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Confocal fluorescent study of the fish blood flukes: the serotonergic elements and ultrastructure of the nervous system of adult *Sanguinicola plehnae* (Digenea: Sanguinicolidae)

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Abstract: The first data on the neurochemical and ultrastructural organisation of the nervous system of the fish blood fluke, suckerless adult *Sanguinicola plehnae* Warren et Bullard in Warren, Poddubnaya, Zhokhov, Reyda, Choudhury et Bullard, 2023 (Digenea: Aporocotylidae) from the circulatory system of pike, *Esox lucius* Linnaeus are presented. Based on 5-HT-IP staining, the simple, uniformly developed orthogonal pattern of *S. plehnae* nervous system is revealed. The ventral and dorsal nerve cords originate from the brain lobes, but the lateral nerve cords originate from anterior nerves at the level of the large serotonergic neurons. In addition, several pairs of such large 5-HT-IP neurons (22–23.5 µm in diameter) are revealed along the ventral nerve cords. Unusual spindle-shaped 5-HT-IP perikarya (7.8–19.8 µm in diameter) are observed along each ventral and lateral nerve cords. The neuroblasts and developing neurons are seen between neurites in *S. plehnae* along with neuron somata scattered around neuropil periphery, evidencing the renewal of neuron somata population in adult digeneans. The morphological variability of both the orthogonal pattern and neuron somata and types of neurovesicles in adult digeneans are discussed.

Keywords: CNS topography, immunocytochemistry, 5-HT-IP, TRITC-phalloidin, SEM, TEM, neuropil, neurites, neuronal somata

Fish blood flukes infect the circulatory system of both marine and freshwater fishes and some of them pose serious threats to aquaculture (Bullard and Overstreet 2002). They represent an early branching and diverse digenean clade within the superfamily Schistosomatoidea (Olson et al. 2003, Pérez-Ponce de León and Hernández-Mena 2019). Five morphologically distinct lineages of fish blood flukes are known: the Chimaerohemecidae Yamaguti, 1971 infect Chondrichthyes; Acipensericolidae Warren et Bullard, 2023 infect Acipenseriformes; Sanguinicolidae Poche, 1926 infect freshwater Teleostei; Elopicolidae Warren et Bullard, 2023 infect Elopomorpha and Aporocotylidae Odhner, 1912 infect marine Teleostei (Warren and Bullard 2023).

Data on the anatomy and ultrastructure of their nervous system are limited to the study of non-neuronal supporting cells of the central nervous system in marine aporocotylid, *Aporocotyle simplex* Odhner, 1900 (Poddubnaya and Gibson 2020) and sensory receptors of *Sanguinicola inermis* Plehn, 1905 (Poddubnaya et al. 2020). In cercaria of *S. inermis* the distribution of neuroactive substances was investigated by McMichael-Phillips et al. (1996). Miniature species of the genus *Sanguinicola* Plehn, 1905 (1–3 mm long) are deprived of the oral and ventral suckers, have dorsoventrally flattened body, marginal spines are arranged in a single column, minute pore-like mouth, small pharynx, straight oesophagus, short 4–5 X-shaped caeca, testis with bilateral lobes, separate posterodorsal male and female genital pores (Poddubnaya et al. 2022, 2023, Warren et al. 2023).

The nervous system of the digeneans contains a variety of signalling molecules, including serotonin (5-hydroxytryptamine, 5-HT), an important molecule in metazoans that is involved in regulation of a variety of biological processes (Day and Maule 1999, Halton and Maule 2004, Okaty et al. 2019, Jones et al. 2020, Wan et al. 2020). The neurochemical organisation of adult digenean nervous system was investigated by Fairweather et al. (1987), Gustafsson (1987), Magee et al. (1989), McKay et al. (1990, 1991), Day et al. (1994a, b), Gustafsson et al. (2001), Stewart et al. (2003), Šebelová et al. (2004), Leksomboon et al. (2012a), Patocka et al. (2014), Kreshchenko et al. (2020), Nefedova et al. (2021), Denisova et al. (2024), Terenina et al. (2024). The innervations of musculature of the

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attachment, digestive and reproductive organs of digeneans by serotonergic nerve elements were investigated by Gustafsson et al. (2002), Šebelova et al. (2004) and Terenina et al. (2022a, 2024).

Few papers on digenean neurocytology in adults have been published for the species of the order Plagiorchiida La Rue, 1957, fasciolid *Fasciola hepatica* Linnaeus, 1758 (Sukhdeo et al. 1988), opisthorchiid *Opisthorchis viverrini* (Poirier, 1886) (Leksomboon et al. 2012b) and renicolid *Renicola parvicaudatus* (Stunkard et Shaw, 1931) (Denisova et al. 2024).

The aim of the present study was to perform the immunocytochemical demonstration of serotonin and detailed transmission electron microscopical (TEM) observation of the nervous system of fish blood flukes in freshwater teleost-infecting species, *Sanguinicola plehnae* Warren et Bullard in Warren, Poddubnaya, Zhokhov, Reyda, Choudhury et Bullard, 2023 from the branchial arteries of the pike, *Esox lucius* Linnaeus. Data on the nervous system organisation of fish blood flukes given in taxonomic descriptions are either absent or scanty (Smith 2002, Warren and Bullard 2023, Warren et al. 2023). Additional morphological characteristics may be suitable for establishing apomorphic traits for each lineage of fish blood flukes, including knowledge of the organisation of their nervous system.

MATERIALS AND METHODS

Seventeen adult specimens of *Sanguinicola plehnae* were collected from the *bulbus arteriosus* leading from the heart of naturally infected pikes *Esox lucius* (Esocidae) from the Upper Volga River Basin, Russia in October and December, 2022–2023. Twelve of ninety five pike sampled were collectively infected by seventeen specimens of *S. plehnae*.

Immunocytochemistry

For immunocytochemical investigation of the nervous system, eight alive worms were fixed in 4% paraformaldehyde (PFA, MP Biomedicals, Irvine, CA, USA) in 0.1 M phosphate buffer (PBS, pH 7.4, Sigma Aldrich, Merck, USA) at 4 °C for 12 hours. With the purpose of fixed *S. plehnae* conservation during transportation, the samples were transferred to PBS buffer with 10% sucrose (Sigma) and kept at 4 °C until stained. The 5-HT-immunopositive (5-HT-IP) nerve structures were identified using indirect immunocytochemical method. Preparations of the worms fixed in paraformaldehyde were incubated with PBS-T, containing 0.1 M PBS, 0.1% Triton X-100, 0.1% sodium azide and 0.1% BSA for 24 hours, and further with a rabbit anti-5-HT (Immunostar, Hudson, Wisconsin, USA, Product ID: 20080, RRID: AB_572263) primary antibodies in dilution of 1 : 500 for four days at 4 °C.

After washing in PBS-T, samples were transferred to the secondary fluorescently-labeled AlexaFluor488 immunoglobulines (goat anti-rabbit IgG (H+L), Abcam, USA, Cat No. ab150077, RRID: AB_2630356) for next four days, in dilution 1 : 400. After final washing in PBS worms were placed on slides and covered with a drop of 75% glycerol (Sigma) under cover slips. They were examined under microscope directly or additionally stained with phalloidin. All procedures were performed in the dark room at 4 °C. Controls included: (1) omitting the primary antibodies and (2) using non-immune serum instead of primary antiserum. Both controls were negative and indicate the absence of the staining. The measurements were performed randomly on the microscopic images obtained from the paraformaldehyde fixed preparations of the worms.

Histochemical staining

TRITC (tetramethylrhodamine B isothiocyanate)-labeled phalloidin (Sigma Aldrich, St. Louis, Missouri, USA, Cat No. P1951) was used for musculature staining. After immunostaining with anti-serotonin, specimens were stained with TRITC-phalloidin in dilution of 1 : 200. The duration of phalloidin staining was 6 hr at room temperature. Whole-mount samples were washed with PBS three times 5 min, and mounted in PBS/glycerol (1 : 9, Sigma) for investigation.

Confocal laser scanning microscopy

Samples were studied with confocal laser scanning microscope (CLSM) Leica TCS SP5 (Leica Microsystem, Wetzlar, Germany). The microphotographs presented are either a maximal projection of a total of 16 to 48 consequent optical sections reconstructed, or as an optical section (or a snapshot). The images were saved in the TIFF format. To create the illustrations, the Adobe Photoshop CS2 9.0 was used (Adobe Systems, Inc.).

Morphological parameters of serotonergic components were measured in different body regions on images obtained from the best staining preparations by a program AxioVisionRel 4.8.1.0. (Carl Zeiss Oberkochen, Germany). At least five measurements were performed for each value.

Transmission and scanning electron microscopy (TEM and SEM)

For electron microscopy, nine alive specimens of *S. plehnae* were fixed using 3% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2) for 20 days at 5 °C, rinsed three times for 20 min periods in the same buffer, and postfixed in 1% osmium tetroxide for 1 h. Fixed worms were dehydrated in a graded ethanol series, with a final change to absolute acetone. After fixation and dehydration for SEM, three specimens were critical-point-dried using a HCP-2 critical point dryer (Hitachi, Tokyo, Japan). Later the specimens were mounted on stubs, sputter-coated using FC 1600 Auto Fine Coater (JEOL Ltd., Tokyo, Japan) with gold-palladium and examined using a JEOL-JEM - 6510LV microscope at 15 kV.

For TEM, six specimens were embedded in a mixture of Araldit and Epon using the instructions provided by the Araldite/Embed-812 EM Embedding Kit (EMS) (Sigma Aldrich, Buenos Aires, Argentina). Ultrathin sections (50–90 nm in thickness) were cut on a Leica MZ6 ultramicrotome (Leica Microsystems, Wetzlar, Germany) mounted on formvar coated copper slots and stained in uranyl acetate and lead citrate before being examined with a JEOL JEM 1011 electron microscope (JEOL, Ltd., Tokyo, Japan) at 80 kV.

RESULTS

The phalloidin-fluorescence staining of adult *Sanguinicola plehnae*

The outer surface (distal tegumental cytoplasm) of the body is typically lined with two muscle layers running in circular and longitudinal directions, in addition to the diagonal

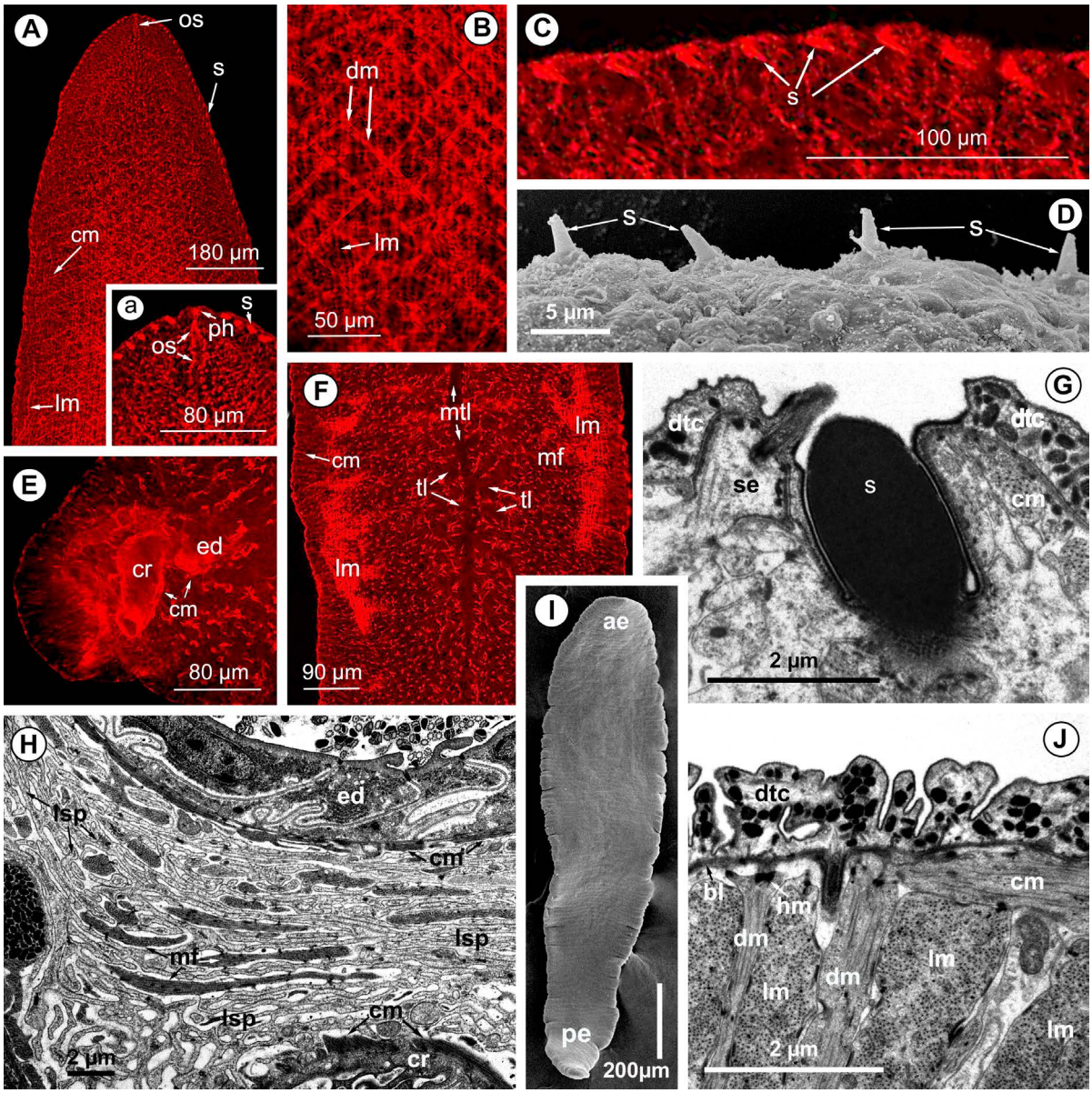

Fig. 1. *Sanguinicola plehnae* Warren et Bullard, 2023 from *Esox lucius* Linnaeus: confocal images of study of TRITC-phalloidin staining (A–C, E, F), SEM (D, I) and TEM (G, H, J) microphotographs. **A** – ventral view of anterior body, showing lateral spines, two body wall muscle layers and longitudinal muscles surrounding oesophagus; **a** – miniature pharynx and oesophagus; **B** – arrangement of diagonal and longitudinal muscle fibres of body wall; **C**, **D** – lateral spines stained with phalloidin (C) and SEM image (D); **E** – posterior body end, showing cirrus and ejaculatory duct surrounded by circular muscle fibres and area around cirrus stained with phalloidin; **F** – ventral view of the middle body, showing lobular testis and circular and longitudinal muscle fibres of the body wall; **G** – distal region of spine extending above the body tegumental surface; note ciliated sensory ending near spine; **H** – section around cirrus; note gathering of lamellar sarcoplasmic processes; **I** – total view of worm's body; **J** – outer body surface, showing circular, longitudinal and diagonal muscle fibres. *Abbreviations*: ae – anterior end of the body; bl – basal lamina; cm – circular muscles; cr – cirrus; dm – diagonal muscles; dtc – distal tegumental cytoplasm; ed – ejaculatory duct; hm – hemidesmosome; lm – longitudinal muscles; lsp – lamellar sarcoplasmic processes; mf – muscle fibres; mtl – middle testicular lumen; os – oesophagus; pe – posterior end of the body; ph – pharynx; s – spine; se – sensory ending; tl – testicular lobes.

muscle fibres localised along the dorso-ventral axis of dorsoventrally flattened *Sanguinicola* body (Fig. 1A,B,F,J). Diagonal muscle fibres are attached to the basal lamina beneath the distal tegumental cytoplasm by hemidesmosomes (Fig. 1J). The longitudinal muscle fibres of about 3.0 µm in width are sometimes located in pairs. This paired pattern is visible in the middle region of the body. The distance between strongly developed diagonal muscles is approximately 17–25 µm.

The anterior body extremity has a diminutive anteroventral mouth and associated ventral pharynx below it (Fig. 1Aa). In confocal image of the ventral body stained with TRITC-phalloidin the longitudinal muscle fibres extend along the border of the oesophagus (Figs. 1A,a, 2B). Along lateral body margins a single column of deeply rooted spines is clearly seen in images stained with phalloidin (Figs. 1A,C, 2B) and scanning (Fig. 1D) and transmission (Fig. 1G) electron micrographs. In Fig. 3D short X-shaped

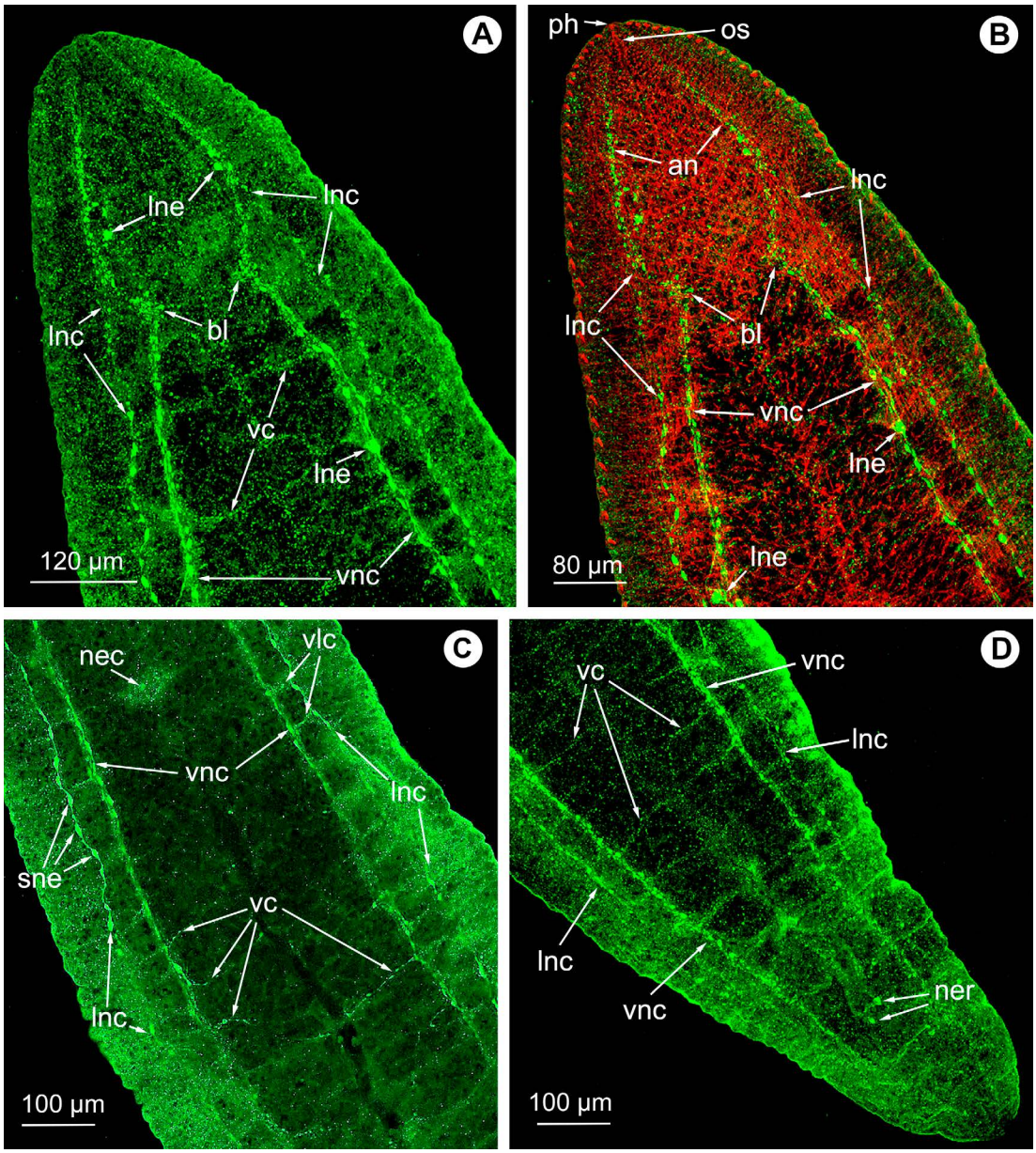

Fig. 2. A general view of serotonergic compartments of the nervous system (in green) and musculature (in red), CLSM images of *Sanguinicola plehnae* Warren et Bullard, 2023 from *Esox lucius* Linnaeus. **A** – anterior body with 5-HT-IP staining in the brain lobes, anterior nerves with large neurons, ventral and lateral nerve cords and transverse commissures, a maxprojection; **B** – anterior body region, the same body plane, double staining with anti-serotonin (nerves) and TRITC-phalloidin (muscles), a maxprojection; **C** – middle body region, staining with anti-serotonin; the ventral and lateral nerve cords with the serotonergic spindle-shaped neurons along the nerve cords, note the ventral and ventrolateral nerve commissures, a stack of three consecutive optical sections; **D** – posterior body end, staining with anti-serotonin, the ventral nerve cords, the ventral commissures, the lateral nerve cords are visible, note neurons located near distal compartments of the reproductive system. *Abbreviations*: an – anterior nerve; bl – brain lobes; lnc – lateral nerve cords; lne – large neuron; nec – neuron near caeca; ner – neurons near reproductive organs; os – oesophagus; ph – pharynx; sne – spindle-shaped neurons; vc – ventral commissures; vlc – ventrolateral commissures; vnc – ventral nerve cords.

caeca areintensely stained with phalloidin. In phalloidin stained image (Figs. 1F, 3D) an outline of an irregularly-shaped, lobular testis is clearly visible. Numerous closely packed testicular lobes arranged in two rows relative to the middle region of the testis are seen, and around every lobe a diffuse arrangement of muscle fibres is revealed (Figs. 1F, 3D). At the posterior end both the cirrus and ejaculatory duct are surrounded by regular circular muscle fibres (Figs. 1E,H, 3H,I). Also, a large area of phalloidin staining was observed around the cirrus (Fig. 1E). Transmission electron microscopy (TEM) shows that the cirrus is surrounded by modified sarcoplasmic processes in the form of lamellar outgrowths with muscle fibres in their extended parts (Fig. 1H).

The topography of the nervous system of adult *S. plehnae* based on 5-HT immunostaining

A general morphology of the serotonergic nervous system can be observed on Figure 2A–D. Two brain lobes are connected by a brain commissure, the width of which is ap-

proximately 9–10 μm and the length is 100–113 μm (Fig. 3A). The distance from the brain commissure to the anterior body tip is 96–114 μm. Several serotonergic globular structures are observed along the periphery of each brain lobe (Fig. 3A,a). The optical sections show the presence of three pairs of 5-HT-IP longitudinal nerve cords – ventral, lateral and dorsal. Visually the ventral cords are slightly prominent (Figs. 2A,C,D, 3A,E,G).

The distance between the ventral cords is about 187–236 μm in the anterior and about of 196–236 μm in the posterior region of the body. The lateral cords are significantly narrower than the ventral ones and lie on the ventral side of the body (Figs. 2A,D, 3G). The distance between the ventral and lateral cords varies from 30 to 65 μm in different body regions. From each brain lobe, a rather thick anterior nerve is running towards the anterior body end (Figs. 2B, 3A,B,E). The arches of anterior nerves are about of 218–239 μm long. At their base the anterior nerves thickness (12–14 μm) is comparable to the thickness of ventral cords and further towards the apical body end they become thinner. Along each anterior nerve four serotonergic neurons are revealed at a distance of about half of the nerve length in the region of middle-posterior oesophagus (Fig. 3B,E,e). The largest neurons are about 17–23 μm in size and three others are about of 9–11 μm. The distance between two large 5-HT-IP neurons in a pair of anterior nerves is 127–144 μm. These serotonergic neurons are lying at different tissue levels and are visible on the consecutive optical sections of the anterior region of the worm. In about 7 μm 5-HT-IP perikaryon can be observed in the region of X-shaped caeca (Fig. 2C).

The brain lobes give rise to a pair of posterior ventral nerve cords (Fig. 2A,C,D). The lateral cords are not rising from the brain (as the ventral ones) but rather merge with the anterior nerves from both body sides at the level of large serotonergic neurons (Fig. 3E). In the ventral and lateral cords of *S. plehnae* there are approximately 4–5 large 5-HT-IP neurons with a diameter of perikarya of about 22–23.5 μm (Figs. 2A,B, 3A,D,E). Along each ventral and lateral nerve cord, a row of numerous regularly distributed 5-HT-IP neurons is visible (Figs. 2C, 3C). The shape of these neurons is mostly elongated or spindle-shaped (Figs. 2C, 3C,D). The size of their perikarya varies from 8 μm to 20 μm in the ventral cords and from 9 μm to 18 μm in the lateral cords. Narrow dorsal nerve cords run along the body on the dorsal side. Several spindle-shaped serotonergic neurons can also be observed in the dorsal nerve cords (Fig. 3F). The distance between the dorsal cords is about of 225–290 μm and the distance from the lateral body margins to the dorsal cords varies from 107 μm in the tail region to 192 μm in the middle body.

The transversal ventral commissures run along the ventral side between the ventral nerve cords at a distance from 40 to 80 μm. They are well observed in anterior (Fig. 2A), middle (Fig. 2C) and posterior (Figs. 2D, 3G) body regions. The ventral and lateral cords are also connected by the ventrolateral commissures (Figs. 2C, 3C–E), the distance between which varies from 48 to 95 μm. The 5-HT-IP dorsal nerve commissures were not observed on the whole-mount preparations (Fig. 3F). At the posterior body end, from four to six 5-HT-IP neurons measuring 5–7 μm are identified in the region of terminal ducts of the male and female reproductive systems (Fig. 3H,I).

Ultrastructure of the neuropil and neuronal somata in the brain and nerve cords of adult *S. plehnae*

At the ultrastructural level, a pair of brain lobes, the brain commissure connecting them and lateral nerve cords consist of compact, irregular neurites of different content and size, forming the so-called neuropil (Fig. 4). The large, light, axonal neurites contain neurotubules, neurofilaments, elements of the smooth endoplasmic reticulum and mitochondria (Fig. 4A,C,F,G). The thin dendritic neurites of the neuropil contain the neuronal vesicles of different morphological types. Four types of vesicles of the neuropil neurites are found in *S. plehnae*: small clear vesicles of synaptic type (scv) (Fig. 4B,D,E,H), dense-core vesicles (dcv) (Fig. 4B,D,E), large dense vesicles (ldv) (Fig. 4A,G) and large lucent vesicles (llv) (Fig. 4B,F,H).

A variety of contacts is observed throughout the neuropil of the brain and nerve cords, including single and shared (one presynaptic ending is connected with two or three postsynaptic ones) synapses, last of which may be multiple and serial kinds (see terminology of Gustafsson 1984) (Fig. 4B,D,E,H). The most synapses of *S. plehnae* contain abundant small clear and dense core vesicles in their presynaptic terminals (Fig. 4B,D,E). There are neurites with three vesicular types (scv, dcv, llv) (Fig. 4F,H). The postsynaptic paramembraneous densities extend the whole length of the synaptic cleft of all synaptic types (Fig. 4B,D). Generally, synaptic vesicles are not observed in the vicinity of the postsynaptic densities. The synaptic junctions of neurites and muscle cells are observed (Fig. 4F,f).

There are no specialised glia-like structures along the periphery of the brain and nerve cords; instead, the neuron somata, myocytes and other cell types aggregate along their peripheral areas (Figs. 4A,C, 5C–G). Usually, the perikarya of the neurosecretory cells are large, possessing ovoid, large, euchromatic nucleus with distinct nucleolus (Fig. 5A,C,F). Their nuclei are surrounded by perinuclear cytoplasm, containing vesicles of different kinds (Fig. 5A,C,E,F). Their neurites extend along brain and nerve cords and terminate in the neuropil (Fig. 5B,E). Among neurosecretory cells, there are neurons located at some distance from the brain and nerve cords, the cytoplasm of which is filled only with one type of vesicles, large dense vesicles or dense-core vesicles (Fig. 5A,F). However, both small clear vesicles and dense-core vesicles are present in the perinuclear cytoplasm of the most peripheral neuron perikarya (Fig. 5E, G).

In addition, the brain and the lateral nerve cords contain number of undifferentiated perikarya (neuroblasts) and those at the initial stages of their differentiation (Fig. 5B,D), with sizes ranging from 3 to 5 μm. Neuroblasts are characterised by ovoid nucleus with large aggregates of dense chromatin and dense perinuclear cytoplasm filled with free ribosomes (Fig. 5B). A small number of synaptic vesicles and cytoplasmic processes are observed

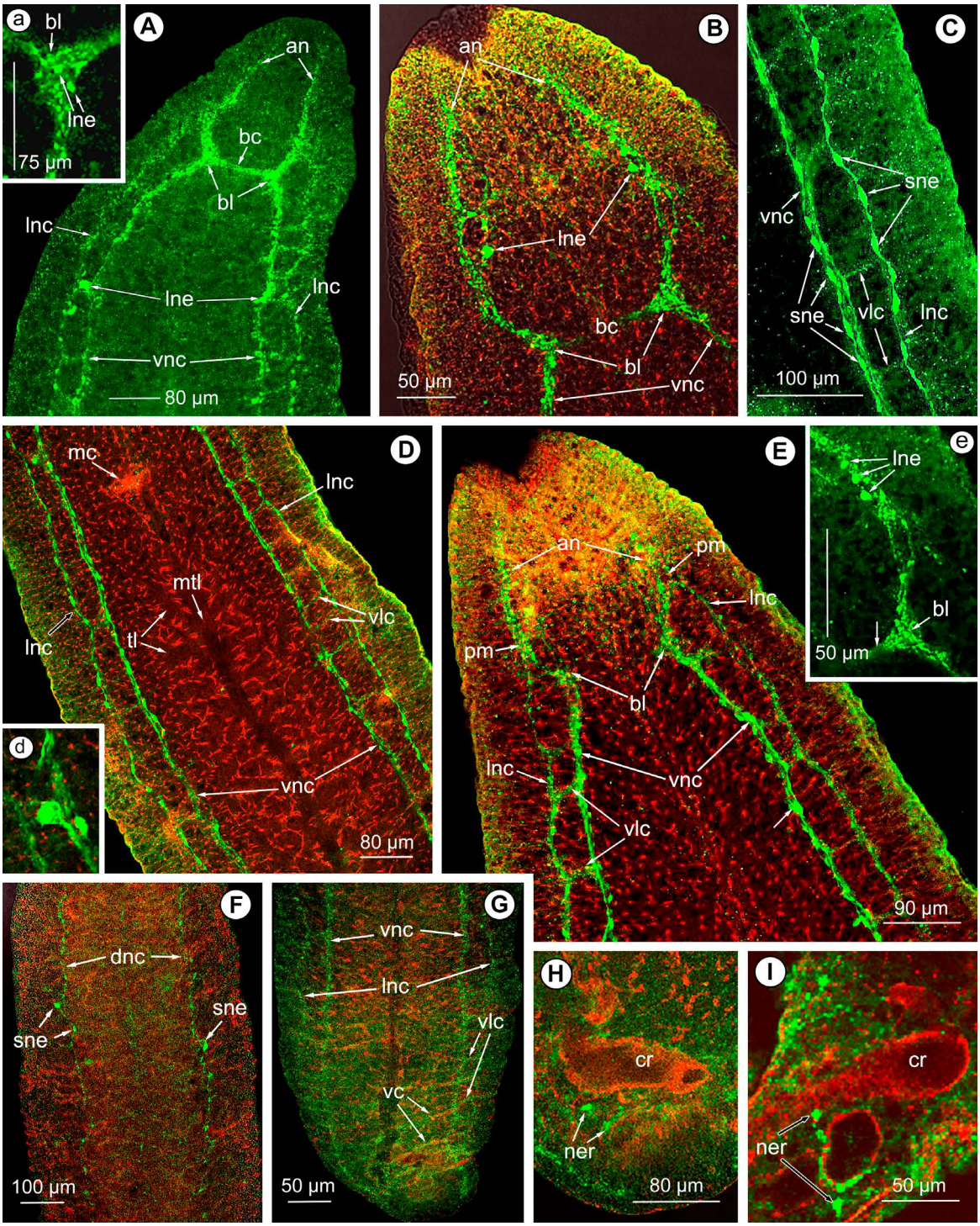

Fig. 3. Serotonergic nerve components distribution in *Sanguinicola plehnae* Warren et Bullard, 2023 from *Esox lucius* Linnaeus (in green) and TRITC-phalloidin staining (in red), CLSM images; A, B, E – anterior, C, D, F – middle and G–I – posterior body regions. **A** – serotonin-IP staining in brain lobes, brain commissure, anterior nerves, ventral and lateral nerve cords, note large 5-HT-IP neurons in the nerve cords; **a** – detail of the brain lobe; **B** – light and CLSM image, anterior nerves with large serotonergic neurons; note the brain lobes, brain commissure and the ventral cords, a snapshot; **C** – ventral and lateral nerve cords with rows of spindle-shaped neurons and ventrolateral nerve commissures connecting these cords; **D** – ventral and lateral nerve cords with ventrolateral commissures; note testicular lumen, muscle fibres along outline of testicular lobs and intensely stained caecal muscles, a snapshot; **d** – large serotonergic neuron located between two nerve cords; an optical section; **E** – the point of connection of lateral cords and the anterior nerve, a stack of four consecutive optical ctions; **e** – brain lobe and serotonergic neurons in the anterior nerve, a snapshot; **F** – 5-HT-IP staining in thin dorsal nerve cords of the middle body region; note a serotonergic neurons, a snapshot; **G** – 5-HT-IP staining of the ventral and lateral nerve cords in posterior body end, a snapshot; **H** – serotonergic neurons near the male terminal organ, a snapshot; **I** –serotonergic neurons near ejaculatory duct, an optical section. *Abbreviations*: an – anterior nerve; bc – brain commissure; bl – brain lobes; cr – cirrus; dnc – dorsal nerve cords; lnc – lateral nerve cords; lne – large neuron; mc – muscles of caeca; mtl – middle testicular lumen; ner – neurons near reproductive structures; pm – point of merging of lateral nerve cords; sne – spindle–shaped neurons; tl – testicular lobes; vc – ventral commissures; vlc – ventrolateral commissures; vnc – ventral nerve cords.

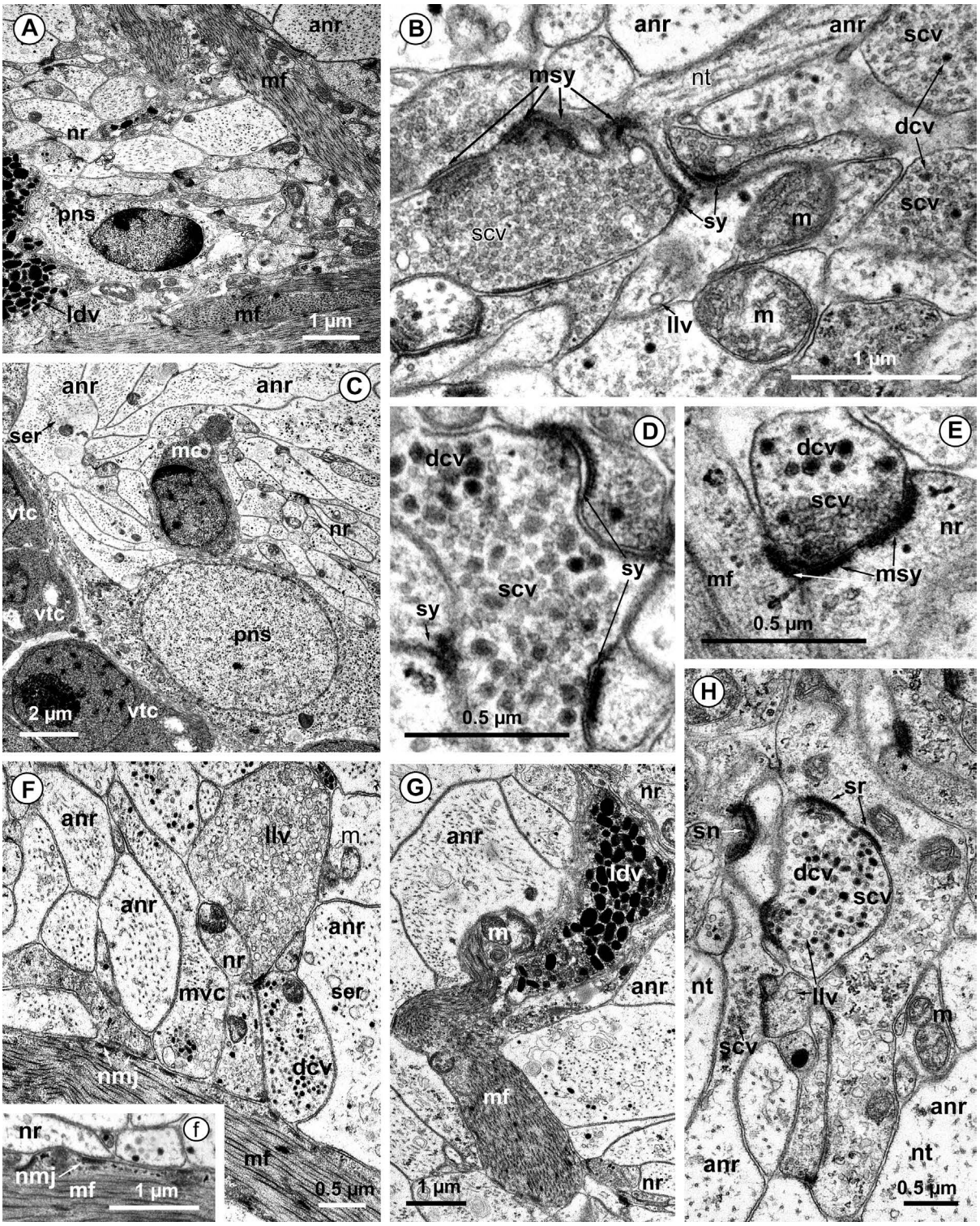

Fig. 4. Ultrastructure of the neuropil of the brain and longitudinal nerve cords of *Sanguinicola plehnae* Warren et Bullard, 2023 from *Esox lucius* Linnaeus. **A** – part of the brain, note neurosecretory cell and muscle fibres along and within neuropil; **B** – brain neuropil showing the tightly packed neurites and variety of synapses between them; **C** – part of the ventral nerve cord showing different kinds of the neurites and muscle cell between neurites, neuronal somata and vitelline follicular cells along periphery; **D** – shared synapse containing small clear and dense-core vesicles in presynaptic and postsynaptic neurites, postsynaptic density and distinct cleft are noted; **E** – multiply synapse showing one presynaptic neurite connected with postsynaptic neurite and sarcoplasmic muscle fibres; **F** – nerve cord neuropil showing neuromuscular junctions, **f** – neuromuscular junctions; **G** – brain neuropil showing muscle fibres, neurite containing large dense vesicles and a number of axonal neurites; **H** – brain neuropil showing different kinds of synapses and three kinds of vesicles in presynaptic terminal of neurite. *Abbreviations*: anr – axonal neurites; dcv – dense-core vesicles; ldv – large dense vesicles; llv – large lucent vesicles; m – mitochondrion; mc – muscle cell; mf – muscle fibres; msy – multiple synapse; mvc – mixed vesicular content; nmj – neuromuscular junctions; nr – neurites; nt – neurotubules; pns – perikaryon of neurosecretory neuron; scv – small clear vesicles; ser – smooth endoplasmic reticulum; sn – single synapse; sr – serial synapse; sy – synapse; vtc – vitelline cell.

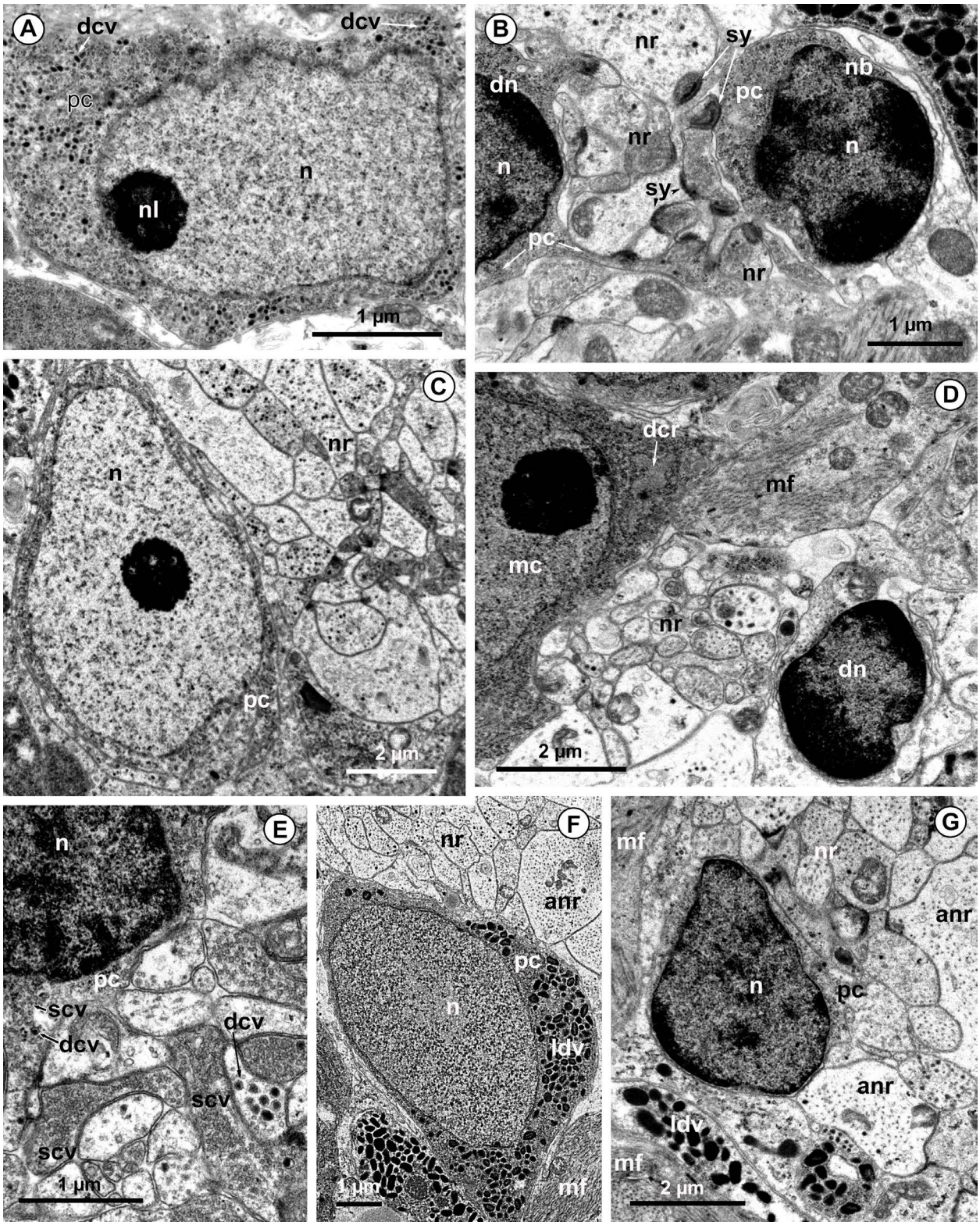

Fig. 5. Ultrastructure of the neuron somata of the brain and nerve cords in *Sanguinicola plehnae* Warren et Bullard, 2023 from *Esox lucius* Linnaeus. **A** – large neurosecretory perikaryon; note dense-core vesicles in perinuclear cytoplasm; **B** – ventral nerve cord; note neuroblast, developing neurons and numerous synapses between neurites; **C** – neuron somata of brain; **D** – myocyte and sarcoplasmic process with muscle fibres along neuropil periphery; note developing neuron; **E** – perinuclear cytoplasm of the brain neurosecretory perikaryon containing small clear and dense-core vesicles, neurites filled with these vesicles; **F** – perikaryon producing large dense vesicles; **G** – neuronal somata and neurites with different kinds of vesicles. *Abbreviations*: anr – axonal neurites; dcr – dilated cisternae of sarcoplasmic reticulum; dcv – dense-core vesicles; dn – developing neuron; ldv – large dense vesicles; mc – muscle cell; mf – muscle fibres; n – nucleus; nb – neuroblast; nl – nucleolus; np – neuropil; nr – neurites; pc – perinuclear cytoplasm; scv – small clear vesicles; sy – synapse.

in developing neurons (Fig. 5B). Synaptic junctions are present between cytoplasmic plasma membrane of neuroblasts and surrounding neurits (Fig. 5B).

The muscle cells have densely stained irregularly ovoid nucleus and dense perinuclear cytoplasm exhibiting dilated cisternae of sarcoplasmic reticulum (Fig. 5D). The sarcoplasmic processes with muscle fibres are observed around neuropil (Figs. 4A, 5D) and also such processes deeply embedded in the neuropil of the brain and nerve cords (Fig. 4A,F,G). Solitary muscle cells are observed within neuropil of the nerve cords (Fig. 4C).

DISCUSSION

So far nothing is known on the detailed pattern of the nervous system of adult fish blood flukes, an early branching and diverse digenean group. The immunocytochemical data shows the presence of serotonin-immunoreactive elements in the nervous system of adult *Sanguinicola plehnae* (present study) and cercaria of *Sanguinicola inermis* (see McMichael-Phillips et al. 1996). Available data indicate the presence of 5-HT-IP components in the nervous system of all digeneans studied to date (McKay et al. 1991, Gustafsson et al. 2001, Stewart et al. 2003, Tolstenkov et al. 2010, Kreshchenko et al. 2020, Denisova et al. 2024, Terenina et al. 2024).

Based on neurochemical data, we may assume that the nervous system of adults of *Sanguinicola* is arranged in an orthogonal plan possessing bilobed brain connected by one brain commissure. Each brain lobe gives rise to the anterior nerve, which runs towards the anterior body end. Three pairs of the longitudinal nerve cords run in parallel along the worm's body to the posterior end; ventral and lateral cords located on the ventral body side and dorsal located on the dorsal side over the ventral cords. Interestingly, the ventral and dorsal nerve cords originate from the brain lobes; lateral cords pass away from the brain merging with anterior nerves at the level of large serotonergic neurons. Thin transverse commissures connect in a regular pattern along the ventral body side; between ventral and lateral nerve cords ventrolateral commissures are present.

The orthogonal pattern of the nervous system is the basic plan for heterogeneous flatworm taxa (Halton and Maule 2004). The neuroanatomy of *S. plehnae* comprises of orthogonal arrangement corresponding to that reported for other species of the Digenea studied to data (Denisova et al. 2024, Terenina et al. 2024). However, the morphological variations of the neuroanatomy in flatworms have been discussed by Kotikova (1969, 1991), Joffe (1990) and Gustafsson et al. (2002); they depend on the specialised shape of the body. Concerning digenean species, the division of the body into forebody (locomotor portion) and hindbody (genital portion) in most studied species affects the structure of their nervous system (Kotikova 1969, Joffe 1990).

In the anterior body portion of adult digeneans the longitudinal nerve cords are thickened and connected by commissures while in the posterior portion they are poorly developed and commissures are absent like in *Sphaerostoma bramae* Müller, 1776; *Podocotyle atomon* (Rudolphi, 1802); *Haematoloechus* sp. (Kotikova 1969), *Dicrocoelium lanceolatum* Stiles et Hassall, 1896 (Kotikova et al. 1990), *Diplodiscus subclavatus* (Pallas, 1760) and *Fellodistomum fellis* (Olsson, 1868) (Kotikova and Joffe 1990). In *Fasciola hepatica* the typical orthogonal nervous system of the anterior part of the body modifies into a dense and reticulate nervous system behind the ventral sucker (Kotikova et al. 1990). Unexpected regularity in neuroanatomy was observed in *Haplometra cylindracea* (Zeder, 1800) (McKay et al. 1990), in which the nerve cords displayed a distinctly rectangular pattern anteriorly, which is replaced by a stellate arrangement towards the posterior end of the worms.

The studied sanguinicolid, *S. plehnae*, lacks oral and ventral suckers, has miniature X-shaped caeca and is characterised by uniformly developed nervous system (present study). Unlike in sanguinicolids, in digeneans with developed oral sucker anterior nerves are increased in number and branched, giving rise to radial neurities of the oral sucker, like in *H. cylindracea* (McKay et al. 1990), *F. hepatica* and *D. lanceatum* (Kotikova et al. 1990, Terenina et al. 2022b), *Bucephaloides gracilescens* (Rudolphi, 1819) (Stewart et al. 2003), *Opisthorchis viverrini* (Leksomboon et al. 2012a), and *Renicola parvicaudatus* (Denisova et al. 2024). Also, the variations of the orthogonal nervous system of digeneans may depend on the quantitative number and disposition of the circular commissures and the way of innervation of their ventral sucker (Kotikova and Joffe 1990). Interestingly and unlike sanguinicolids, in all studied digenean species three pairs of longitudinal nerve cords emerge from the brain. As shown in the present study, in *S. plehnae* only ventral and dorsal nerve cords emerge from brain, lateral ones originate from the anterior nerves.

In general, the immunocytochemical data obtained in the present work corresponds to the published information available for other digeneans and confirms the presence of serotonergic nerve structures in the digeneans as a conservative feature (Šebelová et al. 2004). Along with this, some specific features should be noted regarding the presence and distribution of serotonergic components in the nervous system of *S. plehnae*. Clearly distinguished large 5-HT-IP neurons have been identified in anterior nerves going to the mouth opening. Several pairs of large 5-HT-IP neurons are seen along the ventral nerve cords. It should be noted that along each ventral and lateral nerve cords a very unusual "chain" of the spindle-shaped 5-HT-IP cell bodies has been detected.

Interestingly, confocal study of *S. plehnae* stained with TRITC-phalloidin has clearly shown the deeply rooted tegumental spines, distributed in transverse rows along the lateral body margin. As revealed previously the spines of freshwater and marine fish blood flukes are different from those of other digeneans (Poddubnaya et al. 2019, 2020). These spines are localised deep beneath the body wall and most of the length of their body is surrounded by sarcoplasmic process filled with myofilaments. As suggested by Poddubnaya et al. (2019, 2020) sanguinicolid and aporocotylid spines may be derivatives of the filamentous actin-like material of the muscle cells in contrast with the tegumental origin for spines of other digeneans. The inten-

sively stained spines of *S. plehnae* with TRITC-phalloidin fully support the abovementioned opinion.

In *Sanguinicola* an X-shaped caecaum is restricted to the anterior half of the body; this area displays an intensive TRITC-phalloidin staining. In *S. plehnae* phalloidin staining has been observed around irregular-shaped testicular lobules, which extend longitudinally along the middle of the body from the posterior margin of the intestinal caecaum to the ovary (see Poddubnaya et al. 2022). The intensive phalloidin staining has been observed around the cirrus, surrounded by a large area of modified lamellar-like sarcoplasmic processes with muscle fibres in their extended parts. Available information related to other digeneans gives evidence of their well-developed muscle system, playing an important role in body movement, host attachment and function of digestive and reproductive organs (Stewart et al. 2003, Halton and Maule 2004, Šebelová et al. 2004, Terenina et al. 2022a, b).

Generally, ultrastructure of the nervous system of *S. plehnae* corresponds to that reported for adult digeneans and consists of an extensive neuropil of tightly assembled neurites surrounded by neuron somata located along neuropil periphery (Leksomboon et al. 2012b, Denisova et al. 2024). However, Gustafsson (1992) in her review of the neuroanatomy of parasitic flatworms indicated the presence of a single somata inside their neuropil, similar to that of *F. hepatica* (Sukhdeo et al. 1988) and *S. plehnae* (present study). As shown in the present study, the neuroblasts and developing neuron perikarya of *S. plehnae* are found within the neuropil among neurites, which may indicate the renewal of the population of neuron somata in adult digeneans.

No true glia-like structures were detected in studied fish blood flukes, the sanguinicolid *S. plehnae* (present study) and the aporocotylid *Aporocotyle simplex* (Poddubnaya and Gibson 2020). Instead, their muscle cells aggregate along the periphery of CNS and solitary muscle perikarya and separate muscle fibres are frequently observed within the neuropil among neurites. Previous studies on digenean glia-like cells described 'mesenchymal' cells and their processes, which encircled the nervous cords of *F. hepatica* (Sukhdeo and Sukhdeo 1994) and *O. viverrini* (Leksomboon et al. 2012b). In *Renicola parvicaudatus* (Denisova et al. 2024) the myocytes were observed around the nerve cords. The muscle fibres were observed in the brain and nerve cords of all abovementioned adult digeneans. It should be noted that the true glial cells possess some specific characteristics such as the presence of filaments and their relationship with the nerve cells (Sukhdeo and Sukhdeo 1994). As has been assumed previously for aporocotylids (Poddubnaya and Gibson 2020, Poddubnaya et al. 2021), the muscle cells may conform to such characteristics due to the presence of numerous filaments in their sarcoplasm and the occurrence of the neuromuscular junctions.

In general, the ultrastructural features of neurons and neurites are very similar to those characterising the nervous system of the neodermatans (Gustafsson 1992). The same kinds of neuron somata were revealed in *S. plehnae*, producing four types of neurovesicles: small clear vesicles (scv), dense-core vesicles (dcv), large dense vesicles (ldv) and large lucent vesicles (llv). In *S. plehnae* the majority of presynaptic terminals of neurites contain small clear and dense-core vesicles; neurites with three types of vesicles (scv, dcv and llv) are also present. In *S. plehnae* both the large neuron perikarya filled with dense large vesicles and neuron somata filled with dense-core vesicles contain one type of the vesicles, but the majority of peripheral neuron perikarya contain both small clear vesicles and dense-core vesicles in their neuroplasm.

The variability in the number of vesicular types was recorded in studied adult digeneans. Additionally to nerve vesicles, neuron perikarya of *R. parvicaudatus* contain large osmiophilic granules (Denisova et al. 2024). Three types of vesicles were distinguished in neuron somata of cercaria of *Schistosoma mansoni* Sambon, 1907 (Cousin and Dorsey 1991), four types of vesicles were revealed in neuron perikarya of *F. hepatica* (Sukhdeo et al. 1988) and five neuronal vesicular types in *O. viverrini* (Leksomboon et al. 2012b).

The great morphological variability of fish blood flukes of five morphologically distinct lineages gives an excellent opportunity to gain more insight into the structure/function relationships of their nervous system through detailed mapping of the presence/absence of the oral sucker and pharynx, the location and distribution of unique spines, the shape and number of their testes and caeca. As fish blood flukes represent an early branching group of the Digenea, there is a possibility to trace the variations in the organisation of their nervous system for the species belonging to each morphological lineage. Simple, uniformly developed nervous system is revealed for the sanguinicolid *S. plehnae* lacking attachment suckers and possessing miniature caeca in the anterior portion of the body. Such pattern may consider as apomorphic trait for *Sanguinicola* species.

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