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First study of vitellogenesis of the broad fish tapeworm *Diphyllobothrium latum* (Cestoda, Diphyllobothriidea), a human parasite with extreme fecundity

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ABSTRACT

In the present study, the process of vitellogenesis of one of the most prolific organisms, the broad tapeworm, *Diphyllobothrium latum*, the causative agent of human diphyllobothriosis, was studied for the first time using transmission electron microscopy. Cytochemical staining with periodic acid-thiosemicarbazide-silver proteinate for detection of glycogen was applied. Starting from the periphery toward the center of the vitelline follicle four stages of vitellocytes are differentiated: immature vitellocytes, early maturing vitellocytes, advanced maturing and mature vitellocytes. Differentiation into mature vitellocytes involves the formation of shell globule clusters containing shell globules, large amount of saturated lipid droplets and glycogen. A peculiar ultrastructural feature of *D. latum* vitellogenesis is the presence of lamellar bodies in the cytoplasm of mature vitellocytes. This feature is similar to that present in the closely related caryophyllideans and spathebothriideans. Despite the great similarity observed in the embryonic development of diphylobothriideans, caryophyllideans and spathebothriideans, and the fact that their vitellocytes share a feature not reported from other cestode groups, there are substantial differences in the morphology of vitelline clusters, types, amount and localization of their nutritive reserves.

1. Introduction

The Neoophora, i.e. a group that includes most flatworms (Lophotrochozoa: Platyhelminthes), is unique among animals in having a female germ cell lineage, which divides into two cell lines – ovarium and vitellarium – which become spatially and functionally segregated into separate organs [1]. The vitellarium line forms vitellocytes, which generally possess glycogen, yolk and shell globules needed for later development [1].

Fecundity, i.e. reproductive potential, most easily measured as daily production of eggs, of human-infecting tapeworms (Cestoda) is extremely high, being one of the highest within all animals. Besides strobilization, i.e. serial repetition of a large number (up to several thousands) of proglottids, i.e. sets of genital organs, this extraordinary fecundity is facilitated by effective production of vitellocytes. The vitellocytes have two important functions in the embryogenesis of cestodes: (1) protein synthesis for the formation of a thick and hard egg-shell or a delicate vitelline capsule and (2) lipid accumulation and glycogenesis as a food source for the developing embryo [2,3].

Broad fish tapeworm, *Diphyllobothrium latum* (Linnaeus, 1758), is the most important causative agent of diphyllobothriosis, which itself is not a life-threatening disease, but is considered the most important fish-borne zoonosis caused by a cestode parasite, with up to 20 million persons estimated to be infected worldwide [4]. This cestode has extremely high fecundity and one worm is estimated to produce up to 1 million eggs per day [5]. Ultrastructure of its genital system is only partly known, even though the first ultrastructural studies were carried out in the 1960s [6–8]. However, no ultrastructural data exist on a very important component of the process of reproduction, i.e. vitellogenesis. In fact, little is known about the vitellogenesis in the whole order Diphyllobothriidea, which includes usually large-sized parasites of mammals, including man, birds, reptiles and amphibians [9].

The only study of vitelline cells and their role in the formation of the egg-shell, based on light microscopical observation, was published more than 50 years ago [10]. However, no details of the process of maturation of vitelline cells of broad fish tapeworms are presented in this pioneer, but now much outdated study. In the present study, a complete ultrastructural description of the process of vitellogenesis in the broad fish tapeworm is provided, with the main goals to characterize features that may have contributed to extraordinary fecundity of this human-infecting parasite and to provide ultrastructural characters that might unravel the evolution of morphological and physiological adaptations of cestodes related to their reproductive biology.

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2. Materials and methods

Adult specimens of *D. latum* studied by TEM were collected from the intestine of golden hamster (*Mesocricetus auratus*) experimentally infected with plerocercoids from the musculature of naturally infected perch (*Perca fluviatilis*) collected from Lake Iseo, Italy, on February 5, 2013. The tapeworms from freshly killed host were rinsed in 0.9% NaCl solution. Fragments of strobila containing mature and gravid proglottids were fixed in cold (4 °C) 1.5% glutaraldehyde and 1.5% paraformaldehyde solutions in 0.1 M Hepes (pH 7.4) and stored for 4 weeks at 4 °C. After washing with 0.1 M Hepes (pH 7.4), they were post-fixed in cold (4 °C) 1% osmium tetraoxide (OsO_4) in the same buffer for 1 h, dehydrated in a graded series of acetone, embedded in Spurr's epoxy resin and polymerized at 62 °C for 48 h. Ultrathin sections (60–90 nm in thickness) were cut on a Leica Ultracut UCT ultramicrotome, placed on copper grids and stained sequentially with uranyl acetate and lead citrate according to Reynolds [11].

The Thiéry technique [12] was applied for detection of glycogen. Gold grids with ultrathin sections were treated in periodic acid, thiosemicarbazide and silver proteinate (PA-TSC-SP) as follows: (1) 10% PA for 30 min, rinsed in distilled water, (2) TSC for 24 h, rinsed in acetic solutions and distilled water, (3) 1% SP for 30 min in the dark, and rinsed in distilled water.

Grids with ultrathin sections were examined under a JEOL 1010 transmission electron microscope at 80 kV (Laboratory of Electron Microscopy, Institute of Parasitology, České Budějovice). The voucher material (whole mounts of the same specimen) is deposited at Helminthological Collection of the Institute of Parasitology, Academy of Sciences of the Czech Republic (IPCAS No. C-650).

3. Results

Vitellarium of *D. latum* is formed by numerous follicles situated in two lateral bands in the cortical parenchyma. Each vitelline follicle is composed of vitelline cells in various stages of maturation that are surrounded by interstitial tissue consisting of cytoplasmic processes and nuclei (Fig. 1A). The interstitial nuclei are situated in the central part or at the periphery of the follicle and contain electron-dense clumps of heterochromatin. The cytoplasm of the interstitial tissue is filled with a few small mitochondria and vesicular inclusions (Fig. 1B). The follicles are enveloped by a basal lamina (Fig. 1A). Four stages of vitellocyte development can be differentiated: immature vitellocytes (stage I), early stage of maturation (stage II), advanced stage of maturation (stage III) and mature vitellocytes (stage IV).

3.1. Immature vitellocytes (Figs. 1A,C and 4I)

Immature vitellocytes of *D. latum* possess a large, centrally situated nucleus and a small amount of cytoplasm (Figs. 1C; 4I). They occur predominantly at the periphery of the vitelline follicle and progressively mature toward the center of the follicle (Fig. 1A). The nucleus of immature vitelline cells exhibits irregular dense clumps of heterochromatin. At this stage of development, the nucleolus is not observed. Numerous free ribosomes and mitochondria of different sizes are visible in the perinuclear cytoplasm, and there are no evident endoplasmic reticulum or Golgi complexes.

3.2. Early stage of maturation (Figs. 1D–F, 2A,B,E and 4IIA,B)

At this stage of maturation, vitelline cells are distinguished by increase of cytoplasm volume, development of cytoplasmic organelles and appearance of individual shell globules. The cells contain a large nucleus with numerous clumps of heterochromatin and a roundish nucleolus (Figs. 1D; 4IIA). Cytoplasm matrix possesses Golgi complex, which is made up of a few flattened membrane-enclosed sacs and long parallel cisternae of granular endoplasmic reticulum (GER) (Figs. 1E, F; 2A, B,E). These organelles are associated with the formation of shell globules and shell globule clusters. Initially, small round individual shell globules (ca. 0.3 μm in diameter) appear inside the electron-translucent vesicles of Golgi origin. Usually, they are confined to the cell periphery (Fig. 1D). The vesicles further gradually increases in size and fuse, thus forming larger membrane-bound shell globule clusters embedded in an electron-lucent matrix (Fig. 1F). The number of shell globules within clusters varies widely. At this stage, the clusters are made up of 2–5 shell globules (Figs. 1F; 2A, B). In more mature cells, the number of shell globules in clusters reach up to 30. A few electron-lucent lipid droplets are present in the cytoplasm (Figs. 2A; 4IIB).

3.3. Advanced stage of maturation (Figs. 2C,D, F, G and 4III)

During this stage the cytoplasm is abundant with cellular organelles such as granular endoplasmic reticulum, lipid inclusions and glycogen granules, which denotes high secretory activity of the vitellocytes. Vitelline cells present also the nucleus with electron dense clumps of heterochromatin (Figs. 2C, F; 4III). Shell globule clusters are composed of numerous individual shell globules of various sizes (from 0.4 μm to 0.9 μm in diameter) (Fig. 2D). The concentric rows of granular endoplasmic reticulum are often found in close association with the shell globule clusters and lipid droplets (Fig. 2D, G). The cytoplasm of vitellocytes is provided with large amount of glycogen granules scattered among the lipid droplets (Fig. 2G).

3.4. Mature vitellocytes (Figs. 2H, 3A–F and 4IV)

Mature vitellocytes are large ovoid in shape and their cytoplasm is rich in shell globule clusters, electron-lucent lipid droplets, glycogen granules and lamellar bodies. Shell globule clusters can consist of more than 35 loosely packed shell globules (Figs. 2H; 3A, F; 4IV). Large amount of glycogen granules and lipid droplets (up to 1.4 μm in diameter) are observed throughout the cytoplasm (Figs. 2H; 3F). The presence of glycogen in vitelline cells has been revealed using PA-TSC-SP cytochemical technique (Fig. 3E). A remarkable feature of some mature vitelline cells is the presence of dark lamellar bodies (ca. 0.6 μm) (Fig. 3A–D).

4. Discussion

One of the most remarkable features of the reproductive system of flatworms (Neoophora) is the presence of specialized cells, vitellocytes, participating in the process of egg-shell formation. The embryonic development in cestodes of the recently established order Diphyllobothriidea [9] is polylecithal and occurs in external aquatic environment, similar to other lower eucestodes, i.e. caryophyllideans, bothriocephalideans, spathebothriideans, diphyllideans and trypanorhynchs. Diphyllobothriidean cestodes produce a large number of polylecithal eggs, which are characterized by a thick and hard egg-shell. A great amount of nutritive reserves (lipids and glycogen) is required during embryonic development of these cestodes, which allows them to remain in the water until the embryonation is complete [3]. Therefore, the ultrastructural aspects of the maturation of vitelline cells participating in the egg-shell formation are considered an important step toward better understanding of the reproductive biology of cestodes.

The first detailed study on vitellogenesis in broad fish tapeworm, *D. latum*, which is a human parasite with extreme reproductive potential, demonstrated that the process follows the general pattern previously described in other investigated representatives of evolutionary more basal cestodes [13–27]. However, numerous studies in the past 10 years have indicated that there are some important distinction regarding the morphology, chemical nature and amounts of vitelline inclusions.