Received: 14 May 2021 | Revised: 29 June 2021 | Accepted: 30 June 2021

DOI: 10.1111/jfd.13495

RESEARCH ARTICLE

Journal of Fish Diseases WILEY

Optimization of tools for the detection and identification of *Cryptocotyle* metacercariae in fish: Digestion method and viability studies

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Funding information
SMRE Doctoral School; Københavns Universitet; MED-VET-NET; Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail; Hauts-de-France Regional Council

Abstract

Some trematode metacercariae, including marine digeneans belonging to the genus *Cryptocotyle*, induce black spots in target tissues due to the attraction of fish host melanophores. To promote precise quantification of infection, the counting of black spots has to be confirmed by reliable quantification of metacercariae after tissue digestion. This process ensures the isolation of undamaged parasites for morphological and molecular identification. The aim of this work was to optimize the pepsin digestion protocol and to assess the duration of viability of *Cryptocotyle* metacercariae in fish post-mortem (pm). Four digestion protocols were compared by measuring the viability rate of metacercariae. The present study shows that the orbital digestion method was the least destructive for metacercariae and allowed better quantification of *Cryptocotyle* infection. Moreover, morphological identification seemed reliable up to 8 days pm for *Cryptocotyle* infection.

KEYWORDS

Cryptocotyle, morphological identification, pepsin digestion, viability of metacercariae

1 | INTRODUCTION

Digenean trematodes are parasitic worms that have a complex life cycle with several successive hosts. In most cases, molluscs act as the first intermediate host and fish as the second intermediate host, whereafter marine birds complete the life cycle upon eating parasitized fish. Adult digeneans develop into the adult stage in the avian gastrointestinal tract; eggs are released with host faeces and are ingested by the first intermediate host in which sporocysts and rediae develop, subsequently releasing free-swimming cercariae that go on to infect fish. Following penetration, the parasites encyst and evolve to the metacercarial stage protected by a proteinaceous cyst wall.

The family Heterophyidae comprises 13 genera with zoonotic potential (*Acanthotrema*, *Apophallus*, *Ascotyle*, *Centrocestus*, *Cryptocotyle*, *Haplorchis*, *Heterophyes*, *Heterophyopsis*, *Metagonimus*, *Procerovum*, *Pygidiopsis*, *Stellantchasmus*, *Stictodora* [Chai & Jung, 2017; Chai, 2014; Chai et al., 2005; Hung et al., 2013; Warren, 1953]). Some heterophyid cercariae induce cutaneous black spots following penetration and encystation in the intermediate host. This phenomenon is observed for genera such as *Apophallus* (Sándor et al., 2017), *Cryptocotyle* (Chapman & Hunter, 1954; Sindermann & Farrin, 1962), *Haplorchis* (Paperna & Dzikowski, 2006) and *Stellantchasmus* (Chai et al., 2016) (World Register of Marine Species, www.marinespecies.org). The present work focuses on the genus *Cryptocotyle* (Lühe, 1899).

Trematodes are commonly isolated using digestion method. For black spot diseases, infection is often estimated by counting the number of spots. However, the number of black spots may under-evaluate parasite contamination (Lysne et al., 1995). In this study, methods were assessed for recovering *Cryptocotyle* metacercariae based on available bibliographic references (Borges et al., 2015; Casalins et al., 2020; Heuch et al., 2011; Lysne et al., 1995; McDaniel, 1966). Pepsin digestion was optimized to quantify the number of metacercariae precisely and to compare it to the number of cutaneous black spots. Furthermore, the life span of metacercariae in fish has not yet been documented (Lysne et al., 1995). Then, the viability of metacercariae for up to 15 days after the death of the fish host was estimated. The length of time during which metacercaria can still be morphologically identified after fish post-mortem (pm) was determined. The overall goal was to develop a set of methods to quantify and morphologically identify *Cryptocotyle* metacercariae in fish.

2 | MATERIALS AND METHODS

2.1 | Sample collection and parasitological observations

Four batches of 5 to 50 kg of whiting (*Merlangius merlangus*) ($n \approx 300$ in total) were caught in the English Channel between March and November 2020. They were obtained from a local fishmonger and from an angler in Boulogne-sur-Mer (France). The individuals were fished during the night before the reception at the laboratory. The day of reception was considered as day one post-mortem (pm). The fish were examined with the naked eye for presence of cutaneous black spots. Only parasitized fish with more than 10 black spots per fish were selected. For these individuals, to assess the infection level, the location and number of black spots were observed and counted under a magnifying glass for each area on the fish body according to Buchmann (2007) (Figure 1).

Then, fish were measured, weighed, eviscerated and stored at 1°C (±1) in a refrigerated incubator (Binder GmbH) until use for experiments on the optimization of digestion protocols and assessment of metacercariae viability.

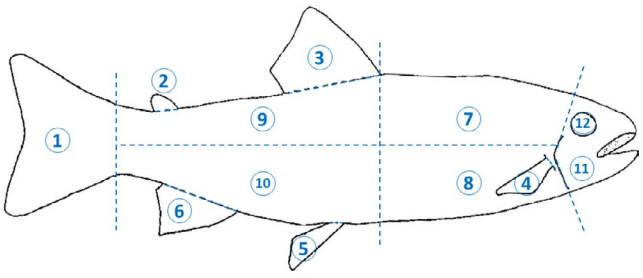

FIGURE 1 Definition of the different areas of a fish individual (Buchmann, 2007)

2.2 | Optimization of artificial pepsin digestion protocols

Four digestion protocols (named D1, D2, D3 and D4) were compared (Figure 2). They were performed simultaneously seven days after fish death. One whole fish fillet (covering 7, 8, 9 and 10 areas, Figure 1) was sampled for the D1 and one for D3 protocols. A 3 × 3 cm piece of tissue from the most infected areas of one of the fillet (taken from 7 to 10 areas, Figure 1) was used for the D2 protocol and one for the D4 protocol. Numbers of black spots per sample (according to digestion protocols) were recorded. For all four methods, the samples (3 × 3 cm samples or whole fillet) were skinned and the subcutaneous muscle with a thickness of approximately 5 mm was removed. Skin and subcutaneous muscle were digested separately in a pepsin/HCl/saline solution (Borges et al., 2015) at 37°C (±1) for 1 hr. For the D1 and D2 protocols, digestion was carried out in 50-ml beakers with magnetic stirring (200 rpm, round magnet L = 40 mm) (Velp Scientifica). For the D3 and D4 protocols, digestion was performed in wells of a 6-well culture plate on an orbital agitator (240 rpm) (IKA).

All digested samples were transferred to Petri dishes. Metacercariae were collected by pipetting under an Olympus SZX16 stereomicroscope (Olympus Corporation). They were kept in phosphate-buffered saline (PBS) (pH = 7.3) at room temperature for no more than 1 hr.

Then, metacercariae recovered from each digested sample were transferred to prewarmed 2% porcine trypsin in PBS according to Duflot et al. (2021) at 37°C (±1) for no more than 1 hr to induce cyst walls to open and live metacercariae to emerge. Under microscopic observation, the parasites were considered alive when they showed signs of movement and their membrane had been breached but not necessarily sloughed off (McDaniel, 1966). The number of live and dead parasites was recorded. All the metacercariae were then stored in 96% ethanol at 1°C (±1).

2.3 | Assessment of metacercariae viability

Fish individuals were stored at 1°C (±1) in a refrigerated incubator starting from day one pm and were analysed on different sequential days pm to cover the period from 1 to 15 days pm ($n = 41$ fish in total). Parasite detection was carried out using the pepsin digestion and trypsin excystment method selected based on the results of the optimization of the pepsin digestion step. The viability rate was also determined as described above.

For each fish, metacercariae stored in 96% ethanol were transferred to Petri dishes. They were observed and photographed under an Olympus SZX16 stereomicroscope with an Olympus UC90 camera (Olympus Corporation). Then, a selection of one third of the samples was rinsed in distilled water, stained with haematoxylin and mounted on slides in a glycerine–gelatine medium (Buchmann, 2007). Parasite morphology and the state of organs were observed (x100 magnification) and photographed under the Olympus IX73 microscope with an Olympus SC50 camera.

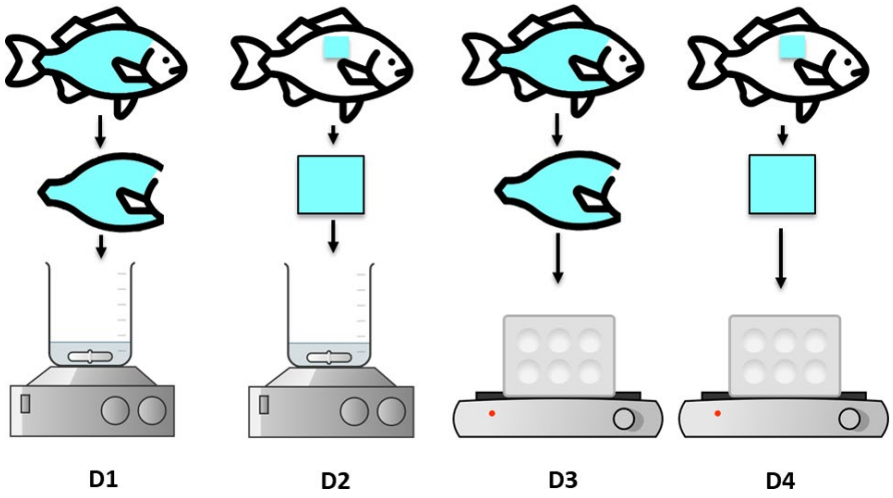

FIGURE 2 Schematic of the digestion protocols on whole fillets (D1, D3) and 3 × 3 cm tissue samples (D2, D4) under magnetic stirring (D1, D2) or orbital agitation (D3, D4); adapted from Chemix 3.32.2 (Codelite Ltd, 2007–2021)

TABLE 1 Number of black spots counted and metacercariae isolated following the four digestion protocols (n = 3 fish). No.:number

Digestion method	No. of black spots in analysed area(s)	No. of recovered metacercariae	No. of viable recovered metacercariae	Viability rate (%)
D1	51	51	46	90
D2	10	10	8	80
D3	42	74	43	58
D4	6	6	6	100

Note: The viability rate is the percentage of the number of viable recovered parasites to the number of recovered metacercariae.

3 | RESULTS

3.1 | Epidemiological data

The selected infected individuals with more than 10 black spots (n = 42) had a mean total length of 29.3 (24.6–37.2) cm and a mean weight of 190.1 (103.9–326.9) g. The mean observed intensity of infection of the selected whiting was 74 (range 11–430) black spots per fish. Areas 7 and 9 were the most infected with, respectively, 9 (0–54) and 12 (1–71) black spots on average (Figure 1). The outer muscle layer was less infected than the skin (Appendix 1). Indeed, metacercariae were mainly recovered from the skin digestion solution. The ratio of black spots to metacercariae for each individual was generally one black spot to one metacercaria (1:1). However, some samples showed fewer recovered metacercariae than black spots (>1) and some displayed the opposite relationship (<1) (Appendix 1).

3.2 | Digestion method

The number of black spots recorded before digestion and the number of recovered metacercariae showed a ratio of 1:1 for the D1, D2 and D4 protocols, but for the D3 protocol, more metacercariae were recovered than counted black spots, and the ratio was 1:1.7 (Table 1). Thus, for each counted black spot, at least one metacercaria was found in fillets (D1 and D3) and in 3 × 3 cm tissue samples (D2 and D4).

The four digestion protocols led to different viability rates. The best result was obtained with the D4 protocol for which all sampled metacercariae were alive. For the D3 protocol, only 58% of metacercariae were alive. This result may be due to intra-individual variation in fish or in parasites (a hostile microenvironment in the fish due to more effective immune responses and/or age of metacercariae). In addition, in the digestion solutions from the D1 and D2 protocols, some empty cysts as well as partial or fragmented metacercariae were observed (Figure 3). The D4 digestion protocol producing the highest viability rate was therefore selected. Moreover, this protocol is based on 3 × 3 cm tissue samples, making for a quick and efficient method.

3.3 | Viability of metacercariae

Viability was studied using the D4 digestion protocol. Numbers of live parasites after pepsin digestion and trypsin excystment were counted on different days of analysis for up to 15 days (shown in Appendix 1). Variability in viability was observed; however, there was a global tendency for a decrease in the proportion of viable parasites with increasing days after fish death (Figure 4).

For each day of analysis, viable parasites were first observed in ethanol and then after staining (Figure 5). In ethanol on a black

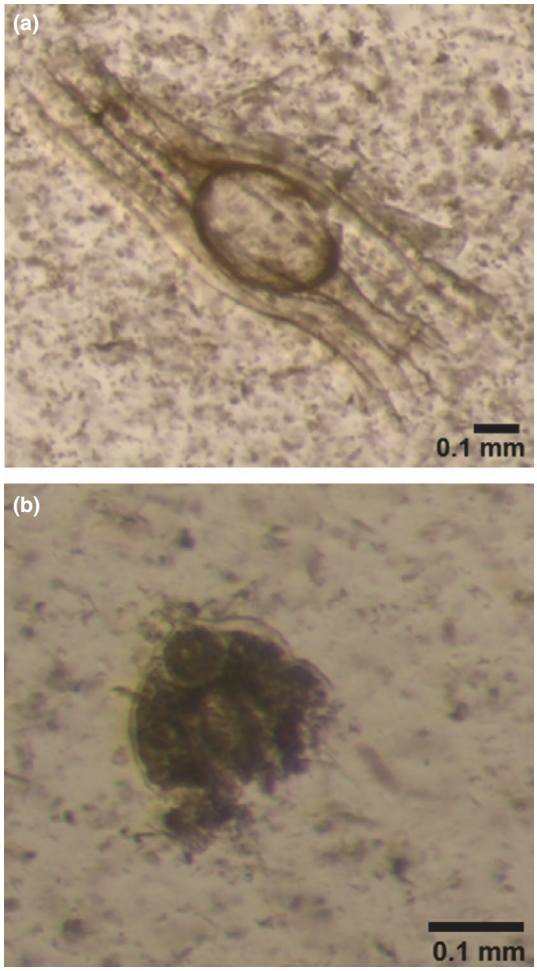

FIGURE 3 Empty cyst (a) and partially degraded metacercaria (b) observed with the D1 and D2 digestion protocols

background, parasite colour was white, and some organs could be distinguished. After staining the slides, the organs appeared differentially stained.

With both observation methods, after day 13, many parasites had irregular outlines and organs became blurred and undistinguishable. From day 8 p.m., parasites were fragile and delicate. It was difficult to stain and fix them on slides, many cracked during rinsing or haematoxylin staining. Furthermore, for those successfully mounted after day 8, the pharynx and intestinal tract looked wider than on previous days. Then, after day 8, some parasite outlines were folded over (e.g., Figure 5, bottom, day 9 and day 14). Thus, after more than 8 days pm, metacercarial morphology was damaged. Organs and outlines were modified, and morphological identification became difficult and unreliable.

4 | DISCUSSION

Development of quantitative and reliable tools to detect and identify cutaneous parasites, such as those from the genus *Cryptocotyle*, help to improve knowledge and understanding of this parasite. Observations of the location of infections confirmed that the dorsal areas (areas 7 and 9 most infected) were the sites of predilection for *Cryptocotyle* infection. Metacercariae were mainly found in the skin layer of the studied fish species. As previously documented in Atlantic cod, although the skin is the preferred microhabitat, the outer body musculature may carry a significant part of the parasite infrapopulation (Borges et al., 2015). McQueen et al. (1973) described the presence of parasites in the dermis and muscles or in the paraspinal connective tissues; in gadoids, Van den Broek (1979) observed that parasite infection was concentrated above the lateral line. McQueen et al. (1973) hypothesized that cercariae preferentially infect thin epidermises that are easy to penetrate and thus that require less energy. For *Merlangius merlangus*, the dermis appears thicker on the ventral part of the fish body than above the lateral line.

The difference in the number of observed black spots and the number of recovered metacercariae for some fish confirmed previous observations (Lysne et al., 1995) whereby, at low levels of infection, the number of black spots can underestimate the number of metacercariae. For instance, in infections with *Cryptocotyle lingua* in *Pleuronectes platessa*, pigmented spots around metacercariae appear in 10 to 20 days post-infection (McQueen et al., 1973). Furthermore, metacercariae in fish muscle contained fewer melanin cells around them than those in the dermis (McQueen et al., 1973). These remarks showed the necessity of precaution in data interpretation. Proper analysis requires recording the number of black spots and the number of metacercariae post-digestion.

The digestion protocols tested in this study showed that magnetic stirrer can destroy some encysted metacercariae during digestion when parasite detection cannot be performed immediately after fish death. Indeed, in D1 and D2 digestion juice preparations, partial structures of metacercariae were found suggesting partial degradation of some metacercariae during digestion. In most studies, trematodes are isolated directly after host death. Grinding followed by pepsin digestion is the most frequently used method (Chai et al., 2019; Dai et al., 2020; Sohn et al., 2018). Grinding may damage some metacercariae and increase inaccuracy in the quantification. Citric acid can be used instead of HCl for the acidification of pepsin solution, with concentrations of more than 5% of citric acid leading to best recovery of viable metacercariae (Kim et al., 2013); however, efficient fish muscle digestion needs over 3 hr. Finally, for the digestion step, orbital agitation in a pepsin solution proved to be gentle, relatively quick and allowed better preservation of metacercariae, providing for better observation of parasites. Furthermore, viability rates on 3 × 3 cm samples and on whole fillets appeared equivalent; however, the digestion of whole fillets is time-consuming. Therefore, the D2 and D4 protocols offered better efficiency than the D1 and D3 protocols. To conclude, the D4 method was selected for the study of viability because it was more efficient, allowed a better preservation of parasite structures and a good assessment of the ratio between number of black spots and recovered metacercariae.

Metacercariae need to be excysted for their morphological identification. However, only live metacercariae can excyst following

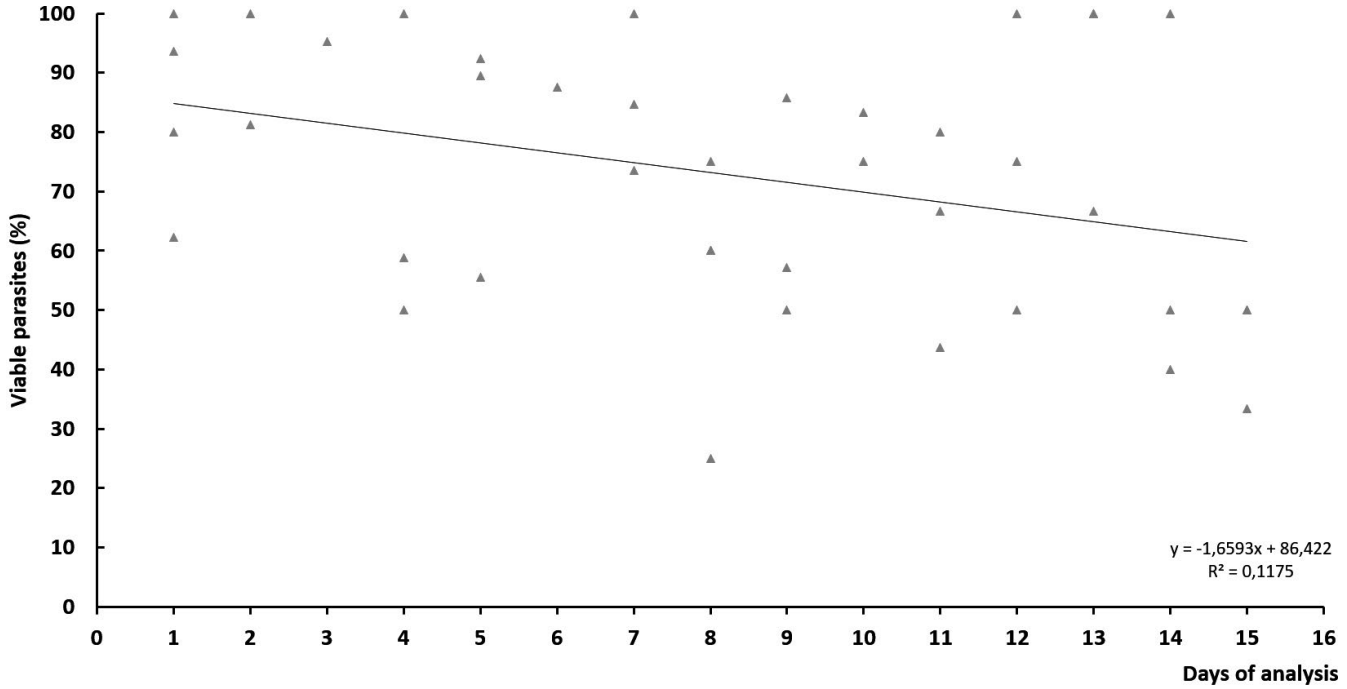

FIGURE 4 Change in the proportion of viable parasites on sequential days post-mortem. The solid line shows the linear regression of data

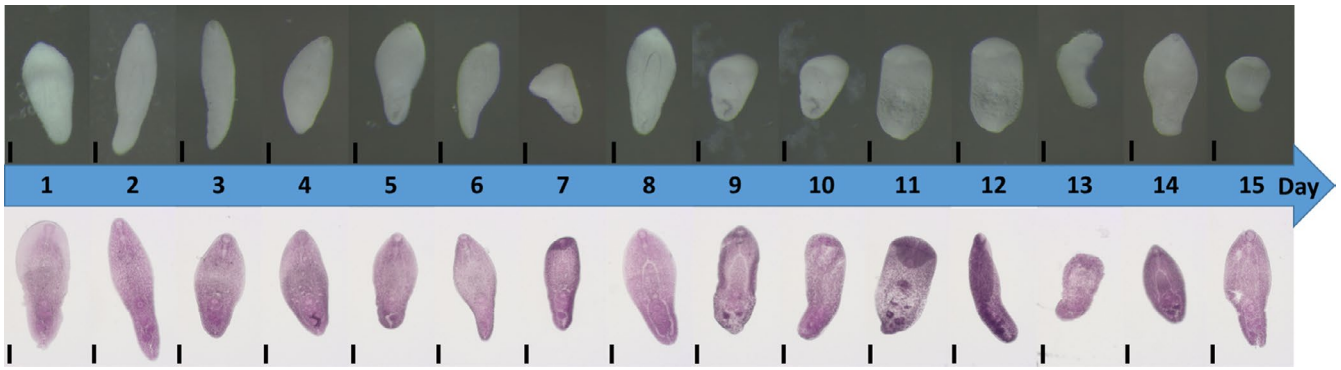

FIGURE 5 Observation of viable parasites in ethanol (top) and after staining and fixation on glass slides (bottom) on each day after fish death (i.e., 1: 1 day post-mortem). Scale: 100 μm

trypsin digestion. The study of the viability of *Cryptocotyle* after fish death showed that live parasites were recovered up to 15 days pm. Nevertheless, beyond 8 days pm, the overall shape and organs of metacercariae were too damaged to morphologically identify the parasites, because many parasites were fragile and crushed when mounted on glass slides after day 8. In conclusion, *Cryptocotyle* metacercariae can be morphologically identified up to 8 days pm, allowing the conservation of fish up to 8 days post-catch before analysis. To our knowledge, no equivalent viability study has previously been carried out on trematodes. Recently, some authors studied both the viability and the infectivity for different pathogens, mixing several techniques in parallel. Bajelan et al. (2020) studied the effects of a potential treatment on *Toxoplasma gondii* by flow cytometry for the viability and by cell cultures for the infectivity. MacInnis et al. (2020) evaluated the effects of environmental factors such as temperatures on the viability and infectivity of *Nosema ceranae* with staining procedures and experimental infections on bees. However, the assessment of infectivity usually relies on experimental infections and/or in vitro models. Such methods are not easily available for *Cryptocotyle* metacercariae. The validation of these tools will be a prerequisite before any study on infectivity assessment after either food/culinary treatments or keeping the fish host after its death may be carried out.

In summary, the present study defined protocols for the detection and identification of *Cryptocotyle* metacercariae. These methods are amenable to large-scale epidemiological studies, now making it possible to acquire parasite prevalence, intensity and identification data.

ACKNOWLEDGEMENTS

This research was funded by the French Agency for Food, Environmental and Occupational Health & Safety; the Hauts-de-France Regional Council; MED VET NET Organisation; the SMRE Doctoral School; and the Laboratory of Aquatic Pathobiology of the University of Copenhagen. The authors are indebted to F. Lebon and Ifremer for sharing their microscope for our studies. We also thank M. Leblond who fished the whiting samples. The authors would like to thank D. Pottratz from Anses and C. Engel-Gautier from Coup de Puce Expansion for English-language editing. Moreover, the authors are thankful to the two anonymous referees who helped to greatly improve this manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data are included in the manuscript or in the Appendix 1.

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How to cite this article: Duflot, M., Midelet, G., Bourgau, O., Buchmann, K., & Gay, M. (2021). Optimization of tools for the detection and identification of *Cryptocotyle* metacercariae in fish: Digestion method and viability studies. *Journal of Fish Diseases*, 44, 1777–1784. https://doi.org/10.1111/jfd.13495

APPENDIX 1

Data from the viability study of metacercariae in fish on sequential days post-mortem. The number of black spots and the number of metacercariae were determined from 3 × 3 cm tissue samples. Bold letters indicate cases in which the ratio showed more recovered metacercariae than observed black spots. Underlined figures indicate a ratio showing fewer recovered metacercariae than the number of black spots, No.: number.

Individual label	Day	No. of black spots	No. of live metacercariae in skin	No. of dead metacercariae in skin	No. of live metacercariae in muscle	No. of dead metacercariae in muscle	Total No. of recovered metacercariae	Ratio No. black spot: No. metacercariae	Viable parasites (%)
T_86	1	170	133	9	0	0	142	1.2	94
T_87	3	16	20	1	0	0	21	**0.8**	95
T_88	5	10	17	2	0	0	19	**0.5**	89
T_89	7	27	25	8	0	1	34	**0.8**	74
T_90	9	4	3	3	0	0	6	**0.7**	50
T_91	1	19	26	13	2	4	45	**0.4**	62
T_92	2	7	7	0	1	0	8	**0.9**	100
T_93	4	14	10	5	0	2	17	**0.8**	59
T_133	2	11	13	3	0	0	16	**0.7**	81
T_134	5	8	5	4	0	0	9	**0.9**	56
T_135	6	9	7	1	0	0	8	1.1	88
T_136	8	7	5	4	1	0	10	**0.7**	60
T_137	1	5	5	0	0	0	5	1.0	100
T_149	1	15	12	3	0	0	15	1.0	80
T_138	4	4	4	0	0	0	4	1.0	100
T_150	4	5	6	6	0	0	12	**0.4**	50
T_160	5	13	12	0	0	1	13	1.0	92
T_139	7	10	11	2	0	0	13	**0.8**	85
T_151	7	6	6	0	0	0	6	1.0	100
T_141	8	19	14	4	1	1	20	1.0	75
T_152	8	5	3	2	0	0	5	1.0	60
T_161	8	4	1	3	0	0	4	1.0	25
T_142	9	7	4	2	0	1	7	1.0	57
T_153	9	8	6	1	0	0	7	1.1	86
T_143	10	6	5	1	0	0	6	1.0	83
T_154	10	4	3	1	0	0	4	1.0	75
T_144	11	5	4	1	0	0	5	1.0	80
T_155	11	16	7	8	0	1	16	1.0	44
T_162	11	3	2	1	0	0	3	1.0	67

(Continiues)

Individual label	Day	No. of black spots	No. of live metacercariae in skin	No. of dead metacercariae in skin	No. of live metacercariae in muscle	No. of dead metacercariae in muscle	Total No. of recovered metacercariae	Ratio No. black spot: No. metacercariae	Viable parasites (%)
T_145	12	4	3	1	0	0	4	1.0	75
T_156	12	5	3	0	0	0	3	1.7	100
T_163	12	5	3	1	0	2	6	**0.8**	50
T_146	13	4	4	0	0	0	4	1.0	100
T_157	13	3	2	1	0	0	3	1.0	67
T_164	13	4	4	0	0	0	4	1.0	100
T_147	14	5	2	3	0	0	5	1.0	40
T_158	14	4	2	2	0	0	4	1.0	50
T_165	14	2	2	0	0	0	2	1.0	100
T_148	15	4	2	2	0	0	4	1.0	50
T_159	15	5	2	2	0	0	4	1.3	50
T_166	15	3	1	2	0	0	3	1.0	33