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VERSITA

The spatial relationship between the musculature and the 5-HT and FMRFamide immunoreactivities in cercaria, metacercaria and adult *Opisthorchis felineus* (Digenea)

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Abstract

The organisation of the neuromuscular system in cercariae, metacercariae and adult *Opisthorchis felineus* was studied. The patterns of nerves immunoreactive (IR) to antibodies towards serotonin (5-HT) and FMRFamide are described in relation to the musculature, stained with TRITC-conjugated phalloidin. The general organisation of the musculature in the body wall, suckers, pharynx, intestine and sphincter of the excretory pore remains the same from the larval stages to the adult worms. However, the diameter of the individual muscle fibres increases distinctly in the adult worms. The general pattern of 5-HT IR fibres in cercariae, metacercariae and adult *O. felineus* remains the same. Despite the large increase in body size, the number of 5-HT IR neurones remains almost the same in the cercariae and metacercariae and only a modest increase in number of neurones was observed in the adult worms. Thus the proportion of 5-HT IR neurones/body mass is greatest in the actively moving cercariae. Anti-FMRFamide stains the nervous system strongly.

Keywords

Opisthorchis felineus, serotonin, FMRFamide, neuromuscular system, larvae, adult

Introduction

The aminergic, cholinergic and peptidergic nervous system (NS) and different kinds of sensory endings and their distribution in both larval and adult flukes have been described with histochemical, immunocytochemical and ultrastructural methods (see Terenina and Gustafsson 2003a, b). However, studies covering all stages from redia to adult of the same species are still rare. Recently, comprehensive investigations of the musculature and associated innervation of adult and intermolluscan stages of two flukes, *Echinostoma caproni* and *Echinoparyphium aconiatum*, have been performed (Šebelová *et al*. 2004, Terenina *et al*. 2006). The muscle systems and the aminergic and peptidergic NS of daughter rediae, cercariae, metacercariae and pre- and post-ovigerous adults have been described using scanning electron microscopy (SEM) and immunocytochemistry (ICC) coupled to confocal scanning laser microscopy (CSLM).

Opisthorchis felineus Rivolta, 1884 (Opisthorchidae Braun, 1901), or the cat liver fluke, infects the liver of mammals. Opisthorchiasis, the disease caused by *O. felineus*, is very dangerous and widely spread in Russia. It is estimated that 1.5 million people in Russia are infected with this parasite. Inhabitants of Siberia acquire the infection by consuming raw, slightly salted and frozen fish. In humans, *O. felineus* invades the gallbladder, liver and pancreas. If not treated in the early stages, opisthorchiasis may cause cirrhosis of the liver and an increased risk of liver cancer. In children opisthorchiasis may be asymptomatic. Two weeks after infection the flukes enter the biliary tract. Symptoms of infection include fever, general malaise, skin rash, and gastrointestinal disturbances. Severe anemia and liver damage may also incapacitate the infected person for 1–2 months. Figure 1 shows the life cycle of *O. felineus*.

Numerous parasiticides are employed to treat infection with parasitic worms. Some parasiticides are known to target the neuromuscular system (NMS) of helminth parasites. The hope lies in finding novel chemotherapeutic agents that act specifically on some neuronal signalling pathway of the worm, with minimal side effects on the host. The NMSs of parasitic

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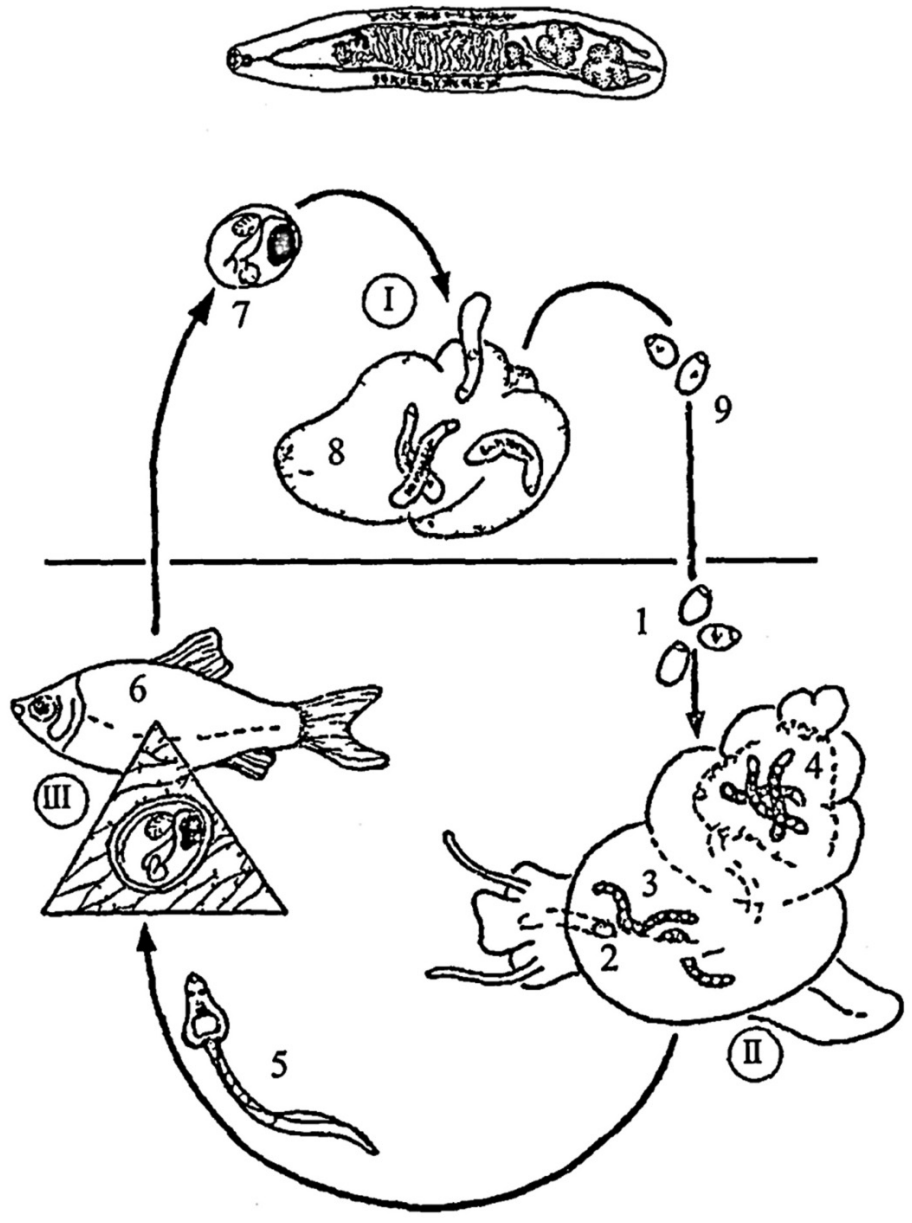


Fig. 1. The life cycle of *Opisthorchis felineus* according to Be'er (2005). **I.** Definitive host (human, wild and domestic carnivorous and omnivorous animals, rodents). **II.** First intermediate host (Bithyniidae molluscs). **III.** Second intermediate host (Cyprinidae fish). **1.** Eggs with developed miracidia. **2.** Miracidia. **3.** Sporocysts. **4.** Rediae and developing cercariae in mollusc. **5.** Free-swimming cercariae. **6.** Metacercariae in the musculature of fish. **7.** Excysted metacercariae in the duodenum of the definitive host. **8.** Adult flukes in the liver of the definitive host. **9.** Embryonated eggs in the environment

flatworms are highly complex, both morphologically involving different types of neurones, and biochemically, containing many putative signal molecules (Halton and Maule 2004). Parasites have attachment organs to secure their place in the host. They produce large numbers of eggs to secure their survival. The NMS has evolved around these organs.

To our knowledge, the NS of adult *O. felineus* was first described by Kolmogorova (1959). She used methylene blue staining and found nerve fibres extending to the body wall, indicating a sensory function. Both the cholinergic and the aminergic NS of adult *O. felineus* have been described and biogenic amines have been identified by spectrofluorimetry (Kotikova *et al*. 1985, Joffe *et al*.1988, Shishov *et al*. 1988). Two preliminary reports on the pattern of nerves immunoreactive (IR) to antibodies towards 5-HT and towards the neuropeptide FMRFamide in *O. felineus* were recently given (Tolstenkov *et al*. 2008, Terenina *et al*. 2008). These reports contain no figures.

The central nervous system (CNS) of adult *O. felineus* consists of a pair of cerebral ganglia connected by a dorsal com-

missure, the so called bilobed brain, and a pair of main nerve cords (MCs) extending along the ventral surface from the cerebral ganglia to the posterior end of the worm. The peripheral nervous system (PNS) consists of two pairs of minor nerve cords, connecting commissures and nerves associated with the suckers, the intestine and the reproductive organs.

This study is the first detailed investigation of the organisation and development of the NMS in cercariae, metacercariae and adult *O. felineus*. The patterns of nerves IR to antibodies towards 5-HT and FMRFamide are described using ICC and CSLM. The musculature is stained with TRITC-conjugated phalloidin.

Materials and methods

Cercariae, metacercariae and adult *Opisthorchis felineus* Rivolta, 1884 (Opisthorchidae Braun, 1901) were used in this work. The cercariae were removed from infected snails *Bithynia troscheli* (Chanti-Mansijsk, Russia) and the metacercaria from ides *Leuciscus idus* (L.) (Tobolsk, Russia). The encysted metacercariae were excysted in alkaline trypsin-bile salt medium at room temperature according to Ursone and Fried (1995). Adult worms (size 8–10 mm in length) were recovered from experimentally infected golden hamster 50 days post infection. The material was fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer at 4°C. For storage it was transferred to the same buffer with 10% sucrose. The material was processed as whole mounts in Eppendorf tubes.

Immunocytochemistry and staining with TRITC-conjugated phalloidin

Whole mounts were stained with rabbit-anti-5-HT (Incstar, USA) (1:500) or rabbit-anti-FMRFamide (1:500) according to the method described by Coons *et al.* (1955). The whole mounts were incubated with the primary antibody during 5 days at 4°C, then they were washed with phosphate buffered saline-triton (PBS-T), and incubated with the secondary antibody swine anti-rabbit FITC (fluorescein isothiocyanate) (DAKO) 1:50 for a further 5 days at 4°C. In order to study the spatial relationship between nerves and musculature, the immunochemical staining was followed by staining the actin fibres with TRITC (rhodamine B isothiocyanate)-conjugated phalloidin (Sigma) (1:200) for 1 h at 4°C according to Wahlberg (1998). After a final wash in PBS-T, the specimens were mounted in 50% glycerol-PBS for examination with a Leica TCS 4D confocal scanning laser microscope coupled to a Leitz Aristoplan. Controls included omission of the primary antibody, substitution of the primary antibody with non-immune rabbit serum. The immunochemical staining and the microscopy were performed at the Department of Biology, Åbo Akademi University, Finland.

Results

The NMS in cercariae of O. felineus

According to Be'er (2005) the body of the cercariae measures approximately 0.16 × 0.08 mm and the oral sucker is about 0.03 mm in diameter. The tail is about 0.4 mm long and 0.03 mm wide at its base. A thin membrane, which starts dorsally and ends ventrally in the middle of the tail, enlarges the surface.

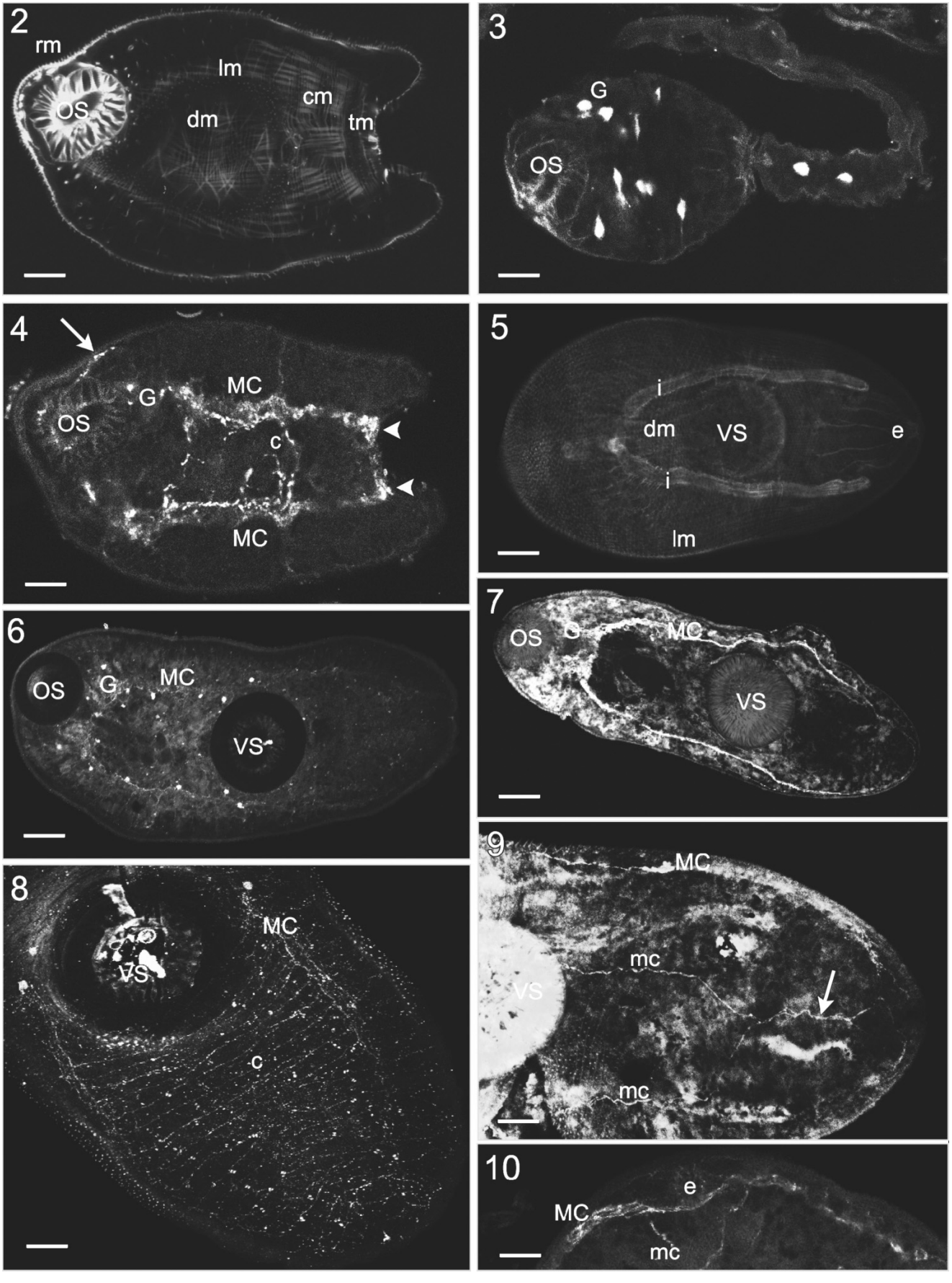
Phalloidin staining of *O. felineus* cercariae revealed a body wall musculature consisting of a well-organised outer circular muscle layer (fibre diameter 0.3–0.5 μm) and an intermediate longitudinal muscle layer (fibre diameter 0.5–0.8 μm) (Fig. 2). The distance between the individual muscle fibres in both layers is <1 μm. In addition, an inner layer of diagonal muscle fibres (diameter 0.5 μm) occurs. The distance between the diagonal muscle fibres is 5.0–7.0 μm. In the oral sucker the radial muscles dominate (Fig. 2). Equatorial muscles were also observed. The ventral sucker is undeveloped. However, radial muscle fibres directed towards the ventral sucker can be seen already at this early stage. At the attachment point of the tail, many radial muscle fibres are present in the body (Fig. 2). In the tail outer circular and inner longitudinal muscle fibres occur.

The 5-HT IR nervous system

The 5-HT IR nerve fibres occur in bilobed brain, the main nerve cords (MCs) and in the tail (Fig. 3). Three 5-HT IR neurones (size 3 × 5 μm) were observed in each cerebral ganglion, one in the commissure connecting the ganglia and one on each lateral side of the pharynx. The MCs extend from the cerebral ganglia to the posterior end of the body. Four 5-HT IR neurones lie along each MC. Three of them are located close to the rudiment of the ventral sucker. At the posterior end of the body two small 5-HT IR neurones occur. In the upper part of the tail, two 5-HT IR neurones (size 3 × 5 μm) were demonstrated (Fig. 3). Thus in cercaria of *O. felineus*, nineteen 5-HT IR neurones were observed in the body and 2 in the tail. The controls were negative.

The FMRFamide IR nervous system

The NS stains strongly with anti-FMRFamide. Staining was observed in the cerebral ganglia, the MCs, and in many commissures (Fig. 4). From each cerebral ganglion FMRFamide IR nerves extend along the lateral sides of the pharynx to the oral sucker. The MCs (about 5 μm in diameter) extend from each cerebral ganglion to the posterior end of the body. The MCs are connected by many FMRFamide IR commissures. In the region of the rudimentary ventral sucker, many thin FMRFamide IR fibres and two neurones were observed. At the posterior end of the body, a group of FMRFamide IR neurones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occur (Fig. 4). The FMRFamide IR neurones are less than 2 × 3 μm and difficult to count. The controls were negative.

The NMS of metacercariae of O. felineus

According to Sudarikov *et al*. (2002) the excysted metacercariae measure approximately 0.64 × 0.16 mm. Thin spikes cover the surface from the anterior end to the posterior border of the ventral sucker. The oral sucker measures 0.08 × 0.06 mm, the pharynx 0.04 × 0.02 mm, the oesophagus 0.08 mm and the ventral sucker 0.08 in diameter. The centre of the ventral sucker is located about 0.38 mm from the anterior end of body, i.e. at 60% of the length of the body (Sudarikov *et al*. 2002).

Phalloidin staining of *O. felineus* metacercariae revealed a body wall musculature consisting of an outer, dense layer of circular muscles (fibre diameter 0.5 μm), and an intermediate layer of longitudinal muscles (fibre diameter 0.5 μm). The distance between the individual fibres in the circular layer is <1 μm and in the longitudinal layer 1–2 μm. In addition, an inner layer of diagonal muscle fibres (fibre diameter 0.5–0.7 μm) occurs. The distance between the diagonal muscle fibres is 5.0–7.0 μm (Fig. 5). Radial and equatorial muscle fibres form the oral and ventral suckers. Many muscle bands from the lateral sides anchor the ventral sucker (Fig. 5). The excretory pore has a sphincter formed by radial and equatorial muscle fibres.

The 5-HT IR nervous system

Nonspecific autofluorescence impaired the ICC staining of metacercariae of *O. felineus* with anti-5-HT. However, 5-HT IR nerve fibres were demonstrated in the cerebral ganglia, the MCs, the minor cords and the commissures (Fig. 6). Three 5-HT IR neurones (size 5 μm in diameter) were observed in each cerebral ganglion, two in the dorsal commissure connecting the ganglia and one on each lateral side of the pharynx. In each MC, four 5-HT IR neurones (size 5 μm in diameter) form an almost symmetrical pattern (Fig. 6). Three of these neurones lie close to the ventral sucker. In the vicinity of the excretory pore, two small 5-HT IR neurones were observed. This means that in all, twenty 5-HT IR neurones were observed in the body of metacercaria of *O. felineus*. The controls were negative.

The FMRFamide IR nervous system

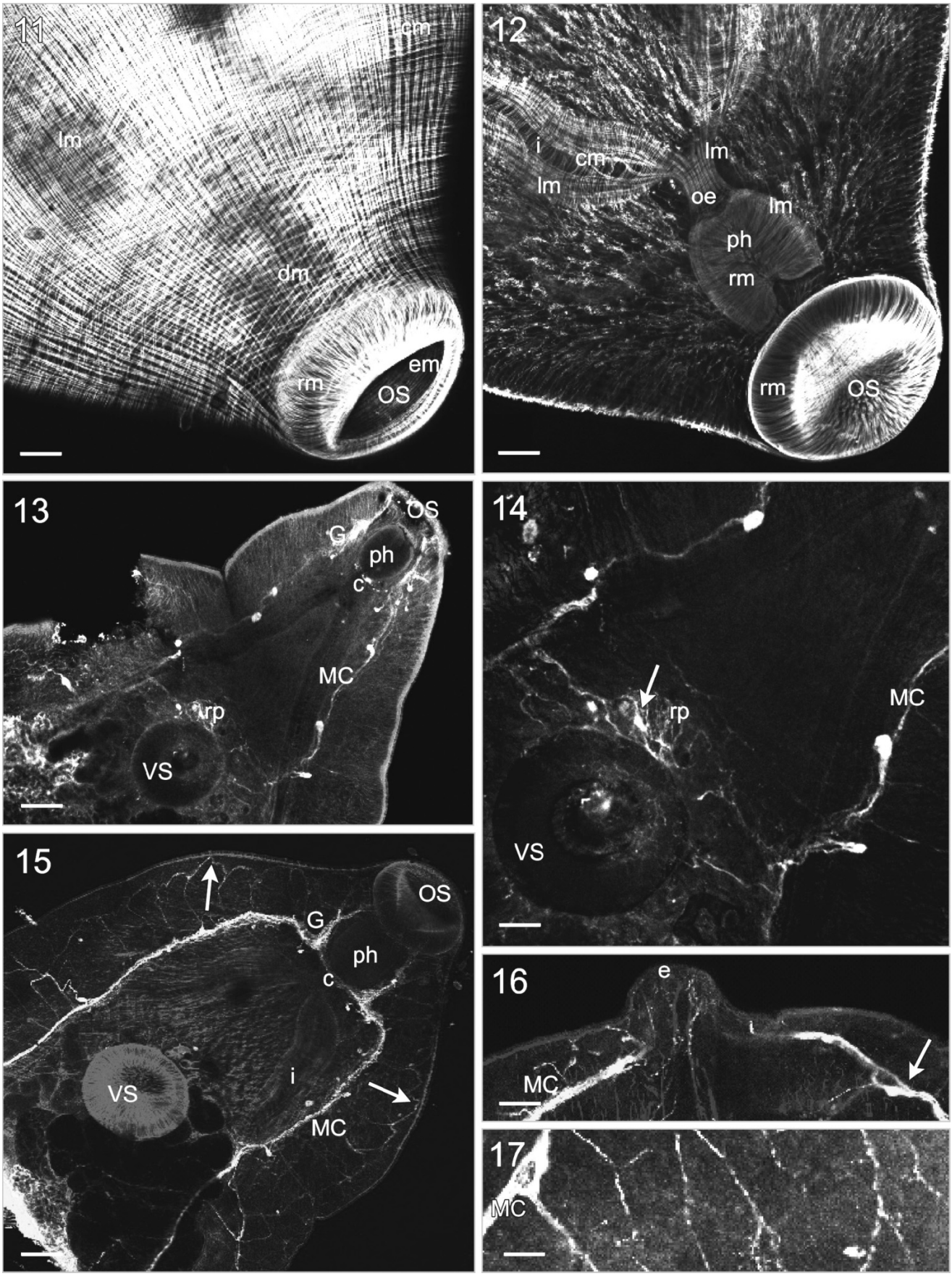
The NS stains strongly with anti-FMRFamide. Staining was observed in the cerebral ganglia, the MCs, the minor cords and in many commissures (Figs 7–10). FMRFamide IR nerve fibres extend from the cerebral ganglia in an anterior direction along the lateral sides of the pharynx up to the oral sucker. The MCs measure about 5 μm in diameter and run along the ventral surface to the excretory pore (Fig. 8). On the dorsal side, starting from the posterior level of the ventral sucker a pair of FMRFamide IR minor cords were observed. They extend in a posterior direction and fuse together for a short distance after which they divide into two again, just before the excretory pore (Figs 9 and 10). Near the excretory pore the MCs form a ring-like structure (Fig. 10). FMRFamide IR nerve fibres from the MCs innervate the ventral sucker. The FMRFamide IR neurones (size 5 μm in diameter) were particularly observed in the anterior part of the metacercariae. The total number FMRFamide IR neurones were not counted. The controls were negative.

The NMS of adult O. felineus

The body of the adult *O. felineus* is lanceolate. Worms taken from infected humans measure 5.4–10.2 × 0.8–1.9 mm. The diameter of both suckers is 0.23 mm. Worms from infected dogs, cats and hamsters are smaller (Be'er 2005). The adult worms used in this study were 8–10 mm in length.

The phalloidin staining revealed a body wall musculature consisting of an outer, dense layer of circular muscles (fibre diameter 1–2 μm) and an intermediate, layer of longitudinal muscles (fibre diameter about 2 μm). The distance between the individual fibres in both layers varies between 1–2 μm. In addition, an inner layer of diagonal muscles (fibre diameter 1–2 μm) occurs. The distance between the diagonal muscle fibres varies between 5–15 μm (Fig. 11). The oral and ventral suckers consist of well-developed radial and equatorial muscle fibres (Fig. 12). Several bands of muscles attach the ventral sucker and reproductive pore to the body wall. The very strong pharynx contains radial and longitudinal muscle fibres. The oesophagus branches into two, forming the intestine, which runs to the posterior end of the worm. The musculature in the oesophagus and intestine consists of circular and longitudinal fibres (Fig. 12).

Figs 2–4. Confocal images of the NMS in *O. felineus* cercaria. Bar = 10 μm. **2.** Max projection showing circular (cm), longitudinal (lm) and diagonal (dm) muscles. Oral sucker (OS) with radial muscles (rm) and muscles at the attachment point of the tail (tm). **3.** Pattern of 5-HT IR neurones. Ganglion (G), oral sucker (OS). Note two 5-HT IR neurones in the tail. **4.** Image pair of Fig. 2 showing the pattern of FMRFamide IR nerves. Ganglion (G), main nerve cord (MC), oral sucker (OS) with innervating nerve (arrow), commissure (c) close to developing ventral sucker, small FMRFamide IR neurones (arrow heads). **Figs 5–10.** Confocal images of the NMS in *O. felineus* metacercaria. **5.** Max projection showing longitudinal (lm) and diagonal (dm) muscles. Ventral sucker (VS), intestine (i), excretory pore (e). Bar = 50 μm. **6.** Max projection showing the pattern of 5-HT IR nerve cells and fibres. Ganglion (G), main nerve cord (MC), oral sucker (OS), ventral sucker (VS). Bar = 50 μm. **7.** Max projection showing the pattern of FMRFamide IR nerve fibres. Ganglion (G), main nerve cord (MC), oral sucker (OS), ventral sucker (VS). Bar = 50 μm. **8.** Pattern of FMRFamide IR nerves in the posterior part. Main nerve cord (MC), commissure (c), ventral sucker (VS). Bar = 25 μm. **9.** Pattern of FMRFamide IR nerves on the dorsal surface. Main nerve cord (MC), two minor cords (mc), joining together (arrow), ventral sucker (VS). Bar = 25 μm. **10.** Large magnification of posterior end showing the FMRFamide IR nerves in main nerve cord (MC) close to excretory pore (e) and a pair of minor nerve cords (mc). Bar = 20 μm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The 5-HT IR nervous system

The 5-HT IR nerve fibres occur in the cerebral ganglia, the MCs, the minor cords and the commissures (Fig. 13). The cerebral ganglia and the MCs consist of a densely interwoven fibrillar network of axons and dendrites. The cerebral ganglia, measuring 75–50 μm, are located on either side of the pharynx, close to its base. The ganglia are connected by a dorsal commissure at the base of the pharynx. The 5-HT IR MCs originate as multifibre rootlets from the cerebral ganglia and measure 25–30 μm in diameter in the anterior part of the worm. They become slightly thinner towards the posterior end. The PNS consists of a pair of dorsal and lateral minor cords, a series of thin commissures and nerves associated to the suckers, intestine and the reproductive organs.

The 5-HT IR neurones are large (size 15–25 × 30–50 μm) and usually bipolar. Three to four large 5-HT IR neurones were observed in each cerebral ganglion, two smaller (size 10 × 20 μm) in the cerebral commissure and one (size 10 × 20 μm) on each lateral side of the pharynx. 5-HT IR nerves extend from the cerebral ganglia along the lateral surface of the pharynx up to the oral sucker. In the MCs, the 5-HT IR neurones form an almost symmetrical pattern (Fig. 13). At the anterior and posterior levels of the ventral sucker, two 5-HT IR neurones occur in each MC (Fig. 13). Thin nerve fibres extend from the MCs to the ventral sucker and to the region of the reproductive pore. A rich nerve plexus composed of 5-HT IR fibres, including two 5-HT IR neurones, surrounds the reproductive pore (Figs 13 and 14). Thin 5-HT IR fibres occur on the inside of the ventral sucker. Two 5-HT IR neurones were observed close to the excretory pore. In all, twenty-six 5-HT IR neurones were identified in adult *O. felineus*. The controls were negative.

The FMRFamide IR nervous system

The NS stains strongly with anti-FMRFamide. Staining was observed in the cerebral ganglia, the MCs, the minor cords and the commissures (Fig. 15). The FMRFamide IR neurones (size 15–20 × 10–20 μm) are often bipolar. Three to four FMRFamide IR neurones were observed in each cerebral ganglion and one (size 10 × 20 μm) on each lateral side of the pharynx. FMRFamide IR nerves extend from each cerebral ganglion along the lateral surface of the pharynx up to the oral sucker (Fig. 15). Closer to the oral sucker, these nerves split into many fine branches. In the MCs (approximately 20 μm in diameter), the FMRFamide IR neurones (size 10–20 μm) form an almost symmetrical pattern (Fig. 15). At the anterior and posterior levels of the ventral sucker, two FMRFamide IR neurones occur in each MC (Fig. 15). Thin nerve fibres extend from the MCs to the ventral sucker, forming a nerve plexus inside the sucker. Close to the reproductive pore an FMRFamide IR plexus, including one neurone, was observed. The FMRFamide IR MCs extend to the excretory pore (Fig. 16). On each side of the excretory pore one FMRFamide IR neurone was observed. A short distance from the excretory pore a group of small FMRFamide IR neurones were revealed in each MC. The number of FMRFamide neurones is estimated to twenty-five. The FMRFamide IR minor cords can be followed only to the level of the ventral sucker. A very extensive sub-tegumental network IR to FMRFamide was noted in the body wall (Figs 15–17). The controls were negative.

Discussion

The musculature

During the development of *O. felineus* from cercariae to metacercariae and further on to adult worm a very large increase in size takes place. The general organisation of the musculature in the body wall, suckers, pharynx, intestine and sphincter of the excretory pore remains the same from the larval stages to the adult worm. However, the diameter of the individual muscle fibres increases distinctly in the adult worm (Table I). The body musculature of the cercariae, metacercariae and adult of *O. felineus* follows the same pattern as that of *Diplostomum pseudospathaceum* (Czubaj and Niewiadomska 1997), *Apatemon cobitidis proterorhini* and *Bucephaloides gracilescens* (Stewart *et al*. 2003a, b), *Echinostoma caproni* (Šebelová *et al*. 2004) and *Echinoparyphium aconiatum* (Terenina *et al*. 2006). Šebelová *et al*. (2004) discussed pattern of movement in *Echinostoma caproni* and pointed out "that contraction of circular and longitudinal muscles, respectively extends and shortens the worm; while diagonal muscles act to provide side-to-side movement and a degree of torsion".

Figs 11–17. Confocal images of the NMS in adult *O. felineus*. **11.** Max projection showing circular (cm), longitudinal (lm) and diagonal (dm) muscles. Oral sucker (OS) with radial (rm) and equatorial muscles (em). Bar = 50 μm. **12.** Frontal section showing oral sucker (OS) with radial muscles (rm), pharynx (ph) with radial and longitudinal muscles (lm), oesophagus (oe) with longitudinal muscles, and intestine (i) with circular (cm) and longitudinal muscles. Bar = 50 μm. **13.** Frontal section showing the pattern of 5-HT IR nerve fibres and cells. Oral sucker (OS), pharynx (ph), ganglion (G), brain commissure (c), main nerve cord (MC) with 5-HT IR neurones, ventral sucker (VS), reproductive pore (rp). Bar = 100 μm. **14.** Larger magnification of 5-HT IR neurones (arrow) close to the ventral sucker (VS) and reproductive pore (rp). Main nerve cord (MC). Bar = 50 μm. **15.** Max projection showing the pattern of FMRFamide IR nerve fibres and cells. Oral sucker (OS), pharynx (ph), intestine (i), ganglion (G), brain commissure (c), main nerve cord (MC), ventral sucker (VS). Note nerves extending from the main nerve cords to the surface (arrows). Bar = 100 μm. **16.** Pattern of FMRFamide IR nerves close to the excretory pore (e). Main nerve cord (MC) with bipolar neurone (arrow). Bar = 50 μm. **17.** Pattern of FMRFamide IR subtegumental network. Main nerve cord (MC). Bar = 50 μm

Table I. The diameter of muscle fibres (μm) in the body wall of cercaria, metacercaria and adult *Opisthorchis felineus*

	Circular	Longitudinal	Diagonal	Distance between diagonal fibres (μm)
Cercaria	0.3–0.5	0.5–0.8	0.5	5.0–7.0
Metacercaria	0.5	0.5	0.5	5.0–7.0
Adult	1.0–2.0	2.0	1.0–2.0	5.0–15.0

The 5-HT IR nervous system

Despite the large increase in body size, the general pattern of 5-HT IR neurones and fibres in cercariae, metacercariae and adult of *O. felineus* remains the same (Fig. 18A-C). Already at the cercariae stage, the organisation of the NS follows the orthogonal system, typical for flatworms (Reisinger 1972). The brain is bipolar. The two MCs start as multifibre outgrowths (rootlets) from the cerebral ganglion and can be distinguished by their size and large 5-HT IR marker neurones (Reuter and Gustafsson 1995). The minor cords are thinner and have less pronounced contact with the cerebral ganglia. The number of 5-HT IR neurones remains almost the same in the cercariae and metacercariae and only a modest increase in number of neurones was observed in the adult worms (Fig. 18A-C). This means that the proportion of neurones/body mass is greatest in the cercariae, which are highly motile. 5-HT is generally regarded as the main excitatory neurotransmitter of motor activity in flatworms (see Halton and Maule 2004). The pattern of 5-HT IR neurones in cercariae of *O. felineus* is similar to those in cercariae of *Cryptocotyle lingua*, *Cercariae emasculans* (Pan *et al*. 1994), *E. caproni* (Šebelová *et al*. 2004) and metacercariae of *D. spathaceum* and *Cotylurus erraticus* (Barton *et al*. 1993). The presence of two 5-HT IR neurones in the tail of cercariae has been reported from a. o. *Diplostomum chromatophorum* (Terenina and Gustafsson 2003b), *E. caproni* (Šebelová *et al*. 2004) and *Echinoparyphium aconiatum* (Terenina *et al*. 2006). The symmetrical pattern of 5-HT-IR neurones in the anterior part of adult *O. felineus* is similar to that observed by Shishov *et al*. (1988) in their study of biogenic amines of adult *O. felineus*. They observed fluorescence characteristic of indolamine in five pairs of neurones symmetrically situated in the anterior part. Furthermore, fluorescence characteristic of catecholamines was observed in 3 pairs of symmetrically situated neurones in the anterior part and in a few smaller neurones in the posterior part of the worm. 5-HT IR neurones often exhibit a marked bilateral symmetry in their arrangement (Halton 2004). In many instances, the distribution pattern of 5-HT staining in flatworms is distinct from that of the cholinergic and peptidergic neuronal pathways, but parallels the staining pattern for catecholamines (Shishov 1991).

A distinct increase in the size of the 5-HT IR neurones was observed in the adult worms (Table II). Two size classes of 5-HT IR neurones were observed in adult *O. felineus*. A similar increase in size of 5-HT IR neurones was observed in cercariae, metacercariae and adult *E. caproni* (Table II) (Šebelová *et al*. 2004). The modest increase in the number of 5-HT IR neurones in adult *O. felineus* may be compensated partly by the increase in neuronal size. In flatworms, the somata range in size from 4.5 × 3.0 μm, e.g., those in the rostellar ganglia of *Hymenolepis diminuta*, to cells of an order of magnitude larger, exemplified by those in the innervation of the ootype in *Fasciola hepatica* and measuring approximately 45 × 30 μm. Generally the 5-HT IR neurones are larger and more distinct than the cholinergic and peptidergic neurones (Gustafsson and Halton 2001). The increased size of the 5-HT IR neurones cells during the development of *O. felineus* falls within the range mentioned above.

Table II. Size of 5-HT IR neurones (μm) in *Opisthorchis felineus* and *Echinostoma caproni*

	O. felineus	*E. caproni*
Redia		6 × 12
Cercariae	3 × 5	6 × 6
Metacercariae	5 × 5	6 × 6
Adult: 1. large	15–25 × 30–50	20–60 in diameter
2. small	10 × 20	

The FMRFamide IR nervous system

The immunostaining for FMRFamide was more intense than that for 5-HT in all stages of *O. felineus*. The FMRFamide IR nerve fibres are also more widely expressed than the 5-HT IR nerve fibres. Similar patterns have been obtained in *D. spathaceum*, *C. erraticus*, *Apatemon cobitidis proterorhini*, *Bucephaloides gracilescens* and *E. caproni* (Barton *et al*. 1993; Stewart *et al*. 2003a, b; Šebelová *et al*. 2004). According to Halton and Gustafsson (1996) the aminergic and peptidergic pathways are quite separate and distinctive in construction. Unfortunately, double staining with anti-5-HT and anti-FMRFamide was not performed in this study. Even though the exact number of FMRFamide IR neurones could not be assessed, the same trend as for the 5-HT IR NS prevails, i.e. the proportion of neurones/body mass is greatest in the highly motile cercariae (Fig.18D-F). FMRFamide belongs to the FMRFamide related neuropeptide family (FaRP) and has been shown to be myoexcitatory in a concentration-dependent manner when applied exogenously to isolated muscle cells or muscle strips from free-living and parasitic flatworms (Day and Maule 1999). The FMRFamide IR minor cords observed on the dorsal side and in the posterior part of the metacercariae of

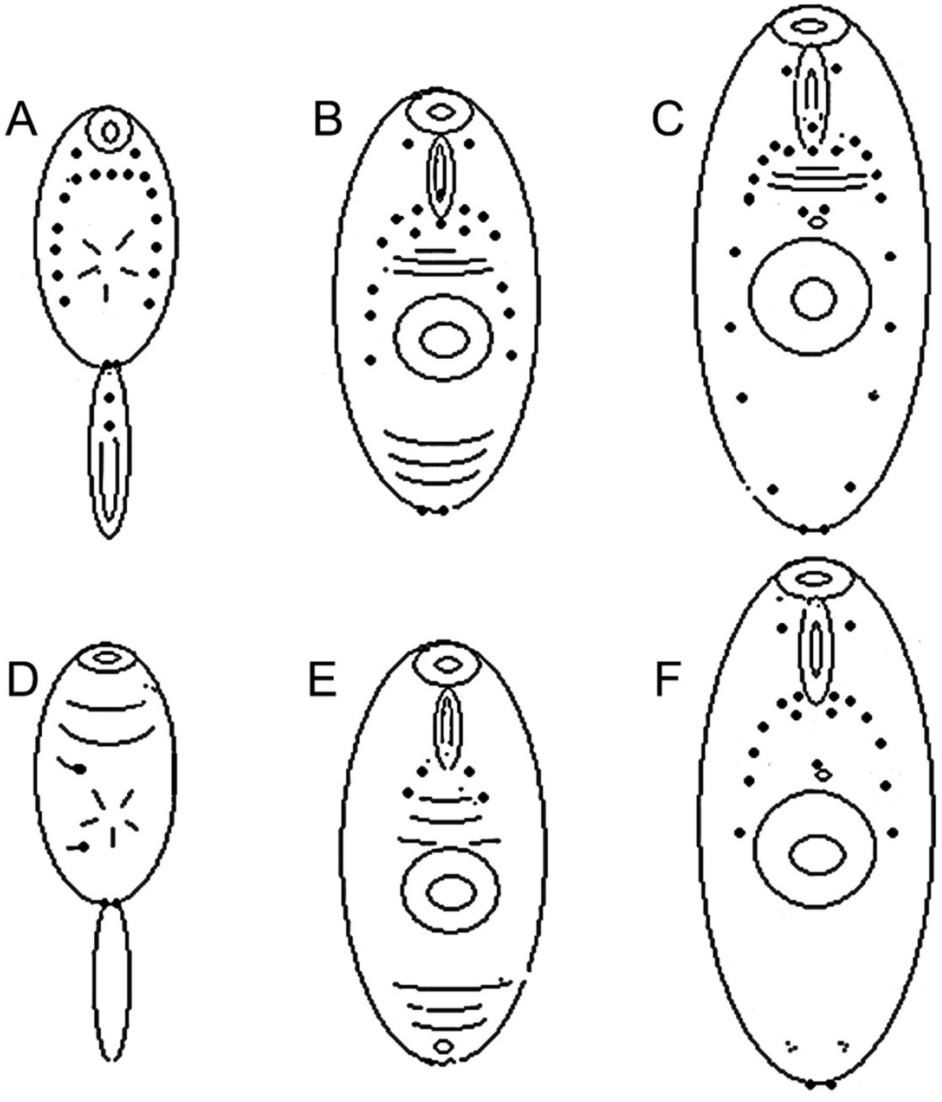

Fig. 18. Schematic drawing showing the number and pattern of 5-HT IR neurones in cercaria (**A**), metacercaria (**B**) and adult (**C**) *Opisthorchis felineus*. Schematic drawing showing the pattern of FMRFamide IR neurones in cercaria (**D**), metacercaria (**E**) and adult (**F**) *O. felineus*

O. felineus are similar to the cholinergic cords described from adult *O. felineus* by Joffe *et al*. (1988). The distribution patterns of peptidergic and cholinergic pathways often overlap (Halton and Maule 2004).

The ventral sucker

In cercariae of *O. felineus*, no ventral sucker was observed. This in accordance with observations made by Be'er (2005) and holds true also for cercariae of *O. viverrini* (Be'er 2005). Generally the ventral sucker and its neuromuscular system are well-developed in cercariae (see Terenina and Gustafsson 2003b, Šebelová *et al*. 2004, Terenina *et al*. 2006). In the free-living flatworm *Girardia tigrina*, the development of the neuromuscular system in the regenerating pharynx was followed during 6 days (Kreshchenko *et al*. 1999). Antisera raised against 5-HT and the native planarian neuropeptide GYIR-Famide, which belongs to the FaRPs neuropeptides (see Halton 2004), were used. A striking parallelism between the appearance of the GYIRFamide IR nerve fibres and muscle fibres stained with TRITC conjugated phalloidin was observed. The peptidergic neurones and fibres and the muscle fibres developed side by side, adjacent to each other. The 5-HT IR neurones and fibres developed later and *de novo* in the tip of the regenerating pharynx. The results suggest that the contact between the muscle fibres and the peptidergic nerves is essential for the development and function of the pharynx. The situation resembles that in *O. felineus* where many FMR-Famide IR nerve fibres, but no 5-HT IR fibres, extend from the MCs towards the developing ventral sucker.

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