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Molecular-genetic and morphological analysis of the nature of the orthonectid plasmodium (Orthonectida)

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1. Introduction

1.1. Relevance of the study

Parasitism is extremely widespread in nature [1,2]. Throughout evolution, parasitism has independently evolved numerous times in various taxa [3]. Parasitic organisms have a significant impact on the ecology, evolution, and behavior of hosts. They control population sizes, regulate the dynamics and structure of ecosystems, participate in energy exchange, and contribute to trophic interactions within ecosystems [1]. Parasites are among crucial components of the biosphere, emphasizing the need for research on the transition to parasitism in different taxa, the evolution of parasitism, and parasitic adaptations.

Studying the origin of parasitism in certain groups is complicated by the absence of extant "transitional forms." Groups such as Myxozoa, Rhizocephala, Microsporidia, and Orthonectida, fall into this category. Orthonectids were traditionally considered a primitive intermediate form between protists and true multicellular organisms [4–7]. However, recent molecular analyses have confirmed their placement within Spiralia [8–13]. Orthonectids have diverged significantly from other spiralians, undergone secondary simplification, and become endoparasites.

Orthonectids possess an intricate life cycle with the alternation of asexual and sexual generations. The parasitic plasmodium, which infiltrates host tissues, represents their asexual generation. The plasmodium cytoplasm contains not only typical cellular organelles but also individual cells and developing embryos of the following sexual stage, which is a unique feature to Bilateria. Orthonectid males and females are then released into the ambient environment, where they copulate and produce larvae, which infect new hosts.

Although the vast majority of Orthonectida-related studies consider various aspects of morphology of sexual stages and their relevance to phylogeny [14–21], the main evolutionary gain of orthonectids is the parasitic plasmodium. Free-living stages exist in the ambient environment for a few days engaged only in sexual reproduction and dissemination, while the parasitic plasmodium performs almost all essential functions: feeding, growth, maintaining the development of sexual orthonectid individuals, interaction with the host and spreading across its tissues. Despite the plasmodium being orthonectid main adaptation to a parasitic lifestyle, various aspects of its development, functioning and interactions with the host remain unresolved. There is still no consensus on the origin of the plasmodium: whether it is an altered host cell or a parasitic organism that develops in the host extracellular environment [22–25].

Over the past 40 years, only four articles on the morphology of the orthonectid plasmodium have been published, and there have been no original works on this topic in the last 15 years. The limited number of studies devoted to orthonectid plasmodium do not allow to answer many questions regarding the origin and functioning of this peculiar parasitic organism. A comprehensive investigation of the orthonectid parasitic stage using modern morphological and molecular methods will enhance the current knowledge of orthonectid parasitism and ultimately address the long-standing question about the nature of the plasmodium.

1.2. The goal and the objectives

The **goal** of this study is to conduct a comprehensive analysis of the nature of the orthonectid plasmodium using modern molecular-genetic and morphological methods.

The **objectives** are:

1. Collecting nemerteans *Lineus ruber*, infected with orthonectids *Intoshia linei*, from their natural habitats, and maintaining them in laboratory conditions
2. Description of the fine structure of the *I. linei* plasmodium using morphological methods (optical microscopy and histology, confocal microscopy and immunohistochemistry, transmission electron microscopy, serial block-face electron microscopy)
3. Identification and characterization of orthonectid genes expressed explicitly in the *I. linei* parasitic stage using RNA-seq analysis

1.3. Theoretical and practical significance

Currently, investigating the evolutionary origin of parasitism remains a major challenge for evolutionary biologists and parasitologists. With the advancement of modern techniques, efforts have increased to understand how the transition from a free-living organism to a parasitic one occurred. In a broad sense, research on the orthonectid parasitic stage is of fundamental biological interest as it aims to explore the nature of parasitism in one of the most intricate parasitic groups. On a more specific level, this study plays a vital role in enhancing the understanding of the biology of orthonectids, contributing to the resolution of long-standing questions regarding the origin and nature of the orthonectid plasmodium.

Orthonectids infect a diverse array of marine invertebrates, contributing to various marine ecosystems. The investigation of orthonectid parasitism holds importance for marine ecology and mariculture. As this study provides insights into a variety of parasite-associated orthonectid genes, its practical implications extend to the potential identification of new targets for antiparasitic drugs and

the development of defense systems for safeguarding agricultural animals and plants against parasitic invasions.

1.4. Scientific novelty

The study of the orthonectid parasitic stage has been notably limited, with research focused on this aspect being conducted only in four orthonectid species. Prior investigations primarily employed optical and transmission electron microscopy to describe the structure of the plasmodium. Throughout the 20th and 21st centuries, only four original articles have addressed the orthonectid parasitic stage. The most recent article dedicated to the orthonectid plasmodium was published in 2009. The current study introduces a significant leap in scientific exploration, employing advanced methodologies such as immunohistochemistry, serial block-face microscopy, and RNA-seq analysis. Importantly, these methods are applied for the first time to investigate the orthonectid parasitic stage. Consequently, the research contributes original data on the development and functioning of the orthonectid plasmodium. The study also provides the first insights into the host-parasite relationship of orthonectids.

1.5. Methodology

All methods used in this study are described in more detail in the section “Materials and Methods” and in the published articles [26,27].

The study was conducted on the parasitic stage of the orthonectid *Intoshia linei* Giard, 1877 [5], a parasite of the nemertean *Lineus ruber* Müller, 1774 [28] (Nemertea: Pilidiophora: Heteronemertea). Infected hosts were observed using stereomicroscopy and kept in laboratory conditions until fixation.

The description of the plasmodium fine structure was performed using optical microscopy (paraplast sections: Azan trichrome stain, semi-thin resin-embedded sections: methylene blue stain), immunohistochemistry (frozen sections: incubation in DAPI, TRITC-conjugated phalloidin, antibodies against acetylated α-tubulin and serotonin), transmission electron microscopy and serial block-face electron microscopy.

For the analysis of the plasmodium-specific orthonectid genes, RNA of three samples (infected host, uninfected host, sexual stages) was isolated and sequenced. Bioinformatic analysis, encompassing read quality control, transcriptome assembly, mapping, differential expression analysis, and protein functional annotation, was performed to identify orthonectid genes and their corresponding proteins expressed explicitly during the parasitic stage of the orthonectid life cycle.

1.6. Approbation of the study and publications

The results of this study were presented on the following conferences:

1. Skalon, E. K. (2017). The orthonectid (Orthonectida) plasmodium research. 1st Scientific Student Session of SPbSU ERS “Belomorskaia”. February 10, 2017. St Petersburg, Russia.
2. Skalon, E., & Slyusarev, G. (2018). Are there any plasmodial nuclei in the plasmodium of orthonectids (Orthonectida)? 2nd Scientific Student Session of SPbSU ERS “Belomorskaia”. February 9, 2018. St Petersburg, Russia.
3. Skalon, E. K. (2018). The origin of the plasmodium of Orthonectida: IHS and ultrastructural data. International Youth Science Forum "Lomonosov-2018". April 9-13, 2018. Moscow, Russia.
4. Skalon, E., & Slyusarev, G. (2019). The “plasmodium” of orthonectids — what is it? 3rd Scientific Student Session of SPbSU ERS “Belomorskaia”. February 8, 2019. St Petersburg, Russia.
5. Skalon, E., Slyusarev, G., & Bondarenko, N. (2021). Discovering orthonectids plasmodium-specific genes (Bilateria: Orthonectida). International Conference “Bioinformatics: From Algorithms to Applications". August 12-13, 2021. St Petersburg, Russia.
6. Skalon, E., Bondarenko, N., & Slyusarev, G. (2021). New insights into the origin of the orthonectids' parasitic plasmodium (Bilateria: Orthonectida). 13th European Multicolloquium of Parasitology. October 12-15, 2021. Belgrade, Serbia.

As a result of this study, three articles were published in the international peer-reviewed journals indexed in Web of Science Core Collection and Scopus.

1. Skalon, E. K., Starunov, V. V., Bondarenko, N. I., Slyusarev, G. S. (2023). Plasmodium structure of Intoshia linei (Orthonectida). Journal of Morphology, 284(7), [e21602]. https://doi.org/10.1002/jmor.21602
2. Slyusarev, G. S., Skalon, E. K., Starunov, V. V. (2023). Evolution of Orthonectida body plan. Evolution and Development. https://doi.org/10.1111/ede.12462
3. Skalon, E.K., Starunov, V.V., Slyusarev, G.S. RNA-seq analysis of parasitism by *Intoshia linei* (Orthonectida) reveals protein effectors of defence, communication, feeding and growth. Journal of experimental zoology. Part B, Molecular and developmental evolution. https://doi.org/10.1002/jez.b.23247

1.7. Author’s personal contribution

The author of this work took an active part in all phases of study: goal and objectives setting, field work, including specimen collection, obtaining and interpretation of the results, writing of scientific manuscripts and presentation of the results at the conferences. Literature search and analysis, experimental work (optical, electron and confocal microscopy), bioinformatic analysis were performed personally by the author. Fixation, sectioning and staining for histological examination, as well as RNA isolation and sequencing were performed by Viktor Starunov. Several schemes were collaboratively prepared by the author of this work, V. Starunov, A. Burnusuz and G. Slyusarev.

1.8. Main results

1. The morphology of the orthonectid *I. linei* plasmodium was examined. The plasmodium was studied using immunohistochemical methods, as well as serial scanning electron microscopy for the first time [26, p. 3]. Novel results on the structure of the plasmodium were also obtained using optical and transmission electron microscopy [26, p. 3]. The fine structure of the plasmodium was described in detail [26, pp. 3-7]. Literature analysis and comparison of the details of the structure of an orthonectid plasmodium and a myxozoan plasmodium were conducted [26, pp. 7-8]. Based on the morphological data obtained, an analysis of the nature of the orthonectid plasmodium was carried out [26, pp. 9-11]. A scheme for the development of the orthonectid plasmodium and its dissemination across the host tissues was proposed [26, p. 11]. The results were published in [26]. The author's personal contribution in obtaining these results: material collection, sample preparation, analysis of morphological data, literature review, interpretation of results, and article writing.

2. The molecular-genetic analysis of the orthonectid *I. linei* plasmodium was performed. RNA sequencing data of the plasmodium were obtained for the first time [27, p. 2]. Proteins corresponding to genes of *I. linei* expressed exclusively in the parasitic stage were characterized [27, p. 3]. Potential molecular effectors involved in various processes related to the functioning of the orthonectid plasmodium were identified [27, pp. 3-5]. Literature data on proteins playing a key role in the activity of other endoparasites were analyzed [27, pp. 4-5]. The results were published in [27]. The author's personal contribution in obtaining these results: material collection, bioinformatic analysis, literature review, interpretation of results, and article writing.

3. Based on literature data, it was shown that plasmodia of all studied orthonectid species have a similar body plan [21, p. 6]. Characteristic features of orthonectid plasmodium organization were described [21, p. 6]. The results were published in [21]. The author's personal contribution in obtaining

these results: literature analysis, interpretation of results, writing the section of the article dedicated to orthonectid plasmodium.

1.9. Principal findings presented for defense

1. Orthonectid plasmodium is a tissue parasite. It is a multinuclear organism containing in its cytoplasm numerous reproductive cells that give rise to sexual individuals, and orthonectid sexual individuals on various developmental stages.

2. Orthonectid genes expressed explicitly in the plasmodium stage and corresponding proteins include effectors known for other endoparasites and are crucial for plasmodium survival within the host. They are involved in various key processes such as growth within the host tissues, obtaining cues from the host, defense against the host, nutrients absorption, and support of sexual stage development.

2. Literature review

2.1. The first description of Orthonectida

The first record of orthonectids dates back to 1869. Werner Keferstein discovered enigmatic oval animals in turbellarians [29]. Five years later, McIntosh [30] observed small ciliated animals during the dissection of a nemertine. Both researchers provided detailed illustrations of the invertebrates they found, but did not accompany them with any descriptions. The group Orthonectida was formally described only in 1877 by Alfred Giard. He described his mysterious discovery from the ophiuran *Ophiocoma neglecta* (now *Amphipholis squamata*) [5,31]. Giard discovered mysterious animals resembling large ciliates in bursal sacs upon dissecting the central disk of the ophiuran. He assigned to these parasitic organisms the rank of species, naming them "*Rhopalura ophiocomae*". In the same article, he defined two more orthonectid species that had been found before but not yet described: *Intoshia linei* from the nemertean *Lineus gesserensis* (now *Lineus ruber*) and *Intoshia leptoplanae* from the turbellarian *Leptoplana tremellaris*. He organized all these newly identified species into a distinct group named "Orthonectida" due to their ability to swim in a straight line. Giard initially described orthonectids as multicellular planuliform bilayered animals covered with cilia and having a metameric structure [6]. Alfred Giard was the first to consider whether the simple structure of orthonectids is a primary state or if it developed due to secondary reduction and adaptation to a parasitic lifestyle. It is important to highlight that, despite the limited methods available in the late 19th century, Giard successfully classified orthonectids as a distinct and high-ranking group, a classification that remains undisputed to this day.

2.2. The phylogenetic position of orthonectids

The phylogenetic position of orthonectids remains unclear and debate about it continues to this day. Traditionally, the group was considered primitive and classified into a phylum Mesozoa, evolutionary intermediate forms between Protozoa and Metazoa, together with other enigmatic parasitic group Dicyemida [4–7].

However, the monophyly and sometimes even the validity of Mesozoa was questioned, first by Caullery and Mesnil [32], and later by other researchers [16,33–35]. Even though Caullery and Mesnil noted the resemblance between orthonectids and dicyemids, drawing parallels between the life cycles of these animals, these researchers suggested that orthonectids underwent secondary reduction due to

their parasitic lifestyle [32]. Then, an 18S rDNA sequence analysis confirmed that Mesozoa is polyphyletic and orthonectids represent triploblastic phyla of uncertain phylogenetic affinity [35].

As an alternative to Dicyemida, different groups of invertebrates were proposed to be a sister group of orthonectids, first based on their morphology [36,37]. In the 21st century, close phylogenetic relationship between annelids and orthonectids was supported by the analyses of mitochondrial and nuclear genes [8,10–12]. However, some recent phylogenetic surveys have revived the classical hypothesis on monophyletic Mesozoa (Orthonectida + Dicyemida) according to transcriptomic and genomic analyses, and the phylum was positioned either sister to Gastrotricha [9], or to Platyhelminthes/Gnathifera [13].

2.3. Orthonectid life cycle

Ilya Ilyich Mechnikov has made a significant contribution to the understanding of orthonectid life cycle. He was the first who described that orthonectid embryos and adults are developing inside the so-called “protoplasmic tubes” (“protoplasmatische Schläuche”) or “plasmodial sacs” (“die Plasmodiumsäcke”) within the host tissues [36]. In this study, Mechnikov made initial hypotheses about how the life cycle of orthonectids unfolds. He proposed that the fertilization of female orthonectid takes place in the ambient environment. Afterwards, the fertilized female infects the brittle star *Amphiura squamata* (in the case of the species *Rhopalura giardii*, as described by Mechnikov) and undergoes a complete transformation into what he termed a "plasmodial sac". The embryos developing within the fertilized female were reminiscent to Mechnikov of embryos he had observed inside the "plasmodial sac".

In the late 19th - early 20th centuries, Maurice Caullery and Félix Mesnil proposed the scheme of the orthonectid life cycle [32,38]. According to them, sexual orthonectid individuals develop within the so-called plasmodium (“un plasmode”) located within the host tissues. Fully developed, sexual individuals exit the host and copulate in the ambient environment. An unknown form then infects a new host and turns into a plasmodium [38]. They considered the multinucleate plasmodial stage as parasitic and outlined its independence, as it can grow and reproduce within the host, generating ciliated sexual forms.

In 1908, Maurice Caullery, in collaboration with Alphonse Lavallée [39], published a detailed description of the embryonic development of orthonectids, starting from the female's fertilization to the formation of a motile ciliated larva. This work revealed the presence of a dispersal form that infects the host and transforms into a plasmodium. The existence of the larva, originating from the fertilized

female, was subsequently confirmed by Atkins [40] and Nakano [41]. However, a detailed description of its structure is still lacking to this day.

The proposed scheme of orthonectid life cycle was confirmed through a series of experiments conducted by the authors mentioned above [42]. The hosts *Amphipholis squamata* were held together with the larvae of the orthonectid *Rhopalura ophiocomae*. Over different time intervals, the authors observed larvae infecting the bursal sacs of the brittle stars, as well as young plasmodia within the walls of the host gonads.

In the mid-20th century, orthonectids were defined as “the forms in which the sexual generation is formed asexually from germ cells produced in a parasitic plasmodium” [40]. The understanding of the orthonectid life cycle currently remains at the same level. Orthonectids are obligate endoparasites with direct monoxenous life cycle: the parasitic stage parasitize on one host, and free-living stage produce larvae infecting new hosts. The life cycle is characterized by the alternation of asexual and sexual generations also called metagenesis. The asexual generation is represented by the parasitic plasmodium, an endoparasite infiltrating host tissues. The following sexual generation, represented by worm-like ciliated males and females, develops within the plasmodium cytoplasm from reproductive cells. Males and females exit the host by beating their cilia and moving along the protrusions of the plasmodium directed to the host surface. They copulate in the sea water and produce dispersal larvae, which then infect new hosts through the epithelium and develop into the plasmodium, although the exact mechanism of this process is still unknown [43] (Fig. 1).

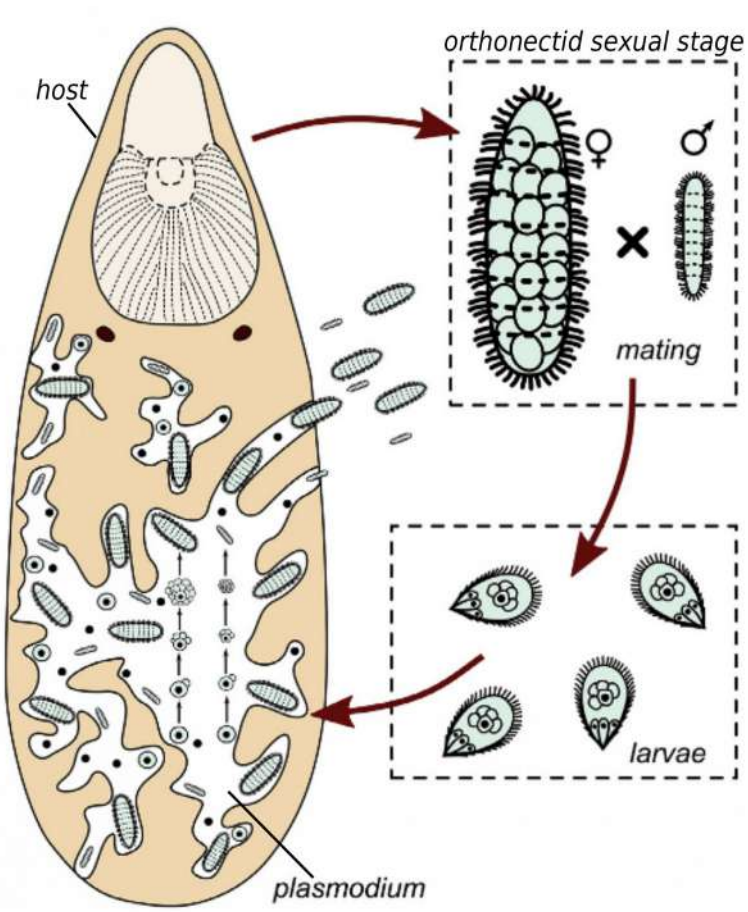

Figure 1. Orthonectid life cycle. From Slyusarev et al., 2020, with changes.

2.4. Sexual orthonectid individuals and dispersal larvae

In early works [31], sexual orthonectid females and males were referred to as "planuliform", emphasizing the exceptional simplicity of their structure. It was noted that, in adult orthonectids, only the ectoderm and endoderm were presented, while the mesoderm remained rudimentary. It was assumed that sexual males and females moved through the water solely by ciliary locomotion, thus sexual orthonectid individuals were compared to ciliates. In Caullery's recent work [44], it is mentioned that, in the case of *Rhopalura ophicomae*, orthonectids do not possess a muscular system at all, and there was no mention of the presence of a nervous system. For a long time, such views on the morphology of adult individuals dominated, distorting the understanding of the phylogenetic position of orthonectids and their overall level of organization.

In later works by Kozloff [14,15], a description of contractile cells in *Rhopalura ophicomae* was presented. Subsequently, Slyusarev [45] provided an account of such cells and also observed that female orthonectids may vary in shape depending on the degree of contraction. In the early 21st century, a fully developed muscular system was demonstrated through phalloidin staining of actin

filaments [17]. The nervous system of orthonectids was also discovered and demonstrated using immunohistochemistry methods [18]. New data obtained through electron and fluorescent microscopy have significantly changed the understanding of orthonectid organization level. Orthonectids were no longer viewed as primitive "planuliform" organisms, and their relationship with more evolutionarily advanced invertebrates was no longer in doubt.

Currently, detailed descriptions of females and males of several orthonectid species are available [14–20]. Both females and males exhibit a worm-like form, with lengths ranging from 25 to 250 μm depending on the species and sex. Their bodies consist of cells of four types: epithelial (ciliated and non-ciliated), muscular, nervous cells, and gametes. The body is covered with a cuticle. Sexual orthonectid individuals lack excretory, digestive, and circulatory systems. Males and females remain alive in sea water for a short period (presumably, a few days), during which they copulate and produce the next stage, dispersal larvae [43].

Orthonectid larvae have been observed by researchers only a few times and are described as spherical organisms approximately 15 μm in length, covered with cilia [40,41].

2.5. The parasitic plasmodium

The first description of the orthonectid plasmodium was made by Metschnikoff in 1881. Mechnikov noted that sexual orthonectid individuals are located within the host body in multinuclear sacs that he called "die Plasmodiumsäcke" [36]. From that point on, the parasitic stage of orthonectids in the scientific community became known as the "plasmodium". However, there is still no consensus on the origin of the orthonectid plasmodium.

The number of studies dedicated to the parasitic stage of orthonectids is limited. Detailed morphological investigations into the orthonectid plasmodium have been undertaken by Kozloff [22,23] and Slyusarev with co-authors [24,25]. Questions about the functioning and origin of the plasmodium have also been addressed by Caullery and Lavallée [42,44].

In the early 20th century, a series of experimental infections conducted by Caullery and Lavallée allowed researchers to reconstruct a possible scheme of the development of the parasitic stage [42]. The authors worked with orthonectid *Rhopalura ophicomae* and brittle star *Amphiura squamata* (now *Amphipholis squamata*). They hypothesized that the orthonectid larva infects the host by penetrating the organism through bursal slits. Several internal cells of the larva are then released and actively penetrate the epithelium of the bursa. The next stage is the young plasmodium, in which the number of nuclei gradually increases. The nuclei of the plasmodium are differentiated: some nuclei are called vegetative and serve to sustain the life of the parasitic organism; others are reproductive.

Cytoplasm surrounds the reproductive nuclei, forming reproductive cells in the plasmodium cytoplasm, which later give rise to orthonectid embryos. According to the authors, the plasmodium mostly serves a trophic function, acting as an intermediary between the host organism and the developing sexual individuals.

In the late 20th century, Kozloff proposed an alternative interpretation of the nature of orthonectid plasmodium, which gained significant support in the scientific community. In his first work dedicated to the plasmodium of the abovementioned species *Rhopalura ophicomae [22]*, Kozloff did not confirm the presence of nuclei in the cytoplasm of the orthonectid plasmodium, as previously indicated by Caullery and other authors [38,44]. According to Kozloff's hypothesis, infectious cells from the larva penetrate through the epithelium of bursal slits or the intestine of the brittle star and infiltrate the host muscle cells located between the epithelium and the coelomic lining. The infected host cell undergoes hypertrophy, and orthonectid reproductive cells (germinal in Kozloff's terminology) within it give rise to orthonectid embryos. Thus, Kozloff insisted that the plasmodium is not a derivative of orthonectids but a modified host cell, and orthonectids are intracellular parasites.

Kozloff's second work on the orthonectid plasmodium [23] confirmed and expanded upon the ideas presented in the first work. The study was focused on orthonectids *Ciliocincta sabellariae*, which parasitize on polychaete *Sabellaria cementarium*. In this work, Kozloff explained the presence of several isolated nuclei in the plasmodium cytoplasm either by artifacts or by the dissociation of the reproductive or embryonic cells whose cytoplasm and cell membrane has disappeared.

Slyusarev and Miller, who mainly worked with orthonectids *Intoshia variabili*, parasites of the turbellarians *Macrorhynchus crocea* (now *Graffiellus croceus*), advocated a different interpretation of the nature of the plasmodium [24]. They demonstrated the presence of free nuclei in the plasmodium cytoplasm, distinct from the host nuclei, using both electron microscopy and, later, fluorescent staining with DAPI [46]. According to the authors, the orthonectid plasmodium has a parasitic nature and develops in the host extracellular environment. The plasmodium was described as a shapeless multinucleated mass of cytoplasm covered by two plasma membranes and containing its nuclei as well as reproductive cells, embryos and sexual orthonectid individuals on different developmental stages. The plasmodium forms numerous fingerlike extensions penetrating the host body that may cause the dissociation of host tissues [43]. The plasmodium consumes surrounding host cells through phagocytosis and pinocytosis [25]. It was also reported that penetrating the host gonads by plasmodium did not lead to the castration of the host [40], but infection of the hosts ganglia injured the hosts and might presumably change their behavior [22].

The scientific community currently lacks a consensus regarding the nature of the orthonectid plasmodium.

3. Material and Methods

All methods used in this study are described in full detail in the published articles [26,27]. Below is a recap of the main procedures.

3.1. Material collection

The material for this study were orthonectids *Intoshia linei* Giard, 1877 parasitizing nemerteans *Lineus ruber* Müller, 1774 (Nemertea: Pilidiophora: Heteronemertea). The hosts, 150-200 specimens per year, were collected in August 2017–2023 on the rocky shore of the Barents Sea near the marine biological station Dalnie Zelentsi (69°07' N, 36°05' E). The hosts were then kept in Petri dishes with filtered seawater at 4°C and examined for orthonectid infection under a stereomicroscope. Depending on a year, from 7 to 10% of the collected specimens were infested by orthonectids. The emission of the free-living stage was triggered by raising the temperature of the water up to 15-20°C.

3.2. Morphological analysis

The process of orthonectid emission from infected hosts was observed under a stereomicroscope. Infected specimens of *L. ruber* were relaxed in 7,5% MgCl2 and fixed depending on a method.

3.2.1. Histology

Infected hosts were fixed in Bouin’s fluid, dehydrated and embedded in Paraplast. 5 μm thick serial sections were cut using a Leica Autocut microtome, mounted on glass slides, stained with Azan trichrome and observed under a Leica DM2500 microscope provided with a Nikon DS-Fi3 camera.

3.2.2. Immunohistochemistry

Infected hosts were fixed in 4% paraformaldehyde with phosphate buffered saline (PBS), then washed in PBS with Triton X-100 (PBT), embedded in Tissue-Tek O.C.T. and cut using Leica CM3050S cryostat. 15-20 μm thick sections were then transferred to glass slides, washed in PBS, blocked in PBT containing 1% bovine serum albumin and incubated in primary antibodies against acetylated α-tubulin and serotonin overnight. Afterwards, sections were washed in PBT and incubated with secondary antibodies overnight. After immunolabeling, sections were stained with TRITC-conjugated phalloidin and DAPI. Sections were mounted in Mowiol and observed using a Leica TCS SPE laser confocal microscope.

3.2.3. Transmission electron microscopy

Infected hosts were fixed in 2.5% glutaraldehyde with 0.1 M cacodylate buffer, pH 7.3, with 6.85% sucrose, then transferred to 1% OsO4 with the same buffer. After fixation, specimens were rinsed in 0.1 M cacodylate buffer, thereafter dehydrated in a graded series of ethanol, and embedded in Epon 812. Sections were cut on a Leica EM UC7 ultramicrotome with a diamond knife. Semi-thin sections were stained with 1% methylene blue and observed under a Leica DM2500 optical microscope equipped with a Nikon DS-Fi3 camera. Ultra-thin sections were observed under a Jeol JEM-1400 STEM transmission electron microscope after staining with uranyl acetate and lead citrate.

3.2.4. Serial block-face electron microscopy

Infected hosts were fixed in 2.5% glutaraldehyde with 2mM calcium chloride in 0.15M cacodylate buffer, pH 7.4, then rinsed and transferred to the mix of 4% OsO4 and 3% potassium ferrocyanide in 0.3M cacodylate buffer with 4mM calcium chloride. After fixation, samples were rinsed and incubated in thiocarbohydrazide solution. After rinsing, samples were transferred to 2% osmium tetroxide in ddH2O and then to 1% uranyl acetate. After *en bloc* lead aspartate staining, samples were rinsed and dehydrated in a graded series of ethanol and acetone, and embedded in Epon 812 hard. Samples were observed under a Thermo Scientific Volumescope 2 scanning electron microscope. Resulting stacks were aligned with the TrackEM2 ImageJ2 plugin.

All images were processed with ImageJ2, GIMP v.2.10.36 and Inkscape v.1.3.2. All schemes were made in Inkscape v.1.3.2.

3.3. Molecular-genetic analysis

Total RNA was extracted from three distinct samples, namely: 1) the sexual stage of *I. linei*, encompassing both male and female individuals released from the host into the water (2 replicates); an entire infected *L. ruber* hosting a parasitic plasmodium (1 replicate), and an entire uninfected *L. ruber* (2 replicates) (Fig. 2). RNA-seq libraries for all samples were prepared following the TruSeq library preparation protocol by Illumina. 100-bp paired-end reads were generated using the Illumina HiSeq2500 sequencing system, and subsequent evaluation and trimming were performed. De-novo assembly and assessment of the transcriptome of uninfected *L. ruber* were conducted. To filter out sequences of host origin from reads of the infected host, they were firstly mapped to the resulting *L. ruber* transcripts and then to the composite genome of *I. linei* (NCBI: GCA_001642005.1) and *Lineus longissimus (*Ensembl: GCA_910592395.2), the closest available relative of *L. ruber*, as recommended for dual-seq experiments [47,48]. Remaining reads of orthonectids from the host were quantified for

each genomic feature of *I. linei*. The mapping, filtering, and quantification steps were repeated for reads of *I. linei* sexual individuals. The orthonectids within the infected host comprise a mixed population of parasitic plasmodium and sexual individuals (Fig. 2), and to identify genes expressed only in the plasmodium, differential expression analysis was conducted between two conditions: sexual orthonectid individuals released from the host and the orthonectids (plasmodium + sexual stage) from the infected host. Genes exhibiting no detectable expression in the sexual stage released from the host were considered likewise unexpressed in the sexual orthonectid individuals sequenced together with the infected host. Therefore, genes showing significant expression in orthonectids from the infected host but not during the sexual stage were presumed to be specific to the orthonectid plasmodium. Despite the limited number of replicates (two in one group and one in the other), it was sufficient for DESeq2 to estimate dispersion across all conditions and produce meaningful results [49]. All samples had high sequencing depth increasing the power of the DE analysis [50]. Low number of replicates may lead to reduced sensitivity, however, it has little effect on strongly changing and highly expressed genes [49–51]. Although some lowly expressed plasmodium-specific genes might not have been captured in the analysis, the sample size did not compromise the credibility of the identified genes. *I. linei* hypothetical proteins corresponding to the detected plasmodium-specific genes were characterized based on cellular localization, secretion potential, and the number of transmembrane domains. Additionally, we identified their conserved domains, families, and superfamilies.

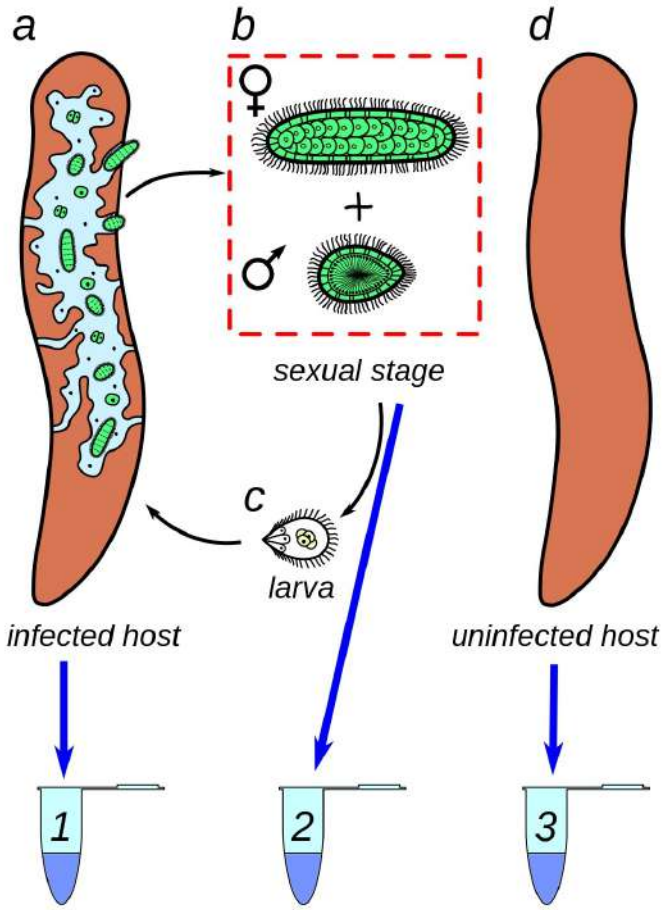

Figure 2. Schematic diagram of orthonectid *Intoshia linei* life cycle indicating samples used for RNA-seq analysis. (a) Infected nemertean *Lineus ruber* (brown) with orthonectid plasmodium (light-blue) parasitizing its tissues, the first sample. The plasmodium harbors the development of cilia-covered sexual males and females (green). (b) Fully matured sexual orthonectid individuals egress from the plasmodium into the ambient environment, the second sample. Free-swimming ciliated males and females copulate; males die right after the copulation, and females remain alive for several days until the release of developed larvae. (c) Larvae develop from fertilized eggs and emerge from females to seawater, infect a host and transform into plasmodia (this process has not been described yet). (d) Nemertean *Lineus ruber* (brown), not infected by orthonectids, the third sample. From [27].

4. Results

4.1. Morphological analysis

4.1.1. Overall plasmodium morphology

The orthonectid plasmodium was visible through the body wall of *Lineus ruber* with the naked eye (Fig. 3A-C). It was stretched along the anterior-posterior axis of the host (Fig. 3A). The plasmodium lacked a clearly defined shape and formed fingerlike extensions of varying diameters and lengths. Some extensions were notably thin, measuring up to 3 μm in diameter (Fig. 4B, 5A, 7A, 9A).

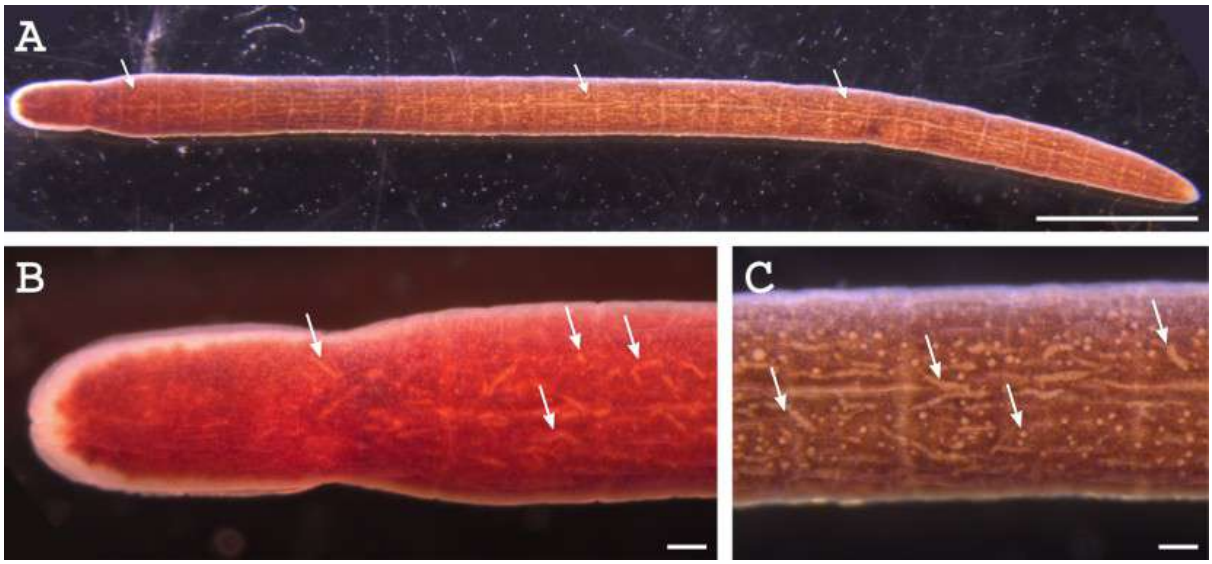

Figure 3. Photographs of *Lineus ruber*, nemertean host infected by *Intoshia linei*, taken under a stereomicroscope. (A) The entire infected specimen. (B) Detail of the head of the infected host. (C) Detail of the body of the infected host. The arrows are pointing to visible parts of the plasmodium. Scale bars: 1 cm in A and 1 mm in B, C. From [26].

Penetrating all internal organs of the host, the plasmodium typically inhabited the host parenchyma and muscle tissue, occasionally infecting the nervous system (Fig. 4A, 9A-B). The plasmodium could occupy a substantial part of the host body (Fig. 4A).

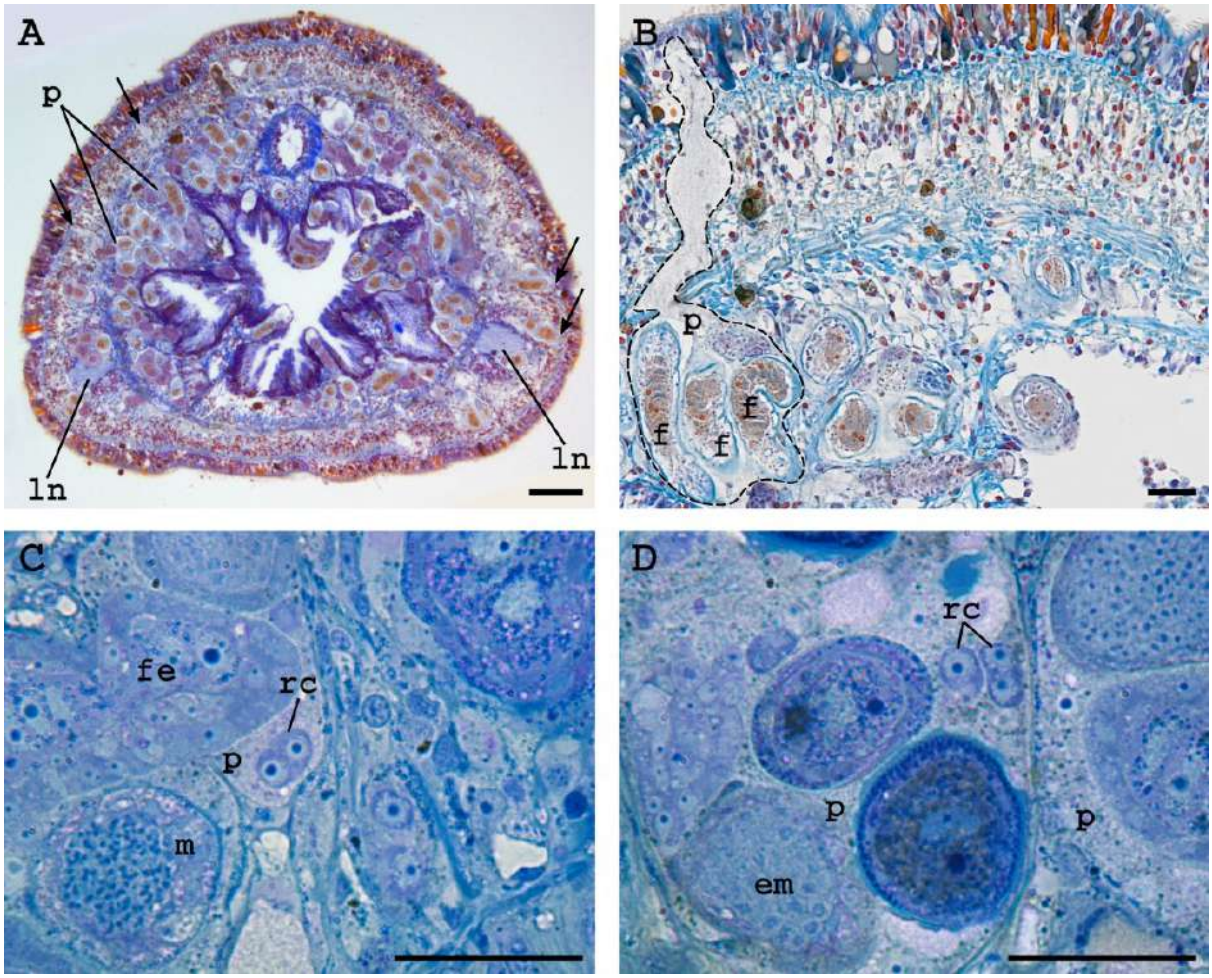

Figure 4. Cross-sections of the infected *Lineus ruber*. (A) Paraffin section; female and male orthonectids are developing in the plasmodium. (B) Paraffin section; plasmodium extension. (C, D) Semi-thin resin sections; double reproductive cells in the plasmodium cytoplasm. em, orthonectid embryo; f, female orthonectid; fe, female orthonectid embryo; ln, lateral nerve cord of the host; m, male orthonectid with spermia; p, orthonectid plasmodium; rc, reproductive cells. Arrows (on A) and dashed lines (on B) indicate plasmodium extensions directed to the surface of the host body for the exit of mature individuals. Scale bars: 100 μm in A and 50 μm in B, C, and D. From [26].

Microvilli-like projections were observed in some areas of the surface of the plasmodium, often perpendicular to its envelope (Fig. 5B, 6B-D, 8B). The envelope of the plasmodium consisted of two membranes of equal density and thickness (Fig. 5C-D).

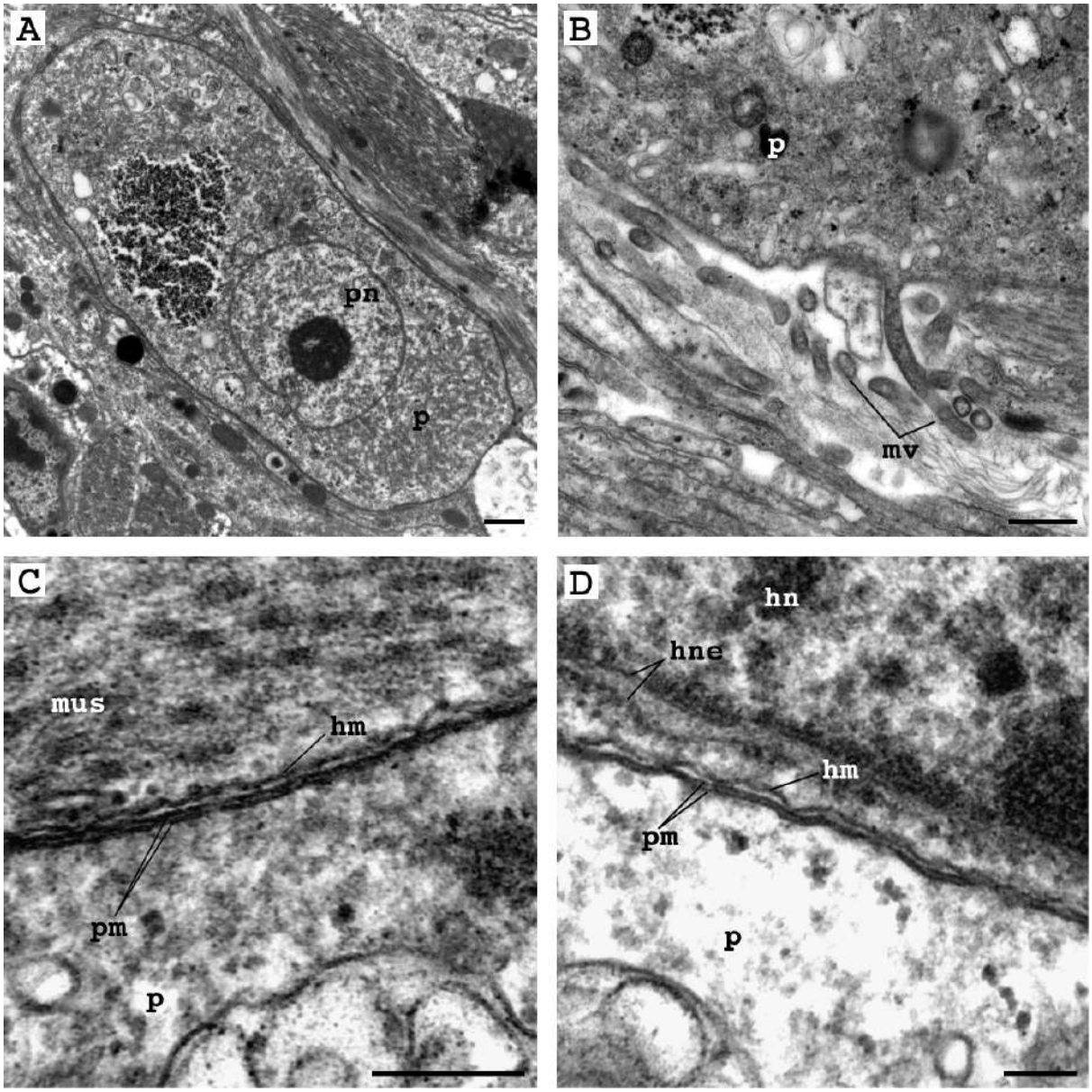

Figure 5. Electron micrographs of the plasmodium extensions and the surface of the plasmodium. (A) Thin plasmodium extension with nucleus. (B) The surface of the plasmodium bears microvilli-like projections. (C, D) The envelope of the plasmodium consists of a double membrane. hm, host membrane; hn, host nucleus; hne, host nuclear envelope; mus, host muscle cell; mv, plasmodium microvilli-like projections; p, plasmodium; pm, plasmodium membrane; pn, plasmodium nucleus. Scale bars: 1 μm in A, B, 200 nm in C, D. From [26].

4.1.2. Plasmodium surface

In a series of block-face scanning electron microscopy images, it was evident that the surface of the terminal area of the plasmodium extension bore a significant number of microvilli-like projections, many of which were long (up to 4 μm) Fig. 6). These microvilli were invaginating the membrane of the host cell, likely facilitating the absorption of nutrients.

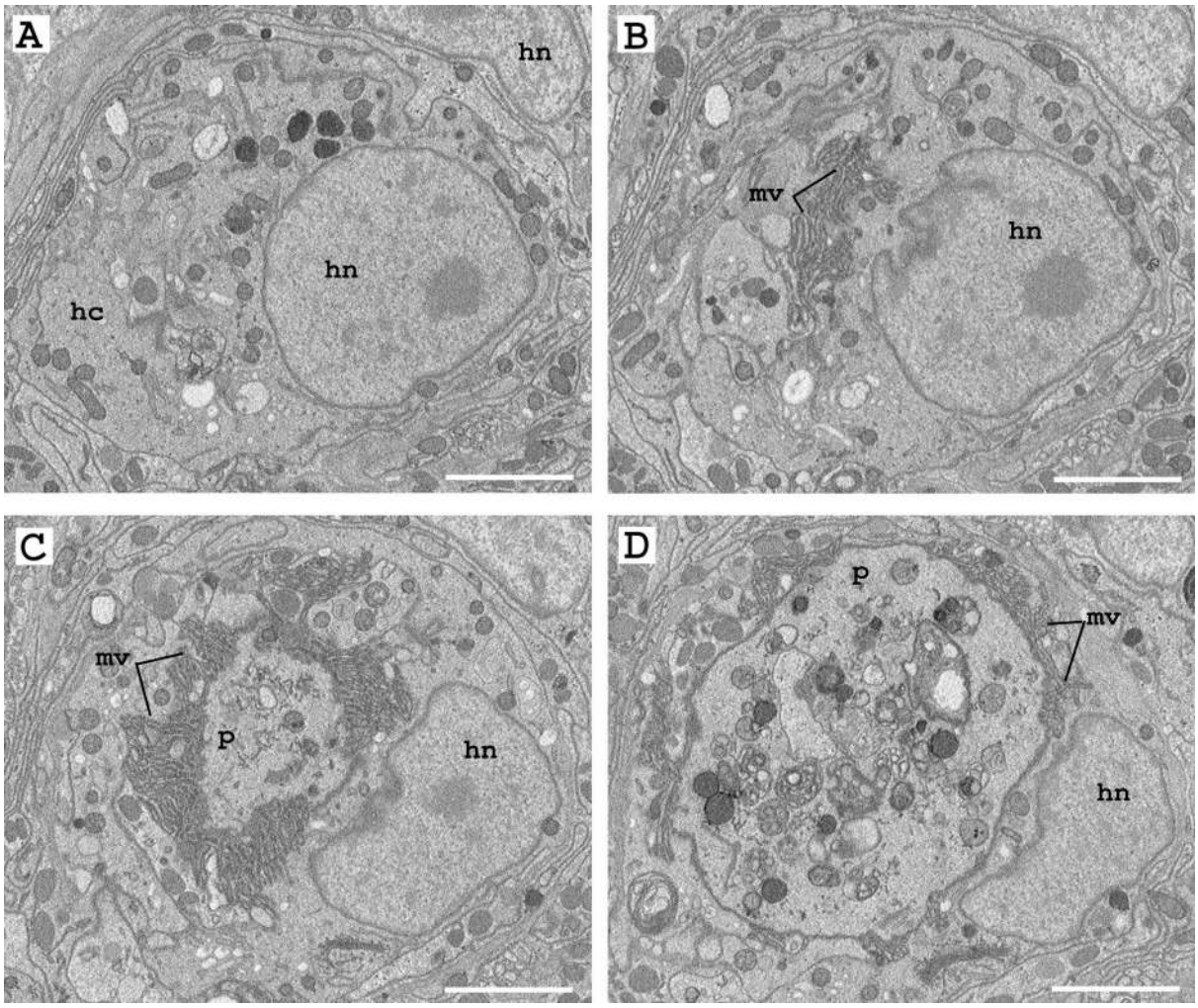

Figure 6. Serial block-face electron micrographs of the terminal area of the plasmodium extension. (A) Host cell with the nucleus. (B) Microvilli-like projections of the plasmodium extension appear in sight. (C, D) The plasmodium cytoplasm appears in sight. hc, host cell; hn, host nucleus; mv, plasmodium microvilli-like projections; p, plasmodium. Scale bars: 5 μm.

4.1.3. Plasmodium cytoplasm

The cytoplasm of the plasmodium contained numerous nuclei (Fig. 5A, 7A, C-D, 9, 11A, C). The nuclei within the plasmodium differed from the nuclei of host cells by size and shape (Fig. 7A, C-D, 11A). Evenly distributed throughout the plasmodium cytoplasm, these nuclei were also present in the thin extensions of the plasmodium (Fig. 5A). Characterized by a rounded shape (approximately 2-2.5 μm in diameter), they contained a dense, well-defined nucleolus (Fig. 5A). Some of plasmodium nuclei undergoing mitosis, likely in metaphase, were observed (Fig. 7D).

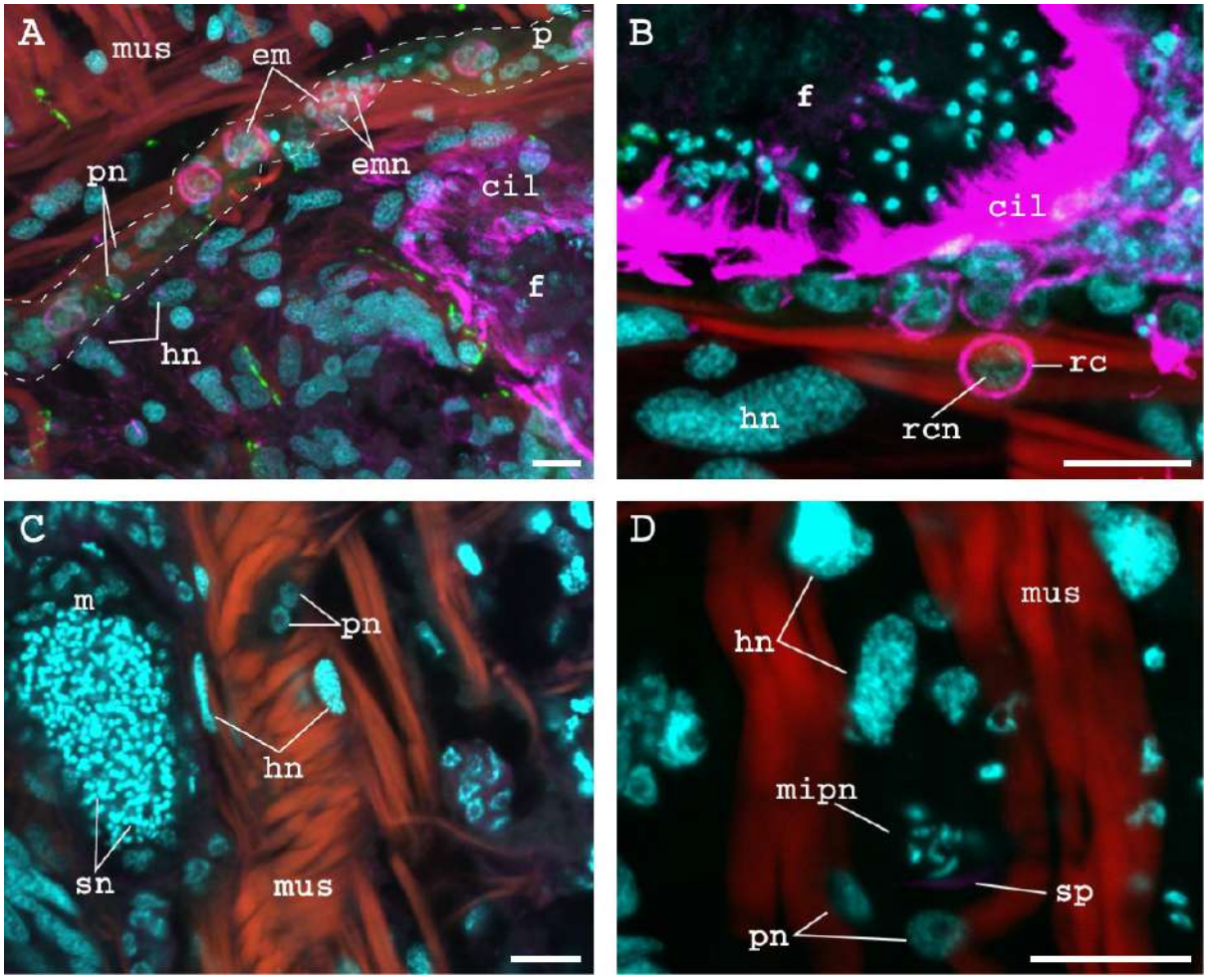

Figure 7. Cryosections of the infected *Lineus ruber*, stained with DAPI (cyan), TRITC-phalloidin (red), α-tubulin antibodies (magenta) and serotonin antibodies (green). (A) Plasmodium extension with nuclei and developing embryos. (B) Reproductive cells in the plasmodium. (C) Plasmodium extension penetrates host muscles. (D) Plasmodium nucleus during mitosis. cil, cilia of mature orthonectid; p, plasmodium; pn, plasmodium nucleus; em, orthonectid embryo, emn, nucleus of the orthonectid embryo; f, orthonectid female; hn, host nucleus; mus, host muscles; m, orthonectid male; mipn, plasmodium nucleus during mitosis; rc, reproductive cell; rcn, reproductive cell nucleus; sn, sperm nuclei of orthonectid male; sp, microtubules of the mitotic spindle. Dashed lines indicate the borders of the plasmodium extension. Scale bars: 10 µm. From [26].

Various membrane-bounded bodies were abundant in the plasmodium cytoplasm (Fig. 8A-B, D, Fig. 9B, Fig. 10A-B). This included plain vesicles, lipid droplets, complex vesicles composed of smaller rounded vesicles, multilamellar and multivesicular bodies, endosomes and spherical electron-dense lysosomes (Fig. 8A-B, D, Fig. 9B, Fig. 10A-B). Many of these vesicles were covered with two membranes. Clusters of multiple vesicles were noted in both the extracellular environment and the plasmodium cytoplasm. One cluster, tightly adherent to the host muscle cell and the plasmodium, was

located in the extracellular space (Fig. 8A). Another cluster, bulging the plasmodium membrane, seemed to be released from the plasmodium into the extracellular space near the host nerve trunk (Fig. 9B).

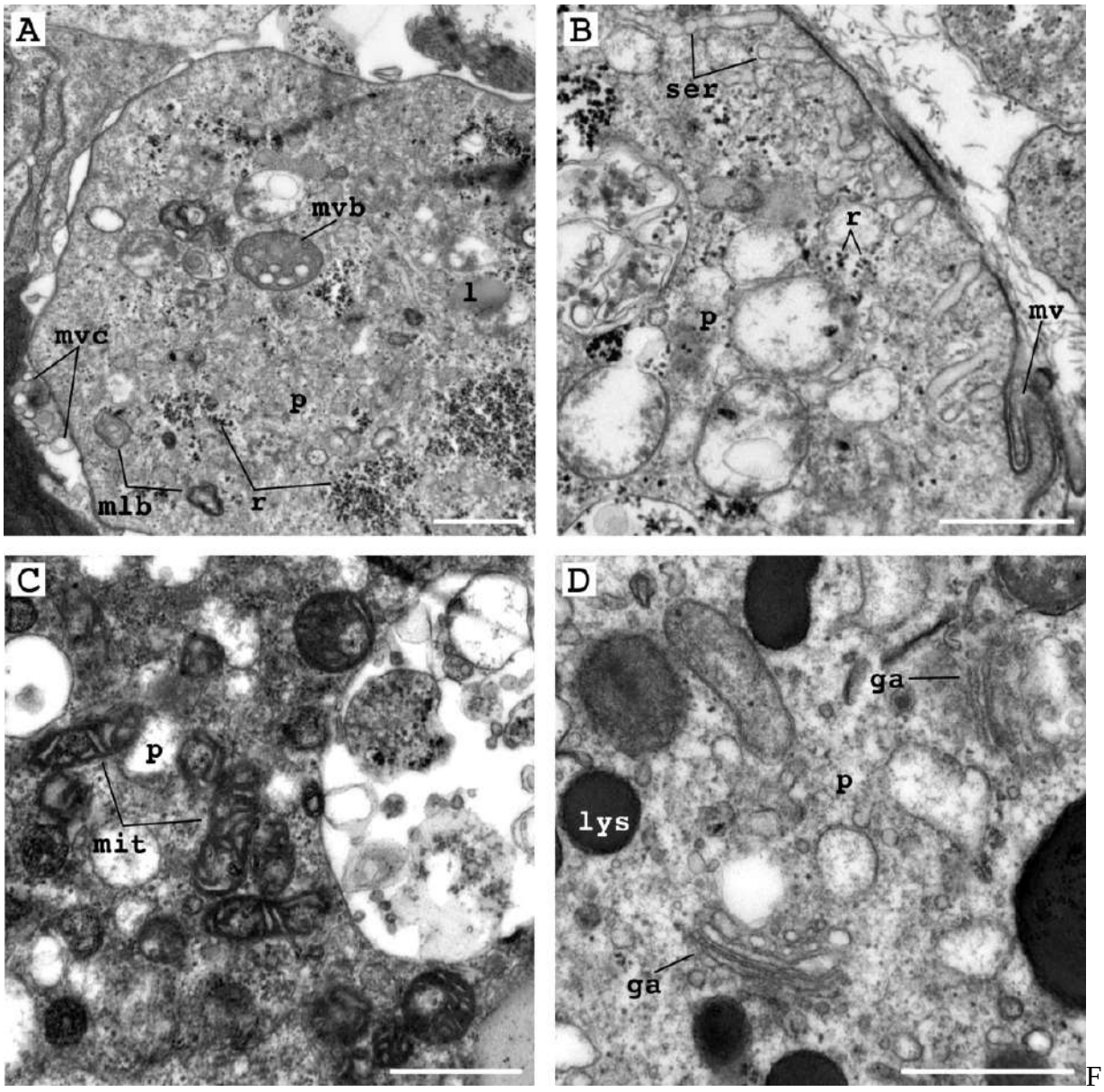

Figure 8. Electron micrographs of the cytoplasm of the plasmodium. (A) Various membrane-bounded bodies in the plasmodium cytoplasm and a cluster of vesicles in the extracellular space. (B) SER tubular network of the plasmodium. (C) Plasmodium mitochondria. (D) Plasmodium Golgi complexes. ga, Golgi apparatus; l, lipid droplet; lys, lysosome; mit, plasmodium mitochondria; mlb, multilamellar body; mv, microvilli-like projections; mvb, multivesicular body; mvc, cluster of multiple vesicles; p, plasmodium; r, ribosome; ser, the tubular network of the smooth endoplasmic reticulum. Scale bar: 1 µm in A, 5 µm in B, and 500 nm in C, D. From [26].

A tubular network of smooth endoplasmic reticulum was associated with the plasmodium surface (Fig. 8B, 10), and numerous free ribosomes were dispersed throughout the cytoplasm (Fig. 8A-B). Small mitochondria with a dense matrix were scattered in the cytoplasm (Fig. 8C), and Golgi complexes were also present (Fig. 8D).

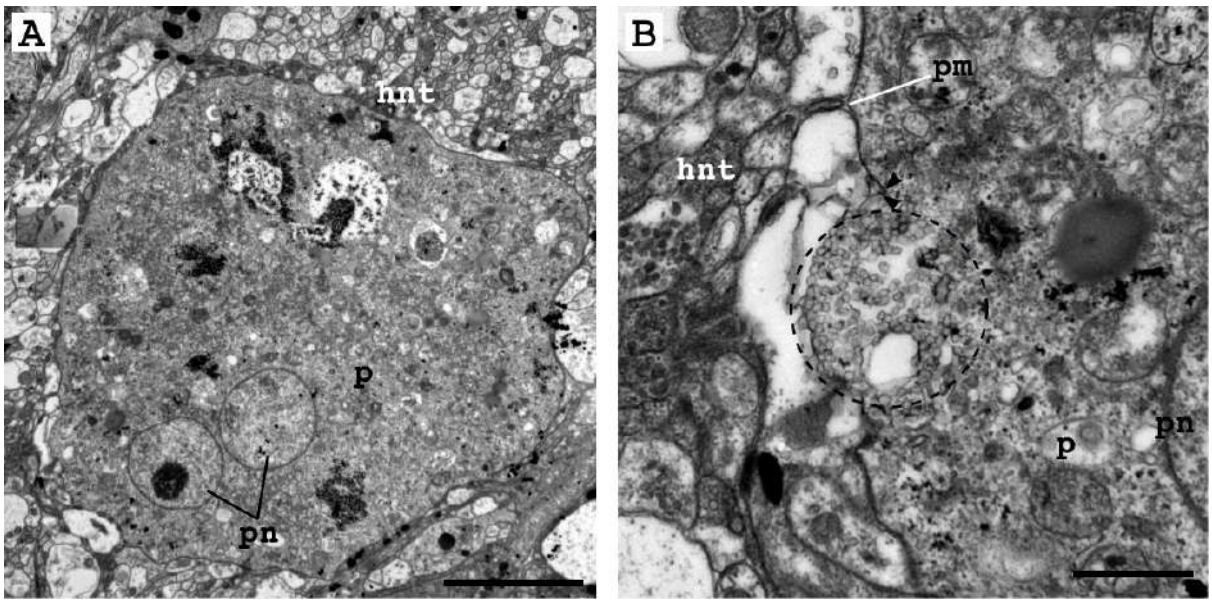

Figure 9. Electron micrographs of the plasmodium extension penetrating the host nerve trunk. (A) An overall view of the plasmodium extension with two nuclei within the cytoplasm in the host nerve trunk. (B) Bulging of the plasmodium membrane by multiple vesicles appearing to be released in the extracellular space of the host nerve trunk. hnt, host nerve trunk; p, plasmodium; pm, plasmodium membrane; pn, plasmodium nucleus; v, plasmodium vesicles. Dashed lines encircle the cluster of multiple vesicles within the plasmodium cytoplasm. Arrowheads point to the plasmodium membrane bulging. Scale bars: 5 µm in A, 1 µm in B. From [26].

The formation of clathrin-coated vesicles through the invagination of the plasmodium plasma membrane was observed (Fig. 10A). Additionally, the formation of the phagosome was documented in the plasmodium extension growing through the host's muscle tissue (Fig. 10B). In both cases, a well-developed tubular network was observed in these parts of the plasmodium cytoplasm, closely interacting with the plasmodium plasma membrane (Fig. 10).

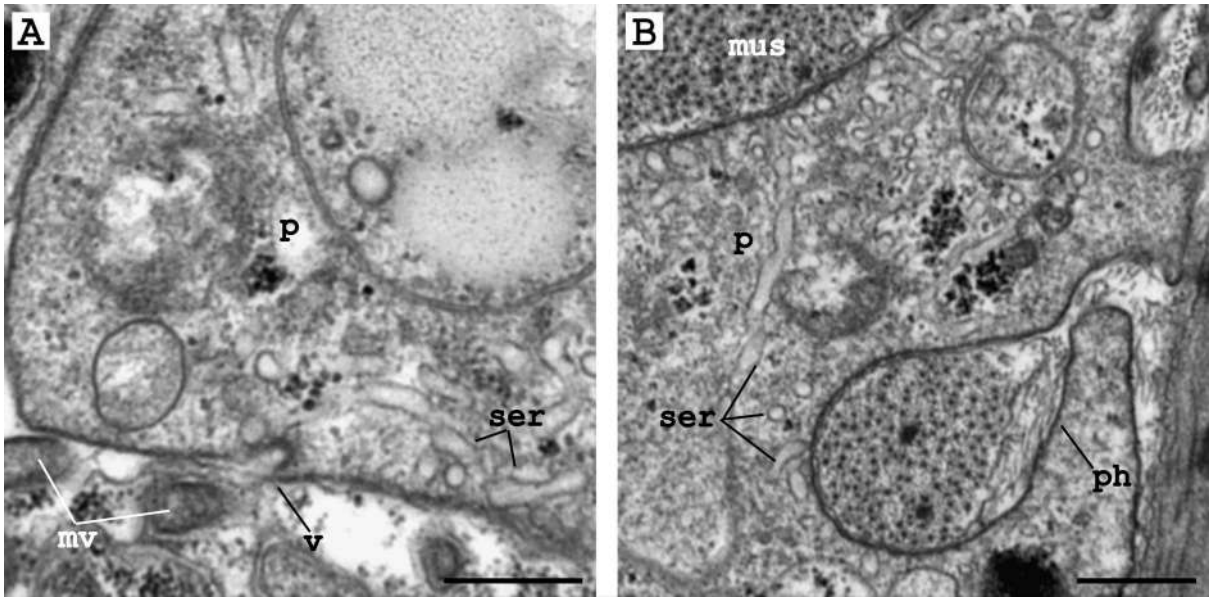

Figure 10. Electron micrographs of the endocytosis in the plasmodium. (A) The formation of the coated vesicle. (B) The formation of the phagosome. mv; microvilli; mus; host muscle cell; p, plasmodium; ph; phagosome; ser, tubular network; v, coated vesicle. Scale bars: 500 nm in A, 1 μm in B.

In mature plasmodia, a substantial portion of the internal volume of the cytoplasm was occupied by developing embryos and nearly mature males and females (Fig. 11C, 12A, C).

4.1.4. Reproductive cells

Reproductive cells (germinal, as per Kozloff's [22,23] terminology), featuring well-defined nuclei were discernible within the plasmodium. These small cells, approximately 3 μm in diameter, possessed a centrally located nucleus (Fig. 4C-D, 11A). Tubulin often formed a visible ring around these cells (Fig. 7B). The plasmodium cytoplasm contained single-cell stages (Fig. 4C, 7B), slightly larger two-cell stages (Fig. 4C-D, 11A), and four to many cell stages (i.e., embryos) (Fig. 4D, 7A, 11C).

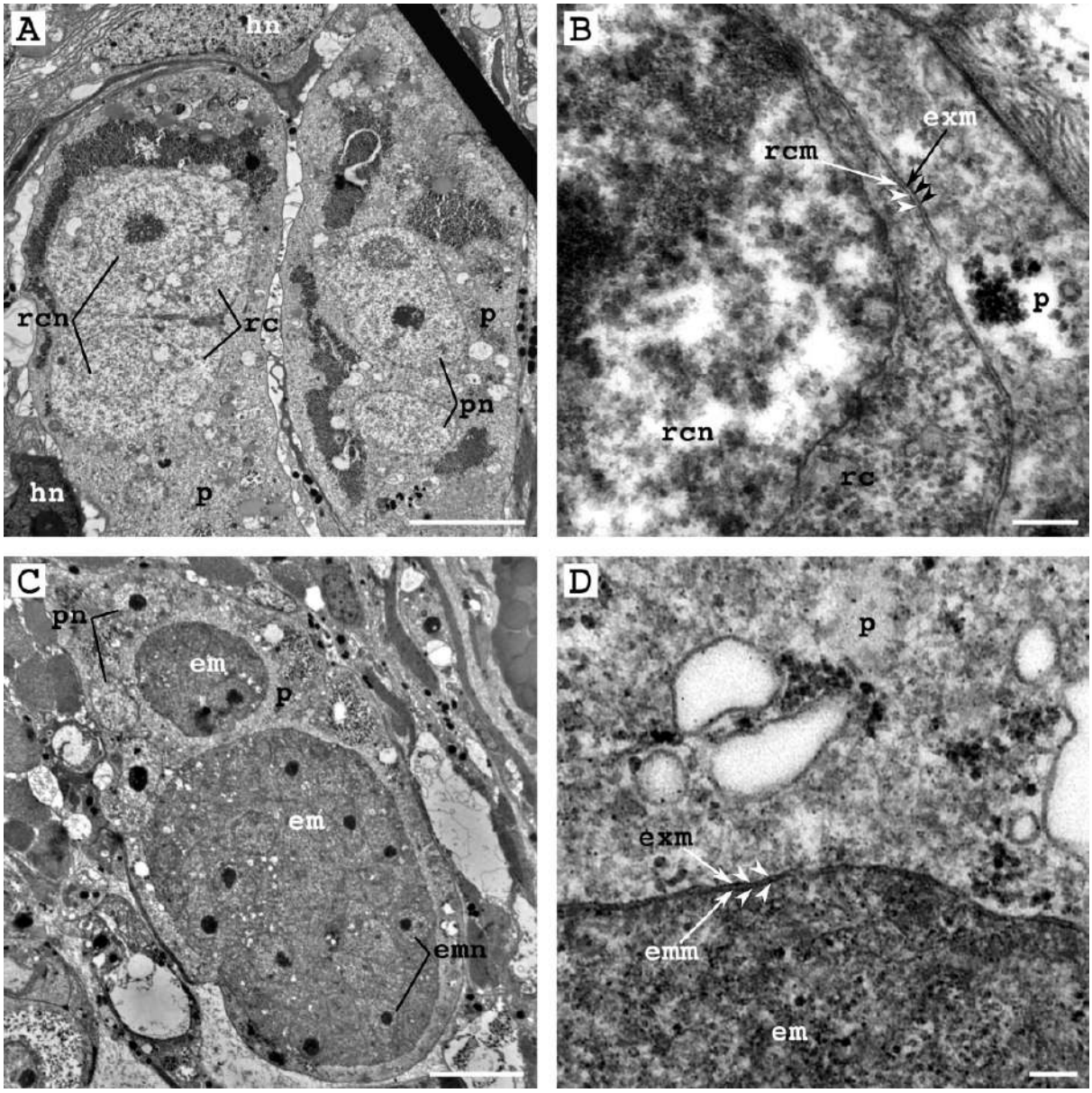

Figure 11. Electron micrographs of reproductive cells and orthonectid embryos in the plasmodium cytoplasm. (A) Reproductive cells and nuclei in the plasmodium. (B) Reproductive cells are separated from the plasmodium cytoplasm by an additional plasma membrane. (C) Orthonectid embryos in the plasmodium. (D) Embryos are separated from the plasmodium cytoplasm by an additional plasma membrane. em, orthonectid embryo; emm, cell membrane of the orthonectid embryo; emn, nucleus of the embryo; exm, extra membrane separating reproductive cells and embryos from the plasmodium cytoplasm; hn, host nucleus; p, plasmodium; pn, plasmodium nucleus; rc, reproductive cell; rcm, plasma membrane of the reproductive cell; rcn, reproductive cell nucleus. Scale bars: 5 μm in A, C, 200 nm in B, D. From [26].

Reproductive cells were enclosed by an additional membrane separating them from the plasmodium cytoplasm (Fig. 9B). This extra membrane persisted around developing embryos until

their full formation (Fig. 9D, 10B, D) and subsequently ruptured, presumably when cilia of mature orthonectid individuals began to beat (Fig. 10B, D).

4.1.5. Orthonectid males and females

Within the cytoplasm of the *I. linei* plasmodium, both orthonectid females (Fig. 4B-C, 7A-B, 12A, C) and males (Fig. 4C, 7C) were formed through divisions of reproductive cells. The average female-to-male ratio was approximately 1:1. Mature individuals could be recognized by the presence of a dense cilia layer surrounding the organism (Fig. 7A-B, 12A, C). Males could be distinguished from females by the presence of sperm nuclei with highly condensed chromatin (Fig. 4C, 7C). Mature males exhibited a length roughly half that of females.

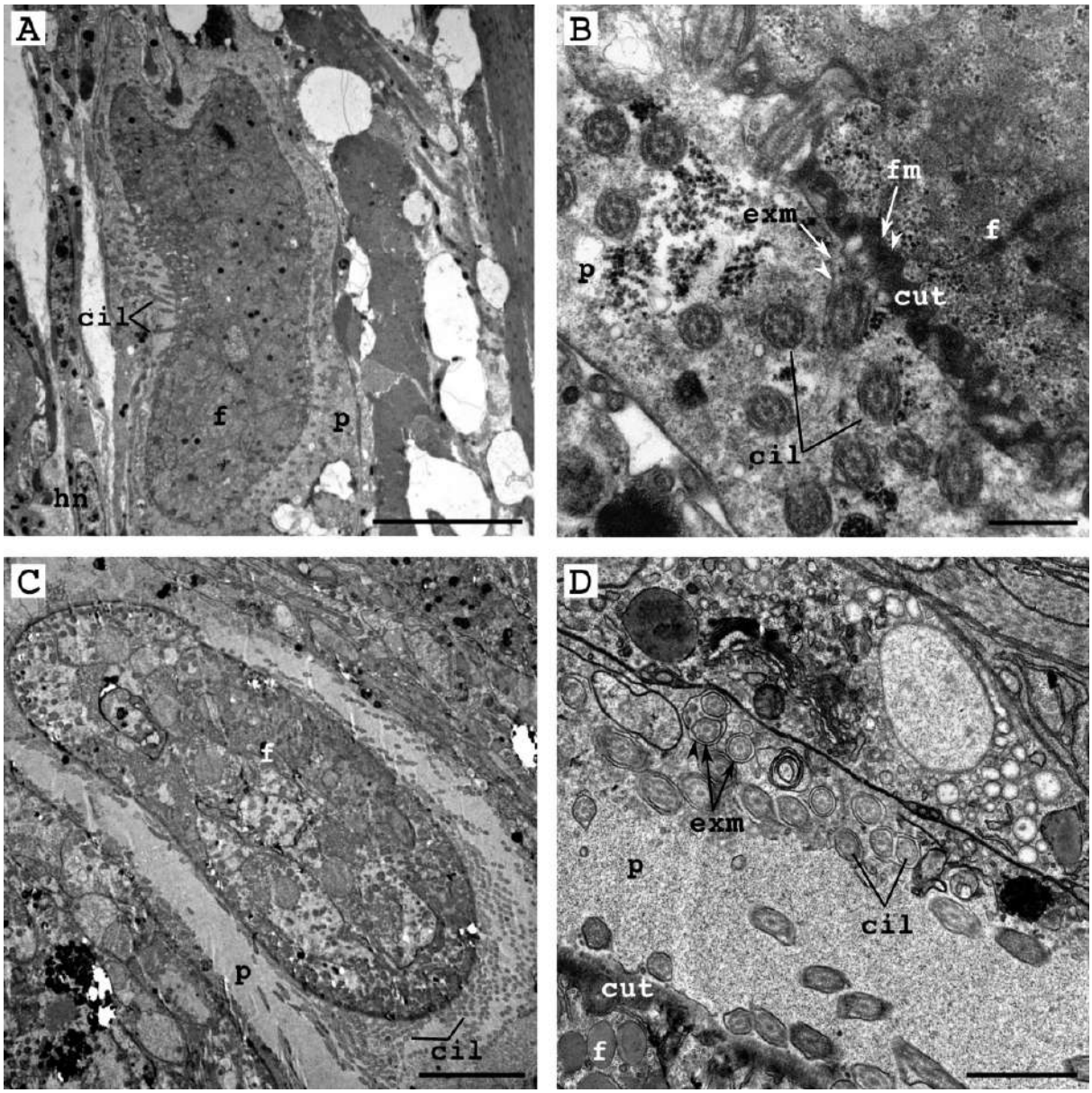

Figure 12. Electron micrographs of orthonectid matures in the plasmodium cytoplasm and the protrusion formed by the plasmodium for the release of mature orthonectid individuals. (A) Cilia-covered ortonectid female in the plasmodium cytoplasm. (B) Matures are separated from the plasmodium cytoplasm by an additional plasma membrane. (C) The cytoplasm of the protrusion formed for the exit of mature orthonectids contains almost no organelles. (D) An extra membrane is retained around some cilia of fully developed orthonectid female. cil, cilia of female orthonectid; cut, orthonecid cuticle; exm, extra membrane separating mature orthonectids from the plasmodium cytoplasm; f, orthonectid female; fm, cell membrane of the female; hn, host nucleus; p, plasmodium. 10 μm in A, 500 nm in B, 5 μm in C, 1 μm in D. From [26].

4.1.6. Mechanism of the exit of mature orthonectids from the host

The process of the release of sexual orthonectid individuals was observed through a stereomicroscope. The plasmodium formed cytoplasmic protrusions directed to the surface of the host body for the exit of mature individuals (Fig. 4A-B). These protrusions were evident across the entire host body, more often on the dorsal and lateral sides, and had the diameter of approximately 50 μm. Penetrating the host ciliary epithelium, these extensions contacted the ambient environment. Notably, the cytoplasm of these extensions showed almost no organelles (Fig. 12C-D). Our observations indicated that once the extensions were fully developed, mature males and females began to beat their cilia and move along the extensions, coming out one by one.

4.2. Molecular-genetic analysis

Illumina HiSeq sequencing of the infected host *L. ruber* yielded a total of 31 million paired reads. Filtering from host reads and any other contamination resulted in the removal of 12 million paired reads of non-*I. linei* origin. Differential expression analysis between orthonectids from the infected host and released sexual stages showed 119 genes which were presumed to be expressed only in the orthonectid plasmodium.

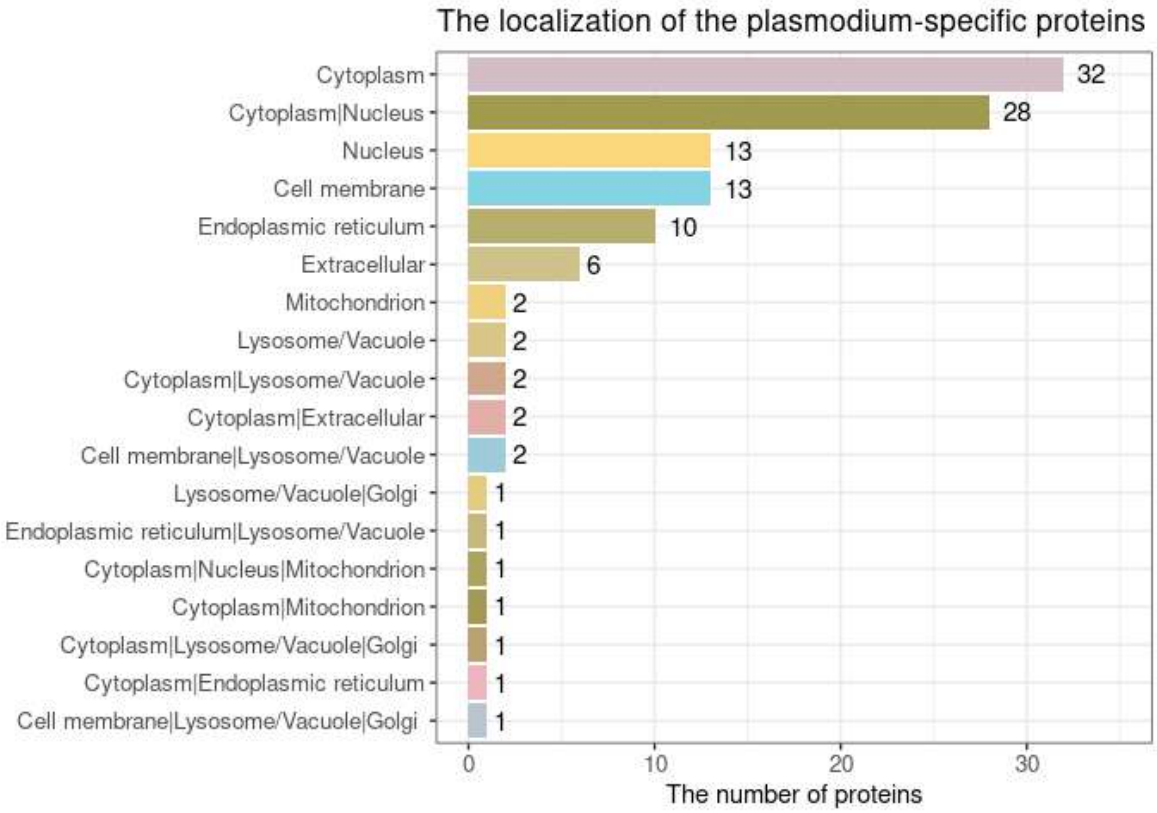

Figure 13. The subcellular localization of the plasmodium-specific orthonectid proteins. The size of each bar represents the number of the plasmodium-associated proteins attributed to different subcellular localizations. From [27].

Most of the proteins associated with the plasmodium were predicted to have cytoplasmic or nuclear localization sites (Fig. 13). 11 proteins were linked to excretory/secretory pathways, while topology prediction revealed that 25 proteins possessed transmembrane domains, indicating their roles in transport, signaling, or attachment (Fig. 14). 9 out of them had only one transmembrane helix, the remaining had two or more transmembrane domains.

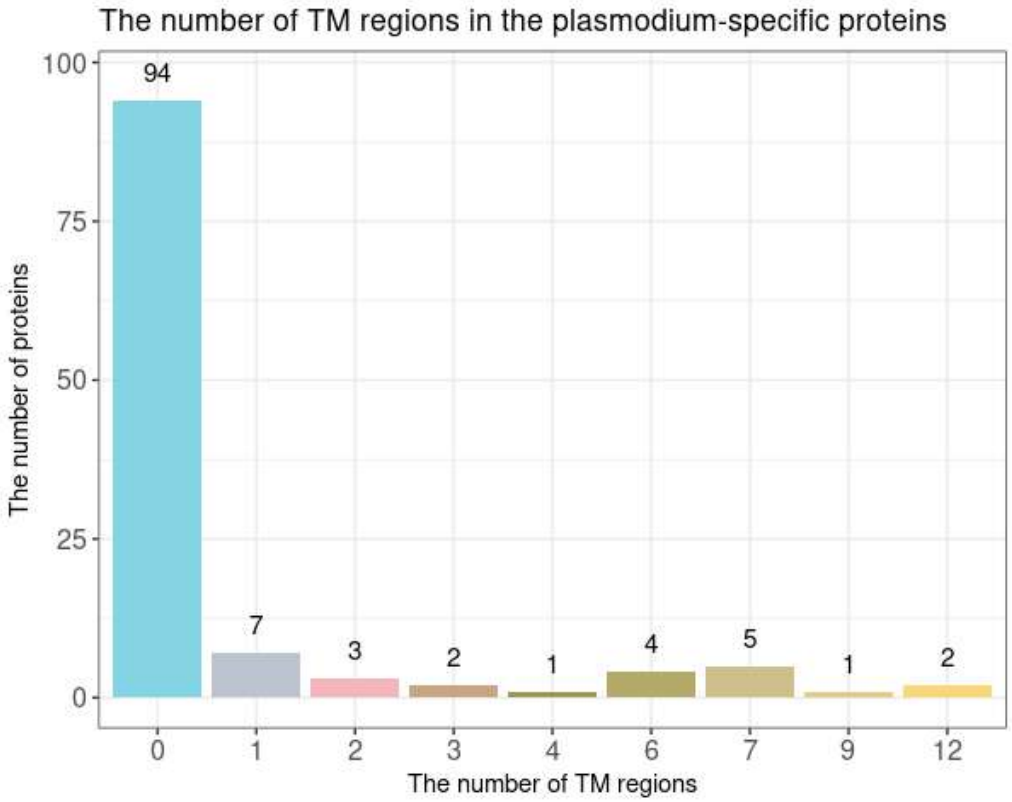

Figure 14. Transmembrane (TM) regions in the plasmodium-specific orthonectid proteins. The size of each bar represents the number of proteins possessing a certain number of TM domains. From [27].

Gene Ontology categories were assigned to 60 of the plasmodium proteins, with the majority being linked to cellular processes, catalytic activity, metabolic processes, transport, and regulation (Fig. 15).

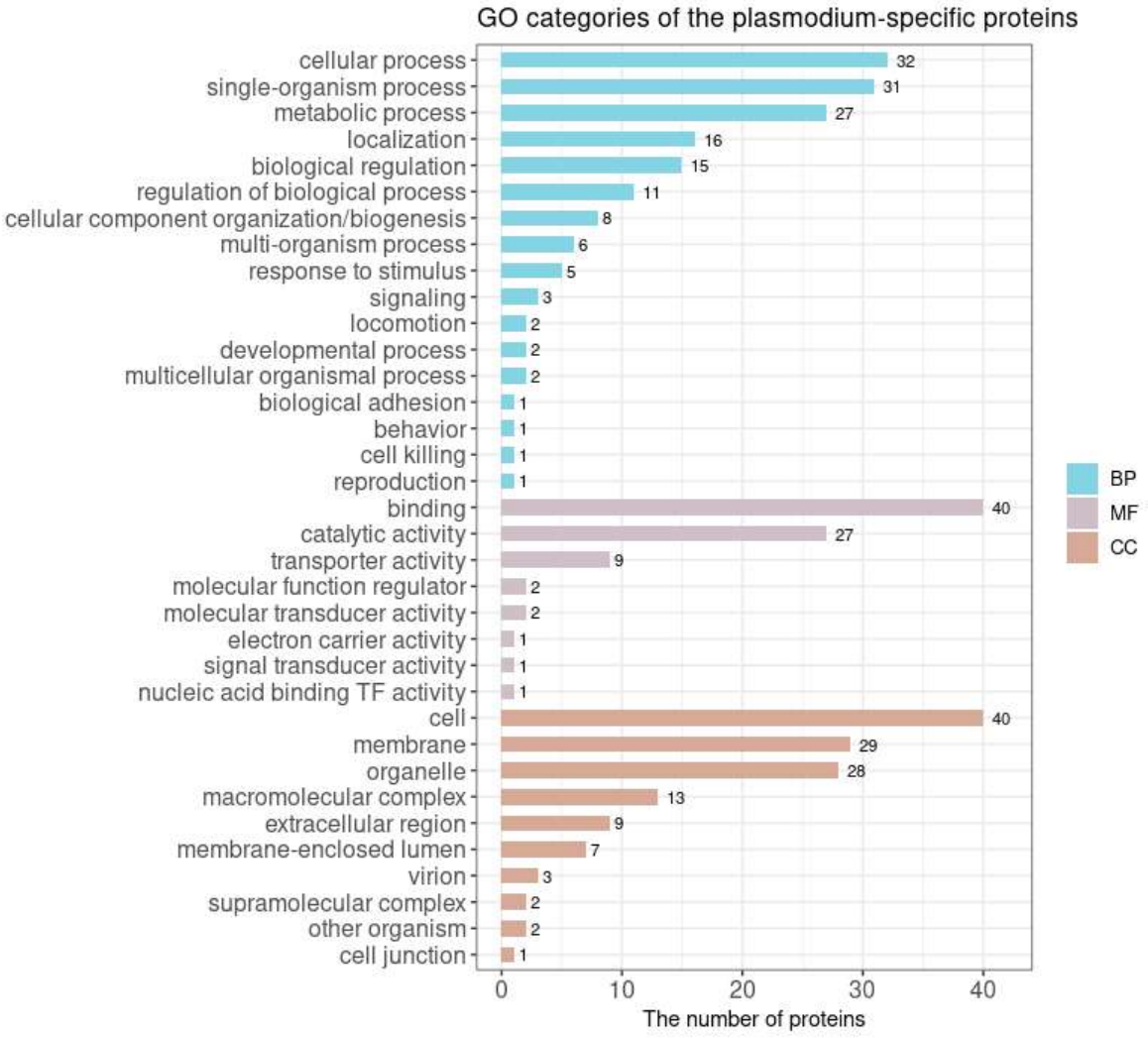

Figure 15. Gene Ontology (GO) categories of the plasmodium-specific orthonectid proteins. The size of each bar represents the number of proteins assigned to a certain GO category among Biological Process (BP) subontology, Molecular Function (MF) subontology and Cellular Component (CC) subontology. From [27].

35 proteins were identified as orphans, lacking homology to any sequenced organism except Orthonectida, and 13 of them exhibited sequence similarity to other orthonectid *Intoshia variabili*. Nearly all the remaining proteins (82) were assigned to specific protein families and superfamilies through structural and functional domain annotation. Two proteins had homologs in the NCBI nr database but lacked known domains. The combined annotation tables can be found in Supplementary Materials to [27] (Supplementary Table S6 and Supplementary Table S7).

5. Discussion

5.1. The morphology of the plasmodium

The main evolutionary trend of orthonectids associated with transitioning to parasitism is the transfer of the key phase of the orthonectid life cycle to the parasitic stage, which remains alive for a prolonged period. Meanwhile, the free-living stage, involved only in the sexual reproduction and dissemination, undergoes significant reduction and miniaturization [21].

Orthonectid plasmodia of studied species (*Rhopalura ophiocomae*, *Ciliocincta sabellariae*, *Intoshia variabili*, *I. linei*) exhibit a similar structure [21–26]. All of them lack a defined shape and are enveloped by two membranes. The surface of the plasmodia bears microvilli-like projections. Plasmodia cytoplasm of these species contains not only cell organelles but also reproductive cells and sexual orthonectid individuals at various stages of embryonic development. Reproductive cells and sexual stages are separated from the plasmodia cytoplasm by an additional membrane (Fig. 16). Similar features in the structure of these four species provide grounds to assume that the plasmodia of other orthonectid species may be organized in the same way [21].

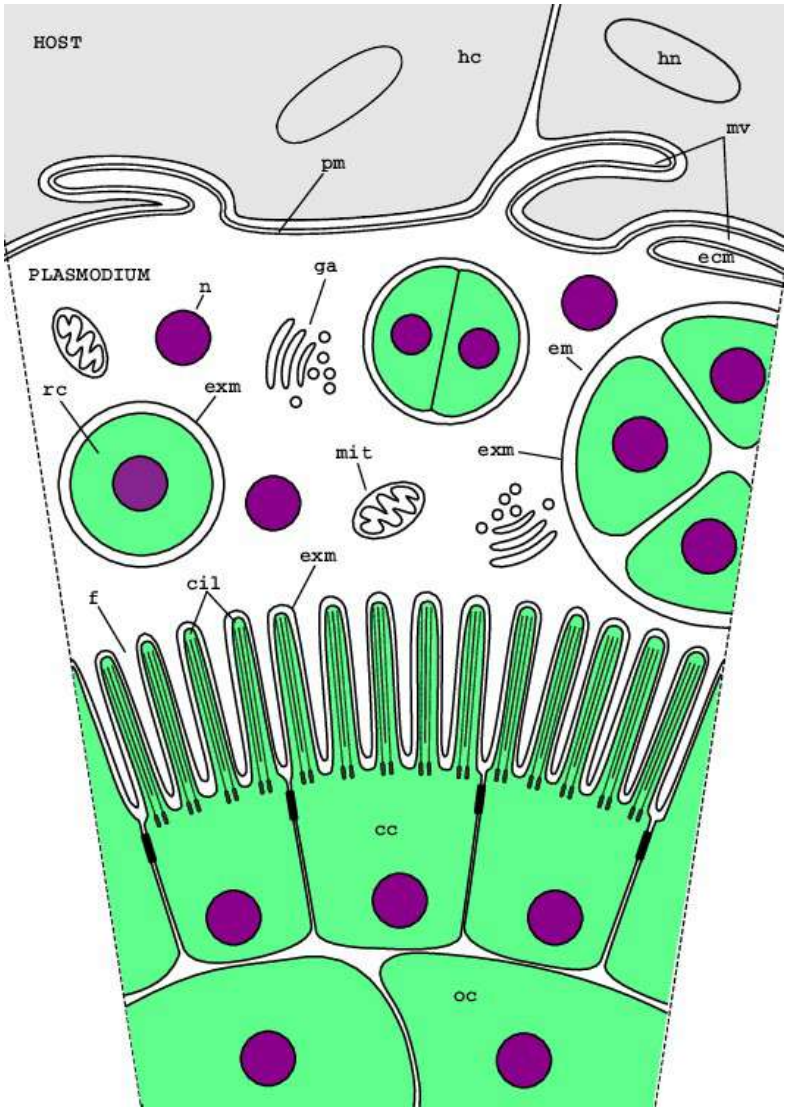

Figure 16. The schematic representation of a portion of the plasmodium cytoplasm with a developed ciliated female prior to release. cc, female ciliated cell; cil, female cilia; ecm, extracellular environment; exm, extra membrane separating orthonectid sexual generation from the plasmodium cytoplasm; f, orthonectid female; ga, Golgi apparatus; hc, host cell; hn, host nucleus; mit, plasmodium mitochondria; mv, microvilli-like projections; n, nucleus; oc, female oocyte; pm, plasmodium double membrane; rc, reproductive cell. From [26].

The cellular envelope comprising two plasma membranes has been observed in some intracellular parasitic protists, such as Microsporidia and Apicomplexa (*Toxoplasma*, *Plasmodium*, *Babesia*, etc.). In these groups, the parasite is often separated from the host cell by the protective membrane of the sporophorous (SPOV) or parasitophorous (PV) vacuole [52,53]. The additional membrane surrounding the orthonectid plasmodium most likely differs from SPOV/PV because it distinguishes the plasmodium not from the cytoplasm of the host cell but from the intercellular matrix

of the host. Furthermore, the two membranes of the plasmodium are closely associated and function as one unit, likely both originating from the parasite and maintained by the plasmodium itself.

The occurrence of a double membrane envelope has also been observed in some myxozoans. Specifically, two unit membranes, presumed to be of parasite origin, have been reported in myxozoan species such as *Henneguya* and *Myxobolus* [54–60]. Plasmodia enveloped by a double membrane are not typical for Myxozoa, and the presence of a second membrane may vary among members of a single species depending on a type of a tissue parasitised by myxozoan plasmodia [54,57,61]. The function of a double-layered envelope and the mechanism underlying its formation in Myxozoa remain uncertain. The similar organizational level observed in the parasitic stages of Myxozoa and Orthonectida allows for drawing parallels between these taxa. Considering the data indicating that the additional membrane in myxozoans may form based on the parasite's location within the host, it is plausible that the additional membrane of myxozoans and orthonectids could be considered a functional trait and is associated with the parasite's survival within the host. Numerous membranous structures within the plasmodium cytoplasm, such as vesicles and cords, many of them double-layered, could potentially serve as a source for constructing the double-membrane envelope.

The orthonectid plasmodium actively obtains nutrients from the host for maintaining both the developing sexual generation and itself. The presence of microvillous surface extensions likely increases the interaction area between the orthonectid plasmodium and the host, facilitating the influx of nutrients from the host. A similar surface structure has been observed in myxozoan plasmodia, where it has been associated with the trophic function [54,62]. The well-developed tubular network associated with the surface of the orthonectid plasmodium resembles pinocytotic canals, which are known for some myxozoan plasmodia where they play a role in feeding [25,63,64]. Presumably, this network plays a role in the pinocytosis of the plasmodium. The processes of receptor-mediated endocytosis and phagocytosis in the plasmodium were also documented. The cytoplasm of the orthonectid plasmodium is rich in various cell organelles involved in endocytic/exocytic pathways, such as numerous vesicles, granules, phagosomes, lysosomes, multilamellar, and multivesicular bodies, indicating the intense nutrition processes take place in the plasmodium cytoplasm.

We observed groups of vesicles, with one cluster seemingly released from the plasmodium into the extracellular environment of host nerve cells, and another located in the extracellular space between the host muscle cell and the plasmodium. The first scenario resembles the release of vesicles from human telocytes [65]: vesicles gather in a cluster near the plasma membrane, causing it to bulge, and then emerge from the cell enclosed by the envelope formed by the cell membrane. Following the disruption of the envelope, the vesicles are released into the extracellular space. If a similar process

occurs in orthonectids, the envelope of the vesicle cluster would likely be double-layered, similar to the plasmodium envelope.

In the second scenario, extracellular vesicles could originate from either the host or the plasmodium, and both possibilities may suggest interactions between the host and the parasite. The production of extracellular vesicles by parasites has been documented in various parasitic diseases where these vesicles play a crucial role in modulating host immune responses [66,67]. While the knowledge about invertebrate immune defense is limited, invertebrates exhibit several mechanisms of innate immune response, as recently confirmed for *Lineus ruber* as well [68,69]. Potentially, extracellular vesicles from orthonectids could enhance parasite survival by protecting the plasmodium from the host. Haloti [70] suggested that the orthonectid plasmodium might produce lytic enzymes that are exposed to host cells. As potential cargo of vesicles, these enzymes could be released into the extracellular space, facilitating the growth and dissemination of the plasmodium within host tissues.

The vesicles released by the plasmodium near the host nerve trunk may also indicate a direct parasitic influence on the host nervous system, as observed in some parasitic protozoa, digeneans, parasitic insects, and Rhizocephala [71–74]. Conversely, host-derived vesicles released into the extracellular environment might mitigate the pathogenesis of the parasitic disease [75,76].

The initial documentation of the plasmodium nuclei dates back to the species *Rhopalura ophiocomae* ([42], p. 700, fig. 596). Our results reveal that the cytoplasm of *I. linei* plasmodium features numerous nuclei that closely resemble those found in reproductive cells and embryos. Furthermore, these plasmodium nuclei exhibit marked distinctions from the nuclei present in the host cells. We observed the nuclei of the plasmodium during the metaphase of mitosis for the first time.

The cytoplasm of the *I. linei* plasmodium harbors reproductive cells, as well as orthonectid males and females on different stages of embryonic development. An additional membrane bilayer separates the reproductive cells and developing embryos from the cytoplasm of the *I. linei* plasmodium. The same has been previously observed in *Ciliocincta sabellariae* ([23], p. 156, fig. 4A) and *I. variabili* [25]. The origin of this extra membrane surrounding reproductive cells and embryos will be elaborated upon in the section 3 of the Discussion.

The presence of cells in the cytoplasm of orthonectid plasmodium again allows drawing parallels with Myxozoa plasmodia. The trophic stages of some Myxozoa in the intermediate vertebrate host are represented by plasmodia that contain generative cells in their cytoplasm, which, as a result of sporogony (asexual reproduction), form multicellular myxospores. Subsequently, the spores are released into the external environment and infect their definitive invertebrate hosts [62].

5.2. The plasmodium-specific orthonectid genes and corresponding proteins

Parasitism has independently and repeatedly evolved in various clades [3]. Despite the phylogenetic diversity of parasites, convergent traits of different parasites have been observed at morphological, functional and genomic levels [3,77–80]. Molecular adaptations shared by various endoparasites, regardless of their phylogenetic positions, are typically associated with the processes of entering the host, defense against host immunity, host-parasite communication, and nutrient absorption. Common molecular effectors of endoparasites from different phyla are usually revealed by the comparison of genes or proteins enriched specifically in parasitic stages and in free-living stages or free-living relatives [77–80]. This study represents the first exploration of orthonectid parasitic stage from a molecular perspective, and the presumed role of the hypothetical proteins associated with the plasmodium is based on existing studies on genes associated with parasitism in various clades [27].

The majority of the annotated plasmodium-specific proteins are enzymes and transporters. This aligns with the established image of a plasmodium as a trophic stage that sustains the embryonic development of a sexual stage within its cytoplasm [22–26]. The abundance of proteins related to endocytosis, exocytosis, and vesicle transport is consistent with morphological observations showing that these processes actively take place in the plasmodium cytoplasm [24,26]. The identified metabolic enzymes most likely support the high metabolic rate required for plasmodium growth and the development of sexual stages. The prevalence of excretory/secretory proteins, transporters, and receptors in the resulting annotation suggests the existence of intense host-parasite interactions, an area that has not been thoroughly explored until now.

The plasmodium, developing in the host extracellular space in close contact with the host cells, needs to possess specific mechanisms for interacting with the surrounding environment. Proteins typically associated with that type of interaction include, for example, excretory/secretory proteins, which could be released by the parasite into the host tissues or cells [66,67]. The resulting set of plasmodium-specific proteins reveals several proteins that could be exposed to the host because of their excretory/secretory (ES) potential. Of particular interest is the C-type lectin homolog, recognized for its importance in parasite immune evasion [81] and innate immune defense in invertebrates in general [82,83]. *L. ruber* possesses various innate immunity pathways [68], and the C-type lectin in orthonectids might play a role in interacting with their components. Another potential ES protein of the plasmodium belongs to the α/β-hydrolase superfamily, whose members have been reported in parasitic trematodes, nematodes, and protists (*Cryptosporidium*, *Plasmodium*), suggesting a significant role in parasite development and survival by catalyzing the degradation of endogenous and host lipids [84,85]. A potential member of the Heat Shock Protein 70 (HSP70) family, proteins involved in an

adaptive response to environmental changes, frequently found in parasitic extracellular vesicles, was also identified [86,87]. Furthermore, a possible member of the pepsin-like aspartic proteases superfamily was detected as a potential ES protein of the plasmodium. As the plasmodium grows within the host and infiltrates its tissues [24,26], it is essential for the parasite to have a mechanism to disrupt host cell-cell adhesion. Secreted proteases found in the secretome of many endoparasites [66,86] could potentially be involved in this process.

There were plasmodium-associated proteins that were not categorized as ES proteins in this analysis but may still play a potential role in host-parasite interactions. These include homologs of serpin (serine protease inhibitor protein), cellular and retroviral pepsin-like aspartate protease, SUEL-type lectin, and a metallopeptidase containing a reprolysin domain found in nematocyst proteomes of certain Myxozoa [88].

ES proteins become exposed to the host through the release of extracellular vesicles from the cytoplasm of the parasitic cell [66,67] The cytoplasm of the orthonectid plasmodium contains numerous vesicles, multivesicular bodies, and clusters of vesicles which are released to the host [24,26]. In the analysis of plasmodium-associated proteins, we identified several homologs of proteins previously investigated in the context of parasitic extracellular vesicles [87]. Enolase, a multifunctional enzyme involved in glycolysis, has been confirmed as a host-interacting molecule in trematodes, nematodes, and protists, and it is associated with extracellular vesicle biogenesis [89,90]. Tetraspanins, integral membrane proteins with the ability to interact with various transmembrane and cytosolic proteins, are suggested to be crucial in extracellular vesicle biogenesis, sorting, and trafficking in helminths [91–93]. Additionally, homologs of other vesicle-associated proteins that may be involved in vesicle biogenesis were identified, including SNARE proteins and Rab GTPases, which play a significant role in vesicle secretion [94].

Another category of proteins potentially participating in host-parasite communication includes membrane proteins. Often associated with the surface of the parasite, these proteins may function as receptors that respond to host stimuli, initiating parasite cellular responses. Alternatively, they might serve as selective transporters, facilitating the import/export of chemicals between the parasite and the host [78]. Occasionally, membrane proteins such as SNARE and tetraspanins may be associated with vesicle membranes. Membrane proteins constitute nearly one-third of the total *I. linei* proteins associated with the plasmodium stage. Although the putative functions of most of them remain unknown, some proteins have identified homologs among other invertebrates and parasites.

To the plasmodium membrane proteins were regarded two potential members of the seven-transmembrane G protein-coupled receptor (GPCR) superfamily, often found in abundance in endoparasites where they play a role in responding to host cues [95]. Additionally, we discovered five

prospective members of the MFS general substrate transporter superfamily and a homologue of the Co/Zn/Cd efflux system component. As the plasmodium grows within the host, it likely possesses the necessary machinery to receive signals from surrounding host tissues through membrane receptors. Besides, membrane proteins of the plasmodium surface could transport small solutes across its outer membrane. Some of the revealed membrane proteins could be associated with the membrane that separates the developing sexual generation from the plasmodium cytoplasm, facilitating communication between the plasmodium and embryos.

Endocytosis plays a pivotal role in the pathogenesis of numerous internal parasites [96]. The process of phagocytosis has been observed in the orthonectid parasitic stage in the current study and by Slyusarev and Cherkasov [25], alongside the presence of various endosomes, lysosomes, multivesicular bodies, and multilamellar bodies within the plasmodium cytoplasm [24,26]. This study for the first time documented the clathrin-dependent vesicle formation in the plasmodium, which is a form of endocytosis. All these observations suggest active endo/exocytosis processes in the plasmodium [24,26]. The analysis identified several plasmodium-specific proteins potentially involved in the endo- and exocytosis. These proteins include homologs of epsin, pleckstrin homology domain-containing protein, adaptor protein complex mu1 subunit, proteases, lectins, SNARE protein, Rab GTPase, stomatin, tetraspanin, BAR domain-containing protein, and G protein-coupled receptors (GPCRs) [97].

Approximately one-fourth of the identified plasmodium-specific *I. linei* proteins currently show no homology to any known species except Orthonectida and lack known conserved domains. This aligns with the divergent genetic nature of orthonectids [8,98]. Orthonectids have been considered relatives to various phyla, and recent phylogenetic analyses underscore the uncertainty of their relationship with the existing phyla [8–13]. Orphan genes typically exhibit a narrow phylogenetic distribution, evolve rapidly, and facilitate lineage-specific adaptations [99]. While some plasmodium-specific orphans may eventually acquire homologs as more organisms undergo sequencing, others may contribute to the unique adaptations of orthonectids to parasitism.

5.3. The nature of the orthonectid plasmodium

There are two points of view on the origin of the orthonectid plasmodium. Traditionally, the orthonectid plasmodium was viewed as a tissue parasite that harbors the development of sexual orthonectid individuals within its cytoplasm [36,38,42,44]. This view of the nature of the plasmodium persisted until the release of Kozloff's works [22,23]. According to Kozloff's terminology, only the reproductive cells (referred to as germinal cells) of the plasmodium are considered part of the parasite

itself, while the plasmodium cytoplasm is essentially the cytoplasm of a modified host cell. Kozloff suggested that orthonectid reproductive cells enter the host cell, leading to its hypertrophy. The formation of males and females occurs through the division of these reproductive cells, and the organism previously identified as a "plasmodium" is, in reality, a modified host cell. In other words, although the multiplication of reproductive cells and the development of adults occur within it, the plasmodium itself is not a stage in the orthonectid life cycle; rather, orthonectids are considered intracellular parasites.

As per Kozloff view [22,23], after the orthonectid larva enters the host, the parasitic cells from the larva infect the host cell. The orthonectid cells undergo division, giving rise to the sexual generation. Any free nuclei observed within the plasmodium cytoplasm are either considered artifacts [22,23] or are originated from dissociated reproductive cells whose cytoplasm and cell membrane have disappeared (see [23] p. 157, fig. 5). The fate of the host nucleus remains unclear - Kozloff [23] suggests it might be "shifted away" from the plasmodium but does not provide any supporting micrographs for this perspective.

Slyusarev and Miller [24] rejected Kozloff's [22] suggestion that the plasmodium has the host origin. Kozloff's idea at that time was based on the absence of visible orthonectid nuclei within the plasmodium cytoplasm. Slyusarev and Miller dismissed this proposition by presenting three arguments against it. These arguments included (1) the identification of free nuclei in the plasmodium cytoplasm of *I. variabili* [24] and *R. ophiocomae* [42], (2) the resemblance between the appearance of the free nuclei within the plasmodium cytoplasm and those present inside embryos, and (3) the observation of specialized modified host cells separating the plasmodium from the host tissues, exhibiting nuclei significantly different from those of the plasmodium. These arguments are still relevant in the case of the *I. linei* plasmodium [26]. Using various morphological methods, we observed well-preserved plasmodium nuclei, even during metaphase of mitosis, without any signs of disintegration, challenging Kozloff's view that these nuclei are artifacts [22,23].

Kozloff [23] proposed an alternative explanation that free nuclei occasionally observed in the plasmodium cytoplasm are remnants of deteriorated reproductive or embryonic cells. According to this hypothesis, such isolated orthonectid nuclei would continue functioning in the cytoplasm of the infected host cell, ensuring its prolonged viability. While there is *in vitro* evidence that the nucleus of one metazoan species can function in the cytoplasm of the other metazoan species (interspecies nuclear transfer, [100]), such a phenomenon is not documented in living Metazoa. Therefore, the only conceivable scenario positing the host origin of the plasmodium implies the host origin of the plasmodium nuclei, with only reproductive cells and embryos originating from the parasite. In this scenario, the resulting structure might resemble xenomas, parasite-induced hypertrophic host cell

complexes observed in hosts infected with certain Microsporidia, Apicomplexa, Myxozoa, etc. [101–104]. Intracellular parasitic infections causing host cell hypertrophy often lead to enlargement and fragmentation of the host cell nucleus. However, *I. linei* plasmodium nuclei do not exhibit abnormality, hypertrophy, or fragmentation. Their morphology remains indistinguishable from the nuclei of other orthonectid cells, making it unlikely that the host nuclei underwent such drastic changes. Considering all available data, the nuclei and the cytoplasm of the plasmodium, enclosed by a membrane, belongs to *I. linei*, affirming the parasitic origin of the orthonectid plasmodium.

Proteins corresponding to orthonectid genes, expressed only in the plasmodium stage and identified through *in silico* analysis, participate in the processes of plasmodium growth within the host, host-parasite interactions, and endo/exocytosis [27]. The presence of genes associated with endoparasitism in other organisms within the orthonectid genome, and the expression of these genes in the infected host, further corroborate that the orthonectid plasmodium has a parasitic nature. The host could not express orthonectid genes, and embryos and generative cells do not require the expression of a gene set with such functions. Furthermore, the presence of processes in the plasmodium, confirmed by RNA sequencing analysis, is also supported by morphological analysis of the plasmodium.

The evidence is robust and convincing to assume that the plasmodium is a tissue parasite where sexual orthonectid individuals undergo development. We propose a possible scheme outlining the process of plasmodium development. The proposed mechanism provides an explanation for the abundance of nuclei within the plasmodium cytoplasm and the presence of an extra membrane enveloping generative cells and embryos, all without contradicting fundamental biological principles (Fig. 17).

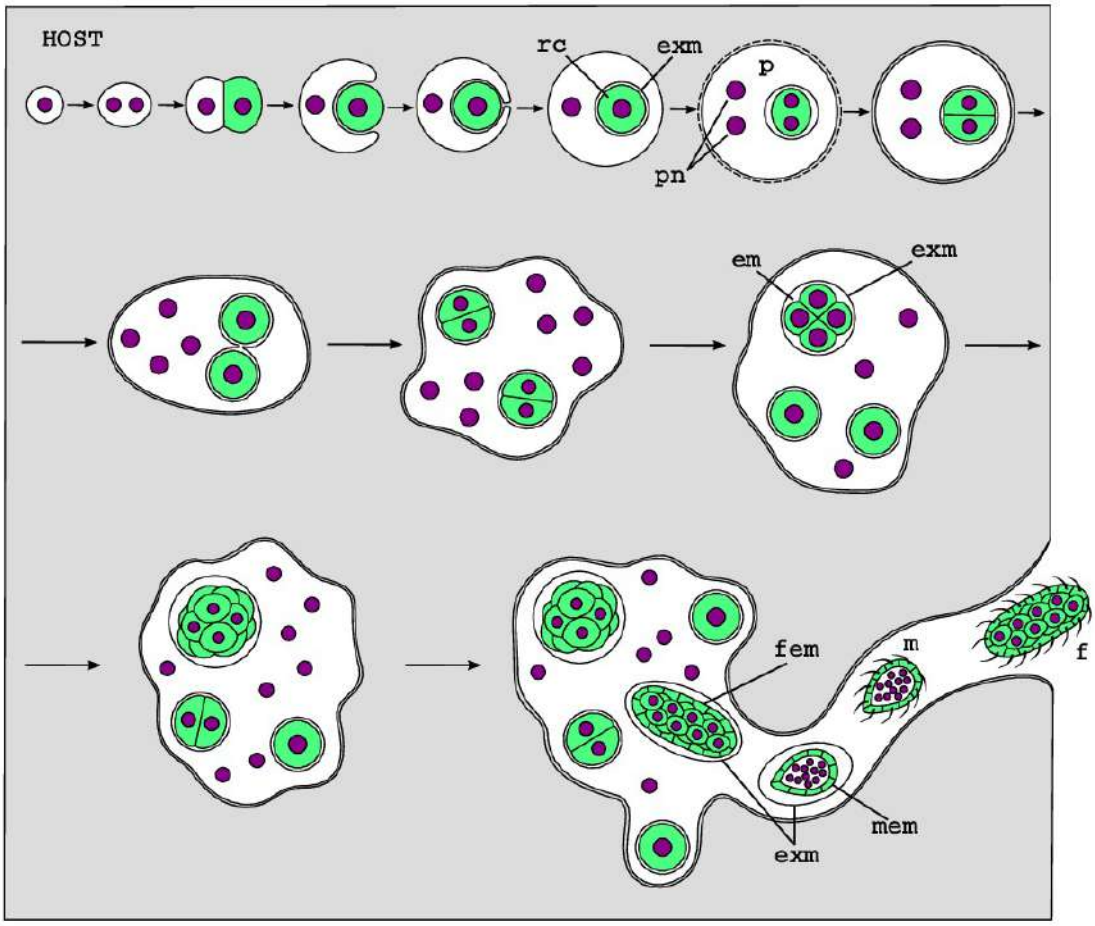

Figure 17. Possible mechanism of the plasmodium formation and development. em, orthonectid embryo; exm, extra membrane separating orthonectid sexual generation from the plasmodium cytoplasm; f, mature orthonectid female; fem, orthonectid female embryo; m, mature orthonectid male; mem, orthonectid male embryo; n, nucleus; p, plasmodium; rc, reproductive cell. Dashed line shows the first documented occurrence of the double-layered plasmodium membrane. From [26].

Upon infecting the host, the larva's undifferentiated cells disseminate within the host tissues without infiltrating the host cells. Subsequently, each parasitic cell undergoes division, with one of the daughter cells enveloping the other, forming a cell-within-cell complex. This complex has been previously reported in Cnidaria and is common in Myxozoa, parasitic cnidarians [105–107]. The outer cell undergoes multiple nuclear divisions without subsequent cytokinesis, and its cytoplasm transforms into the cytoplasm of the plasmodium. Simultaneously, the inner cell, now enclosed by an additional membrane due to being engulfed by the outer cell, also divides. This division gives rise to reproductive cells and embryos, each separated from the plasmodium cytoplasm by an extra membrane. The resulting structure is the orthonectid plasmodium: a multinuclear organism that develops in

extracellular compartments of the host and contains numerous cells, embryos, and nearly mature sexual orthonectid individuals (Fig. 16, 17).

The morphological analysis suggests two potential mechanisms for the dissemination of the parasitic plasmodium within host tissues. In one scenario, it is hypothesized that the plasmodium generates elongated, fingerlike extensions, which subsequently detach, giving rise to daughter plasmodia (Fig. 18, A). Alternatively, in another scenario, individual cells exit the plasmodium and subsequently undergo transformation into daughter plasmodia (Fig. 18, B). The latter mechanism was supported by earlier observations of the *Rhopalura ophiocomae* plasmodium. The egress of reproductive cells from the plasmodium into the host tissues was initially reported by Caullery and Lavalle [42] and later confirmed by Haloti. [108].

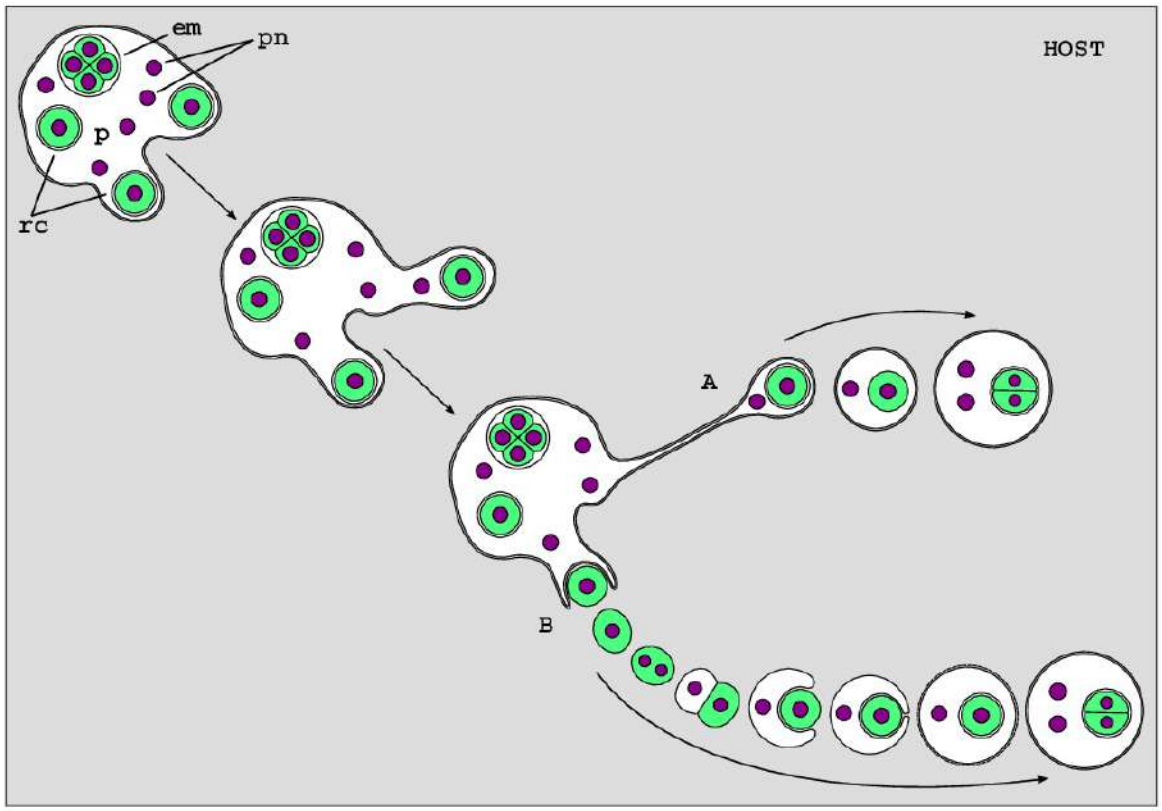

Figure 18. Possible mechanisms of plasmodia spreading across host tissues. (A) The detachment of an extension from the plasmodium. (B) The detachment of a reproductive cell from the plasmodium. em, orthonectid embryo; p, plasmodium; pn, plasmodium nucleus; rc, reproductive cell. Dashed line shows the first documented occurrence of the double-layered plasmodium membrane. From [26].

6. Conclusion

The *I. linei* plasmodium is a shapeless multinucleated organism, enclosed by a double membrane, which isolates it from host tissues. In addition to numerous nuclei, its cytoplasm contains organelles typical for other bilaterians, along with reproductive cells and maturing sexual specimens. Reproductive cells, as well as developing orthonectid males and females, are enveloped by an extra membrane. Endo- and exocytosis are actively occurring processes within the plasmodium cytoplasm. The plasmodium consumes host substances through pinocytosis, phagocytosis, and receptor-mediated endocytosis, as well as directly through the plasma membrane, which forms numerous microvilli to increase the contacting surface area. The interaction between the host and plasmodium is facilitated by extracellular vesicles. The plasmodium forms protrusions directed towards the host body surface and used by mature sexual individuals for egress from the host.

Specific features of the plasmodium prevent its direct comparison with other bilaterians. However, convergent structures, such as a double membrane envelope, microvilli on the surface, well-developed tubular network, and the presence of cells within the plasmodium cytoplasm, are observed in some myxozoan plasmodia. This suggests convergence in the organization of these parasites.

The first-ever RNA-seq analysis of the plasmodium revealed a set of the plasmodium-specific protein-coding genes and corresponding hypothetical proteins that distinguish the parasitic plasmodium stage from the sexual stage of the *I. linei*. Out of 119 plasmodium-specific proteins, 82 have inferred functions based on known domains. 35 of the detected proteins are orphans, at least part of which may reflect the unique evolutionary adaptations of orthonectids to parasitism.

Certain identified proteins are recognized as effector molecules typical for other endoparasites, indicating a convergence. Proteins specific to the plasmodium may play roles in defending against the host, host-parasite communication, feeding and nutrient uptake, growth within the host, and support of the development of the sexual stage. The presence of active endo- and exocytosis and the interaction with the host through extracellular vesicles is also supported by morphological data. The molecular mechanisms associated with these processes in orthonectids have not been previously described, and the specific protein effectors were unknown until now.

The previous observations of numerous plasmodium nuclei within its cytoplasm were confirmed in this study. The cytoplasm surrounding these nuclei belongs to the parasite itself, while outside the plasmodium envelope lies the host extracellular matrix. Parasite-associated orthonectid genes are expressed in the plasmodium to maintain its functioning within the host. The hypothesis

suggested by Kozloff, implying the host origin of the plasmodium cytoplasm, is not supported. The plasmodium is a tissue parasite, representing the parasitic stage in the life cycle of orthonectids.

A possible mechanism for the formation of the plasmodium involves the dispersion of parasitic larval cells throughout host tissues, followed by the generation of a cell-within-cell complex. The cytoplasm of the plasmodium originates from the outer cell, which undergoes multiple nuclear divisions without cytokinesis, while the inner cell divides, giving rise to reproductive cells and embryos. A potential mechanism for the spread of plasmodia across host tissues involves either the egress of the reproductive cell or the detachment of the plasmodium extension from the plasmodium.

7. Principal findings

1. Based on the analysis of all the obtained data, it can be inferred that the orthonectid plasmodium is not a modified host cell, as Kozloff suggested [22,23]; instead, it has a parasitic origin. It develops as a stage of the orthonectid life cycle in the host extracellular space.

2. The orthonectid genes expressed in the plasmodium and potentially involved in the host-parasite relationship have been identified and are recognized in other endoparasites. One of the mechanisms of host-plasmodium interaction involves secretion of extracellular vesicles.

3. The plasmodium consumes host substances through pinocytosis, phagocytosis and receptor-mediated endocytosis. The protein effectors involved in these processes have been identified.

4. The mechanisms encompassing the generation of cell-within-cell complexes were suggested to explain the processes of formation, development, and dissemination of the orthonectid plasmodium across the host tissues.

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