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Viability of *Cryptocotyle lingua* metacercariae from Atlantic cod (*Gadus morhua*) after exposure to freezing and heating in the temperature range from −80 °C to 100 °C

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ABSTRACT

The presence of parasites in fish products is a problem that concerns consumers and authorities due to the potential hazards it may cause. The majority of studies on the viability of parasites in marine fish products currently focus on nematodes of the family Anisakidae whereas only few are concerned with trematodes. In this study on the heterophyid trematode *Cryptocotyle lingua* (identified by morphometric and molecular techniques) we isolated metacercariae from Atlantic cod (*Gadus morhua*) and incubated the parasites in cod muscle tissue at different temperatures ranging from −80 °C to 100 °C and subsequently tested their viability. SEM images were made to assess the physical damage caused to parasites exposed to different temperatures. Temperatures between 50 °C and 100 °C and between −80 °C and −20 °C killed the metacercariae present in fish flesh in less than 2 h. Controls kept at 5 °C survived for nine days. Extreme freezing temperatures caused minimal visual physical damage to cysts, but the tegument of metacercariae was severely affected at all temperatures when incubated for long periods.

1. Introduction

Concerns with the quality and safety of fish products are growing among consumers and legislators (FAO, 1998; Huss, Ababouch, & Gram, 2003) but global changes in the way consumers perceive quality have led to introduction of dishes based on inadequately processed fish products. This, however, increases the risk of obtaining food-borne diseases including parasitoses (Grunert, 2005; Orlandi, Chu, Bier, & Jackson, 2002; Portes Santos, Lopes, Costa, & Santos, 2013). Among these are infections caused by nematodes (Audicana & Kennedy, 2008) but also fish-borne trematode infections are known to affect about 40 million people worldwide. Trematodes in fish reported as human health hazards comprise metacercariae within the families Heterophyidae, Echinostomatidae and Opisthorchiidae (Abdussalam, Käferstein, & Mott, 1995; Fried, Graczyk, & Tamang, 2004; Healy, 1970). Many of the human cases have been reported from Asia where a number of studies have focused on the biology and control (including food processing technology) of several of the endemic representatives within these taxa such as species of *Clonorchis* and *Opisthorchis*. Our knowledge on similar aspects of parasites from temperate and Arctic regions, such as the heterophyid fluke *Cryptocotyle lingua*, is on the other hand far from complete. Snails of the genus *Littorina* act as first intermediate hosts (Lambert, Corliss, Sha, & Smalls, 2012; Mattheus, Montgomery, & Hanna, 1985) and a wide range of teleost fish species act as second intermediate hosts (van den Broek, 1979; Stunkard, 1929). Fish species reported with *C. lingua* infections in Scandinavian waters include Atlantic cod (Buchmann, 1986; Hemmingsen, Lombardo, & MacKenzie, 1991; Heuch et al., 2011; Køie, 1984; Mellegaard & Lang, 1999), dab *Limanda limanda* (Køie, 1983), three-spined stickleback *Gasterosteus aculeatus* (Barber, 2003), Arctic char, *Salvelinus alpinus* (Kristoffersen, 1988) and herring *Clupea harengus* (Tolonen & Karlsbakk, 2003). The adult form is found in the intestine of fish eating birds (Guildal, 1968) but there are reports of adult *C. lingua* infecting mammals such as foxes (Saeed, Maddox-Hyttel, Monrad, & Kapel, 2006), dogs (Christensen

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& Roth, 1949) and cats (Christensen & Roth, 1946). Human infections have been reported from Greenland and Alaska (Babbott, Frye, & Gordon, 1961; Rausch, Scott, & Rausch, 1967). Despite the documented ability of the parasite to infect mammals and humans no studies have been carried out on how food processing techniques affect viability of these metacercariae. In order to provide consumers with safer food products, legislators have provided specific procedures on processing and treatment of fish products in order to avoid parasites (EFSA, 2004, 2010; Huss et al., 2003). However, the current legislation is based on studies on species from other geographic regions (Egypt, China, Thailand and Russia) (Fan, 1998; Fattakhov, 1989; Hamed & Elias, 1970; Kruatrachue, Chitramvong, Upathan, Vichary, & Viyanant, 1982; Tesana, Kaewkes, & Phinlaor, 1986). This calls for investigations into effects of heating or freezing techniques on survival of metacercariae from North-European waters. We have therefore performed a controlled study to describe the lethal effect of cold and heat exposure (temperatures ranging from −80 °C to 100 °C) on *C. lingua* metacercariae from Atlantic cod.

2. Material and methods

2.1. Fish

Atlantic cod (*Gadus morhua*) ($N = 25$) freshly caught in Danish waters were obtained from a local fish monger in Copenhagen. Fish had a mean weight (gutted) of 2.074 (SD 0.513) kg, and a mean total length of 63.4 (SD 6.2) cm.

2.2. Site of metacercariae in cod skin and fillet layers

The cod musculature was removed from the fish skeleton and the two fillets recovered from each fish were skinned. A longitudinal sagittal section divided the fillet into the outer and inner fillet layers, each with a thickness of approximately 1 cm. In order to determine site and parasite density in cod skin and the underlying outer/inner muscle layers similarly sized pieces of the skin, outer muscle and inner muscle of seven cod were measured, weighed and then digested as described in Section 2.4. The musculature used was the part between the neck and the posterior end of the first dorsal fin, and the dorsal midline and the lateral line, which is the most heavily infected part of the skin/musculature (Lysne, Hemmingsen, & Skorping, 1994). The isolated metacercariae of each sample were counted and used to make an estimation of the number of metacercariae per gram of muscle/skin or per area of the skin.

2.3. Artificial digestion

Cod skin or musculature samples (a maximum of 50 g) were digested in standardized volumes (500 mL) of pepsin solution (1 L tap water, 6 mL HCl (≥37%), 9 g NaCl, 10 g pepsin (2000 FIP u/g)) in 1 L glass beakers (Ø = 95 mm) and magnetic stirring (200 rpm, magnet $L = 40$ mm) at 37 °C for 2 h. Digested samples were poured through a 50 μm sieve and isolated metacercariae were collected by flushing with saline (0.9% NaCl) and stored in phosphate buffered saline (PBS) (pH 6.2) until further use. In order to produce a standard population of encysted metacercariae for subsequent studies, a pool of metacercariae (>2000 specimens) collected from 3 individual cod was prepared in a Petri dish.

2.4. Incubation of isolated metacercariae in fillet sandwiches at various temperatures

Twenty isolated metacercarial cysts were mounted in fillet sandwiches i.e. parasites were pipetted between two fillet slices (each measuring 24 × 24 × 2–3 mm) of parasite-free cod muscle tissue and placed in Petri dishes (Ø = 60 mm) for incubation at different temperatures ranging from −80 °C to 100 °C (Table 1a). Duration of incubation (i.e. sampling time points) ranged from 1 min to 10 days dependent on the temperature (see Table 1a). All incubations were performed in triplicate. Petri dishes prepared for low incubation temperatures (<0 °C) were incubated without lid. Petri dishes prepared for high incubation temperatures (>0 °C) were supplied with 500 μL of deionized water and a lid in order to avoid desiccation. Subsequent to incubation, metacercariae were collected and their viability assessed.

Table 1
1a and 1b. Mean number (i.e. mean of triplicates of 10 metacercariae each) of dead metacercariae at different sampling time points and temperatures. Red coloration indicates death of all metacercariae. (1a) Metacercariae were isolated by pepsin digestion and mounted between cod tissue slices prior to incubation. (1b) Metacercariae were kept *in situ* as infections in cod tissue during incubation.

1a

Temperature °C	Sampling time points																				
	Minutes						Hours							Days							
	1	5	10	15	20	30	1	2	3	4	6	12	24	2	3	4	5	6	7	8	9	10
100	0.0	0.3	10.0	10.0	10.0																	
90	0.0	0.3	2.0	10.0	10.0	10.0																
80	0.0	0.0	5.3	10.0	10.0	10.0																
70	-	0.0	0.7	10.0	10.0	10.0	10.0															
60	-	0.0	0.0	0.0	0.0	10.0	10.0	10.0														
50	-	0.0	0.0	0.0	0.0	0.0	0.0	10.0	10.0													
5	-	-	-	-	-	-	-	-	-	-	-	-	0.0	0.0	0.0	0.0	0.0	3.7	8.0	9.0	8.3	10.0
-20	-	-	-	-	-	0.0	8.7	10.0	10.0	-	-	10.0	10.0	10.0								
-40	-	-	-	0.3	-	4.0	10.0	10.0	10.0	10.0												
-60	-	0.0	0.0	0.0	2.5	10.0	10.0	10.0														
-80	0.0	0.0	0.0	9.3	10.0	10.0																

1b

Temperature °C	Sampling time points												
	Minutes						Hours						
	1	5	10	15	20	30	1	2	3	4	6	12	24
70	-	0.0	1.0	9.0	-	10.0	-	10.0					
60	-	0.0	0.0	3.7	3.7	10.0	10.0	10.0					
-20	-	-	-	-	-	0.0	8.3	9.7	-	-	10.0	10.0	10.0
-40	-	-	-	0.0	-	4.3	10.0	10.0	10.0				

2.5. Incubation of non-isolated metacercariae in situ in skin and musculature

Approx. 5 g of infected cod tissue (skin including 4–6 mm of underlying muscle tissue) from three different cod were placed in Petri dishes and incubated at selected temperatures for different time periods (Table 1b). All incubations were made in triplicate. Deionized water and lid was applied as described above (2.5).

2.6. Recovery of metacercariae from incubated cod tissue

After incubation (according to 2.6), each sample (approx. 5 g) was digested in standardized volumes (80 mL) of pepsin solution (see 2.4) in 100 mL glass beakers ($Ø = 50$ mm) and magnetic stirring (300 rpm, magnet $L = 30$ mm) at 37 °C for 1 h. Digested samples were poured through a 50 μm sieve and isolated metacercariae were collected by flushing with saline (0.9% NaCl), placed in PBS (pH 6.2) and their viability tested.

2.7. Assessment of viability

Ten metacercariae recovered from each sample were transferred to 1 mL of 0.25% porcine trypsin (13,000–20,000 BAEE units/mg) (Sigma–Aldrich, Denmark) in PBS (pH 7.4) at room temperature (24 ± 1 °C) for no more than 1 h. To check the viability of metacercariae, the excysted parasites that were not moving were stained in a 1:1 solution of PBS (pH 7.4) and 0.4% Trypan Blue (Gibco, Scotland) for at least 3 min. Excysted metacercariae were considered dead when they stained by Trypan Blue. The same criteria could not be applied to non-excysted specimens, since Trypan Blue stains the cyst wall of both dead and live metacercariae. The viability of the cysts was in this case determined by assessing the amount of change in general morphology with brownish coloration and fading of morphological features indicating death (Fig. 1A). In this paper we refer to excystment as both: the active process by which a live metacercaria emerge from its cyst at favorable conditions and the release of the dead worm after a digestion of the cyst. All metacercariae were stored in 96% ethanol. Ten untreated metacercariae were excysted in triplicate as positive controls and stored in 96% ethanol for further use in molecular identification. Pilot trials showed that the viability of *C. lingua* metacercariae was not compromised by any of the two digestion protocols applied (2.4 and 2.7), not even when extending time of digestion to the double for both procedures (i.e. 4 and 2 h, respectively) (data not shown).

2.8. Light microscopy (LM)

Ten freshly isolated cysts were mounted on slides in saline (0.9% NaCl) and ten excysted metacercariae were fixed in hot neutral buffered 4% formalin and mounted on slides in PBS. The parasites were measured and photographed in a Leica DMLB microscope with a Leica DC300 camera (Fig. 1B). The parasites used for the measurements were uncompressed while parasites used for the photos were flattened under coverslip pressure in order to bring as many morphological features into focus as possible. Measurements are presented in micrometers (μm) as the average followed by the minimum and maximum inside brackets.

2.9. Scanning electron microscopy (SEM)

Ten cysts and ten excysted specimens were taken from each sample and fixed in 2.5% PBS-buffered glutaraldehyde. The samples were washed in PBS, brought through a graded series of alcohol and acetone for dehydration. Critical-point drying was done using an Autosamdri-815(A) (Tousimis, Maryland, USA) on automatic sequence, after which the specimens were mounted on SEM stubs, sputter-coated and observed in a JEOL JSM-6335-F (FE-SEM, Tokyo, Japan) microscope. All images were digitally processed using standard software.

2.10. Molecular identification

Individual excysted metacercariae preserved in 96% ethanol were air-dried and lysed at 55 °C in 30 μL of lysis reagent [Tween 20 (0.45%), proteinase K (60 μL/mL) 10 mMTris and 1 mM EDTA]. Complete lysis was confirmed by microscopy, whereafter the protease was inactivated at 95 °C for 10 min (Thuy, Kania, & Buchmann, 2010). PCR reactions were performed in a volume of 60 μL containing 2 μL lysate as template, 1 unit of BIOTAQ™ DNA polymerase (DNA-Technology), 1 μM dNTP, 1.5 μM $MgCl_2$, and 1 μM of the primers. In order to amplify the ITS region the primers Diplost_F3 (5′- AGGAATTCCTGGTAAGTGCAAG–3′)/Diplost_R4 (5′- TATGCTTAA ATTCAGCGGGT–3′) were used (Galazzo, Dayanandan, Marcogliese, & McLaughlin, 2002), and to amplify the mt DNA-Cox1 region the primers COI2575F (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′)/COI302 1R (5′- TAAAGAAAGAACATAATGAAAATG–3′) were used (Bowles, Blair, & McManus, 1992). PCR was carried out in a Biometra T3 thermocycler (Fisher Scientific) using the following cycling parameters: ITS primers - pre-denaturation at 94 °C for 5 min, 45 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min and post-elongation of 72 °C for 7 min. COI primers – pre-denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 50.9 °C for 30 s, 72 °C for 40 s and post-elongation of 72 °C for 7 min. The products were analyzed by ethidium bromide-stained 1.5% agarose gel electrophoresis. PCR products were purified using the illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare) and sequenced at Macrogen Inc. (South Korea) using the PCR primers.

3. Results

3.1. Identification

The metacercariae (encysted and excysted) were identified as *C. lingua* (Creplin) based on morphometric and molecular parameters. Measurements based on ten cysts and ten metacercariae. The cysts were 258.3 (225–282.5) μm long by 202.3 (190–212.5) μm wide (Fig. 1B). The body of excysted specimens was covered with scale-like spines; total body length 635.5 (575–675) μm and largest width 192.5 (175–205) μm. Oral sucker subterminal, 52.5 (50–55) μm long and 58.5 (60–55) μm wide. Prepharynx short; pharynx 38 (30–40) μm long by 30 (35–25) μm wide. Ventral sucker small, 24.5 (30–20) μm long and 24.5 (30–20) μm wide. The measurements are in accordance with previous descriptions of *C. lingua* metacercariae (Linton, 1915; Stunkard, 1929, 1930). COI region sequences and ITS region sequences were deposited in GenBank under the accession numbers KJ711861-KJ711866 and KJ641518-KJ641524, respectively. These are the first sequences of *C. lingua* for the ITS region deposited in GenBank.

The sequences obtained in this study ($N = 6$) were identical over 387 nt compared. They showed 99% identity to the *C. lingua* COI sequences available in GenBank (EU876333–EU876528).

3.2. Density of parasites in cod tissue

The cod were infected with an average of 45.8 (SD 35.6) metacercariae per cm^2 of skin and 561.4 (SD 480.2) metacercariae per gram of skin. The outer muscle layer was less infected compared to the skin presenting an average of 2.4 (SD 2.5) metacercariae per gram of muscle (Fig. 2). No metacercariae were found in the inner muscle layers.

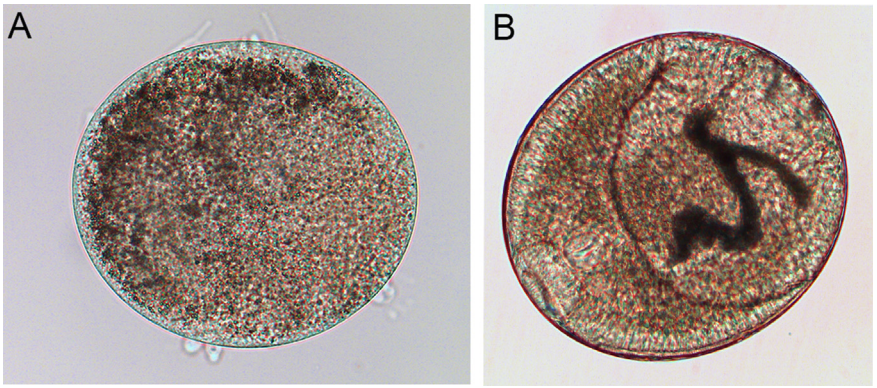

Fig. 1. Encysted *Cryptocotyle lingua* metacercariae. (A) Dead encysted metacercaria (rendered non-viable by incubation at −20 °C for 2 h) showing loss of morphological features. (B) Live encysted metacercaria presenting oral sucker, pharynx and excretory bladder.

3.3. Survival of metacercariae

Untreated metacercariae (no freezing or heating) showed 100% excystation and viability after incubation for 1 h to the applied trypsin solution. Metacercariae were killed in less than 20 min at 70 °C, 80 °C, 90 °C and 100 °C whereas it took 2 h at 50 °C. For freezing temperatures at −80 °C, 20 min were necessary to kill all the metacercariae but 2 h were needed at −20 °C to −60 °C. The metacercariae were rendered non-viable after 2 h of exposure to all temperatures tested. This applied both for experiments in which isolated metacercariae were placed between fish slices as well as experiments where metacercariae in naturally infected tissue were incubated *in situ*. Controls kept at 5 °C survived for up to nine days (Table 1a and b). There were no differences (with regard to viability) between the experiments *in situ* and those with isolated metacercariae artificially mounted between fish slices.

3.4. Damage to the cyst wall of encysted metacercariae associated with incubation

The extreme freezing temperature −80 °C did not cause any visual damage (evaluated by LM and SEM) to the cyst wall even after 30 min of exposure when all metacercariae were already dead (Fig. 3A). The cysts of the metacercariae exposed to −20 °C for 30 min presented holes, cracks and started to "peel". The same type of damage was observed in cysts exposed to 70 °C for 5 min and 1 h (Fig. 3B–D). These damages might have been exacerbated by the SEM preparation.

3.5. Damage to excysted metacercariae associated with incubation

Metacercariae exposed to −80 °C for 1 min and excysted afterward presented an undamaged tegument while metacercariae exposed for 30 min to the same temperature lost most of the morphological characters (Fig. 3E, G and H). The same damage pattern in the tegument was observed in metacercariae exposed to −20 °C for 1 h (Fig. 3F). The excystation of metacercariae following exposure to heating temperatures was not as efficient as the excystation of metacercariae exposed to freezing temperatures. SEM micrographs of the broken cysts of metacercariae exposed to 70 °C showed cracks and loss of scales in the tegument of the metacercariae inside (Fig. 3B and D).

4. Discussion

The present study on both *in situ* and isolated *C. lingua* metacercariae from Atlantic cod has demonstrated that 2 h incubation at temperatures ranging from 50 °C to 100 °C and from −20 °C to −80 °C is sufficient to kill these parasites.

Previous studies on the viability of metacercariae after heating or freezing reported a higher resistance of the parasites (Fan, 1998; Hamed & Elias, 1970). It was previously pointed out (FDA, 2001, chap. 5) that effectiveness of frozen storage and heating depends on several factors. These include species of parasite, fat content and fillet thickness.

We have determined the needed incubation conditions for *C. lingua* from Danish waters in a lean fish *G. morhua* and we advocate performance of specific tests for various geographic isolates of zoonotic metacercariae in various host types with different fish meat quality. This will provide a more reliable recommendation and knowledge base of the local legislation for products of different origins.

At high temperatures the presence or absence of water during the incubations seems to be an important factor regarding determination of the incubation time required for inactivation of metacercariae. In pilot experiments carried out for this study the incubation of metacercariae at 70 °C for 1 h was not sufficient to kill *C. lingua* metacercariae, but when deionized water was added to the samples before incubation, the cysts were not viable after 20 min. In a study carried out on *Heterophyes* sp. heating without water present showed survival of metacercariae up to 3 h at 50 °C

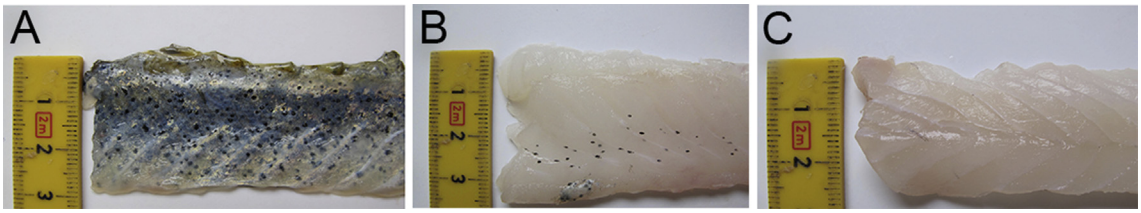

Fig. 2. *Cryptocotyle lingua* metacercariae in cod skin and muscle. (A) Skin viewed from the inside showing numerous black spots (i.e. pigment associated with encysted metacercariae). (B) Outer muscle showing few black spots. (C) Inner muscle free from black spots. Scale bar indicates centimeters (cm).

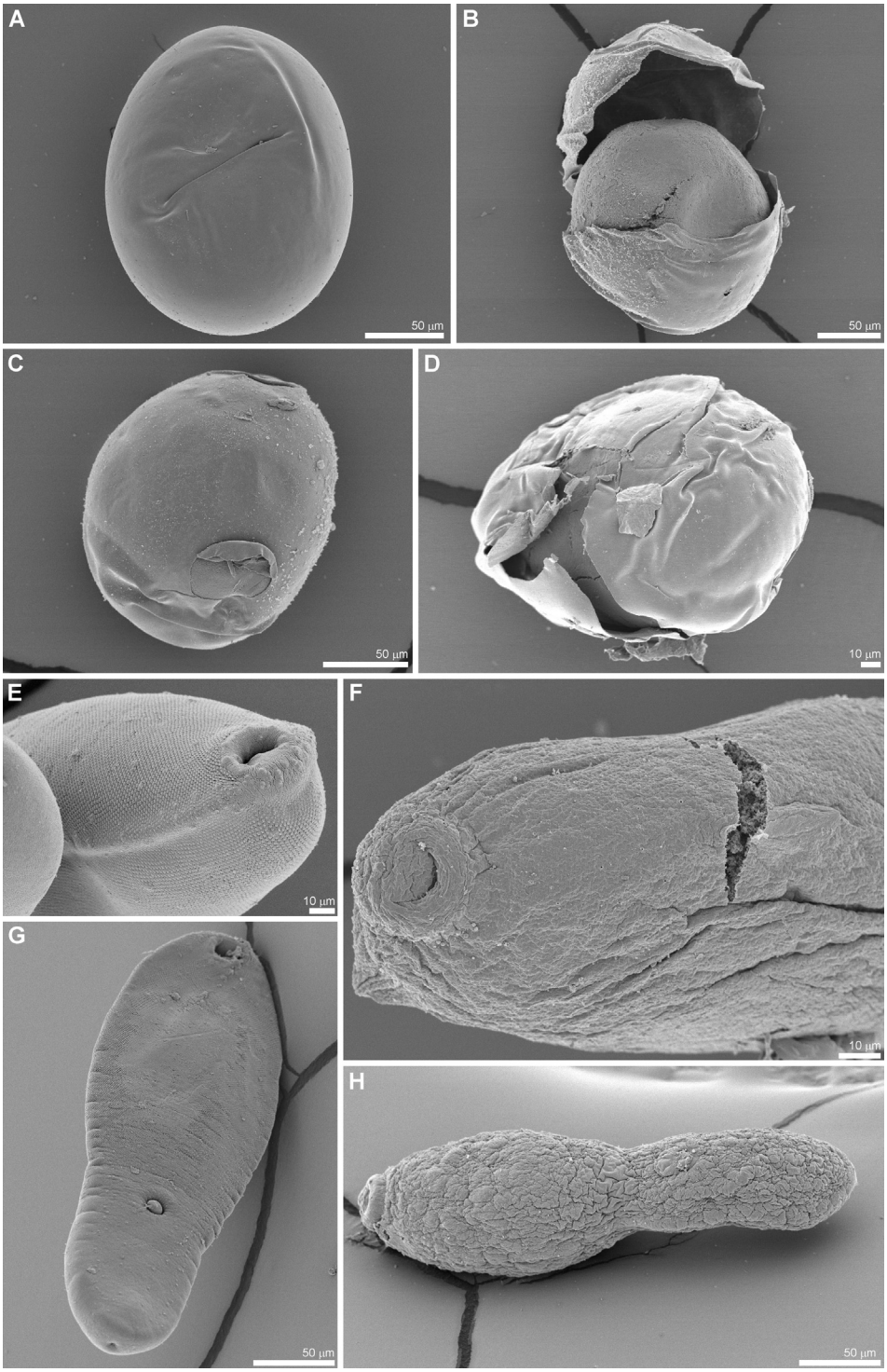

Fig. 3. SEM microscopy of *Cryptocotyle lingua* exposed to freezing and heating. (A) Cyst exposed to −80 °C for 30 min. (B) Cyst exposed to −20 °C for 30 min. (C) Cyst exposed to 70 °C for 5 min. (D) Cyst exposed to 70 °C for 1 h (E) and (G) Excysted metacercaria exposed to −80 °C for 1 min. (F) Excysted metacercaria exposed to −20 °C for 1 h. (H) Excysted metacercaria exposed to −80 °C for 30 min.

and after 10 min at 100 °C (Hamed & Elias, 1970) suggesting that the presence of water in our study facilitated heat conductance.

Another study from Thailand showed that no viable metacercariae of *Opistorchis viverrini* were found in fish grilled at 80 °C for 12 min (Prasongwatana, Laummaunwai, Boonmars, & Pinlaor, 2012). In a study from S. Korea the metacercariae of *Clonorchis sinensis* were not viable after 5 min of exposure to 80 °C (Cho, Chu, Rim, & Hwang, 2002). Different results are also found with regard to the time needed to kill metacercariae of different species exposed to freezing temperatures. Studies on *C. sinensis* and *Heterophyes* sp. exposed to −20 °C showed that metacercariae of these species lost viability after seven days and 33 h, respectively (Fan, 1998; Hamed & Elias, 1970). However, it is possible that the metacercariae of a northern species such as *C. lingua* is more

susceptible to high temperatures than those of tropical and subtropical trematodes. The current legislation requires freezing of fish or fish products at a temperature not higher than −20 °C in all parts of the product for at least 24 h (EFSA, 2004). This procedure is recommended for some fishes when destined for raw or almost raw consumption as well as for some species of fish going through processing such as cold smoking below 60 °C (Beldsoe & Oria, 2001; EFSA, 2004, 2010). Although this treatment is sufficient to inactivate *C. lingua* metacercariae, cod is not present in the list of fish species that should go through freezing before smoking processes (EFSA, 2010). Based on this investigation we recommend the industry to freeze cod before cold smoking procedures are performed.

Heat-killed metacercariae (60–100 °C) in cysts did not excyst in trypsin even when exposed for more than 1 h. However, freeze-killed metacercariae did excyst, albeit slower than live metacercariae. It may suggest that active movements of the metacercariae are involved in the excystation process. This has been suggested before (Chung, Kong, Joo, Cho, & Kang, 1995; Erasmus & Bennett, 1965) although McDaniel (1966) disputed this. In our study, live metacercariae excysted faster than the dead ones, but the dead metacercariae (killed by freezing) did excyst in less than 1 h of exposure to trypsin solution. Thus, movements of *C. lingua* metacercariae seem to help in the process of excystation but are not essential for its completion.

The role of excretory-secretory products (including proteases) from metacercariae in the excystation process is not totally understood yet, but some studies show the active secretion of products during the excystation in some species of metacercariae (Chung et al., 1995; Johnston & Halton, 1981; Li et al., 2004). We suggest that excystment of freeze-killed but not heat-killed metacercariae may be due to an importance of excretory-secretory products, particularly proteases. These may be inactivated by heating, but may still be active in freeze-killed metacercariae.

In this study cysts exposed to the temperature of −20 °C for 30 min showed more cyst wall damages than the ones exposed to −80 °C for the same amount of time. Thus the duration of incubation seems to be less important than the temperature in which the cysts were incubated. Metacercariae exposed for the shorter period of time to −20 °C (30 min) and 70 °C (5 min) presented as much damages as the metacercariae exposed for longer periods (1 h). This could in part be attributed to the SEM preparations. However, the SEM methodology alone would not cause the kind of damage observed if the cysts have not been weakened before. Freezing can cause damage to parasites both by a desiccation caused by the formation of ice crystals in the cysts accompanied by an accumulation of external solutes, and through mechanical damage caused by ice crystals forming in the parasite tissues. At modest subzero temperatures, a lower rate of ice formation leads to the formation of larger ice crystals. The dehydration, on the other hand, may be more efficient at more extreme subzero temperatures when the rate of solute accumulation grows faster (Gill, 2006). The excysted dead parasites were clearly shrunken probably due to the desiccation induced during the freezing process. Formation of intracellular ice crystals may also have been responsible for mechanical damages especially at more extreme freezing temperatures.

The present work should be considered a part of a new catalog to be created on susceptibility to heating and freezing of a range of trematode species from various geographic regions.

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