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Functional Role of Vitelline Glands and Mehlis Gland in the Process of Resistant Egg Shell Formation in Trematodes

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Abstract: The study of the functional morphology of the reproductive system of trematodes, as well as its individual parts, provides an opportunity to solve an important problem that plays a huge role in theoretical and practical terms as the directed regulation of the fecundity of endoparasites. The article is devoted to the study of the functional role of vitelline glands and Mehlis glands in the process of formation of resistant egg shells on the example of the *Parastrigea robusta* trematode. The functional mechanisms are revealed on the basis of histomorphological and ultrastructural studies, underlying the provision of trematode eggshell formation processes and the role of each of the mentioned departments. The sequence of appearance and maturation of shell material in the vitelline follicles during vitellogenesis has been determined. The role of the Mehlis gland is substantiated on the basis of electron microscopy methods and their functional morphological analysis of ultrastructural and micromorphological data. The obtained data reveals information on the structural and functional unity of the mechanisms underlying the formation of a resistant trematode eggshell, the role of vitelline glands, and Mehlis gland in providing a "large number of eggs". According to the results of histological and electron microscopic studies of vitelline glands and Mehlis gland of the *Parastrigea robusta* trematode, a general scheme of the formation of trematode eggshell was formed. As a result, the obtained knowledge on the peculiarities of the structure and maturation of the vitelline glands and Mehlis gland of *Parastrigea robusta* will significantly complement the knowledge on the structural features of the female reproductive system of trematodes and the fertility of endoparasites. The results of the work can be used for further research in the selection of anthelmintic agents that affect vitelline glands and Mehlis glands, which will lead to directed regulation of the fertility level of endoparasites.

Keywords: Trematode, Micromorphology, Ultrastructure, Vitelline Glands, Mehlis Gland

Introduction

Adaptation mechanisms determine the ability of any organism to effectively master the ecological niche that was developed in the course of a long evolutionary process (Cutmore *et al*., 2014). Parasitic organisms are not exceptional, so their ecological niches, places of localization in the host are an environment of living beings characterized by special properties determined by the physiological functions of organs, tissues, and cells (Lozano-Cobo *et al*., 2022).

The Trematoda class includes exclusively parasitic forms of flatworms (Li *et al*., 2022). In this regard, they have developed a variety of morphological and functional adaptations to a parasitic lifestyle (Fischer *et al*., 2017; Hoai, 2020). One of them is high fecundity, which makes it possible to quickly and widely disperse and find hosts (De Santi *et al*., 2018). Therefore, the study of the reproductive apparatus of helminths is the main problem in parasitology.

The study of the reproductive system of trematodes became interesting in the 20th century. Despite the fact that the micromorphological features of trematodes' reproductive system have been studied since the beginning of the 20th century and continue to be studied at the present time, detailed descriptions have been made for only a small number of species (Ahuir-Baraja *et al*., 2015; Conn *et al*., 2018; Bruschi, 2022).

Understanding the adaptive mechanisms underlying the provision of a large number of eggs is important from the point of view of applied and fundamental aspects of the problem (Dkhil *et al.*, 2015). The anatomical connection of Mehlis` gland and vitelline cells with the proximal parts of the female reproductive system of trematodes suggests that they are involved in the formation of eggshells (Puljas and Burazin, 2022). Despite numerous studies, the process of formation and stabilization of the shell has not been fully elucidated. Scientists put forward various proposals on the functioning of the vitelline glands and Mehlis gland, as the main structural components involved in the egg shell formation (Ualiyeva *et al*., 2022).

The *Parastrigea robusta* (Szidat, 1928) trematode, belonging to the order Strigeida La Rue, 1926, the family Strigeidae (Railliet, 1919) was chosen as the object of study and it was obtained from the digestive system (intestines) of common pochard (*Aythya ferina*).

Parastrigea robusta is a trematode with a differentiated body. The evolutionary point of view draws on the fact that trematodes with a differentiated body are considered an advanced group of organisms, therefore, the study of the structure of the Mehlis gland and vitelline glands in this species is especially interesting for science.

The goal of the research is to study the functional role of the vitelline glands and Mehlis glands in the process of resistant eggshell formation on the example of the *Parastrigea robusta* trematode.

Materials and Methods

Trematodes were collected by the method of incomplete helminthological dissection of the common pochard (*Aythya ferina*).

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

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

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

- Picric acid (saturated solution) 50 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 (Ovcharenko *et al*., 2013). 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 Maddie TPC-15 host 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 embedded in 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.

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. 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 2 h, followed by double washing for 10-15 min with a cacodylate buffer (pH 7.4) (Bozzola, 2014).

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

Proportions of the peon 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., 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

Vitelline Glands of Parastrigea Robusta

Micromorphology. The vitelline follicles of *Parastrigea robusta* are located in the anterior and posterior sections of the trematode. The diameter of the vitelline follicle is 56.6-93.3 × 66.6-83.3 microns (Fig. 1).

The vitelline follicle contains vitelline cells that are at different stages of development (Fig. 1). Immature vitelline cells are characterized by weak structural cellular differentiation. The cells contain a large nucleus, which occupies the main part of the cell and is stained with hematoxylin-eosin in a dark purple color. The cytoplasm becomes light pink after staining. As the shell material accumulates in the vitelline cells, the basophilia of the cytoplasm decreases.

In the course of vitelline cell maturation, the size of the nucleus does not change and cell growth occurs because of an increase in cytoplasm volume with vitelline granules located in it. In mature vitelline cells, almost the entire space is occupied by the shell material, except for the nucleus. The diameter of mature cells is 18.6-23.3 microns, while 4.5-4.7 microns is occupied by the nucleus. In the course of hematoxylin-eosin staining according to the Ehrlich method, shell granules are not stained and have a light yellow tint in the photographs (Fig. 1).

Ultrastructure. The vitelline cells that make up the follicles are separated by thin processes of parenchymal tissue, which is located nearby. As a result of maturation, cells undergo structural and morphological changes.

Our studies demonstrate that vitelline cells in the course of maturation undergo 4 stages (Fig. 2).

The cells of the 1st stage are small. Most part of the cell volume is occupied by the nuclear apparatus. During this stage, the chromatin is in a condensed state. It is represented by heterochromatin, which is localized mainly along the periphery of the nucleus. The cytoplasm contains a large number of free ribosomes and polysomes, there are several mitochondria and small areas occupied by the granular endoplasmic reticulum. In the vitelline cells of this stage, shell material was not detected.

The 2nd stage of differentiation is characterized by noticeable rearrangements occurring in the cell: The amount of euchromatin in the nuclei increases, in most of the vitelline cells of the follicle the areas with euchromatin and heterochromatin are almost in equal proportion, which is a sign of the activation of synthetic processes. In cells at this stage, single shell granules are already visible.

The cells of the 3rd stage increase in size because of the synthesis of shell material. The nucleus has an irregular shape and is located in the central part of the cell. There is a structural change in the nuclear material, resulting in an increase in the active form of chromatin euchromatin. The granular endoplasmic reticulum, which contains many ribosomes, branches off from the nucleus.

The cytoplasm is characterized by granularity and contains extensive networks of granular endoplasmic reticulum with numerous ribosomes. Mitochondria are localized mainly near the shell granules, which undergo synthesis. Growing shell globules move to the cell periphery. Larger vitelline granules are formed as a result of the fusion of small ones. The cytoplasm contains a large number of free ribosomes and glycogen grains and it is characterized by a granular structure.

The cell at the 4th stage of differentiation completes the process of synthesis of shell globules. Vitelline cells at this stage have a maximum size. The nucleus is rounded and contains a well-structured nucleolus, chromatin is in an inactive state, which is represented mainly by heterochromatin. Vitelline globules contain a large number of shell granules. Vitelline globules occupy 1/5 of the cell volume.

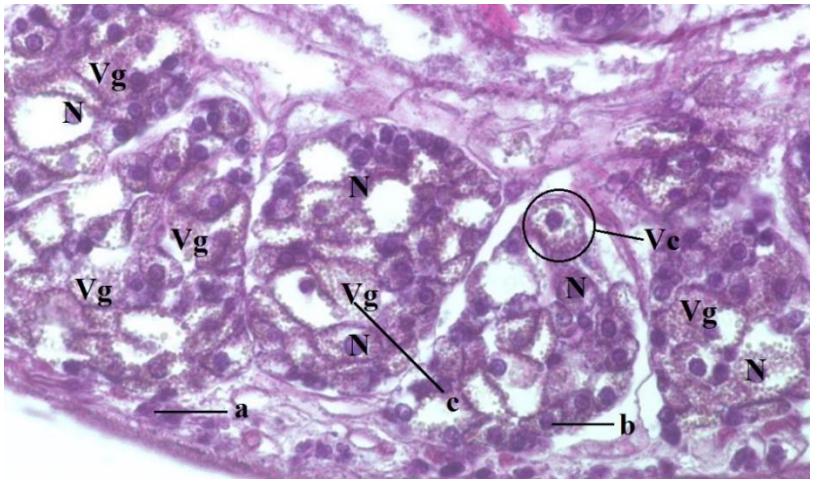

Fig. 1: Vitelline follicles of *Parastrigea robusta* (× 600); Vc-vitelline cell; Vg-shell globules; N-nucleus; (a) immature; (b) developing; (c) mature vitelline cells

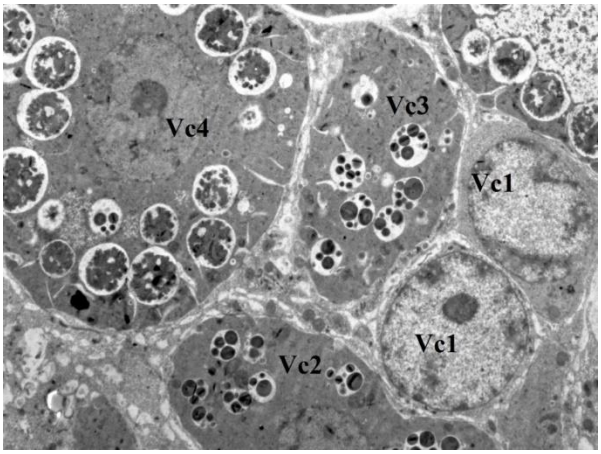

Fig. 2: *Parastrigea robusta* vitelline follicles site (× 8000); Vc 1-vitelline cell of the 1st stage of development; Vc 2-vitelline cell of the 2nd stage of development; Vc 3-vitelline cell of the 3rd stage of development; Vc 4-vitelline cell of the 4th stage of development

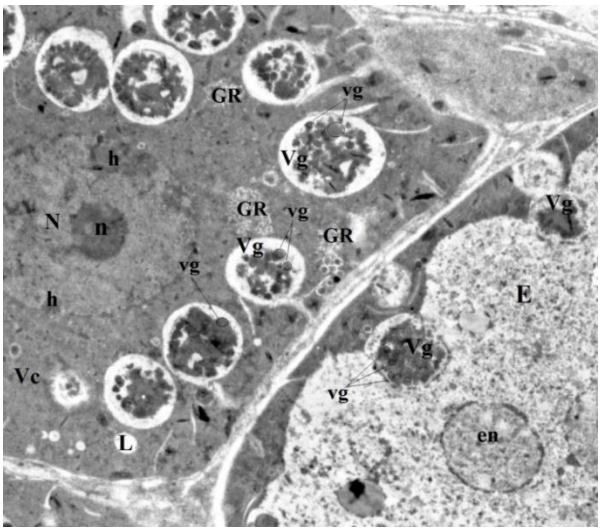

Fig. 3: Mature vitelline cell near the developing egg of *Parastrigea robusta* (× 12000); Vc-vitelline cell; N-nucleus; N-nucleole; H-heterochromatin; Vg-shell globules; Vg-shell granules; GR-granular endoplasmic reticulum; L-lipid drop; E-forming egg; En-enzymes

After finishing the process of cell maturation in the vitelline follicle, mature cells move through the vitelline ducts to the area of the vitelline reservoir, from where they enter the ootype, where the formation of a resistant eggshell occurs. Vitelline cells are in close contact with the egg membrane. Vitelline globules are visible on the periphery of the egg, they contain shell material (Fig. 3).

Mehlis Gland of Parastrigea Robusta

Micromorphology. The Mehlis gland is located on the posterior segment of the trematode`s body. The gland is localized between the testes. Mehlis gland cells are represented in a small amount and completely surround the ootype (Fig. 4). The size of the gland varies between 85.7-214.3 microns (Fig. 4).

The Mehlis gland consists of two cell types (Fig. 4). The cells of the first type are localized near the ootype and surround it. Cells are drop shaped or elongated, the size of which is 5.7-8.6 microns. The nucleus is large (2.3-4.3 microns), and slightly displaced from the center of the cell. The glandular cells are in contact with the forming egg membrane and vitelline cells located near the ootype in the proximal uterus (Fig. 5).

The second type of Mehlis` gland cells occupies a distant or peripheral location relative to the ootype. Cells of this type are represented by an elongated shape and the largest sizes, where nuclei are 2.3-4.3 microns and cell diameter is 10-14.3 microns. Light microscopy images show that the cytoplasm contains various vesicles (Fig. 6).

Ultrastructure. Pictures taken with an electron microscope demonstrate that the Mehlis` gland cells are separated from each other and each cell is covered with its own membrane. In the immature cells of the Mehlis gland, the nucleus is large and localized in the central part of the cell. The nucleolemma is represented by two layers; large areas of electron dense heterochromatin are also visible in the nucleus (Fig. 7). In the course of the maturation of glandular cells, heterochromatin is replaced by active euchromatin, which has a light color. The nucleus in mature cells occupies the largest part, which is about half of the cell (Fig. 7).

The cytoplasmic matrix contains a large number of granular endoplasmic reticulum channels and numerous clusters of ribosomes from which polysomes are formed. Ribosomes are mainly concentrated on the membranes of the endoplasmic reticulum. Mitochondria were found in the hyaloplasm of the Mehlis` gland cell, but there was no Golgi apparatus.

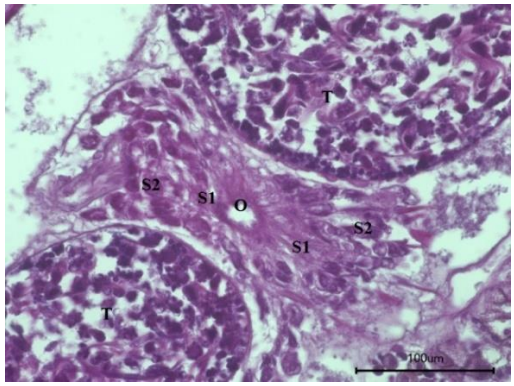

Fig. 4: Mehlis gland of *Parastrigea robusta* trematode (× 500); T-testis; O-ootype; S1 1st type of secretory cells of Mehlis gland; S2 sec type of secretory cells of Mehlis gland

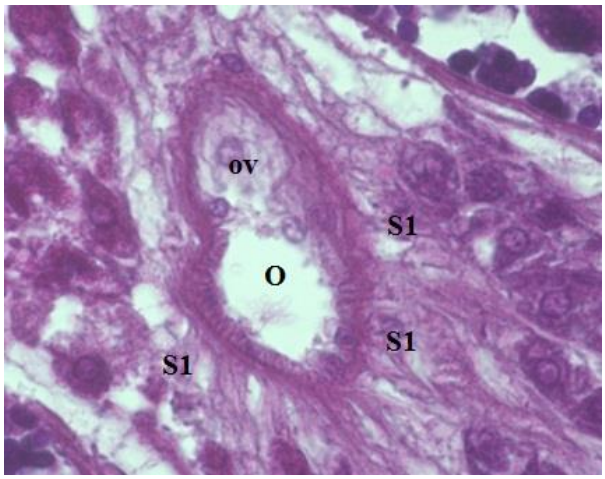

Fig. 5: Forming egg of *Parastrigea robusta* (× 600); O-ootype; S1 1st type of secretory cells of Mehlis gland; OV-ovum

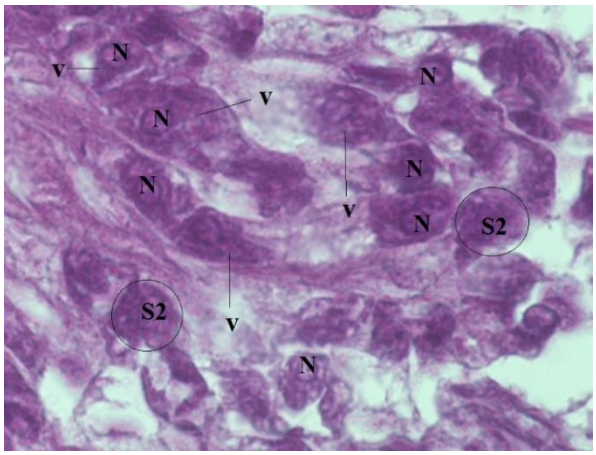

Fig. 6: Second type of Mehlis gland cells of *Parastrigea robusta* (× 600); S2 sec type of secretory cells of Mehlis gland; N-nucleus; V-vesicles

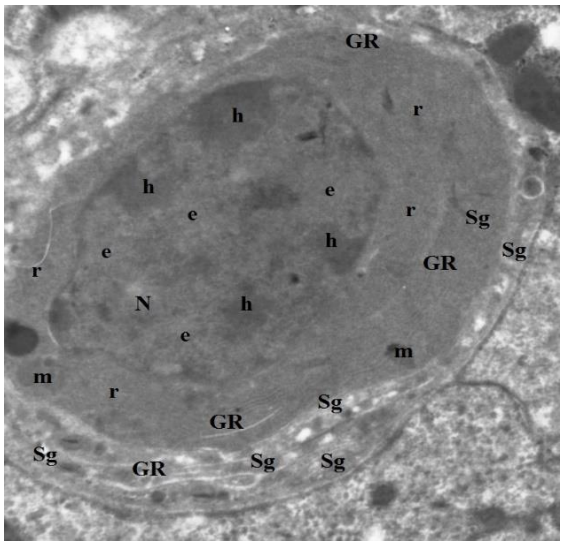

Fig. 7: First type of Mehlis` gland cells of *Parastrigea robusta* (× 14000); N-nucleus; e-euchromatin; h-heterochromatin; GR-granular endoplasmic reticulum; m-mitochondria; Sg-secretory granules; r-ribosomes

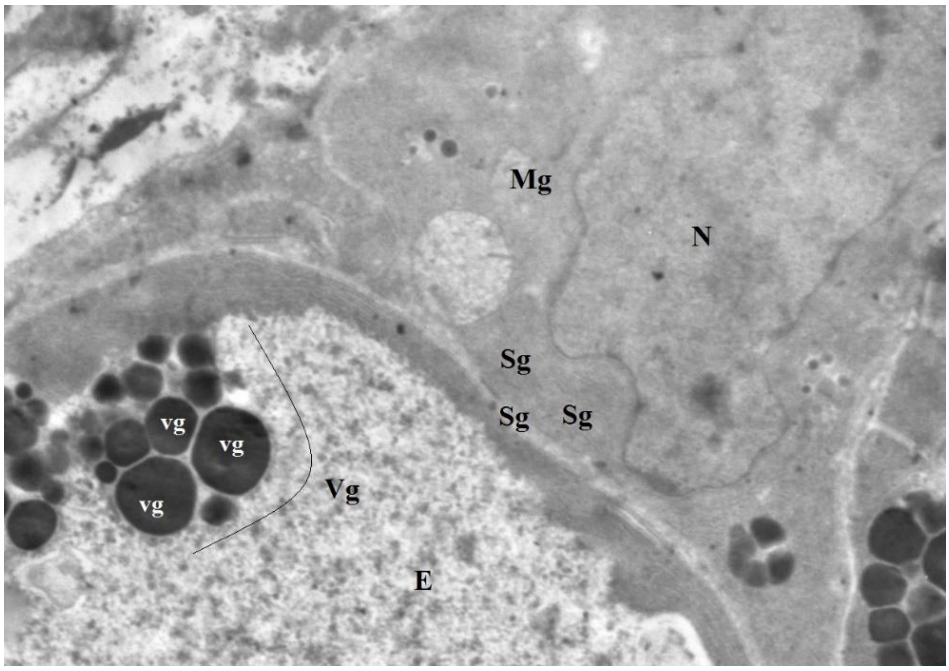

Fig. 8: *Parastrigea robusta* Mehlis gland cell and forming egg site (× 12000); Mg-Mehlis gland cell; Sg-cluster of secretory granules; E-forming egg; Vg-shell globules; Vg-shell granules

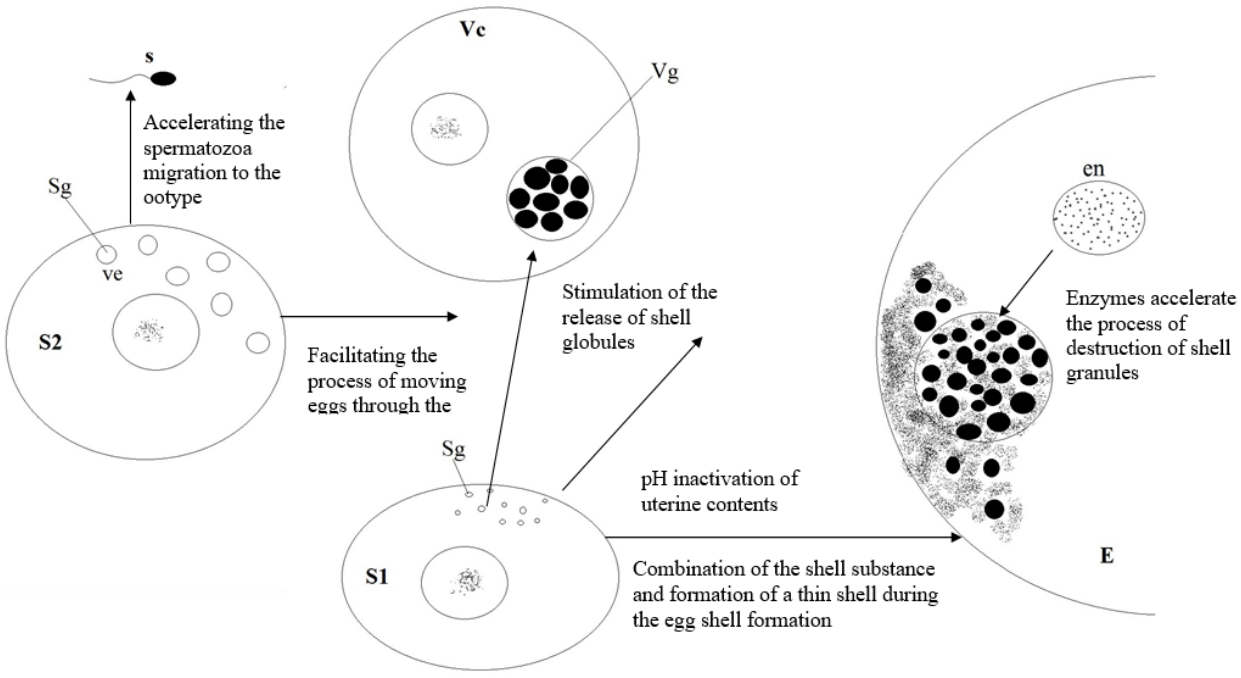

Fig. 9: Scheme of the process of shell formation of trematode eggs; Note: Compiled by the author; Vc-vitelline cell; S1 1st type of Mehlis gland cell; S2 sec type of Mehlis gland cell; E-forming egg; Sg-secretory granules; Ve-vesicles; En-enzymes

These glandular cells are located in the ootype region and are associated with the forming egg. We consider them to be Mehlis` gland cells of the first type due to the fact that they are without vacuoles and vesicles (Fig. 8).

According to the results of histological and electron microscopic studies of the *P. robusta* trematode vitelline glands and the Mehlis gland, a general scheme of the process of eggshell formation was constructed (Fig 9).

Discussion

P. robusta parasitizes in the digestive tract, where helminths undergo constant chemical and mechanical action from the host organ; therefore, such helminths have a complex life cycle. These circumstances, which were explained from the point of view of biology and ecology of parasitic worms, in the course of evolution have led to the fact that they need greater sexual productivity. This is due to the fact, that after entering the external environment not all eggs undergo all stages of development and find intermediate and definitive hosts. Only a few of them reach the stage of Marita. Therefore, to ensure the survival of the species and offspring, it is necessary to produce a large number of eggs.

High sexual productivity is also provided by the reliable protection of the embryo in the egg, for which a shell resistant to external environmental conditions is formed.

The process of synthesis of the eggshell membrane in the studied trematode takes place in the ootype. The vitelline ducts open into this ootype and supply mature vitelline cells. Classical literary sources show that the ootype receives spermatozoa from the seminal receptacle and egg from the ovary.

Our studies have shown that vitelline cells go through 4 stages of development: Undifferentiated cells, developing cells with initial signs of synthetic activity, developing cells with active protein synthesis, and fully mature vitelline cells.

Mature vitelline cells of *P. robusta* accumulate in the vitelline reservoir. The shell globules of mature vitelline follicles, enter the ootype after migration from the vitelline reservoir, from where they enter the forming egg and start to form the shell as a result of the tanning process. This is confirmed by the work of other researchers (Shaimardanov, 2002; Grebenshhikov and Budancov, 2011).

Our studies have shown that shell globules with shell granules are localized on the periphery of the egg near the primary membrane. The cytoplasm of a fertilized egg of *P. robusta* is represented by a granular structure with a moderate electron density. We consider the structures in the thin-walled cytoplasm to be structures containing enzymes. They are localized throughout the egg, including near the vitelline globules. We believe that under the action of the above-mentioned enzymes, the process of destruction of the vitelline granules is accelerated and the shell material is released. Shell substances released during the destruction of granules are necessary for the process of trematode eggs shell formation.

The involvement of the Mehlis gland in the process of formation of the eggshell is obvious. Our studies show that the Mehlis gland cells of the 1st type of the *P. robusta* trematode are localized in the ootype region next to the forming egg and mature vitelline cells that migrate from the vitelline reservoir. Substances secreted by the glandular cells form the eggshell.

The cells of the Mehlis gland are in contact with mature vitelline cells, which is confirmed by the fact that substances secreted by glandular cells are visible in the intercellular space. We believe that these substances are stimulants for the release of shell material from vitelline cells.

Vitelline globules are located along the periphery of the forming egg. The shell granules that make up the vitelline globules are smaller than mature vitelline cells. In our opinion, vitelline globules are destroyed as the shell material is released, which is used to form a resistant eggshell.

The 2nd type of Mehlis gland cells is localized distally from the ootype and consists of vacuoles and vesicles with different electron densities. Smyth J. and Clegg K. in their studies found mucopolysaccharides in the secretion of these cells, which confirms the mucus-like consistency of such substances (Shaimardanov, 2002). These cells improve the process of formed egg migration along the uterine loops.

We guess that the Mehlis gland secret can also affect the speed of migration of spermatozoa to the egg and the activity of the fertilization process.

The process of eggshell formation has a direct relationship with the functional peculiarities of the vitelline glands and Mehlis glands. From a functional point of view, the vitelline granules are ready and contain mature shell material (precursor proteins-not tanned proteins). Vitelline granules, which are in the ootype or proximal uterus, release proteins for the tanning process under the agent's action and possible pH changes in the ootype or proximal uterus.

Conclusion

Based on the study of the *P. robusta* trematode female reproductive system, the following conclusions are presented.

Studies of the *P. robusta* trematode female reproductive system demonstrate the involvement of the vitelline glands and Mehlis gland in the process of formation of the eggshell, which occurs in the ootype.

Vitelline cells are formed in vitelline follicles located on the body sides of a parasitic worm.

The shell material of mature vitelline cells is released under the influence of stimulants secreted by the first type of Mehlis gland cells and forms a resistant shell membrane.

The main functions of the Mehlis gland are:

1) Activation of the process of shell material release from vitelline cells
2) Formation of a resistant eggshell as a result of the shell material layering
3) Facilitating the process of egg migration along the uterine loops because of "mucosal" substances secreted by cells of the second type. The mucoid component of the Mehlis` gland secret acts as a lubricant and facilitates the migration of eggs along the uterine loops

The obtained data on the micromorphology, ultrastructure, and functions of the *P. robusta* trematode vitelline glands and Mehlis` gland will supplement the knowledge of the features of the resistant egg shell formation process, which provides resistance to the second order environment effects. The results of work on the micromorphology and ultrastructure of structural elements of the female reproductive system of trematodes in normal conditions can be used for further research in the selection of anthelmintic agents that affect vitelline glands, Mehlis gland, eggshell tanning processes, which will lead to directed regulation of the fertility level of endoparasites.

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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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