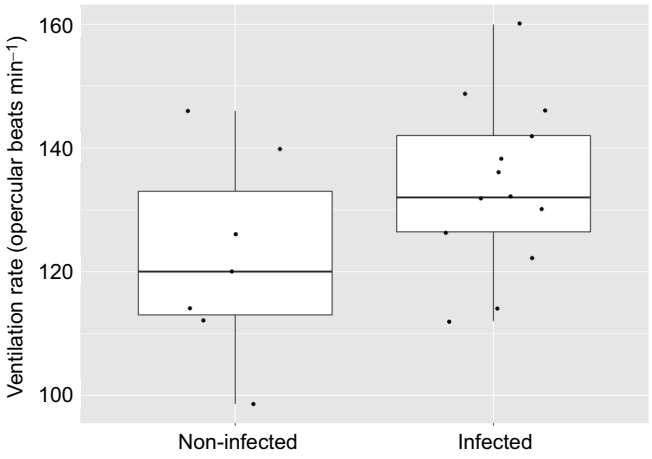

Fig. 3. Ventilation rates do not differ significantly between non-infected and infected threespine sticklebacks. Ventilation rate, monitored as the number of opercular movements per minute, ranged between 99 and 146 opercular beats min^{-1} in non-infected fish (n=7) and between 112 and 160 opercular beats min^{-1} in *S. solidus*-infected fish (n=13). The lower and upper margins correspond to the 25th and 75th percentiles and the vertical bars extend to the largest value no further than 1.5 times the interquartile range. Two-sided t-test, t=−1.57, P=0.15, n=20.

We found a significant correlation between Ct values (which are inversely correlated to the amount of *S. solidus* eDNA) and the total parasite mass found within a fish (one-sided Spearman correlation, ρ=−0.41, P=0.01; Fig. 2). This result suggests that in the future this approach could be used in a quantitative way to estimate not only the presence but also the total mass of parasites within a host. The correlation is non-linear and there is large variation in total parasite mass for a given Ct value and thus further refinement would be needed. Our protocol could be improved by lowering the ranges of Ct values for both standard curves and eDNA samples. We suggest increasing the concentration of the starting material by resuspending the DNA pellet in a smaller volume (40 µl instead of 80 µl for eDNA samples). Therefore, our method in its current state gives a very reliable presence/absence response and its use for quantitative assessment looks promising for the future.

Variation of ventilation rate between uninfected and infected sticklebacks

We measured the ventilation rate of 20 fish, 3 times over 3 consecutive days, and then determined that 13 of these were infected. The infected individuals harboured from 1 to 5 worms with a total mass ranging between 618 and 2356 mg (PI between 13.2 and 40.4). One infected individual died after the first ventilation measurement and we could use the videos for only the first 2 days for one of the non-infected fish. We had 3 ventilation measurements for all other individuals. Repeatability of ventilation across 3 days for a given fish was 0.41 (Lessells and Boag, 1987). As ventilation rates were repeatable over this time scale, we used ventilation rates measured on the first day in subsequent analysis, to reflect how this method would be used to quickly detect infection. Ventilation rates estimated over 30 s and reported as the number of opercular movements per minute ranged between 99 and 146 opercular beats min^{-1} (average 122 opercular beats min^{-1}) in uninfected fish and between 112 and 160 opercular beats min^{-1} (average 134 opercular beats min^{-1}) in *S. solidus*-infected fish (Fig. 3). Ventilation rates of uninfected fish were in the same range as found in previous studies of freshwater sticklebacks (Bell et al., 2010; Di Poi et al., 2016). We found that ventilation rates did not differ significantly between non-infected and infected individuals (two-sided t-test, t=−1.57, P=0.15, n=20; Fig. 3). Ventilation was positively correlated to the PI, but this covariation was not significant (one-sided Spearman correlation, ρ=0.35, P=0.07, n=20). There was a substantial overlap in ventilation rate between the two groups, which prevented the use of this trait to discriminate between *S. solidus*-infected and healthy individuals. Therefore, the power of this simple measurement is low and can only be used as a complementary indicator of host infection by *S. solidus*.

We expect the eDNA method presented here to find applications beyond the *S. solidus*–threespine stickleback system. Indeed, several fish species have parasites in their abdominal cavity (Hoffman, 1999). To apply our method to these systems, the main 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 morphologically based methods already used in these systems.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.S.B., N.A.-H.; Methodology: C.S.B., N.A.-H.; Validation: N.A.-H.; Formal analysis: C.S.B., N.A.-H. Investigation: N.A.-H.; Resources: N.A.-H.; Data curation: C.S.B.; Writing - original draft: C.S.B.; Writing - review & editing: C.S.B., N.A.-H.; Visualization: N.A.-H.; Supervision: N.A.-H.; Project administration: N.A.-H.; Funding acquisition: N.A.-H.

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