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Pathogenicity of *Clinostomum complanatum* (Digenea: Clinostomidae) in naturally infected chub (*Squalius cephalus*) and common carp (*Cyprinus carpio*)

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ABSTRACT

Metacercariae of the digenetic trematode *Clinostomum complanatum* Rudolphi, 1814 were observed in chub (*Squalius cephalus*) and common carp (*Cyprinus carpio*) from the Orljava River (Croatia). Both the prevalence and infection intensity were higher in the chub (prevalence 23.4%, average intensity 70.5) than in the common carp (5.9%, 4.5). In general, the metacercariae were located in the wall of the anterior part of the digestive tract and branchial cavity, the supraocular region, and the hypaxial musculature near the paired fins. Histology revealed the obvious affinity of the parasite for striated muscles and connective tissue. *In situ*, the metacercariae were surrounded by well-vascularized connective tissue capsules composed primarily of collagen fibers, fibroblasts, and fibrocytes. This study provides some insight into the parasite's pathogenicity and the relationship between host and parasite.

Key words: *Clinostomum complanatum*; metacercariae; pathogenicity; chub; common carp; freshwater fish

Introduction

Clinostomum complanatum Rudolphi, 1814 is a well-known representative of the taxonomically complex genus *Clinostomum* Leidy, 1856 (MATTHEWS and CRIBB, 1998). The parasite is widely distributed in the western Palearctic region (LOCKE et al., 2019), and it deserves special attention because of its zoonotic potential (LOCKE et al., 2015).

The life cycle of *C. complanatum* involves freshwater snails as the first intermediate hosts,

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fish and amphibians as the second intermediate hosts (CAFFARA et al., 2014), and ardeid birds as the main definitive hosts (LOCKE et al., 2019). Many freshwater fish species of different families (including members of the Cyprinidae, Cobitidae, Centrarchidae, Percidae, and Odontobutidae) have been identified as suitable intermediate hosts (CAFFARA et al., 2011; ANTAL et al., 2015; LOCKE et al., 2015; GAGLIO et al., 2016; FEDORČÁK et al., 2019; LOCKE et al., 2019). Metacercariae of *C. complanatum* show low site specificity and are found in different tissues and organs, typically in the anterior part of the body (ANTAL et al., 2015; GAGLIO et al., 2016; SIMSEK et al., 2018; FEDORČÁK et al., 2019). At the site of infection, *Clinostomum* metacercariae are enclosed by a connective tissue capsule of host origin, with no parasite-induced acellular inner wall (HUNTER and DALTON, 1939; LO et al., 1985; KALANTAN et al., 1986; LARSON et al., 1988; EIRAS et al., 1999; GAGLIO et al., 2016; MONTES et al., 2020). These metacercariae, colloquially known as "yellow grubs", are recognized as a serious pathogen, capable of inducing oxidative stress and tissue injury (BELLO et al., 2000), eventually causing host death (FORNEY, 1955; SCHWARTZ, 1956; TORRES and PRICE, 1971; LO et al., 1985). Moreover, *Clinostomum* infection in farmed loach (*Misgurnus anguillicaudatus*), ayu (*Plecoglossus altivelis*), and goldfish (*Carassius auratus*) has been associated with high mortality and significant economic losses (LIU, 1979; LO et al., 1981; YASUMOTO et al., 2018).

C. complanatum was recorded in Croatian waters for the first time in 2012 (GJURČEVIĆ et al., 2014). The present study expands on those initial findings by analyzing the parasite's pathogenicity and its relationship with the host.

Materials and methods

Case history. The occurrence of nodular yellow foci in freshwater fish from the Orljava River in the basin of the Sava River was reported by a local fisherman. A survey was conducted of different fish species caught by anglers over a three-month period (between July and September). A total of 129 specimens, representing 10 species of 4 families (Cyprinidae, Siluridae, Esocidae, and Percidae) were caught during the survey: 47 chub (*Squalius cephalus*), 34 common carp (*Cyprinus carpio*), 16 Prussian carp (*Carassius gibelio*), 8 European catfish (*Silurus glanis*), 7 pike (*Esox lucius*), 6 asp (*Aspius aspius*), 5 rudd (*Scardinius erythrophthalmus*), 3 perch (*Perca fluviatilis*), 2 bream (*Abramis brama*), and 1 pikeperch (*Sander lucioperca*). The fish were caught at a number of localities, and the locality with the affected common carp was a channel connected to a nearby carp farm.

Immediately after capture, all collected fish were screened for the presence of *Clinostomum* metacercariae on site. The precise location of the metacercariae was noted for each infected fish. The intensity of infection was evaluated, and the prevalence was calculated. The weight and length of the infected host species were determined, and the condition factor (CF) was calculated according to the following equation:

$$CF = \text{wet weight [g]} \times 100 / \text{total length}^3 \text{ [cm]}.$$

Differences in CF between infected and uninfected chub or common carp were assessed for significance using the Mann-Whitney rank sum test (SigmaPlot, version 11.0). The level of significance was set at $P<0.05$.

For species identification and histological examination, three infected chub and one infected common carp were transported alive in a plastic bag with oxygenated river water to the Laboratory for Fish Diseases at the Faculty of Veterinary Medicine. Upon arrival at the laboratory, the fish were transferred to a 100-L glass aquarium with dechlorinated, aerated tap water. Within 30 min after transfer, they were euthanized in a separate tank by immersion in a 350 mg/L solution of MS-222 (Sigma-Aldrich, St. Louis, USA) buffered with 700 mg/L sodium bicarbonate. The study was approved by the Institutional Ethics Committee (Faculty of Veterinary Medicine; No. 251-61-01/139-20-28).

Species identification. Metacercariae were removed using dissecting needles under an Olympus SZX7 stereoscope, and fixed in 70% ethanol. For morphological determination, fixed metacercariae (n = 9) were cleared in lactophenol (GUSTINELLI

et al., 2010) and measured using an Olympus DP 12 digital camera and Cell B software (Soft Imaging System). Measurements of metacercariae are presented in µm, and are given as: range (mean ± standard deviation). A specimen of *Clinostomum* metacercaria was deposited in the Platyhelminthes collection of the Croatian Natural History Museum (No. 079). Additionally, some of the metacercariae collected were fixed in 4% neutral-buffered formalin and processed for histology. The 5-µm thick serial sections of metacercariae were stained with haematoxylin and eosin (H&E), Mallory's aniline blue, and Masson's trichrome.

The total DNA of a *Clinostomum* specimen sampled from chub was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. PCR was conducted to amplify the internal transcribed spacer (ITS) region using diplostomid-specific ITS primers (GUSTINELLI et al., 2010). Template DNA (5 µl) was added to the reaction mixtures containing 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 20 pmol of each primer, 1 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, USA) and nuclease-free water, to give a final volume of 50 µl. The amplicon was sequenced in both directions (Macrogen Europe, Netherlands) using the same primers as in the PCR. The sequence was manually edited and searched against public databases using BLAST (ALTSCHUL et al., 1990).

Histopathology. Tissue samples containing metacercariae were dissected and fixed in 10% neutral-buffered formalin, embedded in paraffin, and sliced into 5-µm sections. Sections were stained with H&E, periodic acid-Schiff (PAS), or toluidine blue (TB). Several sections were also stained with Mallory's aniline blue and Masson's trichrome to visualize collagen, the Van Gieson and Verhoeff-Van Gieson methods to visualize elastic fibers, or the Gridley modification of the silver impregnation method to visualize reticular fibers. Sections were analysed by light microscopy using an Olympus BX41.

Results

Of the 10 species of freshwater fish collected from the Orljava River, *Clinostomum* infection was found in only two cyprinid species, chub and common carp. The prevalence of infection was 23.4% (11/47) in chub and 5.9% (2/34) in common carp. The intensity of infection ranged from 11 to 136 metacercariae per infected chub (average intensity = 70.5), and from 2 to 7 metacercariae per infected common carp (average intensity = 4.5). At the time of capture, the affected fish appeared to be 'healthy'. Importantly, the CF did not vary significantly between infected and uninfected chub or common carp ($P>0.05$). Upon arrival at the laboratory, however, two heavily infected chub exhibited behavioral changes (i.e. flashing and erratic swimming).

Gross pathological examination. Clinical examination of the infected fish revealed the presence of *Clinostomum* metacercariae in different body areas. In the chub, metacercariae were predominantly found in the wall of the anterior part of the digestive tract (buccal cavity, pharynx, and oesophagus) and branchial cavity (including the base of gill arches). In cases of intense infection, they were also present in the supraocular region and hypaxial musculature near the base of the pectoral fins. In the common carp, however, metacercariae were only observed in the hypaxial musculature, anterior to the pelvic fins.

Morphological and molecular identification of metacercariae. The measurements and description of the metacercariae from the chub and common carp (Fig. 1) were as follows: body elongated, oval, tongue-shaped, 2859 – 4101 (3661 ± 472) long and 1340 – 1514 (1424 ± 67) wide, with a constriction at the level of ventral sucker. Tegumental spines visible only in histological sections, not present across the entire surface. Oral sucker 221 – 329 (248 ± 31) long and 330 – 503 (364 ± 56) wide. Oral collar well developed, 609 – 715 (658 ± 34) wide. Ventral sucker slightly oval and muscular, located in the anterior third of body, 490 – 726

Fig. 1. *Clinostomum complanatum* metacercaria. Scale bar = 500 µm. (a) Metacercaria cleared in lactophenol. (b) Histological section of metacercaria stained with Mallory's aniline blue. Note the presence of numerous gland cells (*) around the foregut, between the oral and ventral sucker.

(607 ± 81) long and 530 – 764 (617 ± 71) wide. Distance between oral and ventral sucker 610 – 805 (701 ± 61). Forebody gland cells present, reach the level of ventral sucker. Intestinal bifurcation anterior to ventral sucker. Caeca long, with lateral pouches posterior to ventral sucker, terminate at posterior end of body. Caecal content yellowish. Genital complex intercaecal and post-equatorial. Testes more or less lobed and triangular. Anterior testis almost entirely on the left of the median line, 190 – 301 (226 ± 32) by 200 – 268 (230 ± 21). Posterior testis median, 200 – 267 (229 ± 19) by 230 – 326 (288 ± 26). Distance between testes 210 – 384 (250 ± 66). Efferent ducts emerge from right corner of testes, enter into posterior part of cirrus pouch. Cirrus pouch on the right of median line and anterior testis, 228 – 295 (272 ± 21) by 100 – 139 (112 ± 11). Located at distinct distance from anterior testis. Genital pore lateral to the right margin of anterior testis. Ovary oval, laterally intertesticular and posterior to cirrus pouch, 74 – 139 (102 ± 21) long and 71 – 112 (93 ± 15) wide. Uteroduct extends from intertesticular space, passes along the left side of anterior testis and enters into uterine sac. Uterine sac narrow and straight, between anterior testis and ventral sucker. Metraterm anterior to cirrus pouch, joins uterine sac a short distance anterior to anterior testis. Excretory vesicle V-shaped, between caeca and posterolateral body margin.

On the basis of the morphological characters observed, the species was identified as *C. complanatum*. PCR amplification and sequencing of 1012 bp of the ITS region ITS1+5.8S+ITS2 confirmed the presence of *Clinostomum* genus. The nucleotide sequence obtained was deposited in GenBank under accession number JX235337 and showed 100% identity to published sequences of *C. complanatum* of European origin (Italy and Romania; JF718623, JF718624, MK796829, MK796830, and MK811210).

Histopathology. Microscopically, the metacercariae of *C. complanatum* were located in the striated muscles of the head (near the bucco-pharyngeal and branchial cavity wall) and trunk, and rarely, in periocular connective tissue and connective tissue beneath the branchial cavity epithelium. *In situ*, metacercariae were typically found encapsulated by connective tissue in variable

arrangements, from loose to more dense. These connective tissue capsules, 7 – 203 µm thick, contained many small blood vessels and were composed of collagen fibers with fibroblasts and fibrocytes scattered among them. Isolated muscle fibers were also observed, but only at the periphery of the capsules. Occasionally, muscle fibers were surrounded by a clearly visible network of reticular fibers, whereas no elastic fibers were noted within the capsule.

Inside the capsule, the majority of metacercariae appeared to be situated freely, without direct contact with the hosts' encapsulation. In contrast, in the chub with abnormal swimming behaviour, many of the metacercariae were found attached to the host tissue by their ventral suckers. In these fish, caecal content was visible in the capsule cavity, and several non-encapsulated metacercariae were observed. The non-encapsulated metacercariae were located superficially, immediately underneath the epithelium (Fig. 2).

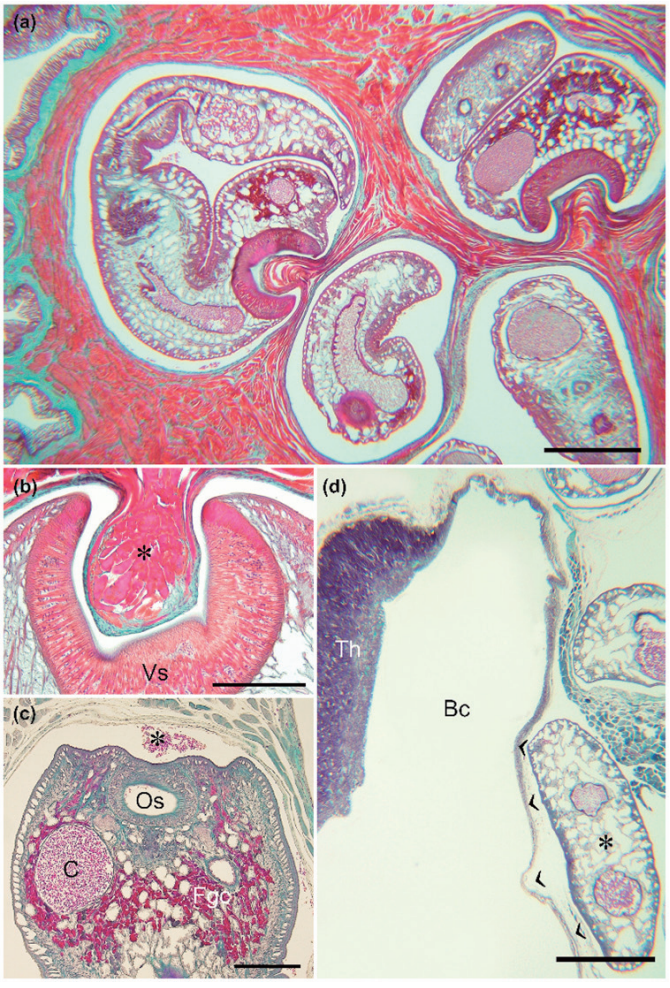

Fig. 2. Spontaneous activation of *Clinostomum complanatum* metacercariae in chub. (a) Histological section of a chub esophagus showing encapsulated metacercariae in the striated muscles. Two metacercariae are firmly attached to the host tissue by their ventral suckers (Masson's trichrome). (b) High magnification of the attachment site. At the point of attachment, hypercontraction of muscle fibers (*) is evident (Masson's trichrome). (c) Forebody of the encapsulated metacercaria. Caecal content (*) is clearly visible in the capsule cavity, near the oral sucker (Mallory's aniline blue). (d) Histological section of the dorsal wall of the branchial cavity showing non-encapsulated metacercaria (*) just opposite the thymus. Note the protrusion and marked thinning of the mucosal epithelium (arrowheads) close to the parasite (Mallory's aniline blue). Abbreviations: Vs – ventral sucker; Os – oral sucker; C – caecum; Fgc – forebody gland cells; Bc – branchial cavity; Th – thymus. Scale bars: a, d = 500 µm; b, c = 200 µm.

Around the encapsulated metacercariae, and especially in the area between adjacent capsules, muscle fibers were usually atrophic. Muscle fiber atrophy was associated with mild to moderate endomysial fibrosis. Within heavily affected muscles, segmental necrosis and regeneration of muscle fibers in a multifocal polyphasic pattern were evident. Damaged fiber segments displayed hypercontraction and fragmentation, accompanied by invasion of macrophages. Regeneration of muscle fibers was evident from myosatellite cell proliferation, basophilia and the presence of a central row of nuclei (Fig. 3).

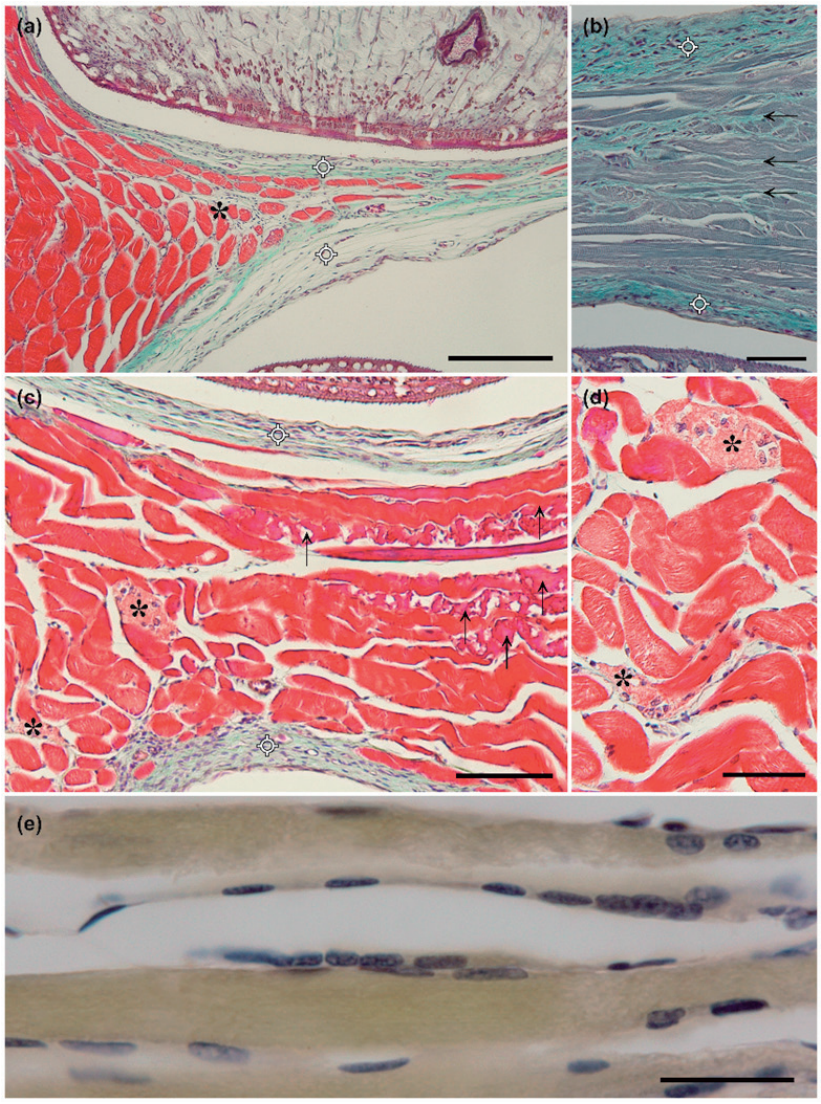

Fig. 3. Histopathological changes associated with encapsulated *Clinostomum complanatum* metacercariae. (a) Histological section of a common carp hypaxial musculature. In the area between adjacent capsules (☼), muscle fiber atrophy with mild endomysial fibrosis (*) is evident (Masson's trichrome). (b) High magnification of endomysial fibrosis (arrow) in the area between adjacent capsules (☼) (Mallory's aniline blue). (c) Segmental necrosis of muscle fibers between two capsules (☼) in the buccal cavity wall of chub. Note the fragmentation (arrow) and invasion of the muscle fibers by macrophages (*) (Masson's trichrome). (d) Invasion of damaged segments of muscle fibers by macrophages (*) is clearly visible at higher magnification (Masson's trichrome). (e) Regeneration of muscle fibers. Note the activation of myosatellite cells (Van Gieson). Scale bars: a = 200 µm; b, d = 50 µm; c = 100 µm; e = 20 µm.

In areas of extensive myonecrosis, mild to moderate mononuclear cell infiltration was also observed. Mild hemorrhages were noted close to the non-encapsulated metacercariae.

Discussion

A survey of 129 freshwater fish from the Orljava River (representing 10 species of 4 families) demonstrated the presence of *C. complanatum* metacercariae in only two species, chub and common carp, both in the family Cyprinidae. This is not surprising since *Clinostomum* usually infects two or more fish species in a given locality (TORRES and PRICE, 1971; AOHAGI et al., 1992; DIAS et al., 2003; DE LIMA et al., 2014; WANG et al., 2017). *C. complanatum* has also been reported in chub in Italy (CAFFARA et al., 2011) and Turkey (SIMSEK et al., 2018). These findings, as well as the high prevalence and infection intensity observed in the present study (prevalence 23.4%; average intensity 70.5) strongly indicate that *C. complanatum* is a common parasite of chub in the western Palearctic region. The preference of *C. complanatum* for this species in the present study could be explained by the abundance of chub in the river and their preference for a specific habitat during the dry season: small and shallow tributaries, channels, pools, and old meanders. This type of habitat, with slow or no water current, is suitable for freshwater snails, and attractive to ardeid birds.

C. complanatum has low site specificity and is typically found in the anterior part of the body (FEDORČÁK et al., 2019). Consistent with this, in the present study metacercariae were found in the wall of the anterior part of the digestive tract and branchial cavity, the supraocular region, and the hypaxial musculature near the paired fins. Histological examination revealed *C. complanatum* showing affinity with striated muscles and, to a lesser extent, connective tissue. Therefore, it seems reasonable to assume that cercariae enter the host through the mucous membrane of the bucco-pharyngeal and branchial cavity, or through the skin on the head and near the base of the fins (where scales are absent), from which they migrate to the striated muscles or connective tissue. These sites are rich in glucose and other dissolved organic nutrients (LARSON et al., 1988), and there the cercariae develop into metacercariae.

The host reaction to *C. complanatum* in our study involved marked encapsulating fibroplasia. Specifically, the encapsulating connective tissue structures (capsules) were well-vascularized and composed primarily of collagen fibers, fibroblasts, and fibrocytes. The arrangement of the connective tissue was variable (from loose to more dense), sometimes even within a single capsule. When metacercariae were located in the striated muscles, individual muscle fibers were occasionally found incorporated into the capsule. The observed structure of these capsules corresponds to earlier descriptions by HUNTER and DALTON (1939), LO et al. (1985), LARSON et al. (1988), and MONTES et al. (2020), although there are minor differences. The structure of '*Clinostomum* capsules' depends on their maturity (HUNTER and DALTON, 1939), host species (HUNTER and DALTON, 1939), and site of infection (LARSON et al., 1988). Therefore, some structural variations are to be expected, including the amount, arrangement, and cellular composition of the connective tissue (HUNTER and DALTON, 1939). Regardless of possible variations, *Clinostomum* capsules are sufficiently permeable to permit the diffusion of glucose to the tegumental surface of encapsulated metacercariae (LARSON et al., 1988).

Inside the capsule, the majority of metacercariae were situated freely. However, in the chub with abnormal swimming behaviour, many of the metacercariae were firmly attached to the host tissue, and their caecal content could occasionally be seen in the capsule cavity, indicating metacercarial activation. The finding of non-encapsulated metacercariae in these fish represents clear evidence of spontaneous activation. This observation is in accordance with previously published data (LO et al., 1987). When activated outside their definitive host, *Clinostomum* metacercariae vomit their caecal content, which contains proteolytic enzyme(s) synthesized by their gland cells. The protease released disintegrates the capsule and facilitates the efficient migration of metacercariae out of the fish (LO et al., 1987). This, in turn, causes destruction of adjacent tissue (LO et al., 1981; LO

et al., 1985; LO et al., 1987; YASUMOTO et al., 2018), ultimately leading to abnormal swimming behaviour (TORRES and PRICE, 1971; LO et al., 1985), abdominal perforation (TORRES and PRICE, 1971; LO et al., 1981; YASUMOTO et al., 2018), and the death of the fish (TORRES and PRICE, 1971; LO et al., 1981; LO et al., 1985; LO et al., 1987; YASUMOTO et al., 2018). There are several possible reasons for activation of metacercariae outside their definitive host, such as high water temperature (> 33 °C) (LO et al., 1987; YASUMOTO et al., 2018), a rapid increase in water temperature (LO et al., 1987), and exposure of the fish to pesticides (LO et al., 1981; LO et al., 1985). In the present study, the metacercariae may have been activated by an increase in water temperature during transport to the laboratory.

Generally, encapsulated metacercariae in our study caused pressure atrophy of adjacent muscle fibers, as well as mild to moderate endomysial fibrosis. Similar findings were also reported in other fish species infected with *Clinostomum*, such as banded sunfish (*Enneacanthus obesus*) (HUNTER and DALTON, 1939), loach (LO et al., 1985), and Italian spined loach (*Cobitis bilineata*) (GAGLIO et al., 2016). However, to our knowledge, multifocal polyphasic lesions have never been associated with encapsulated *Clinostomum* metacercariae. This type of lesion is a result of ongoing insults occurring over a prolonged period (VALENTINE and McGAVIN, 2007; COOPER and VALENTINE, 2016). The fact that encapsulated metacercariae cause polyphasic injury of the affected muscles indicates that the strategy of this digenetic trematode is to focus on 'minimal' but continued damage to the host, which subsequently leads to behavioural alterations. This strategy facilitates predation of infected fish by the definitive avian host and ensures that the parasite can complete its life cycle. This supposition is supported by several studies (EIRAS et al., 1999; BELLÓ et al., 2000; DIAS et al., 2003).

In conclusion, *C. complanatum* is a common parasite of chub in the western Palearctic region. Due to its zoonotic potential, pathogenicity, and low host specificity, this species of *Clinostomum* may represent an important problem for aquaculture and the fishing industry. Fortunately, over the last 20 years, there have only been a few documented reports of such zoonotic agents in freshwater fish in Croatia (GJURČEVIĆ et al., 2007; GJURČEVIĆ et al., 2020).

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SAŽETAK

Metacerkarije dvodomnog metilja *Clinostomum complanatum* Rudolphi, 1814 utvrđene su u klena (*Squalius cephalus*) i šarana (*Cyprinus carpio*) podrijetlom iz rijeke Orljave (Republika Hrvatska). Prevalencija i intenzitet invazije veći su u klena (prevalencija 23,4% i srednji intenzitet 70,5), nego u šarana (5,9% i 4,5). Općenito, metacerkarije se smještaju u stjenku prednjeg dijela probavne cijevi i škržne šupljine, područje oko oka i hipaksijalno mišićje oko parnih peraja. Histološki nalaz pokazuje afinitet metilja za skeletno mišićno tkivo i vezivno tkivo. *In situ*, metacerkarije okružuje dobro vaskularizirana vezivnotkivna kapsula građena pretežno od kolagenih vlakana, fibroblasta i fibrocita. Ovo istraživanje doprinosi razumijevanju patogenosti parazita i daje uvid u odnos između domaćina i parazita.

Ključne riječi: *Clinostomum complanatum*, metacerkarije, patogenost, klen, šaran, slatkovodna riba