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HELMINTHOLOGY - ORIGINAL PAPER

Architecture of the nervous system in metacercariae of *Diplostomum pseudospathaceum* Niewiadomska, 1984 (Digenea)

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Abstract

The development of metacercariae of *Diplostomum pseudospathaceum* Niewiadomska, 1984 is accompanied by profound morphological transformations often characterized as metamorphosis, which makes these metacercariae an interesting case for studying the morphogenesis of the digenean nervous system. Although the nervous system of *D. pseudospathaceum* is one of the most extensively studied among digeneans, there are still gaps in our knowledge regarding the distribution patterns of some neuroactive substances, most notably neuropeptides. The present study addresses these gaps by studying pre-infective metacercariae of *D. pseudospathaceum* using immunochemical staining and confocal microscopy to characterize the distribution patterns of serotonin (5-HT) and two major groups of flatworm neuropeptides, FMRFamide-related (FaRPs) and substance P-related (SP) peptides. The general morphology of the nervous system was examined with antibodies to alpha-tubulin. The nervous system of the metacercariae was shown to conform to the most common morphology of the nervous system in the hermaphroditic generation, with three pairs of posterior nerve cords and four pairs of anterior nerves. The patterns of FaRP- and 5-HT immunoreactivity (IR) were similar to those revealed in earlier studies by cholinesterase activity, which is in accordance with the known role of these neurotransmitters in controlling muscle activity in flatworms. The SP-IR nervous system was significantly different and consisted of mostly bipolar cells presumably acting as mechanoreceptors. The architecture of the nervous system in *D. pseudospathaceum* metacercariae is discussed in comparison to that in cercariae of *D. pseudospathaceum* and metacercariae of related digenean species.

Keywords Nervous system · Metacercariae · 5-HT · Substance P · FMRFamide · Alpha-tubulin

Introduction

In the digenean life cycle, metacercaria is an intermediate larval form between free-living cercariae and adult worms, which is generally viewed as a metabolically dormant stage. Metacercariae, however, are not always a quiescent, encysted stage; in some digenean groups, most notably in Diplostomidae Poirier, 1886, metacercariae remain morphogenetically and metabolically active for a substantial part of their life in the second intermediate host. Diplostomid metacercariae begin their development with penetration into the second intermediate host and extensive migration of the diplostomula to their specific sites (see, for example, Shigin 1986; Höglund 1991; Matisz and Goater 2010; Matisz et al. 2010). Further development is accompanied by a significant growth of the larvae and by a series of profound morphogenetic transformations often described as metamorphosis (Shigin 1986; Ginetsinskaya 1988; Galaktionov and Dobrovolskij 2003). These processes have been studied the most in members of the genera *Diplostomum* and *Ornithodiplostomum* (Niewiadomska and Moczoń 1984; Shigin 1986; Podvyaznaya 1999, 2013; Conn et al. 2008; Matisz and Goater 2010; Matisz et al. 2010; Petrov and Podvyaznaya 2016a, 2016b). The studies have shown that over the course of metacercarial development some transitory larval structures adapted for penetration and migration undergo degeneration. These structures include penetration glands and cercarial spines, of which the largest and most densely set form spination of the protrusible proboscis (Shigin 1986; Conn et al. 2008; Petrov and Podvyaznaya 2016b). Other

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structures may become transformed into those of the adult worms: the anterior organ into the oral sucker and the glandular cercarial intestine into typical digenean caeca (Podvyaznaya 2013; Petrov and Podvyaznaya 2016a). Diplostomid metacercariae begin to develop specialized musculo-glandular organs (lappets and the holdfast) that become fully functional in adult worms, and the primordium of the hindbody, which in adult worms contains reproductive organs. Both in *Diplostomum* and *Ornithodiplostomum*, substantial developmental changes were observed in the tegument (Podvyaznaya 1999; Goater et al. 2005; Conn et al. 2008), suggesting that it takes part in food absorption. In *Diplostomum*, massive morphogenetic transformations were shown for the excretory (Komiya 1938; Shigin 1986; Niewiadomska 1996; Niewiadomska and Czubaj 2000) and muscular systems (Petrov and Podvyaznaya 2016a). There is evidence (Niewiadomska and Moczoń 1984; Niewiadomska et al. 1996) that the nervous system also undergoes extensive changes manifested in the gradual complexification of the initially simple cercarial nervous system.

Given the integrative role of the nervous system, its detailed study in diplostomid metacercariae may shed more light on the morphogenetic processes and the metabolic activity during metacercarial development in this group. The present study examined the nervous system of pre-infective metacercariae of *Diplostomum pseudospathaceum* Niewiadomska, 1984, very common pathogenic parasites of many species of freshwater Palaearctic fish (Niewiadomska 1996). The architecture of the nervous system and the distribution patterns of neuroactive substances were studied by immunochemical staining with antibodies to alpha-tubulin, serotonin (5-HT), FMRFamide, and substance P (SP). The nervous system of *D. pseudospathaceum* is one of the most extensively studied among digeneans (Niewiadomska and Moczoń 1982, 1984, 1987, 1990; Czubaj and Niewiadomska 1990, 1991, 1996; Niewiadomska and Czubaj 1996; Niewiadomska et al. 1996; Tolstenkov et al. 2012), providing ample morphological data with which to compare the results of the present study. Since immunochemistry and confocal microscopy offer advantages in increased specificity of neuronal staining and improved understanding of the three-dimensional architecture of the nervous system, the use of these methods in the present study allowed us to expand on the findings of the earlier studies, which employed primarily histochemical and electron-microscopy methods. Another purpose of the present study was to bridge the existing gaps in the knowledge of the nervous system in *D. pseudospathaceum*, one of which is the paucity of information on the distribution of neuroactive peptides. Although the neurobiological studies in flatworms have traditionally been focused on classical neurotransmitters, there is now substantial evidence that neuropeptides play a pivotal, or even central, role in the biology of these animals (Gustafsson et al. 2002; Halton and Maule 2004; McVeigh et al. 2005). Flatworms possess a variety of neuropeptides that were implicated in controlling muscular contractions, sensory functions, development, and reproduction (Gustafsson et al. 2002; Halton and Maule 2004). The present study examined in detail the distribution patterns of two major groups of flatworm neuropeptides, FMRFamide-related peptides (FaRPs) and SP-related peptides. While FaRPs in flatworms were demonstrated to be myoexcitatory (Halton and Maule 2004; Ribeiro et al. 2005), the role of SP-related peptides is much less studied and comparison of their distribution with that of other neuroactive substances may provide further insight into their possible function in digeneans.

Material and methods

Metacercariae of *D. pseudospathaceum* were extracted from eye lenses of the common roach *Rutilus rutilus* (*Linnaeus*, 1758) caught in Lake Pertozero in the outskirts of Petrozavodsk (62° 10′N, 33° 58′E). Species and the developmental stage of the metacercariae were identified as described in Petrov and Podvyaznaya (2016a). All worms selected for this study were late-stage metacercariae, which was evidenced by the presence of well-developed holdfast and lappets, but they were not infective metacercariae, because the definitive tegumental spination was not fully formed. These metacercariae corresponded in their morphology to the larvae aged 22 days post-infection in Niewiadomska and Moczoń (1984) or approaching 20 days post-infection in Petrov and Podvyaznaya (2016a); in the following text, they are referred to as pre-infective. Metacercariae were fixed for 15–17 h with 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS), and were kept in 0.01 M PBS containing 0.1% (*w*/*v*) sodium azide. The worms were permeabilized for 2–4 h with 0.25% Triton X-100 in 0.01 M PBS (Tr-PBS) and blocked with Image-iT FX Signal Enhancer (Molecular Probes, Eugene, OR, USA) for 1 h. Blocked metacercariae were rinsed 4 × 15 min in Tr-PBS, incubated for 10–12 h in FMRFamide (1: 600; Abcam, Cambridge, MA, USA), 5-HT (1: 600; Sigma-Aldrich, St. Louis, MO, USA), or substance P (1:400; Immunostar, Hudson, WI, USA) antibodies, rinsed again 4 × 15 min in Tr-PBs and incubated for 5 h in Chromeo 488-conjugated secondary antibodies (1: 300) (Abcam, Cambridge, UK). For alpha-tubulin, the metacercariae were blocked and rinsed as described above and then were incubated for 8–10 h in fluorescein isothiocyanate (FITC)-conjugated alpha-tubulin (1:250; Sigma-Aldrich, St. Louis, MO, USA) antibodies. After incubation, the worms were rinsed in Tr-PBS for 15 min and stained 2–3 h with tetramethylrhodamine B isothiocyanate (TRITC)-conjugated phalloidin (1:150, Sigma-Aldrich, St. Louis, MO, USA), then rinsed once in 0.01 M PBS, and mounted on slides

with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA). Staining specificity was checked by omitting primary antibodies. Confocal images were collected on a Leica TCS SP5 confocal laser scanning microscope. All confocal images were presented as maximum intensity projections of the *Z*-stacks.

Results

General morphology of the nervous system The brain is situated at the level of the esophagus and is composed of a pair of cerebral ganglia (*cg*; Figs. 1b–d and 3a, b, d) connected by a dorsal, supraesophageal commissure (*se*; Figs. 1b, d, 2c, and 4a, b). The metacercariae have three pairs of longitudinal cords in the ventral, lateral, and dorsal positions. The ventral cords (*vc*; Figs. 1a–c, 2a, c, 3a, b, d, g, and 4a) are by far the most prominent; arising from the ventrolateral sides of the cerebral ganglia, they extend laterally, then turn posteriorly, and run longitudinally to the posterior end of the body, where they are connected by a commissure (*pcv*; Figs. 2a, e, 3a, g, and 4a). Extending from this commissure is a pair of posterior nerves (*vh*; Figs. 2a and 3a–g) that run into the hindbody primordium to end in a nerve ring (*re*; Fig. 3c) at the tip of the primordium around the excretory pore. A pair of nerves (*an*; Fig. 2a, e) arise medially from the ventral cords to terminate in a nerve ring (*ar*; Figs. 2a, e and 3a, b, f) around the

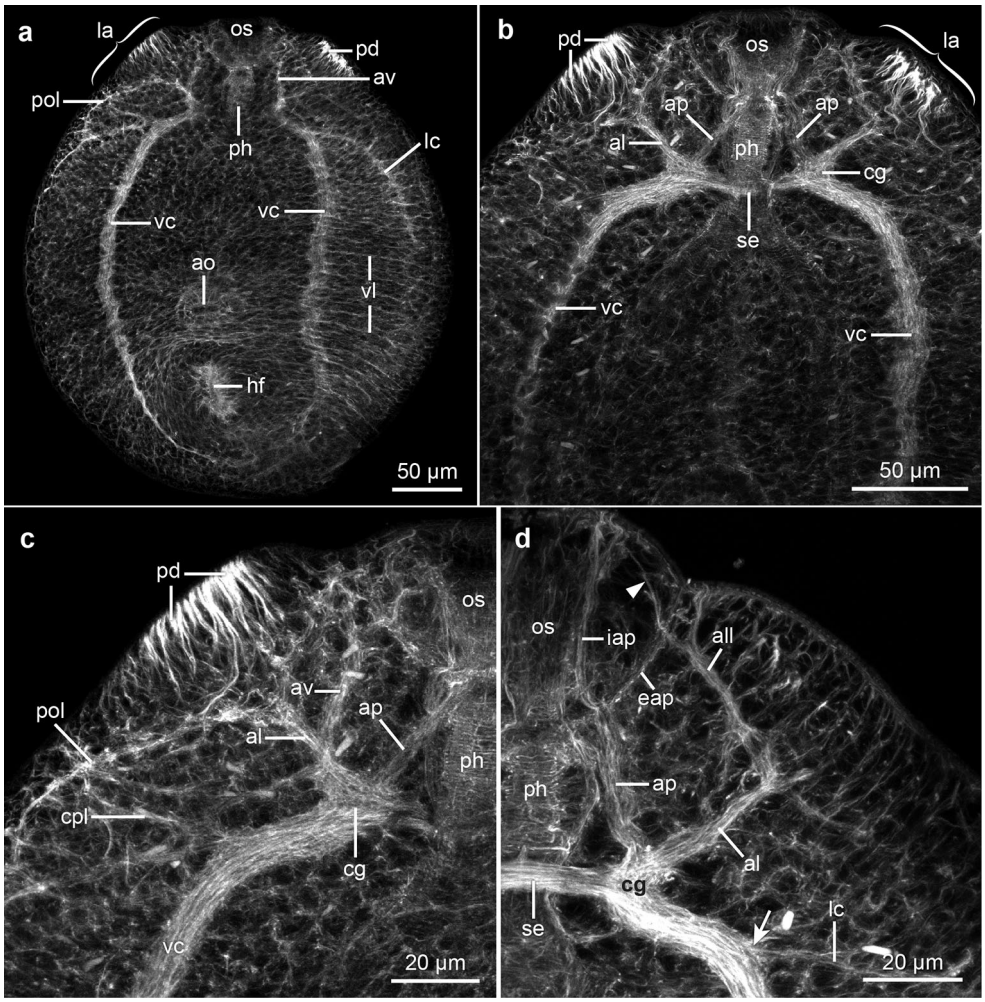

Fig. 1 Confocal images of the nervous system of *Diplostomum pseudospathaceum* stained with antibodies to alpha-tubulin. **a** General view of the body close to the ventral side. **b** Anterior half of the forebody. **c**, **d** Anterior end of the forebody showing the anterior nerves and their branches. Arrow indicates the point of origin of a lateral cord. Arrowhead shows the anterolateral branch of a lateral anterior nerve inside the wall of the oral sucker. al lateral anterior nerve, all anterolateral branch of lateral anterior nerve, ao acetabular opening, ap pharyngeal anterior nerve, av ventral anterior nerve, cg cerebral ganglion, cpl commissure of posterolateral branch of lateral anterior nerve, eap external branch of pharyngeal anterior nerve, hf holdfast, iap internal branch of pharyngeal anterior nerve, la lappet, lc lateral nerve cord, os oral sucker, pd projections of lateral anterior nerve in lappet, ph pharynx, pol posterolateral branch of lateral anterior nerve, se supraesophageal cerebral commissure, vc ventral nerve cord, vl commissures between ventral and lateral cords

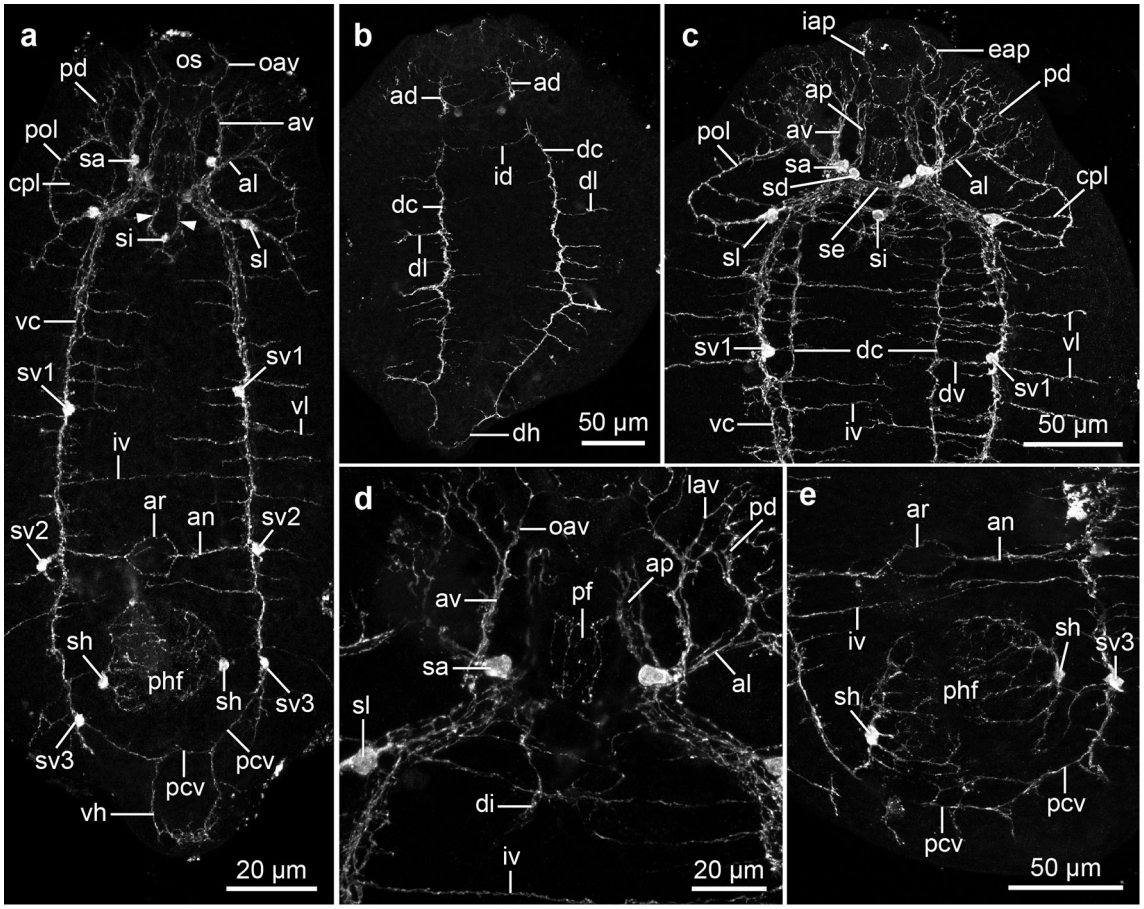

Fig. 2 Confocal images of the 5-HT-IR nervous system of *Diplostomum pseudospathaceum*. **a** General view of the body close to the ventral side. Arrowheads show a pair of neurites of the intestinal nerve cell (*si*) extending into the brain. **b** General view of the body close to the dorsal side. **c** Anterior half of the body. **d** Anterior end of the body showing the anterior nerves and the nerve plexus in the digestive tract. **e** Posterior end of the body showing the innervation of the acetabulum and the holdfast. ad dorsal anterior nerve, al lateral anterior nerve, an acetabular nerve, ap pharyngeal anterior nerve, ar acetabular nerve ring, av ventral anterior nerve, cpl commissure of posterolateral branch of lateral anterior nerve, dc dorsal nerve cord, dh dorsal longitudinal nerve in hindbody primordium, di dorsal intestinal nerve, dl commissures between dorsal and lateral cords, eap external branch of pharyngeal anterior nerve, iap internal branch of pharyngeal anterior nerve, id interconnecting commissures of dorsal cords, iv interconnecting commissures of ventral cords, lav lappet branch of ventral anterior nerve, oav oral branch of ventral anterior nerve, os oral sucker, pcv posterior commissure of ventral cords, pd projections of lateral anterior nerve in lappet, pf nerve plexus of pharynx, phf nerve plexus of holdfast, pol posterolateral branch of lateral anterior nerve, sa anterior cerebral 5-HT-IR cells, sd dorsal 5-HT-IR cells in cerebral ganglia, se supraesophageal cerebral commissure, sh 5-HT-IR cell bodies in holdfast, si 5-HT-IR cell bodies in intestine, sl lateral cerebral 5-HT-IR cells, sv1–3 5-HT-IR cell bodies of ventral cord, vc ventral nerve cord, vh ventral longitudinal nerve in hindbody primordium, vl commissures between ventral and lateral cords

opening of the acetabulum. There are several other nerves that connect the ventral cords and the nerve ring of the acetabulum, but they are very thin and appear indistinct in most specimens. The lateral cords (*lc*; Figs. 1a, d and 3a, g) originate from the ventrolateral sides of the brain together with the ventral cords and both cords initially run as a single bundle, but at the point where the ventral cords bend posteriorly (*arrow*; Fig. 1d), the lateral cords diverge from them extending in the lateral direction and then turn to run longitudinally throughout the forebody. The dorsal cords (*dc*; Figs. 2b, c and 3c, e) extend dorsally from the cerebral ganglia, then bend posteriorly, and run longitudinally to the end of the forebody, where they merge with the lateral cords. The merged cords (*dh*; Figs. 2b and 3c) pass into the hindbody primordium and end at the nerve ring of the excretory pore (*re*; Fig. 3c). All three pairs of cords and the two longitudinal nerves in the hindbody primordium are joined by closely set, regular commissures, which tend to anastomose with each other at the lateral body sides (*arrowheads*; Fig. 3c). The commissures link the lateral cords with the ventral (*vl*; Figs. 1a, 2a, c, and 3a) and dorsal cords (*dl*; Figs. 2b and 3c, e, g), interconnect the contralateral ventral (*iv*; Figs. 2a, c–e and 3a) and dorsal (*id*; Figs. 2b and 3c, e) cords, and there are also commissures directly connecting the ventral and dorsal cords, which can only be clearly seen by 5-HT IR (*dv*; Fig. 2c). There are 22 commissures dorsally and 26 ventrally (16 pre-acetabular and 10 post-acetabular); the

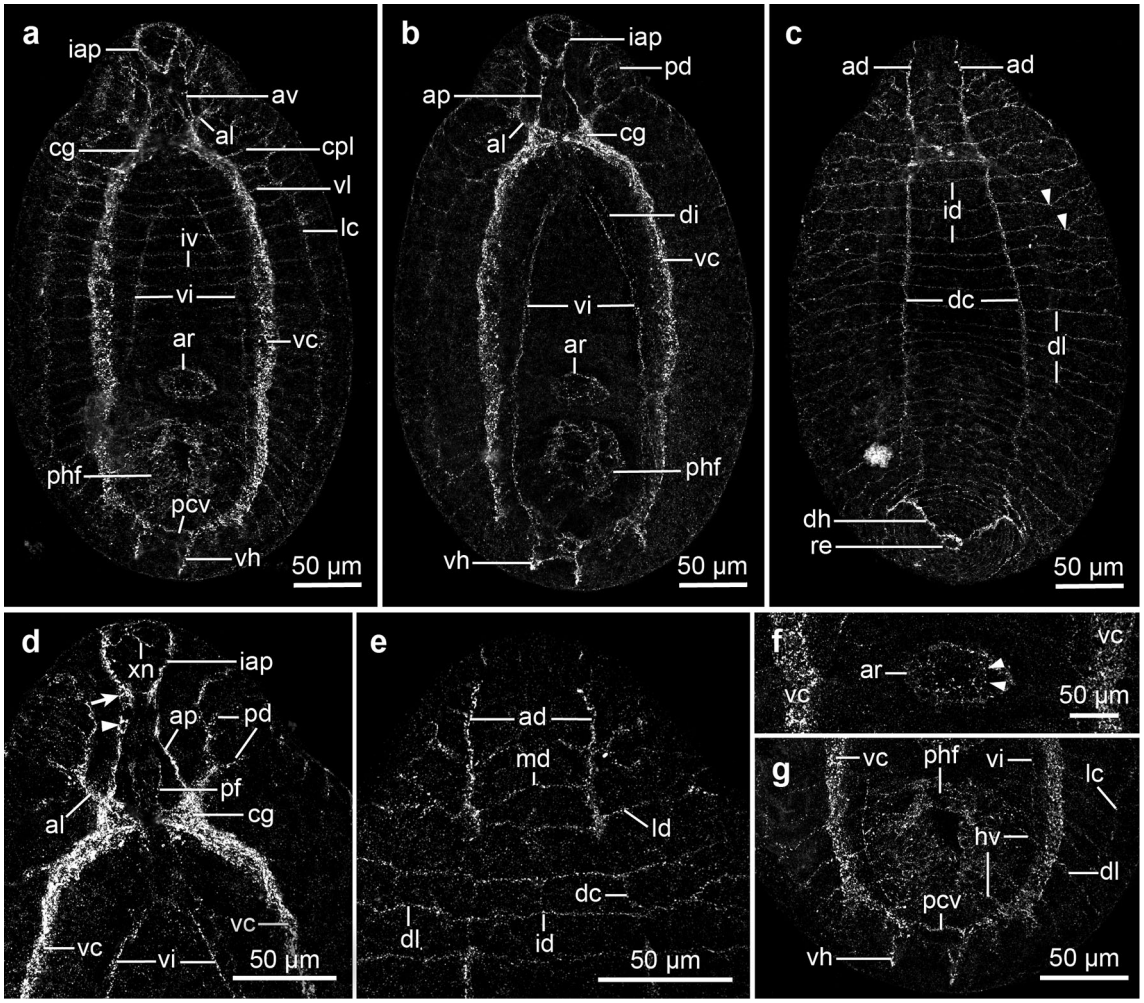

Fig. 3 Confocal images of the FaRP-IR nervous system of *Diplostomum pseudospathaceum*. **a**, **b** General view of the body close to the ventral side. **c** General view of the body close to the dorsal side. **d** Anterior end of the body close to the ventral side showing the pharyngeal and lateral anterior nerves and their branches. **e** Anterior end of the body close to the dorsal side showing the dorsal anterior nerves. **f** Ventral view of the acetabulum. Arrowheads indicate the FaRP-IR granules arranged in a circle around the acetabular opening. **g** Posterior end of the forebody showing the innervation of the holdfast. ad dorsal anterior nerve, al lateral anterior nerve, ar acetabular nerve ring, ap pharyngeal anterior nerve, av ventral anterior nerve, cg cerebral ganglion, cpl commissure of posterolateral branch of lateral anterior nerve, dc dorsal nerve cord, dh dorsal longitudinal nerve in hindbody primordium, di dorsal intestinal nerve, dl commissures between dorsal and lateral cords, iap internal branch of pharyngeal anterior nerve, id interconnecting commissures of dorsal cords, iv interconnecting commissures of ventral cords, lc lateral nerve cord, ld lateral commissure of dorsal anterior nerve, md medial commissure of dorsal anterior nerve, pcv posterior commissure of ventral cords, pd projections of lateral anterior nerve in lappet, pf nerve plexus of pharynx, phf nerve plexus of holdfast, re nerve ring of excretory pore, vc ventral nerve cord, vh ventral longitudinal nerve in hindbody primordium, vi ventral intestinal nerve, vl commissures between ventral and lateral cords, xn X-shaped neurites in oral sucker

commissures in the hindbody primordium (Fig. 3c) are too closely spaced to be counted with certainty.

The anterior end of the forebody is innervated by four pairs of nerves: lateral anterior (*al*; Figs. 1b–d, 2a, c, d, and 3a, b, d), ventral anterior (*av*; Figs. 1a, c, 2a, c, d, 3a, and 4a), pharyngeal anterior (*ap*; Figs. 1b–d, 2c, d, and 3b, d), and dorsal anterior (*ad*; Figs. 2b and 3c, e). The lateral, ventral, and pharyngeal anterior nerves arise by a single root from the anterolateral corners of the cerebral ganglia. The lateral anterior nerves extend anterolaterally towards the lappets; reaching these organs, they split into several smaller nerves (*pd*; Figs. 1a–c, 2a, c, d and 3b, d) branching out inside the lappets. Near the lappets, the lateral nerve gives off two branches (posterolateral and anterolateral). The posterolateral branch (*pol*; Figs. 1c and 2a, c) runs posteriorly and forms a loop with the lateral nerve cord. This branch is connected with three transverse nerves (*cpl*; Figs. 1c, 2a, c, and 3a) to the ventral cord. The anterolateral branch (*all*; Fig. 1d) proceeds forward and enters the wall of the oral sucker (*arrowhead*; Fig. 1d), continuing to its tip. The pharyngeal anterior nerve runs forward along the pharynx and, approaching the oral sucker, divides into two smaller branches. One of these branches (*iap*; Figs. 1d, 2c, and 3a, b, d) enters the interior of the oral sucker, the other (*eap*; Figs. 1d and 2c) runs along its wall. The ventral anterior nerve first

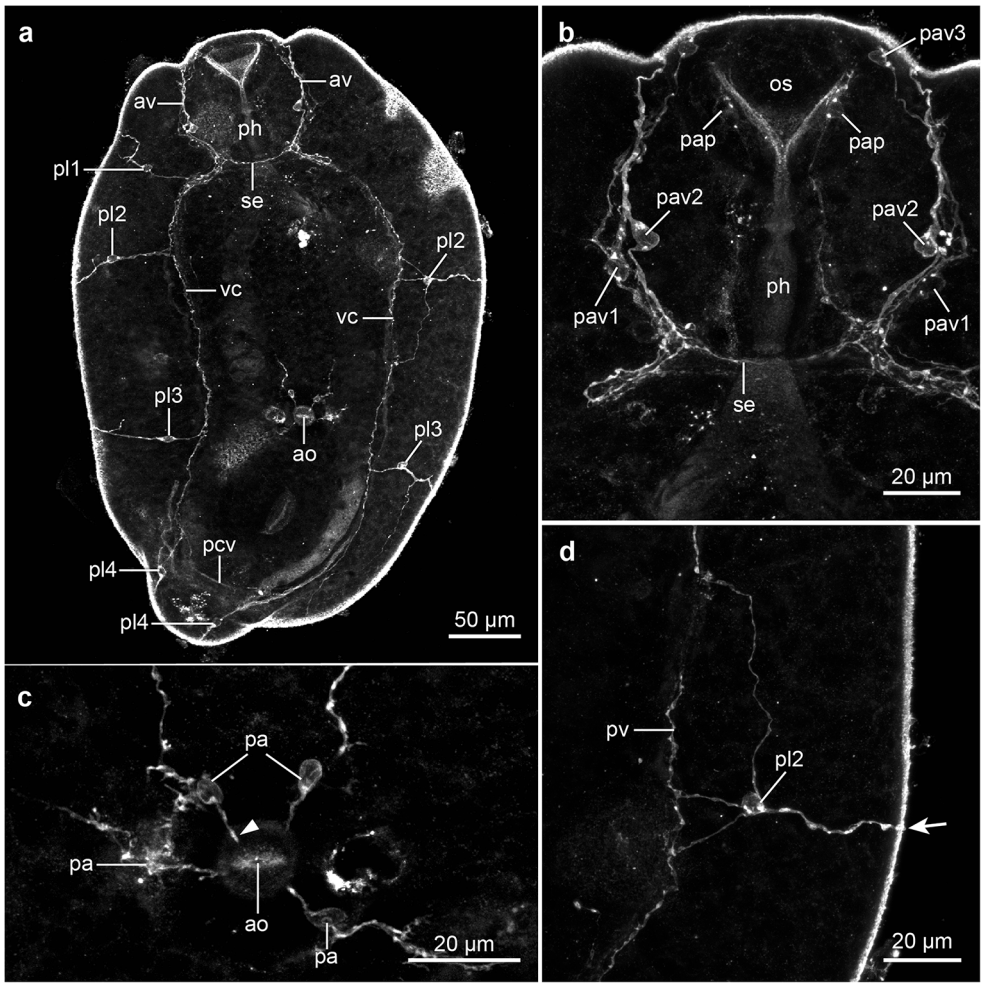

Fig. 4 Confocal images of the SP-IR nervous system of *Diplostomum pseudospathaceum*. **a** General view of the body close to the ventral side. **b** Anterior end of the body showing the nerve cells in the lateral anterior nerves. **c** Ventral view of the acetabulum showing nerve cells around the acetabular opening with dendrites ending in a large varicosity at the rim of the opening (*arrowhead*). **d** Lateral nerve cell (*pl2*) with axons reaching a ventral nerve cord and the dendrite ending in a small protrusion at the body surface (*arrowhead*). ao acetabular opening, av ventral anterior nerve, os oral sucker, pa SP-IR cells in acetabulum, pap SP-IR cells in pharyngeal anterior nerve, pav1–3 SP-IR cells in ventral anterior nerve, pcv posterior commissure of ventral cords, ph pharynx, pl1–4 lateral SP-IR cells, pv SP-IR neurites in ventral cord, se supraesophageal cerebral commissure, vc ventral nerve cord

courses ventrally, then turns forward, and supplies with its branches both the lappets (*lav*; Fig. 2d) and the rim of the oral sucker (*oav*; Fig. 2a, d). Some of the neurites of this nerve travel over the tip of the oral sucker, then turn back, and join the pharyngeal anterior nerve. The dorsal anterior nerves run dorsally from the cerebral ganglia, then continue forward to the anterior tip of the body. These nerves are interconnected by three medial commissures (*md*; Fig. 3e) and three lateral commissures (*ld*; Fig. 3e) connect them to the lateral anterior nerves.

The digestive tract is innervated by three pairs of thin nerves originating from the medial side of the cerebral ganglia. One pair passes anteriorly on either side of the pharynx where it forms a plexus (*pf*; Figs. 2d and 3d), the other two run posteriorly within the wall of each branch of the gut: one in the dorsal (*di*; Figs. 2d and 3b) and the other in the ventral wall (*vi*; Fig. 3a, b, d, g). The holdfast (*hf*; Fig. 1a) is supplied by seven nerves (*hv*; Fig. 3g) from the ventral cords; these nerves pass into the nerve plexus (*phf*; Figs. 2a, e and 3a, b, g) that lines the walls of the holdfast.

Serotonin 5-HT-IR neurites (Fig. 2) are present in the brain, in the ventral and dorsal nerve cords, and in all anterior nerves and their branches, but absent in the lateral cords, except in their anterior portions that form a loop with the posterior branch of the lateral anterior nerve. The 5-HT IR reveals most commissures of the nerve cords, including those that connect the ventral and the dorsal cords (*dv*; Fig. 2c), which are not visualized by the other immunostains used. The 5-HT-IR nervous system includes one unpaired and 14 paired cell bodies; all of these cells appear to be multipolar. Anteriorly, one pair (*sd*; Fig. 2c) is situated dorsally in the cerebral ganglia flanking the dorsal commissure, another (*sa*; Fig. 2a, c, d) sits at the roots of the lateral anterior nerves, and the third (*sl*; Fig. 2a, c, d) is located at the origin of the lateral nerve cord. There are three pairs of cell bodies along the ventral cords: the anterior pair (*sv1*; Fig. 2a, c) is situated about one third of the cord's length from the brain, the middle pair (*sv2*; Fig. 2a) is at the level of the acetabulum, and the posterior pair (*sv3*; Fig. 2a, e) is at the level of the holdfast. One of the neurites of each of these cells extends towards the dorsal nerve cord. The middle pair also sends neurites medially (*an*; Fig. 2a, e) to form a nerve ring (*ar*; Fig. 2a, e) in the ventral wall of the acetabulum, which in most specimens has a distinct hexagonal shape (Fig. 2a). The holdfast is flanked with a pair of large cell bodies (*sh*;

Fig. 2a, e) that project three branching neurites into the nerve net in the wall of the holdfast and one neurite into the ventral cord. The unpaired cell body (*si*; Fig. 2a, c) is located in the dorsal wall of the intestine at the bifurcation of the gut branches and sends two ramifying neurites along their dorsal wall (*di*; Fig. 2d) and a pair of anterior neurites (*arrowheads*; Fig. 2a) into the supraesophageal commissure. This cell forms a nerve net over the wall of the esophagus and a finer plexus (*pf*; Fig. 2d) in the pharynx.

FaRPs FaRP IR (Fig. 3) is present in the brain, in the anterior nerves and their branches, in the intestinal nerves, in all nerve cords, and throughout their commissures both in the forebody and in the hindbody primordium. The pharyngeal anterior nerves project FaRP-IR neurites (*xn*; Fig. 3d) over the dorsal wall of the oral sucker showing an X-shaped pattern consistently present in all the individuals studied. FaRP IR also forms a nerve ring (*ar*; Fig. 3f) in the ventral wall of the acetabulum, and there is another ring of disjointed granules (*arrowheads*; Fig. 3f) around the rim of the acetabular opening. In the wall of the holdfast, FaRP-IR neurites appear to build an irregular plexus (*phf*; Fig. 3a, b, g) connected to the ventral cords with seven transverse nerves (*hv*; Fig. 3g). In general, FaRP immunostaining produces a more patchy and punctate pattern than the other antibodies used and individual FaRP-IR neurites and cell bodies are difficult to differentiate with confidence. The only cell bodies that can be tentatively identified are two pairs of somata in the pharyngeal anterior nerve: one pair (*arrow*; Fig. 3d) is located in the wall of the oral sucker and the other (*arrowhead*; Fig. 3d) immediately posterior to the sucker.

Substance P SP-related peptides (Fig. 4) are consistently present both in the somata of the nerve cells and along the length of their major neurites allowing the overall cell morphology to be seen for most of the SP-IR neurons. SP-IR neurites extend throughout the ventral nerve cords and in the ventral and pharyngeal anterior nerves, while the lateral and dorsal cords show no detectable SP immunoreactivity. The only commissures that have SP-IR neurites are the dorsal cerebral commissure (*se*; Fig. 4a, b) and the posterior commissure (*pcv*; Fig. 4a) connecting the ventral cords. The ventral anterior nerves (*av*; Fig. 4a) contain three bipolar cells: two with large somata in the proximal portion of the nerve (*pav1–2*; Fig. 4b) and the third (*pav3*; Fig. 4b) inside the muscular wall of the oral sucker. The pharyngeal anterior nerve contains one small bipolar cell with the soma located in the wall of the sucker (*pap*; Fig. 4b). All the cells of the anterior nerves have one neurite reaching into the cerebral ganglia and the other ending in a terminal varicosity at the surface of the oral sucker.

The somata of seven large SP-IR neurons (*pl1–4*; Fig. 4a, d) lie laterally on the ventral side of the body; some of these cells appear bipolar, while others are multipolar (*pl2*; Fig. 4d). One or several neurites of each of these cells extends medially and continue inside the ventral nerve cords, the opposite neurite (dendrite) runs laterally to the integument to terminate in a small protrusion at the body surface (*arrow*; Fig. 4d). The anterior-most of these cells (*pl1*; Fig. 4a) appears unpaired in all three individuals studied and is located on the left side of the body at the anterior end of the nerve cord; the dendrite of this neuron ends at the surface immediately posterior to the lappet. The remaining six neurons are paired: the first pair (*pl2*; Fig. 4a, d) is located about 1/3 and the second (*pl3*; Fig. 4a) about 2/3 of the length from the anterior end of the body, the posterior-most pair (*pl4*; Fig. 4a) is positioned at the posterior end of the body, and its dendrites reach the surface near the end of the hindbody. The opening of the acetabulum is surrounded by a group of four bipolar cells (*pa*; Fig. 4c), with the somata situated just outside the sucker and the dendrites ending in a large varicosity at the rim of the acetabular opening (*arrowhead*; Fig. 4c). Although there are four of these neurons in all three individual studied, they are spaced around the opening in such a way as to suggest that there are in fact six cells arranged in a hexagonal pattern.

Discussion

Metacercariae of *D. pseudospathaceum* have been studied earlier for the distribution patterns of acetylcholine (Niewiadomska and Moczoń 1984; Niewiadomska et al. 1996) and catecholamines (Niewiadomska et al. 1996), and there have also been several studies of metacercariae in other *Diplostomum* species for 5-HT, acetylcholine, and a range of neuropeptides including FaRP- and SP-related peptides (Barton et al. 1993; Niewiadomska et al. 1993; Solis-Soto and De Jong-Brink 1995). Niewiadomska and Moczoń (1984) have provided the most detailed description of the cholinergic nervous system across several stages of metacercariae including the pre-infective stage of about the same age as that used in our study (22-day-old metacercariae in Niewiadomska and Moczoń 1984). The distribution of the cholinergic nervous system agrees in most details with the general morphology of the nervous system as revealed by alpha-tubulin IR in our study, with only a few minor differences. The cholinesterase activity in Niewiadomska and Moczoń (1984) showed several equally thick nerves innervating the acetabulum, while in our study under alpha-tubulin staining one of these nerves was significantly more prominent than the others and 5-HT IR was present in only one acetabular nerve. Niewiadomska and Moczoń (1984) described connectives between the dorsal anterior nerves and the dorsal cords, but in our study these connectives were not visualized by any of the four neural markers used. The presence of the connectives may be an

artifact caused by the roots of the dorsal anterior nerves located in close proximity to the dorsal cords.

Serotonin In *Diplostomum*, the serotonergic nervous system has previously been characterized for the cercariae of *D. pseudospathaceum* (Tolstenkov et al. 2012) and a closely related *D. spathaceum* (Rudolphi, 1819) (Gustafsson 1988) and for the metacercariae of another closely related species, *Diplostomum paracaudatum* (Iles, 1959) (Niewiadomska et al. 1993). Like pre-infective metacercariae in our study, cercariae of *D. pseudospathaceum* have three pairs of neurons in the cerebral ganglia, but only two pairs of cells along the ventral cords compared to the three pairs in pre-infective metacercariae. It seems likely from the arrangement of the cells that the third (posterior-most) pair of neurons along the ventral cords becomes differentiated over the course of metacercarial development in the post-acetabular area with the posterior growth of the forebody. The unpaired cell body in the intestinal wall of pre-infective metacercariae was not observed in cercariae. Apparently, this neuron is formed later in development, as the glandular cercarial gut is transformed into the functional gut of metacercariae. Another pair of cells added later in development are the neurons that innervate the holdfast.

Cercariae of *D. spathaceum* are similar to those of *D. pseudospathaceum* in having two pairs of cells along the ventral cords, but differ in having five pairs of neurons in the cerebral ganglia (Gustafsson 1988). However, the comparison of the topographical positions of neurons between these species suggests that two posterior pairs of cerebral neurons in *D. spathaceum* may actually be the anterior nerve cells of the ventral cords. If this interpretation is correct, the number of cerebral neurons is the same in both species (three pairs), but the number of cells along the ventral cords in cercariae of *D. spathaceum* is twice that in *D. pseudospathaceum* (four vs. two pairs).

In *D. paracaudatum,* 5-HT IR has been studied in metacercariae of about the same age as those in our study (Niewiadomska et al. 1993) offering the opportunity for between-species comparison across the same development stages. *Diplostomum paracaudatum* has four pairs of cells in the brain (two dorsal, one medial, and one lateral) and six pairs along the ventral cords. One pair of neurons in *D. paracaudatum* is associated with the holdfast as in pre-infective metacercariae of *D. pseudospathaceum*, but there is no unpaired intestinal cell. The comparison of cell arrangement suggests that the medial and lateral neurons of *D. paracaudatum* are homologous to sl cells and slc cells of *D. pseudospathaceum*, respectively (*sl* and *slc* in Fig. 2), and that the two dorsal cells in *D. paracaudatum* correspond to one dorsal cell in *D. pseudospathaceum* (*sd* in Fig. 2). The condition with three pairs of neurons in the brain appears to be plesiomorphic for Diplostomidae as the same number was found outside this family in a closely related family Strigeidae Railliet, 1919: in cercariae of *Cotylurus szidati* Lutz, 1928 (Tolstenkov et al. 2012) and metacercariae of *Cotylurus* sp. (Terenina and Gustafsson 2014), suggesting that the dorsal cells were duplicated in the lineage leading to *D. paracaudatum*.

The number of 5-HT-IR cells along the ventral cords in *Cotylurus* Szidat, 1928 is two pairs in cercariae (Tolstenkov et al. 2012) and six pairs in metacercariae (Terenina and Gustafsson 2014), and therefore, members of Strigeidae and Diplostomidae may have either two (cercariae of *D. pseudospathaceum* and *C. szidati*), three (metacercariae of *D. pseudospathaceum*), four (cercariae of *D. spathaceum*), or six (metacercariae of *D. paracaudatum* and *Cotylurus* sp.) pairs of cells associated with the ventral cords. In metacercariae with four or six pairs of cells, the cells usually cluster on either side of the body in groups of two (Gustafsson 1988; Niewiadomska et al. 1993), raising the possibility that these numbers have resulted from cell duplication events. It is therefore likely that two processes can take place concurrently in these digeneans in the course of cercarial and metacercarial development: addition of cells posteriorly along the ventral cords (from two to three pairs) and cell duplication (from two to four pairs or from three to six pairs). In *Cotylurus*, both processes seem to occur, raising the cell number along the ventral cords from two pairs in cercariae to six pairs in metacercariae. As six pairs of cells are present in metacercariae of both Strigeidae and Diplostomidae, this condition is likely to be plesiomorphic, with the three cell pairs in pre-infective metacercariae of *D. pseudospathaceum* originating by exclusion of cell duplication event from metacercarial development.

FaRPs The distribution of RFamide-IR cells in pre-infective metacercariae of *D. pseudospathaceum* is generally similar to that of acetylcholine (Niewiadomska and Moczoń 1984) and conforms to the patterns of FaRP IR found in cercariae of *D. pseudospathaceum* (Tolstenkov et al. 2012) and in metacercariae of other species of *Diplostomum* (Barton et al. 1993; Solis-Soto and De Jong-Brink 1995). Our results show the lack of any unequivocal immunoreactivity in the cell bodies, which is in sharp contrast with the other studies that revealed numerous neuronal somata, especially in the anterior sucker and along the ventral nerves (Solis-Soto and De Jong-Brink 1995). This apparent lack of immunoreactivity may be due to the patchiness of staining patterns in our study, which make cell bodies indistinct from neurites, or may reflect the actual distribution of FaRPs that could be significantly different depending on the morphogenetic status of the worm.

SP-related peptides In the present study, SP-IR elements were shown to consist primarily of bipolar neurons with their axons running inside the ventral cords or anterior nerves and their

dendrites extending towards the lateral surface of the body or the surface of the suckers. The morphology of these cells clearly suggests their role as sensory, probably contact, receptors, which agrees with the predominantly sensory function of substance P in mammals, where it is involved in pain, nociception, and stress mechanisms (see review in DeVane 2001). In flatworms, SP-related peptides have been identified in different turbellarian taxa and in all major parasitic groups including digeneans (see review in Fairweather and Halton 1991; Halton et al. 1994). In digeneans, SP-IR has been demonstrated in *Fasciola hepatica* (Linnaeus, 1758) (Magee et al. 1989), *Gorgoderina vitelliloba* (Olsson, 1876) (McKay et al. 1991), *Haplometra cylindracea* (Zeder, 1800) (McKay et al. 1990), *Schistosoma mansoni* Sambon, 1907 (Gustafsson 1987), an unidentified species of *Diplostomum* (Solis-Soto and De Jong-Brink 1995), and *Diplostomum spathaceum* (Barton et al. 1993).

The age of the metacercariae used in the two studies on substance P in *Diplostomum* species (Barton et al. 1993; Solis-Soto and De Jong-Brink 1995) was not specified, but judging from the general morphology and the presence of the holdfast and lappets, they were about the same age as the pre-infective metacercariae in our study. Both studies give only a brief overview of the SP-IR distribution, but, in general, their descriptions are consistent with our results: they showed prominent SP-IR in the ventral nerve cords and Solis-Soto and De Jong-Brink (1995) observed neurons similar in morphology and arrangement to the lateral sensory cells of *D. pseudospathaceum* (see Fig. 11 in Solis-Soto and De Jong-Brink 1995). In contrast with our results, Solis-Soto and De Jong-Brink (1995) reported that in *D. spathaceum* SP-IR was present in the holdfast, but absent in the acetabulum, while in *D. pseudospathaceum* SP-IR cells were shown in the acetabulum and no immunoreactivity was observed in the holdfast. Since *D. spathaceum* and *D. pseudospathaceum* are very closely related species, this inconsistency in staining patterns cannot be explained by taxonomic differences and is likely to be caused by the possible cross-reactivity of the antibodies with other tachykinins or by differences in physiological or morphogenetic status of the metacercariae.

Bipolar sensory cells connecting the nerve cords to the lateral body surface appear to be one of the characteristic elements of the SP-IR nervous system in flatworms. In Neodermata, these cells were observed in *Schistosoma mansoni* (Gustafsson 1987) and *Diphyllobothrium dendriticum* (Nitzsch, 1824) (Gustafsson et al. 1993) and neurons of similar morphology were described using histological techniques (Bettendorf 1897; Havet 1900). In free-living flatworms, Reuter (1994) observed bipolar cells at the anterior end of *Microstomum lineare* (Müller OF, 1773), but most SP-IR cells in these turbellarians appear to be unipolar.

A curious feature of SP-IR in our study was an asymmetry in staining patterns of lateral sensory cells and acetabular neurons. Although there is no reason to believe that these results reflect the actual asymmetry in the arrangement of SP-IR cells, the consistency of staining patterns across the specimens indicates that this phenomenon may be associated with the asynchronous development of the neurons.

Conclusion

The overall architecture of the nervous system in pre-infective metacercariae of *D. pseudospathaceum* conforms to the most common (and most likely plesiomorphic) morphology of the nervous system in the digenean hermaphroditic generation, with three pairs of posterior nerve cords and four pairs of anterior nerves (see reviews in Bullock and Horridge 1965; Biserova 2015). The patterns shown by FaRP and 5-HT IR are similar to those revealed by cholinesterase activity, which is much in line with the known role that these three neurotransmitters play in controlling muscle activity in flatworms (Halton and Maule 2004; Ribeiro et al. 2005). The comparison of the arrangement of 5-HT-IR neurons between cercarial and metacercarial stages of Diplostomidae and members of a closely related family Strigeidae suggests the possibility that metacercarial development in these families is accompanied by addition and duplication of cells along the ventral nerve cords and that the evolution within *Diplostomum* may be associated with cell duplication events in the brain.

The SP-ergic nervous system shows a markedly different distribution than 5-HT and FaRPs and consists of bipolar and multipolar cells presumably acting as mechanoreceptors. The arrangement of the SP-IR cells in *D. pseudospathaceum* indicates that SP-related peptides may function as true sensory neurotransmitters in digeneans.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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