Ultrastructural evidence for the participation of muscle cells in the formation of extracellular matrices in aporocotylid blood flukes (Digenea)

Larisa G. Poddubnaya[1] [ORCID 0000-0003-0472-9846], Alexander E. Zhokhov[1], Willy Hemmingsen[2], David I. Gibson [ORCID 0000-0002-2908-491X][3]

[1] *I. D. Papanin Institute for Biology of Inland Waters, Russian Academy of Sciences, 152742 Borok, Yaroslavl Province, Russia*

[2]*Department of Arctic and Marine Biology, Faculty of Biosciences, Fisheries and Economics, University of Tromsø, Norway*

[3] *Department of Life Sciences, Natural History Museum, Cromwell Road, London SW7 5BD, UK*

Corresponding author:

Larisa Poddubnaya, I.D. Papanin Institute for Biology of Inland Waters, Russian Academy of Sciences, 152742 Borok, Yaroslavl Province, Russia

E-mail address: poddubnaya@ibiw.ru

Abstract

The muscle cells and extracellular matrices (ECMs) of two teleost-infecting blood flukes belonging to distinct evolutionary lineages of the Aporocotylidae (Digenea) were examined using Transmission Electron Microscopy. Four morphotypes of muscle cells were found in the freshwater species *Sanguinicola* sp., but were considered to be various different developmental stages of a single cisternic type. In the marine species *Aporocotyle simplex*, three types of muscle cells were apparent, one of which is cisternic. The first ultrastructural evidence is presented for the exocytosis of the moderately dense contents of dilated cisternae of cisternic muscle cells into the extracellular space in both *Sanguinicola* sp. and *A. simplex*. The basal matrices of aporocotylids support various types of epithelia. In *Sanguinicola* sp., beneath the distal tegumental cytoplasm, there is a thin *lamina densa*, whereas the intestinal epithelium is supported by a *lamina reticularis*. In *A. simplex,* both a thin *lamina densa* and a thick *lamina reticularis* underlie the distal cytoplasm of the tegument and are present at the periphery of the ovary, but beneath the epithelial lining of the caeca and both genital and excretory ducts there is only a *lamina reticularis*. Significant variation in the development and amount of the ECM in marine and freshwater aporocotylids is described, since *A. simplex* has a much better developed ECM than occurs in *Sanguinicola* sp. Moreover, thin myofilaments of muscle fibres participate in the ECM formation in *A. simplex* and represent its dominant component. The presence of two mechanisms for ECM formation in *A. simplex*, as opposed to a single mechanism in *Sanguinicola* sp., may represent further evidence for the affiliation of these two taxa to divergent evolutionary lineages. The data presented are discussed in relation to available information on these structures in other neodermatan groups.

Key words: TEM, Aporocotylidae, muscle cells, extracellular matrix, freshwater *Sanguinicola*, marine *Aporocotyle simplex*

1. Introduction

Traditionally, the parenchymal body of flatworms (Platyhelminthes) contains, beneath their surface epithelia, a number of different types of mesenchymal cells embedded in the extracellular matrix (ECM) that appear to act as a structural substitute for true connective tissue (Pedersen, 1991). The ECM of flatworms can be divided into basal matrices which occur beneath various epithelia and those matrices, which occur between the mesenchymal cells (Conn, 1993). Great variation in the structure of both types of matrix has been revealed in different species of free-living and neodermatan platyhelminths (Pedersen, 1991; Conn, 1993). Pedersen (1991), Conn (1993) and Ehlers (1995) have summarized the ultrastructural data available at that time on mesenchymal cells in both free-living and parasitic flatworms and noted that the heterogeneous muscle cells (myocytes) of flatworms comprise one component of their 'parenchyma'. In relation to this, Lindroos and co-authors have provided data on the ultrastructural, cytochemical and immunocytochemical features of the ECM in both free-living and parasitic flatworms (Lindroos, 1984; Lindroos and Wikgren, 1987; Lindroos and Still, 1988; Lindroos and Reuter, 1991). In the case of the Digenea, there is only fragmentary ultrastructural information on muscle cells, parenchyma and the ECM (Threadgold and Gallagher, 1966; Lumsden and Foor, 1968; Wilson, 1969; Abbas and Cain, 1987; Stitt et al., 1992; Fujino et al., 1996; Stoitsova and Gochilova, 1997; Poddubnaya et al., 2020a; Poddubnaya and Gibson, 2020).

The typical morphological features used in flatworm taxonomy and systematics differentiate families, genera and species; however, those features are seemingly less informative regarding deep phylogenetic relationships among flatworm lineages. Investigations of the morphological diversity of muscle cells and the ECM in a wide variety of digeneans are important for any understanding of the evolutionary origin of the so-called

cytoskeletal components in members of the Neodermata. The acquisition of such knowledge is especially relevant in the case of members of the most basal groups in each neodermatan class. The present study is the first to explore patterns in the cytoskeletal components of two quite different species of the Aporocotylidae, one of the most basal digenean families (Olson et al., 2003; Orélis-Ribeiro et al., 2014; Cribb et al., 2017), i.e., the marine species *Aporocotyle simplex* Odhner, 1900 and freshwater species *Sanguinicola* sp.[1], and their correlation with known information on this type of structure in other neodermatan groups. In this respect, the fine structural details of muscle cells are relevant in terms of any discussion on the origin of the ECM.

2. Materials and methods

Specimens of *Sanguinicola* sp. were collected from the branchial arteries leading from the heart of naturally infected pike *Esox lucius* (Linnaeus, 1758) (Esocidae) from the small Sutka River in the Upper Volga River Basin, Russia. Specimens of *Aporocotyle simplex* were obtained from the branchial arteries of the long rough dab *Hippoglossoides platessoides* (Fabricius, 1780) (Pleuronectidae) trawled from the Norwegian Sea off Tromsø, Norway. For transmission electron microscopy (TEM) live specimens were fixed using 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 7 days at 5°C, rinsed three times for 10 min periods in the same buffer and postfixed in 1% osmium tetroxide for 1 h. Fixed specimens were dehydrated in a graded ethanol series, with a final change to absolute acetone and then critical-point dried with liquid CO_2. Later, specimens were embedded in Araldite/EMbed-812

[1] This taxon is the same species as that referred to as *Sanguinicola inermis* Plehn, 1905 in a previous paper (Poddubnaya et al., 2020b). Subsequent work on this parasite has suggested that this material may represent a new species. Morphometrical and molecular studies are currently under way with a view to describing this taxon.

EM Embedding kit (EMS). Ultrathin sections were then stained with uranyl acetate and lead citrate, and examined using a JEM 1011 microscope operating at 80 kV.

3. Results

3.1. Muscle cells of Sanguinicola *sp.*

Different morphotypes of muscle cells can be distinguished based on their shape, size and cytoplasmic composition (Fig. 1). The first cell morphotype observed is associated with the tegumental musculature. These cells have the morphological characteristics of undifferentiated cells, i.e., a large nucleus surrounded by a thin area of dense perinuclear cytoplasm (Fig. 1A, B). Dense areas of central and peripheral heterochromatin are present within the nucleus (Fig. 1A, B), numerous ribosomes are scattered throughout the perinuclear cytoplasm and a solitary mitochondrion may be present (Fig. 1A, B). These elongate-oval cells can be seen to possess one or two sarcoplasmic extensions containing muscle fibres (Fig. 1A, B).

Another muscle cell morphotype occurs well below the tegumental layer, occupying a medial region of the worm's body. The large, ovoid nucleus of this type contains uniformly scattered euchromatin and a little heterochromatin (Fig. 1C, D, E). Three morphological modifications of these cells are apparent. The first variation is characterized by the presence of a few dilated cisternae of the granular endoplasmic reticulum filled with a substance of moderate electron density (Fig. 1C). An additional cell variation can be distinguished by a greater volume of perinuclear sarcoplasm, an accumulation of mitochondria and the presence of both oval and rounded dense vesicles in an expanded region of cytoplasm. A sarcoplasmic

extension, containing muscle fibres, arises from this expanded region of cytoplasm (Fig. 1D). Dictyosomes of the Golgi complex occur in the muscle cell cytoplasm, close to which small, dense vesicles (~ 0.2 µm in diameter) congregate (Fig. 1D, insert). Sarcoplasmic extensions of the muscle cells contain muscle fibres, large mitochondria, sarcotubules, a small number of lipid droplets and a few glycogen granules (Fig. 1F, G). Glycogen, stained by uranyl acetate, is found in a small area among the myofilaments (Fig. 1F, G). A third muscle cell variation is elongate in outline and has a large volume of perinuclear sarcoplasm, within which pale areas devoid of ribosomes appear, but sarcoplasmic extensions with muscle fibres are absent (Fig. 1E). In these cells, numerous vesicular cisternae of the sarcoplasmic reticulum occupy a peripheral position close to the sarcolemma of the cell (Fig. 1E).

3.2. Extracellular matrix of Sanguinicola *sp.*

As in all neodermatans, the body of the worm is bounded by a distal tegumental cytoplasm limited by both surface and basal membranes (Fig. 2A, D). The basal membrane is supported by a basal matrix (also commonly referred to as a ‘basement membrane’). In *Sanguinciola* sp., it represents a thin, closely packed network of dense fibrils (often referred to as the *lamina densa*) (Fig. 2A, C, D). Beneath this are outer and inner muscle fibres running in circular and longitudinal directions, in addition to diagonal muscle fibres localized along the dorso-ventral axis of the worm (Fig. 2A, D). All multidirectional muscle fibres are delimited by a sarcolemma, close to which large mitochondria are apparent (Fig. 2A, D). In one section, the distal cytoplasm of the tegument and the muscles lie in close proximity (Fig. 2A, C); in others, large gaps are observed between them (Fig. 2D). The extracellular spaces beneath the tegumental layer, which are present between the tegumental muscles and between various other kinds of the cells within the body of the worm, appear almost empty (Fig. 2A,

D, E) apart from occasional membranous whorls (Fig. 2G), vesicles and some other moderately-dense material (Figs. 1A, 2G, H, I). Infrequently distributed hemidesmosomes occur on opposite sides of the body at positions where the diagonal muscles are connected directly to the basal matrix of the tegumental cytoplasm by anchoring fibrils (Fig. 2B, D). A basal matrix occurs around the epithelial lining of the digestive and genital ducts and resembles a thin, loosely packed network of fibrils (often referred to as a *lamina reticularis*) (Fig. 2F). Separate sarcoplasmic extensions filled with muscle fibres surround the nerve cords (Fig. 2J). There is evidence of exocytotic activity from muscle cells into the extracellular spaces (Fig. 2G, H, I) as the contents of dilated cisternae of the sarcoplasmic reticulum are released (Fig. 2G, H, I).

3.3. Muscle cells of Aporocotyle simplex

The first of three types of muscle cells occupies a central position in the body of the worm (Fig. 3A – D). These cells are elongate, irregular in outline and have an irregularly ovoid nucleus containing aggregates of dense heterochromatin (Fig. 3A, B). The perinuclear cytoplasm includes many free ribosomes and dilated, sac-like cisternae of the sarcoplasmic reticulum which are filled with a moderately dense, amorphous material. These dilated cisternae are distributed both along the periphery of the perinuclear cytoplasm and close to the sarcolemma (Fig. 3A, B). A fusion between the cisternae and the sarcolemma is readily observed, and the exocytosis of their moderately dense contents into the extracellular space is clearly apparent (Fig. 3A – D). The thin sarcoplasmic extensions of these cells contain numerous ribosomes and a collection of myofibrils in their expanded areas (Fig. 3A, B).

The second type of muscle cell occurs in the region of the cerebral ganglia, nerve cords and oesophagus. Their irregularly shaped nuclei contain aggregates of dense

heterochromatin, and their dense, perinuclear cytoplasm can be distinguished by the presence of large, dense areas exhibiting a typical, linear patterned, crystalline structure morphologically similar to the filamentous form of actin in digenean spines (Fig. 3F, G). The sarcoplasmic extensions of such cells contain a few muscle fibres, and their sarcoplasm includes an accumulation of moderately electron-dense lipid droplets surrounded by membranous whorls (Fig. 3E, G). Additionally, close to the oesophagus, their muscle fibres are surrounded by a narrow, branching region of dense, homogeneous sarcoplasm (Fig. 5G).

Muscle cells of the third type are visible beneath the tegument, lying adjacent to the organs and ducts of the body (Fig. 3I). Their large nuclei are irregular in outline and contain numerous clumps of central and peripheral heterohromatin (Fig. 3I). The perinuclear cytoplasm contains an electron-dense sarcoplasm filled with free ribosomes. Dilated cisternae of the sarcoplasmic reticulum are absent (Fig. 3I). Associated sarcoplasmic extensions have a fairly electron lucent sarcoplasm, within which are muscle fibres, a few lipid droplets and mitochondria (Fig. 3I). These extensions may be greatly flattened in the narrow spaces between vitellocytes and may give rise to long, lamellate projections, the accumulation of which is apparent in the extracellular space surrounding the reproductive ducts (Fig. 4E, G, H).

The sarcoplasmic extensions of all three types of muscle cell fill the body regions between the different organs and ducts. Fig. 3H shows an area between the testes containing tightly packed sarcoplasmic extensions from different types of muscle cell, all of which are surrounded by a moderately dense extracellular matrix.

3.4. Extracellular matrix of Aporocotyle simplex

The ECM is extensive in this species and continuous throughout the body of the worm (Figs. 4, 5). It consists of an amorphous substance in which abundant filaments are distributed (Figs. 4C, 5A – G). A large extracellular zone, richly supplied with ECM (Fig. 4B), occurs beneath the distal tegumental cytoplasm and around the tegumental muscles. Immediately beneath the basal plasma membrane of the distal tegumental layer, a dense, narrow lamina-like network of ECM may also be apparent; this is identified as a *lamina densa* (Fig. 4C). Between the *lamina densa* and the neighbouring circular muscles, a wide zone of ECM occurs in the form of a thick network, which is itself surrounded by the tegumental muscle fibres of circular and longitudinal muscles (Fig. 4B, C). Diagonal muscles situated along the dorso-ventral axis of the body are not apparent; instead, numerous muscle bosses occur laterally throughout the length of the body, each of which bear tegumental spines embedded in the diagonal muscle bundles (see Poddubnaya et al. 2019). At the tapering ends of these diagonal muscle fibres are hemidesmosomes connecting them to the *lamina densa* (Fig. 4F). An extensive ECM surrounds the epithelial lining of the intestinal caeca, genital organs, reproductive ducts and excretory ducts, and also fills the extracellular zone between various kinds of cells within the body of the worm (Fig. 4D – J). The nucleate epithelium of the intestine, the epithelial lining of the male and female reproductive ducts and that of the excretory ducts are supported by large areas of ECM, within which a few muscle fibres can be seen (Fig. 4D, E, G, H). Numerous flattened sarcoplasmic extensions arranged in parallel rows are present beneath the ECM close to the reproductive ducts (Fig. 4E). The ECM has a similar appearance in the different regions of the body. In addition to the large ECM zone, a thin *lamina densa* occurs only in association with the ovary (Fig. 4I, J).

A connection between the musculature and the ECM occurs around muscle fibres (Fig. 5A – D). Myofibres of the subtegumentary circular and longitudinal layers are surrounded by closely adhering extracellular filaments, so the outline of the sarcolemma is obscured (Figs.

4B, C, 5A, B, D, G). Our TEM observations showed that dense, oval concentrations of myofilaments are present along the margins of the muscle fibres (Fig. 5B, C, G, H). Gradually these aggregations bud off from the muscle fibres and independent clusters are visible in the extracellular zones between the myofilaments (Fig. 5B – D, G, H). In such places, these aggregations gradually become loosely packed and break down into separate filaments which are morphologically similar to those that fill the extracellular matrix (Fig. 5C, D, H). Areas with a small number of thin myofilaments can also be seen within the muscle fibres (Fig. 5E); here degenerating muscle fibres containing a few myofilaments and empty areas are also present (Fig. 5F).

4. Discussion

4. 1. Muscle cells of aporocotylids

The present study has shown that various types of muscle cells occur in both freshwater *Sanguinicola* sp. and marine *Aporocotyle simplex*. We suggest that the four apparent muscle cell morphotypes in the freshwater aporocotylid *Sanguinicola* sp. represent different developmental stages of a single type of muscle cell (i.e. the cisternic type of Conn, 1993). These morphotypes are distinguishable by their degree of development, ranging from those possessing the morphological characteristics of undifferentiated cells (myoblasts) to those having degenerating areas in their sarcoplasm. The development of these cells is accompanied by successive morphological modifications to the volume of the sarcoplasm, the number of dilated cisternae in the sarcoplasmic reticulum and the production of electron-dense vesicles associated with the Golgi complexes.

Nevertheless, in the marine aporocotylid *A. simplex*, three distinct types of muscle cell are apparent, one type of which is cisternic, similar to the fully-formed muscle cells of *Sanguinicola* sp., but differing in that the sarcoplasm lacks both Golgi complexes and electron-dense vesicles. The second type of muscle cell in *A. simplex* is especially interesting due to the presence in the sarcoplasm of large, dense areas of a linear patterned, crystalline structure morphologically similar to the filamentous form of actin in digenean spines (Cohen et al., 1982; Abbas and Cain, 1987; Stitt et al., 1992; Poddubnaya et al., 2019). It is also worth noting that actin in a filamentous form is associated with the actin filaments of flatworm smooth muscle fibres (Reger, 1976; Stitt et al., 1992; Sulbarán et al., 2015; Grano-Maldonado et al., 2018). Actin is a common protein in muscle cells, and hexagonally packed actin filaments have been shown to occur in the spines of both schistosomatid and fasciolid digeneans (Cohen et al., 1982; Abbas and Cain, 1987; Stitt et al., 1992). The concentration of such muscle cells around nerve ganglia and nerve trunks and within the muscular sheath of the oesophagus in *A. simplex* indicates a role in the support of and/or isolation of the functions of these structures. Our previous study on glial cells in *A. simplex* suggested a possible switching of glial functions to muscle cells containing actin (Poddubnaya and Gibson, 2020). In the case of the third type of muscle cell in *A. simplex*, both broad and flattened sarcoplasmic extensions occupy areas between different organs and ducts in the worm's body, and contain a sarcoplasm with sporadic areas of muscle fibres and a few lipid droplets. Similar flattened sarcoplasmic extensions of muscle cells, with long, narrow processes, have also observed in tapeworms (Conn, 1988; Świderski and Tkach, 1997; Willms et al., 2003; Poddubnaya et al., 2005; Poddubnaya and Mackiewicz, 2009; Korneva et al., 2016). In cestodes, such flattened sarcoplasmic extensions have been described around movable attachment organs, reproductive ducts with an accumulation of reproductive material and the terminal regions of the male and female reproductive system. According to Conn (1993),

three types of muscle cells occur in flatworms, i.e., secretory, storage and secretory/storage, and the sarcoplasm of the latter two types contains glycogen and lipid droplets. Glycogen-rich sarcoplasmic extensions with two forms of glycogen (single granules and rosettes) have been recorded for a number of tapeworms (Lumsden and Byram, 1967; Kuperman, 1988; Conn, 1988, 1993). On the other hand, monogenean muscle cells appear to store only small amounts of glycogen (Halton, 1967). In the freshwater and marine aporocotylids studied, glycogen was found to be stored only within the muscle fibres. The same type of glycogen storage has also been reported from a polyclad turbellarian by MacRae (1965). In digeneans, two kinds of 'parenchymal cell' with morphologically and functionally different types of mitochondria and different types of peroxidase activity have been found in the paragonimid *Paragonimus ohirai* by Fujino et al. (1996). Based on the ultrastructural features of the muscle cells described in *P. ohirai*, we can assume that one cell type has a tegumental localization and contains mitochondria with an aerobic metabolism. This type is comparable with the first developmental stage of muscle cells in *Sanguinicola* sp., considered by us to be undifferentiated cells. The second type of muscle cell in *P. ohirai* occupies a large proportion of the body volume and has anaerobic metabolism; this type corresponds morphologically to the fully-formed muscle cells of *Sanguinicola* sp. A single type of muscle cells (referred to as 'parenchymal cells') has been shown to occur in the fasciolid digenean *Fasciola hepatica* by Threadgold and Gallagher (1966). Pedersen (1991), Conn (1993) and Ehlers (1995) have all commented on the absence of true parenchymal cells in platyhelminths and their functional replacement by muscle cells, the multifunctional nature of which includes a fibroblastic activity and a role in the production of an extracellular matrix. Conn (1993) supported the idea that this fibroblastic activity is a characteristic of the cisternic type of muscle cell. In such muscle cells, the dilated cisternae of the sarcoplasmic reticulum have a sac-like shape and are filled with an amorphous material of moderate electron density. They are encountered

immediately beneath the sarcolemma (Conn 1993). Such a position for dilated sarcoplasmic cisternae has also been demonstrated in tapeworms (Lumsden and Foor, 1968; Conn and Rocco, 1989) and now in the aporocotylid digeneans (Present study). Furthermore, we have presented the first ultrastructural evidence for the exocytosis of the moderately dense contents of dilated cisternae into the extracellular zone in both the freshwater *Sanguinicola* sp. and the marine *Aporocotyle simplex*.

4.2. Extracellular matrices of aporocotylids

Basal matrices, widely referred to as basement membranes, of members of the Platyhelminthes are extracellular structures organized to support epithelia. In reviews on the structure and composition of the basal matrix system in both free-living (Pedersen, 1991) and parasitic (Threadgold, 1984; Conn, 1993) flatworms, the basal matrix is described as comprising a *lamina densa* (i.e. a dense basal lamina) and a *lamina reticularis* (i.e. an interstitial filamentous layer). A similar basic pattern is found in the basal matrix of a digenean in a detailed study of *Fasciola hepatica* by Stoitsova and Gorchilova (1997). In aporocotylid digeneans, we found that, in the freshwater *Sanguinicola* sp., a thin *lamina densa* is present beneath the syncytial distal cytoplasm of the tegument, whereas the intestinal epithelium is supported by a *lamina reticularis*. In the marine *Aporocotyle simplex*, a thin *lamina densa* and a thick *lamina reticularis* underlie the distal cytoplasm of the tegument and on the periphery of the ovary, but beneath the epithelial lining of the caeca and both genital and excretory ducts only a *lamina reticularis* is present. It is worth noting that Lindroos (1984) assumed that the fibrous basal lamina of the tegument in the cestode *Diphyllobothrium dendriticum* forms a large extracellular ‘compartment’ which also penetrates the muscle layers and is continuous throughout the worm. Subsequently, this view was supported by

Conn (1988, 1993), who suspected a myofibroblast origin for both the basal matrices and the ECM in neodermatans. This view is in full agreement with our own data on the ECM of the marine *A. simplex*. As we have shown in this species, a filamentous substance similar to the contents of a *lamina reticularis* fills the extracellular space and has a close relationship with subtegumentary circular and longitudinal muscle fibres. Moreover, for the first time, we have demonstrated morphological evidence indicating the participation of myofilaments in the formation of the ECM via the release of small aggregations of thin (actin) filaments from smooth muscle fibres and their subsequent breakdown into separate filaments, the fine morphology of which is identical to those of the ECM. In this respect, it is worth noting that, in the digeneans *Schistosoma mansoni* and *Fasciola hepatica*, the cytoskeletal protein actin has been demonstrated to occur beneath the distal cytoplasm of the tegument in the circular and longitudinal muscle layers (Abbas and Cain, 1987; Stitt et al., 1992).

The present study has shown the existence of significant variation in the development and amount of ECM in marine and freshwater aporocotylids, such that *Aporocotyle simplex* has a much better developed ECM than occurs in *Sanguinicola* sp. However, it is difficult to determine the reason for this difference, although in flatworms ECMs are known to be dynamic and structurally diverse units which depend not only on the species but also on the life-cycle stage (Pedersen, 1991; Conn, 1993). It is interesting that, in tapeworms, Conn and Rocco (1989) suggested a possible correlation between the abundance of dilated cisternae in the sarcoplasmic reticulum and the number or type of ECM components. For example, gravid specimens of the cestode *Oochoristica anolis* exhibited very few ECM components and lacked muscle cells with dilated cisternae (Conn and Etges, 1984). However, in the adult aporocotylid species studied here, both have muscle cells with dilated sarcoplasmic cisternae, but in *Sanguinicola* sp. the ECM components are scarce and only basal matrix components have been observed, whereas the ECM of *A. simplex* is well

developed and contains abundant filamentous components. The exocytosis of the contents of dilated cisternae into the extracellular zone occurs in both aporocotylids. Additionally, in *A. simplex,* thin myofilaments of muscle fibres participate in ECM formation and represent its dominant component. The presence of two mechanisms for ECM formation in the marine *A. simplex* and a single mechanism in the freshwater *Sanguinicola* sp. may be explained by the affiliation of these species to two different evolutionary lineages within the Aporocotylidae (i.e. marine teleost-infecting and freshwater teleost-infecting blood fluke lineages, as suggested by Cribb et al., 2017). Nevertheless, more investigations of a greater number of species from a wider range of aporocotylid genera are needed to confirm these ideas.

5. Conclusions

The present TEM study supports current opinions on the role of muscle in the ECM production of neodermatan worms, providing for the first time ultrastructural evidence for the participation of muscle cells in the formation of the ECM in two fish blood flukes belonging to different evolutionary lines of the Aporocotylidae. Two mechanisms for EMC formation are shown to occur in the marine teleost-infecting species *Aporocotyle simplex,* as opposed to a single mechanism in the freshwater teleost-infecting species *Sangiuinicola* sp. This finding may represent further evidence for the affiliation of these two taxa to divergent evolutionary lineages and may also explain different variations in the muscle cells of marine and freshwater aporocotylids.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank the staff of the RV 'Johan Ruud', belonging to Tromsø University (Norway), for their help with the fishing. Our thanks go to the staff of the Centre of Electron Microscopy at the I.D. Papanin Institute for the Biology of Inland Waters (Borok, Russia) for technical assistance. The study was funded by the Russian Foundation for Basic Research (RFBR) via Research Project No. 20-04-00086A.

References

Abbas, M.K., Cain, G.D., 1987. Actin and intermediate-sized filaments of the spines and cytoskeleton of *Schistosoma mansoni*. Parasitol. Res. 73, 66–74. https://doi.org/10.1007/bf00536338

Cohen, C., Reinhard, B., Castellani, B., Norton, P., Stirewalt, M., 1982. Schistosome surface spines are 'crystals' of actin. J. Cell Biol. 95, 987–988. https://doi.org/10.1083/jcb.95.3.987

Conn, D.B., 1988. The role of cellular parenchyma and extracellular matrix in the histogenesis of the paruterine organ of *Mesocestoides lineatus* (Platyhelminthes: Cestoda). J. Morphol. 197, 303–314. https://doi.org/10.1002/jmor.1051970305

Conn, D.B., 1993. The biology of flatworms (Platyhelminthes): parenchyma cells and extracellular matrices. Trans. Am. Microsc. Soc. 112, 241–261. https://doi.org/10.2307/3226561

Conn, D.B., Etges, F.J., 1984. Fine structure and histochemistry of the parenchyma and uterine egg capsules of *Oochoristica anolis* (Cestoda: Linstowiidae). Z. ParasitKde. 70, 769–779. https://doi.org/10.1007/bf00927130

Conn, D.B., Rocco, L.J., 1989. Fine structure of the cellular parenchyma and extracellular matrix of *Ophiotaenia loennbergii* (Cestoda: Proteocephalidea). Acta Zool. 70, 105–110. https://doi.org/10.1111/j.1463-6395.1989.tb01059.x

Cribb, T.H., Chick, R.C., O'Connor, W., O'Connor, S., Johnson, D., Sewell, K.B., Cutmore, S.C., 2017. Evidence that blood flukes (Trematoda: Aporocotylidae) of chondrichthyans infect bivalves as intermediate hosts: indications of an ancient diversification of the Schistosomatoidea. Int. J. Parasitol. 47, 885–891. https://doi.org/10.1016/j.ijpara.2017.05.008

Ehlers U., (1995) The basic organization of the Plathelminthes. Hydrobiologia 305, 21-26. https://doi.org/10.1007/bf00036358

Fujino T., Takamiya S., Fucuda K., Aoki T., 1996. Two types of parenchymal cells in the lung fluke *Paragonimus ohirai* (Digenea: Troglotrematidae) characterized by the cytochemistry of their mitochondria. Compar. Biochem. Physiol. 113B, 387–394. https://doi.org/10.1016/s1383-5769(97)82552-4

Grano-Maldonado, M.I., Bruno de Sousa, C., Roriquez-Santiago, M., 2018. First insights into the ultrastructure of myosin and actin bands using transmission electron microscopy in *Gyrodactylus* (Monogenea). J. Microsc. Ultrastruct. 6, 177–181.

Halton, D.W., 1967. Studies on glycogen deposition in Trematoda. Compar. Biochem. Physiol. 23, 113–120. https://doi.org/10.1016/0010-406x(67)90478-1

Korneva, J.V., Kornienko, S.A., Jones, M.K., 2016. Fine structure of uterus and non-functioning paruterine organ in *Orthoskrjabinia junlanae* (Cestoda, Cyclophyllidea). Parasitol. Res. 115, 2449–2457. https://doi.org/10.1007/s00436-016-4997-2

Kuperman, B.I., 1988. Functional Morphology of Lower Cestodes. Nauka, Leningrad, 166 pp. (In Russian.)

Lindroos, P., 1984. Observations on the extracellular spaces and intercellular junctions in *Diphyllobothrium dendriticum* (Cestoda). Acta Zool. 65, 153–158. https://doi.org/10.1111/j.1463-6395.1984.tb00820.x

Lindroos, P., Reuter, M., 1991. Extracellular matrix in some microturbellarians. Hydrobiologia 227, 283–290. https://doi.org/10.1007/978-94-011-2775-2_40

Lindroos, P., Still, M.J., 1988. Extracellular matrix components in *Polycelis nigra* (Turbellaria, Tricladida). Fortschr. Zool. 36, 157–162.

Lindroos P, Wikgren M. 1987. Extracellular matrix in platyhelminths with special reference to the presence of fibronectin. Acta Zool. 68: 147-151. https://doi.org/10.1111/j.1463-6395.1987.tb00885.x

Lumsden, R.D., Byram, J., 1967. The ultrastructure of cestode muscle. J. Parasitol. 53, 326–342. https://doi.org/10.2307/3276584

Lumsden, R.D., Foor, W.E., 1968. Electron microscopy of schistosome cercarial muscle. J. Parasitol. 54, 780–794. https://doi.org/10.2307/3277040

MacRae, E.K., 1965. The fine structure of muscle in a marine turbellarian. Z. Zellforsch. Mikrosk. Anat. 68, 348–362. https://doi.org/10.1007/bf00342552

Odhner, T., 1900. *Aporocotyle simplex* n. g. n. sp., ein neuer Typus von ektoparasitischen Trematoden. Centralbl. Bakteriol., 1. Abt., 27 (2), 62–66.

Olson, P.D., Cribb, T.H., Tkach, V.V., Bray, R.A., Littlewood, D.T.J., 2003. Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). Int. J. Parasitol. 33, 733–755. https://doi.org/10.1016/s0020-7519(03)00049-3

Orélis-Ribeiro, R., Arias C.R., Halanych K.M., Cribb T.H., Bullard S.A., 2014. Diversity and ancestry of flatworms infecting blood of nontetrapod craniates "fishes". Adv. Parasitol. 85, 1–62. https://doi.org/10.1016/B978-0-12-800182-0.00001-5

Pedersen, K.J., 1991. Invited review: Structure and composition of the basement membranes and other basal matrix systems in selected invertebrates. Acta Zool. 72, 181-201. https://doi.org/10.1111/j.1463-6395.1991.tb01196.x

Poddubnaya, L.G., Gibson, D.I., 2020. Are glial cells of the Digenea (Platyhelminthes) muscle cells? Parasitol. Res. 119, 317–319. https://doi.org/10.1007/s00436-019-06490-9

Poddubnaya, L.G., Hemmingsen, W., Bruňanská, M., Gibson, D.I., 2020a. Interrelations of vitelline and muscle cells within the vitelline follicles of the blood fluke *Aporocotyle simplex* (Digenea, Aporocotylidae) and morphological evidence for the modification of vitelline material for eggshell formation. Parasitol. Res. 119, 3967–3976. https://doi.org/10.1007/s00436-020-06849-3

Poddubnaya, L.G., Hemmingsen, W., Poddubny S.A., Gibson, D.I., 2019. Unique ultrastructural characteristics of the tegument of the digenean blood fluke *Aporocotyle simplex* Odhner, 1900 (Digenea: Aporocotylidae), a parasite of flatfishes. Parasitol. Res. 118, 2801–2810. https://doi.org/10.1007/s00436-019-06436-1

Poddubnaya, L.G., Mackiewicz, J.S., 2009. Ultrastructure of the cirrus sac of echinophallid tapeworms (Cestoda, Bothriocephalidea) and the terminology of cirrus hard structures. Int. J. Parasitol. 39, 381–390. https://doi.org/10.1016/j.ijpara.2008.07.008

Poddubnaya, L.G., Mackiewicz, J.S., Bruňanská, M., Dezfuli, S., 2005. Fine structure of the male reproductive ducts, vagina and seminal receptacle of *Cyathocephalus truncatus* (Cestoda: Spathebothriidea). Folia Parasitol. 52, 241–250. https://doi.org/10.14411/fp.2005.032

Poddubnaya, L.G., Zhokhov, A.E., Gibson D.I., 2020b. Ultrastructural features of aporocotylid blood flukes: The tegument and sensory receptors of *Sanguinicola inermis* Plehn, 1905 from the pike *Esox lucius*, with a comparative analysis of their traits within the Neodermata. Zool. Anz. 289, 108–117. https://doi.org/10.1016/j.jcz.2020.10.001

Reger, J.F., 1976. Studies on the fine structure of cercarial tail muscle of *Schistosoma* sp. (Trematoda). J. Ultrastruct. Res. 57, 77–86. https://doi.org/10.1016/s0022-5320(76)80057-3

Stitt, A.W., Fairweather, I., Trudgett, C.F., Anderson, S.M.L., 1992. Localization of actin in the liver fluke, *Fasciola hepatica*. Parasitol. Res. 78, 96–102. https://doi.org/10.1007/bf00931648

Stoitsova, S., Gochilova, L., 1997. Ultrastructure of the tegumental basement membrane of *Fasciola hepatica* (Trematoda). Acta Zool.78, 171–175. https://doi.org/10.1111/j.1463-6395.1997.tb01136.x

Sulbarán, G., Alamo, L., Pinto, A., Marquez, G., Méndez, F., Padrón, R., Craig, R., 2015. An invertebrate smooth muscle with striated muscle myosin filaments. PNAS 112, E5660–E5668. https://doi.org/10.1016/j.bpj.2013.11.911

Świderski, Z., Tkach, V., 1997. Differentiation and ultrastructure of the paruterine organs and paruterine capsules, in the nematotaeniid cestode *Nematotaenia dispar* (Goeze, 1782) Lühe, 1910, a parasite of amphibians. Int. J. Parasitol. 27, 635–644. https://doi.org/10.1016/s0020-7519(96)00185-3

Threadgold, L.T., 1984. Parasitic Platyhelminthes. *In*: J. Bereiter-Hahn, A.G. Matoltsky & K.S. Richards (eds.), *Biology of the Tegument*. *Vol. 1 Invertebrates*. Berlin, Heidelberg, Springer-Verlag, pp. 132–211.

Threadgold, L.T., Gallagher, S.S.E., 1966. Electron microscope studies of *Fasciola hepatica* – I. Ultrastructure and interrelationship of the parenchymal cells. Parasitology 56, 299–304. https://doi.org/10.1017/s0031182000070888

Willms, K., Robert, L., Caro, J.A., 2003. Ultrastructure of smooth muscle, gap junctions and glycogen distribution in *Taenia solium* tapeworms from experimentally infected hamsters. Parasitol. Res. 89, 308–316. https://doi.org/10.1007/s00436-002-0733-1

Wilson, R.A., 1969. Fine structure and organization of the musculature in the miracidium of *Fasciola hepatica*. J. Parasitol. 55, 1153–1161. https://doi.org/10.2307/3277247

Figure captions

Fig. 1. Ultrastructural characteristics of the muscle cells (myocytes) of *Sanguinicola* sp. (A, B) Tegumental muscle cell with a sarcoplasmic extension filled with muscle fibres. (C) Muscle cell showing a few dilated cisternae of the sarcoplasmic reticulum in the perinuclear cytoplasm and one sarcoplasmic extension filled with muscle fibres. (D) Muscle cell showing an accumulation of mitochondria and dense vesicles plus a sarcoplasmic extension containing muscle fibres; insert, detail of the sarcoplasm showing a Golgi complex and dense, rounded vesicles. (E) Muscle cells with pale areas of perinuclear cytoplasm and the absence of any sarcoplasmic extensions. (F, G) Sarcoplasmic extensions showing muscle fibres, large mitochondria, lipid droplet and a few glycogen granules, note the membranous whorls in the extracellular zone. *Abbreviations*: dc, dilated cisternae of sarcoplasmic reticulum; dv, dense vesicles; ez, extracellular zone; gc, Golgi complex; gl, glycogen granules; h, heterochromatin; ld, lipid droplet; m, mitochondrion; mf, muscle fibres; mw, membranous whorl; n, nucleus; pa, pale areas; pc, perinuclear cytoplasm; se, sarcoplasmic extension. *Scale bars*: A – E = 2 µm, insert = 0.5 µm; F, G = 1 µm.

Fig. 2. Extracellular matrix (ECM) of *Sanguinicola* sp. (A) Sagittal section of the tegument showing the distal tegumental cytoplasm, beneath which are circular muscle fibres in close vicinity, note the large extracellular zones between the muscle layers. (B) Hemidesmosomes connecting diagonal muscle fibres to the basal matrix. (C) Basal region of the distal cytoplasm of the tegument showing its basal membrane, a thin layer of basal matrix (*lamina densa*) and closely situated muscle fibres. (D) Transverse section of the tegument showing a thin basal matrix beneath the tegumental cytoplasm and both longitudinal and diagonal muscle fibres which are attached to the basal matrix by hemidesmosomes. (E) Extracellular

zone between the muscle and vitelline cells. (F) Basal matrix (*lamina reticularis*) around the epithelial lining of an intestinal caecum. (G, H, I) Exocytosis of the contents of dilated cisternae through the sarcolemma into an extracellular zone. (J) Region of the nerve cord surrounded by muscle fibres. *Abbreviations*: bm, basal membrane; cm, circular muscles; dc, dilated cisternae of sarcoplasmic reticulum; dm, diagonal muscles; edc, exocytosis of contents of dilate cisterna; ez, extracellular zone; hd, hemidesmosome; ie, intestinal epithelium; ld, *lamina densa* of basal matrix; lm, longitudinal muscles; m, mitochondrion; mf, muscle fibres; mm, moderately dense material; mw, membranous whorl; nc, nerve cord; pc, perinuclear cytoplasm; tc, tegumental cytoplasm; v, vesicles. *Scale bars*: A – F, I = 1 µm; G, H, J = 0.5 µm.

Fig. 3. Muscle cells of *Aporocotyle simplex*. (A – D) First type of muscle cell. (A, B) Muscle cells, note the sac-like cisternae of the sarcoplasmic reticulum distributed along the cell periphery. (C, D) Exocytosis of the contents of sac-like cisternae into the extracellular zone. (E – G). The second type of muscle cell. (E) Sarcoplasmic extensions showing lipid droplets surrounded by membranous whorls. (F) Linear pattern in the structure of the perinuclear sarcoplasm. (G) Large, dense areas of the perinuclear sarcoplasm with a linear patterned structure. (H) Extracellular zone between the testes, note the sarcoplasmic extensions of the second and third muscle cell types which are surrounded by the moderately dense contents of the ECM. (I) Third type of muscle cell showing sarcoplasmic extensions with electron-lucent cytoplasm and muscle fibres. *Abbreviations*: dc, dilated cisternae of sarcoplasmic reticulum; ecm, extracellular matrix; edc, exocytosis of contents of dilated cisternae; h, heterochromatin; lcp, linear patterned crystalline structure; ld, lipid droplet; m, mitochondrion; mf, muscle fibres; mw, membranous whorl; n, nucleus; pc, perinuclear cytoplasm; se, sarcoplasmic extension; se2, sarcoplasmic extension of second type of muscle cell; se3, sarcoplasmic

extension of third type of muscle cell. *Scale bars*: A, B, G – I = 2 µm; C, D = 0.5 µm; E, F = 1 µm.

Fig. 4. Extracellular matrix (ECM) of *Aporocotyle simplex*. (A) Section through a spine boss, note the network of diagonal fibres surrounding each spine within the boss. (B) Area beneath the distal tegumental cytoplasm, the extracellular zone of which is filled with abundant, evenly distributed filaments. (C) ECM network beneath the thin *lamina densa*. (D) ECM beneath the caecal epithelium of the intestine. (E) Flattened sarcoplasmic extensions filling the zone between two female reproductive ducts, note the extracellular matrix and small number of muscle fibres surrounding one duct. (F) Hemidesmosomes connecting the diagonal muscle fibres of a boss to the *lamina densa*. (G) ECM surrounding a female reproductive duct. (H) Thick EMC, sparsely distributed muscle fibres and flattened sarcoplasmic extensions around an excretory duct. (I) Muscle cells close to the ovary, note the *lamina densa* and thick EMC. (J) *Lamina densa* and thick EMC network surrounding the ovary. *Abbreviations:* bm, basal membrane; cm, circular muscles; dm, diagonal muscle fibres; ecm, extracellular matrix; ede, excretory duct epithelium; fse, flattened sarcoplasmic extensions; gde, genital duct epithelium; hd, hemidesmosome; ie, intestinal epithelium; ld, *lamina densa* of basal matrix; lm, longitudinal muscles; mc, muscle cell; mf, muscle fibres; mw, membranous whorl; n, nucleus; ov, ovary; pc, perinuclear cytoplasm; se, sarcoplasmic extension; sl, surface lamellae; tc, tegumental cytoplasm. *Scale bars*: A = 5 µm; B, D, H, I = 2 µm; C, E, F, G, J = 1 µm.

Fig. 5. Connection between the tegumental musculature and the ECM in *Aporocotyle simplex*. (A) Circular and longitudinal tegumental muscle fibres closely surrounded by ECM. (B) Two concentrations of myofilaments along the margin of a muscle fibre and one similar concentration in the extracellular zone. (C) Concentration of myofilaments budding off from a

muscle fibre, note the loosely packed aggregation of myofilaments degenerating into separate filaments in the extracellular zone. (D) More detail of an aggregation of myofilaments degenerating into separate filaments. (E) Muscle fibre with a region of sparsely distributed independent myofilaments. (F) Muscle fibre with degenerating myofilaments. (G) Margin of muscle fibre with an oval aggregation of myofilaments. (H) Muscle fibre with a marginal aggregation of myofilaments, note two additional aggregations in the extracellular zone with independent filaments around them. (I) Muscle fibre surrounded by a dense, narrow, branching region of homogeneous sarcoplasm around oesophagus. *Abbreviations*: bne, narrow, branching sarcoplasmic extensions; dmf, degenerating muscle fibre; ef, extracellular filaments; fc, filamentous concentration; mf, muscle fibre; sf, isolated filaments. *Scale bars*: A, E, F = 1 µm; B – D, G, H = 0.5 µm; I = 2 µm.