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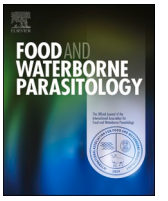

Viability of *Metagonimus romanicus* (Ciurea, 1915) metacercariae after physico-chemical treatments

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ABSTRACT

Digenean (Heterophyidae) trematodes include several zoonotic species such as the genus *Metagonimus* (Katsurada, 1912). *Metagonimus romanicus* (Ciurea, 1915) is a species widely distributed in Europe, whose metacercariae can be found on cyprinoids from the River Danube in Hungary. The aim of the study was to measure the viability of *Metagonimus romanicus* metacercariae by physical (freezing, heating, desiccation) and chemical (acetic acid and NaCl solutions) treatments. The methods were chosen as a model for procedures commonly used in traditional food preservation (such as freezing, salting, pickling and smoking) to measure the survival rate of metacercariae under different conditions. Most physical treatments (freezing of metacercariae at −20 °C, keeping them at 40 °C and 60 °C and desiccation) and chemical treatments (2.5%, 5%, 10% acetic acid solution and 5% and 10% NaCl solution) killed the metacercariae in a relatively short time (from 30 min to 6 days depending on the treatment) so their effects eliminated the risk of zoonotic infection. On the other hand, the metacercariae survived at room temperature and at 4 °C up to one month. Therefore, storing unprepared fish in domestic refrigerators cannot prevent infection with metacercariae in humans.

1. Introduction

Digenean trematode species are responsible for millions of infections in humans around the world, and members of the families Echinostomidae, Heterophyidae and Opisthorchiidae (Abdussalam et al., 1995; Keiser and Utzinger, 2009; WHO, 2011) can be the causative agents of these diseases through the consumption of raw or undercooked fish. The genus *Metagonimus* Katsurada, 1912 from the family Heterophyidae is one of the most common trematodes that cause zoonotic infections. Seven species have been shown to infect humans (Chai and Yung., 2024). In Europe, *M. yokogawai* (Katsurada, 1912) has been reported from Bulgaria, Czechia, Hungary, Serbia, and Spain (Molnár, 1969; Yu and Mott, 1994; Rácz and Zemankovics, 2002; Chai et al., 2009; Pornruseetairatn et al., 2016; Molnár and Baska, 2017). Based on molecular data, Cech et al. (2023) considered, that *Metagonimus* sp. in Europe does not belong to the species *M. yokogawai*, and it is probably identical to *Metagonimus romanicus* (Ciurea, 1915), which was reported from Romania and Hungary in the first half of the 20th century (Ciurea, 1915; Prettenhoffer, 1930). Recently, it was confirmed by both morphology and sequence data by Scholz et al. (2024).

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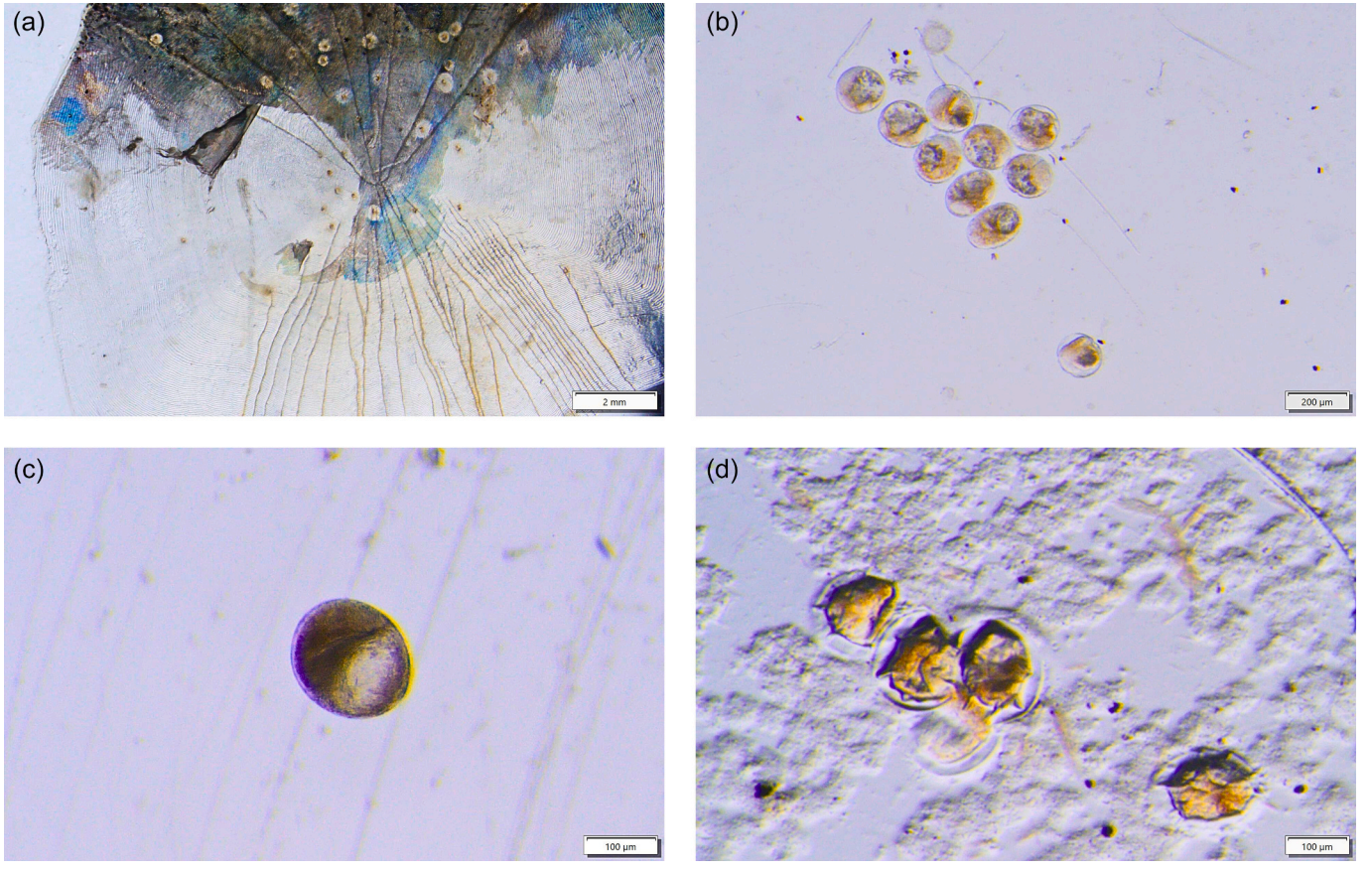

Fig. 1. a, *Metagonimus romanicus* metacercariae on a scale of a chub (*Squalius cephalus*) under stereomicroscope b, live *Metagonimus romanicus* metacercariae in the well of a 12-well microplate c, dead *Metagonimus romanicus* metacercariae showing amorphous body, granular and vesicular morphology due to acetic acid treatment d, dessicated *Metagonimus romanicus* metacercariae showing deformed body shape.

Seven species of the genus *Metagonimus* have been detected in humans (Chai and Jung, 2024), with *M. yokogawai*, the type species of the genus, causing the most cases. Metagonimiasis in humans is induced by consumption of raw or undercooked fish containing metacercariae (Sohn, 2009; Chai and Jung, 2017). The ingested parasite irritates the mucosa of the middle part of the small intestine and can cause villous atrophy and inflammatory reactions (Chai, 1979). Symptoms of mild infection may include abdominal pain, spasms, diarrhoea, lethargy, and fatigue, while chronic disease is associated with inflammation of the small intestinal mucosa and atrophy of the intestinal villi followed by malabsorption and weight loss. Infections of humans by *Metagonimus romanicus* are not reported from Europe, but Scholz et al. (2024) have listed several species of mammalian definitive hosts, suggesting a potential risk of infection for mammalian species.

Survival of metacercariae under different physical and chemical conditions may influence the transmission to people. As for as human cuisine is concerned, one of the main functions of cooking and preservation methods is to prevent infection by eliminating parasites. Survivability during food processing has been tested in the case of *Opisthorchis* sp. Blanchard, 1895, *Clonorchis sinensis* Looss, 1907, *Cryptocotyle lingua* (Creplin, 1825), *Paragonimus westermani*, Kerbert, 1878 *Holostephanus* sp. Szidat, 1936 (Fattakhov, 1989; Fan, 1998; Borges et al., 2014; Kim et al., 2017; Sándor et al., 2020). Abdallah et al. (2009) examined seven different species including cyathocotylid, diplostomatid, heterophyid and clinostomid species. *Metagonimus yokogawai* metacercariae collected from fish in the Danube River fishes were previously investigated during viability experiments, but only by freezing at −26 °C, desiccation and acetic acid treatment (Rácz and Zemankovics, 2002). It should be noted, that the identification of the metacercariae in the study as *M. yokogawai* is probably incorrect and it should be regarded as *Metagonimus romanicus*.

We investigated the survivability of *Metagonimus* metacercariae exposed to various physico-chemical treatments to model the effects of different food preservation methods such as freezing, cold and hot smoking, marinating, pickling, and salting on their viability.

2. Materials and methods

2.1. Sample collection

The fish were caught with an electrofishing machine (Samus 725MP) from a small tributary (Bükkös-patak) of the Danube River at Szentendre (N 47.663910, E 19.078625) and then transported alive in plastic bags with oxygenated water to the laboratory of the Fish Pathology and Parasitology Research Group of the HUN-REN Veterinary Medical Research. Metacercariae were isolated from the scales of two heavily infected chub specimens (*Squalius cephalus*) (Fig. 1.a). The larger individual (38 cm, 1820 g) had an average of 19.3 (± 10.3) metacercariae per scale; the highest number was 49 (Fig. 1.a). The smaller specimen (19 cm, 187 g) had an average of 1.8 (± 1.6) to 6 metacercariae per scale. The average number of metacercariae was estimated by counting the metacercariae of 100 randomly selected scales. The metacercariae were counted individually and then averaged with standard derivation calculation.

2.2. Artificial digestion

The fish were anaesthetized by adding clove oil to water of the aquarium, whereupon fish were decapitated. The scales of the fish were removed, and used to isolate the metacercariae by enzymatic incubation in 0.5% pepsin solution (2 l tap water, 10 g of 1:10.000 NF powder-based pepsin and 16 ml of 25% hydrochloric acid) for 40 min with stirring at a temperature of 40 °C (Sándor et al., 2020). The intact cysts were pipetted into 12-well microplates. The metacercariae were counted and examined under a stereomicroscope (Olympus SZX16) for viability based on the presence of dynamic movements and the dark excretory system.

2.3. Sensitivity of metacercariae to physico-chemical treatments

The metacercariae isolated by artificial digestion were placed into 12-well microplates, ten metacercariae in each well (Fig. 1.b). Each trial was performed in a single microplate with 120 metacercariae per test. The treatments were started at 10:00 am on the experimental days and several examination points were established (minutes: 1, 5, 10, 15, 20, 30; hours: 1, 2, 3, 4, 6, 12; days: 1, 2, 4, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26). After 26 days, the experiment was terminated, as only the control group contained living metacercariae. At each examination point, the wells were examined with an SZX16 stereomicroscope (Olympus, Tokyo, Japan). The number of surviving and dead metacercariae was recorded. The intact structure and movement of the metacercariae within the capsule were used as viability criteria. Dead metacercariae inside the cyst were characterized by an amorphous body, granular, vesicular morphology or the complete absence of movement (Fig. 1.c). Desiccated metacercariae showed no movement and their bodies were also shrunken (Fig. 1.d). The control group was also kept in 0.9% physiological saline solution at 4 °C. Here, we investigated how physical (freezing, heat, desiccation) and chemical (acetic acid and NaCl solutions) treatments (procedures commonly applied in the industry) affect the survival of metacercariae. The freezing experiment was carried out at −18 °C, as most household freezers operate at this temperature. The metacercariae were kept in plastic plates with the addition of physiological saline, and then thawed at −18 °C to record the viability of the metacercariae during freezing. To imitate cold- and hot-smoking procedures, the metacercariae were placed in wells (with and without physiological saline for each temperature) and then subjected to heat treatment at 20, 40 and 60 °C, in an INCO CO_2 Incubator (Memmert, Büchenbach, Germany). The combined use of heat treatment with physiological saline solution only reflected the effects of temperature, but without a liquid medium, dehydration affects metacercariae in addition to temperature. To model the effect of pickling, metacercariae were treated with 2.5%, 5% and 10% acetic acid at room temperature. Metacercariae were also treated with 5% and 10% sodium chloride (NaCl) solution to determine the outcome of preservation by wet salting (e.g.

Table 1
P-values of the pairwise multiple comparison of different (physical and chemical) treatments on the survival of metacercariae.

Treatment	control	−20 °C saline	20 °C saline	40 °C saline	60 °C saline	20 °C desiccated	40 °C desiccated	60 °C desiccated	acetic acid 10%	acetic acid 5%	acetic acid 2.5%	NaCl 10%
−20 °C saline	<0.0001	–	–	–	–	–	–	–	–	–	–	–
20 °C saline	<0.0001	<0.0001	–	–	–	–	–	–	–	–	–	–
40 °C saline	<0.0001	0.000158	<0.0001	–	–	–	–	–	–	–	–	–
60 °C saline	<0.0001	<0.0001	<0.0001	<0.0001	–	–	–	–	–	–	–	–
20 °C desiccated	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	–	–	–	–	–	–	–
40 °C desiccated	<0.0001	<0.0001	<0.0001	<0.0001	0.1226	<0.0001	–	–	–	–	–	–
60 °C desiccated	<0.0001	<0.0001	<0.0001	<0.0001	0.0084	<0.0001	<0.0001	–	–	–	–	–
acetic acid 10%	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	–	–	–	–
acetic acid 5%	<0.0001	<0.0001	<0.0001	<0.0001	0.0243	<0.0001	0.3024	0.0003	<0.0001	–	–	–
acetic acid 2.5%	<0.0001	<0.0001	<0.0001	<0.0001	0.0013	<0.0001	0.0509	<0.0001	<0.0001	0.6937	–	–
NaCl 10%	<0.0001	<0.0001	<0.0001	<0.0001	0.0087	<0.0001	0.1485	<0.0001	<0.0001	0.7218	0.7676	–
NaCl 5%	<0.0001	0.0002	<0.0001	0.7597	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

canned fish and salted fish).

2.4. Data analysis

The efficacy of the different treatments was compared by survival analysis in the R environment (version 4.1.2) (R Core Team, 2021) using the packages "survival 3.2.13" (Therneau, 2021) and "survminer 0.4.9" (Kassambara et al., 2021). In the model, the treatment type and the number of surviving metacercarial individuals at continuous time points were used as predictor and response variables, respectively. Since some metacercariae in the control group outlived the experiment, we used our model with censoring (Crawley, 2015). In case of significant differences between treatments, a pairwise multiple comparison with "BH" correction (Benjamini and Hochberg, 1995) was performed.

3. Results

The survival analysis showed that there was a significant difference in the survival of metacercariae between the treatments ($\chi 2 = 1197$, DF = 12, $P < 0.0001$). According to the pairwise multiple comparison, the differences between each of the treated groups and the untreated (i.e., control) group were significant (Table 1). The number of surviving metacercariae in the case of physical and chemical treatment and the time points are shown in Table 2. and Table 3, as well as in Fig. 2. and Fig. 3.

The metacercariae in the control group survived for >26 days, 42 (35%) of 120 were still alive at the endpoint.

Each heat treatment was significantly different from the others (Table 2, Fig. 2). The higher treatment temperatures led to a faster mortality of the metacercariae. Death of the examined parasite larvae was completed within 2 h at 60 °C, while at 40 °C and 20 °C some of them were able to survive for 2 and 16 days, respectively.

Desiccation increased the destruction rate of metacercariae even more at all temperatures (Table 2, Fig. 2). Drying at 60 °C was the most effective treatment for inactivating the metacercariae, as they were completely destroyed within 1 h, whereas drying at 40 °C and 20 °C was only effective after 6 h and 24 h, respectively.

Freezing had a slower effect on the decay of the metacercariae, 5 individuals (4.2%) were still alive after four days.

In the chemical treatments, the more concentrated solutions generally had a greater effect on the viability. There were significant differences between the 10% acetic acid solution and the more dilute solutions (5% and 2.5%) in the inactivation of metacercariae. The efficacy of the latter two treatments is almost identical (Table 3.). A notable difference can also be observed between the two different concentrations (10% and 5%) of NaCl solution. As for the chemical treatments, the fastest effect was obtained with the 10% acetic acid solution, which killed all metacercariae within 30 min (Table 3., Fig. 3.). All chemical treatments required 6 h, with the exception of the 5% NaCl solution, in which 10% of the metacercariae were still alive after 2 days.

4. Discussion

All physical and chemical treatments were effective in the elimination of living metacercariae. As expected, the higher

Table 2
Number of surviving metacercariae at different control points during the physical treatments (freezing, heating with/without liquid media).

Treatment		4 °C (control)	20 °C	40 °C	60 °C	20 °C	40 °C	60 °C	−20 °C
		In saline solution				Without saline solution (desiccated)			In saline
		Number of surviving metacercariae							
Elapsed time	Start (0 min)	120	120	120	120	120	120	120	120
Minutes	1	120	119	115	101	115	114	114	117
	5	116	118	94	72	111	88	110	103
	10	112	117	87	64	106	70	80	96
	30	110	115	83	49	86	55	1	91
Hours	1	109	114	81	21	77	29	0	90
	2	106	114	75	0	49	6	0	83
	6	104	113	71	0	23	0	0	78
	24	97	109	32	0	0	0	0	53
Days	2	92	104	6	0	0	0	0	33
	4	83	90	0	0	0	0	0	5
	6	80	83	0	0	0	0	0	0
	8	76	81	0	0	0	0	0	0
	10	73	72	0	0	0	0	0	0
	12	73	42	0	0	0	0	0	0
	14	72	13	0	0	0	0	0	0
	16	62	3	0	0	0	0	0	0
	18	58	0	0	0	0	0	0	0
	20	54	0	0	0	0	0	0	0
	22	49	0	0	0	0	0	0	0
	24	45	0	0	0	0	0	0	0
	26	42	0	0	0	0	0	0	0

Table 3
Number of surviving metacercariae at different control points during the chemical treatments (different concentration of acetic acid and NaCl) at room temperature (20 °C).

Treatment		In saline	In acetic acid solution			In NaCl solution	
		control	10%	5%	2.5%	10%	5%
		Number of surviving metacercariae					
Elapsed time	Start (0 min)	120	120	120	120	120	120
minutes	1	120	82	112	106	96	112
	5	116	60	94	83	88	103
	10	112	37	74	70	73	89
	30	110	0	46	64	62	83
hours	1	109	0	26	41	25	76
	2	106	0	17	16	19	67
	6	104	0	2	0	0	62
	24	97	0	0	0	0	24
days	2	92	0	0	0	0	13
	4	83	0	0	0	0	0
	6	80	0	0	0	0	0
	8	76	0	0	0	0	0
	10	73	0	0	0	0	0
	12	73	0	0	0	0	0
	14	72	0	0	0	0	0
	16	62	0	0	0	0	0
	18	58	0	0	0	0	0
	20	54	0	0	0	0	0
	22	49	0	0	0	0	0
	24	45	0	0	0	0	0
	26	42	0	0	0	0	0

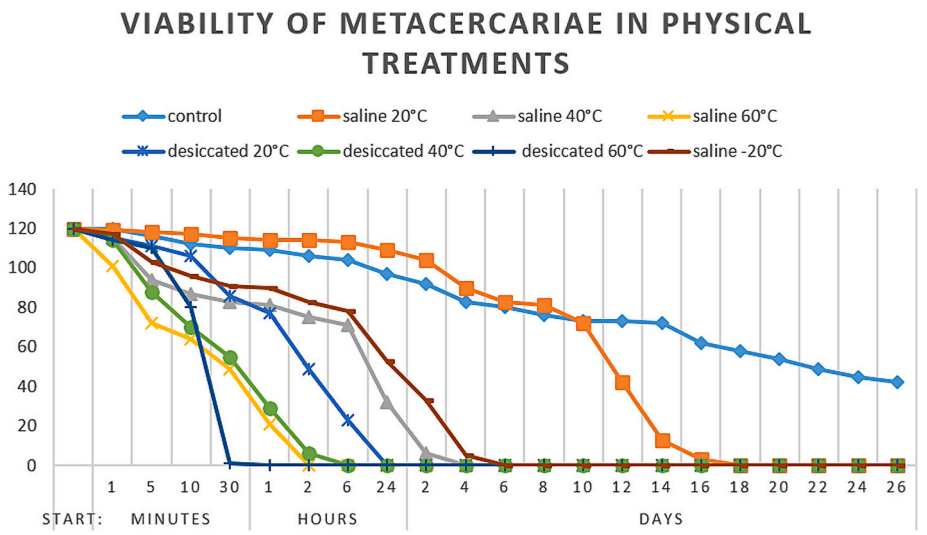

Fig. 2. Reduction in the number of live *Metagonimus romanicus* metacercariae during physical treatments (freezing at − 20 °C, heating at 20, 40, and 60 °C, heating and desiccation at 20, 40, and 60 °C).

concentrations (10%) of acetic acid and NaCl and the different temperature conditions (like −20 or 60 °C) both led to a rapid death of metacercariae (Figs. 2. and 3.). In general, the treatments eliminated the metacercariae within one week, but at −20 °C and in a 5% NaCl solution it took longer than with the other treatments. Storing samples at 4 °C (control) or 20 °C allow the metacercariae survive for more than a week, which may pose a risk of infection for anyone who accidentally consumes a scale.

Formerly, Rácz and Zemankovics (2002) investigated the viability of *M. romanicus* (referring to the species as *M. yokogawai*), but only by freezing at −26 °C, desiccation and the different concentrations of acetic acid. The effect of acetic acid correlated with our results; the different dilutions (10%, 5%, 2.5%) killed the metacercariae within a few minutes to 6 h, depending on the concentration. Rácz and Zemankovics (2002) documented the effect of lower concentrations up to a 128-fold dilution of 10% acetic acid solution, at which the viability of the metacercariae was extended to 4 days. Desiccation had a rapid effect in both studies (Rácz and Zemankovics, 2002 and the present study), with the last metacercariae dying within one day. A slight difference was observed during the freezing, as all the metacercariae in our experiment lost their viability within 6 h, while Rácz and Zemankovics (2002) detected living

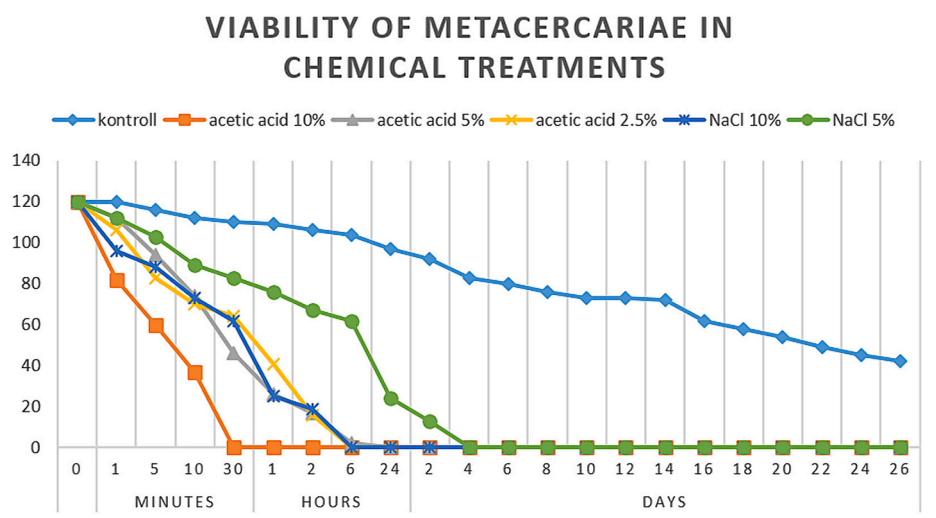

Fig. 3. Reduction in the number of live *Metagonimus romanicus* metacercariae during chemical treatments (2.5%, 5%, 10% acetic acid and 5%, 10% NaCl).

metacercariae in a low percentage (10%) after 24 h. In both studies, the metacercariae survived at 4 °C for up to one month, therefore storing unprepared fish in domestic refrigerators is not a suitable method for preventing metacercariae infections in humans.

Regarding the viability of other species of digenean trematodes, Fattakhov (1989) found that metacercariae of *Opisthorchis felineus* (Rivolta, 1884) were able to survive at −28 °C, −35 °C and − 40 °C for 20, 8 and 2 h, respectively. In contrast, Borges et al. (2014) documented that metacercariae of *Cryptocotyle lingua* died after 2 h at −20 °C, and after 1 h at −40 °C. In Italy, several human infections occurred from the consumption of raw or uncooked tench (*Tinca tinca*) and *Coregonus* sp. that were stored at −10 °C for three days before serving, then soaked in a mixture of vinegar and wine for 24 h (Armignacco et al., 2008; Traverso et al., 2012). Remarkable differences were found in NaCl tolerance. The metacercariae of *Opisthorchis viverrini* (Poirier, 1886) die within 24 h in a 13.6% NaCl solution (Kruatrachue et al., 1982), while the metacercariae of *Haplorchis taichui* (Nishigori, 1924) remained alive in 15% salt solution at room temperature for 14 days and in the refrigerator for 21 days (Kaenjampa et al., 2017). Sándor et al. (2020) attempted to model the effects of different fish preserving practices (salting, pickling and smoking) with various physical and chemical treatments in relation to cyathocotylid metacercariae. Similar to our results, the trematode in their experiments generally lost their viability in a relatively short time, whereas the untreated metacercariae in the control group (stored at 4 °C) survived for over a week. It cannot be ruled out that the isolation of the metacercariae from the scales had a limited effect on the viability in the experiment. On the other hand, it should be noted that Rácz and Zemankovics (2002) examined the metacercariae on the scales in their experiments, and received similar results with regard to the survivability of the metacercariae.

The significance of the viability experiments lies in the fact that seven species of the genus *Metagonimus*, namely *M. yokogawai (Katsuradai, 1912), M. takahashii* Suzuki, 1930, *M. miyatai* Saito, Chai, Kim, Lee et Rim, 1997, *M. suifunensis* Shumenko, Tatonova et Besprozvannykh, 2017, *M. katsuradai* Izumi, 1935, *M. minutus* Katsuta, 1932, and *M. pusillus* Tatonova, Shumenko et Besprozvannykh, 2018, have been reported as pathogens in human infections in Asia (Chai and Jung, 2024). According to Chai et al. (2015) the tolerable parasitic load of an infected person can have a wide range. After the recovery of adult flukes from 11 patients, the average number of worms was 6383.9/case, but it ranged from 141 to 44,320. The authors stated that these patients "had varying degrees of gastrointestinal symptoms, including abdominal discomfort and indigestion", but they do not give a linear relationship between the number of parasites and the severity of symptoms, although Chai (2015) clearly states that the severity of clinical symptoms is closely related to the individual worm burden. Extremely high levels of intestinal flukes, in particular, *Haplorchis taichui* have been documented from Laos, with an average number of 12,078.6 worms per person (range 6–129,238 worms per person), but the villagers' symptoms have been not described in detail. In the case of liver flukes, it has been documented that fewer than hundreds of *Clonorchis sinensis* metacercariae usually do not trigger symptoms, but several hundred to thousands of worms may cause patients to seek medical help (Rim, 1990). It can be assumed that accidental consumption of metacercariae is unlikely to pose a risk of serious disease to humans, as only several pieces of scales contain the amount of metacercariae (over a hundred) that can cause mild or severe symptoms.

At the same time, no human cases associated with *M. romanicus* (formerly mentioned as *M. yokogawai)* have been yet documented in Europe. However, four definitive mammalian hosts: domestic dog, *Canis familiaris* L, European jackal *Canis aureus moreoticus* Saint-Hilaire, *Vulpes vulpes* (L.) and cat, *Felis catus* L. have been shown to be infected with *M. romanicus*, and experimentally three laboratory hosts: domestic pig *Sus domesticus*, European polecat *Mustela putorius* and golden hamster *Mesocricetus auratus* have also been documented (Scholz et al., 2024).

Infection of humans has not been observed in the case of *M. romanicus*, and it is also not very likely given the culinary habits in Europe (where raw fish is traditionally not eaten). However, it can be assumed that *M. romanicus*, like other species of the genus, also has zoonotic potential, as indicated by the widespread infection of members of the mammalian orders (Carnivora, Rodentia and Artiodactylia). In addition, pets in close proximity of humans (e.g. cats and dogs) can also be the target of metacercarial infection.

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CRediT authorship contribution statement

Martina Gyöngy: Writing – review & editing, Writing – original draft, Visualization, Investigation. **Boglárka Sellyei:** Writing – review & editing, Investigation. **István Czeglédi:** Software, Methodology, Data curation. **Csaba Székely:** Writing – review & editing. **Gábor Cech:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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