# Comparative analysis of the cytoarchitecture of the excretory bladder of adult Digenea (Platyhelminthes) with consideration of the presence of mineralized excretory corpuscles in marine and freshwater adult worms

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**Abstract**

The ultrastructural differences are shown between the cytoarchitecture of the excretory bladder and excretory inclusions in four digenean species, two azygiids, the marine *Otodistomum cestoides* and the freshwater *Azygia lucii*, the marine derogenid *Derogenes varicus* and the freshwater allocreadiid *Acrolichanus auriculatus*. The unusual cytoarchitecture of the bladder epithelium of the azygiid digeneans, consisting along its entire length of two alternating, morphologically different zones, tegumental and cellular excretory epithelial zones, connected by septate junctions, has recorded for the first time for the Digenea and, in general, for the Neodermata. It, possible, suggests the participation of the tegumental distal cytoplasmic layer in the formation of their excretory bladder epithelium. Like most digeneans, the excretory bladder of *A. auriculatus* and *D. varicus* has a syncytial epithelial lining. Based on available literature and our own results, we can confirm the presence of the excretory corpuscles in adult marine digeneans and their absence from freshwater species, regardless of the digenean localization in their host. The present study shown that in marine digeneans, the excretory corpuscles are associated with specialized excretory cells or excretory syncytial epithelium. Ultrastructural data were obtained on the possible growth of the excretory bladder epithelium due to the migration of undifferentiated cells into the epithelial lining in studied marine species. We may assume that the bladder epithelium of marine adult digeneans specializes, in addition to the excretory function, in osmoregulatory function.

**Key words**: TEM SEM Excretory bladder *Otodistomum cestoides* *Azygia lucii* *Derogenes varicus* *Acrolichanus auriculatus*

## 1. Introduction

The processes of biomineralization take place in the neodermatan flatworms. As a result of such biomineralization, mineralized structures made of concentric layers ('excretory concretions', 'excretory corpuscles' or 'calcareous corpuscles') were described in adult and larval Trematoda (see Martin and Bils, 1964; Gibson, 1973; Mattison et al., 1992) and in Cestoda (see McCullough and Fairweather, 1987). Formation of such structures in the Neodermata has been divided into two types, occurring extracellularly, in the lumen of the excretory bladder in trematodes (see Martin and Bils, 1964), rarely in the lumen of the excretory ducts in some cestodes (Etges and Marinakis, 1991; Vargas-Parada et al., 1999), and intracellularly, in the 'parenchymal or mesenchymal' cells in most cestodes (see McCullough and Fairweather, 1987). Concerning the chemical nature of neodermatan corpuscles, available data indicates that these mineralized structures in cestodes consist of an organic matrix (proteins) together with a high level of inorganic concentric material, mainly calcium, magnesium and phosphorus (Yamane et al., 1988; Smith and Richards, 1993; Yang, 2000). Numerous possible functions have been ascribed to these corpuscles in tapeworms (see Chowdhury and De Rycke, 1977), but still their presence/absence and functions in adult marine and freshwater trematodes are poorly understood (see Gibson 1973, Mattison et al., 1992).

There is little information on the fine structure of the excretory bladder in adult marine and freshwater trematodes. It has been shown that the trematodes have a syncytial bladder epithelium and distally the bladder lining is connected to the excretory pore tegument by septate junctions (Gibson, 1973; Bennett, 1977; Powell, 1979; Soboleva et al., 1988; Podvyaznaya, 1989; Mattison et al., 1992).

The ultrastructure of the excretory bladder of azygiid trematodes has not been studied previously. The azygiid bladder possesses a number of fine structural characteristics that

differ from those of other trematodes, which is particularly interesting. The ultrastructural investigation of the excretory bladder of two adult azygiid species, the marine *Otodistomum cestoides* (Van Beneden, 1870) Odhner, 1911 and the freshwater *Azygia lucii* (Müller, 1776) Lühe, 1909, are presented in this study. In addition to the previous few studies, we studied the excretory bladder of the marine derogenid *Derogenes varicus* (Müller, 1784) Looss, 1901 and the freshwater allocreadiid *Acrolichanus auriculatus* (Wedl, 1858) for comparative analysis.

## 2. Material and methods

Specimens of *O. cestoides* were obtained from the stomach and intestine of the elasmobranch *Amblyraja radiata* (Donovan, 1808) trawled from the Norwegian Sea off Tromsø, Norway, during June 2017. Specimens of *A. lucii* were obtained from the stomach of the northern pike *Esox lucius* (Linnaeus, 1758) trawled from the Rybinsk reservoir of the Upper Volga River during October 2021. Additionally, specimens of *D. varicus* were obtained from the intestine of the long rough dab *Hippoglossoides platessoides* (Fabricius, 1780) trawled from the Norwegian Sea off Tromsø, Norway, during June 2017, and specimens of *A. auriculatus* were obtained from the intestine of the sterlet sturgeon *Acipenser ruthenus* from River Irtysh of the Siberian Ob River Basin, Russia during summer 2022.

For electron microscopy, the specimens of *O. cestoides*, *A. lucii*, *D. varicus* and *A. auriculatus* were fixed directly in 3% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2) for 5 days at 5° C, rinsed twice for 20 min in the same buffer, and postfixed in 1% osmium tetroxide for 1 hr. Fixed specimens were dehydrated in a graded ethanol series with a final change to absolute acetone. After fixation and dehydration, for scanning electron microscopy (SEM) seven specimens of *O. cestoides* were critical-point-dried desiccated using a HCP-2 critical point dryer (Hitachi, Tokyo, Japan). Four dried big specimens were cut with

a blade longitudinally for topographical examination of their internal structure. Later, all specimens were mounted on stubs, sputter-coated using an JFC 1600 Auto Fine Coater (JEOL Ltd., Tokyo, Japan) with gold-palladium (15-20 µm in thickness), and examined using a JEOL-JSM-6510LV microscope (JOEL Ltd., Tokyo, Japan) at 30kV. For transmission electron microscopy (TEM), five specimens from each trematode species, *O. cestoides*, *A. lucii*, *D. varicus* and *A. auriculatus* 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 JOEL JEM 1011 microscope (JOEL Ltd., Tokyo, Japan) at 80 kV.

## 3. Results

### *3.1. Excretory bladder of marine Otodistomum cestoides*

The excretory bladder of *O. cestoides* is Y-shaped. The terminal excretory pore leads into a long bladder stem (Fig. 1A, D), which bifurcates at the level of the post-testicular region into two ducts (bladder arms), which extend anteriorly to near the anterior of the body. Along its entire length, the excretory bladder epithelium consists of two zones, namely excretory bladder cells and tegumental distal cytoplasm, which are interconnected by septate junctions (Figs. 1G, H and 2A - C).

The lumen of the long bladder stem of adult *O. cestoides* contains excretory corpuscles and lipid droplets (Fig. 1B, C, E, F, G, I). A smaller number of these inclusions is present in the bifurcated bladder ducts (Fig. 2G). Excretory corpuscles are rounded or slightly oval in shape and vary in diameter from 0.5 µm in emerging corpuscles to 5.5 µm in formed ones

(Fig. 1B, C, E, F, G, I). The excretory corpuscles are built up of concentric rings of granular or fibrous material of variable density (Figs. 1I and 2G, I). The inner structure of individual corpuscles varies considerably depending upon the degree of their development (Figs. 1H, J and 2G, I). The outer surface of corpuscles is covered by a thin layer of flocculent material (Fig. 2H, I). Lipid droplets of large adult worms are slightly oval in shape and vary in size from 2.2 x 2.0 to 9.0 x 8.5 μm (Fig. 1F, G). These lipid droplets contain heterogeneous content, consisting of a mixture of moderately electron-dense and electron-lucent material (Fig. 1F, G). In smaller adult worms the rounded lipid droplets are electron-dense and vary in diameter from 0.7 to 5.0 μm (Fig. 1C).

The epithelial lining of the excretory bladder cells varies in thickness from 1.0 to 6.5 μm. Its luminal plasma membrane is elevated by surface protrusions of different sizes and shapes and is thrown up into lamellae projecting into the lumen to form a lamellar layer between 3.8 and to 5.8 μm high above its epithelial surface (Figs. 1F, I and 2I). The lamellae are abundant in the bladder stem and less so in the bifurcated bladder arms (Fig. 2G). In each excretory bladder cell there is a large nucleus, extensive endoplasmic reticulum, numerous ribosomes, occasional Golgi complexes, small mitochondria, and a few small electron-dense and electron-lucent vesicles (Figs. 1I and 2A, E). Lipid droplets of different sizes occur within cell cytoplasm (Figs. 1C, F and 2C, E, G). Occasionally, they may be present inside the nucleus of excretory bladder cells and in the cytoplasm of such cells there are myelin-like whorls; such cells possess features to suggest that they are undergoing degeneration (Fig. 2F). Different stages of lipid droplet protrusion into the bladder lumen may be observed in their final stage when they are released into the bladder lumen (Fig. 1C, F). Lipids discharge into the lumen by apocrine secretion during which the lamellae in contact with the droplets form a base surrounding the droplet (Figs. 1F, I and 2G, I). The formation of the excretory corpuscles is associated with the surface lamellae of the excretory cells; between lamellae are small

gatherings of finely dispersed material (Figs. 1C, I and 2H, I). In addition, secretory inclusions of different kinds (small electron-lucent vesicles and electron-dense bodies) were observed in the bladder excretory cells (Figs. 1I and 2E, I). The vesicles are discharged into the lumen via exocytosis associated with excretory cells (Fig. 2I). Within the bladder lumen, these inclusions may be attached to finely dispersed material, around which the corpuscle material becomes organized into concentric layers (Fig. 2H, I).

The anucleate zones of the tegumental cytoplasm of the bladder epithelium are structurally similar to that of the tegumental body cytoplasm, differing in its thickness from 3.5 to 9.0 μm in the bladder epithelium while the tegumental body lining is from 25 ± 38 μm thick. The surface of the tegumental epithelium is smooth and folded (Figs. 1G and 2A - C). The tegumental cytoplasm contains numerous small tegumental vesicles of electron-lucent, moderately dense and dense content; occasionally a lipid droplet may be observed in its apical part close to adjacent excretory cells (Fig. 2A). The excretory bladder cells are joined to the tegumental epithelium by septate junctions, which may see along the whole bladder length (Figs. 1G, H and 2A – C). The bladder epithelium is supported by basal lamina and muscle fibres (Figs. 1F, G, I and 2A - C).

It is of interest to note that throughout the bladder epithelium, undifferentiated excretory cells may localized beneath and close to the bladder epithelial cytoplasm (Fig. 2B – D). These cells are characterized by a large nucleus and a thin area of perinuclear cytoplasm (Fig. 2B, D). Thereafter the gradual intrusion of undifferentiated cells may be observed throughout the bladder stem epithelium (Fig. 2B, C).

*3.2. Excretory bladder of freshwater Azygia lucii*

The excretory bladder of *A. lucii* is Y-shaped. The terminal excretory pore leads into a long terminal stem, bifurcating at the level of posterior testis into two bladder arms, which extend

anteriorly to near the anterior body extremity. The distal portion of the terminal stem is formed with the body tegumental, syncytial, anucleate lining, containing the same cytoplasmic inclusions and smooth luminal surface similar to those observed in the body tegument (Fig. 3A, D). However, the thickness of the body lining varies from 9.0 to 15.0 μm while the excretory tegumental lining varies from 3.0 to 5.0 μm in thickness. The narrow lumen of the distal terminal stem is filled with electron-lucent vesicles (Fig. 3D). Subsequent excretory bladder lining consists of two alternating zones, which are distinguished morphologically (Fig. 3B, C, E, F, H). It includes the tegumental and excretory bladder epithelial zones connected by septate junctions (Fig. B, E, H). The luminal surface of anucleate tegumental lining is smooth and slightly folded, varying in thickness from 1.0 to 4.5 μm. Electron-dense rod-shaped bodies and small vesicles of different content fill the tegumental cytoplasmic lining (Fig. 3B, E, H).

The folded excretory bladder epithelial lining is from 0.8 to 1.5 μm in thickness and cellular (Fig. 3B, E, H). The excretory cell epithelial surface is distinguished by lamellar outgrowths often widened apically and from 1.2 to 3.0 μm long (Fig. 3B, C, E). Each excretory cell contains a large nucleus, which often bulges into the bladder lumen, thereby increasing the epithelial thickness to 8.5 μm (Fig. 3E, H). Wherein, thin prolongations of the tegumental cytoplasm remain from both sides of such excretory cell, and septate junctions are prominent between the different plasma membranes (Fig. 3C, E, H). The excretory cell cytoplasm is recognized by the presence of clusters of large, rounded electron-lucent vesicles, Golgi complexes and mitochondria (Fig. 1B, I). Individual electron-dense lipid droplets (about 2.8 μm in diameter) may observe in the excretory cell cytoplasm and bladder lumen of proximal bladder arms (Fig. 3G, J). The bladder epithelial lining is surrounded by basal lamina and muscle fibres (Fig. 3B, E). Small lumps of electron-dense material are occasionally observed in the lumen of the bladder lining close to the excretory cells (Fig. 3E).

### *3.3. Excretory bladder of freshwater Acrolichanus auriculatus*

*A. auriculatus* has an I-shaped excretory bladder, which extends into the seminal receptacle. An epithelial lining around and within the terminal excretory pore is lined with tegument (Fig. 4A, B, D). The epithelial lining of the excretory pore possesses the same ultrastructural characteristics such as distal syncytial cytoplasm of the body tegument, characterized by the presence of a smooth surface with a thin electron-dense layer on the surface membrane, a large apical mitochondrion in each fold of the cytoplasmic lining, and the presence of several cytoplasmic electron-dense rod-shaped bodies and electron-lucent vesicles (Fig. 4B). Below the distal tegumental cytoplasm, a thin moderately dense basal lamina and a large layer of the basal interstitial matrix, in which circular and longitudinal muscle fibres are embedded, are present (Fig. 4B, D). This is distinguished from the body tegument by increased folding of the tegumental lining of the pore and pore cavity, which extends into the worm's body up to the connection with the excretory bladder epithelium (Fig. 4 C – E). Two different kinds of the abovementioned epithelial linings are connected by septate junctions (Fig. 4C). The enlarged lumen of the excretory bladder of *A. auriculatus* is lined with a thin syncytial epithelial lining varying in thickness from 0.2 to 0.5 μm, the luminal surface of which forms finger-like protrusions from 0.4 to 0.6 μm long (Fig. 4C, E, F). The mitochondria, a few moderately dense rounded vesicles and ribosomal gatherings may be observed in the syncytial cytoplasm (Fig. 4F). No lipid droplets are to be seen in the bladder epithelial lining or in the bladder lumen (Fig. 4E, F), although a few electron-dense lipid droplets (about 0.4 μm in diameter) may be observed within the cytoplasm of the epithelial lining of the excretory ducts. Excretory corpuscles are absent from the lumen of the excretory bladder as well as from the excretory ducts.

*3.4. Excretory bladder of marine Derogenes varicus*

The excretory bladder of *D. varicus* is Y-shaped. It bifurcates at the level of the gonads and its two arms extend to the level of the esophagus. The excretory pore is terminal leading into the terminal duct within the worm's body (Fig. 4G). The latter is continuous with the tegumental syncytial cytoplasm of the body and its luminal surface is relatively smooth (Fig. 4H, K). The thickness of this cytoplasmic lining varies from 3.0 to 6.0 μm and the epithelial cytoplasmic matrix is filled with small electron-dense and moderately-dense bodies and vesicles of heterogeneous content (Fig, 4H, K). In addition, vacuoles with homogeneous, moderately dense content may be observed in the tegumental cytoplasm, and within the bladder lumen free portions of tegumental cytoplasm exhibit degenerative changes (Fig. 4H, K). Also, the transition zone between the short terminal duct and the distal portion of the excretory bladder epithelium consists of mixed tegumental and excretory bladder epithelium (Fig. 4H, K). The different epithelial linings are connected with each other via apical septate junctions (Fig. 4K). The bladder epithelium is a syncytial layer, forming abundant foldings covered with lamellae, due to which its thickness varies from 0.3 to 1.8 μm (Fig. 4H, K, L). The large epithelial nuclei are scattered along the whole excretory bladder length (Fig. 4I, L). They are usually located in protruding epithelial thickenings extending deep into the bladder lumen (Fig. 4I). Not infrequently, the protruding nucleus surrounded by cytoplasmic inclusions projects deep into the bladder lumen and connects with the epithelium by thin cytoplasmic processes, and free cytoplasmic fragments with a nucleus may be seen within the excretory bladder lumen (Fig.4I, L). Numerous large, rounded vesicles, possessing moderately dense, uniformly distributed fine granular material with point inclusions of electron-dense material, are dominant inclusions of the cytoplasmic epithelial lining of the excretory bladder (Fig. 4K, insert, L). The diameter of individual vesicles varies between 0.3 – 1.0 μm. Larger vesicles arise by the fusion of smaller ones (Fig. 4K). Moreover, there are

vacuole-like structures up to 3.0 μm in diameter in the bladder epithelial cytoplasm (Fig. 4I, K, L). Fusion of vesicles with vacuole-like structures can be observed (Fig. 4I, K). Morphologically, the content of these structures resembles those of the abovementioned vesicles, although there are some electron-dense inclusions within them (Fig. 4I, K, L). We saw clear morphological evidence to suggest that these structures discharge into the bladder lumen, where they are present (Fig. 4I, K, L). Close to the excretory bladder epithelial lining, between lamellar projections, there are a number of excretory corpuscles (Fig. 4H – J, L). Most rounded excretory corpuscles of *D. varicus* are distinguished by few concentrically arranged peripheral layers with a central non-lamellated area of flocculent material, the presence of a thin layer of flocculent material on its outer surface, and with a diameter of from 0.4 to 1.2 μm (Fig. 4J). The present study indicates that the corpuscle precursor is accumulations of electron-dense, finely dispersed material scattered anywhere between the surface lamellae of the excretory bladder epithelial lining (Fig. 4J). The bladder epithelial lining is supported by the basal lamina and two muscle layers of circular and longitudinal muscles (Fig. 4H, I, L). Undifferentiated cells may be observed beneath the distal bladder epithelium (Fig. 4L).

## 4. Discussion

### *4.1. Variations of the cytoarchitecture of the bladder epithelium in the Digenea*

The present study of the ultrastructural organization of the excretory bladder of four adult digenean species belonging to the families Azygiidae, Allocreadiidae and Derogenidae demonstrates the differences in the fine structure of their excretory bladder. From the available literature, in the case of most larval and adult digeneans previously studied, the excretory bladder epithelium is a nucleated syncytial lining (Erasmus 1967; Gibson 1973;

Soboleva et al. 1988; Podvyaznaya 1989; Mattison et al. 1992; Niewiadomska and Czubaj 2000). In common with most digeneans, the excretory bladders of the examined allocreadiid *Acrolichanus auriculatus* and derogenid *Derogenes varicus* have syncytial epithelial lining. Like in most digeneans, in *A. auriculatus* the syncytial bladder epithelium connects with the tegumental syncytial cytoplasm, which leads from the excretory pore, by a junctional complex. However, there are some variations in the interaction of these two epithelial types in the distal bladder part of the digeneans. In *D. varicus* (present study) as well as in the previously studied allassogonoporid *Allassogonoporus amphoraeiormes* (Podvyaznaya 1989) the tegumental syncytial lining is replaced gradually with epithelium of the excretory bladder, where alternating different types of epithelia are present to form some septate junction contacts between them.

Interestingly, in the studied two azygiid species*, Otodistomum cestoides* and *Azygia lucii*, along the entire bladder length its epithelial lining consists of two alternating, morphologically different zones, tegumental and excretory, which are interconnected by septate junctions. Such cytoarchitecture of the bladder epithelium is described for the first time in the Digenea as well as in the Neodermata. Here it is appropriate to talk about the participation of tegument in the formation of the epithelial wall of the excretory bladder of azygiids. It should be emphasized that two types of the excretory bladder were found in digenean cercariae, with non-epithelialized and epithelialized walls (see La Rue 1957). As the present study has shown, the walls of the large excretory bladder of azygiids are clearly epithelialized and formed by two epithelial types, tegumental distal cytoplasm mixed with specialized excretory cells, whereas in other digenean species studied to date the excretory bladder is lined with a nucleated syncytial epithelial wall as a continuation of the walls of the main collecting excretory ducts (Erasmus 1967; Soboleva et al. 1988, Mattison et al 1992). Interestingly, schistosomatids do not have the excretory bladder instead there is an excretory

atrium, the walls of which are derivatives of the tegumental distal cytoplasm of the body surface (see Powell 1979). Concerning bladder epithelial growth in azygiids, we may assume the existence of intercalary growth when the excretory cells are formed from undifferentiated cells, possibly representing embryonic excretory cells. In *O. cestoides* we observed the presence of undifferentiated cells immediately beneath the bladder epithelial lining. Occasionally, the enveloping of undifferentiated cells by the invaginated basal lamina may observed, which we may considere as a stage of their gradual intrusion into the bladder epithelium. The same mechanism associated with growth of the excretory bladder in developing juveniles of the fasciolid *Fasciola hepatica* observed by Bennett (1977). Moreover, in *O. cestoides* the excretory cells may observed in different stages of their development throughout the bladder epithelium. Also, such mechanism of cell renewal is known to occur in the formation of the caecal epithelia of polyopistocotylean and polystomatid monogeneans (see Tinsley 1973; Brennan and Ramasamy 1995; Poddubnaya et al. 2015).

Variations in the ultrastructural organization of the bladder epithelial lining of different digenean species have been observed. For example, in the distal bladder part of the marine *D. varicus* the epithelial nuclei surrounded by a cytoplasmic area may project deep into the lumen with its subsequent elimination by a holocrine-like process, and no degradation of the bladder epithelium was observed. Probably, this is a result of renewal of the distal bladder epithelial lining. The presence of undifferentiated cells below the distal bladder epithelium may support such occurrence. However, bulging nucleated portions are also present in the bladder epithelium of *A. lucii* (present study), *A. amphoraeiormes* (Podvyaznaya 1989) and in the cercarial excretory bladder of *Bucephaloides gracilescens* (Podvyaznaya and Galaktionov 2004), but in these species an elimination of nucleated portion into the bladder lumen was not observed.

The surface lamellae have been interpreted as common luminal projections of the epithelial excretory bladder wall (Erasmus 1967; Gibson 1973; Bennett 1977; Powell 1979; Soboleva et al. 1988; Mattison et al. 1992; Podvyaznaya and Galaktionov 2004). This is also true for the studied azygiid and derogenid species (present study), but in the allocreadiid *A. auriculatus* the luminal plasma membrane is formed into finger-like protrusions (present study). Mattison et al. (1992) speculated that the surface lamellae of the digenean excretory bladder may facilitate fluid and nutrient reabsorption. Moreover, histochemical tests have shown that acid and alkaline phosphatases have localized on the bladder lamellae (Erasmus 1967; Mattison et al 1992). Powell (1979) commented that the highly folded and lamellated bladder epithelial surface strongly suggests surface area amplification for secretion/absorption in the adult digeneans.

*4.2. Variations in the excretory inclusions of the bladder epithelium in marine and freshwater adult digeneans*

The results of the present paper add to the previous scanty knowledge on the excretory inclusions of adult digeneans inhabiting marine and freshwater definitive hosts. Only one study was previously published on the excretory bladder of a marine species, *Podocotyle staffordi,* obtained from the gut of the flounder *Platichthys flesus* (Gibson 1973), in which the author indicated the presence of excretory corpuscles in the bladder lumen. In our study two additional marine digenean species have been investigated, *Otodistomum cestoides* and *D. varicus.* As in *P. staffordi*, excretory corpuscles are the main component of the bladder lumen in both species investigated by us and these corpuscles are associated with specialized excretory bladder epithelium. As noted by Gibson (1973), the digeneans parasitic in the gut of marine fishes are subject to an environment of varying osmolarity. It should be emphasized that among the three marine species studied to date, the largest amount of excretory

corpuscles with most concentric layers was detected in *O. cestoides* parasitic in elasmobranchs. Interestingly, the concentration of NaCl in body fluids of marine teleosts is approximately equal to that of seawater, and they actively secrete salt and retain water to maintain osmotic homeostasis. Wherein, the marine teleosts keep their body fluids at a lower level by absorbing the water and monovalent ions in the gut and then excreting the latter via the gills (Kültz 2015). On the contrary, in elasmobranchs NaCl concentration is less than half that of seawater and the osmotic gap is filled by active accumulation of compatible organic osmolytes, maintaining the difference in NaCl content (relative to seawater) by active NaCl secretion via the rectal gland (Kültz 2015). In *O. cestoides* the extensive excretory bladder is equipped with specialized secretion/absorption excretory cells throughout its entire length, constantly changing the dynamics of their numbers due to the differentiation of surrounding undifferentiated cells. Such a mechanism is reminiscent of the adaptation of teleost fish to changing water salinity due to the presence of specialized chloride cells at the base of the gills (Zavarzin, 1985). The main mechanism for adaptation to water salinity is an increase in the number of chloride cells due to the rapid maturation of undifferentiated cells and their differentiation. With an increased concentration of ions in tissue fluids, maturation of cellular elements occurs with a simultaneous outbreak of proliferation of undifferentiated cells and rapid differentiation of the resulting cells (Zavarzin 1985). As shown in our results, in the second widely distributed species of marine teleosts, *D. varicus*, the lumen of the excretory bladder contains numerous small excretory corpuscles possessing few concentric layers or not layered. We also observed morphological evidence of the secretory activity of the distal bladder epithelium in *D. varicus*. So, in the studied marine digeneans *O. cestoides* and *D. varicus*, the presence of undifferentiated cells immediately beneath the bladder epithelial lining and their possible intrusion into the bladder epithelium may assume as a possible permanent process of its renovation in marine adult digeneans.

On the other hand, based on available literature and our present investigation, we may confirm that in freshwater adult digeneans the excretory concretions are not present. This statement is true for the following species: the azygiid *Azygia lucii* from the stomach of pikes and the allocreadiid *A. auriculatus* from the intestine of sterlets (present study), the fasciolid *Fasciola hepatica* from the mouse (Pantelouris and Threadgold 1963, Bennett 1977); the cyathocotylid *Cyathocotyle bushiensis* from the gut of the duck (Erasmus 1967); the ochetosomatid *Ochetosoma aniarum* from the mouth of water snakes (Powell 1979); the brachylaimid *Brachylaimus aequans* from the intestine of laboratory mice (Soboleva et al. 1988); the lecithodendriid *Prosthodendrium mirabile* and the allassogonopoid, *Allassogonoporus amphoraeformis* from the intestine of bats (Podvyaznaya 1989). In spite of the variety of the digenean localization in their host, none of them are affected by salinity as much as marine digeneans.

We support the opinion of Gibson (1973) that excretory corpuscles of adult marine digeneans may help with osmoregulation due to the high salt content of the intestine environment and this is a reason why excretory cells of marine digeneans have a mobile mechanism to regulate the formation and quantity of excretory corpuscles. It is appropriate to note that in the marine aporocotylid digenean *Aporocotyle simplex*, blood parasites of the marine long rough dab, there are no excretory corpuscles (unpublished data by Poddubnaya L.) due to the presence of osmoregulatory proteins in the fish blood (Khlebovich 1974). Therefore, the protein systems of the blood of the marine fish are organized in accordance with the level of mineralization of their external aquatic environment, taking into account the salt composition of the internal liquid environment (Khlebovich 1974).

It is generally recognized that osmoregulatory and excretory functions are based on the processes of active ion transport and the processes of endo- and ectocytosis in animals (Zavarzin 1985). This is also true for the flatworms, including the flukes, in which the

osmoregulatory and excretory functions are realized by the epithelium of the protonephridial excretory ducts. The specialization of their excretory cells comes down to the hypertrophy of cellular organelles responsible for transmembrane transport and hypertrophy of surface membranes. The distal part of the protonephridial system, the excretory bladder, ensures the regulation of water and salt metabolism in flatworms. As shown in our results, the excretory bladder of adult marine digeneans provides adaptive regulation of salt metabolism in extreme salt conditions due to the formation of mineralized excretory corpuscles.

It should be noted that the excretory bladder epithelium is a highly secretory epithelium showing a change in the type of excretory product during parasite development (see Gibson 1973, 1974; Powell 1972, 1977, 1979). It would appear that in the metacercarial stage the appearance of the excretory corpuscles has been observed in most digenean metacercariae studied to date (Martin and Bils 1964; Erasmus 1967; Bennett 1977; Powell 1979; Mattison et al. 1992; Niewiadomska and Czubaj 2000). According to Erasmus (1967) the corpuscle calcium carbonate of cyathocotylid metacercariae of *Cyathocotyle bushiensis* may be derived from the host's fluids, since calcium carbonate is the main material of mollusk shells (Wilbur 1960) and may be available to larval digeneans from their first intermediate hosts. The corpuscle material may be used for the formation of metacercarial cyst (Leong and Howell 1971, Benjamin and James 1987).

Excretory lipid droplets are not always present in the excretory bladder of the adult digeneans. In the examined digeneans, lipid accumulation occurred in the bladder epithelium and bladder lumen of the marine azygiid *O. cestoides*, but in the freshwater azygiid *A. lucii* not numerous lipid droplets only were observed in the bladder epithelial cytoplasm and in the bladder lumen of the bladder arms. In both azygiids the lipid droplets increase in size due to the merging of droplets of different sizes into one. For *O. cestoides* we have clear morphological evidence to suggest that lipid droplets are produced in the bladder epithelium

and discharged into the bladder lumen by apocrine secretion. Not unfrequently, an accumulation of 3 - 5 lipid droplets within the nucleus of the excretory cells was seen in *O. cestoides*. Previously, lipid excretion has been recorded in the adult cyathocotylid *C. bushiensis* (Erasmus 1967), the fasciolid *F. hepatica* (Bennett 1977), the lecithodendriids *P. mirabile* and *P. ascidia* (Podvyaznaya 1989), and the paramphistomids, *Paramphistomum epiclitum* and *Fischoederius elongates* (Mattison et al. 1992). No lipid droplets were observed in the examined adult freshwater allocreadiid *A. auriculatus* and in the marine derogenid *D. varicus* (present study), in the adult marine *P. staffordi* (Gibson 1973) and the brachylaimid *B. aequans* (Soboleva et al. 1988). Excretory lipid droplets are regularly excreted from the digenean body through the excretory pore (Ginetsinskaya 1968). Lipid droplets have not been observed in the metacercariae of most digeneans (Martin and Bils 1964; Erasmus 1967; Benett 1977; Mattison et al. 1992), but they can consider to be the waste products of adult digeneans only.

## 5. Conclusions

Based on previous and present studies we may make several conclusions. The unusual cytoarchitecture of the bladder epithelium of the studied azygiid digeneans, consisting of two alternating, morphologically different zones, tegumental and cellular excretory epithelial zones, is recorded for the first time for the Digenea and, in general, for the Neodermata. Such cytoarchitecture may indicate a participation of the tegument in the formation of the excretory bladder epithelium of the Azygiidae, taking into account that the protonephridial system of flatworms is an organ of ectodermal origin. The obtained ultrastructural results may assume that the excretory bladder epithelium of marine adult digeneans specializes, in addition to the excretory function, in osmoregulatory function. Based on the available literature and our own

results we can confirm the presence of excretory corpuscles in adult marine digeneans and their absence from freshwater species, regardless of the digenean localization in their host. We support Gibson's opinion that excretory corpuscles of marine adult digeneans help with the osmoregulation under conditions of increased salinity. Among marine species studied to date, the largest amount of excretory corpuscles with most concentric layers was detected in *O. cestoides* infecting elasmobranchs. Our morphological data let us to assume that the growth of the excretory bladder epithelium via migration of undifferentiated cells into the bladder epithelial lining in marine species may be considered as an adaptive mechanism of marine digeneans to an environment of varying osmolarity.

## 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.


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## Author contribution

L.P. and W.H. collected parasites at sea during scientific trip on RV 'Johan Ruud' (Norway). W.H. performed light microscopy and parasite's identification. L.P. performed scanning and transmission electron microscopy, prepared figures. L.P., K.M. and W.H. wrote the main manuscript text and analyzed data. All authors revised the text and approved the final draft.

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## Figure captions

**Fig. 1.** Scanning (A, B, D, E) and transmission (C, F, G, H, I) electron microscopic view of the excretory bladder of marine *Otodistomum cestoides*. (A) SEM view of the posterior end, note excretory pore. (B) Section through bladder stem, note excretory corpuscles and lipid droplets within its lumen. (C) Part of the bladder of the smaller worm, note electron-dense lipid droplets and excretory corpuscles. (D) SEM section through posterior body end showing portions of the long bladder stem. (E) Lumen of excretory bladder stem, showing numerous excretory corpuscles and released lipid droplets. (F, I) Portions of the excretory bladder of the larger worm showing heterogeneous lipid droplets in the epithelial lining and free lipid droplets within bladder lumen along with excretory corpuscles. (G) Part of the bladder epithelium, showing excretory bladder cell and tegumental zones, connected by septate junctions. (H) Septate junction. Scale bars: A, D = 500 μm; B = 20 μm; C, F, G, I = 2 μm; E = 10 μm; H = 0.2 μm.

*Abbreviations to all figures*: bl, basal lamina; cp, corpuscle's precursor; db, dense bodies; dl, dense surface layer; dm, dense excretory material; ec, excretory corpuscles; ebc, epithelium of the excretory bladder zone; ebl, excretory bladder lumen; ebs, excretory bladder stem; ed, excretory duct; ep, excretory pore; exp, excretory product; fm, flocculent material of corpuscle outer surface; fp, finger-like protrusion; gc, Golgi complex; ld, lipid droplet; m, mitochondrion; mf, muscle fibres; mw, myeline-like whorls; n, nucleus; ref, released fragment of the bladder epithelial lining; sj, septate junction; sl, surface lamellae; ss, smooth surface; r, ribosomal gathering; tbc, epithelium of the tegumental bladder zone; tc, tegumental cell; tf, tegumental fold; tl, lumen of the tegumental lining; tsl, tegumental syncytial lining; unc, undifferentiated cell;. v, vesicles; vc, vacuole.

**Fig. 2.** Fine structure of the excretory bladder of marine *Otodistomum cestoides*. (A) Two epithelial bladder zones, note lipid droplets in tegumental zone and excretory corpuscles associated with excretory bladder zone. (B, C) Excretory bladder epithelium showing lipid droplets and excretory corpuscles associated with excretory bladder zone, note undifferentiated excretory cell beneath excretory bladder zone. (D) Undifferentiated cell beneath and close to tegumental epithelial zone. (E) Lipid droplet in the excretory cell cytoplasm. (F) Lipid droplets within epithelial nucleus, note myeline-like whorls. (G) Thin epithelial lining of the bifurcated bladder duct. (H, I) Emerging excretory corpuscles between surface lamellae of excretory bladder cell, note vesicles and small gatherings of finely dispersed material. Scale bars: A – C = 2 μm; D, F, G = 1 μm; E, H, I = 0.5 μm.

**Fig. 3**. Fine structure of the excretory bladder of freshwater *Azygia lucii*. (A) Tegumental cytoplasm of the distal terminal stem of the excretory bladder. (B) Two different zones of the excretory bladder epithelium showing smooth tegumental surface of the tegumental zone and lamellated surface of excretory bladder zone, note septate junction between zones. (C) Two different epithelial (tegumental and excretory) zones, connected by apical septate junctions. (D) Narrow lumen of the terminal stem filled with vesicles. (E, F) Parts of the bladder epithelium, showing excretory bladder nucleated zones alternate with tegumental zones. Note, smooth tegumental and lamellar excretory surfaces connected by septate junctions. (G) Portion of the bladder epithelium near oral sucker, note two zones and dense lipid droplet associated with excretory bladder zone. (H) Protruded nucleated excretory zone surrounded by tegumental zone (I) Excretory bladder cytoplasm, note vesicles and Golgi complex. (J) Lipid droplets within lumen of proximal portion of excretory bladder localized near oral sucker and within adjacent excretory ducts. Scale bars: A, E, F, J = 2 μm; B, C, G = 1 μm; D = 0.5 μm; H = 5 μm; I = 0.2 μm.

**Fig. 4.** Fine structure of the excretory bladder of freshwater *Acrolichanus auriculatus* (A–F) and marine *Derogenes varicus* (G–L). (A) SEM view of the excretory pore of *A. auriculatus*. (B) Folded tegument of the terminal duct. (C) Transition zone of tegumental and excretory bladder epithelial linings, note septate junction between morphologically different epithelia. (D) Excretory pore cavity, note folded tegument. (E) View of terminal duct and enlarged portion of the excretory bladder. (F) Portion of excretory bladder epithelium, note finger-like surface protrusions. (G) SEM view of the excretory pore of *D. varicus*. (H) Transition epithelial zone between terminal duct and distal portion of the excretory bladder, note mixed different epithelial linings. (I) Protruded nucleus of the bladder epithelium. (J) Excretory corpuscles. (K) Two epithelia connected by apical septate junctions, note their different cytoplasmic inclusions, insert, dominant heterogeneous vesicles of the bladder cytoplasmic lining. (L) Portion of the excretory bladder showing luminal content filled with excretory corpuscles, note free epithelial fragment with nucleus within lumen and undifferentiated cells beneath bladder epithelial lining. Scale bars: A = 10 μm; B, C, J, K = 1 μm; D, H, I, L = 2 μm; E = 5 μm; F, insert = 0.2 μm; G = 50 μm.