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Ultrastructure of the Plasmodial Development of *Myxobolus insignis* (Myxozoa), Infecting the Amazonian Fish *Semaprochilodus insignis* (Prochilodontidae)

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Abstract. This study used light and electron microscopy to describe a myxosporean, polysporic, histozoic plasmodium infecting the gill filaments of the freshwater teleost, *Semaprochilodus insignis*, specimens of which were collected from the Trombetas River (Central Amazonian Region, Brazil). Ultrastructural analyses of the fish-infecting spores identified the parasite as *Myxobolus insignis*, an organism that occurs within whitish unequal-sized plasmodia located in the intralamellar epithelium of the gill. Based on the observed morphological and ultrastructural features of the plasmodia in this study three stages in the plasmodial evolution were distinguished, related to the sporogonic stages of *Myxobolus insignis*. The plasmodium walls were also found to constitute a number of layers of fibroblasts, surrounded by collagen fibres, which displayed different morphological arrangements according to the different phases of evolution. This represents the first time such ultrastructural features have been described in detail for *Myxobolus insignis* plasmodia and offers potentially significant points of comparison with plasmodia from other species of myxosporea.

Key words: Amazonian Region, fish, gill, plasmodium, myxosporean, parasite, ultrastructure.

INTRODUCTION

Numerous descriptions of myxosporean species have been reported in fish from different geographic areas; however, few papers have examined the developmental evolution of the plasmodium during sporogony (Azevedo *et al*. 2011). Among the myxosporeans, the genus *Myxobolus* is a common species of the most important pathogen that infects fishes world-wide, and it has therefore received reasonably extensive scholarly attention (Lom and Dyková 2006).

The great majority of myxosporea parasitizing Brazilian host freshwater fish have been defined simply

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through diagrammatic drawings of spores based upon observations using light microscopy (Kent and Hoffman 1984; Molnár and Békési 1993; Gioia and Cordeiro 1996; Molnár *et al.* 1998; Cellere *et al*. 2002; Eiras *et al.* 2005a, b, 2007; Martins and Onaka 2006). Only a few *Myxobolus* spp. have had their developmental stages described through ultrastructural studies (Casal *et al.* 1996, 2002, 2006; Azevedo *et al*. 2002, 2009, 2010, 2011, 2012; Tajdari *et al*. 2005; Adriano *et al*. 2006, 2009a, b; Milanin *et al.* 2010). In spite of the great variety of species among the Brazilian fauna that have been described previously through the above methods we have found few examples of studies concerning the evolution of myxosporean plasmodia during the sporogony and spore maturing process (Rocha *et al.* 2011). In this paper, therefore, we describe for the first time ultrastructural aspects of the plasmodium evolution of *Myxobolus insignis* found in the gill of a teleost collected from the Amazonian region. This myxosporean was first described based on light microscopic data by Eiras *et al*. (2005b) and, recently, ultrastructurally by Azevedo *et al*. 2012.

MATERIAL AND METHODS

During a parasitological survey expedition in the central Amazon River in January 2012, numerous fish of different species were collected. Among these were specimens of *Semaprochilodus insignis* Jardine, 1841 (Teleostei, Prochilodontidae) (15–21 cm long) (Brazilian common name "Jaraqui"). These fish were collected from the Trombetas River (a tributary of the central region of the Amazon River) (01°45′S, 55°53′W), near the city of Oriximiná (State of Pará), located about 550 km downstream from the city of Manaus and about 770 km upstream from the Amazon River mouth, Brazil. The fish were transported alive to the laboratory of the Campus of the Federal Fluminense University located in the city of Oriximiná.

Fish which died during the trip were placed in ice (for 2–3 h), while the live fish were later anesthetized using MS 222 solution (Sandoz Lab) prior to dissection. Infected fish contained several plasmodia in the form of whitish cysts located in the base of the gill filaments. Numerous developmental stages, including immature and mature spores, were examined in fresh mounts with a light microscope equipped with Nomarski differential interference-contrast optics (LM-DIC). For transmission electron microscopy (TEM), small parasitized fragments of infected tissues were fixed in 5% glutaraldehyde in a 0.2 M sodium cacodylate buffer (pH 7.4) at 4°C for 20–24 h, washed overnight with the same buffer, and post-fixed in 2% OsO_4 buffered with 0.2 M sodium cacodylate for 4 h at the same temperature. The infected fragments were dehydrated in an ascending ethanol series, propylene oxide and embedded in Epon. Semi-thin sections were stained with methylene blue-Azur II and observed under LM-DIC optics. Ultra-thin sections, cut with a diamond knife, were stained with both aqueous uranyl acetate and lead citrate and observed in a JEOL 100CXII TEM, operated at 60 kV.

RESULTS

The principal organs of the specimens of *Semaprochilodus insignis* were observed both macroscopically and microscopically by LM-DIC, in order to detect the presence of possible eventual parasites. It was observed that some gills were infected by numerous myxozoan plasmodia containing different developmental stages, including immature and mature spores. The following description of this parasite is based upon the observed ultrastructural aspects of the plasmodia and their evolution during the sporogenetic process (Figs 1–8).

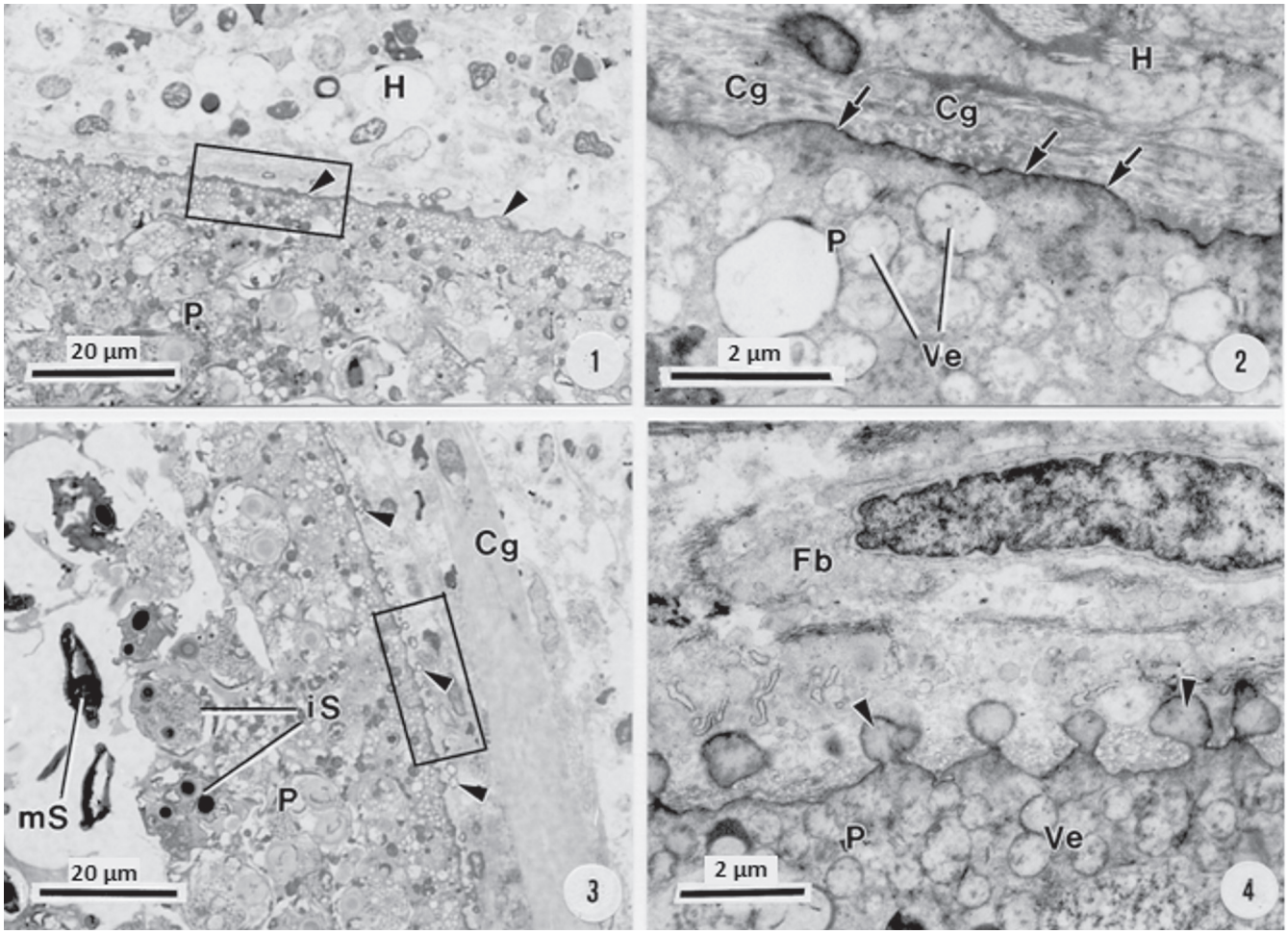
Description of the plasmodia

Several irregular plasmodia with variable dimensions were observed macroscopically as whitish cysts in the base of the secondary gill lamellae. These plasmodia were classified according to the different sporogenetic developmental phases as falling into three types:

Stage 1. Plasmodia were the smallest (up to ~ 110–150 μm in diameter), with a spherical to ellipsoidal morphology and containing the earliest sporogenetic developmental stages without spores. These early stage plasmodia had a smooth membrane, without microvilli or pseudopodia, with only a few, small, irregular projections (Fig. 1). The peripheral region of the plasmodia was occupied by a thin layer of fibroblasts surrounded by collagen fibres. The cortical zone of the plasmodia contained early sporogenic cells (nuclei and generative cells) along with numerous electron-lucent vacuoles and vesicles, these latter seeming sometimes fused with the plasmodium wall (Figs 1, 2). Internally, in the central zone of the plasmodia, some developmental stages were observed, although not yet spores (Fig. 1).

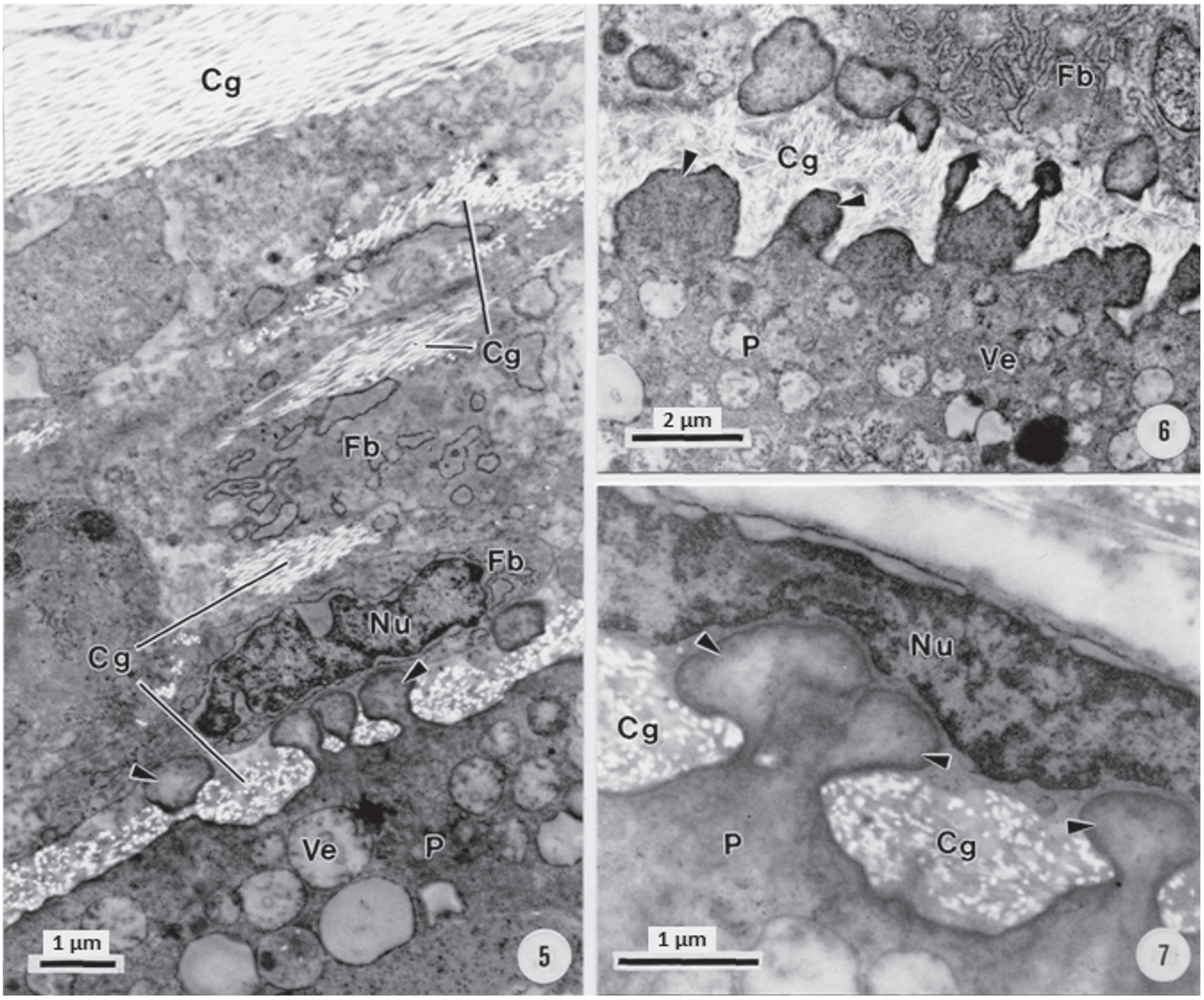
Stage 2. Plasmodia were categorised as comprising those up to about 200–300 μm in diameter, corresponding to the intermediate stages of plasmodial evolution. During this stage the plasmodia membrane developed microvillus-like structures with apically hemispherical ends (Figs 3, 4). The internal region of the plasmodia contained several sporogenetic developmental stages along with some immature and, rarely, mature spores.

Stage 3. Plasmodia were categorised as being those from ~ 350 μm in diameter up to ~ 650 μm, corre-



Figs 1–4. Light and ultrastructural aspects of the periphery of plasmodia of *Myxobolus insignis* infecting the freshwater teleost *Semaprochilodus insignis* from the Amazon River, showing the evolution of the plasmalemma during the three phases. **1 –** light micrograph showing the smooth plasmalemma (arrowheads) of the plasmodium (P) in contact with the surrounding cells of the host (H). This aspect corresponds to stage 1 of the plasmodium development; **2** – ultrastructural aspect of the area boxed in Fig. 1, observed with higher magnification showing several collagen fibres (Cg) at the periphery of the plasmodium (P) and some vesicles (Ve) in the cortical zone of the plasmodium; **3** – light micrograph showing the plasmalemma containing several small microvilli (arrowheads). Internally, some immature (iS) and mature spores (mS) and vesicles are present and, externally, a layer of collagen fibres (Cg). This aspect corresponds to stage 2 of the plasmodium development; **4** – ultrastructural aspect of the plasmalemma and their microvilli (arrowheads) in contact with surrounding fibroblasts (Fb). Among the microvilli numerous collagen fibres are present. The cortical zone of the plasmodium (P) contains numerous vesicles (Ve), some of which seem to fuse with the plasmalemma.

sponding to the oldest stages of plasmodial evolution and containing, therefore, the oldest stages of the sporogenetic process. During these developmental stages and the maturing process of the spores, the plasmodium membrane was observed to become denser and thicker, transforming into a wall in which numerous complex microvilli or pseudopodia could be observed (Figs 5, 6). The oldest plasmodia tended to present a sinuous and irregular outline, formed by numerous microvilli and pseudopodia-like structures with a distinct morphology (frequently with a mushroom-like aspect) in which bud-like structures at the extremity projected towards the external region of the wall (Figs 5–7). These microvilli, containing the spherical bud-like structures at the apical end, were in close contact with the fibroblasts and with the first peripheral collagen fibril layers (Fig. 7). Meanwhile, the external region surrounding the plasmodia became denser and thicker through the



Figs 5–7. Ultrastructural aspects of the periphery of the plasmodia (P) of *Myxobolus insignis* n. sp. infecting the freshwater teleost *Semaprochilodus insignis* from the Amazon River showing the different aspects of the microvilli (arrowheads) and collagen fibre arrangements (Cg) located among the fibroblasts (Fb), some of which are showing their nuclei (Nu). The cortical zone of the plasmodium contains numerous vesicles (Ve).

development of several continuous layers of collagen fibrils intermingled with some fibroblasts (Figs 6, 7).

The schematic drawing (Fig. 8) represents the morphological evolution and differentiation of the peripheral region of the plasmodia according to our observations of the three sampled stages.

DISCUSSION

Considering that the myxozoan described in the present paper was collected from the gills of the Amazonian fish *Semaprochilodus insignis*, and taking into account the observed morphological aspects of the spores, we concluded that the parasite isolated in our study was the same as that previously described as *Myxobolus insignis* through the light based observational study

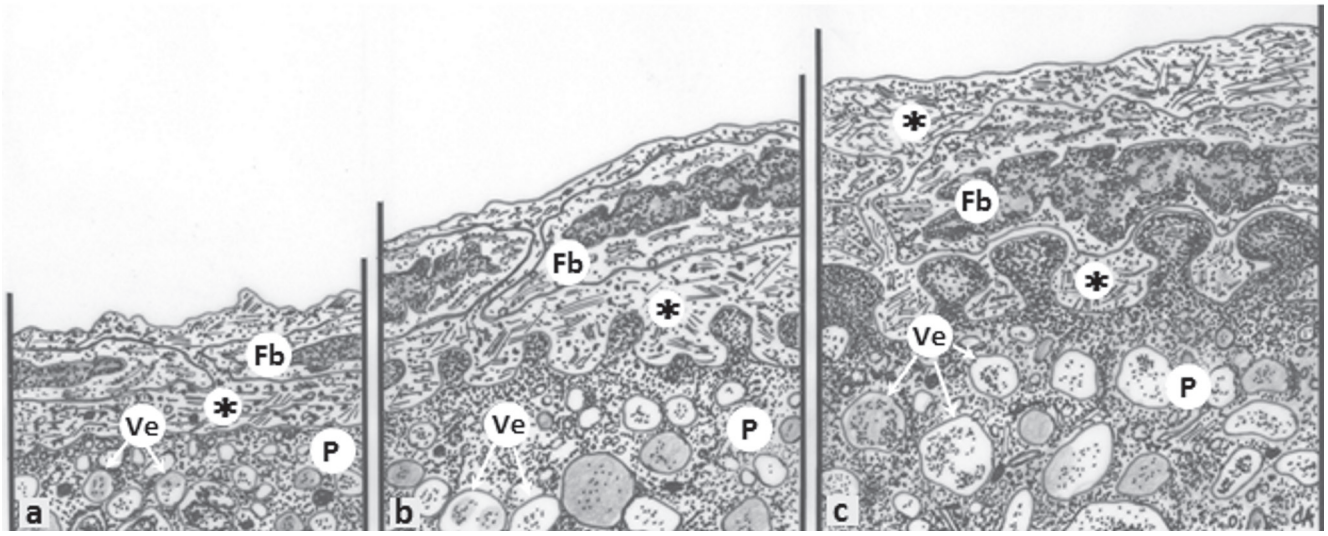


Fig. 8. Schematic drawing of the plasmodial evolution of the periphery of plasmodia (P) of *Myxobolus insignis* n. sp. showing the differentiation of the plasmalemma during the three stages (a – Stage 1; b – Stage 2 and c – Stage 3) during sporogenesis (Fb – fibroblasts, P – plasmodia, Ve – vesicles, * – collagen fibres).

of Eiras *et al.* (2005b), and the ultrastructural observations of the parasitical spores conducted by Azevedo *et al*. (2012). In addition, the ultrastructural aspects of the spores observed in the present work were similar to those observed previously by Azevedo *et al.* (2012).

Studies concerning the morphological description of *Myxobolus* spp. spores have often failed to report any information about their corresponding plasmodia or their evolution, reporting only the presence of plasmodia without any detailed characterisation, particularly in relation to the plasmodial evolution during sporogenesis (Molnár and Békési 1993; Casal *et al*. 1996, 2002; Adriano *et al.* 2002; Azevedo *et al*. 2002, 2011, 2012; Tajdari *et al.* 2005). This study, therefore, addresses this issue in respect to *Myxobolus insignis*, and develops the previous work of Azevedo *et al.* (2012), by describing in detail the ultrastructure of the plasmodia associated with *Myxobolus insignis* (Eiras *et al.* 2005b) and by demonstrating that these plasmodia differ both in size and in other morphological aspects from other myxosporean species (Canning *et al*. 1999, Rocha *et al.* 2011). In particular, the study shows that, through careful observation and description of the peripheral membrane and the developmental stages of sporogenesis, it is possible to identify three distinct plasmodial stages corresponding to the sequential phases of the plasmodial evolution. This conclusion parallels similar observations that have been reported in some other myxosporeans (Lom and Dyková 2006, Canning *et al.* 1999, Azevedo *et al*. 2011, Rocha *et al.* 2011).

To summarise the observations in this study in the context of their implications for our wider understanding of myxosporean plasmodia, we noted that the plasmodial wall of *Myxobolus insignis* showed a very irregular outline containing numerous pseudopodia projecting towards the periphery of the host cells, as well as collagen fibril layers, sometimes compressing in the cytoplasm of the host cells. We infer that this development of pseudopodia during the maturation process of the spores serves to enlarge the surface of the plasmodia to enable more efficient nutrient uptake as the plasmodia grow.

Whilst a similar structure has been observed in plasmodia of *Myxidium trachinorum,* for the most part plasmodia of both *Myxobolus* spp., and other myxosporeans, present a smooth outline utilising numerous pinocytotic channels for the same function (Current 1979; Current *et al.* 1979; Casal *et al*. 1996, 2002, 2006; Matos *et al.* 2005; Azevedo *et al.* 2011). Furthermore, the morphology of the plasmodia wall in this case, involving microvilli that contain bud-like structures at their extremities, which in turn were generally intruded into the peripheral host cell, has not previously been described in this group of parasites, although this feature also seems to relate

to the enlargement of the contact surface for the purposes of nutrition. Comparing our results obtained from disporic *M. insignis* with other disporic or polysporic species revealed significant morphological differences in plasmodial ultrastructure, mainly their attachment to the host cells and surface evolution of the plasmodial plasmalemma. Our results are similar, however, to those obtained in *Myxidium trachinorum* infecting the gallbladder of the lesser weever fish collected in the south and south-west of England (Canning *et al.* 1999) and in *Triangulamyxa psittaca* infecting the urinary bladder of the freshwater fish (*Collomeus psittacus*) collected in the Amazon River (Rocha *et al*. 2011).

The presence of some surrounding layers of collagen fibres which appeared among the surrounding fibroblasts is, however, a more frequent phenomenon that occurs in the periphery of myxosporean plasmodia, mainly in the final phase of the plasmodium maturation (Current 1979, Current *et al.* 1979, Azevedo and Matos 2002, Casal *et al*. 2006, Azevedo *et al*. 2009).

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