Parasitology Research (2023) 122:1435–1443
https://doi.org/10.1007/s00436-023-07843-1

BRIEF REPORT

A qPCR-based method to detect the eel parasitic nematode *Anguillicola crassus* in intermediate and final hosts

Chloé Suzanne Berger[1] · Bérénice Bougas[1] · Guillaume Côté[2] · Jean-François Dumont[2] · Louis Bernatchez[1]

Received: 5 December 2022 / Accepted: 5 April 2023 / Published online: 18 April 2023
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Abstract

Being able to systematically detect parasitic infection, even when no visual signs of infection are present, is crucial to the establishment of accurate conservation policies. The nematode *Anguillicola crassus* infects the swimbladder of anguillid species and is a potential threat for eel populations. In North America, naïve hosts such as the American eel *Anguilla rostrata* are affected by this infection. The accidental introduction of *A. crassus* following restocking programs may contribute to the actual decline of the American eel in Canada. We present a quantitative real time PCR-based method to detect *A. crassus* infection in final and intermediate hosts. We tested two protocols on samples from different geographical origins in Canada: 1) a general detection of *A. crassus* DNA in pools of young final hosts (glass eels) or crustacean intermediate hosts 2) a detection at the individual scale by analyzing swim bladders from elvers, or from adult yellow and silver eels. The DNA of *A. crassus* was detected in one pool of zooplankton (intermediate host) from the Richelieu River (Montérégie-Québec), as well as in individual swim bladders of 13 elvers from Grande and Petite Trinité rivers (Côte-Nord-Québec). We suggest that our qPCR approach could be used in a quantitative way to estimate the parasitic burden in individual swim bladders of elvers. Our method, which goes beyond most of previous developed protocols that restricted the diagnosis of *A. crassus* to the moment when it was fully established in its final host, should help to detect early *A. crassus* infection in nature.

Keywords Parasite · *Anguillicola crassus* · Eel · Invasive species · qPCR

Introduction

Conservation of aquatic biodiversity requires being able to rapidly detect parasitic infection in species occurring in those ecosystems. One parasite that represents a potential threat for eel populations is the nematode *Anguillicola crassus*. This parasite, which infects the swimbladder lumen of anguillid eels, is endemic to East Asia and was first reported in the Japanese eel *Anguilla japonica* (Kuwahara et al. 1974). While *A. crasssus* does not induce serious pathological effects on original hosts, it has been shown that eel species in the non-native distribution range are strongly affected by the nematode infection (Knopf 2006). Negative effects were reported on the morphology (*e.g.* Lefebvre et al. 2004; Pegg et al. 2015), physiology (*e.g.* Kelly et al. 2000; Sokolowski and Dove 2006; Würtz and Taraschewski 2000; Würtz et al. 1996) and behaviour (Fazio et al. 2012) of the European eel *Anguilla anguilla* and the American eel *Anguilla rostrata*.

In Canada, the American eel is a species of conservation concern and is identified as "Threatened" by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC 2012; Pratt et al. 2019). In the province of Quebec, the American eel is included on the list of species likely to be designated as threatened or vulnerable. A recent scientific advice on the status of the American eel in Quebec confirmed that the abundance of the eel stock in this area is regarded as very concerning (COSCIAN 2019). Specifically in the St. Lawrence watershed, the total number of recruiting American eels has been dramatically decreasing since the 1980s (Pratt et al. 2019). To counteract this loss, a conservation stocking program was put in place in 2006. Thus, more

Section Editor: Matthew Wayland

✉ Chloé Suzanne Berger
chloe.berger.1@ulaval.ca

[1] Institut de Biologie Intégrative Et Des Systèmes (IBIS), Université Laval, Québec, QC, Canada

[2] Ministère de L'Environnement, de La Lutte Contre Les Changements Climatiques, de La Faune Et Des Parcs (MELCCFP), Québec, QC, Canada

than 6.6 million eels and elvers were translocated from New Brunswick and Nova Scotia into the St. Lawrence River watershed between 2005 and 2010 (Pratt et al. 2019). These translocation programs probably led to the accidental introduction of *A. crassus* in the St. Lawrence River watershed, through infected intermediate hosts like plankton or translocated eels. The first record of the parasite presence in the St-Lawrence watershed was confirmed in 2010 (Pratt et al. 2019). Since that, infection prevalence and intensity have greatly increased in resident yellow-stage eels from the upper St. Lawrence and Ontario lake (Pratt et al. 2019). On the other hand, silver-phase outmigrant eels seem to be less intensely infected both in terms of prevalence and intensity. The parasite monitoring in the Quebec commercial fishery started in 2015, and at this point, only the translocated eels were infected by the parasite. In 2021, both translocated and naturally-recruited eels were infected (Landry-Massicotte et al. 2022). According to the conservation context of American eels in the St. Lawrence watershed and the interest to resume possible stocking projects, there is an urgent need to develop a method that would allow determining whether or not translocated eels come from a source environment where *A. crassus* is present, therefore preventing the translocation of infected eels in recipient populations.

Because of the morphological deformations of infected swim bladders (including wall thickness and transparency, lesions, inflammation, haemorrhaging and fibrosis (Woo and Buchmann 2012)), visual examination of this organ has been the most common method used to detect infection (Haenen and Van Banning 1990). The visual screen can be performed using either microscopy or imaging (Beregi et al. 1998; Frisch et al. 2016). However, even if microscopical detection for *A. crassus* has already been performed at larval stages (L3 and L4) (Dangel et al. 2013), this visual approach may be less reliable on elvers and glass eels when they are infected by early larval-stages or by small or juvenile nematodes, which do not induce apparent symptoms on the swim bladder. This limitation may be exacerbated in young elvers that were recently infected. Furthermore, this method can only be applied when the parasite has reached its final anguillid host. To our knowledge, no protocol allows the detection of *A. crassus* in its intermediate hosts, which include small crustaceans on which eels feed (De Charleroy et al. 1990).

Recent years have been marked by the emergence of molecular based approaches for the detection of *A. crassus* in eels. Two protocols based on DNA amplification by PCR from fecal materials were recently developed to detect the parasite in *A. anguilla* (De Noia et al. 2022; Jousseaume et al. 2021). These methods are certainly useful as they have the advantage of being non-lethal. Yet, their application is limited to the final host, and the detection window is restricted to the moment when *A. crassus* releases eggs in the swimbladder, which pass through the eel's digestive tract (De Charleroy et al. 1990).

Our objective was to develop a method that would detect *A. crassus* infection in final and intermediate hosts, even when no visual signs of infection are present. Using a quantitative real time qPCR-based method, we explored the infection status of final and intermediate hosts from different geographical origins in Canada that would represent a potential source for translocation. We tested two different protocols: the first one was based on a general detection of the DNA of *A. crassus* in pools of young, final hosts (glass eels), or in pools of crustacean intermediate hosts (gammarids or zooplankton). The second one aimed at detecting *A. crassus* DNA at the individual level, by analyzing swim bladders from young elvers, or from adult yellow and silver eels. In the context of fish management policies, our results will help to determine if the eels living in the targeted regions could serve as a source for translocation in depleted areas.

Materials and methods

Sampling

In 2020 and 2021, a total of 90 samples—including pools of gammarids and pools of zooplankton, pools of glass eels, as well as swim bladders from young elvers or adults of yellow eels and silver eels—from different geographical origins were sampled by the Ministère de l'Environnement, de la Lutte contre les Changements Climatiques, de la Faune et des Parcs (MELCCFP), Québec, QC, Canada (Table 1). Gammarids were collected in July 2020 on the Sault-au-Cochon River estuary in Côte-Nord area (QC, Canada) with a 1 m diameter × 2.5 m length push-net with Nitex 750 micron mesh size, mounted on a 18' John-boat style ship. Soft water zooplankton was caught in the Richelieu River in Montérégie (QC, Canada) in June 2021. Glass eels came from Newfoundland (2020 and 2021) and Nova Scotia (2021) (Canada) commercial fishing companies. All elvers were collected in 2021 on Grande Trinité and Petite Trinité Rivers in Côte-Nord (QC, Canada), following regular MELCCFP annual recruitment monitoring. Eel traps and 4" × 3" portable fish nets were used to catch the elvers on these two rivers, respectively. Yellow eels came from electrofishing projects realized in 2021 on the Richelieu River in Montérégie and on the St-Pierre lake in Mauricie (QC, Canada), and the only silver-phase eel used in this study was found dead in lac Sergent, approx. 25 km west from Quebec City (QC, Canada). All plankton species and glass eels were killed by 95% ethanol immersion. Elvers and yellow-phase eels were euthanized by a high-concentration eugenol solution overdose. Swim bladders were obtained after fish dissection using PTFE-coated, anti-magnetic stainless steel tweezers. After each collection, tweezers were dipped

Table 1 Summary of the 90 samples that were tested for the presence of *A. crassus* DNA using a qPCR-based approach in final and intermediate hosts

Sample type	Number of pools/samples	Sampling time	Sampling localization	Coordinates
Pools of intermediate host: Gammarids	2 pools	July 28 2020	Sault-au-Cochon River, Côte-Nord, QC, Canada	48.72563064725399, -69.07895967032695
Pools of intermediate host: Zooplankton	6 pools	June 15–16 2021	Richelieu River, Montérégie, QC, Canada	45.175181, -73.256036
Pools of final host: Glass eels	4 pools	2020	Newfoundland, Canada (commercial fishing companies)	NA
Pools of final host: Glass eels	2 pools	March 24 2021 April 8 2021	East river, Nova Scotia, Canada Salmon River, Nova Scotia, Canada	44.58606320113199, -64.1687098734186 45.36983670233677, -63.255280658899416
Pools of final host: Glass eels	2 pools	April 25 2021 July 4 2021	Meteghan River, Nova Scotia, Canada	44.21902075847468, -66.10077778014939
Pools of final host: Glass eels	4 pools	May 17–26 2021	Seal Brook, Newfoundland, Canada	48.29285161917848, -58.7564508098479
Pools of final host: Glass eels	8 pools	May 17–26 2021	Farmer Brook, Newfoundland, Canada	47.66553222903096, -58.50037536404313
Individual swim bladders: Elvers	33 swim bladders	June 23–30 2021 July 5–15-28–31 2021 August 3–6-18–20 2021	Grande Trinité and Petite Trinité Rivers, Côte-Nord, QC, Canada	49.52639388813291, -67.2456024574947
Individual swim bladders: Yellow eels (adult)	5 swim bladders	August 9–13 2021	Electrofishing project by MELCCFP realized on the Richelieu River, Montérégie, QC, Canada Saint-Pierre lake, Mauricie, Centre-du-Québec, QC, Canada	46.213669, -72.814804
Individual swim bladder: Silver eels (adult)	1 swim bladder	June 21 2021	Lac Sergent, Capitale-Nationale, QC, Canada	46.86165476677816, -71.72071665942238
Individual swim bladders Yellow eels (adult)	23 swim bladders	June 7–18 2021	Electrofishing project by MELCCFP realized on the Richelieu River, Montérégie, QC, Canada	45.55945234065063, -73.11735613192543

in a sodium hypochlorite solution then rinsed in fresh water. Each swim bladder was thoroughly screened under a microscope for the presence of *A. crassus* and/or for the presence of abnormalities in swim bladders. Only one swim bladder appeared to harbor a parasite (Fig. 1), while no external sign of infection was reported in the other pools nor individual samples. Samples were kept in ethanol 95% and sent at the IBIS (Institut de Biologie Intégrative et des Systèmes), Université Laval, Québec, QC, Canada, for molecular analyses.

DNA extraction

In order to develop the qPCR method, we used six *A. crassus* adult nematodes that were sampled in October and November 2021 from commercially harvested silver eels in Rivière-Ouelle/Kamouraska area (QC, Canada) and yellow eels commercially harvested in the Magdalen Islands (QC, Canada). Their masses ranged from 875 to 2000 mg. Parasites were weighted to the nearest 0.1 mg and kept in 95% ethanol before extraction. The DNA of these six *A. crassus* nematodes was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to a previously developed protocol (Goldberg et al. 2011; Spens et al. 2017) with some modifications (Supp. Mat. 1). Digestion was performed on whole tissue for each worm (whole individual).

DNA from pools of elvers, intermediate hosts and from swim bladders was extracted using the same DNeasy Blood and Tissue Kit (Qiagen) with some modifications (Supp. Mat. 1). Pools of elvers and intermediate hosts were composed of a mixture of at least 50 individuals. For these pools, as well as for yellow and silver eel (adult eels) swim bladders (without intestine), digestion was performed on 2 g of mixture, while digestion was performed on whole tissue for elver (young) swim bladders (without intestine). For mixtures, we decided to use only 2 g for digestion as the final quantity of extracted DNA was primarily dependent on the amount of material used for digestion (so that more material input would have solely increased DNA concentrations

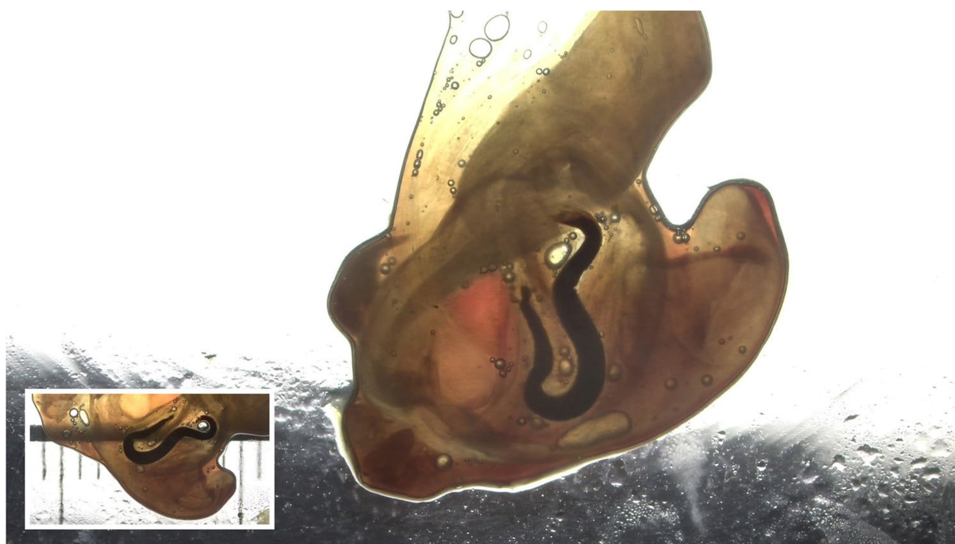

Fig. 1 Microscopic visualization of the swimbladder that harboured visual signs of infection by *A. crassus*. The swimbladder was dissected from an elver of the Grande Trinité river, Côte-Nord, QC, Canada that was caught on July 5th 2021 (ID: Trinité July 5 2021 #1; eel length 241 mm). The parasite appeared to be at stage L4 of development (De Charleroy et al. 1990). Background scale (mm) in box indicates parasitic size between 8–9 mm. Picture by the Ministère de l'Environnement, de la Lutte contre les Changements Climatiques, de la Faune et des Parcs

without affecting the results), and as 2 g would prevent column clogging during protocol. Purity of extracted DNA was confirmed by the ratios of absorbance at 260/280 nm with a NanoDrop 1000 spectrophotometer (Thermo Scientific). Instruments were rinsed in bleach between each sample to avoid DNA cross-contamination (except for the pools of gammarids, zooplankton and eels of Newfoundland (2020), for which the equipment was washed abundantly with clear water and 95% ethanol between each pool). DNA was stored at -20 °C until amplification. Negative extraction controls were included at each extraction batch to account for possible contamination.

Test for the specificity and efficiency of the qPCR primers and probe

The qPCR method was used to test for the specificity and efficiency of the primers and probe targeting *A. crassus* DNA. We used two methods: i) The qPCR Fast Sybr Green technology, which tests the specificity and efficiency of primers. We thus tested a pair of primers targeting *A. crassus* DNA: *A. crassus* F and R (Table 2). The pair targeted a 303 base pair sequence within the cytochrome oxidase I mitochondrial DNA (COI mtDNA) region that is specific to *A. crassus* (Grabner et al. 2012; Jamison et al. In preparation). Annealing temperature was validated using Primer Express 3.0 (Life Technologies) and cross-amplification to unrelated species was verified using Primer Blast (Ye et al. 2012) (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were validated for amplification of targeted species (six distinct nematodes). Each qPCR reaction was carried out in triplicates. Amplification was performed on a 7500 Fast Real-Time PCR machine (Life Technologies) in a final volume of 20 µL including 1 µL of each primer (10 µM), 10 µL of PowerUp SYBR Green Master mix (Life Technologies), 6 µL of H2O and 2 µL of DNA under these conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. ii) The TaqMan technology, which tests the specificity of the primers and the probe. With this approach, we tested the pair of primers F and R, using the same DNA extracts from six different parasites, coupled with their probe (Table 2). Probe was designed using the COI sequences of *A. crassus* in Geneious 9.0.5 (https://www.geneious.com/). Each reaction was carried out in triplicates. The amplification was performed on a PCR 7500 Fast Real-Time machine (Life Technologies) in a final volume of 20 µL including 1.8 µL of each primer (10 µM), 0.5 µL of the probe (10 µM), 10 µL of the Environmental Master Mix 2.0 (Life Technologies), 3.9 µL of H2O and 2 µL of DNA under these conditions: 2 min at 50 °C, 10 min at 95 °C, 50 cycles of 15 s at 95 °C and 60 s at 60 °C. Because we expected the DNA of *A. crassus* to be at small concentrations in the tested samples,

Table 2 Primers and probe tested to detect *A. crassus* DNA by qPCR. The pair targets the COI region of the species. F stands for Forward and R for Reverse. Tm is the melting temperature

Primer Name	Primer sequence	Primer Tm	Probe sequence	Probe Tm
A. crassus F	TTAGGGGCTCCT GATATAAGT TTTCC	64.7	ACTGGTTGTGGTACTAGTT	53.0
A. crassus R	GCCAATAACACT CAAAGGAGG ATAAA	63.1		

we used Environmental Master Mix as it is commonly used in environmental DNA studies to detect very low levels of DNA molecules in samples with potential high levels of inhibitors (Schenekar et al 2020).

Assay sensitivity

A standard curve experiment was performed following the same condition as described for the TaqMan assay. A synthetic DNA template of 500 base pairs (Integrated DNA Technologies Inc.) including the target amplicon sequence was designed from the COI, and used to estimate by serial dilution the detection limit of the primer pair F and R, *i.e.* until the fluorescence signal corresponding to one molecule is reached (Forootan et al. 2017). From the stock, diluted at 1.00E + 10 copies/µL, a nine-level dilution series (1000, 500, 250, 50, 10, 4, 2, 1, and 0.5 copies per reaction) was prepared in a sterile yeast tRNA (100 ng/µL) solution. Yeast tRNA acts as a coprecipitant that is essential for quantitative recovery of small amounts of nucleic acids in dilute solutions (ThermoFisher). Ten replicates of each dilution were run to determine, for the pair of primers F and R and probe, the amplification efficiency and the limit of detection (LOD) defined as the lowest standard concentration at which 95% of technical replicates amplify (Bustin et al. 2009; Klymus et al. 2020).

We also tested the sensitivity of primers and probe for detection of *A. crassus* DNA in host at different ratios (from 1:1 to 1:10,000). This approach was performed using the TaqMan technology with the pair of primers F and R and their probe, according to the qPCR program described previously. We tested five parasite DNA: host DNA ratios (1:1; 1:10; 1:100; 1:1000 and 1:10,000, with the first ratio at concentrations of 20 ng/µL: 20 ng/µL). Three individual parasites were tested, except for the last ratio where only one parasite was tested as this ratio required high quantities of host DNA (10,000 times more concentrated than parasitic DNA) and that the quantity of host DNA available for this study was limited. We used DNA of *A. crassus* extracted in this study (from whole worm tissue) and DNA from an American eel *A. rostrata* (white muscle) that was previously extracted (Hernandez et al. 2020). Each reaction was carried out in triplicates.

qPCR analysis

All samples were tested by qPCR, according to the TaqMan method previously used with the F and R primers and probe. For the reaction, we added SPUD as an internal positive control for the detection of inhibitors in nucleic acid preparations (Nolan et al 2006), as well as a standard curve with synthetic DNA to quantify the number of molecules in the positive samples. For each sample and the extraction controls, the presence of *A. crassus* DNA was tested on six replicates. Amplification was performed in a final volume of 20 µL including 1.8 µL of each primer (10 µM), 0.5 µL of probe (10 µM), 10 µL of Environmental Master Mix 2.0 (Life Technologies), 3.9 µL of SPUD and 2 µL of DNA according to these conditions: 2 min at 50 °C, 10 min at 95 °C, 50 cycles of 15 s at 95 °C and 60 s at 60 °C. The presence of *A. crassus* DNA was confirmed when amplification was detected before the detection limit was reached. Negative qPCR controls were included on each plate.

Results and discussion

Negative controls

All negative extraction and qPCR controls showed no positive amplification indicating the absence of contamination during sample extraction and amplification. During qPCR, SPUD controls confirmed the absence of inhibitors in nucleic acid preparations.

Primers/probe specificity and efficiency

The DNA of *Anguillicola crassus* was successfully amplified with the pair of primers tested (F and R) using both the qPCR Fast Sybr Green (Ct mean 25.48. ± SD 0.53) and TaqMan (Ct mean 27.65 ± SD 1.45) technologies on six distinct *A. crassus* samples. We also performed *in silico* analysis to confirm that the pair of primers did not amplify any other DNA sequences than the ones of *A. crassus*. We used the Primer BLAST tool from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and chose the nr database as a template for broadest coverage. Complete matches were only found for *A. crassus*. Partial matches were detected on other parasitic *Anguillicoloides* species (including *Anguillicoloides australiensis* and *Anguillicola papernai*) but there were at least four mismatches between their sequences and the primers. No matches were found with eels.

Limit of detection

Using the TaqMan protocol, we were able to detect *A. crassus* DNA in eel DNA, down to a ratio as low as 1:10,000. Each ratio (from 1:1 to 1:10,000) was separated by 2.5 cycles of amplification (Ct means ranging from 25.18 ± SD 0.24 to 37.90 ± SD 0.23). Furthermore, the standard curve assay, made with a synthetic DNA template including the target amplicon sequence diluted at different concentrations, had mean Ct values ranging from 29.07 ± SD 0.07 (highest concentration, 1000 copies/µL of standard dilution) to 39.56 ± SD 1.01 (lowest concentration, 0.5 copies/µL of standard dilution) ($R2 = 0.98$, Slope = -3.33, y-inter = 39.28,

Eff% = 99.69). The LOD value was four copies/μL of standard dilution and the pair of primers was able to detect eight molecules with 100% efficiency. The threshold of one molecule was detected at 40 Ct. In our dataset, six samples exceeded 40 Ct. They were considered as potential artifact of PCR and were eliminated from further analyses.

Detection of *Anguillicola crassus* DNA in intermediate and final hosts

For intermediate hosts, the DNA of *A. crassus* was detected in one pool of zooplankton from the Richelieu River (Montérégie, Québec) in mid-June 2021 (Table 3, DNA molecules /g of extracted tissues: 4.08). No *A. crassus* DNA was detected in other pools of zooplankton nor gammarids.

For final hosts, the DNA of *A. crassus* was not detected in pools of young hosts (glass eels). However, at the individual level, we were able to detect *A. crassus* DNA in individual swim bladders of 13 elvers from Grande Trinité and Petite Trinité Rivers (Côte-Nord, Québec). Specifically, *A. crassus* was detected at four sampling dates during Summer 2021: June 23 (3/9 positive swim bladders, DNA molecules per gram of extracted tissues: 0.54—1.52), June 30 (4/4 positive swim bladders, DNA molecules per gram of extracted tissues: 0.43 – 2.91), July 5 (2/2 positive swim bladders, DNA molecules per gram of extracted tissues: 0.58 – 1 350 258.18) and July 15 (4/9 positive swim bladders, DNA molecules per gram of extracted tissues: 0.87 – 2.35) (Table 3). All the swim bladders sampled in June 30 and July 5 showed positive detection.

Our results are in accordance with previous reports of the presence of *A. crassus* in the Richelieu River, which received thousands of translocated eels from New Brunswick and Nova Scotia in the early 2000s (Engler-Palma et al. 2013; Pratt et al. 2019). To our knowledge, we report with the Trinité River the northernmost record of *A. crassus* in Quebec. Depending on water temperature, the occurrence of *A. crassus* is known to increase in intermediate hosts and elvers from March to Mid-June, and to reach its peak in elvers and adult eels in July (Hein et al. 2016). This could specifically explain the highest detection level we observed early July in the Trinité River (Table 3, Trinité July 5 2021 #1).

The positive detection of *A. crassus* DNA using two different protocols—first with a screen in pools of intermediate hosts (zooplankton), then with the analysis of swim bladders from elvers—highlights the strength of the qPCR approach both at general and individual levels. The qPCR method presented here appears to be more sensitive than visual screens, as small samples (such as swim bladders of elvers) that were first defined as non-infected under a microscope were actually positive to *A. crassus* with our qPCR analyses (Table 4). Specifically, only one swim bladder from an elver was reported as infected by visual observation. This swim bladder was also positive for *A. crassus* DNA (Table 3 and Fig. 1, Trinité July 5 2021 #1). The associated parasite was already at stage L4 of development (length ~ 9 mm), which explains the success of the visual screen and the very high level of DNA molecules detected in this swim bladder sample compared to the others. For the other swim bladders, the qPCR approach was powerful enough to detect DNA molecules even at low concentrations in the absence of visual signs.

For the pools of glass eels sampled in Nova Scotia, we did not detect the DNA of *A. crassus* despite the fact that natural infections have been confirmed during summer of 2007 in this area by histological examination of glass eels (Threader

Table 3 qPCR detection of *Anguillicola crassus* DNA in one pool of zooplankton (intermediate host) from the Richelieu River (Montérégie, Québec), and in individual swim bladders of 13 elvers from Grande Trinité and Petite Trinité rivers (Côte-Nord, Québec). Ct: qPCR cycle threshold. DNA molecules / g: DNA concentration that is expressed per gram of extracted tissue. SD: standard deviation. For each sample, the mean represents the average Ct or the average number of DNA molecules for the six qPCR replicates. The number of positive qPCR replicates (maximum of 6) is indicated

Sample name	Sample type	Ct mean	Mean DNA molecules / g	SD	Number of qPCR positive /6
Richelieu June 2021	pool of zooplankton	38.38	4.08	0	1
Trinité June 23 2021 #1	elver swimbladder	39.28	0.54	0	1
Trinité June 23 2021 #2	elver swimbladder	39.04	0.70	0	1
Trinité June 23 2021 #3	elver swimbladder	39.37	1.52	0.13	2
Trinité June 30 2021 #1	elver swimbladder	37.82	0.97	0	1
Trinité June 30 2021 #2	elver swimbladder	38.81	1.37	0.14	4
Trinité June 30 2021 #3	elver swimbladder	38.73	0.43	0	1
Trinité June 30 2021 #4	elver swimbladder	37.62	2.91	0.33	4
Trinité July 5 2021 #1	elver swimbladder	18.27	1 350 258.18	53 498.08	6
Trinité July 5 2021 #2	elver swimbladder	38.53	0.58	0.16	2
Trinité July 15 2021 #1	elver swimbladder	39.31	2.35	0	1
Trinité July 15 2021 #2	elver swimbladder	39.74	1.37	0	1
Trinité July 15 2021 #3	elver swimbladder	39.89	0.87	0	1
Trinité July 15 2021 #4	elver swimbladder	38.61	1.87	0	1

Table 4 Comparison of the visual and qPCR methods to detect *A. crassus* in young elvers

Sampling localization	Sample type	Tissue	% positive to *A. crassus* with visual method	% positive to *A. crassus* with qPCR method	% positive to *A. crassus* (visual + qPCR)
Grande Trinité and Petite Trinité rivers	Elvers	Individual swim bladder	3.03% (1/33)	39.39% (13/33)	39.39% (13/33)

et al. 2011) and macroscopic examination of yellow eel swim bladders (Rockwell et al. 2009). As these samples were collected early during the season (March–April), it is possible that these young elvers did not begin to feed on infected intermediate hosts at the time of sampling (Jessop 1998). For example, it was previously reported that glass eels start to feed in Petite Trinité River only at stage 3 (VIA2 -VIA3, reached in late June by the time they moved into estuaries) with 17% of eels having different preys in their stomach. Most glass eels at stage 4 (VIA4-VIB, 75%) and elvers at stage 5 (VII, 98%) (reached in early August) had food in their stomach (Dutil et al. 1989; Nilo and Fortin 2001).

Finally, we found a significant positive correlation between the levels of DNA molecules from *A. crassus* detected in the swim bladder of an elver and the anguillid length (Fig. 2) (Spearman correlation, $R = 0.83$, $p = 0.0014$). If we consider the elver length as a proxy of its age, our results suggest that longer (*i.e.* older) elvers were probably infected earlier than smaller (*i.e.* younger) individuals, which would explain why longer eels harbour a higher parasitic burden with more developed parasites. Our qPCR approach could therefore be used in a quantitative way to estimate the parasitic burden in individual swim bladders of elvers. However, the method at this point cannot determinate if a high level of DNA molecules detected is due to a few big nematodes and/or many small parasites, as we were generally not able to visually check for the presence of the parasites.

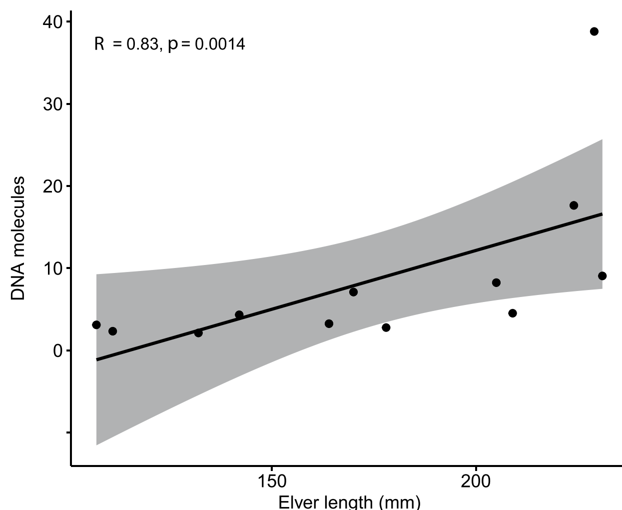

Fig. 2 DNA concentrations of *Anguillicola crassus* detected in individual swim bladders relative to elver length. For each swim bladder, the total quantity of DNA molecules detected in that sample was plotted. A Spearman correlation test was performed using the ggplot2 package (Wickham 2009)

Our study gives an additional support to the usefulness of the qPCR technology to detect parasitic infection in fishes (*e.g.* myxosporean parasite in Rainbow trout *Oncorhynchus mykiss* (Cavender et al. 2004); cestode in Threespine stickleback *Gasterosteus aculeatus* (Berger and Aubin-Horth 2018); nematode in Zebrafish *Danio rerio* (Norris et al. 2020)), and has the advantage of being applicable across different developmental/host stages of the parasite. We therefore hope that the method presented here will be helpful to fisheries management issues, specifically in the context of eel stocking programs. Its application to intermediate hosts would be particularly useful for the early detection of infected stocks in Canada, therefore preventing the introduction of already infected eels in recipient populations.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s00436-023-07843-1.

Acknowledgements The authors thank the MELCCFP regional teams from Gaspésie-îles-de-la-Madeleine, Bas-St-Laurent and Mauricie-Centre-du-Québec. Special thanks to William Cayer-Blais, Vanessa Cauchon and Andréanne Masson.

Authors' contribution Guillaume Côté (G.C.), Jean-François Dumont (J-F.D.) and Louis Bernatchez (L.B.) designed the study. G.C. and J-F.D. performed field sampling. Chloé Suzanne Berger (C.S.B.) and Bérénice Bougas (B.B.) performed the laboratory work. C.S.B. analyzed the results. C.S.B. and B.B. wrote the manuscript with collaboration from all the authors.

Funding This work was funded by the Ministère de L'Environnement, de La Lutte Contre Les Changements Climatiques, de La Faune Et Des Parcs (MELCCFP), Québec, QC, Canada.

Data availability All data and material are available in the results and supplementary material.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflicts of interest The authors declare no conflict of interest.

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