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Structural Organization of Vitelline Cells of Trematode with Undifferentiated Body of *Azygia Lucii* (Muller, 1776)

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Abstract: It is known that the class Trematoda includes exclusively parasitic forms of flatworms. In this regard, they have formed a variety of morphological and functional adaptations to a parasitic lifestyle. One of them is the colossal fecundity, which provides the possibility of rapid and wide settlement and detection of hosts. Therefore, the study of the sexual reproduction of helminths is the central problem of parasitology. The study of the functional morphology of the reproductive system of trematodes in general and its parts, is of particular interest in solving such an important problem in theoretical and practical terms as the directed regulation of the fecundity of end parasites. The present article provides information on the functional morphology of the vitelline cells of the *Azygia lucii* trematode, describing in detail the micromorphology and ultrastructure of the vitelline cells and the development stages of vitelline cells in the course of vitellogenesis. As a result, the knowledge gained on the peculiarities of the structure and maturation of *Azygia lucii* vitelline s will significantly supplement the knowledge about the peculiarities of the structure of the female reproductive system of Strigeidae trematodes and the fecundity of end parasites.

Keywords: Trematode, *Azygia lucii*, Micromorphology, Ultrastructure, Vitelline Cells

Introduction

Parasitism is a widespread form of the existence of organisms in the biosphere. This is because parasitic organisms have a huge range of various adaptations that ensure their existence both in the host body and in the external environment (Terentyeva and Kostevich, 2009; Zhavoronkova and Novak, 2015; Ualiyeva *et al*., 2017; Aleuy and Kutz, 2020). Among adaptations, the main ones are those that significantly increase the reproductive activity of parasites. At any degree of reduction in helminths of certain organs and their systems, the sexual reproduction system in all cases invariably reaches a high level of development, complexity and specialization. The high reproductive potential of helminths compensates for the high level of their elimination at various stages of existence. Even though the micromorphological features of the sexual system of trematodes have been studied since the beginning of the 20th century, detailed descriptions have been made only for a small number of species out of 15,000 knowns (Kurochkin, 1985). This article describes the structural and functional morphology of the organ of the female reproductive system involved in the formation of the shell of the eggs of a trematode with an undifferentiated body of *Azygia lucii*.

Materials and Methods

The research object is the *Azygia lucii* trematode, belonging to the order Strigeida, the family Azygiidae from the digestive system (intestines) of pike (*Esox lucii*).

Fixing mixtures and the mode of fixing helminths were selected depending on the goals of the study.

For histological examination, the material was fixed in Buen fluid and 10% neutral formalin, which were used to fix wholemounts and tissue pieces (Gaponov *et al*., 2009).

Buen fluid is represented by the following composition (Gaponov *et al*., 2009):

- Picric acid (saturated solution) - 150 mL
- Formalin (40% formaldehyde solution) - 50 mL
- Glacial acetic acid - 10 mL

For light-optical microscopy of finished micro-preparations, the fixed helminthological material (trematodes) has passed several stages. The processing of the study material began with washing the trematodes in 70% alcohol for one day. The trematodes were placed in special baskets with pads for biopsy material. The work was carried out using a Medide TPC-15 his to processor for histologic diagnosis of tissue, where the studied material passed the tissue dehydration and paraffinization stages according to the Standard 1 program.

As a result, the material was completely dehydrated and filled with paraffin.

Slices with a thickness of 5-7 microns were obtained using a rotary microtome, pre-pouring the material into paraffin blocks. Slices, fixed on slides were stained with hematoxylin-eosin according to the Ehrlich method (Ehrlich, 1886).

The finished whole mounts were examined using a Keyence Bz-9000 light microscope with further photographing of the slices at different magnifications.

The ultrastructure was studied by transmission electron microscopy (Salnikova *et al*., 2016). Ultrathin slices were prepared according to the B. Weekly method (Weekly, 1975). For electron microscopic examination, the material was fixed in 3% glutaric dialdehyde on a cacodylate buffer (pH 7.4) at 4°C. After two rinses for 10-15 min with a cacodylate buffer (pH 7.4), the material was fixed in a 1% solution of osmium tetroxide using the same buffer for two hours, followed by double washing for 10-15 min with a cacodylate buffer (pH 7.4) (Weekly, 1975).

During dehydration, the material was contrasted with uranyl acetate in 70% alcohol. A mixture of epon-araldite resins was used as a filling medium.

Proportions of the epon-araldite mixture were as follows:

- Epon 812 - 4 g
- Araldite 502 - 2 g
- Epon DDSA - 9 g
- DMP-30 catalyst - 8 drops

The preparations impregnation scheme:

- A mixture of resins:

1) Absolute acetone 1:3 - 4 h
2) Absolute acetone 1:3 - 4 h
3) Absolute acetone 1:3 - 4 h

- A mixture of resins - from 12 to 24 h
- A new mixture of resins - from 12 to 24 h

Polymerization of the preparations was carried out for 1.5-2 days at 60°C.

Ultrathin slices 60-100 nM thick were obtained using an Ultrotome III ultramicrotome (LKB, Sweden). Slices, prepared according to Reynolds E. (1963), were additionally contrasted with lead citrate for 10 min at room temperature and with a 2% solution of uranyl acetate prepared on 50% ethanol for 10-20 min at 37°C. The obtained preparations were examined employing a JEM-100 CXII electron microscope (JEOL, Japan) with an aperture of 25-30 microns at an accelerating voltage of 80 kV.

All illustrative material presented is original.

Results

Micromorphology

The vitelline glands of *Azygia lucii* are located on the sides of the body along the outer edges of the intestinal trunks (Fig. 1). They extend from the posterior end of the abdominal sucker, at a distance approximately equal to the diameter of the sucker and stretch beyond the border of the posterior spermarium. Vitelline glands have a follicular structure. The follicles, elongated or oval, have an indistinctly marked thin connective tissue membrane, apparently formed by the apophyses of parenchymal cells. The dimensions of the vitelline follicles are 58.8-100 x 52.9-105.9 microns in diameter.

A common vitelline duct stretches along the vitelline cells, which is approached by thin channels of vitelline follicles extending from the median ends of the vitelline cells (Fig. 2). In the anterior and posterior parts of the body, the ducts connect to the main vitelline-duct, which ends with the vitelline reservoir located near the ecotype. The vitelline reservoir has a spherical shape, its dimensions are 68.6 × 107.1 microns. It is filled with mature vitelline cells containing formed shell globules. Shell globules fall into the ecotype, where they participate in forming the shell of a complex egg.

Vitelline follicles are represented by more or less dense groups of vitelline cells that differ in shape and structure. The vitelline cells in the follicles are located close to each other, but there are also follicles in which the vitelline cells lie freely. The space between them is filled with parenchymal processes.

Vitelline cells have different stages of development: From the immature vitelline cells to mature, formed ones. Differentiated mature vitelline cells are found throughout the vitelline follicle, mainly localized in its central part. Undifferentiated or immature vitelline cells occupy a parietal peripheral location in the follicle. Most of the vitelline follicle is occupied by mature or developing vitelline cells (Fig. 3).

Immature vitelline cells have smaller dimensions compared to mature differentiated cells, whose diameter reaches a maximum and is 11.4 × 17.1 microns. The differentiation of vitelline cells takes place in several

stages, which are characterized by intense secretory activity resulting in forming shell granules that merge and form shell globules. The size of the nuclei practically does not change at all stages of differentiation. The nuclei in mature cells are mostly displaced to the periphery and have dimensions of 2.9 × 5.7 microns in diameter. This morphology of the nuclei is associated with a large content of shell globules in mature vitelline cells, which push the nucleus to the periphery.

When stained with hematoxylin-eosin dye according to the Ehrlich method, the nuclei of vitelline cells acquire a dark purple color, while the cytoplasm turns pink.

Ultrastructure

Vitelline cells that form follicles have different stages of development: Immature (undifferentiated), developing and mature (specialized). As the vitelline cells develop from undifferentiated to specialized, the cells of the vitelline follicles undergo four stages of differentiation, whose morphological development is characterized by an increase in cell size and the formation of shell globules.

The cells of the 1st stage are represented by undifferentiated cells of small size. The cell nucleus has a large size and occupies the main part of the cell. The nuclei have a rounded shape and contain diffuse nucleoli and large areas of electron-dense condensed chromatin, i.e., heterochromatin. The cell cytoplasm at this stage is homogeneous and has a fine-grained structure. Among the cell organs, small mitochondria are rarely identified and areas of a smooth endoplasmic network are encountered. The cytoplasm contains a large number of free-lying ribosomes. Inclusions in the form of glycogen and lipids are not detected.

The cells of the 2nd stage have larger sizes compared to the cells of the 1st stage of differentiation and the size of the nuclei remains unchanged. The cell nuclei at this stage have a lower electron density, which is associated with a growing area of actively decondensed euchromatin. The most noticeable changes are associated with the cytoplasm. At this stage, single shell granules are already beginning to form. They are small in size, rounded in shape and separated from the cytoplasm by a membrane. The shell granules are surrounded by a network of granular endoplasmic reticulum and mitochondria. A large number of ribosomes and polysomal complexes formed by them are concentrated in the hyaloplasm (Fig. 4).

The cells of the 3rd stage increase in size. The increase in the diameter of the cells occurs by increasing the volume of the cytoplasm in relation to the nuclear apparatus of the cell, however, the size of the nucleus remains unchanged. The nuclei of these cells are large, rounded, or oval. The nucleus has a typical two-membrane nucleolemma dotted with ribosomes. The karyolemma is permeated with nuclear pores.

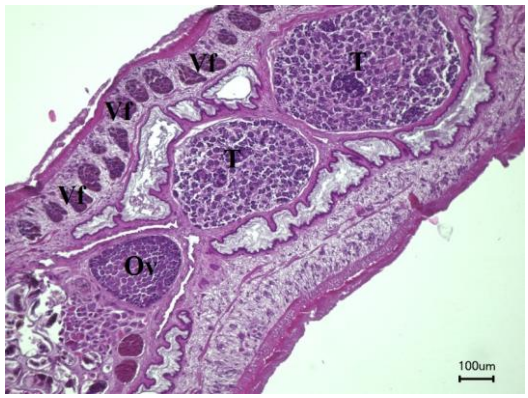

Fig. 1: Section of the reproductive system of Azygia lucii (x400); Note: Hematoxylin-eosin staining according to the Ehrlich method; Vf – vitelline follicle; T – spermarium; Ov – ovary

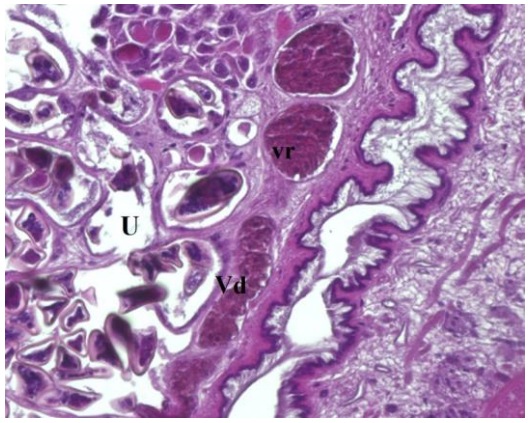

Fig. 2: Common vitelline duct and vitelline reservoir of Azygia lucii (x500) Note: Hematoxylin-eosin staining according to the Ehrlich method; vr – vitelline reservoir; Vd – vitelline -duct; U – uterus with eggs

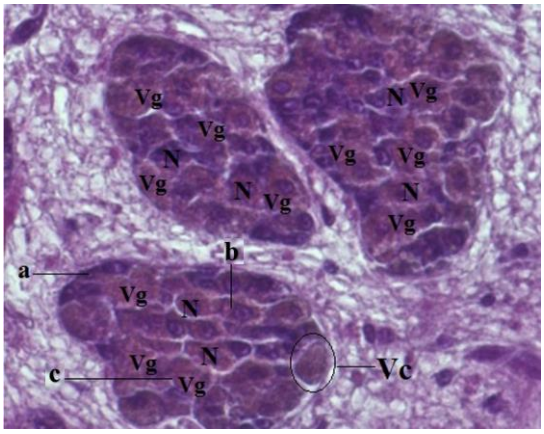

Fig. 3: Vitelline follicles of Azygia lucii (x600); Note: Hematoxylin-eosin staining according to the Ehrlich method; Vc – vitelline cell; N – nucleus; Vg – shell globules; (a) immature; (b) developing; (c) mature vitelline cells

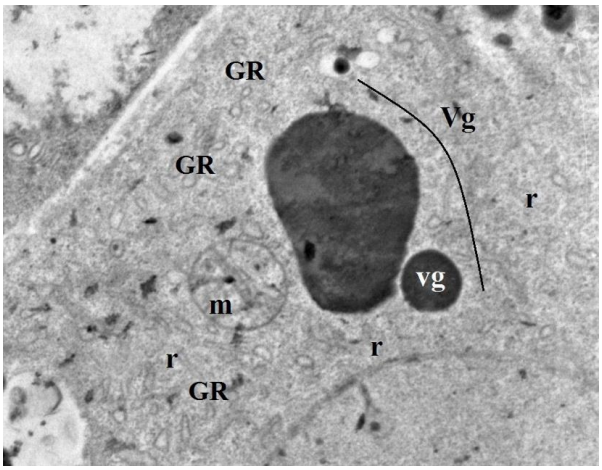

Fig. 4: Azygia lucii vitelline cell site at the 2nd stage of development (x12,000); vg – shell granules; Vg – forming shell globules; m – mitochondria; GR – granular endoplasmic reticulum; r – ribosomes

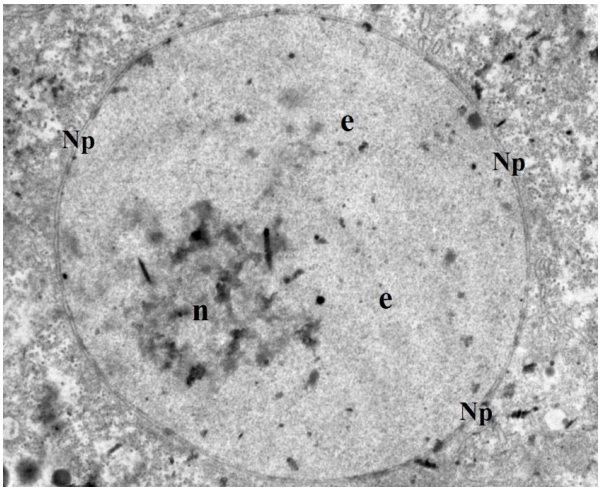

Fig. 5: Azygia lucii nucleus at the 3rd stage of development (x13,000); Np - nuclear pore; n - nucleole; e - euchromatin

The karyoplasm of the nuclei contains one diffuse, poorly distinguishable nucleolus, most often shifted to the periphery of the nucleus. The electron-light structure of euchromatin indicates the active secretory activity of the cell (Fig. 5).

The number of channels of the granular endoplasmic reticulum located mainly around the synthesized shell granules increases. A large number of ribosomes are concentrated on the rough endoplasmic reticulum. Mitochondria are localized in the places of formation and synthesis of shell granules. Mitochondria are enlarged in size, have a light matrix with weakly expressed cristas (Fig. 6).

This stage is characterized by the growth of shell granules and their merge into globules. The shell globules are isolated, surrounded by a thin membrane. Globules contain a different number of granules, depending on maturity and development (Fig. 6). Mature globules are filled with shell granules of various sizes of a rounded shape. There is an electron-light space between the granules. As the granules are synthesized, the shell material accumulates and round granules are formed. A single vitelline globule contains granules of different sizes. The larger the shell granule, the more pronounced its boundaries are. Flattened membrane sacs and a system of vesicles that make up the Golgi apparatus are visible around the maturing vitelline globules (Fig. 6). No Golgi apparatus was detected in the area of mature shell globules. The cytoplasm contains glycogen grains.

This stage is distinguished by forming and developing shell globules, developed granular endoplasmic reticulum, mitochondria and an abundant content of free polyribosomes in the cytoplasm (Fig. 7).

The cells of the 4th stage are represented by mature vitelline cells. They mainly occupy the peripheral part of the vitelline follicle (Fig. 8). The cells reach their maximum size and are characterized by maturity and functional specialization. On average, the number of vitelline globules in the vitelline cell is up to 15 large globules consisting of numerous shell granules of various shapes. As a result of the increase in the shell globules in the cell, the nuclei are "squeezed" and shifted to the periphery of the cell. No synthetic processes are observed in mature vitelline cells. The karyoplasm of the nucleus has strands of inactive condensed heterochromatin. The content of ribosomes is also reduced in quantity. The channels of the rough endoplasmic network are localized between the shell granules, the Golgi complex is not detected at this stage.

Shell globules of mature vitelline follicles, moving along the vitelline ducts, enter the ecotype, from where they enter the forming egg. In the egg, shell globules in an undisturbed state containing shell granules are localized along the periphery, adjacent to the eggshell (Fig. 9). The egg cytoplasm has a granular structure of moderate electron density. In the cytoplasm, there are clusters, apparently, of enzymes, surrounded by a thin membrane. They are found throughout the entire cross-section of the egg, including near the shell globules (Fig. 9). The eggshell is at the stage of formation. The elements (shell granules) of the collapsed shell globules are visible, forming a homogeneous structure in the area of the eggshell. Shell globules, located in the egg have a larger number of granules inside the globule, compared with mature vitelline globules of a mature vitelline cell (Fig. 9).

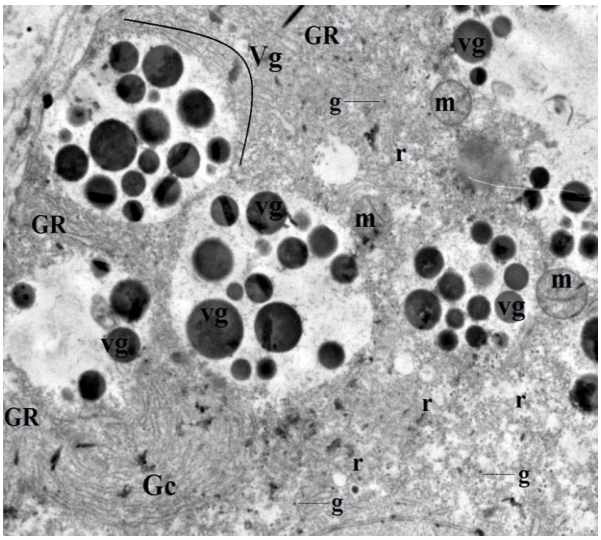

Fig. 6: Azygia lucii vitelline cell site at the 3rd stage of development (x12,000) vg – shell granules; Vg – shell globules; m – mitochondria; GR – granular endoplasmic reticulum; m – mitochondria; Gc – Golgi apparatus; r – ribosomes; g – glycogen grains

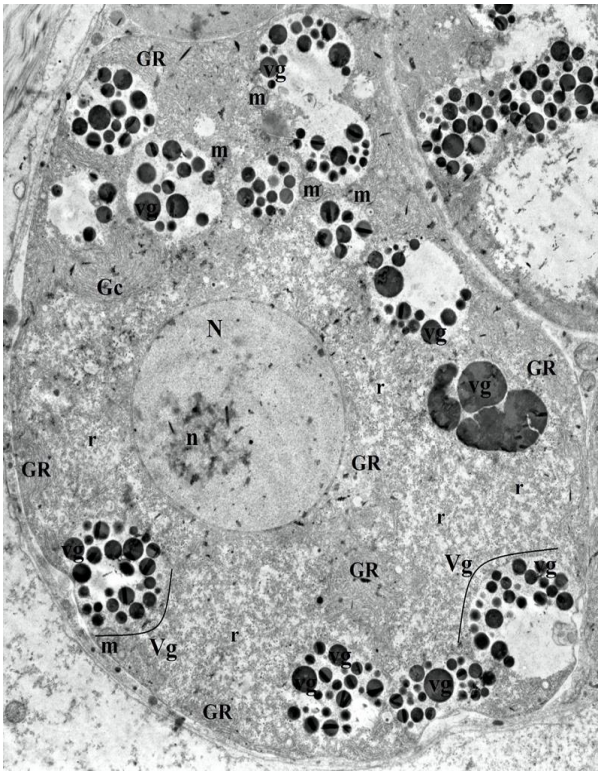

Fig. 7: *Azygia lucii* vitelline cell at the 3rd stage of development (x12,000) vg – shell granules; Vg – shell globules; m – mitochondria; N – nucleus; n – nucleole; GR – granular endoplasmic reticulum; Gc – Golgi apparatus; r – ribosomes

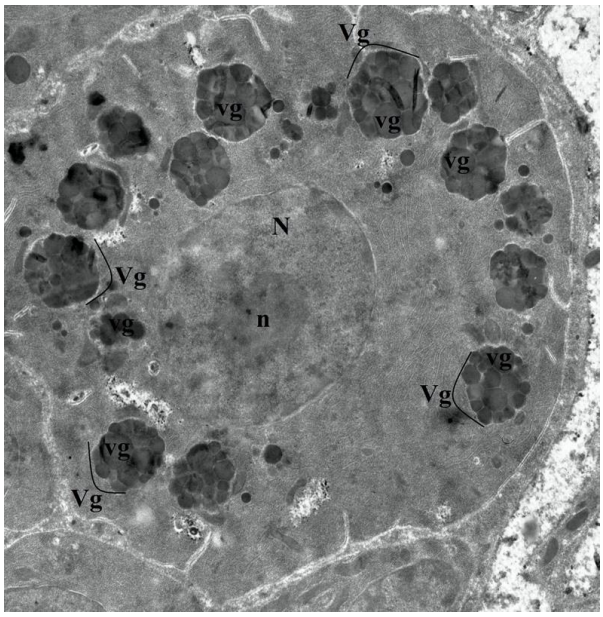

Fig. 8: Mature vitelline cell of Azygia lucii (x1,000) Vg – shell globules; vg – shell granules; N – nucleus; n – nucleole

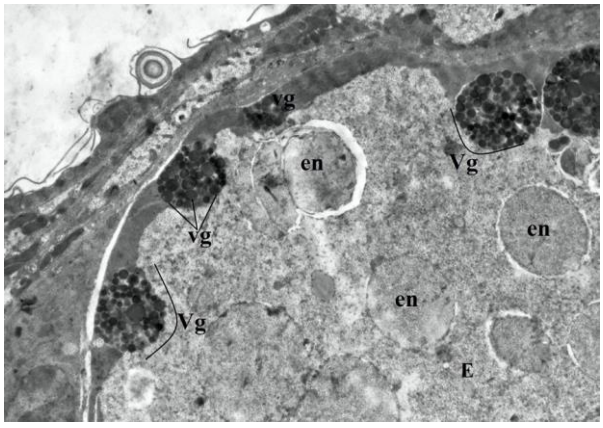

Fig. 9: The site of the forming egg of Azygia lucii (x14,000) E – forming egg; Vg – shell globules; vg – shell granules; en – enzymes

Discussion

The vitelline follicles of *Azygia lucii* do not have clear boundaries. At the ultrastructural level, it was determined that the follicles of *Azygia lucii* do not have their own follicular envelope. Its role is performed by the processes of parenchymal cells.

The vitelline glands of the trematode under study are localized laterally to the lines of the helminth body along the external intestinal trunks.

Vitelline cells of *Azygia lucii* undergo several stages during vitellogenesis, characterized by certain structural features. Four stages of differentiation are distinguished:

Stage 1 – immature cells, stages 2-3 – developing cells, stage 4 – mature specialized cells. Such differentiation and sequence of vitelline cell maturation are described in other trematode species (Shaimardanov, 2002) and by us in *A. lucii*.

We have found the existence of a certain pattern in the location of vitelline cells in the follicle, depending on the degree of maturity. Thus, in *A. lucii*, mature vitelline cells are found throughout the entire section of the vitelline follicles, while immature cells are localized in the peripheral zone.

Most of the vitelline follicles are occupied by mature or developing vitelline cells. The accumulation of cells of different degrees of maturity in the vitelline follicle, in our opinion, is associated with the constant outflow of mature cells into the vitelline ducts and the displacement of the remaining cells in the follicle, that is, the forming processes are fully completed in the vitelline follicle.

A small number of immature vitelline cells, with obvious signs of lack of specialization, can occur not only in the marginal zone of the follicle but also in different parts of it. We believe that these are stem cells that give rise to new cells resulting from division. The increase in the number of vitelline cells is mitotic. Mitosis of vitelline cells was described in *Probalitrema californiense* (family Gorgoderidae) and *F. hepatica* (family Fasciolidae) (Shaimardanov, 2002).

Our research suggests that the vitelline cells in the vitelline follicle are at different stages of maturation. We have established a morphometric pattern that depends on the degree of cell maturity. Thus, young vitelline cells have the smallest size among the vitelline cells in the follicle. As the shell material is synthesized, the cytoplasm volumes grow due to an increase in membrane organoids and the accumulation of vitelline granules. The largest cells are mature vitelline cells containing the maximum amount of shell material. Other authors also indicate an increase in the size during the maturation and differentiation of vitelline cells (Shaimardanov, 2002; Erasmus, 1975).

The Structure of Vitelline Cells the Cytoplasm

When histological preparations were stained with hematoxylin-eosin dye according to the Ehrlich method, the cytoplasm of vitelline cells showed basophilia, which is associated with a significant content of ribonucleic acids (RNA, DNA). Strong basophilia is characteristic of the cytoplasm of immature vitelline cells. In mature cells, basophilia decreases due to a reduction in the number of RNA-containing structures.

Our studies have shown that in *A. lucii*, the cytoplasm shows strong basophilia at the stage of maturation of vitelline cells, especially, this is inherent in the 2nd stage, at which the cytoplasm of cells contains the maximum number of RNA-containing structures. As the formation of shell globules and their accumulation in the cytoplasm matrix is completed, the basophilia of cells decreases.

Developing cells of *A. lucii* have a large number of free ribosomes and polysomes. As the vitelline cells mature, their number decreases, which is associated with a decrease in the synthetic functions of the cell and the accumulation of shell material.

The Nucleus

The nuclei of the vitelline cells of the trematode under study have a structure typical of eukaryotic cells. The karyolemma has a dual-membrane structure. The outer membrane passes directly into the endoplasmic reticulum. The nuclear envelope is permeated with numerous pores through which metabolic processes are carried out. The nuclei of the studied trematode species have one nucleole each.

The nuclei of young immature metabolically inactive vitelline cells have condensed chromatin, i.e., heterochromatin. It has the appearance of characteristic dark spots, usually located closer to the nucleus shell. As the cells mature, a tendency to reduce the area with condensed chromatin and increase the amount of decondensed electron-light euchromatin was noted. An increase in RNA synthesis in cells is morphologically manifested by an increase in the zones of decondensed loose euchromatin (Taylor *et al*., 2016).

An indicator of the metabolic activity of vitelline cells is the state of the membrane cell structures, i.e., the endoplasmic reticulum, the Golgi apparatus and the mitochondria. In the trematode species studied, these structures have structural differences, which perhaps is due to the systematic position of helminths. We also do not exclude the physiological condition of these subcellular structures.

Granular Endoplasmic Reticulum

The granular endoplasmic reticulum is one of the markers of synthetic processes in the cell (Taylor *et al*., 2016). The vitelline cells of *A. lucii* have all the signs of active synthetic processes and clusters of well-developed channels of the granular endoplasmic reticulum.

According to our observations and based on the analysis of the literature, the shell material begins to form in the channels of the granular endoplasmic reticulum, whose walls host a large number of ribosomes and polysomes. It is the ribosomes associated with the endoplasmic reticulum that provide the synthesis of the material, necessary to the construction of shell granules. Electron microscopic studies show the presence of channels of the granular endoplasmic reticulum in the forming shell granules.

Agranular Endoplasmic Reticulum

No smooth endoplasmic reticulum was found in the vitelline cells of the studied trematode. Perhaps, due to the high content of free ribosomes and polysomes in the cytoplasm of vitelline cells, the smooth endoplasmic reticulum looks like a rough one.

The Golgi Apparatus

The Golgi complex in vitelline cells is represented by flattened channels of the granular endoplasmic reticulum packed and concentrated in the synthesis of shell granules. Dictyosomes, the so-called Golgi bubbles were found. Thus, the Golgi apparatus tanks are a marker of areas of the cytoplasm of vitelline cells with active synthetic processes.

Mitochondria

The mitochondria of *A. lucii* are concentrated in the field of synthesis of shell granules, which indicates the role of energy suppliers in the synthesis of shell granules. They are absent in mature cells.

Shell Globules

Globules of *A. lucii* contain a different number of granules, depending on maturity and development. Mature globules are filled with shell granules of various sizes of a rounded shape. There is an electron-light space between the granules. As the granules are synthesized, the shell material accumulates and large granules are formed. A single vitelline globule contains granules of different sizes. The larger the shell granule, the more pronounced its boundaries are. Flattened membrane sacs and a system of vesicles that make up the Golgi apparatus are concentrated around the maturing vitelline globules. The Golgi apparatus is absent in the area of mature shell globules.

Based on the results of the present study, a general scheme of the stages of *A. lucii* vitelline cells formation was compiled (Fig. 10).

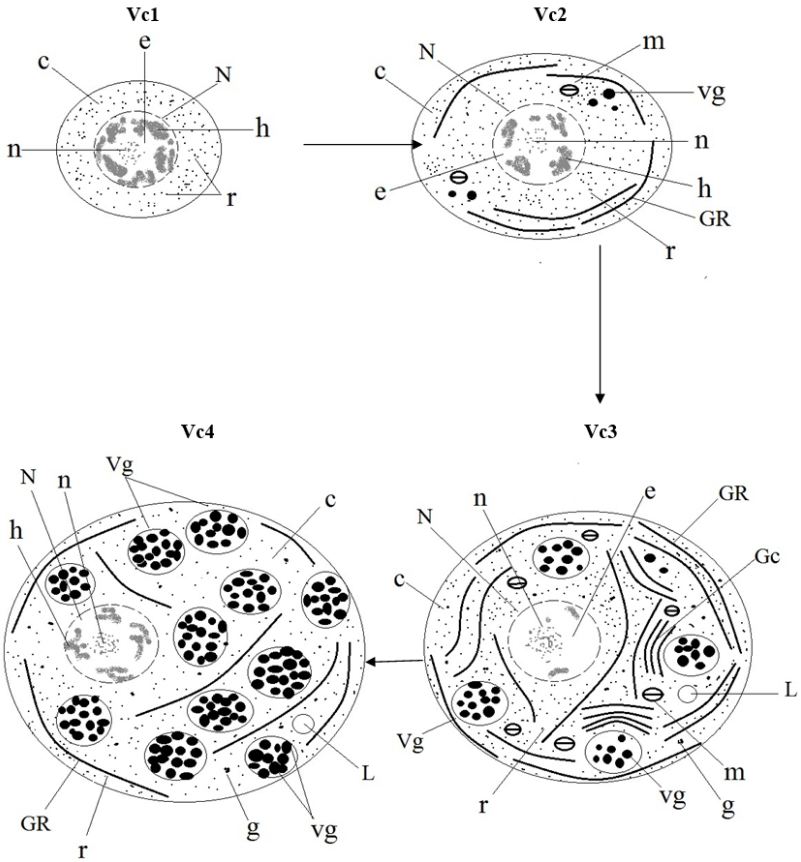

Fig. 10: Diagram of the formation stages of vitelline cells; Note: Compiled by the authors Vc 1 – vitelline cell of the 1st stage of development; Vc 2 – vitelline cell of the 2nd stage of development; Vc 3 – vitelline cell of the 3rd stage of development; Vc 4 – vitelline cell of the 4th stage of development; c – cytoplasm; vg – shell granules; Vg – shell globules; N – nucleus; n – nucleole; h – heterochromatin; e – euchromatin; GR – granular endoplasmic reticulum; Gc – Golgi apparatus; m – mitochondria; r – ribosomes; L – lipid drop; g – glycogen grains

Conclusion

Azygia lucii vitelline cells undergo four stages of development during vitellogenesis, which are characterized by structural and functional features, namely, stage 1 - immature non-specialized vitelline cells; stages 2-3 - cells at the stage of maturation and synthesis of vitelline granules; stage 4 - mature specialized vitelline cells.

The nuclei of immature metabolically inactive vitelline cells are morphologically characterized by the presence of condensed chromatin. The amount of euchromatin increases as it matures. To provide a large number of eggs with shell material, vitelline cells have a developed system of Rough Endoplasmic Reticulum (RER). The ultrastructural manifestation of active forming processes in vitelline cells is the formation of shell globules (spherical structures) consisting of shell granules. The number and morphological characteristics of shell granules in the shell globules are determined by the number of eggs produced by the helminth.

Studies have shown that the distribution of mature vitelline cells in the vitelline follicles is typical for members of the Trematoda class, regardless of the differentiation of the body and the systematic position of the helminth.

The performed micro morphological and ultrastructural study of *Azygia lucii* vitelline cells supplemented the knowledge about the structural features of the female reproductive system of trematodes of the family Strigidae.

Author's Contributions

All authors equally contributed in this study.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all other authors have read and approved the manuscript and no ethical issues have been involved.

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