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ORIGINAL PAPER

The New Data on the Serotonin and FMRFamide Localization in the Nervous System of *Opisthorchis felineus* Metacercaria

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Abstract

Background Trematoda *Opisthorchis felineus* Rivolta, 1884 is the causative agent of dangerous parasite disease—opisthorchiasis, widespread in the Russian Federation. The details of the neuroanatomical localization of the serotoninergic and FMRFamidergic neurotransmitter elements as well as their functional roles remain not studied enough in both adult and larval forms of *O. felineus*. The studies in this area are important in term of the development of a new pharmacological strategy of the struggle with the causative agent of opisthorchiasis affecting the neuronal signal substances and the function of its nervous system.

Purpose The aim of this work was the immunocytochemical study of the neurotransmitters serotonin (5-HT, 5-Hydroxitryptamine) and neuropeptide FMRFamide localization in the nervous system of the opisthorchiasis causative agent—*O. felineus* metacercaria. To study the relationship between the detected neurotransmitters and the muscular elements of the parasite, the muscle staining was carried out simultaneously using fluorophore-conjugated phalloidin.

Methods The localization of 5-HTergic and FMRFamidergic nerve structures was determined by immunocytochemical method. The staining samples were analyzed using a fluorescent and confocal laser scanning microscopies.

Results The new data on the presence and distribution of the serotonin-immunopositive (IP)- and FMRFa-IP components in the central and peripheral departments of the nervous system of *O. felineus* metacercaria has been obtained. Besides that a number of the new anatomical details of the nervous system organization and of the innervation of the organs and tissues in the investigated parasite have been revealed.

Conclusion The data obtained on the presence and localization of the 5-HTergic and peptidergic (FMRFamide) components in central and peripheral departments of the nervous system of *O. felineus* metacercaria elaborated and expanded the existing information about the nervous system as well as the innervations of the tissues and organs in the causative agent of opistchorchiasis.

Keywords Trematoda · *Opisthorchis felineus* · Metacercaria · Nervous system · Serotonin (5-HT) · FMRFamide

Introduction

Trematoda *Opisthorchis felineus* Rivolta, 1884 (cat or Siberian fluke) is the causative agent of a dangerous parasite disease—opisthorchiasis, widespread in the Russian Federation. The scientific interest to the opisthorchiasis is not occasional as it is connected with the high social importance of this disease. It is known that the largest natural center of opisthorchiasis is associated with the Ob-Irtysh rivers basin in Western Siberia [49]. The epidemiology and distribution of opisthorchiasis in humans, fish, mollusks, and other animals in Western Siberia has been reviewed by [Yurlova et al. [62]] in detail.

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The complex life cycle of *O. felineus* includes three hosts, two of which are the intermediate hosts (the mollusks of Bithyniidae family and the carp fishes) and one is the definitive one (humans and carnivores). Opisthorchis eggs disseminated into water are swallowed by mollusks in the intestine of which the moving miracidia larvae are hatched. In the mollusk body the miracidia give rise to next life cycle stage: the sporocysts and redia in which the free swimming cercariae larvae are developed. The stage of sporocysts formation and the formation of cercaria in redia is a parthenogenetic step in the life cycle in the development of opistorchis. The active cercariae larvae form from germinal balls of the redia and leave it through the birth pore after their maturation. Then they migrate inside the mollusk body and finally exit into water, where they actively swim for some time. After meeting with the fishes of the carp family the cercariae attach to them and penetrate into the muscles, where they develop into the next stage—the metacercaria.

The metacercariae reach the invasive stage and infect the definitive hosts—domestic and wild carnivorous, omnivorous animals and humans. The infection with *O. felineus* occurs when humans eat the raw, weak-salted or insufficiently thermally treated fish containing metacercaria of the parasite in their muscles. In the human stomach and anterior regions of the intestine the metacercariae exit the cysts and enter through the bile ducts into the gallbladder and liver bile ducts. Here the parasites became sexually mature and begin the egg laying [4]. Opisthorchiases is characterized by variety of pathological processes in human organs and tissue. The infection caused by cat's fluke leads to the development of angiocholitis, chronicle hepatitis, pancreatitis, liver cirrhosis, and anemia. Opisthorchiasis can initiate the formation of the primary liver and pancreas cancers [51].

Currently, a number of drugs are used for opisthorchiasis treatment [2]. However, it should be noted that the emergence of the resistance to the existing chemotherapeutic drugs and the need to fight the helminthes infections in humans and animals require a search for new substances which should be highly effective and less toxic for the host while influencing the parasite organs and tissues.

One of the areas of science which make it possible to influence the causative agents of helminthes infections is the study of neurochemical basis of parasitic worms' life activity. Such knowledge allows using the helminthes nervous system as a target for chemotherapy of the disease. Thus, the investigations in this direction are important not only from a point of view of fundamental biology problems of parasites' nervous system evolution, but also in terms of the development of new effective anthelmintic drugs specifically influencing the neurobiological mechanisms of parasite.

As is well known, the nervous system of trematoda is well developed and plays an important role in regulation and coordination of the various functions of the parasites. Flatworms occupy a pivotal position in animal evolution when the cephalization and an organized nervous system were first appeared. The flatworm central nervous system (CNS) has an archaic brain located in the head region and paired longitudinal nerve cords, which are cross-linked at regular intervals by transverse commissures. In turn, the CNS is linked to a simple peripheral nervous system (PNS), consisting of simple nerve cords and nerve plexuses that supply all the major body structures, in particular the somatic musculatures, the alimentary tract and reproductive organs [47].

In the nervous system of trematodes, including *O. felineus*, a number of neuronal signaling molecules have been identified, such as serotonin and FMRFamide-like neuropeptides, which plays regulatory role in helminthes [17, 18, 21, 55–57, 59].

Serotonin (5-HT, 5-Hydroxitryptamine) is the dominant biogenic amine in flatworms. In trematodes, this substance was identified by biochemical methods in tissue extracts of *Schistosoma haematobium, S. japonicum, S. mansoni* [5, 8], *Haplometra cylindracea, O. felineus, Azygia lucii, Codonocephalus urnigerus* [55]. Immunocytochemical data confirmed the presence of serotonin in the central and peripheral departments of the nervous system of all trematodes studied so far [21, 56].

Among the several families of the regulatory peptides found in flatworms [24], a special attention was paid to FMRFamide-like peptides [41, 43], which were immunocytochemically identified in the nervous system of a several flatworm species [20, 21]. The important role of these signaling substances in the regulation of the muscle function was suggested [13, 34].

At the same time, it should be noted that details of the neuroanatomical localization of serotoninergic and FMRFamidergic nerve elements, and their functional roles remain poorly understood for both adults and larvae of trematodes. This also is the case for causative agent of the opisthorchiasis *O. felineus*, in regard of which few data are available as to the organization and function of their nervous system [59]. The aim of our work was an immunocytochemical study of the localization of neurotransmitters serotonin and neuropeptide FMRFamide in the nervous system of causative agent of the opisthorchiasis—*O. felineus* metacercaria. To study the relationship between the detected neurotransmitters and the muscle system elements of the parasite the simultaneous histochemical staining of the muscle fibers was also carried out using phalloidin conjugated with a fluorophore.

As a result of the study and analysis of the data obtained with confocal laser scanning microscopy (CLSM) the new details of the morphology of 5-HT and FMRFamidergic components were found in *O. felineus* metacercaria of the nervous system. Special attention was also paid to the relationship between the detected 5-HT-IP and FMRFa-IP

elements of the nervous system and the muscle fibers to test the hypothesis about their possible regulative function in the muscular activity of *O. felineus* metacercariae.

The data obtained in this work allow expanding our knowledge about the nervous system organization of opisthorchiasis causative agent, as well as about the functional significance of 5-HTergic and FMRFamidergic components of the nervous system of the parasite. In our study a number of new anatomical details on the organization of the nervous system of *O. felineus* metacercaria and the innervations of its organs and tissues have been revealed. The received data on the functional morphology of the nervous system of *O. felineus* metacercaria can be useful in terms of the development of a new pharmacological strategy of fighting the causative agents of helminthiasis affecting the nervous system functioning and its neuronal signaling substances.

Materials and Methods

Metacercariae of *O. felineus* were obtained from naturally infected fish *Leuciscus idus* captured from water reservoir in Tobolsk region, Russia. The muscles of fish were digested in artificial gastric juice [16] and collected metacercariae were excised in 3–5 drops of 0.25% trypsin in 10 ml of normal saline solution (0.85% w/v sodium chloride in distilled water) at 37 °C during 10–20 min. Subsequently, these worms were fixed in a fresh solution of 4% paraformaldehyde (MP Biomedicals, USA) in 0.1 M phosphate buffered saline (PBS, Sigma) at pH 7.4) at the temperature of 4 °C during 12 h and then transferred to 10% sucrose (MP Biopmedicals) on 0.1 M PBS for few days.

Immunocytochemistry

The localization of serotoninergic and FMRFamidergic nervous structures was found using immunocytochemical methods [9]. The samples (whole mounts) were incubated during 5 days in the primary antibodies—rabbit anti-5-HT (Immunostar, USA) (1:500) or rabbit anti-FMRFamide (Peninsula, Belmont, CA, USA) (1:500) dissolved in PBS, containing 1% of Triton X-100 (Sigma) at the temperature 4 °C, then in secondary fluorescently-labeled immunoglobulines (Alexa Fluor 488, goat anti-rabbit IgG (H + L), Molecular Probes, USA) during the next 5 days. The analysis was performed in 7–10 replicas (samples).

Controls included: (1) incubation of samples with only the secondary immunoglobulin without primary antibodies or (2) using of non-immune rabbit serum instead of the primary antiserum. Both controls demonstrated the absence of the non-specific staining.

Staining of Musculature with TRITC-Conjugated Phalloidin

For the musculature staining the TRITC-(tetramethylisothiocyanate)-conjugated phalloidin (Sigma) in dilution in 1:200 was used, the samples were kept in dark for 6–12 h at 4 °C according to the method described by [Wahlberg [60]]. Staining by TRITC-phalloidin allowed to identify and analyze the distribution of the muscle fibers in the body wall, oral and ventral suckers, alimentary tract and excretory system of the parasite.

Fluorescent and Confocal Laser Scanning Microscopy

Specimens were investigated under the fluorescent microscope Leica DM1000 (Leica, Germany) in Center of Parasitology of A.N. Severtsov Institute of Ecology and Evolution RAS, and with the microscope Leica DM6000B equipped with a digital camera DC300F (Leica, Germany) and confocal laser scanning microscope Leica TCS SP5 (Leica, Germany) at the Optical Microscopy and Spectrophotometry Core Facilities, ICB RAS, Federal Research Center "Pushchino Scientific Center for Biological Research of the Russian Academy of Sciences" (Moscow Region, Russia).

A fluorescent filter I3 (excitation spectrum 450–490 nm; emission spectrum 515 nm) was used for the evaluation of Alexa488 fluorophore, while TRITC (tetramethylrhodamine isothiocyanate) was detected with an N2.1 filter (excitation spectrum 515–560 nm; emission spectrum 590 nm). The images were saved in the TIFF format at high resolution. The microphotographs were represented as a total of 16 to 64 consequent optical sections. The optical sections were obtained by sample scanning through 40–70 µm sample thickness and the images were presented as an optical section (or snapshot) or were reconstructed at the maximum fluorescence intensity (the maxprojections). The morphological measurements were carried out with a computer program provided with the CLSM microscope. To create the illustrations, the Adobe Photoshop CS 8.0 was used.

Results

Musculature

The body of *O. felineus* metacercaria is covered by small and densely located spines. The length of the body is ranging from 500 to 650 µm, the width is about 190–220 µm; the diameter of the oral sucker is 67 µm, the diameter of the ventral sucker is 106 µm. The distance between the bottom

edge of the oral sucker and the top edge of the ventral sucker is 190 µm. The general view of metacercaria stained with TRITC-labeled phalloidin is represented in Fig. 1a.

The musculature of the body wall of *O. felineus* metacercaria consists of three muscles layers—the circular, the longitudinal and the diagonal. The musculature of the attachment organs of studied metacercaria, the oral and the ventral suckers, is represented by longitudinal, circular and radial muscle fibers. The radial muscles are especially highly developed (Fig. 1a, c, e). Several muscular bundles extending from the body wall to the ventral sucker can be observed (Fig. 1b, c, e).

In the different parts of the digestive system—pharynx, oesophagus and intestine, the circular muscle fibers were also found (Fig. 1c, Inset d). In the intestine besides the circular muscle fibers the longitudinal muscle filaments were seen. In the posterior end of the excretory system both the circular and longitudinal muscle fibers were observed in the excretory bladder (Fig. 1f, g).

5-HT-Immunopositive Elements in the Nervous System

5-HT-IP staining was observed in the central and peripheral parts of the nervous system of *O. felineus* metacercaria (Fig. 2a–h). 5-HT-IP staining was detected in the paired brain ganglia, in the brain commissure (10 µm in width), in the three pairs of the longitudinal nerve cords—ventral, lateral and dorsal ones.

The brain commissure in metacercaria is located in anterior body region at some distance from the oral sucker and just behind the pharynx. The distance from the central part of the oral sucker to the middle part of 5-HT-IP (or FMRF-IP) staining brain commissure varied from 40 to 80 µm in different investigated samples, which could be related to the size of the metacercaria, as well as to the fixation conditions and other factors.

The ventral nerve cords (diameter about 3 µm) are the most developed among the longitudinal nerve cords (Fig. 2a, b, c). All the longitudinal cords are connected by the numerous thin transversal 5-HT-IP commissures. Approximately 10–12 of the 5-HT-IP commissures have occurred in the anterior part of the body (before the ventral sucker) and the same number of commissures was seen in the posterior part of the body (behind the ventral sucker). The distances between two commissures connecting the ventral nerve cords are about 10–19 µm in the anterior and posterior parts of metacercaria body.

The obtained results are as follows.

- From each brain ganglion the two thick nerve branches (diameter approximately 5 µm) are extended ahead. One of these branches (the anterior nerve) is going towards the oral sucker and another one is running towards the upper region of the lateral nerve cord (Fig. 2a). The branch which is extending to the oral sucker (the anterior nerve) forms the circle around the oral sucker and the 5-HT-IP nerve net in the sucker musculature (Fig. 2h). Two small 5-HT-IP neurons are observed in each anterior nerves following from the brain ganglion to the oral sucker (Fig. 2c). The nerve fibers are running towards the oral sucker from the lateral nerve cord too (Fig. 2b).
- The four 5-HT-IP neurons (5–7 µm in size) are visible in each brain ganglion. The four 5-HT-IP neurons (the same size) are situated along the both ventral cords too (Fig. 2a–c).
- On the both sides of the pharynx located before the brain commissure, two 5-HT-IP neurons (5–7 µm in size) and the nerve fibers connecting them are situated (Fig. 2a, b, c). The nerve net comprising of 5-HT-IP fibers running from the brain commissure is seen in metacercaria pharynx (Fig. 2h);
- From the ventral nerve cords and from the two 5-HT-IP neurons located by each sides of the ventral sucker, the 5-HTergic nerve fibers are extending towards the ventral sucker (Fig. 2d, e). The 5-HT-IP nerve fibers are also directly running from the lateral nerve cords to the ventral sucker (Fig. 2g). These nerve fibers form the net amongst the sucker muscles and the two nerve rings at the sucker outer surface connecting with each other by very thin transversal fibers (Fig. 2f, g). Besides that, the ventral sucker is also supplied by the 5-HTergic nerve fibers going from the transversal nerve commissure connected the ventral nerve cords at the level of the anterior edge of the sucker (Fig. 2b, g). Thus, the ventral sucker is innervated by the 5-HT-IP nerve fibers, which extend from the ventral and lateral nerve cords as well as from the 5-HT-IP neurons situated nearby the sucker, and also by the fibers going from the transverse commissure and connecting the ventral nerve cords.
- At the anterior (upper) edge of the ventral sucker, in the region which corresponds to the localization of the reproductive pore in adult *O. felineus*, a pair of small serotoninergic neurons are seen (Fig. 2c, inset);
- In the outer layer of the body wall musculature of *O. felineus* metacercaria the nerve net comprised of 5-HT-IP fibers is identified.

FMRFamide Immunopositive (FMRFa-IP) Elements in the Nervous System

An intensive FMRFa-IP staining was observed in the central and the peripheral parts of the *O. felineus* metacercaria nervous system (Fig. 3a–j). The positive staining to FMRFamide is found in the major compartments of the central nervous system (CNS): in the brain ganglia, brain commissure, in the

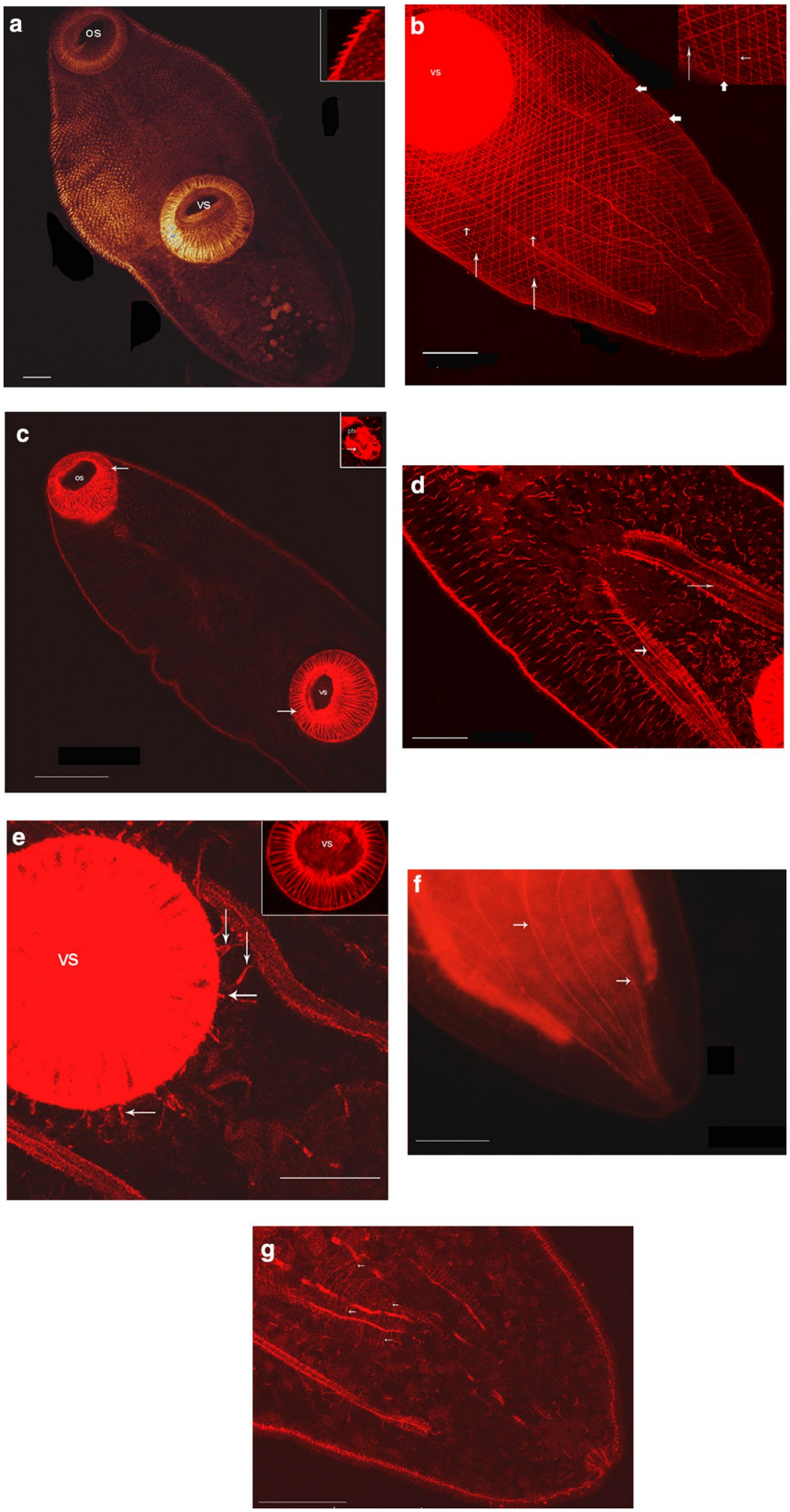
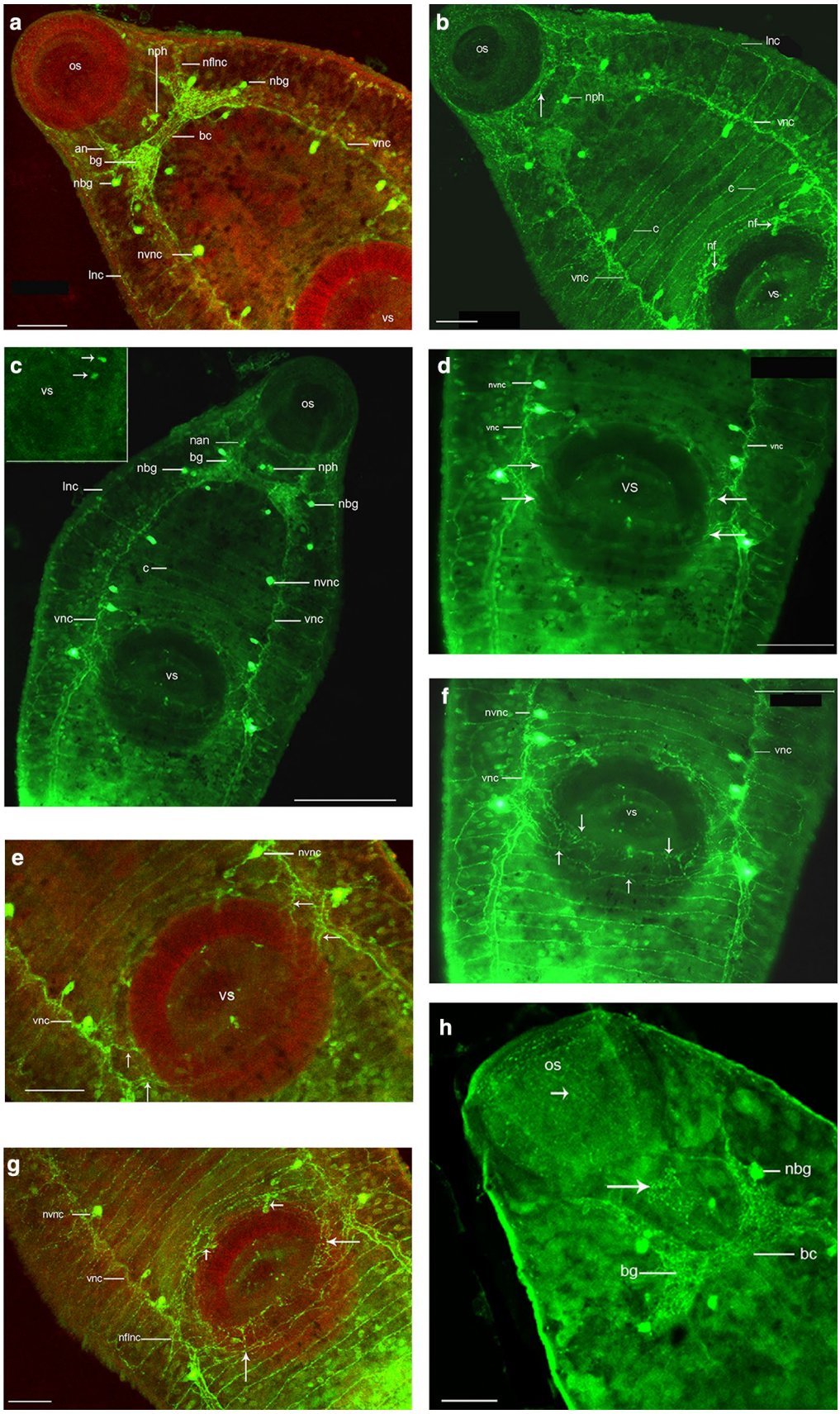


Fig. 1 **a–f** Musculature of *O. felineus* metacercaria stained with TRITC-phalloidin (in red) (CLSM optical sections). **a** Confocal image of phalloidin-stained whole mounts of *O. felineus* metacercaria showing the musculature of the oral and ventral suckers and spines on the body surface, ventral view. Inset: spines on the body surface (arrow), large magnification; os—oral sucker, vs—ventral sucker. Scale bar 30 µm; **b** circular (thick arrows), longitudinal (long arrows) and diagonal (short arrows) muscle fibers in the posterior of body part. Inset: the body musculature, large magnification, ventral view. Vs—ventral sucker. Scale bar 41,7 µm; **c** radial muscle fibers in oral and ventral suckers (arrows). Inset: circular musculature in pharynx (arrow), ventral view; os—oral sucker, vs—ventral sucker, (ph); scale bar 75 µm; **d** circular (long arrow) and longitudinal (short arrow) muscle fibers in intestine, ventral view; scale bar 35 µm; **e** muscle fibers, going to the ventral sucker (arrows); vs—ventral sucker. Inset: radial musculature of the ventral sucker, ventral view; scale bar 35 µm. **f** longitudinal muscle fibers in the excretory bladder (arrows); scale bar 50 µm. **g** circular muscle fibers in the excretory bladder (arrows), scale bar 35 µm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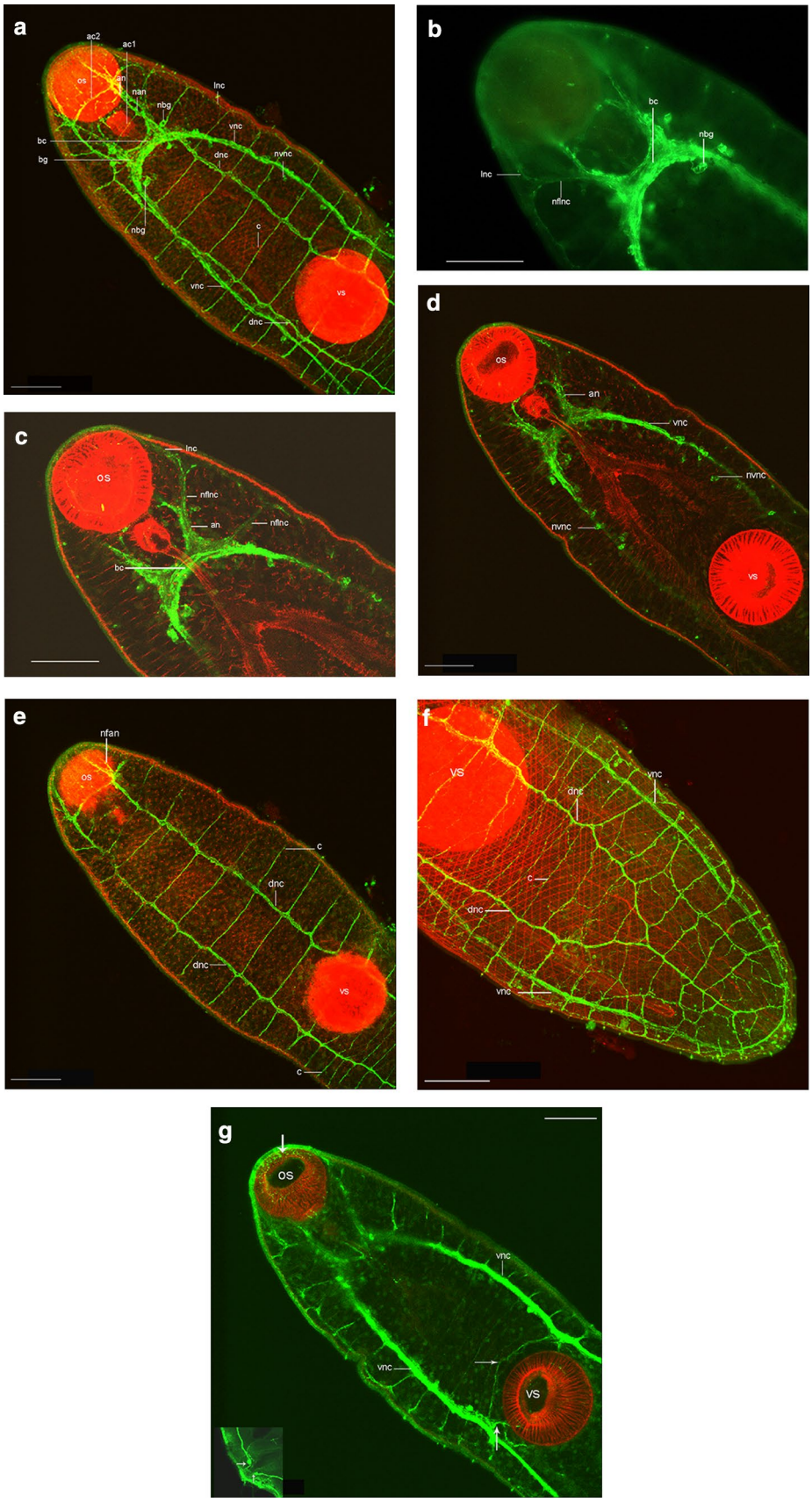
◂**Fig. 2** **a**–**h** 5-HT-immunopositive staining (in green) in nervous system of *O. felineus* metacercaria and TRITC-phalloidin staining (in red) in muscles (CLSM optical sections goes from ventral side of the body to dorsal). **a** Anterior body part with brain ganglia (bg), brain commissure (bc), ventral nerve cord (vnc), lateral nerve cord (lnc), neurons in brain ganglia (nbg), neuron near pharynx (nph), neuron in the vicinity of the ventral cord (nvnc), anterior nerves going to the oral sucker (an), nerve fiber going from anterior nerve to the lateral nerve cord (nflnc). Musculature (in red) stained by TRITC-phalloidin; os—oral sucker, vs—ventral sucker. Scale bar 30 µm; **b** anterior body part with transverse commissures connected the longitudinal nervous cords (c); the nerve fibers visible near the oral sucker (arrow), nerve fibers going from transverse commissure to ventral sucker (nf), ventral nerve cord (vnc), lateral nerve cord (lnc), neurons near pharynx (nph); os—oral sucker, vs—ventral sucker. Scale bar 30 µm. **c** general view of serotoninergic components in the nervous system: brain ganglia (bg), ventral nerve cord (vnc), lateral nerve cord (lnc), neuron in brain ganglia (nbg), neuron near pharynx (nph), neurons in the vicinity of anterior nerve (nan), neuron in the vicinity of the ventral cord (nvnc), transverse commissures (**c**) connected the longitudinal nerve cords; os—oral sucker, vs—ventral sucker. Inset: two small 5-HT–IP neurons in the vicinity of the upper border of the ventral sucker (arrows). Scale bar 100 µm; **d**, **e**, **f**, **g** CLSM optical sections demonstrating the innervation of the ventral sucker with 5-HT–IP fibers : **d**, **e** 5-HT-IP nerve fibers going from neurons and ventral nerve cord to the ventral sucker (arrows). vnc—ventral nerve cord, nvnc—neuron in the vicinity of ventral nerve cord, vs—ventral sucker. The musculature (in red) stained by TRITC-phalloidin (**e**); **f** two nerve circles in the vicinity of the outer border of ventral sucker (arrows); **g** nerve fibers going from transverse commissure to the ventral sucker (short arrows). Visible two nerve circles in the vicinity of the outer border of ventral sucker (long arrows) and nerve fiber going from lateral verve cord to ventral sucker (nflnc). The musculature (in red) stained by TRITC-phalloidin; vnc—ventral nerve cord, nvnc—neuron in the vicinity of ventral nerve cord, vs—ventral sucker. Scale bars: **d**, **f** 50 µm, **e**, **g** 30 µm; **h** 5-HT-IP nerve fibers in pharynx (long arrow) and oral sucker (short arrow); os—oral sucker, bg—brain ganglia, bc—brain commissura, nbg—neuron of brain ganglia. Scale bar 30 µm

lateral cords and in the transversal commissures. It should be noted that the intensity of the FMRFa-IP staining in the fibers comprising the brain commissure was not the same. The FMRFa-IP staining in the nervous system of metacercaria was more intensive as compare with the staining against 5-HT.

The details of FMRFa-IP nerve elements localization in the nervous system of *O. felineus* metacercaria are presented in Fig. 3a–j and are as follows.

- In the region of each brain ganglion four FMRFa-IP neurons with size of 5 µm have been located. From the brain ganglia the two FMRFa-IP nerve braches are running towards the oral sucker forming two anterior nerves (*an*, Fig. 3a). In the basal part of these anterior nerves a pair of FMRFa-IP neurons is localized (*ncan*, Fig. 3a). In the region of these neurons the thin FMRFa-IP commissure connecting the two anterior nerves is present (*ac1*, Fig. 3a). The second commissure connecting the anterior nerves is situated on the level of the oral sucker (*ac2*, Fig. 3a). Both anterior nerves bifurcate in the musculature of the oral sucker, forming a net of FMRFa-IP nerve fibers (Fig. 3a, e, g).
- From the brain ganglia the nerve fibers are running towards the lateral nerve cords, where they end with small varicosities (Fig. 3b, c).
- Three pairs of the longitudinal nerve cords (ventral, lateral and dorsal) are running along the whole metacercaria body (Fig. 1a–k). The ventral nerve cords are the most prominent. The width of the ventral cord in the middle body part is 5–6 µm, the dorsal—3–4 µm, and the lateral—about 2–3 µm. Two dorsal nerve cords connect together at the distance of about 125 µm from the posterior edge of the ventral sucker. Then at the distance of about 35 µm from the excretory pore they are subdivided again into two fibers which surround the excretory opening (Fig. 3f). In the close proximity to the excretory pore two small FMRFa-IP neurons located in the base of the ventral nerve cords are observed (Fig. 3g, inset).
- Approximately 10–12 of the FMRFa-IR commissures have occurred in the anterior part of the body (before the ventral sucker) and the same number of commissures has occurred in the posterior part of the body (behind the ventral sucker).
- The immunostaining of FMRFa-IP neurons was less pronounced than the staining of 5-HT-IP neurons (meaning that the amount of the neuromediators inside the neurons was different, it looks like 5-HT-IP neurons were much more filled with serotonin than the FMRFa-IP neurons with neuropeptides). At the same time FMRFa-IP staining of the ventral cord itself was more intensive than the corresponding 5-HT-IP staining. The width of anti-FMRFamide immunopositively stained components in the ventral cords (5–6 µm) size) nearly twice more than the size of the 5-HT-IP stained elements of the ventral nerve cords.
- The row of the FMRFa-IP neurons (size about 5–7 µm) can be seen along the ventral nerve cords (Fig. 3a, j). Similar neurons were found near the lateral (in the region located behind of the ventral sucker) and the dorsal nerve cords (in the region situated at the level of the ventral sucker).
- The longitudinal nerve cords are connected by transversal nerve commissures (Fig. 3a, e, f). The commissures which connect the ventral cords in the anterior body region are more rarely located than that in the posterior body region. Thus, the distance between the FMRFa-IP commissures was about 25 µm in the anterior part, whereas in the posterior part of the body it was around 15 µm. The transversal commissures in posterior region of the metacercaria body are connected by thin longitudinal and diagonal nerve fibers forming a nerve net (Fig. 3f).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◂ **Fig. 3** **a**–**j** FMRFamide-immunopositive staining (in green) in the nervous system of *O. felineus* metacercaria and TRITC-phalloidin staining (in red) in the muscles. **a** Anterior body part with brain ganglia (bg), brain commissure (bc), neurons of brain ganglion (nbg), ventral nerve cords (vnc), dorsal nerve cords (dnc), lateral nerve cords (lnc), transverse commissures (c), neurons in vicinity of the ventral nerve cord (ncvnc), anterior nerve (an), anterior neurons (nan), anterior commissure 1, connected the anterior neurons (ac1), anterior commissure 2, connected anterior nerves (ac2). The musculature (in red) stained by TRITC-phalloidin. os—oral sucker, vs—ventral sucker. A max projection, scale bar 50 µm; **b**, **c**, **d** CLSM optical sections of an anterior body part (**b**, **c** ventral view, **d** orsal view, optical sections) demonstrating the FMRFa-IP staining in: brain ganglia (bg), brain commissure (bc), the neurons in brain ganglia (ncbg), neurons in the vicinity of the ventral nervous cord (nvnc), anterior nerve (an), ventral nervous cords (vnc), lateral nerve cords (lnc), nerve fiber going to the lateral nervous cord (nflnc); Musculature (in red) stained by TRITC-phalloidin, **c**, **d** scale bars 50 µm; **e**, **f** FMRFa-IP staining in dorsal nerve cords: **e** an anterior body part, dorsal view, optical sections; **f** a posterior body part, dorsal view, a max projection, dnc—dorsal nerve cord, vnc—ventral nerve cord, nfan—nerve fibers of anterior nerve; **c** transverse commissure, os—oral sucker, vs—ventral sucker. Scale bars: **e** 50 µm; **f** 42,5 µm; **g** innervations with FMRFa-IP fibers of the ventral sucker (arrows), ventral view, an optical section. See FMRFa-IP staining in oral sucker (os) too (arrow), vs—ventral sucker, vnc—ventral nerve cord. Inset: two neurons near the excretory pore (arrows). Scale bar 50 µm; **h** two FMRFa-IP neurons in the vicinity of the lower border of ventral sucker (arrows), an optical section, vnc—ventral nervous cord), vs—ventral sucker. Scale bar 100 µm. **i** FMRFa-IP staining in the collateral going from the ventral cord (arrow), ventral view, an optical section, vnc—ventral nerve cord, vs—ventral sucker. Scale bar 30 µm. **j** FMRFa-IP nervous fibers in pharynx, oesophagus and intestine (arrows), ventral view, an optical section. Inset: the nerve fibers in the end part of intestine, vnc—ventral nervous cord, lnc—lateral nervous cord, nvnc—FMRFa-IP neurons located near the ventral nerve cord, bc—brain commissure. Scale bar 30 µm

- From two FMRFa-IP neurons situated in the ventral nerve cord at the level of the middle part of the ventral sucker the FMRFa-IP nerve fibers are directed towards the sucker. They circumflex the ventral sucker on its top and bottom sides giving the fibers into the sucker itself (Fig. 3g, i).
- In the vicinity of the posterior border of the ventral sucker two small bipolar FMRFa-IP neurons giving the processes in the distal and proximal directions of metacercaria body were found (Fig. 3h).
- The ventral nerve cord at the level of the ventral sucker form a collateral, i.e., the fiber which exits from the ventral cord, runs for some distance separately and then enters again into the nerve cord (Fig. 3i).
- FMRFa-IP nerve fibers were identified in different departments of the alimentary system—in pharynx, oesophagus and intestine (Fig. 3j). The thin FMRFa-IP nerve fibers extended from the ventral nerve cord and brain commissure towards the pharynx were detected.
- The thin FMRFa-IP nerve fibers were observed in the area of the location of the excretory pore.

Discussion

In present work the new details of the functional morphology of the nervous and muscle systems of *O. felineus* metacercaria—causative agent of the opisthorchiasis has been revealed with the use of the immunocytochemical and histochemical methods and confocal laser scanning microscopy. The 5-HT-IP and FMRFa-IP components of the parasite nervous system were discovered which were not described previously. The details of the innervations of the metacercaria muscle system by the neurotransmitters studied have been characterized.

Musculature

The muscle system of trematodes is well developed and plays an important role in the contractile activity of the body and in the functioning of the attachment and reproductive organs [21].

The general morphology of the adult and larval trematode muscle system has been described in a number of studies [15, 21, 25, 27, 28, 45, 48, 52, 53, 59, 61]. Three muscle layers were discovered in the body wall: outer circular, intermediate longitudinal and inner diagonal. The outer circular muscle layer is the thinnest and most densely packed, whereas the longitudinal layer is thicker and has the bigger distance between fibers as compared with the circular ones. The inner diagonal muscle fibers are most rarely packed within the body wall. The comparative analysis indicates that the some differences in the sizes of the myofibrils and their density exist in different species of trematodes [56].

The musculature of the attachment organs of trematodes, the oral and the ventral suckers is represented by longitudinal, circular and radial muscle fibers. The contractions of the longitudinal muscles cause the sucker opening, and the contractions of the circular muscles together with the radial fibers localized between outer and inner surface of the sucker close it [21].

Our findings regarding metacercaria *O. felineus* confirm the literature data on the organization of the body wall and attachment organs musculature in trematodes [21, 48, 52, 53]. The latest investigations of the oral sucker musculature using fluorescent actin staining, confocal laser scanning microscopy, scanning electron microscopy and light microscopy of histological sections of eight digenean species (all Plagiorchiida) indicated that the organization of the oral sucker is quite complex and represented by several groups of muscles including up to 14 groups. The musculature of the oral sucker was independent from the body wall and the internal muscles [26].

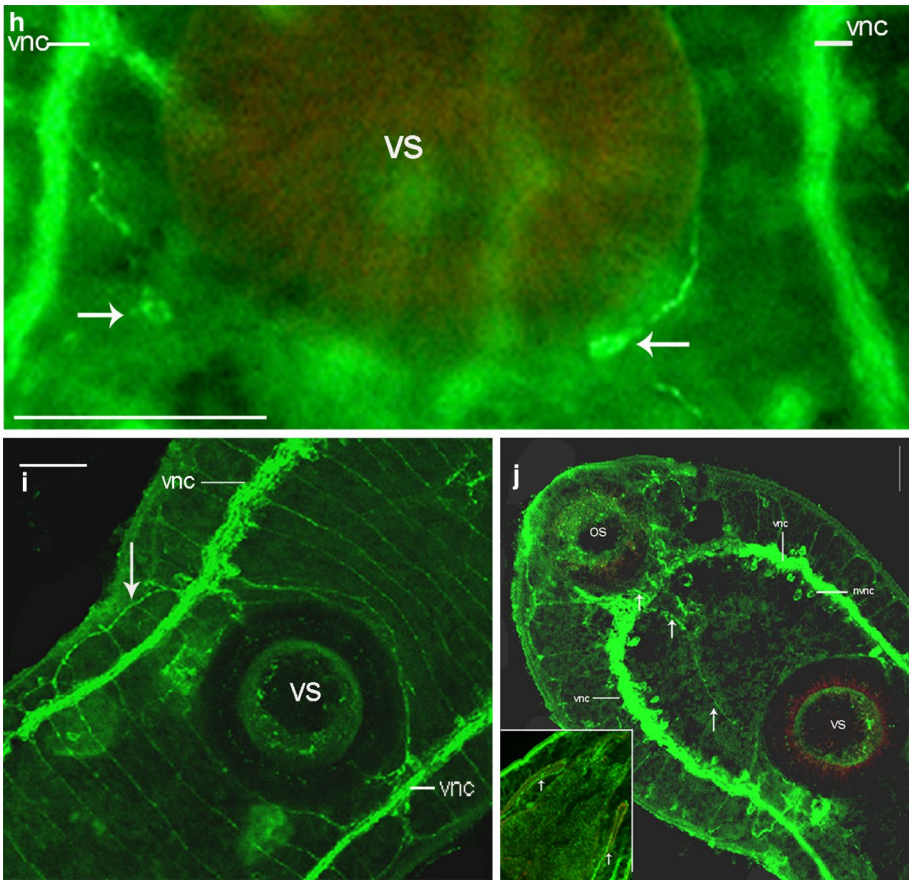

Fig. 3 (continued)

The additional studies are needed for the more detail investigation of the musculature arrangement of the attachment organs in *O. felineus* metacercaria.

In several studies the presence of the well developed muscle bundles running to the ventral sucker from two sides of body was described for a number of adult trematodes (*Allocreadium isoporum, Opisthorchis felineus, Plagiorchis laricola*) [56, 59]. We also observed the presence of well developed muscle bundles running to the ventral sucker from two body sides in *O. felineus* metacercaria.

Our results on the morphology of the muscle system of *O. felineus* metacercaria are generally in agreement with the available literature data on the existence of the circular, longitudinal and diagonal muscle fibers in the body wall of trematodes. At the same time the previously unknown data about presence of muscle elements in the digestive tract compartments, such as pharynx, oesophagus, intestine, as well as in the excretory system of *O. felineus* metacercaria has been received in our study.

The muscle fibers in different departments of the alimentary system were detected in adults and larvae of the several species of trematodes [56]. Thus, the presence of radial and circular muscle fibers was described in the musculature of spherical pharynx of *A. isoporum*. It was also noted that the musculature of the different compartments of the alimentary system (oesophagus, pharynx and branches of intestine) of *Cryptocotyle concavum, Cryptocotyle lingua, Gymnophallus deliciosus, P. laricola, A. isoporum, Paramphistomum cervi* is represented by the outer longitudinal and inner circular muscle fibers [56]. The muscle elements were described in pharynx of cercariae, preovigerous, and ovigerous adults of *Diplodiscus subclavatus* [25], and in the alimentary system compartments (pharynx, oesophagus and intestine) of cercaria and metacercaria of *Diplostomum pseudospathaceum* [45].

The circular and longitudinal muscles were detected in the pharynx of metacercaria of *Echinostoma caproni.* The investigation of the preovigerous adults of this parasite revealed the presence of two muscle layers (circular and longitudinal) in the alimentary tract [48]. In the same time, in the intestine wall of *Schistosoma mansoni* only the circular muscle layer was described [31].

As to *Opisthorchis felineus,* previously the radial and longitudinal muscle fibers were found in the pharynx and the circular and longitudinal muscle fibers were detected in the oesophagus and in intestine of adult worms [59]. In our

study for the first time the presence of the circular and longitudinal muscle fibers has been shown in the intestine of *O. felineus* metacercaria not found earlier in the adult forms of *O. felineus*, but observed in metacercariae of other trematoda species [45, 48]. Thus, our results and the literature data clearly show the presence of the muscle elements in the alimentary system of trematodes metacercaria indicating that musculature of alimentary tract is developed already in larval stages of the parasite, namely, in stage of metacercaria.

Our results about the presence of the longitudinal and circular muscle fibers in the excretory bladder of *O. felineus* metacercaria are in agreement with the data received on preovigerous adults of *D. subclavatus* [25] and *Echinostoma caproni* metacercaria [48].

5-HT- and FMRFa-IP Components in the Nervous System

Serotonin (5-hydroxytryptamine, 5-HT) is a most widespread neuromediator in the animal kingdom and so far was found in all investigated flatworm species [21, 47]. The presence of serotonin in the tissue of trematodes was shown by biochemical researches [1, 5, 6–8, 11, 33, 55 63]. These data were confirmed by immunocytochemical data which showed the localization of 5-HT in the nervous system of trematodes [3, 6, 12, 14, 30, 31, 40, 50, 52, [53]. It is assumed that 5-HT is an excitatory neurotransmitter in parasitic flatworms—cestodes, trematodes and monogenea, causing the contraction of their muscles [32, 38, 44, 54].

Amongst several families of the signal neuropeptides found in flatworms the special attention was paid to FMRF-like peptides, relatively short molecules of which contain from 4 to 20 amino acid residues [42]. Four FMRFamide –like peptides were identified in Platyhelminthes: YIRFamide from *Bdelloura candida,* GYIRFamide from *B. candida, Girardia tigrina*, RYIRFamide from *Artioposthia triangulata* and GNFFRFamide from *Moniezia expansa* [22, 23, 36, 37]. NPF-like peptides were represented by neuropeptide F (NPF, comprising of 39 amino acid residues) isolated from cestoda *Moniezia expansa* [35] and peptide NPF (containing 36 amino acid residues) isolated from planaria *Arthurdendyus triangulatus* [10]. The application of the specific antibodies raised against these peptides discovered the wide distribution of peptide-immunopositive staining in central and peripheral nervous system of Platyhelminthes [19, 20, 21]. The data obtained in regards to the flatworm representatives show that the peptidergic neurons and its fibers were detected in cerebral ganglia, longitudinal nerve cords and transversal commissures. Moreover, the peptidergic innervations of the attachment organs as well as reproductive organs of the flatworms were revealed [14, 17, 19, 21, 29, 31, 39, 40, 52, 59, 58]. It is also reported that the peptidergic nerve fibers are innervating the sensory organs situated in the body wall of the worms. A wide distribution of the neuropeptides among the parasitic flatworms allowed supposing their fundamental role in neuro-muscular physiology of their organisms [13, 21]. However, the whole functional significance of the neuropeptides in the organism of parasitic flatworms remains not completely clear.

It should be noted that the presence, the localization and functional roles of the neuronal substances are mainly studied in adult forms of trematodes [21, 56]. At the same time, a number of data indicate the presence of 5-HT- and FMRFamidergic components in the nervous system of larvae of trematodes, in particular in metacercaria. Thus, 5-HT-IP and FMRFa-IP elements were identified in central and peripheral departments of the metacercaria nervous system of *Apatemon cobitidis proterorhini*, *Echinostoma caproni*, *Biomphalaria glabrata*. *Bucephaloides gracilescens*, *Diplostomum sp., Diplostomum pseudospathaceum*, *Cotylurus erraticus, Helicometra fasciata, Cotylurus sp., Microphallus piriformis, Leucochloridiomorpha lutea* [3, 46, 48, 52, 53, 56, 58]. In these studies the innervation by the 5-HT-IP and FMRFa-IP nerve fibers of the musculature of the attachment organs, a sphincter of the excretory pore, the reproductive organs and the digestive system has been reported.

Immunocytochemical data on localization of serotonin in the nervous system of metacercariae of trematodes were confirmed by biochemical study. Thus, serotonin was isolated by spectrophuorometric method in metacercaria of *Codonocephalus urnigerus* (Strigeidae) in the amount of 0135–0.449 mkg/g of wet tissue [55].

Thus, the available data suggest that the serotoninergic and peptidergic elements of the nervous system are widely distributed not only in adults, but also in larvae (metacercaria) of trematodes, where they take part in the regulation of various functions of the parasite.

A comparative analysis of our and available literature data showed the presence of both similar and different characteristics in the morpho-functional organization of the nervous system in the metacercaria *O. felineus* and other trematodes species, both adults and larvae. The main similarity is the presence of 5-HT-IP and FMRFa-IP components in the nervous system of the *O. felineus* metacercaria the presence of neurotransmitters studied in the central and peripheral compartments of the nervous system of trematodes described in the available literature data. At the same time a number of new anatomical details on the structure of the nervous system and the innervation of the organs and tissues of the parasite were revealed in our work.

Thus, we identified the 5-HT-IP neurons located in the upper region of the ventral sucker, which were not previously described in metacercarias of other trematoda species. These cells probably correspond to the neurons located near the genital pore identified in the adult of *O. felineus* [59]. It can be assumed that 5-HT-IP neurons innervating the distal

regions of the reproductive system of the adult *O. felineus* are already developing in the larval stage (metacercaria), in which the reproductive system has not yet been formed.

The total number of 5-HT-IP neurons (the twenty two of 5-HT-IP neurons) detected in the present study in the body of *O. felineus* metacercariae was higher than previously described [59] (the twenty of 5-HT-IP neurons) due to the detecting of the additional 5-HT-IP neurons located in the brain ganglia, as well as in the vicinity of the oral and ventral suckers.

We have also identified the 5-HT-IP nerve fibers in the pharyngeal musculature of *O. felineus* metacercaria for the first time. The information on the innervation of the muscles of the alimentary system (pharynx, intestine) in *O. felineus* metacercaria correspond to the available data on the adult forms of trematodes *Allocreadium isoporum, Paramphistomum cervi* [56] and *Diplostomum pseudospathaceum* metacercariae [46]. Our and literature data about the innervation of the digestive system of the trematodes indicates that muscles activity which promotes the food movement along the alimentary tract is controlled by 5-HTergic nervous elements in both adult and larval trematodes.

The details of the innervations of the attachment organs of *O. felineus* metacercariae revealed in the present study are of great interest. Some information about the innervation by serotoninergic nerve fibers of the ventral and oral sucker in a number of adult and larvae trematodes is available, for example, *Fasciola hepatica, Parafasciolopsis fasciolaemorpha*, *Haplometra cylindracea,* [18, 39, 55, 58]. The differences in our findings and literature data are that we observed that the innervations of ventral sucker of *O. felineus* metacercaria by serotoninergic nerve fibers was coming not only from the ventral nerve cords, as was found earlier [59], but also from the lateral nerve cords as well as by the fibers extending from the 5-HT-IP nerve cells located nearby the ventral nerve cord. Besides that serotoninergic nerve fibers run towards the ventral sucker from the transversal nerve commissure connecting the ventral cords on the top edge of the ventral sucker. Nearby to the outer surface of the ventral sucker serotoninergic nerve elements form two nerve rings connected by thin 5-HT-IP fibers. The 5-HT-IP nerve fibers were also well visible inside of the ventral sucker musculature.

The obtained results indicated that the serotoninergic nervous system in *O. felineus* metacercaria control the musculature not only of ventral sucker but also that of the oral one, which is innervated by several fibers extending from the head ganglia. Details of the location of these nerve fibers are given in our work.

Our data on the presence of FMRFa-IP staining in the central and peripheral parts of the nervous system of O. *felineus* metacercaria are generally confirm the available literature data concerning of other trematodes species on presence of FMRFa-IP elements in central and peripheral compartments of the nervous system of trematoda larvae [3, 48, 52, 53, 56, 58, 59].

Analyses of the obtained results indicate higher intensity of the FMRFa-IP staining of the longitudinal nerve cords compared with the corresponding 5-HT-IP staining. Thus, the width of the FMRFa-IP components of the ventral nerve cord (5-6 μm) was almost two times larger than the width of the 5-HT-IP nerve elements in the ventral nerve cord. At the same time, it can be noted that the intensity of FMRFa-IP neurons staining was less pronounced than the intensity of 5-HT-IP neurons staining. The differences can be associated with different quantitative content of the neurotransmitters in various compartments of the metacercaria nervous system.

For the first time we have detected the FMRFa-IP nerve fibers in the alimentary system of the *O. felineus* metacercaria, namely in the pharynx, oesophagus and intestine. The study of the innervation of the digestive system by serotoninergic elements has shown the presence of 5-HT-IP nerve fibers only in pharynx, whereas the 5-HT-IP staining has not been observed in oesophagus and intestine. Based on these data it can be assumed that the innervation of the different parts of the digestive system of *O. felineus* metacercaria is provided by different neurotransmitters. However, this question requires a further investigation.

Our findings on the presence of nerve fibers in musculature of the oral and ventral suckers of FMRFa-IP broaden the available information on the innervations of the attachment organs of larvae of trematodes [56, 58, 59].

According to our data the ventral sucker of *O felineus* metacercaria is more intensely innervated by the serotoninergic than by FMRFa-IP fibers. In present work the FMRFa-IP neurons not described previously [59] were also seen in the lateral (in the area behind the ventral sucker) and in the dorsal (at the level of the ventral sucker) nerve cords. Additionally, two small bipolar FMRFa-IP neurons were identified nearby the posterior edge of the ventral sucker. These cells send their nerve processes in the distal and proximal directions of the metacercaria body. It cannot be excluded that these FMRFa-IP neurons and its fibers situated at close proximity to the posterior edge of the ventral sucker are involved in the regulation of the sucker muscle activity. The results of our study indicate also a possible regulation of the muscles of the excretory bladder by the FMRFa-IP nerve fibers.

Obtained in our study morphological data on the nervous system of *O. felineus* metacercariae have an important significance for the development of a new pharmacological strategy of fighting dangerous helminth infections by affecting the function of the nerve system of parasitic flatworms and neuronal signal compounds.

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Compliance with Ethical Standards

Conflict of Interest Authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in the article "The new data on the serotonin and FMRFamide localization in the nervous system of *Opisthorchis felineus* metacercaria" involving animals were in accordance with the ethical standards of the Institution at which the studies were conducted.

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