



**ULTRASTRUCTURAL AND MOLECULAR DESCRIPTION
OF SOME MYXOSPOREANS (PHYLUM MYXOZOA)
INFECTING THE AQUATIC FAUNA**

SÓNIA RAQUEL OLIVEIRA ROCHA

Dissertation for Master in Marine Sciences – Marine Resources

November

2011



SÓNIA RAQUEL OLIVEIRA ROCHA

**ULTRASTRUCTURAL AND MOLECULAR DESCRIPTION OF SOME
MYXOSPOREANS (PHYLUM MYXOZOA) INFECTING THE
AQUATIC FAUNA**

Dissertation for Master's degree in Marine
Sciences – Marine Resources submitted to
the Institute of Biomedical Sciences Abel
Salazar, University of Porto.

Supervisor – Doctor Carlos Azevedo
Category – “Professor Catedrático Jubilado”
Affiliation – Institute of Biomedical Sciences
Abel Salazar, University of Porto.

Nota prévia

Declaro que, como autora desta tese, estive envolvida na realização de todos os procedimentos laboratoriais conduzentes à obtenção dos resultados aqui apresentados pela primeira vez. A minha actividade desenvolveu-se desde a colheita e diagnóstico preliminar de material biológico para amostragem, à execução do processamento protocolar para microscopia óptica, incluindo contraste de interferência diferencial, microscopia eletrónica de transmissão e microscopia eletrónica de varrimento, à realização dos procedimentos laboratoriais necessários para a biologia molecular. O conteúdo desta tese é da minha autoria, embora inclua as recomendações e sugestões positivamente feitas pelo orientador, colaboradores e técnicos. O trabalho realizado e informação obtida resultaram na elaboração de três artigos científicos distintos, aqui apresentados nos capítulos 2, 3 e 4.

Rocha S., Casal G., Matos P., Matos E., Dkhil M. and Azevedo C. 2011: Description of *Triangulamyxa psittaca* sp. nov. (Myxozoa: Myxosporea), a new parasite in the urinary bladder of *Colomesus psittacus* (Teleostei) from the Amazon River, with emphasis on the ultrastructure of plasmodial stages. Acta Protozool. 50: (In press)

Rocha S., Casal G., Al-Quraishy S. and Azevedo C. 2011: Morphological and molecular characterization of *Chloromyxum clavatum* n. sp. (Myxozoa: Myxosporea), infecting the gall bladder of *Raja clavata* (Chondrichthyes) from the Portuguese Atlantic coast. J. Parasitol. (Submitted)

Rocha S., Casal G. and Azevedo C. 2011: Morphological and ultrastructural re-description of *Chloromyxum leydigi* (Myxozoa: Myxosporea) from the gall bladder of marine cartilaginous fish *Torpedo marmorata* (Chondrichthyes: Torpedinidae) from the Portuguese Atlantic coast. (Unsubmitted)

Preliminary Note

I hereby declare that, as the autor of this thesis, I executed the laboratory procedures leading to the acquisition of the results here presented for the first time. My activity included the sampling and preliminary diagnosis of biological material, the execution of specific procedures for light microscopy, including differential interference contrast, transmission electron microscopy and scanning electron microscopy, and the realization of the laboratory procedures necessary for molecular biology. The entire content of this thesis is of my authorship, despite including the positive recommendations and suggestions made by the supervisor, collaborators and technicians. The work developed and the information acquired resulted in the elaboration of three distinct scientific papers here presented in chapters 2, 3, and 4.

Rocha S., Casal G., Matos P., Matos E., Dkhil M. and Azevedo C. 2011: Description of *Triangulamyxa psittaca* sp. nov. (Myxozoa: Myxosporea), a new parasite in the urinary bladder of *Colomesus psittacus* (Teleostei) from the Amazon River, with emphasis on the ultrastructure of plasmodial stages. Acta Protozool. 50: (*In press*)

Rocha S., Casal G., Al-Quraishy S. and Azevedo C. 2011: Morphological and molecular characterization of *Chloromyxum clavatum* n. sp. (Myxozoa: Myxosporea), infecting the gall bladder of *Raja clavata* (Chondrichthyes) from the Portuguese Atlantic coast. J. Parasitol. (*Submitted*)

Rocha S., Casal G. and Azevedo C. 2011: Morphological and ultrastructural re-description of *Chloromyxum leydigi* (Myxozoa: Myxosporea) from the gall bladder of marine cartilaginous fish *Torpedo marmorata* (Chondrichthyes: Torpedinidae) from the Portuguese Atlantic coast. (*Unsubmitted*)

Agradecimentos

A entrega desta tese representa para mim mais uma etapa da minha vida académica e pessoal que termina e que, de forma igual a tudo o que muito se deseja e dificilmente se alcança, tem um sabor simultaneamente doce e amargo. Doce porque sinto-me plenamente satisfeita do meu desempenho nas funções a que me propus e dos objectivos que alcancei, bem como da evolução profissional e pessoal inerente a todo este processo e que hoje me faz seguramente sentir mais completa. Amargo porque apesar de todas as horas que despendi num microscópio ou na bancada do laboratório, do quão perdida me senti no meio de mais de uma centena de artigos por vezes conflituosos, do cansaço das horas de olhos colados num computador para escrever dois ou três parágrafos, tenho a certeza que esta época me deixará imensas saudades das experiências que tive e das pessoas que conheci. Como tal, não posso deixar de agradecer a todos os que incondicionalmente me apoiaram e motivaram.

Ao professor Doutor Carlos Azevedo pela orientação, disponibilidade, confiança e optimismo com que me brindou e que estimularam em mim o “bichinho” para a parasitologia.

À Doutora Graça Casal, que sendo uma pessoa deveras ocupada, não deixou de me prestar a sua valiosa assistência sempre que necessária.

Ao Professor Doutor Alexandre Lobo da Cunha, Director do Departamento de Microscopia, e meu professor de licenciatura, pela sua boa vontade em me acolher no laboratório e simpatia que sempre me dedicou.

À Elsa Oliveira e Ângela Alves que não só me prestaram o seu precioso auxílio sempre com boa disposição, como se tornaram a minha maior e melhor companhia durante todo este período. Sem os vossos conselhos e experiência não teria sido possível terminar esta tese como o faço.

À Mestre Carla Oliveira pela disponibilidade em me orientar na execução dos procedimentos laboratoriais para a biologia molecular.

Às minhas colegas de mestrado Lúcia Barriga Negra, Ângela Ferreira, Cláudia Mendes e Lúcia Sousa por todas as dicas que me dispenderam, e por compreenderem todas as vezes em que ao invés de dizer sim tive que dizer não, em que cheguei atrasada, em que tive que sair mais cedo, e nas quais porventura não tive a minha melhor prestação como amiga.

À minha irmã Gisela, com quem discuto todos os dias mas a quem amo na certeza de que sempre nos apoiaremos ao longo da nossa vida, tanto nos sucessos como nas dificuldades. Afinal de contas, é esse o papel de uma irmã, uma melhor amiga velada pelos laços fraternais.

Aos meus avós maternos, que estiveram sempre presentes em todas as etapas da minha vida com um sorriso ou uma palavra carinhosa, a quem eu amo profundamente e é para mim uma enorme felicidade dar-lhes mais esta alegria e saber que tem orgulho em mim.

Aos meus pais, por lutarem tanto para me providenciarem uma carreira como estudante e profissional, pela liberdade de opções e apoio que sempre me proporcionaram, e pelo amor incondicional que me têm. Bem sei que este é um sonho que realizo conjuntamente com vocês e que constitui igualmente motivo de orgulho próprio.

Ao Miguel, por ser o meu melhor amigo, por me dar todo o seu amor e carinho e por me fazer sentir a melhor pessoa do mundo. Não podia ter conhecido melhor pessoa na minha vida, com tanta integridade e que me apoiasse tanto e me aturasse tanto como tu fazes todos os dias. Desculpa as horas infindáveis que passaste a ouvir falar de mixosporídeos, de cápsulas polares, de planches, de escalas, de DNA e do “ultramicrofone”, como dizes. Sei que para ti os meus sucessos são também os teus sucessos. Portanto, aqui vai a nós!

Preface

As part of my master's degree in Marine Sciences – Marine Resources in the Institute of Biomedical Sciences Abel Salazar of the University of Porto, I developed a work focused in the area of cellular biology, more specifically in parasitology. This type of research has always interested me, since it allows the conjugation of both new and old methodologies and quantitative and qualitative results, thus making the exercise of interpretation much more interesting. With this purpose, I joined the Laboratory of Cellular Biology of the same institute, which develops several projects concerning microparasites infecting the aquatic fauna of different geographical areas, and thus is equipped with all the necessary equipment for both microscopic and molecular studies. Also, the experience demonstrated by the investigators and technicians integrated in those projects reflected a positive receptivity for the development of my work, which culminates with the presentation of this document.

The present thesis attempts to provide fundamental information on the class Myxosporea of the phylum Myxozoa. To date, more than 2000 species of myxosporeans have been found infecting several fish species, and more rarely amphibians, birds, mammals, and even humans. Despite most myxosporeans being harmless, some are serious pathogens that provoke devastating damages in both wild and reared populations of fish. Considering environmental sustainability, the socio-economical importance of the aquaculture industry, as well as other activities associated with the aquatic environment, the acquisition of precise knowledge concerning these species is essential. Furthermore, science for the sake of knowledge should never be disregarded, even if a direct advantage is not perceptible.

For many years, the microscopic dimensions of myxosporean parasites and the lack of appropriate technical support held back scientists from studying the intricacies of the myxosporeans morphological and life cycle adaptations. Nowadays, as new and much more reliable techniques arise, it is possible to discern old problematics and to provide new insights on both established and new species. There exist some monographs and few books on myxosporeans. However, most are very old and outdated; and the more recent ones are more directed towards the pathogenic species with economical impact. Therefore, this thesis summarizes the overall aspects of the class Myxosporea described up until now and presents three new works on species belonging to two of its genera. A resumed presentation of the phylum Myxozoa is given before introducing the class Myxosporea. For this introductory chapter, a summary of taxonomic, morphological and biological aspects is provided, depicting the main events and adaptations of the parasites

life cycle. Although a taxonomic scheme from phylum to genera is followed, taxa beneath the class Myxosporea are not characterized, with the exception of the genus *Triangulamyxa* and the genus *Chloromyxum*. Detailing these genera fits the context of this thesis, since the new data here presented results from studies relating to them. Each of the following three chapters contemplates a specific study and is organized according to the outline of the indexed journal chosen for publication. The last chapter provides a general discussion that gives closure to this thesis, which I hope will be able to demonstrate the surprising and fascinating intricacies of these creatures.

Abstract

Members of the class Myxosporea Bütschli, 1881 are metazoan organisms characterized by the formation of spores composed of one to seven shell valves, one or more sporoplasms, and one to several nematocyst-like polar capsules, each containing an extrudible polar filament coiled along its inner wall. Myxosporeans possess a complex life cycle, in which a myxosporean stage and an actinosporean stage are alternated. Unfortunately, most species do not possess both these stages described. In fact, the number of established myxosporean species greatly overcomes the number of reported actinosporean stages. During the myxosporean stage, Myxosporea are common parasites in the tissues and organ cavities of fish, and less frequently of amphibians, birds and mammals. During the actinosporean stage, they occupy an invertebrate as their host, namely oligochaetes, polychaetes, and more rarely spinculids. The majority of myxosporeans are harmless towards the host metabolic and physiological processes. However, some are pathogens of fishes and provoke more or less serious pathological damages in the host body, often leading to high levels of mortality within affected wild or reared populations. Widely distributed in several geographical areas, the devastating effects of some known myxosporeans, as well as environmental sustainability issues and the development of aquaculture and other important social-economical activities, increased the scientific interest towards these organisms. New information is frequently published but, despite the use of several different methodologies, many taxonomic relationships, life cycle aspects, environmental adaptations, as well as transmission and immunological mechanisms remain unclear for both established and new myxosporean species. Acknowledging the controversy and difficulties associated with this research area, the present thesis summarizes old and provides new developments concerning the taxonomic, morphological, ultrastructural and life cycle aspects of the class Myxosporea. From this class, the genus *Triangulamyxa* Azevedo et al., 2005 and *Chloromyxum* Mingazzini, 1890 are focused. For genus *Triangulamyxa*, a new species is described from the urinary bladder of *Colomesus psittacus* Schneider, 1801, a teleostean of the Amazon River, Brazil. The description of the new species, named *Triangulamyxa psittaca* sp. n., is based on light and transmission electron microscopic observations, and emphasizes the ultrastructural development of the vegetative stages, which morphology is apparently influenced by the adaptation to environmental factors, namely water temperature. For genus *Chloromyxum*, two species are described from the gall bladder of cartilaginous fishes. The first is a new species found in the gall bladder of the thornback ray, *Raja clavata* Linnaeus, 1758, from the Northwest Atlantic coast of Portugal. The description of *Chloromyxum clavatum* sp. n. is based on light, transmission and scanning electron

microscopic observations of the aspects displayed by the spores and vegetative stages, associated with molecular data obtained from sequencing of the 18S rDNA gene. The second consists on the morphological and ultrastructural characterization of *Chloromyxum leydigi* Mingazzini, 1980, a previously sequenced species and focus of taxonomic and phylogenetic controversy. This parasite constitutes the type-species of the genus *Chloromyxum* and, although it has been found infecting the gall bladder of several elasmobranchs, lacks the proper accurate description of its morphological aspects. The re-description here made is based on light and transmission and scanning electron microscopy, and allows the recognition of the morphological features of the different developmental stages of this species in the gall bladder of the spotted torpedo, *Torpedo marmorata* Risso, 1810, from the Portuguese Atlantic coast.

Resumo

A classe Myxosporea Bütschli, 1881 é constituída por organismos metazoários, caracterizados pela formação de esporos compostos por uma a sete valvas, um ou mais esporoplasmas, e uma a várias cápsulas polares similares a nematócistos, cada uma contendo um filamento polar extrusível enrolado em torno da sua parede interna. Os mixosporídios possuem um ciclo de vida complexo, no qual se aternam um estadio de mixosporídio e um estadio de actinosporídio. Infelizmente, a maioria das espécies não possui ambos os estadios descritos. De facto, o número de espécies conhecidas de mixosporídeos supera largamente o número de estadios de actinosporídeos relatados. Durante o estadio de mixosporídeo, os Myxosporea são parasitas comuns nos tecidos e cavidades dos órgãos de peixes, e menos frequentemente em anfíbios, aves e mamíferos. Durante o estadio de actinosporídeo, ocupam um invertebrado como hospedeiro, nomeadamente oligoquetas, poliquetas, e mais raramente sipunculídeos. A grande maioria é inóqua para o metabolismo e processos fisiológicos do hospedeiro. No entanto, alguns são patogéneos de peixes, cuja ação provoca danos patológicos ligeiros ou graves no corpo do hospedeiro e, frequentemente, conduz a elevadas taxas de mortalidade entre populações selvagens ou cultivadas. Distribuídos em diversas áreas geográficas, os efeitos devastadores de algumas espécies de mixosporídeos para o desenvolvimento da aquacultura e outras actividades socio-economicamente importantes, bem como questões de sustentabilidade ambiental, têm resultado no aumento do interesse científico para com estes organismos. Informação nova é regularmente publicada mas, não obstante o uso de variadas metodologias, muitas relações taxonómicas, aspetos específicos do ciclo de vida, adaptações ambientais e mecanismos de transmissão e de defesa imunológica permanecem obscuros tanto em espécies estabelecidas como em espécies novas. Tendo em conta a controvérsia e dificuldades associadas a esta área de investigação, a presente tese sumariza antigos e relata novos desenvolvimentos referentes a aspetos taxonómicos, morfológicos, ultrastruturais e do ciclo de vida da classe Myxosporea. Desta classe, os géneros *Triangulamyxa* Azevedo et al., 2005 e *Chloromyxum* Mingazzini, 1890 são focados. Para o género *Triangulamyxa*, uma nova espécie é descrita da bexiga urinária de *Colomesus psittacus* Schneider, 1801, um peixe teleósteo do Rio Amazonas, Brasil. A descrição da nova espécie, designada *Triangulamyxa psittaca* n. sp., baseia-se em observações de microscopia óptica e eletrónica de transmissão, e enfatiza o desenvolvimento ultrastrutural dos estadios vegetativos, cuja morfologia é aparentemente influenciada pela adaptação a fatores ambientais, nomeadamente à temperatura da água. Para o género *Chloromyxum*, duas espécies são descritas da vesícula biliar de peixes cartilagíneos. A primeira é uma nova

espécie encontrada na vesícula biliar da raia lenga, *Raja clavata* Linnaeus, 1758, da costa Atlântica Noroeste de Portugal. A descrição de *Chloromyxum clavatum* n. sp. baseia-se no reconhecimento de esporos e estádios vegetativos através de observações de microscopia óptica, eletrónica de transmissão e de varrimento, associadas à informação molecular adquirida pela sequenciação do DNA da pequena subunidade ribossomal. A segunda consiste na caracterização morfológica e ultrastrutural de *Chloromyxum leydigi*, uma espécie previamente sequenciada e foco de controvérsia taxonómica e filogenética. Este parasita foi a primeira espécie determinada dentro do género *Chloromyxum* e, apesar de ter sido encontrado na vesícula biliar de diversos elasmobrânquios, falha em possuir uma descrição adequada e precisa dos seus aspetos morfológicos. Nesta tese, observações de microscopia óptica e eletrónica de transmissão e de varrimento permitem o reconhecimento da evolução dos diferentes estádios no desenvolvimento desta espécie na vesícula biliar da tremelga-marmoreada, *Torpedo marmorata* Risso, 1810, da costa Atlântica Portuguesa.

Table of Contents

Preface.....	vii
Abstract.....	ix
Resumo.....	xi
 Chapter 1 – General Introduction.....	 1
1.1. Phylum Myxozoa Grassé, 1970.....	3
1.1.1. General description and taxonomy.....	3
1.1.2. Taxonomic and phylogenetic history.....	5
1.2. Class Myxosporea Bütschli, 1881.....	11
1.2.1. Taxonomy.....	11
1.2.2. Geographical distribution and seasonal variations.....	12
1.2.3. Ultrastructural description.....	13
1.2.4. Life cycle.....	16
1.2.4.1. The actinosporean stage.....	18
1.2.4.2. The myxosporean stage.....	21
1.2.5. Hosts.....	24
1.2.6. Transmission.....	31
1.2.7. Nutrition.....	34
1.2.8. Pathogenicity and host immune response.....	34
1.2.9. Economical and sociological impact.....	37
1.2.10. Diagnosis.....	39
1.3. Genus <i>Triangulamyxa</i> Azevedo et al., 2005.....	42
1.4. Genus <i>Chloromyxum</i> Mingazzini, 1890.....	43
References.....	44
 Chapter 2 - Description of <i>Triangulamyxa psittaca</i> sp. nov. (Myxozoa: Myxosporea), a new parasite in the urinary bladder of <i>Colomesus psittacus</i> (Teleostei) from the Amazon River, with emphasis on the ultrastructure of plasmodial stages.....	 71
 Chapter 3 - Morphological and molecular characterization of <i>Chloromyxum clavatum</i> n. sp. (Myxozoa: Myxosporea), infecting the gall bladder of <i>Raja clavata</i> (Chondrichthyes) from the Portuguese Atlantic coast.....	 91

Chapter 4 - Morphological and ultrastructural re-description of <i>Chloromyxum leydigi</i> (Myxozoa: Myxosporea) from the gall bladder of marine cartilaginous fish <i>Torpedo marmorata</i> (Chondrichthyes: Torpedinidae) from the Portuguese Atlantic coast.....	113
Chapter 5 – Final Remarks.....	133
5.1. General Discussion.....	135
5.2. General Conclusion.....	137
References.....	138

CHAPTER 1

General Introduction

Parasitology is the scientific discipline dealing with the association of two organisms, which may result in disease for the host species. The word “parasite” derives from the Greek language, meaning “situated beside”. Sociologically, it was used in ancient Greece to describe people who sat beside one another. Scientifically, parasites are described as organisms that live through a very close relationship with other organisms, either residing on or within them. The parasite depends on its host in order to perform some or many of its basic life functions, frequently causing harm and sometimes leading to death. The term parasite envelops many species, macroscopic and microscopic, from all taxonomic groups. They are animals or plants, including a diversity of species such as bacteria, yeasts, fungi, algae, protozoa, helminths and arthropods. Throughout history, parasites have always raised interest in the scientific community, as they were associated with diseases and high levels of mortality amongst humans, as well as animals and plants of economical interest. Between the 17th and the 19th centuries, parasitology was restricted to the study of zooparasites, which are parasites species belonging to the animal kingdom. The rest of the parasitic species, classified of plant origin, became subject to the discipline of microbiology. Nowadays, parasitology remains an important area of research in great development. Amongst the animal species of economic interest affected by parasites, fish, molluscs and crustaceans are in the first line of research. Several taxonomic groups of microparasites are described in the mentioned animals. The present thesis considers only one: the parasitic species of the class Myxosporea Bütschli, 1881 of the phylum Myxozoa Grassé, 1970.

1.1. Phylum Myxozoa Grassé, 1970

1.1.1. General description and taxonomy

Myxozoans are microscopic eucariotic organisms, obligate parasites of vertebrates and invertebrates (Morris and Adams 2007), which possess very complicated life cycles characterized by the formation of multicellular spores. Vegetative (trophic) stages are represented by spore-producing multicellular plasmodia. Each spore is constituted by one to seven shell valves, one to several nematocyst-like polar capsules and one or more sporoplasms (amoeboid infective germs). Each capsule contains a polar filament that, when extruded, possesses an anchoring function (Lom 1987; Lom and Dyková 1992, 2006; Andree et al. 1999). As eukaryotic cells, Myxozoa lack centrioles and flagella. Cells junctions are very common and mitochondria have flat, tubular or discoid cristae. Mitosis is closed, with the microtubules of the spindle often persisting as a coherent bundle, after

karyokinesis (Lom and Dyková 2006). Overall, Myxozoa have no gross similarity to other animals (Jiménez-Guri et al. 2007).

The phylum Myxozoa follows a simple taxonomic scheme and comprises only two classes: the class Malacosporea Canning et al., 2000 and the class Myxosporea (Lom and Dyková 1992, 2006).

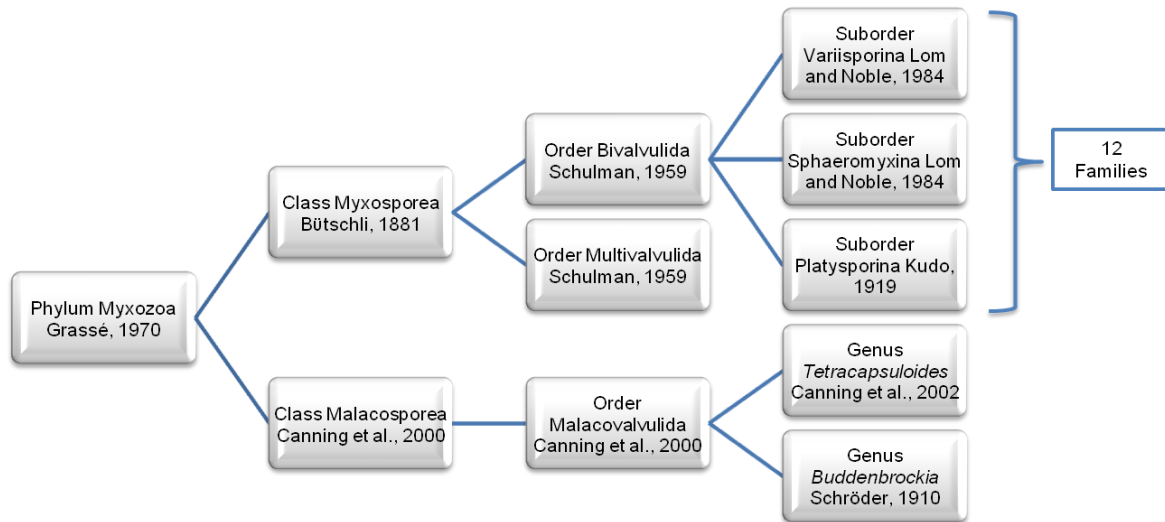


FIGURE 1. Taxonomic scheme of the phylum Myxozoa. The orders Bivalvulida and Multivalvulida of the class Myxosporea divide according to the number of shell valves, two or three to seven, respectively. The order Bivalvulida divides into three suborders, depending on the character of the polar filament and the position of the polar capsules. The order Multivalvulida contains three families, occurring predominantly as histozoic parasites in the skeletal muscles of marine fishes (adapted from Lom and Dyková 2006).

The main criteria used for the classification of myxozoan species is spore morphology (Andree et al. 1999; Lom and Hoffman 2003; Lom and Dyková 2006). Characters for differential diagnosis include spores and polar capsules size and shape, structural aspects and number of the shell valves, organization, direction and number of coils of the polar filament, projections and envelopes of the spores, among others. Vegetative stages usually do not possess sufficient classification features, but the ultrastructural characteristics displayed by the different life cycle stages may provide valuable information, for instance the formation of the spores occurring with or without the development of a pansporoblast (Lom and Noble 1984; Lom and Dyková 1992, 1993, 2006; Lom and Hoffman 2003). A practical key for the determination of myxosporean genera is given by Lom and Dyková (1992, 2006), using the classification criteria of Lom and Noble (1984). Host specificity and site of infection in the host body are often considered for the proper determination and description of new species (Lom and Noble 1984; Lom and Dyková 1992, 2006; Bahri et al. 2003; Eszterbauer 2004; Casal et al. 2009). Some studies actually report taxa to cluster more by development and tissue location than by spore morphology (Kent et al. 2001; Bahri et al. 2003; Eszterbauer 2004). Nevertheless, these criteria are not always

reliable for classification (Andree et al. 1999; Fiala 2006). Morphology is many times insufficient, some myxosporean species are reported to infect more than one host during the myxosporean or the actinosporean stages (O'Grodnick 1979; El-Mansy and Molnár 1997), and some may infect more than just one specific site in the hosts body (Molnár 1991; Redondo et al. 2004). Also, the effects of environmental factors and host species in the development and morphology of the spores remain unclear (Molnár 1991; Andree et al. 1999).

Malacosporean species differ from myxosporean species in its hosts, vegetative stages and by having spores with eight unhardened shell valves. The vegetative stages described in Malacosporea appear in the form of a primitive bilateral worm-like organism or in the form of a closed sac; while in Myxosporea they often appear in the form of an amoeboid structure – the plasmodium (Lom and Dyková 2006; Jiménez-Guri et al. 2007). Myxosporea predominantly infect aquatic oligochaetes as invertebrate hosts and fish as vertebrate hosts, forming two well-supported clades: one of marine taxa and the other of freshwater taxa. The freshwater and marine lineages divide into several clades that follow the tissue tropism of the parasites within the hosts (Andree et al. 1999; Kent et al. 2001; Eszterbauer 2004; Fiala and Dyková 2004; Holzer et al. 2004; Fiala 2006; Bartošová et al. 2009). Malacosporea infect only freshwater bryozoans as invertebrate hosts (Canning et al. 2000; Morris et al. 2002).

1.1.2. *Taxonomic and phylogenetic history*

Early classifications placed Myxozoa together with Microsporidia Sprague, 1977 and along with some members of Apicomplexa Levine, 1970, in the class Sporozoa. As more accurate knowledge was acquired, this class subsequently referred only to apicomplexans, while myxozoans and microsporidians remained together in the phylum Cnidospora Doflein, 1901. Following recognition of profound ultrastructural differences between these organisms, microsporidians warranted their own phylum, Microsporidia, leaving Myxozoa to stand alone as a phylum without recognized phylogenetic relationships (Vossbrinck et al. 1987; Sogin et al. 1989; Siddall et al. 1995).

For a long time, myxozoan origins and phylogenetic position have been the focus of much controversy (Evans et al. 2010), with various hypotheses being considered (Bartošová et al. 2009). Initially, Myxozoa were considered of protozoan nature. However, many authors contested this classification, arguing with observations that contradicted the assignment of myxozoans to protists, such as the presence of characters like multicellularity, septate junctions, collagen and putative nematocysts (Štolc 1899, *in*: Kent et al. 2001; Weill 1938,

in: Kent et al. 2001; Siddall et al. 1995). Their affinities to the metazoans were disputed until the late century, when sequencing of the 18S ribosomal DNA confirmed them as highly modified metazoans (Smothers et al. 1994; Katayama et al. 1995; Schlegel et al. 1996, *in*: Zrzavý 2001; Lom and Dyková 2006), which suffered extreme secondary reduction of body-plan complexity due to their endoparasitic life-styles (Katayama et al. 1995; Okamura et al. 2002). The discovery that the bizarre *Buddenbrockia* was indeed a myxozoan was groundbreaking in determining the true assignment of this phylum (Canning et al. 1996). Nevertheless, Myxozoa remained considered of protozoan nature for more than a hundred years (Lom and Dyková 2006). During this more controversial period in the myxozoans taxonomic history, apologists of the metazoan classification of Myxozoa, considered several possible taxonomic relationships with other groups from Metazoa. Of those, two dramatically different hypotheses have been put forward, one placing them within Cnidaria (Siddall et al. 1995; Zrzavý 2001; Zrzavý and Hypša 2003) and the other within Bilateria (Smothers et al. 1994; Hanelt et al. 1996; Kim et al. 1999; Zrzavý and Hypša 2003).

The first hypothesis places Myxozoa as a sister taxon to Cnidaria or a highly derived cnidarian clade, possibly within Medusozoa (Siddall et al. 1995; Evans et al. 2010). This hypothesis is the most traditional point of view, since Weill (1938) (*in*: Kent et al. 2001) suggested an affinity to the narcomedusan *Polypodium hydriforme* Ussov, 1885, due to the astonishing resembles found between coelozoic myxozoans and some parasitic Cnidaria (Kent et al. 2001). *Polypodium hydriforme* is an aberrant freshwater parasite of sturgeon fish and paddlefish oocytes and, like myxozoans, possesses nematocysts-like polar capsules (Kent et al. 2001; Raikova 2008). Nevertheless, their overall morphology is different, *Polypodium hydriforme* displays several more cnidarian characteristics than myxozoans, namely tentacles and a gut with only one opening (Raikova 2008). This remote hypothesis was later reaffirmed by Siddall (1995), when the combination of results from the fixed alignments of rDNA sequences and morphological data, recovered the Myxozoa and *Polypodium hydriforme* group within Cnidaria (Siddall et al. 1995; Zrzavý et al. 1998; Siddall and Whiting 1999; Zrzavý and Hypša 2003; Evans et al. 2010). Therefore, this

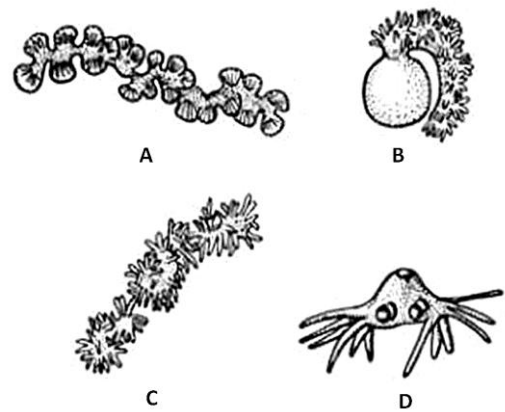


FIGURE 2. Parasitic (A, B) and free-living (C, D) phases of *Polypodium hydriforme*. **(A)** Stolon with internal tentacles inside the egg before spawning. **(B)** Stolon with external tentacles emerging from the egg during spawning. **(C)** Free stolon just after emerging from the egg. **(D)** Free-living *Polypodium* with 12 tentacles and 4 male gonads (adapted from Raikova 2008).

theory is based on phylogenetic and morphological data showing similarities between myxozoans and cnidarians, specifically the myxozoans polar capsules and the cnidarians nematocysts, which indicate a possible phylogenetic parallelism, later supported by molecular analysis of the small subunit rDNA (Jiménez-Guri et al. 2007; Evans et al. 2010). Both the polar capsules and nematocysts are similar in size, possess an operculum and inverted tubules in continuity with the capsule wall, a “stopper” that taps the filament but allows discharge in response to a mechanical stimuli (Weill 1938, *in*: Kent et al. 2001; Yokoyama et al. 1993; Yokoyama and Urawa 1997; Cannon and Wagner 2003; Kallert et al. 2005). Nevertheless, polar capsules differ from nematocysts, as they lack the chemo- and/or mechanosensory structures and neural connections that modulate discharge on those organelles (Westfall 2004). Cannon and Wagner (2003) provide a wide comparison between the morphology and discharge mechanism of the Myxozoa and the Cnidaria.

The second hypothesis places Myxozoa as a sister taxon to Bilateria and is based on molecular biological data collected from 18S rDNA sequences (Smothers et al. 1994; Evans et al. 2010). Bilateria include most metazoans (true animals), excluding cnidarians, ctenophores, sponges and placozoans. In this case, homology between the polar capsules and the nematocysts would be explained by the evolution of nematocyst-like structures previously to the divergence of cnidarians and bilaterians, or an independent arise of those structures (Jiménez-Guri et al. 2007). Most of the small subunit rDNA phylogenetic studies supporting the bilaterian origin of the Myxozoa do not include the *Polypodium hydriforme* sequence. However, those considering such sequence suggest a parallelism to *Polypodium hydriforme* that, together with Myxozoa, forms a clade (Endocnidozoa) recovered as the sister taxon to Bilateria, close to basal clades such as Mesozoa and Nematoda, rather than derived cnidarians (Smothers et al. 1994; Hanelt et al. 1996; Kim et al. 1999; Zrzavý and Hypša 2003). Although supporting this theory, Hanelt et al. (1996) and Kim et al. (1999) also pointed the possible occurrence of long-branch attraction between myxozoans and *Polypodium hydriforme*, since these organisms possess highly divergent DNA sequences. Supporters of the cnidarian origin of Myxozoa, Siddall and Whiting (1999) refused to believe that long-branch attraction could explain the monophyly found between Myxozoa and *Polypodium hydriforme*. Other reports propose the selection of distant outgroups and poor taxonomic sampling as significant reasons leading to the discrepancy between phylogenetic results (Siddall et al. 1995; Kim et al. 1999; Siddal and Whiting 1999). Following their expressed necessity for the application of different tree-building and long-branch extraction methods, associated with a combination of SSU rDNA data with morphological characters, these authors again inferred the

placement of Endocnidozoa within Cnidaria (Siddall et al. 1995; Siddall and Whiting 1999). Zrzavý and Hypša (2003) reanalyzed the *Polypodium* and Myxozoa relationship by recurring to the SSU sequences of 46 metazoan taxa in three different alignments, later combined in a single data matrix, and neutralized “long-branch” artifacts through the “long-branch extraction” technique proposed by Siddall and Whiting (1999). In their results, *Polypodium* did not group with cnidarians, no matter what analytical parameters were considered. Furthermore, they state that the basal-bilaterian position of Endocnidozoa is supported by the improbability of the systematic position of *Polypodium hydriforme* within Narcomedusae, which is exclusively based on parasitism and similarities in early development, despite its morphological appearance being undeniably that of a cnidarian. Other studies have also tried to resolve this issue, namely by removing the long-branched attractor Myxozoa (Evans et al. 2008), but so far have been unsuccessful (Evans et al. 2009). Another molecular data supporting the bilaterian theory was the re-investigation of four bilaterian-like Hox genes (*Myx1*, *Myx2*, *Myx3* e *Myx4*) in two myxozoan species, *Tetracapsula bryozoides* [now revised to *Buddenbrockia plumatellae* (Canning et al. 2002)] and *Myxidium lieberkuehni* (Anderson 1998, in: Jiménez-Guri et al. 2007; Zrzavý and Hypša 2003); until they were latter reported as likely belonging not to the parasite but to the bryozoan host himself. Polymerase chain reaction (PCR) with gene-specific primers amplified the Hox genes from uninfected bryozoans, but not from the myxozoans samples (Jiménez-Guri et al. 2007).

The most interesting and debated report in discerning the true phylogeny of Myxozoa is probably the case of *Buddenbrockia plumatellae*, an aberrant and motile vermiform parasite inhabiting the body cavities of freshwater ectoprocts (Zrzavý and Hypša 2003). Despite looking nothing like a myxozoan, strong evidences affirm this species as a true member of the phylum Myxozoa, including the presence of polar capsules similar to those of malacosporean species, both in the epidermis and in infective spores, as well as

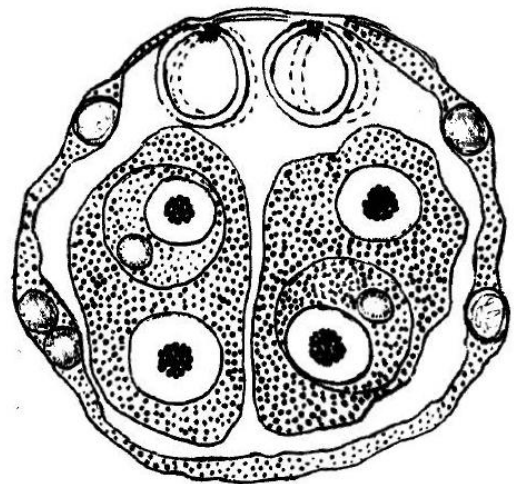


FIGURE 3. Schematic drawing of the Malacospore of *Buddenbrockia plumatellae*, showing the four polar capsules (two are beyond the plane of drawing) and two uninucleate sporoplasms, each with a uninucleate secondary cell. Notice the cytoplasmic wall containing mitochondria and haplosporosomes (adapted from Canning et al. 1996).

a type of septate junctions typically present in Malacosporea (Canning et al. 1996; Okamura et al. 2002; Morris and Adams 2007). They also parasitize the same freshwater bryozoan species, and have similar 18S DNA sequences, suggesting that they are at list

congeneric (Monteiro et al. 2002; Morris et al. 2002; Okamura et al. 2002). Unlike Malacosporea, the body is not sac-like shaped; *Buddenbrockia* body is worm-like shaped due to the presence of four nematode-like blocks of longitudinal muscular cords (Zrzavý and Hypša 2003), which enable the parasite to undergo bending movements in the host coelomic cavity. Current knowledge on this species demonstrates its unusual development, in which unicellular amoeboid-like cells present in the basal lamina of the hosts body wall divide in more complex unconnected cells that develop into tissue layers through the establishment of cell junctions, forming a stage structurally similar to a solid gastrula (McGurk et al. 2006; Morris and Adams 2007; Canning et al. 2008). This structure develops into a vermiform sac (worm) that detaches from the host epithelium into the coelom. The “worm” is composed by an ectodermal layer, a basal lamina, four longitudinal muscles blocks and an inner layer of cells surrounding a body cavity. Those cells enter the cavity and form spores that are released into the host when the parasite body ruptures (Canning et al. 2002; Canning and Okamura 2004; McGurk et al. 2006). The bryozoan releases the spores into the water column by retraction of the zooid, likely through the vestibular pore (Canning et al. 2002; Morris et al. 2002). The developmental stages vary in the different bryozoan hosts (Morris and Adams 2007).

The discovery of *Buddenbrockia plumatellae* as a vermiform stage in malacosporean species was considered evidence of the bilaterian nature of Myxozoa, representing a missing link in myxozoan evolution (Canning et al. 2002; Okamura et al. 2002). Its morphology and body movements are bilaterian-like and quite unlike those of elongate cnidarians (Okamura et al. 2002; Jiménez-Guri et al. 2007; Evans et al. 2010). Most cnidarians move through retraction and peristalsis (Pickens 1988), while *Buddenbrockia plumatellae* sinuous body movements are more similar to those of nematodes and nematophorms (Okamura et al. 2002). Although some cnidarians, such as Stauromedusae, also possess blocks of longitudinal muscles, they are not vermiform (Jiménez-Guri et al. 2007). Bilaterian-like Hox genes characterized in this species also supported its placement in the Bilateria (Anderson et al. 1998, *in*: Jiménez-Guri et al. 2007), although such reports were later contradicted (Jiménez-Guri et al. 2007), as previously mentioned. The triploblastic organization of this parasite remains considered evidence that Myxozoa are related to Bilateria (Smothers et al. 1994; Katayama et al. 1995; Hanelt et al. 1996; Schlegel et al. 1996, *in*: Zrzavý 2001; Kim et al. 1999; Zrzavý and Hypša 2003; Canning and Okamura 2004). On the other hand, *Buddenbrockia* resemblance to bilaterian vermiforms is contradicted by several other characteristics that suggest its placement in Cnidaria. For instance, *Buddenbrockia* has polar capsules resembling the cnidarian nematocysts. Ultrastructural studies report that the four blocks of

longitudinal muscles in this species are, in fact, radially distributed (Okamura et al. 2002) not bilaterally, making *Buddenbrockia* a tetradial worm with one axis of symmetry (Jiménez-Guri et al. 2007). In the same manner, many molecular biology studies support a phylogenetic relationship between *Buddenbrockia plumatellae* and Cnidaria. Jiménez-Guri (2007) published an article in which this subject was targeted through several methodologies. In one of the studies, 129 proteins (29,773 unambiguously aligned amino acid positions) were aligned from *Buddenbrockia* and several other groups of species, including cnidarians, poriferans, ecdysozoans, lophotrochozoans, and deuterostomes, chosen from the basis of the shortest branch lengths of each taxon. The results placed *Buddenbrockia* within the clade Medusozoa, along with Hydrozoa and Scyphozoa, excluding Anthozoa. Therefore, the species would be a cnidarian that during its evolution lost the opening to the gastrovascular cavity and, subsequently, acquired a hydrostatic skeleton. Consequently, such results support the hypothesis that Myxozoa are also within this taxon, on the medusozoan lineage (Jiménez-Guri et al. 2007).

Another hypothesis considers a common ancestor to cnidarians and bilaterians that would have possessed bilateral symmetry and muscular worm shaped body plan (Matus et al. 2006). The controversy of *Buddenbrockia plumatellae* in molecular phylogenetic analysis is probably the result of the genes rapid sequence evolution, causing the appearance of artifactual groupings as well as offering less support to correct groupings (Sanderson and Shaffer 2002). Also contributing to this controversy is the lack of clear cleavage stages in its highly aberrant development and sacculogenesis (Morris and Adams 2007; Canning et al. 2008).

In reality, despite the use of different and innovating technologies, authors remain conflictuous when it comes to resolving the phylogenetic position of Myxozoa (Morris and Adams 2007). Not only due to a paucity in morphological characters but also to the contradictions in biological molecular data, which support both hypotheses, perhaps as a consequence of the highly divergent long-branch rDNA sequences of myxozoans. Missing data, different model choice and inference methods also have an effect in placing highly divergent taxa (Evans et al. 2010). Future studies must include comparative developmental studies and further phylogenetic analyses of a wider range of genes (Morris and Adams 2007). Nevertheless, molecular analysis of 18S rDNA allowed the resolution of many phylogenetic and life cycle questions within this taxon and, consequently, the acquisition of new knowledge concerning myxozoan phylogeny and metazoan affinities important for the study of an early metazoan evolution, as well as for the design of efficient intervention methods in the case of pathogens (Kent et al. 2001; Fiala and Bartošová 2010).

1.2. Class Myxosporea Bütschli, 1881

1.2.1. Taxonomy

Myxosporea were first discovered by Jurine (1825) in the early 19th century, infecting the musculature of a fish host, primarily described by Müller (1841) and classified by Otto Bütschli (1881) as the subclasse Myxosporidia of the then class Sporozoa, along with Sarcosporida (Lom and Dyková 2006). The subsequent taxonomic changes would later determine Myxosporea as a class of the phylum Myxozoa, together with the class Malacosporea. Nowadays, Myxosporea comprises the overwhelming majority of myxozoan species, with about 2180 myxosporean species assigned to about 62 genera (Lom and Dyková 2006). New species are frequently added (Azevedo et al. 2009).

Initially, Malacosporea did not exist and the other class in this phylum was Actinosporea Noble, 1980. For many years the actinosporean stage was not viewed as a sexual developmental stage of the complex life cycle of myxosporeans. In fact, it was not considered a life cycle stage at all, but a completely different class, within the same phylum, named class Actinosporea. The discoveries of Wolf and Markiw (1984) demonstrated that the actinospore is, as mentioned, a stage in the myxosporean life cycle, which lead Kent and Lom (1999) to recommend the suppression of the actinosporean class, with its former genera being deemed invalid (except the genus *Tetractinomyxon* from spinculids) and named only in the vernacular using the collective group names to describe actinosporean stages (Lom et al. 1997; Kent and Lom 1999; Kent et al. 2001; Lom and Dyková 2006). These authors stated that although the actinospore represents the definitive stage in the myxosporean life cycle and contains a sexual process, it is not fulcral for taxonomic and nomenclature purposes, since the International Code for Zoological Nomenclature does not require the use of such parameters; thus proposing the stages found in vertebrates as the only basis for species description. They also considered the existence of a primitive sexual process (autogamy) in the myxospore, as well as the existence of an ancestral vertebrate host, based on the myxozoan proximity to *Polypodium hydriforme*. On the other hand, Lester et al. (1999) considered the suppression of almost all the species and genera of the class Actinosporea premature. Instead, they stated the existence of an ancestral invertebrate host, based on the hypothesis that Myxozoa are not related to Cnidaria but to Bilateria, and denied the existence of a sexual process during the myxospore stage. Hallett et al. (1999) referred to the uncertainty in the host alternation for all myxosporean species and to the possibility of direct fish-fish transmission (Diamant 1997) when stating the prematurity of the class suppression. Nevertheless, the class was indeed suppressed,

leaving only one class in the phylum Myxozoa - the class Myxosporea - until the discovery of Malacosporea (Monteiro et al. 2002; Okamura et al. 2002). Nowadays, eighteen collective groups of actinospores are recognized and used to describe the actinosporean stage: Antonactinomyxon, Aurantiactinomyxon, Echinactinomyxon, Guyenotia, Hexactinomyxon, Hungactinomyxon, Neoactinomyxon, Ormieractinomyxon, Pseudotriactinomyxon, Raabeia, Siedleckiella, Synactinomyxon, Endocapsa, Sphaeractinomyxon, Tetractinomyxon, Tetraspora, Triactinomyxon and Unicapsulactinomyxon (Feist 2008; Rangel et al. 2011). Only the last five collective groups are known from the marine environment (Lom and Dyková 2006).

New molecular data on Myxosporea also led to the suppression of many species, genera and even families within this class. Nowadays, the genus *Kudoa* of the family Kudoidae, assembles the species formally belonging to the three different families Hexacapsulidae, Pentacapsulidae and Septemcapsulidae, that included multivalvulids with more than four valves and polar capsules (Whipps et al. 2004). Another example is the former genus *Leptotheca*, which species are now assigned to the genus *Ceratomyxa* in the case of gall bladder infecting species and genus *Sphaerospora* in the case of urinary system infecting species, due to their unclear dissimilarity to these genera. One species was also assigned to the genus *Ellipsomyxa* and another to the genus *Myxobolus* (Gunter and Adlard 2010). Phylogenetic analyses of this class led to the separation of its genera into two major branches: freshwater and marine myxosporeans (Kent et al. 2001; Fiala and Dyková 2004; Fiala 2006; Bartošová et al. 2009; Fiala and Bartošová 2010). Nevertheless, some genera possess species that constitute exceptions to this separation. *Ceratomyxa shasta*, *Parvicapsula minibicornis*, *Chloromyxum leydigi*, *Sphaeromyxa zaharoni*, as well as some *Myxobolus* and *Henneguya* species, constitute those exceptions (Fiala 2006).

1.2.2. Geographical distribution and seasonal variations

Focusing only on myxosporeans and corresponding literature, these species are showed to possess a wide distribution in different geographic areas (Lom and Dyková 1992; Kent et al. 2001; Casal et al. 2009). The lack of knowledge and effective diagnoses procedures unable the acquisition of a more accurate estimate concerning the pattern of myxosporean distribution. Nevertheless, it is clear that parasites nowadays displaying worldwide range were once restricted to specific geographical areas. The spores possess morphological features that allow dispersion, namely in the aquatic environment; including increased spore surface, projections and mucous envelops (Lom and Noble 1984). Also, myxosporeans display the potential to become established in different geographical areas

via the migration or translocation of the host (Hedrick et al. 1990; Pronin et al. 1997; Bartholomew and Reno 2002). This capacity has determined the worldwide dissemination of diseases associated with myxosporeans, namely through the commercialization of live and dead stocks (O'Grodnick 1979; Bartholomew and Reno 2002; Bartholomew et al. 2005). The parasite migration is more successful in monoxenic species, in heteroxenic species when the intermediate host migrates as well, or in cases of low host specificity (Bauer 1991). However, the lack of information relating to the diversity of myxosporean hosts and geographic range make it difficult to arrive at firm conclusions regarding the possible translocation of this species (Feist 2008). The development of the aquaculture industry highly increased the possibility of dissemination of myxosporean species (O'Grodnick 1979; Lom and Dyková 1992; Bartholomew and Reno 2002; Bartholomew et al. 2005), but subsequently stimulated studies on these parasites.

Myxosporeans display seasonal and annual variations of prevalence due to several biological and physical factors. Although the oligochaete host can release actinospores throughout the entire year, most studies report higher rates of release during the spring and summer periods, which have the highest water temperatures (Lom 1987; El-Mansy et al. 1998; Gay et al. 2001; Özer et al. 2002; Oumouna et al. 2003). Consequently, the prevalence of infection is often highest during the autumn and winter periods. Some studies also report inter annual variations of the parasite in the fish host (Awakura et al. 1995; Molnár 1998; Molnár and Székely 1999; Pampoulie et al. 2001). Therefore, prevalence of infection of a myxosporean species in a specific geographical area depends on both direct physiological and indirect ecological factors. For instance, benthic fish are usually more susceptible than pelagic fish and young fish more than adult fish (Lom and Dyková 1992).

1.2.3. Ultrastructural description

The spores produced during the myxosporean stage present different shapes and structure according to the species. Spores dimensions range between 10-20 µm, although *Myxidium giganteum* is documented to have spores up to 98 µm (Lom and Dyková 1992; Molnár 2002; Ali et al. 2003; Molnár and Székely 2003; Reimschuessel et al. 2003).

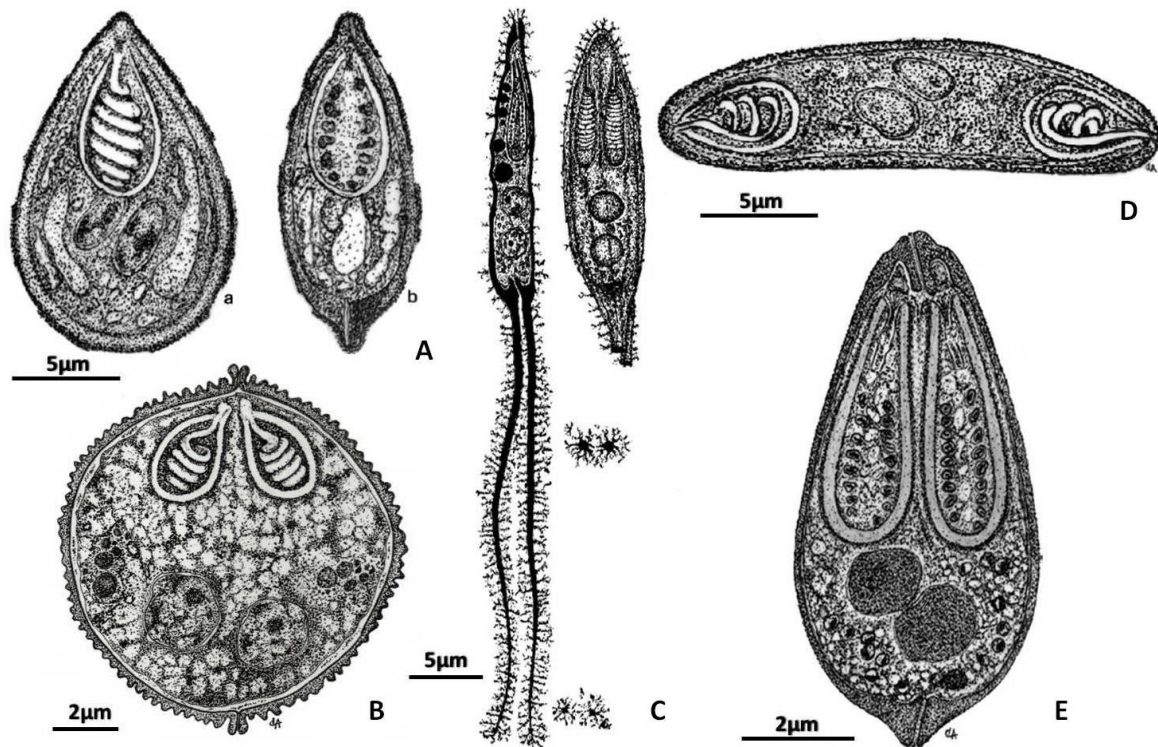


FIGURE 4. Schematic drawings showing the internal organization of some myxosporean spores. **A.** Longitudinal section of the spore of *Thelohanellus rhabdalestus* observed in frontal (a) and lateral (b) view and showing its single polar capsule (courtesy of Azevedo et al. 2011c and Syst. Parasitol.). **B.** Longitudinal section of the spore of *Chloromyxum menticirrhii* in frontal view, showing two of its four polar capsules. Notice the detail on the valvar ridges organization (courtesy of Casal et al. 2009 and Eur. J. Protistol.). **C.** Spore of *Henneguya pilosa*. The internal organization is depicted in longitudinal section (courtesy of Azevedo and Matos 2003 and Folia Parasitol.). **D.** Spore of *Myxidium volitans* displaying fusiform shape and two polar capsules situated at different extremities (courtesy of Azevedo et al. 2011a and Mem. Inst. Oswaldo Cruz, Rio de Janeiro). **E.** Longitudinal section of *Myxobolus sciades* in frontal valvar view (courtesy of Azevedo et al. 2010 and Mem. Inst. Oswaldo Cruz, Rio de Janeiro).

The spore shell is hard and constituted by two to seven shell valves aligned together along a suture line and composed by nonkeratinous proteins. The valves can present a smooth or ridged surface, have several projections, a secreted caudal appendage or even a mucous envelop. The latter often disappears after the spore is released from its host. Studies reveal that the spores are essential for the wide dispersion of the parasitic species and also enhance the probability of ingestion by a new host, since they promote floatability. Within the spores, one to seven polar capsules and one binucleate or two uninucleate sporoplasms (the actual infective germ) can be observed (Lom and Dyková 1992). In this class, sporoplasms contain sporoplasmosomes, but lack the central lucent invagination known in the class Malacosporea (Lom and Dyková 2006). Also, both *Myxobolus* and *Henneguya* present circular inclusions in binucleate sporoplasms (O'Grodnick 1979). The inclusions are named iodophilous vacuoles, constituting polysaccharide reserves in the form of β -glycogen particles, which normally disappear a

few days after the spore is released from the host. Polar capsules are composed by a capsular wall, a polar filament contiguous with the wall and a “stopper” of unknown composition that covers the lumen of the inverted filament.

The capsule wall is very thick and when observed under the electron microscope presents two layers: the inner is electron lucent and resistant to alkaline hydrolysis and the outer is of protein nature. Both layers continue into the polar filament wall. The polar filament is a hollow and terminally closed tube, coiled spirally along each capsule inner wall. This structure is capable of rapid extrusion and, when everted, serves fundamental purposes: attaching the spore to the host and contributing to the separation of the shell valves as well as to the release of the sporoplasm. Extrusion occurs through a cap-like structure located at the apical end of the polar capsule. The cap actually works as a “stopper”, allowing the polar filament extrusion only when digested in the host digestive tract.

Two explanations are considered concerning the discharge mechanism. The first considers that during

capsulogenesis energy is stored; creating an inner pressure that is released when the polar filament everts. The second considers extrusion to be an active calcium-dependent process mediated by proteins (Lom and Dyková 1992; Cannon and Wagner 2003). There are several works exploring the biological, physical and chemical conditions mediating or affecting this process (Hoffman et al. 1965; Yokoyama et al. 1995; El-Matbouli et al. 1999; Wagner et al. 2002b; Kallert et al. 2007).

The great morphological diversity found in the myxospores is less evident in the actinospores, which are usually defined as possessing triradial symmetry, with 3 valves, 3 polar capsules and sometimes caudal projections (Lom and Dyková 1992, 2006). Although actinospores and myxospores are structurally different, some aspects are quite similar. For instance, the polar capsules of the myxospores and the actinospores are very much alike, except in the cap-like structure. In the actinospore, the “stopper” is a granular cone sometimes covered with microtubules that in turn cover the capsulogenic cell membrane and stick into the aperture between the sutural edges. In the myxospore, the extrusion channel is filled with a projection. Lom and Dyková (1992) assume such differences as evidence of the necessity of distinct stimuli in each stage. Also interesting is the fact that a single actinosporean genotype may display two different phenotypes in

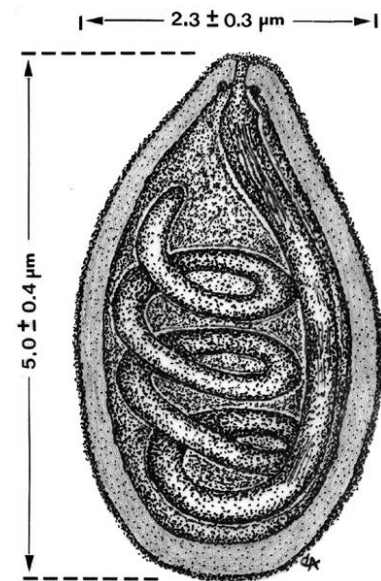


FIGURE 5. Schematic drawing of the polar capsule of *Myxidium volitans* in longitudinal section (courtesy of Azevedo et al. 2011a and Mem. Inst. Oswaldo Cruz, Rio de Janeiro).

the same oligochaete host, possibly distinct designs intended for different fish hosts (Hallett et al. 2002; Holzer et al. 2004; Eszterbauer et al. 2006).

Vegetative stages may be coelozoic or histozoic. Coelozoic species have presporogonic development inside the organs or body cavities, and appear attached to the walls or floating freely in interstitial fluid. Histozoic species may have presporogonic development intra or intercellularly and are considered more evolved than coelozoic species. However, the same parasite can be coelozoic in one host species and histozoic in another host species. In the same manner, some studies describing the complete life cycle of a myxosporean species, report it as coelozoic in one of the life cycle stages and as histozoic in the other. For instance, in the brackish shallow areas of Denmark, *Ellipsomyxa gobii* infects the gall bladder, hepatic and bile ducts of *Pomatochistus microps* during the myxosporean stage, but is found between the musculature of *Nereis spp.* during the actinosporean stage (Lom 1987; Lom and Dyková 1992; Køie et al. 2004). The vegetative stages that occur during the myxospores development vary greatly in shape, structure and dimension. Plasmodia contain several vegetative nuclei and several to many secondary cells, named generative cells, since they are able to produce the spores that eventually initiate a new generation of parasites. Vegetative and generative nuclei are distinguished based on their size, being larger or smaller, according to the species. Also, vegetative nuclei are tetraploid and generative nuclei are diploid. Some plasmodia attain large dimensions, up to several millimetres, thus producing a considerably amount of spores. These type of plasmodia, when histozoic, form cysts by ensheathing in the cellular connective tissue. Other plasmodia are very small and may pervade the host tissues by diffuse infiltration. Histozoic plasmodia are immobile in the tissues, while coelozoic plasmodia may display moving peripheral cellular extensions, (Lom 1987; Lom and Dyková 1992; Molnár 2002; Ali et al. 2003; Molnár and Székely 2003; Reimschuessel et al. 2003).

1.2.4. Life cycle

The first description of the myxosporean life cycle was made by Wolf and Markiw (1984). According to their report, the life cycle of Myxosporea develops in two different hosts, correspondent to two life cycle stages: the myxosporean stage and the actinosporean stage (Wolf and Markiw 1984; Lom and Dyková 1992, 2006; Kent et al. 2001). Their conclusions were based on the existence of two different life cycle stages for *Myxobolus cerebralis*: an actinosporean stage in a tubificid oligochaete (*Tubifex tubifex*) and a myxosporean stage in a salmonid fish; thus allowing the union of what were previously

considered parasites of two separate classes (Myxosporea and Actinosporea) of the phylum Myxozoa (Wolf et al. 1986; El-Matbouli and Hoffmann 1989).

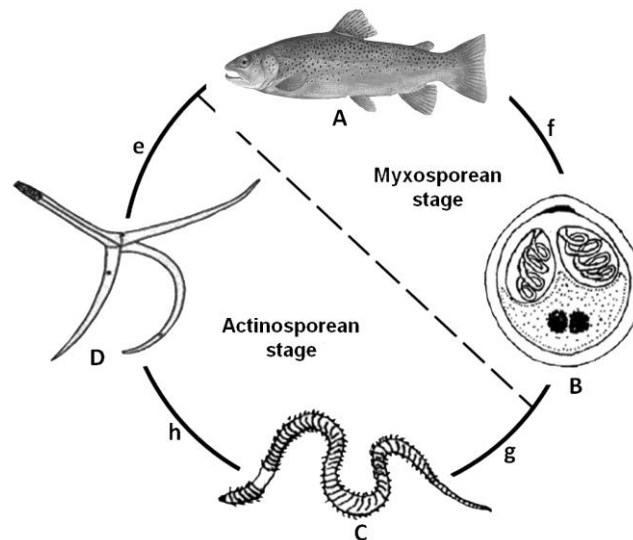


FIGURE 6. Diagram of the life cycle of *Myxobolus cerebralis*. The myxosporean stage development occurs in the salmonid host (A) and culminates in the release (f) of the myxosporean spores (B), which sink to the bottom of the water column (g) and are ingested by the oligochaete host *Tubifex tubifex* (C). The actinosporean stage development takes place (h) and produces the triactinomyxon spores (D) that are water-borne and infective (e) towards the salmonid fish host (adapted from Hedrick et al. 1998).

Although there was initial disbelief in such findings, they were later confirmed by analysis of the 18S ribosomal RNA sequences of the alternate stages in *Myxobolus cerebralis* (Andree et al. 1997). Unfortunately, few myxosporean species have been coupled to their corresponding actinosporean stages (Kent et al. 1996). Also, the few known actinosporean stages are remarkably outnumbered by the known myxosporean stages, especially in the marine environment (Lom 1987). Molecular studies may allow this area of research to develop more (Andree et al. 1997, 1999; Kent et al. 2001). The terms actinospore and myxospore are used to distinguish between the spore stages observed in the invertebrate and vertebrate hosts, respectively, as suggested by Lom et al. (1997).

The actinosporean stage takes place in the definitive host, usually an invertebrate species, namely annelids and more rarely sipunculids, resulting in the production of actinospores through a sexual process. The actinospores from polychaetes and sipunculids are all of the tetractinomyxum type (Ikeda 1912; Hallett et al. 1999; Køie et al. 2004). Triactinomyxons described from marine (Roubal et al. 1997) and freshwater species (El-Mansy and Molnár 1997), probably belong to genera with members in both these environments (e.g. *Myxidium* and *Myxobolus*) (Køie et al. 2004). The myxosporean stage takes place in the temporary host, usually lower vertebrates such as fishes, sometimes

amphibians and reptiles and, extremely rarely, birds and mammals, resulting in the production of myxospores. In this stage, cell-in-cell organization is common, when the endogenously formed cell persists within the original cell (Lom and Dyková 2006; Morris 2010). Previously to the discovery of sexual reproduction in *Tubifex tubifex*, the vertebrate host was insubstantially believed to be the definitive host (Gilbert and Granath 2003). Lom and Dyková (1992) described the differences between the parasites development in the actinosporean and myxosporean host. They pointed out that the gross differences found between the mature spores produced in these stages are misleading, since light and electron microscopic observations of the cell structure demonstrate them as more or less identical. Differences in the appearance of the spores can be attributed to their adaptation to different life styles and hosts. The actinosporean stage is short-lived and planktonic, while the myxosporean stage is long-lived and bentonic (El-Matbouli and Hoffmann 1998). In both the actinosporean and myxosporean host, the spores development is dependent on environmental factors, namely water temperature. Consequently, incubation periods vary according to this parameter (Wolf and Markiw 1985; Markiw 1992b; Blazer et al. 2003; Golomazou et al. 2009; Estensoro et al. 2010). Markiw (1992b) reported that the developmental period of the spores of *Myxobolus cerebralis* could be shortened or lengthened by recurring to temperatures above or below 12.5 °C, respectively. The mechanisms trough which environmental factors influence infection rates and parasite development are not completely clear, as well as other factors also mediating these processes (Hallett et al. 1997; Molnár and Székely 1999; Blazer et al. 2003; Golomazou et al. 2009).

Up to now, more than two thousand myxosporean species have been described, but for only a fraction of these has the life cycle been elucidated (Køie et al. 2004).

1.2.4.1. *The actinosporean stage*

The actinosporean stage development generally follows the same pattern, independently of the site of infection and host species (Ikeda 1912; El-Matbouli and Hoffmann 1998; Lom and Dyková 2006; Meaders and Hendrickson 2009; Rangel et al. 2009, 2011). This stage is described as a succession of three processes: schizogony, gametogony and sporogony (Lom and Dyková 1992; El-Matbouli and Hoffmann 1998; Kent et al. 2001). Schizogony initiates when the myxospores, released by the vertebrate host, are ingested by the annelid host. In the lumen of the annelid gut, the myxospores extrude their polar filaments, anchoring themselves to the gut epithelium. Subsequently, the shell-valves open along the suture line, allowing the binucleate sporoplasm to penetrate between the host cells. Both diploid nuclei undergo several divisions, given rise to two multinucleate cells, which

in turn suffer plasmotomy in order to produce numerous uninucleate cells. These new cells now follow one of two paths: undergo new divisions thus producing additional multinucleate and uninucleate cells or fuse to form binucleate cells that will engage in gametogony. Considering the latter path, the nuclei in the binucleate cell divide (karyogamy) forming a cell with four nuclei. Plasmotomy occurs to form four uninucleate cells; thus producing the pansporocyst, constituted by two enveloping somatic cells and two generative cells, named α and β . The latter suffer three mitotic divisions and one meiotic division. Mitosis repeated three times produces 8 α - and 8 β -diploid gametocytes, which through meiotic division produce 16 haploid gametocytes and 16 polar bodies. Polar bodies are expelled. At the end of gametogony, each gametocyte from de α line unites with another gametocyte from the β line to produce eight zygotes inside the pansporocyst. The somatic cells also divide twice, giving rise to eight surrounding cells. Sporogony begins with each of the 8 zygotes undergoing two mitotic divisions to produce 8 diploid four-cell stages. Three cells are located peripherally and one centrally. Each of the three peripheral cells divides into one valvogenic and one capsulogenic cell. The fourth cell first undergoes endogeneous cleavage, producing an inner cell (sporoplasm germ) within the enveloping vegetative cell. The sporoblast is formed (Lom and Dyková 1992, 2006; El-Matbouli and Hoffmann 1998; Morris 2010; Rangel et al. 2011). Mitotic divisions of the inner cell give rise to a specific number of sporoplasm germs. Valvogenic cells grow thinner and spread to adhere together, completely surrounding the capsulogenic cells and a portion of the sporoplasm. The sporoplasm remains naked in the pansporocyst until reaching the final number of germs (64 in *Myxobolus cerebralis*) (El-Matbouli and Hoffmann 1998). The capsulogenic cells are constituted by a cylindrical microtubule formation surrounded by rough endoplasmic reticulum and some mitochondria. Together, these structures form a club-shaped form externally lined with microtubules, termed polar capsule primordium. The base of the club assumes a rounded shape and the narrow end of the apex begins to grow an elongated coiled tube – the polar filament. In the apex of the polar capsule, a cap-like plug formed from a granular dense substance lined with microtubules and covered by the cell membrane of the capsulogenic cell, covers the mouth of the polar capsule (El-Matbouli and Hoffmann 1998; Rangel et al. 2011). The nucleus of the capsulogenic cell often remains visible at bottom of the polar capsules.

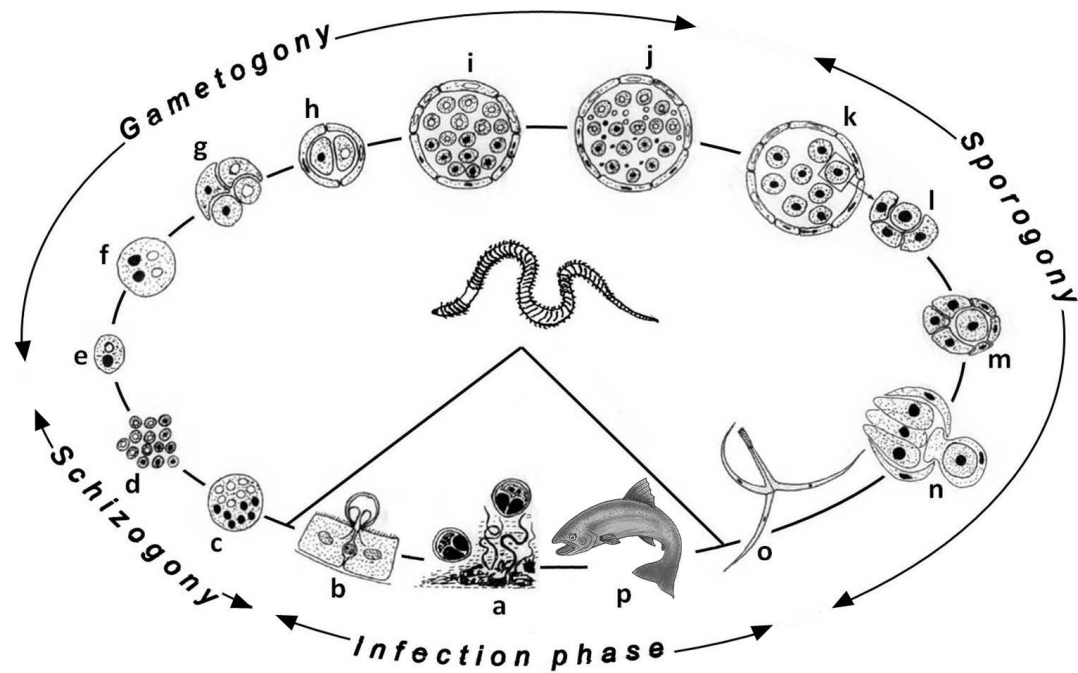


FIGURE 7. Diagram of the actinosporean development of *Myxobolus cerebralis* in the gut epithelial cells of *Tubifex tubifex*. (a) *Tubifex tubifex* ingests the myxospores of *Myxobolus cerebralis*. (b) The polar filament extrudes and anchors the parasite to the gut wall, allowing the binucleate sporoplasm to penetrate between the epithelial cells, when the shell valves open. (c) Interepithelial schizogonic multiplication of the binucleate sporoplasm. (d) Uninucleate one-cell stages. (e) Two uninucleate cells fuse to produce one binucleate cell stage. (f) Mitotic division of both nuclei to produce four-nuclei stage. (g) Plasmotomy occurs to form a four-cell stage, in which two of the four cells begin to envelope the other two cells. (h) The pansporocyst is formed by two somatic cells and two generative cells. (i) Generative cells undergo three mitotic divisions and somatic cells undergo two mitotic divisions, producing 16 diploid gametocytes (8 α and 8 β) enveloped by 8 somatic cells. (j) Meiotic division of the 16 diploid gametocytes produces 16 haploid gametocytes and 16 polar bodies. (k) Copulation of α - and β - gametes produces eight diploid zygotes. (l) The zygote undergoes two mitotic divisions to produce three peripheral cells and an inner cell, thus forming the sporoblast. (m) Three valvogenic and three capsulogenic cells are produced through mitotic division of the three peripheral cells. (n) The valvogenic cells surround the capsulogenic cells, while internal cleavage of the developing sporoplasm cell produces one generative cell enveloped by one somatic cell. The sporoplasm remains naked in the pansporocyst until reaching the final number of germs through several mitotic divisions. (o) Inflated mature triactinomyxon spore. (p) The triactinomyxon infects a salmonid fish and initiates the myxosporean stage development, which produces the myxospores infective towards *Tubifex tubifex* (adapted from El-Matbouli and Hoffmann 1998).

These developmental stages lead to the formation of eight actinospores in each pansporocyst. The morphology of the actinospore varies according to the species, but most possess an anterior spore body that contains three capsules and three shell valves, leaving an opening for the polar capsules apex. In the posterior part of the spore body, the three shell valves extend into very long, hollow and mutually divergent three caudal projections, which occur in most of the actinosporean stages. When the actinospore is released from the host, those projections fill with water from the surrounding area, due to the process of osmosis. In some species, they fuse rather than diverging, forming a style (Lom and Dyková 2006; Rangel et al. 2011).

1.2.4.2. The myxosporean stage

For the myxosporean stage, two developmental processes are considered: presporogonic development and sporogony (Lom 1987; Kent et al. 2001; Lom and Dyková 2006). Presporogonic development is also referred to as extrasporogonic development (Lom 1987). The first takes place when the actinospore, discharged from the annelids gut into the water, comes in contact with the intermediate host epidermis (El-Matbouli and Hoffmann 1989; El-Matbouli et al. 1999). Contact established, the actinospore will then extrude the polar filaments from their polar capsules, anchoring the spore to the host skin. The spore shell valves open, allowing the sporoplasm to exit the spore and entry the host body through the openings of the epidermal and epithelial mucous cells (El-Matbouli et al. 1999; Belem and Pote 2001; Kallert et al. 2007). The next processes occurring in the sporoplasm are presporogonic stages and may be intra or/and intercellular. The sporoplasm undergoes an endogenous cleavage and, as a result, a secondary cell is formed within what is now, the primary cell. The secondary cell suffers numerous mitotic divisions, forming a parasitic aggregate that compresses the host cell nucleus against the plasmalemma of that same cell (El-Matbouli et al. 1995; Kent et al. 2001). When the mitotic divisions are over, the secondary cells will then undergo an endogenous division, forming cell-doublets with an enveloping (secondary cell) and an inner cell (tertiary cell). The cell-doublets rupture first the primary cell and then the host cell, becoming free in the extracellular space and allowing the infection to go deeper or spread through the host body, perhaps repeating the cycle. The release of the cell-doublets marks the beginning of the sporogony (Molnár and Kovács-Gayer 1986; Lom 1987; Lom and Dyková 1992, 2006; Sitjà-Bobadilla and Alvarez-Pellitero 1993b; Morris 2010).

Reached the sporogonic site, this stage initiates with the development of a plasmodium or a pseudoplasmodium, considered the vegetative stages or trophozoites of myxosporean species. As previously mentioned, the plasmodium may be histozoic (often appearing as cysts) or coelozoic (mainly infecting the urinary tract or gall bladder), as well as polysporic (produces many spores), disporic (produces two spores) or monosporic (produces one spore). Species with small monosporic or disporic plasmodia, which produce only one or two spores are considered not to have true plasmodia, but pseudoplasmodia instead. One example is *Sphaerospora truttae*, which asynchronous sporogonic development occurs in disporous pseudoplasmodia (Holzer et al. 2003). Pseudoplasmodia are smaller and contain only one vegetative nucleus. In these cases, generative cells will proliferate and aggregate to form a sporoblast and later produce spores. Some generative cells also

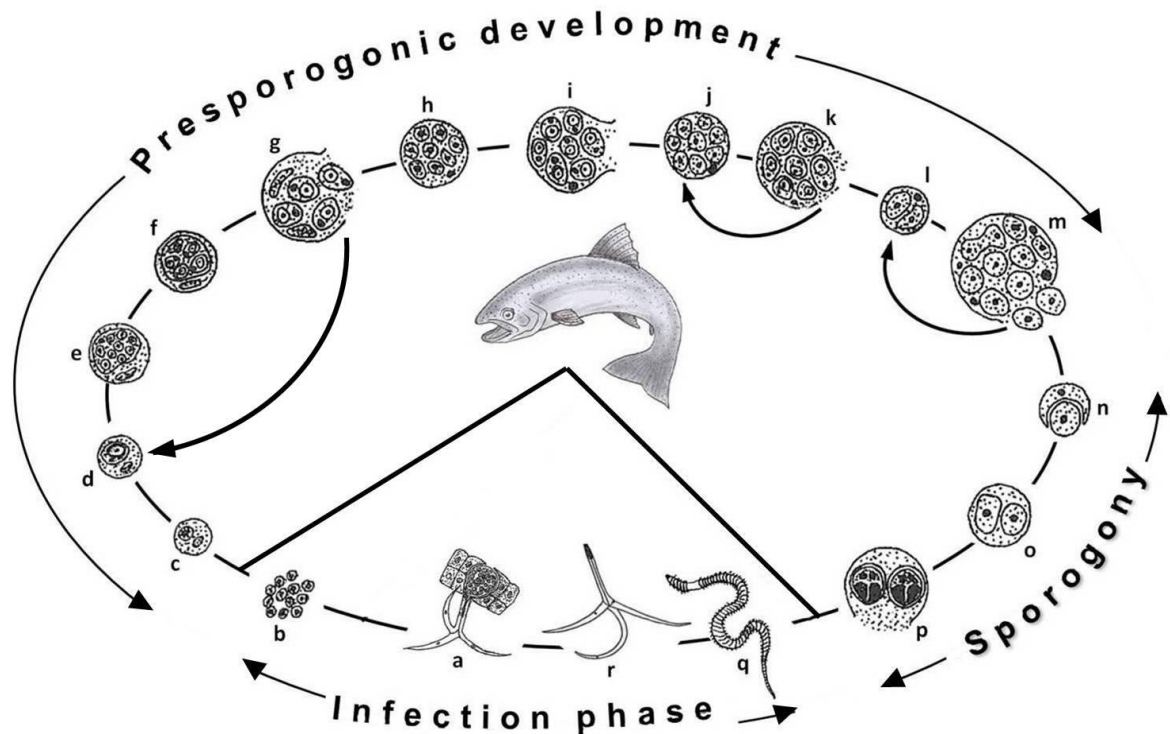


FIGURE 8. Diagram of the myxosporean development of *Myxobolus cerebralis* in the salmonid fish host. **(a)** The triactinomyxon spores contact the salmonid fish host epidermis and gill epithelium. **(b)** The sporoplasms are released. **(c)** 60 minutes post-penetration, the sporoplasms migrate intercellularly. **(d)** The sporoplasm undergoes an endogenous cleavage and, as a result, a secondary cell is formed within what is now, the primary cell. **(e)** Numerous rapid mitotic divisions of the secondary cell lead to the formation of a parasitic aggregate that compresses the nucleus of the primary cell. **(f)** The secondary cells undergo endogenous divisions to produce new cell-doublets with an enveloping and an inner cell (tertiary cell). **(g)** The cell-doublets rupture the membrane of the original primary cell and enter the host cell cytoplasm to migrate to the extracellular space, becoming able to infect new host cells. **(h)** Shortly after exposure the infection is in the subcutis and the cycle is repeated. Endogenous cleavage again forms secondary cells. **(i)** New cell-doublets with an enveloping secondary cell and tertiary inner cell are produced through mitotic divisions. **(j)** and **(k)** Repeating the presporogonic stages allows the parasite to migrate intercellularly in the nervous tissue. **(l)** The cell-doublet is released in the sporogonic site. **(m)** Plasmodia are formed. The primary cell grows and its nucleus divides to produce numerous internal vegetative nuclei. The enveloped cell divides to produce numerous generative cells. Rupture of the primary cell releases the enveloped cells, which may repeat this stage to form new plasmodia or initiate sporogony. **(n)** The enveloped cell unites with another cell thus forming an inner cell, named sporogonic cell, within an enveloping cell, named pericyte. Sporogony is initiated. **(o)** The initial pansporoblast is formed. **(p)** Pansporoblast containing two myxosporean spores of *Myxobolus cerebralis*. **(q)** After release into the water, the spores sink and are ingested by the oligochaete host. **(r)** The actinosporean stage culminates in the production of the triactinomyxon spores that are infective towards the salmonid fish, thus beginning the myxosporean development as described (adapted from Kent et al. 2001).

suffer endogenous division, originating tertiary cells within the original generative cell (Morris 2010). Plasmotomy may also take place at this point of development, increasing the number of presporogonic stages. Mictosporic species with monosporic, disporic or polysporic plasmodia, which produce one, two or several spores, are common in coelozoic species. Polysporic plasmodia, which are very large and contain both many nuclei and generative cells, can occur in coelozoic or histozoic species. Coelozoic

plasmodia divide according to three different processes: plasmotomy, endogenous and exogenous budding. Some species actually possess all of these types. Endogenous budding initiates with the formation of several inner buds and terminates with the release of those same structures as the plasmodium falls apart. Exogenous budding occurs when a portion of the plasmodium cytoplasm is cleaved, separating with several nuclei and generative cells (Lom and Dyková 1992, 2006; Morris 2010). Also, it is important to mention that the generative cells of coelozoic plasmodia often have very well developed pseudopodia, exhibiting slow amoeboid movements. Other cells, large and amoeboid, have been observed and termed lobocytes. The function of lobocytes remains unclear since its discovery, but they are claimed to ingest generative cells and sporoblasts. Villosities can be observed at the plasmodia cell membrane, promoting nutrient uptake (Sitjà-Bobadilla and Alvarez-Pellitero 1993b, 2001; Canning et al. 1999). Sporogony is not synchronized, resulting in simultaneous development of early and advanced stages. Histo zoic plasmodia are commonly within the tissues and, contrarily to coelozoic plasmodia, do not divide. The lack of division processes is compensated with their capacity for growth. Also, contrarily to coelozoic plasmodia, the cell membrane of the plasmodium is not covered by villosities. Instead, many minuscule invaginations and pinocytotic vesicles serve the purpose of nutrient uptake (Current 1979; Current et al. 1979; Cho et al. 2004; Azevedo et al. 2011b). In these species, plasmodia appear within a large fibroblast envelope, forming macroscopic structures named cysts. Although the cysts encase spores at different stages of sporogonic development, sporogony is a more or less synchronized process, so that all the spores mature at the same time. Nevertheless, some species, such as *Sphaerospora truttae*, are reported to possess nonsynchronous sporogony with undifferentiated early sporogonic stages appearing alongside mature spores (Holzer et al. 2003).

Independent to the type, plasmodial development is, ultimately, a more or less similar process, in which the plasmodium results from the primary cell growth and its nucleous division to produce numerous vegetative nuclei. The enveloped cell divides leading to the formation of numerous generative cells that may undergo two paths: repeating the cycle thus forming a new plasmodium (Diamant 1997), or unite with another cell thus forming an inner cell, named sporogonic cell, within an enveloping cell, named pericyte. The last mentioned option initiates sporogony, a stage during which spores are formed directly or through the production of pansporoblasts (Lom 1987; Lom and Dyková 1992; El-Matbouli et al. 1995).

The first - spores formed directly - is less frequent, occurring in the pseudoplasmodia of some genera. The pseudoplasmodium is uninucleate and sporogony begins with the

formation of a sufficient number of cells to compose one to two spores and continues while these same cells assume their predetermined role. The second - spores formed in pansporoblasts - occurs in large plasmodia. Both the pericyte and its enclosed sporogonic cell maintain their cell membranes, so that the latter appears enveloped in a tightly fitted vacuole. The pericyte will then divide in order to produce two cells of the pansporoblast envelope, responsible by nutrient mediation between host and sporogonic cell. The latter undergoes binary fission, giving rise to three different types of sporogonic cells: valvogenic, capsulogenic and sporoplasm. The pericyte containing the sporogonic cell progeny is the so called pansporoblast. Pansporoblasts may be monosporic or more frequently, disporic. Corresponding numbers of the sporogonic derived cells develop into two sporoblasts that later mature into two myxospores. Valvogenic cells develop into the shell valves; capsulogenic cells into the polar capsules and the sporoplasm matures according to the species. Capsulogenesis is homologous in the actinosporean and myxosporean development, with both primordia originating from dilated cisterna of rough endoplasmic reticulum, before assuming a club-like shape (Lom and Puytorac 1965; Lom and Vávra 1965; El-Matbouli et al. 1990; Ali et al. 2003). The events occurring during sporogony are detailed for *Fabespora vermicola* in Weidner and Overstreet (1979).

It remains unknown how sporogonic cells, having the same origin, differentiate into distinct cells. Also important to refer is the persistence of presporogonic development in the intermediate host, even after the formation of myxospores, thus magnifying proliferation (Lom 1987; Lom and Dyková 2006). Lom (1987) and Lom and Dyková (1992) consider the existence of life cycle abortive sequences occurring during sporogenesis and presporogonic development.

1.2.5. Hosts

For a long time, Myxosporea were regarded exclusively as parasites of poikilothermic vertebrates and invertebrates, with body temperatures within a few degrees of the environment. Nowadays, there are several reports proving that such statement is erroneous (Friedrich et al. 2000; Garner et al. 2005; Jirků et al. 2006; Dyková et al. 2007; Prunesco et al. 2007; Bartholomew et al. 2008). Nevertheless, it is true that both freshwater and marine fishes are the commonest hosts during the myxosporean stage (Matos et al. 2005; Bartholomew et al. 2008), with about 3 species reported from Agnatha, 35 species from Chondrichthyes and the rest from Osteichthyes (Lom and Dyková 1992, 2006). Although many freshwater myxosporeans have their complete life cycle described (El-Mansy and Molnár 1997; Hallett et al. 1998; Kent et al. 2001; Lom and Dyková 2006), little is known about the heteroxenous life cycle of marine species (Diamant 1997; Hallett

et al. 1998, 1999; Køie 2002; Køie et al. 2004, 2007, 2008; Rangel et al. 2009, 2011). Approximately 34 myxosporean species have their complete life cycle described from freshwater fishes (Bartholomew et al. 1997; Székely et al. 1998; Holzer et al. 2006; Lom and Dyková 2006; Caffara et al. 2009), while only 4 species have been completely described from marine fishes, all of which possessing a polychaete as the invertebrate host (Køie et al. 2004, 2007, 2008; Rangel et al. 2009). However, there are some species reported to have both marine and freshwater life cycles, but little is known about the actinosporean stages in these cases. Diamant et al. (2006) studied the possibility of a myxosporean species infecting marine fishes, *Enteromyxum leei*, infecting freshwater fishes as well. He experimentally fed *Sparus aurata* gut tissue infected with this parasite to 17 freshwater species and verified that the specimens of four of those species became infected with *Enteromyxum leei*. The prevalence of infection, as well as its location and pathology, were similar to that observed in marine hosts. Normally, when the parasite is ingested it encounters many physiological barriers, namely of the gastrointestinal and immunological system (Chevassus and Dorson 1990; Feist 2008). In this case, there is also an osmotic barrier that is surpassed in 4 of the experimentally infected freshwater fish, which is interpreted as a sign that the osmotic environment within the alimentary tract is not highly divergent between marine and freshwater fishes. Actually, the myxosporean species infecting migratory fish most probably are adapted to the osmotic variability of different environments (Higgins et al. 1993; Moran et al. 1999b). Also, Buddington and Krogh (2004) report that a relatively steady osmotic pressure is maintained between the freshwater and marine clades of teleosts, through neural and hormonal regulatory mechanisms. Therefore, the true barrier for the other 13 species not displaying infection by *Enteromyxum leei* probably results of genetic predispositions or differing anatomical or physiological gastric and immune conditions. Also, the natural host of *Enteromyxum leei* remains unknown and a possibly freshwater origin must not be ruled out (Diamant et al. 2006).

Less frequently, other poikilothermic vertebrates such as amphibians and reptiles are also parasitized (Azevedo et al. 2005; Bartholomew et al. 2008), with about 13 species reported from amphibians and 6 species reported from reptiles (Lom and Dyková 2006), and belonging to the genera *Myxobolus*, *Myxidium*, *Hoferellus*, *Chloromyxum*, *Caudomyxum* and *Sphaerospora* (Eiras 2005). For these non-fish infecting myxosporeans, there is no data concerning their actinosporean stage (Lom and Dyková 2006). Also, host specificity appears to be the exception rather than the rule in these cases (Eiras 2005), remaining unclear if they have broad-host specificity or comprise an assemblage of species (Jirků et al. 2006).

Myxosporean species exceptionally appear in birds and mammals, providing evidence that these parasites may occur in homoeothermic animals and that temperature may not be a barrier in host-switching (Friedrich et al. 2000; Dyková et al. 2007; Prunescu et al. 2007; Bartholomew et al. 2008). A possible explanation is the capability of some warm-water fishes in tolerating high water temperatures. Bartholomew et al. (2008) reported the observation of a myxosporean species infecting the liver and bile ducts of North American waterfowl, namely 6 species of ducks from 5 different locations. Upon combined morphological and molecular research of both developmental stages and mature spores, the myxosporean proved to be a new species. The parasite was named *Myxidium anatum* and constitutes the first report of a bird infecting myxosporean species (Bartholomew et al. 2008). Friedrich et al. (2000) observed myxosporean developmental stages forming xenomas (enlarged intracellularly parasitized host cells) in the brain of the mole *Talpa europaea*, constituting the first putative data of a myxozoan species infecting mammals; spores were not found. Few more studies report the occurrence of myxosporean species in birds and mammals, and several of the same are considered incidental or aberrant host records (Bartholomew et al. 2008). In studies where putative myxozoan developmental stages are observed, identification is unconfirmed due to the lack of either mature spores or molecular evidence. In others where spores are observed, developmental stages fail to indicate the true host status (Friedrich et al. 2000; Moncada et al. 2001). However, the data collected by Prunescu et al. (2007) and Dyková et al. (2007) showed otherwise, with both developmental stages and mature spores of a new myxosporean species appearing in a mammal. Their studies provided the first information of a terrestrial mammal containing the several stages of myxosporean development from plasmodia to spores, and it was reported from shrews, *Sorex araneus* (Soricomorpha), whose liver was infected by *Soricimyxum fegati*, a new myxosporean species at the time (Dyková et al. 2007; Prunescu et al. 2007). Prunescu et al. (2007) even postulates that this species possibly infects a soil-dwelling oligochaete during the actinosporean stage, since the aquatic related intermediate hosts infect aquatic annelids as definitive hosts. In this case, a different path of transmission is suggested, with both the myxospores and actinospores being transmitted by peroral infection (Dyková et al. 2007).

There are also reports of myxosporean species, namely from the genus *Myxobolus*, infecting humans infected with the HIV virus or suffering from intestinal disorders (Boreham et al. 1998; Moncada et al. 2001). However, few evidences suggest that the parasite developed in the human host. The spores are highly resistant to environmental conditions, namely the action of the gastrointestinal fluid. Therefore, they are most likely acquired from the contaminated environment (Boreham et al. 1998). In the cases presented by Boreham et al. (1998), it was reported that the infected humans had

previously eaten infected fish. On the other hand, in the case presented by Moncada et al. (2001), the patient had been imprisoned for 6 months and the *Myxobolus* genus had never before been described from Colombian fish species. The pathogenic role of myxosporeans infecting humans remains dubious and in both cases appears unrelated to the clinical symptoms displayed by the subjects (Boreham et al. 1998; Moncada et al. 2001). Such findings possibly infer that, under certain conditions, myxozoans may become opportunistic parasites of homeothermic vertebrates (Canning and Okamura 2004).

Some myxosporean species were also reported to have their myxosporean stage developed in invertebrate hosts (Rajulu and Radha 1966; Weidner and Overstreet 1979; Yokoyama and Masuda 2001; Lom and Dyková 2006). Weidner and Overstreet (1979) reported *Fabespora vermicola* as the only myxosporean species infecting a platyhelminth, more precisely a member of the subclass Digenea, *Crassicutis archosargi*, which in turn infected the sheepshead, *Archosargus probatocephalus*, an estuarine fish of the Mississippi. Yokoyama and Masuda (2001) reported the occurrence of a *Kudoa* in the arm muscles of the North-Pacific giant octopus *Paroctopus dofleini*, which led to the muscle degeneration referred as “post-mortem myoliquefaction”, a result of the activity of the proteolytic enzymes released by the parasite. It is even possible to find reports of myxosporean species infecting insects during the myxosporean stage, but those are doubtful (Lom and Dyková 2006). For instance, the species described as *Symmetrula cochinealis*, was reported from the fat bodies of the insect *Dactilopius indicus* (Rajulu and Radha 1966).

During the actinosporean stage, myxosporean species parasitize invertebrates as definitive hosts. The most common invertebrate hosts are oligochaetes (El-Mansy et al. 1998), namely tubificids, from both the marine and the freshwater environment. Some marine and freshwater polychaetes (Bartholomew et al. 1997; Køie 2002; Køie et al. 2004, 2007, 2008; Rangel et al. 2009) and more rarely sipunculids were also reported to be infected (Ikeda, 1912). Since Wolf and Markiw (1984) description of the myxozoan life cycle, studies aimed mostly at freshwater species that used oligochaetes as invertebrate hosts, making the environment of infection more restrict than in the myxosporean stage (El-Mansy and Molnár 1997; Hallett et al. 1998; Kent et al. 2001; Lom and Dyková 2006). However, some studies suggest that in the marine environment, polychaetes can be the best candidates for invertebrate hosts of Myxozoa (Køie 2002). Four myxosporean species have their life cycle described from marine species, with polychaetes as the actinosporean hosts: *Ellipsomyxa gobii* from *Nereis diversicolor* (Køie et al. 2004); *Gadimyxa atlantica* from *Spirorbis* spp. (Køie et al. 2007); *Ceratomyxa auerbachii* from

Chone infundibuliformes (Køie et al. 2008); and *Zschokkella mugilis* from *Nereis diversicolor* (Rangel et al. 2009). Other species hosted by polychaetes in the marine environment remain unidentified (Køie 2002; Rangel et al. 2011). There are also two myxosporean species with their life cycle described from freshwater polychaetes: *Ceratomyxa shasta* (Bartholomew et al. 1997) and *Parvicapsula minibicornis* (Bartholomew et al. 2006), both from *Manayunkia speciosa*. Other marine actinospores have been reported in oligochaetes (Hallett et al. 1998, 1999).

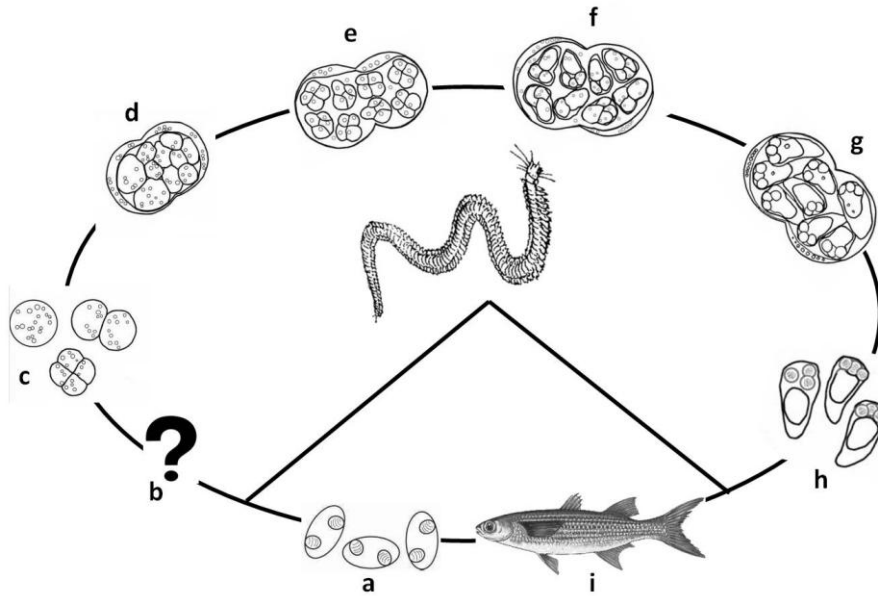


FIGURE 9. Diagram of the actinosporean development of *Zschokkella mugilis* in *Nereis diversicolor*. **(a)** Myxospores of *Z. mugilis*. **(b)** Infection and first stage of the actinosporean development in the host's intestinal epithelium. **(c-h)** Actinosporean development in the host's coelomic cavity. **(c-d)** Gametogony phase. **(e-h)** Sporogony phase. **(h)** Free actinospores. **(i)** The actinospores infect the fish host, in which the myxosporean development occurs to produce the myxospores. The cycle is reinitiated (adapted from Rangel et al. 2009).

Reports of direct transmission between temporary hosts of the same species or even of different species suggest that some myxosporean species may not need an actinosporean development, making the proliferative stages in the myxosporean development responsible for the transmission (Redondo et al. 2004; Diamant et al. 2006; Diamant 1997).

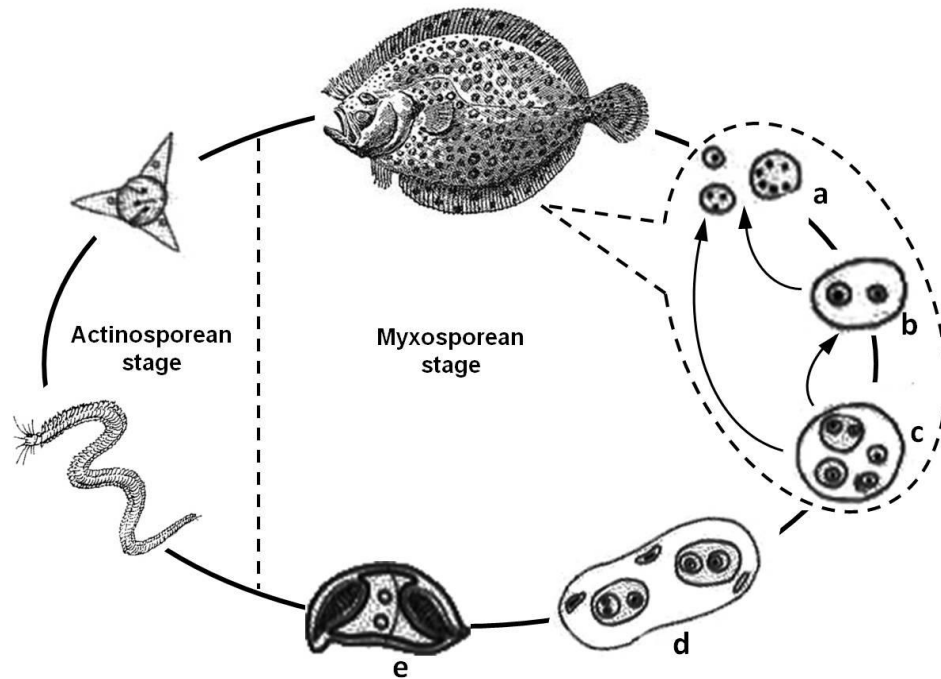


FIGURE 10. Diagram of the hypothetical marine life cycle with fish-to-fish transmission of *Enteromyxum scopthalmi* in turbot *Scophthalmus maximus*. **(a-c)** Proliferative stages responsible for the invasion and proliferation within the fish. **(b-c)** are also the stages responsible for direct transmission to other fish. **(d-e)** Sporogonic stages that continue the life cycle to produce the myxosporean spores infective for the oligochaete host (adapted from Redondo et al. 2004).

Many authors refer host specificity as a form of species determination, but that is not always correct. As previously mentioned, myxosporean species are more likely to have broad-host specificity or comprise an assemblage of host species (Hoffman et al. 1965; Sitjà-Bobadilla and Alvarez-Pellitero 1993b; Diamant et al. 2006; Fiala 2006; Jirků et al. 2006). To be host specific, they could only infect one species in its entire life cycle and that is not the case. Also, host specificity has been experimentally tested in both the actinosporean and the myxosporean stages, with results demonstrating that it is possible for a myxosporean species to infect more than just one specific species (Yokoyama et al. 1995; El-Mansy and Molnár 1997; McGeorge et al. 1997; Özer and Wootten 2002). Yokoyama et al. (1995) reported raabeia-type actinospores of *Myxobolus cultus* responding to various fish mucous as well as bovine submaxillary mucin. McGeorge et al. (1997) and Özer and Wootten (2002) reported polar filament discharge and sporoplasm release of several actinospores to all isolates of mucous belonging to salmon, trout, stickleback and bream. The studies of El-Mansy and Molnár (1997) demonstrated, experimentally, that *Myxobolus hungaricus*, a parasite infecting the gills of the sea bream *Abramis brama*, can infect both *Tubifex tubifex* and *Limnodrilus hoffmeisteri* in the actinosporean stage.

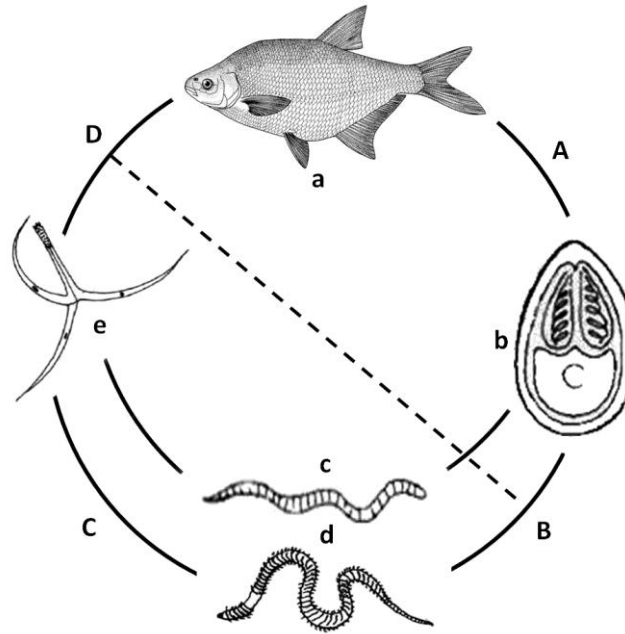


FIGURE 11. Diagram of the life cycle of *Myxobolus hungaricus*. **(a)** Development of the myxospores in the gills of *Abramis brama*. **(A)** The myxospores sink to the bottom of the water column. **(b)** Frontal view of the myxospores of *Myxobolus hungaricus*. **(B)** Ingestion of the myxospores by the oligochaete species. **(c)** Development of the actinospores in *Limnodrilus hoffmeisteri*. **(d)** Development of the actinospores in *Tubifex tubifex*. **(C)** The actinospores are released into the water. **(e)** The waterbourne triactinospores. **(D)** The fish host is infected by contact between the triactinospores at the gills level (adapted from El-Mansy and Molnár 1997).

Chloromyxum fluviatile as been repeatedly reported from the gall bladder of a variety of teleosts, such *Alburnus alburnus*, *Leuciscus cephalus*, *Cyprinus carpio*, *Abramis brama*, among other cyprinids (Lom and Dyková 1993). *Enteromyxum leei*, a common parasite of a wide range of marine fish hosts, was successfully transmitted to freshwater species in the experiments of Diamant et al. (2006). Other studies report the existence of more than one temporary host in the lyfe cycle of some myxosporean species (Weidner and Overstreet 1979; Boreham et al. 1998).

On the contrary, several studies support myxosporean species as specific for a genus or specific groups of fishes, but rarely for just one specific species. *Myxobolus cerebralis* constitutes a case of host specificity for salmonid hosts, although El-Matbouli et al. (1999) postulated that high exposure rates could possibly trigger conditions that allowed the actinospores to penetrate other hosts. Yokoyama et al. (1997) reported *Thelohanellus hovorkai* to distinguish between cyprinid genera. Xiao and Dessler (2000) observed different ratios of sporoplasm release in an array of actinospores to mucous of various fishes, and reported lack of myxosporean development and parasitic developmental stages in non-susceptable species. Tansmission and recognition of hosts is possible trough mechanical and chemical stimuli, namely the abundance of fish mucus in the water (Yokoyama et al. 1993, 1995; El-Matbouli et al. 1999; Özer and Wootten 2002; Kallert et

al. 2005). To understand myxosporean host specificity, it is necessary to determine how a species or genus-specific parasite of this class interacts with non-compatible fish species, namely the fate of the developmental stages that possibly enter in those non-specific species.

1.2.6. Transmission

Since the discovery of Myxosporea, myxospores were believed to mature outside the fish host until they became infective. Infection would occur by ingestion of the mature spores, with no intermediate host necessary. Inside the fish host, spores would release their sporoplasms into the digestive tract as small amoebulas, which crossed the intestinal epithelium and migrated via the blood or lymphatic system to the target organ (Noble 1944; Dyková and Lom 1988). This theory of events remained unclear, since experimental infection of fishes by peroral administration of myxospores was unsuccessful and explained by the necessity of ripening in the water or mud during periods of several months. In light of the new data brought by Wolf and Markiw (1984) discoveries, authors disregarded their previous assumptions in favour of the new view on the myxosporean life cycle that, contrarily to the first, had successful studies supporting it (El-Matbouli and Hoffmann 1989, 1998; Bartholomew et al. 1997; Hedrick et al. 1998; Székely et al. 1998; El-Matbouli et al. 1999; Køie et al. 2004, 2007, 2008; Holzer et al. 2006; Lom and Dyková 2006; Caffara et al. 2009; Rangel et al. 2009). Considering the necessity of developing actinospores in the invertebrate definitive host in order to attain infection capability towards the vertebrate temporary host, several studies relate to the environmental viability of myxospores and actinospores. Again, studies on *Myxobolus cerebralis* were pioneer in demonstrating the resistance displayed by both these spores. Contrarily to the triactinomyxons produced during the actinosporean stage of this species, the myxospores are prepared to face and resist rigorous or changing environmental conditions, retaining infectivity after enzymatic digestion in the host digestive system. Other studies also report them as resistant to several physical parameters, such as freezing and varying pH levels, increasing the probability of survival in the environment (El-Matbouli et al. 1992; Hedrick et al. 2008). Studies relating to the actinospores resistance demonstrate them as being prone to several environmental and ecological factors, including predation, damage, physiological and osmotic stress, while still maintaining their ability to quickly infect the host fish (Markiw 1992a; Yokoyama et al. 1993, 1995; Yokoyama and Urawa 1997; Wagner et al. 2003; Kallert et al. 2007; Kallert and El-Matbouli 2008). As the actinospores constitute the infecting agent for fishes and other vertebrates, their inactivation or

eradication represents an important prevention tool from myxosporean pathogens (Wagner 2002; Wagner et al. 2003; Hedrick et al. 2007).

Some myxosporean species appear to disregard the necessity of actinospores in order to become infective for fishes and other vertebrates. Lom (1987) pointed that the number of known myxosporean stages greatly outnumbers the number of known actinosporean stages, especially in the marine environment. Also, oligochaetes are believed to have evolved from freshwater into the marine environment, while myxosporeans are believed to have evolved from the marine environment into freshwater habitats (Lom and Noble 1984; Fiala and Bartošová 2010), arousing doubts about the participation of an oligochaete in the transmission of many myxosporean species (Diamant 1997). This is the basis of studies concerning possible direct fish-to-fish transmission for several myxosporean species. Horizontal transmission may be a process of surpassing the far less diversity of oligochaetes in the marine environment (Diamant 1997; Diamant et al. 2006). Also, the infective stages in this type of transmission appear to be the vegetative stages, while the myxospores can infect only the invertebrate definitive host. If so, initial transmission might derive from waterborne contamination with actinospores, with direct fish-to-fish transmission occurring only in intensive cultures (Diamant 1997; Yasuda et al. 2002; Diamant et al. 2006). In reality, spontaneous direct fish-to-fish transmission has been demonstrated in both *Enteromyxum leei* (Diamant 1997; Diamant et al. 2006; Golomazou et al. 2006; Estensoro et al. 2010) and *Enteromyxum scophthalmi* (Redondo et al. 2002, 2004), allowing the authors to disregard the participation of the actinosporean host in these species. Yasuda et al. (2002) reported direct fish-to-fish transmission of *Myxidium fugu* and *Myxidium* sp. in the tiger puffer, *Takifugu rubripes*. A possible case of direct transmission by the ingestion of eggs of *Kudoa ovivora* in labrid fishes was also reported by Swearer and Robertson (1999). Other species, such as *Myxobolus cerebralis*, *Kudoa thyrsites* and *Ceratomyxa shasta*, seem unable to transmit directly between vertebrate hosts, since the myxospores appear to infect only the oligochaete and the actinospores only the fish host (Wolf and Markiw 1985; Markiw 1992b; Bartholomew et al. 1997; Moran et al. 1999b).

In both cases, alternating hosts and direct fish-to-fish transmission, studies are unclear in demonstrating the routes of invasion and dispersion of the parasite within the fish. The epidermis, mouth and gills are considered main portals of infection, as well as the upper esophagus and lining of the digestive tract of the fish (Markiw 1989a; Yokoyama and Urawa 1997; El-Matbouli et al. 1999; Belem and Pote 2001; Holzer et al. 2003). Seasonality variations, longevity and circadian rhythm may affect the actinospores capability for infection. Recognition of the target host is believed to be the result of both a mechanostimulant, represented by the movement of the swimming fish, and a

chemoreceptor, probably located in the fish epidermis. This aggregation of stimuli is considered because studies report chemical stimulus alone to be insufficient (Yokoyama et al. 1993, 1995; Yokoyama and Urawa 1997; El-Matbouli et al. 1999; Kallert et al. 2005). Also supporting the recognition pattern are some studies referring to the mechanisms mediating nematocysts excitation, considering the remarkable similarities between this structure of the Cnidaria and the myxosporeans polar capsules (Pantin 1942; Cannon and Wagner 2003). The most important chemical factor influencing transmission rates and host specificity appears to be the composition and quantity of mucous produced by the fish host (Yokoyama et al. 1993, 1995; El-Matbouli et al. 1999; Özer and Wootten 2002; Kallert et al. 2005). Kallert et al. (2005) postulates that different discharge rates result from different threshold concentrations triggering the actinospores receptors, the variability of cues in the mucous of different fishes and varying mechanical sensitivity. Furthermore, they suggest that the recognition present in different species, even higher vertebrates, results from a cue in the mucous signalling that was maintained through those species evolution. Better knowledge of transmission mechanisms is warranted.

Concerning direct fish-to-fish transmission, the main route of entry is through the ingestion of developmental stages from the donor fish by the receptor fish (Redondo et al. 2004), namely through predation or necrophagy of presporogonic proliferative stages (Diamant 1997; Moran et al. 1999b; Redondo et al. 2002), since the parasite remains protected in the host tissue incurring minor exposure to environmental conditions. The unsuccessful experiments of Moran et al. (1999b) for inducing infection by *Kudoa thyrsites* in Atlantic salmon through the intubation of freshly harvested myxospores; again suggest the vegetative stages as the infecting agents in direct fish-to-fish transmission. Once ingested, the parasite must resist the host physical, chemical and enzymatic activities in the alimentary tract. The aggressiveness of these processes varies considerably depending on the fish feeding habit, and the capacity of the parasite to survive these conditions is of fundamental importance. Other studies consider waterborne contamination as a gateway to infection (Diamant 1997; Diamant et al. 2006). Reports on proliferative blood stages conclude them as being implicated in the successful fish-to-fish transmission of some myxosporeans, by experimental inoculation of infected material, ceasing once the target organ is reached. These stages are most common in the genus *Sphaerospora* (Molnár and Kovács-Gayer 1986; Lom 1987; Holzer et al. 2003).

Whatever the route taken, the parasite must reach its target organs in order to develop the infection, with some routes appearing faster than others (Molnár 1988; Redondo et al. 2004). How the parasite recognizes its site of infection remains unclear. Holzer et al. (2003) suggested that the early sporogonic stages of *Sphaerospora truttae* did not

possess specific recognition of the target organ, with prevalence of infection attaining similar percentages for several organs strongly connected to the vascular system.

1.2.7. Nutrition

Plasmodia acquire nutrients actively through the process of osmotrophy, as well as through extracellular digestion in the case of myocytes and cartilage infecting myxosporeans. Some species are able to phagocytise large cells. *Sphaerospora renicola* is an example of this capability since it phagocytises erythrocytes during the blood proliferative stages (Molnár and Kovács-Gayer 1986; Lom 1987; Holzer et al. 2003). When it comes to the nutrition of the plasmodia, pinocytosis has an important role, being common in both histozoic and coelozoic species. Coleozoic plasmodia also often display peripheral cell extensions that develop in order to permit nutritional intake, especially in plasmodia appearing associated or lining the epithelial cells of the organs cavities (Lom 1969; Current et al. 1979; Sitjà-Bobadilla and Alvarez-Pellitero 1993b, 2001; Canning et al. 1999). Studies relating to the myxosporean metabolism are limited, since they are based on the constitution of the cytoplasmatic reserves. Globally, myxosporea are neither obligate aerobes nor obligate anaerobes (Lom and Dyková 1992).

1.2.8. Pathogenicity and host immune response

In the early 20th century, the pathogenic potential of myxosporeans was brought to light (Lom 1987). Economical losses in the aquaculture industry brought to attention the necessity of assessing the host immune response against myxosporean infections, including the different innate and adaptative immune mechanisms, their relationship to natural and acquired resistance and strategies effective in preventing or controlling myxosporoses (Sitjà-Bobadilla 2008). Despite the considerable diversity of described myxosporean species, only a few are important pathogenic agents, causing serious or fatal infections, both for animals and humans. Evolution has promoted a less damageable relationship between host and parasite, thus allowing them to coexist peacefully in most cases (Lom 1987; Lom and Dyková 1992, 2006). Experiments aiming to study the host humoral response to myxosporean infections have demonstrated little or none response by the immunological system, especially for coelozoic species (Griffin and Davies 1978; Velasco et al. 2002). In some cases, the lack of immunological response results from the parasites development in immunoprivileged sites, such as the central nervous system, eyes and gonads (Sitjà-Bobadilla and Alvarez-Pellitero 1993a; El-Matbouli et al. 1995;

Feist 2008; Sitjà-Bobadilla 2008). In others, a more prominent immunitary response may take place, with a strong host tissue response occurring almost immediately and rapidly destroying the parasite (Bartholomew et al. 1989b; Velasco et al. 2002), namely when it infects an atypical site in the host body or an atypical host. In the latter, proliferation may occur, but not complete sporogony (Moran et al. 1999a; Velasco et al. 2002).

Myxosporean infections can occur in any organ of the host body. The amount of damage to those cells and tissues depend on the infecting myxosporean species, its life cycle stage, as well as the intensity of the infection and host response. Therefore, according to those factors, several types of lesions may be observed, ranging from hardly noticeable to lethal. Myxosporean species cause all types of pathological changes: progressive and regressive. Progressive changes include hypertrophy and hyperplasia, or even a combination of both. Hypertrophy affects an organ or parts of it. Hyperplasia normally affects the epithelial linings. The host organism often fails in recovering from these types of pathologies. Regressive changes include dystrophy, atrophy, deposition of calcium salts in necrotic tissues and necrosis. Dystrophy is most common and usually occurs in the epithelial linings of infected organs. Atrophy is frequent in cases of infection by large or highly proliferative plasmodia, both coelozoic and histozoic. Necrosis, especially focal necrosis, is often observed when the parasitic developmental stages take place in intracellular locations (Lom and Dyková 1992). Also important to refer is the enzymatic degradation of tissues in infected fish, provoked by the myxosporean parasite. In fishes displaying diffuse infiltration by small plasmodia or pseudocysts, the muscles appear to lose elasticity. Post-mortem, the tissues rapidly soften and liquefaction occurs, making the flesh appear jellified. Curiously, studies have shown that bacterial lysis is absent and that such occurrences are, in fact, a result of the myxosporean activity in the host body. Myxosporean parasites produce proteolytic enzymes which are actively removed by the host bloodstream or are restrict to the pseudocyst, thus having a localized effect. Post-mortem, such mechanisms are cessed and the enzymes accumulate and diffuse towards other tissues, even the non-infected, causing autolysis (Willis 1949; Moran et al. 1999a; Yokoyama and Masuda 2001; Wang et al. 2005).

Despite the different sintomatology, the host immunological response varies little and the more or less prominent host responses to the parasitic infection are observed at the cell and tissues level. Although the fish imunne response possesses some special features, recent findings establish a functional parallelism between the fish and the mammalian immunitary system. A parasite entering a host is met not only by physiological barriers, but also by cellular factors, such as lymphocytes, macrophages, granulocytes, non-specific cytotoxic cells, and rodlet cells; and several humoral immune factors, including

peroxidases, lysozymes, antiproteases, complement and specific antibodies. Some immune related genes have also been studied (Chevassus and Dorson 1990; Sitjà-Bobadilla 2008). At the cellular level, inflammation of the proliferative type is the main defence mechanism, usually settling when the infecting plasmodia develop mature spores. The inflammatory process destroys the plasmodia and the resulting lesions are usually regenerated by granulation tissue. Histo zoic myxosporean species forming cysts are unnoticed by the host immune system and only after the formation of mature spores will a granulomatous infection settle. The infected tissue is replaced by the host granulation tissue, often leading to complete recovery. Encapsulation by the host connective, fibrotic and epithelial tissue prevents the parasite dissemination into surrounding tissues (Davies and Sienkoswki 1988; Lom and Dyková 1992; Koehler et al. 2004). Rodlet cells appear increased in myxosporean infected tissues discharging their rods, but their specific role remains unknown (Muñoz et al. 2000; Reite 2005; Alvarez-Pellitero et al. 2008). On the other hand, the role of the different types of leukocytes, including phagocytes, monocytes, granulocytes, lymphocytes and macrophages, is well documented in several myxosporean infections (Sitjà-Bobadilla and Alvarez-Pellitero 1993a; Redondo et al. 2004; Karagouni et al. 2005; Bermúdez et al. 2006; Cuesta et al. 2006b; Sitjà-Bobadilla et al. 2006; Alvarez-Pellitero et al. 2008). Among them, phagocytosis constitutes a very important immunological process occurring during myxosporean infection. Melanomacrophages ingest spores and small plasmodia released in the tissue and transport them into the melanomacrophages centres in the spleen, kidney and liver. In these centres they are encapsulated and gradually digested (Lom 1987; Lom and Dyková 1992; Alvarez-Pellitero and Sitjà-Bobadilla 1993; Holzer et al. 2003; Redondo et al. 2004). Redondo et al. (2004) reported the existence of macrophages containing parasitic stages and debris of *Enteromyxum scophthalmi* in the host's intestinal epithelium, subepithelial connective tissue and circulating blood, during the early infection. More rarely, melanization may occur in tissue surrounding the site of infection. The humoral immune response relies on some innate factors that partake directly in the pathogen elimination from the fish host and include peroxidases, lysozymes and complement. The data concerning these factors is quite variable, according to the host-parasite model (Muñoz et al. 2000, 2007; Foott et al. 2004; Karagouni et al. 2005; Cuesta et al. 2006a, 2006b; Sitjà-Bobadilla et al. 2006; Kaltner et al. 2007; Alvarez-Pellitero et al. 2008).

Specific immune response is rarely seen and, for a long time, fish were believed to lack specific antibodies towards myxosporean infections; thus the absences of a stronger host immune response (Bartholomew et al. 1989b; Lom and Dyková 1992). Studies going deeper into this area, discuss the mechanisms through which such equilibrium is made

possible; namely the myxosporean ability to mimic the host antigens, which would allow them to avoid the potential action of antibodies (McArthur and Sengupta 1982; Velasco et al. 2002). More recent studies report the present of specific antibodies and try to understand their role in the immune fish response. The specific immune responses are always accompanied by an array of non-specific immune factors, which constitute the first line of protection (Markiw and Wolf 1974; Griffin and Davies 1978; Wolf and Markiw 1985; Markiw 1989b; Hedrick et al. 1998; Redondo et al. 2002; Sitjà-Bobadilla et al. 2004, 2006, 2007).

When these processes are unable to destroy the infection, the host may face dramatic pathological changes, perhaps leading to death, depending on the parasite species as well as the intensity and site of infection. Many studies report natural resistance of various fish species or strains against myxosporeans. However, they are unable to describe the mechanisms involved in this phenomenon, which result in inter and intra-specific differences (Bartholomew 1998; Hedrick et al. 1998, 2001; Sugiyama et al. 1999; Wagner et al. 2002a; Blazer et al. 2004; Quiroga et al. 2006; Schisler et al. 2006; Sitjà-Bobadilla et al. 2006). The association of acquired immunity with the production of specific antibodies have also been demonstrated for *Enteromyxum scophthalmi* and *Myxobolus cerebralis* (Hedrick et al. 1998; Golomazou et al. 2006; Sitjà-Bobadilla et al. 2007). The different immunological responses displayed by the fish host are probably affected by environmental factors as well (Le Morvan et al. 1998).

A more accurate characterization of the fish immune system and its regulation is crucial for the development of efficient prevention methods concerning myxosporean infections, including immunomodulation and selection of disease-resistant strains. This research area is still undeveloped, being held back by the lack of *in vitro* cultures and the difficulty to set up experimental transmission models (Yokoyama et al. 1995; Redondo et al. 2003; Muñoz et al. 2007; Sitjà-Bobadilla 2008). Molecular studies also appear promising but still lack a data base (Bosworth et al. 2003; Cuesta et al. 2006a; Schisler et al. 2006; Severin and El-Matbouli 2007).

1.2.9. *Economical and sociological impact*

Although almost all known myxosporean species are parasites of fish at some point of their life cycle, the vast majority of these species are non-pathogenic (Lom and Dyková 1992, 2006; Masoumian et al. 1994; Roubal 1994). Nevertheless, some are highly pathogenic and constitute an emerging threat for fisheries and aquaculture (Kent et al. 2001). The parasites may impair the fish growth, damage its tissues and organs or even

cause death. The pathogenic effects depend on the myxosporean species, life cycle stage host immune reaction, intensity of infection and environmental factors. The high densities of fishes exposed to various environmental and manipulation stress factors in fisheries, provide favourable conditions for the rapid spread of infections, sometimes leading to the stock depletion and, consequently, serious economical losses (Lom 1987; Chevassus and Dorson 1990; Alvarez-Pellitero and Sitjà-Bobadilla 1993; Yasuda et al. 2002; Sitjà-Bobadilla 2008). The most notable myxosporean diseases are whirling disease (*Myxobolus cerebralis*) in salmonid fish, proliferative gill disease (*Henneguya ictaluri*) in catfish, and enteromyxosis (*Enteromyxum leei* and *Enteromyxum scophthalmi*) in cultured marine sparids (Kent and Hedrick 1986; Lom and Dyková 1992, 2006; Alvarez-Pellitero and Sitjà-Bobadilla 1993; Kent et al. 1994; Branson et al. 1999; Matos et al. 2005; Bartholomew et al. 2005; Feist 2008; Casal et al. 2009). Infections by *Ceratomyxa* spp., *Kudoa* spp. and *Sphaerospora* spp., among others, are also frequent in both wild marine fishes and mariculture fishes (Molnár and Kovács-Gayer 1986; Lom 1987; Alvarez-Pellitero and Sitjà-Bobadilla 1993; Moran et al. 1999a; Yokoyama and Masuda 2001; Yasuda et al. 2002). Proliferative kidney disease (*Tetracapsuloides bryosalmonae*) is perhaps the second most notorious myxozoan disease, after whirling disease, but is caused by a malascosporean species affecting salmonid fish, not a myxosporean (Kent and Hedrick 1986; Kent et al. 1994; Morris et al. 2000).

Whirling disease is caused by *Myxobolus cerebralis*, one of the best well known and most pathogenic myxosporean species. Clinical signs include whirling movements, severe skeletal deformations and blackening of the tail, leading to high mortality rates in reared and wild salmonid fish. The developmental stages of *Myxobolus cerebralis* digest the cartilage and destroy the structural framework necessary for healthy bone formation, provoking permanent disfiguration. The acute neurological signs occur do to constriction of the brainstem and spinal cord (O'Grodnick 1979; Wolf and Markiw 1985; Lom 1987; Markiw 1992b; Hedrick et al. 1998; Rose et al. 2000). Whirling disease is capable of provoking devastating effects in both reared and wild populations of salmonid fish. The aquaculture industry development stimulated research in control methods that would allow effective prevention against this parasite. However, they are not effectively applied to wild populations, for which whirling disease remains a high level management problem (Hedrick et al. 1998; Wagner 2002; Gilbert and Granath Jr. 2003; Bartholomew et al. 2005). Proliferative gill disease is associated with high mortalities in commercial channel catfish *Ictalurus punctatus*. The presence of *Henneguya ictaluri* in the fish gills provokes branchial inflammation, epithelial hyperplasia, lysis of filamentous cartilages, lamellar fusion and, finally, death as a consequence of the previous clinical pathologies (Belem and Pote 2001; Bosworth et al. 2003; Wise et al. 2008). The enteromyxosis caused by

Enteromyxum leei is one of the most severe pathologies caused by a myxosporean species. It is characterized by the invasion of the gut epithelial mucosa, initially with little or no inflammatory response, and later provoking disruption of the mucosa integrity, desquamation and detachment of the epithelium, leading to the release of host cells, mucous and parasitic stages into the gut lumen (Cuesta et al. 2006a, 2006b; Diamant et al. 2006; Golomazou et al. 2006; Muñoz et al. 2007). Consequently, this parasite leads to serious mortality and economic losses in sparid aquaculture, since this order appear to be the most susceptible, namely *Puntazzo puntazzo* (Diamant et al. 1994; Athanassopoulou et al. 1999; Kent et al. 2001; Padrós et al. 2001). Prevention methods are difficult due to the lack of knowledge concerning its life cycle, as well as the possible occurrence of direct fish-to-fish transmission, as shown by Diamant (1997). The myxosporean *Enteromyxum scophthalmi* is a marine histozoic parasite that causes fatal emaciative disease in farmed turbot, *Scophthalmus maximus*. After invading the fish intestine, this parasite activity leads to acute enteritis, starvation and death, sometimes reaching up to 100% mortality of affected stocks (Branson et al. 1999; Palenzuela et al. 2002; Redondo et al. 2002, 2004; Sitjà-Bobadilla et al. 2004, 2007; Quiroga et al. 2006). The lack of knowledge and effective treatments on different aspects of the life cycle and transmission of myxosporeans, mainly marine species, makes prevention difficult (Moran et al. 1999b; Redondo et al. 2004).

These diseases are a good example of how myxosporean species can cause serious environmental and economic impacts. The recent idea of adding polychaete cultures to fish rearing facilities further enhances the need of effective control methods (Fidalgo-e-Costa 1999; Rangel et al. 2009). New detection methods may help in assessing diagnosis and the acquisition of new knowledge, fulcral to the development and implement off more focused and effective control measures, not only in aquaculture facilities but also in wild affected populations.

1.2.10. Diagnosis

Considering the intricate and diverse life cycle of myxosporean species it is not hard to grasp that diagnosis of infections has always been complicated (Kent et al. 2001), and how essential it is to understand these processes in order to properly classify myxosporean species and more importantly, create techniques that efficiently protect fisheries and other economic significant activities against the pathogenic action of these parasites.

For several centuries, technological impediments made parasitological studies very poor and in the case of microscopic parasites, virtually impossible. Studies were limited to unprecise morphological data and measurements were hardly taken, making the available information somewhat ambiguous (Gunter and Adlard 2010). The inadequacy of these parameters still confounds actual identifications, sometimes making classification very problematic (Jirků et al. 2006; Gunter and Adlard 2010). Thankfully, the 19th and 20th century brought major technological innovations and witnessed consolidation of the discovery of many parasites, identification of their life cycle, and recognition of their related disease syndromes (Wolf and Markiw 1984; Lom and Dyková 1992, 2006; Kent et al. 2001). Nowadays, the characterization of myxosporean parasites rely mainly in light microscopy (LM), namely differential interference contrast (DIC), and transmission electron microscopy (TEM). TEM constitutes the most important tool in describing and classifying these species, since it provides detailed ultrastructural data. However, they remain insufficient as demonstrated by Lom and Dyková (1993) that used scanning electron microscopy (SEM) to more acutely redescribe previously established species of the genus *Chloromyxum*, and clarify several dissimilarities.

The morphological variability typical of many parasitic species, as well as the more or less frequent lack of mature spores for observation, required more specific methods for diagnosis. Immunological studies became usual practice in myxosporean diagnosis, with both monoclonal and polyclonal antibodies being produced against several species, namely pathogenic (Wolf and Markiw 1985; Bartholomew et al. 1989a; Markiw 1989a; Muñoz et al. 1999b; Belem and Pote 2001; Velasco et al. 2002). Lectins are also used in histochemical studies concerning myxosporean species, with significant differences in binding patterns being useful in diagnosis (Muñoz et al. 1999a). Still, these methods are not always precise, since antigens change throughout the parasite life cycle and are not specific to one species alone. Concurrent myxosporean infections within the same host also influence the use of fish serum antibodies, confusing diagnosis (Bartholomew et al. 1989a; Andree et al. 1998; Muñoz et al. 1999a). More recently, molecular biology techniques, such PCR and *in situ* hybridization, became fundamental tools in detecting all life cycle stages of the parasite, offering highly specific diagnostic assays (Moran et al. 1999a; Holzer et al. 2003). Species characterization is also best when recurring to this methodology (Xiao and Desser 2000). The first report of a DNA based assay was described by Bartholomew et al. (1995) for *Ceratomyxa shasta*, and recurred to arbitrary primers in order to amplify unique DNA fragments. Subsequent development of molecular techniques focused on using primers from variable regions of the 18S rRNA gene (Bartholomew et al. 1997; Andree et al. 1998; Palenzuela et al. 1999; Yokoyama et al. 2000). *In situ* hybridization procedures have also been developed, in hope of examining

more accurately the biological context of the parasite (Antonio et al. 1998, 1999; Bosworth et al. 2003). Furthermore, molecular analysis provide more reliable data concerning myxosporean biology and phylogenetic determination (Smothers et al. 1994; Siddall et al. 1995; Hanelt et al. 1996; Andree et al. 1997; Zrzavý et al. 1998; Hallett and Diamant 2001; Zrzavý and Hypša 2003; Holzer et al. 2004; Eszterbauer et al. 2006; Fiala and Bartošová 2010), allowing specific relationships within this class to be resolved (Kent et al. 2001). Molecular studies proved that structure and shape are not always consistent with phylogenetic relationships (Smothers et al. 1994; Andree et al. 1999; Kent et al. 2001; Holzer et al. 2004; Fiala 2006; Bartošová et al. 2009; Fiala and Bartošová 2010), especially considering that some genus such as *Myxobolus* and *Myxidium* are paraphyletic and others, such as *Henneguya*, *Chloromyxum*, *Zschokkella* and *Sphaerospora*, are polyphyletic (Andree et al. 1999; Kent et al. 2001). Fiala (2006) and Fiala and Bartošová (2010) even stated that the phylogeny of Myxosporea, based on SSU rDNA does not correspond to its current taxonomy. Therefore, although not always required, a molecular approach must be encouraged (Andree et al. 1999; Kent et al. 2001; Jirkù et al. 2006).

Despite the obvious practical advantages of molecular techniques, sequencing of the 18S rDNA gene alone may also be insufficient for phylogenetic purposes, since this gene is remarkably variable among closely related taxa. Furthermore, it is often very difficult to purify the parasites from the host tissues (Kent et al. 2001; Zrzavý 2001; Fiala and Bartošová 2010). Although several morphological characters are congruent with the phylogeny resulting from sequencing of the SSU rDNA gene, a more solid taxonomic classification is advised by recurring to the association of two or more molecular markers, namely the LSU rDNA gene, which suffered similar evolution to the SSU rDNA gene (Fiala 2006; Bartošová et al. 2009; Fiala and Bartošová 2010).

The growing amount of knowledge concerning myxosporean parasites surely indicates that major revisions in the taxonomy of this phylum are necessary. An association of comprehensive morphological data and molecular analysis may resolve the problems in this subject, providing accurate evolutionary taxonomic schemes for Myxosporea, as well as more accurate biological information relating to the parasite life cycle, host specificity, transmission and immunological response (Bartholomew et al. 1997; Andree et al. 1999; Hallett and Diamant 2001; Kent et al. 2001; Jirkù et al. 2006; Lom and Dyková 2006; Bartošová et al. 2009; Caffara et al. 2009; Dyková et al. 2009; Fiala and Bartošová 2010; Bartošová and Fiala 2011).

1.3. Genus *Triangulamyxa* Azevedo et al., 2005

The genus *Triangulamyxa* was recently instituted by Azevedo et al. (2005), when describing the new species *Triangulamyxa amazonica* from the intestine of the freshwater teleostean *Sphoeroides testudineus*, from the Amazon River. The genus is assigned to the family Ortholineidae Lom and Noble, 1984, suborder Variisporina Lom and Noble, 1984, order Bivalvulida Shulman, 1959 of the class Myxosporea. The features displayed by the spores of *Triangulamyxa amazonica* were consistent with the characteristics belonging to members of the family Ortholineidae, but not specifically to any of the three genera contained in this family: *Ortholinea* Shulman, 1962; *Neomyxobolus* Chen and Hsieh, 1960; and *Triangula* Chen and Hsieh, 1984. Distinction between these genera is not always easy (Lom and Dyková 1992). However, the morphological aspects observed in *Triangulamyxa amazonica* warranted the determination of a new genus, more similar to the genus *Triangula* than to the other two genera in the family Ortholineidae (Azevedo et al. 2005).

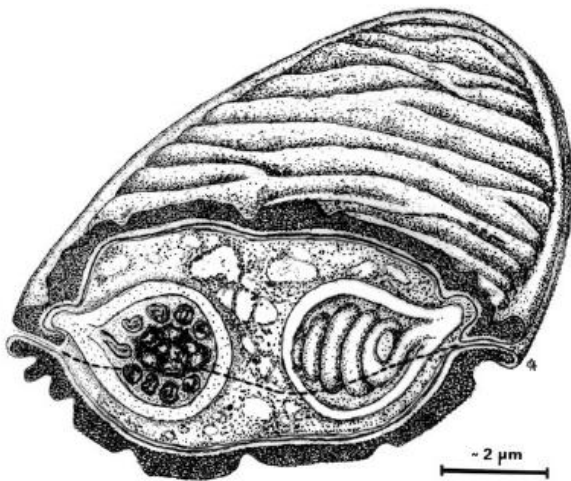


FIGURE 12. Schematic drawing of a transverse section of the spore of *Triangulamyxa amazonica* (adapted from Azevedo et al. 2005).

Triangulamyxa amazonica is the type species of this genus. Spores of the genus *Triangulamyxa* are equilaterally triangular in valvar view with rounded ends, ellipsoidal in transverse section, wider on the anterior end and flattened parallel to a slightly sinuous sutural line. In *Triangulamyxa amazonica* spores were ~8.5 μm long, ~7.6 μm wide and ~3.8 μm thick. Differing from the spores of the genus *Triangula*, the spores' surface of *Triangulamyxa* is not smooth, but ridged instead. Two subspherical polar capsules, appearing drop-shaped, are contained in the

anterior portion of the spores. In *Triangulamyxa amazonica* polar capsules measured 2.5-2.8 μm in diameter, each containing a polar filament coiled in 5-6 turns. The sporoplasm is binucleate and the nuclei are centrally located and surrounded by several sporoplasmosomes.

Polysporic plasmodia are coelozoic in freshwater fishes, and appear irregular, containing a variable number of spores (up to 18 in *Triangulamyxa amazonica*) and various other developmental stages. These structures are found floating free in the lumen or attached to the epithelium of the hosts organ through numerous microvilli with hemidesmosome-like processes in *Triangulamyxa amazonica* (Lom and Dyková 1992; Azevedo et al. 2005).

1.4. Genus *Chloromyxum* Mingazzini, 1890

The genus *Chloromyxum* Mingazzini, 1890 is assigned to the family Chloromyxidae Thélohan, 1982, suborder Variisporina Lom and Noble, 1984, order Bivalvulida Shulman, 1959, of the class Myxosporea. Among the 62 genera belonging to this class, *Chloromyxum* is the fourth largest, containing about 119 documented nominal species (Fiala and Dyková 2004; Lom and Dyková 2006; Bartošová and Fiala 2011), and with new species being frequently added (Azevedo et al. 2009). Parasites of this genus are usually coelozoic in the urinary tract and gall bladder of both freshwater and marine fishes (Fiala and Dyková 2004; Lom and Dyková 2006). They might also be found in non-fish hosts, such as amphibians (Lom and Dyková 1993; Duncan et al. 2004), although these occurrences are less frequent.

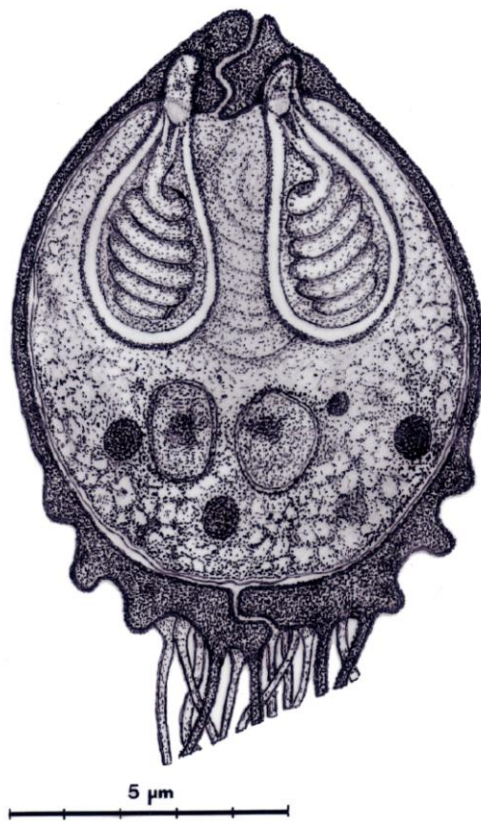


FIGURE 13. Schematic drawing of a longitudinal section in frontal view of the spore of *Chloromyxum riorajum*, with emphasis on the polar capsules and the bundle of basal filamentous projections (adapted from Azevedo et al. 2009).

Chloromyxum species possess spherical or slightly elongated spores with four polar capsules at the apex, which may be of equal or unequal size. The spore wall surface may be straight or irregular with ridges. The pattern of the ridges is often important in the species description as they appear concentric and parallel to the sutural ridge, meridional or wound in loops and irregular whorls, sometimes delimiting a smoother area (Lom and Dyková 1992, 1993, 2006). Sporoplasms are mainly binucleate, but two uninucleate sporoplasms have also been observed. Small plasmodia are monosporic and medium sized plasmodia are polysporic (Lom and Dyková 1992; Fiala and Dyková 2004). Although some characteristics are consistent for all the species within this genus, several differences may be observed in the morphological aspects of the spores and the plasmodia, as well as in the host preference. Such variability may be consequence of the possibly different

phylogenetic origin for the *Chloromyxum* freshwater and marine clades (Fiala and Dyková 2004). Freshwater species are mostly spherical or subspherical with surface ridges, and infect mainly the urinary tract or gall bladder of teleosts (Lom and Dyková 1993; Fiala and

Dyková 2004). Marine species are oval with an attenuated apex and filamentous appendages in the posterior end. They appear exclusively in the gall bladder of elasmobranchs (Kovaleva 1988).

Understanding this polyphyletic genus as the fourth larger within Myxosporea, it is not hard to realize the difficulties that occur when establishing taxonomic relations between specimens of this group. Even more considering some species, such as *Chloromyxum fluviatile*, which are highly variable in its own characteristics and without a safe distinction for separation (Lom and Dyková 1993). In these situations, relying solely in microscopic observations is insufficient. Unfortunately, although molecular technology is helping to improve the establishment of phylogenetic and taxonomic relationships amongst these species, the available data on the SSU rDNA of the genus *Chloromyxum* remains very poor. Further molecular studies are required, namely for the resolution of the phylogenetic position of the marine and freshwater species within this genus (Fiala and Dyková 2004).

References

Ali M. A., Abdel-Baki A. S., Sakran T., Entzeroth R. and Abdel-Ghaffar F. 2003: Light and electron microscopic studies of *Myxobolus stomum* n. sp. (Myxosporea: Myxobolidae) infecting the blackspotted grunt *Plectorhynchus gaterinus* (Forsskal, 1775) in the Red Sea, Egypt. Parasitol. Res. 91: 390-397.

Alvarez-Pellitero P., Palenzuela O. and Stijà-Bobadilla A. 2008: Histopathology and cellular response in *Enteromyxum leei* (Myxozoa) infections of *Diplodus puntazzo* (Teleostei). Parasitol. Int. 57: 110-120.

Alvarez-Pellitero P. and Stijà-Bobadilla A. 1993: Pathology of Myxosporea in marine fish culture. Dis. Aquat. Org. 17: 229-238.

Andree K. B., Gresoviac S. J. and Hedrick R. P. 1997: Small subunit ribosomal RNA sequences unite alternate actinosporean and myxosporean stages of *Myxobolus cerebralis* the causative agent of whirling disease in salmonid fish. J. Eukaryot. Microbiol. 44: 208-215.

Andree K. B., MacConnell E. and Hedrick R. P. 1998: A nested polymerase chain reaction for the detection of genomic DNA of *Myxobolus cerebralis* in rainbow trout *Oncorhynchus mykiss*. Dis. Aquat. Org. 34: 145-154.

Andree K. B., Székely C., Molnár K., Gresoviac S. J. and Hedrick R. P. 1999: Relationships among members of the genus *Myxobolus* (Myxozoa: Bilvalvidae) based on small subunit ribosomal DNA sequences. J. Parasitol. 85: 68-74.

Antonio D. B., Andree K. B., McDowell T. S. and Hedrick R. P. 1998: Detection of *Myxobolus cerebralis* in rainbow trout and oligochaete tissues by using a nonradioactive *in situ* hybridization (ISH) protocol. J. Aquat. Anim. Health 10: 338-347.

Antonio D. B., El-Matbouli M. and Hedrick R. P. 1999: Detection of early developmental stages of *Myxobolus cerebralis* in fish and tubificid oligochaete hosts by *in situ* hybridization. Parasitol. Res. 85: 942-944.

Athanassopoulou F., Prapas T. H. and Roger H. 1999: Diseases of *Puntazzo puntazzo* Cuvier in marine aquaculture systems in Greece. J. Fish Dis. 22: 215-218.

Awakura T., Nagasawa K. and Urawa S. 1995: Occurrence of *Myxobolus arcticus* and *M. neurobius* (Myxozoa: Myxosporea) in masu salmon *Oncorhynchus masou* from northern Japan. Sci. Rep. Hokkaido Fish Hatch. 49: 35-40.

Azevedo C., Casal G., Clemente S. C. S., Lopes L. M. S., Matos P., Abdel-Baki A. A., Oliveira E. and Matos E. 2011a: *Myxidium volitans* sp. nov., a parasite of the gallbladder of the fish, *Dactylopterus volitans* (Teleostei: Triglidae) from the Brazilian Atlantic coast – morphology and pathology. Mem. Inst. Oswaldo Cruz, Rio de Janeiro 106: 557-561.

Azevedo C., Casal G., Garcia P., Matos P., Teles-Grilo L. and Matos E. 2009: Ultrastructural and phylogenetic data of *Chloromyxum riorajum* sp. nov. (Myxozoa), a parasite of the stingray *Rioraja agassizii* in Southern Brazil. Dis. Aquat. Org. 85: 41-51.

Azevedo C., Casal G., Marques D., Silva E. and Matos E. 2011b: Ultrastructure of *Myxobolus brycon* n. sp. (phylum Myxozoa), a parasite of the piraputanga fish *Brycon hilarii* (Teleostei) from Pantanal (Brazil). J. Eukaryot. Microbiol. 58: 88-93.

Azevedo C., Casal G., Mendonça I., Carvalho E., Matos P. and Matos E. 2010: Light and electron microscopy of *Myxobolus sciades* n. sp. (Myxozoa), a parasite of the gills of the Brazilian fish *Sciades herzbergii* (Block, 1794) (Teleostei: Ariidae). Mem. Inst. Oswaldo Cruz, Rio de Janeiro 105: 203-207.

Azevedo C., Corral L. and Matos E. 2005: Ultrastructure of *Triangulamyxa amazonica* n. gen. and n. sp. (Myxozoa, Myxosporea), a parasite of the Amazonian freshwater fish, *Sphoeroides testudineus* (Teleostei, Tetodontidae). Eur. J. Protistol. 41: 57-63.

Azevedo C. and Matos E. 2003: Fine structure of *Henneguya pilosa* sp. n. (Myxozoa: Myxosporea), parasite of *Serrasalmus altuvei* (Characidae), in Brazil. Folia Parasitol. 50: 37-42.

Azevedo C., Samuel N., Saveia A. P., Delgado F. and Casal G. 2011c: Light and electron microscopical data on the spores of *Thelohanellus rhabdalestus* n. sp. (Myxozoa: Myxosporea) , a parasite of a freshwater fish from the Kwanza River, Angola. Syst. Parasitol. 78: 19-25.

Bahri S., Andree K. B. and Hedrick R. P. 2003: Morphological and phylogenetic studies of marine *Myxobolus* spp. from mullet in Ichkeul Lake, Tunisia. J. Eukaryot. Microbiol. 50: 463-470.

Bartholomew J. L. 1998: Host resistance to infection by the myxosporean parasite *Ceratomyxa shasta*: a review. J. Aquat. Anim. Health 10: 112-120.

Bartholomew J. L., Atkinson S. D. and Hallett S. L. 2006: Involvement of *Manayunkia speciosa* (Annelida: Polychaeta: Sabellidae) in the life cycle of *Parvicapsula minibicornis*, a myxozoan parasite of Pacific Salmon. J. Parasitol. 92: 742–748.

Bartholomew J. L., Atkinson S. D., Hallett S. L., Lowenstine L. J., Garner M. M., Gardiner C. H., Rideout B. A., Keel M. K. and Brown J. D. 2008: Myxozoan parasitism in waterfowl. Int. J. Parasitol. 38: 1199-1207.

Bartholomew J. L., Kerans B. L., Hedrick R. P., MacDiarmid S. C. and Winton J. R. 2005: A risk assessment based approach for the management of whirling disease. Rev. Fish. Sci. 13: 205-230.

Bartholomew J. L. and Reno P. W. 2002: The history and dissemination of whirling disease. Am. Fish Soc. Symp. 29: 3-24.

Bartholomew J. L., Rodriguez R. J. and Arakawa C. K. 1995: Development of a DNA probe for the myxosporean parasite *Ceratomyxa shasta*, using the polymerase chain reaction with arbitrary primers. *Dis. Aquat. Org.* 21: 215-220.

Bartholomew J. L., Rohovec J. S. and Fryer J. L. 1989a: Development, characterization, and use of monoclonal and polyclonal antibodies against the myxosporean, *Ceratomyxa shasta*. *J. Eukaryot. Microbiol.* 36: 397-401.

Bartholomew J. L., Smith C. E., Rohovec J. S. and Fryer J. L. 1989b: Characterization of a host response to the myxosporean parasite, *Ceratomyxa Shasta* (Noble), by histology, scanning electron microscopy and immunological techniques. *J. Fish Dis.* 12: 509-522.

Bartholomew J. L., Whipple M. J., Stevens D. G. and Fryer J. L. 1997: The life cycle of *Ceratomyxa shasta*, a myxosporean parasite of salmonids, requires a freshwater polychaete as an alternate host. *J. Parasitol.* 83: 859-868.

Bartošová P. and Fiala I. 2011: Molecular evidence for the existence of cryptic species assemblages of several myxosporeans (Myxozoa). *Parasitol. Res.* 108: 573-583.

Bartošová P., Fiala I. and Hypša V. 2009: Concatenated SSU and LSU rDNA data confirm the main evolutionary trends within myxosporeans (Myxozoa: Myxosporea) and provide an effective tool for their molecular phylogenetics. *Mol. Phylog. Evol.* 53: 81-93.

Bauer O. N. 1991: Spread of parasites and diseases of aquatic organisms by acclimatization: a short review. *J. Fish Biol.* 39: 679-686.

Belem A. M. G. and Pote L. M. 2001: Portals of entry and systemic localization of proliferative gill disease organisms in channel catfish *Ictalurus punctatus*. *Dis. Aquat. Org.* 48: 37-42.

Bermúdez R., Vigliano F., Marcaccini A., Sitjà-Bobadilla A., Quiroga M. I. and Nieto J. M. 2006: Response of Ig-positive cells to *Enteromyxum scophthalmi* (Myxozoa) experimental infection in turbot, *Scophthalmus maximus* (L.): a histopathological and immunohistochemical study. *Fish Shellfish Immunol.* 21: 501-512.

Blazer V. S., Densmore C. L., Schill W. B., Cartwright D. D. and Page S. J. 2004: Comparative susceptibility of Atlantic salmon, lake trout and rainbow trout to *Myxobolus cerebralis* in controlled laboratory exposures. Dis. Aquat. Org. 58: 27-34.

Blazer V. S., Waldrop T. B., Schill W. B., Densmore C. L. and Smith D. 2003: Effects of water temperature and substrate type on spore production and release in eastern *Tubifex tubifex* worms infected with *Myxobolus cerebralis*. J. Parasitol. 89: 21-26.

Boreham R. E., Hendrick S., O'Donoghue P. J. and Stenzel D. J. 1998: Incidental finding of *Myxobolus* spores (Protozoa: Myxozoa) in stool samples from patients with gastrointestinal symptoms. J. Clin. Microbiol. 36: 3728-3730.

Bosworth B. G., Wise D. J., Terhune J. S. and Wolters W. R. 2003: Family and genetic group effects for resistance to proliferative gill disease in channel catfish, blue catfish and channel catfish x blue catfish backcross hybrids. Aquacult. Res. 34: 569-573.

Branson E., Riaza A. and Alvarez-Pellitero P. 1999: Myxosporean infection causing intestinal disease in farmed turbot, *Scophthalmus maximus* (L.), (Teleostei: Scophthalmidae). J. Fish Dis. 22: 395-399.

Buddington R. K. and Krogdahl A. 2004: Hormonal regulation of the fish gastrointestinal tract. Comp. Biochem. Physiol. A 139: 261-271.

Bütschli O. 1881: Myxosporidien. Zool. Jahrb. F. 1: 162-164.

Caffara M., Raimondi E., Florio D., Marcer F., Quaglio F. and Fioravanti M. L. 2009: The life cycle of *Myxobolus lentisuturalis* (Myxozoa: Myxobolidae), from goldfish (*Carassius auratus auratus*), involves a Raabeia-type actinospore. Folia Parasitol. 56: 6-12.

Canning E. U., Curry A., Anderson C. L. and Okamura B. 1999: Ultrastructure of *Myxidium trachinorum* sp. nov. from the gall bladder of the lesser weever fish *Echiichthys vipera*. Parasitol. Res. 85: 910-919.

Canning E. U., Curry A., Feist S. W., Longshaw M. and Okamura B. 2000: A new class and order of myxozoans to accommodate parasites of bryozoans with ultrastructural observations on *Tetracapsula bryosalmonae* (PKX organism). J. Eukaryot. Microbiol. 47: 456-468.

Canning E. U., Curry A. and Okamura B. 2008: Early development of the myxozoan *Buddenbrockia plumatellae* in the bryozoans *Hyalinella punctata* and *Plumatella fungosa*, with comments on taxonomy and systematics of the Myxozoa. *Folia Parasitol.* 55: 241-255.

Canning E. U. and Okamura B. 2004: Biodiversity and evolution of the Myxozoa. *Adv. Parasitol.* 56: 44-131.

Canning E. U., Okamura B. and Curry A. 1996: Development of a myxozoan parasite *Tetracapsula bryozoides* gen. n. et sp. n. in *Cristatella mucedo* (Bryozoa: Phylactolaemata). *Folia Parasitol.* 43: 249-261.

Canning E. U., Tops S., Curry A., Wood T. S. and Okamura B. 2002: Ecology, development and pathogenicity of *Buddenbrockia plumatellae* Schröder, 1910 (Myxozoa, Malacosporea) (syn. *Tetracapsula bryozoides*) and establishment of *Tetracapsuloides* n. gen. for *Tetracapsula bryosalmonae*. *J. Eukaryot. Microbiol.* 49: 280-295.

Cannon Q. and Wagner E. 2003: Comparison of discharge mechanisms of cnidarian Cnidae and myxozoan polar capsules. *Rev. Fish. Sci.* 11: 185-219.

Casal G., Garcia P., Matos P., Monteiro E., Matos E. and Azevedo C. 2009: Fine structure of *Chloromyxum menticirrhi* n. sp. (Myxozoa) infecting the urinary bladder of the marine teleost *Menticirrhus americanus* (Sciaenidae) in Southern Brazil. *Europ. J. Protistol.* 45: 139-146.

Chevassus B. and Dorson M. 1990: Genetics of resistance to disease in fishes. *Aquaculture* 85: 83-107.

Cho J. B., Kwon S. R., Kim S. K., Nam Y. K. and Kim K. H. 2004: Ultrastructure and development of *Ceratomyxa protopsettae* Fujita, 1923 (Myxosporea) in the gall bladder of cultured olive flounder, *Paralichthys olivaceus*. *Acta Protozool.* 43: 241-250.

Cuesta A., Muñoz P., Rodríguez A., Salinas I., Sitjà-Bobadilla A., Alvarez-Pellitero P., Esteban M. A. and Meseguer J. 2006a: Gilthead seabream (*Sparus aurata* L.) innate defence against the parasite *Enteromyxum leei* (Myxozoa). *Parasitology* 132: 95-104.

Cuesta A., Salinas I., Rodríguez A., Muñoz P., Sitjà-Bobadilla A., Alvarez-Pellitero P., Meseguer J. and Esteban M. Á. 2006b: Cell-mediated cytotoxicity is the main innate immune mechanism involved in the cellular defence of gilthead sea bream (Teleostei: Sparidae) against *Enteromyxum leei* (Myxozoa). *Parasite Immunol.* 28: 657-665.

Current W. L. 1979: *Henneguya adiposa* Minchew (Myxosporida) in the channel catfish : ultrastructure of the plasmodium wall and sporogenesis. *J. Protozool.* 26: 209-217.

Current W. L., Janovy Jr. J., Knight S. A. 1979: *Myxosoma funduli* Kudo (Myxosporida) in *Fundulus kansae*: Ultrastructure of plasmodium wall and of sporogenesis. *J. Protozool.* 26: 574-583.

Davies A. J. and Sienkowski I. K. 1988: Further studies on *Zschokkella russelli* Tripathi (Myxozoa: Myxosporea) from *Cilliata mustela* L. (Teleostei: Gadidae), with emphasis on ultrastructural pathology and sporogenesis. *J. Fish Dis.* 11: 325-336.

Diamant A. 1997: Fish-to-fish transmission of a marine myxosporean. *Dis. Aquat. Org.* 30: 99-105.

Diamant A., Lom J. and Dyková I. 1994: *Myxidium leei* n. sp., a pathogenic myxosporean of cultured sea bream *Sparus aurata*. *Dis. Aquat. Org.* 20: 137-141.

Diamant A., Ram S. and Paperna I. 2006: Experimental transmission of *Enteromyxum leei* to freshwater fish. *Dis. Aquat. Org.* 72: 171-178.

Duncan A. E., Garner M. M., Bartholomew J. L., Reichard T. A. and Nordhausen R. W. 2004: Renal myxosporidiasis in Asian horned frog (*Megophrys nasuta*). *J. Zoo Wildl. Med.* 35: 381-386.

Dyková I., Buron I., Fiala I. and Roumillat W. A. 2009: *Kudoa inornata* sp. n. (Myxosporea: Multivalvulida) from the skeletal muscles of *Cynoscion nebulosus* (Teleostei: Sciaenidae). *Folia Parasitol.* 56: 91-98.

Dyková I. and Lom J. 1988: *Chloromyxum reticulatum* (Myxozoa, Myxosporea) in the liver of burbot (*Lota lota* L.) and its migration to the final site of infection. Eur. J. Protistol. 23: 258-261.

Dyková I., Tymi T., Fiala I. and Lom J. 2007: New data on *Soricimyxum fegati* (Myxozoa) including analysis of its phylogenetic position inferred from the SSU rRNA gene sequence. Folia Parasitol. 54: 272-276.

Eiras J. C. 2005: An overview on the myxosporean parasites in amphibians and reptiles. Acta Parasitol. 50: 267-275.

El-Mansy A. and Molnár K. 1997: Development of *Myxobolus hungaricus* (Myxosporea: Myxobolidae) in oligochaete alternate hosts. Dis. Aquat. Org. 31: 227-232.

El-Mansy A., Székely Cs. and Molnár K. 1998: Studies on the occurrence of actinosporean stages of myxosporeans in Lake Balaton, Hungary, with the description of Triactinomyxon, Raabeia and Aurantiactinomyxon types. Acta Vet. Hung. 46: 437-450.

El-Matbouli M., Fischer-Scherl T. and Hoffmann R. W. 1990: Light and electron microscopic studies on *Myxobolus cotti* El-Matbouli and Hoffmann, 1987 infecting the central nervous system of the bullhead (*Cottus gobio*). Parasitol. Res. 76: 219-227.

El-Matbouli M., Fischer-Scherl T. and Hoffmann R. W. 1992: Present knowledge on the life cycle, taxonomy, pathology, and therapy of some *Myxosporea* spp. important for freshwater fish. Annu. Rev. Fish Dis. 2: 367-402.

El-Matbouli M. and Hoffmann R. W. 1989: Experimental transmission of two *Myxobolus* spp. developing bisporogeny via tubificid worms. Parasitol. Res. 75: 461-464.

El-Matbouli M. and Hoffmann R. W. 1998: Light and electron microscopic studies on the chronological development of *Myxobolus cerebralis* to the actinosporean stage in *Tubifex tubifex*. Int. J. Parasitol. 28: 195-217.

El-Matbouli M., Hoffmann R. W. and Mandok C. 1995: Light and electron microscopic observations on the route of the triactinomyxon sporoplasm of *Myxobolus cerebralis* from epidermis into rainbow trout (*Oncorhynchus mykiss*) cartilage. J. Fish Biol. 46: 919-935.

El-Matbouli M., Hoffmann R. W., Schoel H., McDowell T. S. and Hedrick R. P. 1999: Whirling disease: host specificity and interaction between the actinosporean stage of *Myxobolus cerebralis* and rainbow trout *Oncorhynchus mykiss*. Dis. Aquat. Org. 35: 1-12.

Estensoro I., Redondo M. J., Alvarez-Pellitero P. and Sitjà-Bobadilla A. 2010: Novel horizontal transmission route for *Enteromyxum leei* (Myxozoa) by anal intubation of gilthead sea bream *Sparus aurata*. Dis. Aquat. Org. 92: 51-58.

Eszterbauer E. 2004: Genetic relationship among gill-infecting *Myxobolus* species (Myxosporea) of cyprinids: molecular evidence of importance of tissue-specificity. Dis. Aquat. Org. 58: 35-40.

Eszterbauer E., Marton S., Rácz O. Z., Letenyei M. and Molnár K. 2006: Morphological and genetic differences among actinosporean stages of fish-parasitic myxosporeans (Myxozoa): difficulties of species identification. Syst. Parasitol. 65: 97-114.

Evans N. M., Holder M. T., Barbeitos M. S., Okamura B. and Cartwright P. 2010: The phylogenetic position of Myxozoa: exploring conflicting signals in phylogenomic and ribosomal data sets. Mol. Biol. Evol. 27: 2733-2746.

Evans N. M., Lindner A., Raikova E. V., Collins A. G. and Cartwright P. 2008: Phylogenetic placement of the enigmatic parasite, *Polypodium hydriforme*, within the phylum Cnidaria. BMC Evol. Biol. 8: 139.

Evans N. M., Lindner A., Raikova E. V., Collins A. G. and Cartwright P. 2009: Correction: phylogenetic placement of the enigmatic parasite, *Polypodium hydriforme*, within the phylum Cnidaria. BMC Evol. Biol. 9: 165.

Feist S. W. 2008: Myxozoan diseases in: Eiras J. C., Segner H., Wahli T. and Kapoor B. G. 2008: Fish diseases, volume 2. Science Publishers, Enfield, NH, USA. 613 pp.

Fiala I. 2006: The phylogeny of Myxosporea (Myxozoa) based on small subunit ribosomal RNA gene analysis. Int. J. Parasitol. 36: 1521-1534.

Fiala I. and Bartošová P. 2010: History of myxozoan character evolution on the basis of rDNA and EF-2 data. BMC Evol. Biol. 10: 228.

Fiala I. and Dyková I. 2004: The phylogeny of marine and freshwater species of the genus *Chloromyxum* Mingazzini, 1890 (Myxosporea: Bivalvulida) based on small subunit ribosomal RNA gene sequences. *Folia Parasitol.* 51: 211-214.

Fidalgo-e-Costa P. 1999: Reproduction and growth in captivity of the polychaete *Nereis diversicolor* O. F. Müller, 1776, using two different kinds of sediment: preliminary assays. *Bol. Inst. Esp. Oceanogr.* 15: 351-355.

Foott J. S., Harmon R. and Stone R. 2004: Effect of water temperature on non-specific immune function and ceratomyxosis in juvenile chinook salmon and steelhead from the Klamath River. *Calif. Fish and Game.* 90: 71-84.

Friedrich C., Ingolic E., Freitag B., Kastberger G., Hohmann V., Skofitsch G., Neumeister U. And Kepka O. 2000: A myxozoan-like parasite causing xenomas in the brain of the mole, *Talpa europaea* L., 1758 (Vertebrata, Mammalia). *Parasitology* 121: 438-492.

Garner M. M., Bartholomew J. L., Whipps C. M., Nordhausen R. W. and Raiti P. 2005: Renal myxozoanosis in crowned river turtles *Hardella thurjii*: description of the putative agent *Myxidium hardella* n. sp. by histopathology, electron microscopy, and DNA sequencing. *Vet. Pathol.* 42: 589-595.

Gay M., Okamura B. and Kinkelin P. 2001: Evidence that infective stages of *Tetracapsula bryosalmonae* for rainbow trout *Oncorhynchus mykiss* are present throughout the year. *Dis. Aquat. Org.* 46: 31-40.

Gilbert M. A. and Granath W. O. Jr. 2003: Whirling disease of salmonid fish: life cycle, biology, and disease. *J. Parasitol.* 89: 658-667.

Golomazou E., Athanassopoulou F., Karagouni E. and Kokkokiris L. 2009: The effect of seasonality on the health and growth of a newly recorded *Myxobolus* species infecting cultured sharpsnout seabream (*Diplodus puntazzo* C.). *Turk. J. Vet. Anim. Sci.* 33: 1-5.

Golomazou E., Athanassopoulou F., Karagouni E., Tsagozis P., Tsantilas H. and Vagianou S. 2006: Experimental transmission of *Enteromyxum leei* Diamant, Lom and

Dyková, 1994 in sharpsnout sea bream, *Diplodus puntazzo* C. and the effect on some innate immune parameters. Aquaculture 260: 44-53.

Griffin B. R. and Davis E. M. 1978: *Myxosoma cerebralis*: Detection of circulating antibodies in infected rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board Can. 35: 1186-1190.

Gunter N. and Adlard R. 2010: The demise of *Leptotheca* Thélohan, 1895 (Myxozoa: Myxosporea: Ceratomyxidae) and assignment of its species to *Ceratomyxa* Thélohan, 1892 (Myxosporea: Ceratomyxidae), *Ellipsomyxa* Køie, 2003 (Myxosporea: Ceratomyxidae), *Myxobolus* Bütschli, 1882 and *Sphaerospora* Thélohan, 1892 (Myxosporea: Sphaerosporidae). Syst. Parasitol. 75: 81-104.

Hallett S. L., Atkinson S. D. and El-Matbouli M. 2002: Molecular characterization of two aurantiactinomyxon (Myxozoa) phenotypes reveals one genotype. J. Fish Dis. 25: 627-631.

Hallett S. L. and Diamant A. 2001: Ultrastructure and small-subunit ribosomal DNA sequence of *Henneguya lesteri* n. sp. (Myxosporea), a parasite of sand whiting *Sillago analis* (Sillaginidae) from the coast of Queensland, Australia. Dis. Aquat. Org. 46: 197-212.

Hallett S. L., Erséus C. and Lester R. J. G. 1999: Actinosporeans (Myxozoa) from marine oligochaetes of the Great Barrier Reef. Syst. Parasitol. 44: 49-57.

Hallett S. L., O'Donoghue P. J. and Lester R. J. G. 1997: Infections by *Kudoa ciliatae* (Myxozoa: Myxosporea) in Indo-Pacific whiting *Sillago* spp. Dis. Aquat. Org. 30: 11-16.

Hallett S. L., O'Donoghue P. J. and Lester R. J. G. 1998: Structure and development of a marine actinosporean, *Sphaeractinomyxon ersei* n. sp. (Myxozoa). J. Eukaryot. Microbiol. 45: 142-150.

Hanelt B., Van Schyndel D., Adema C. M., Lewis L. A. and Loker E. S. 1996: The phylogenetic position of *Rhopalura ophiocoma* (Orthonectida) based on 18S Ribosomal DNA sequence analysis. Mol. Biol. Evol. 13: 1187-1191.

Hedrick R. P., El-Matbouli M., Adkinson M. A. and MacConnell E. 1998: Whirling disease: re-emergence among wild trout. *Immunol. Rev.* 166: 365-376.

Hedrick R. P., Groff J. M. and MacDowell T. S. 1990: Gill sphaerosporosis in goldfish (*Carassius auratus*). *J. Wildl. Dis.* 26: 558-560.

Hedrick R. P., McDowell T. S., Mukkatira K., Georgiadis M. P. and MacConnell E. 2001: Salmonids resistant to *Ceratomyxa shasta* are susceptible to experimentally induced infections with *Myxobolus cerebralis*. *J. Aquat. Anim. Health* 13: 35-42.

Hedrick R. P., McDowell T. S., Mukkatira K., MacConnell E. and Petri B. 2008: Effects of freezing, drying, ultraviolet irradiation, chlorine, and quaternary ammonium treatments on the infectivity of myxospores of *Myxobolus cerebralis* for *Tubifex tubifex*. *J. Aquat. Anim. Health* 20: 116-125.

Hedrick R. P., Petri B., McDowell T. S., Mukkatira K. and Sealey L. J. 2007: Evaluation of a range of doses of ultraviolet irradiation to inactivate waterborne actinospore stages of *Myxobolus cerebralis*. *Dis. Aquat. Org.* 74: 113-118.

Higgins M. J., Margolis L. and Kent M. L. 1993: Arrested development in a freshwater myxosporean, *Myxidium salvelini*, following transfer of its host, the sockeye salmon (*Oncorhynchus nerka*), to sea water. *J. Parasitol.* 79: 403-407.

Hoffman G. L., Putz R. E. and Dunbar C. E. 1965: Studies on *Myxosoma cartilaginis* n. sp. (Protozoa: Myxosporidea) of centrarchid fish and a synopsis of the *Myxosoma* of North American freshwater fishes. *J. Protozool.* 12: 319-332.

Holzer A. S., Sommerville C. and Wootten R. 2003: Tracing the route of *Sphaerospora truttae* from the entry locus to the target organ of the host, *Salmo salar* L., using an optimized and specific *in situ* hybridisation technique. *J. Fish Dis.* 26: 647-655.

Holzer A. S., Sommerville C. and Wootten R. 2004: Molecular relationships and phylogeny in a community of myxosporeans and actinosporeans based on their 18S rDNA sequences. *Int. J. Parasitol.* 34: 1099-1111.

Holzer A. S., Sommerville C. and Wootten R. 2006: Molecular identity, phylogeny and life cycle of *Chloromyxum schurovi* Shul'man and Leshko, 2003. Parasitol. Res. 99: 90-96.

Ikeda J. 1912: Studies on some sporozoan parasites of sipunculoids. I. The life history of a new Actinomyxidian, *Tetractinomyxon intermedium* g. et sp. nov. Arch. Protistenkd. 25: 240-242.

Jiménez-Guri E., Philippe H., Okamura B. and Holland P. W. H. 2007: *Buddenbrockia* is a cnidarian worm. Sci. 317: 116-118.

Jirků M., Bolek M. G., Whipps C. M., Janovy J., Kent M. L. and Modrý D. 2006: A new species of *Myxidium* (Myxosporea: Myxidiidae), from the western chorus frog, *Pseudacris triseriata triseriata*, and Blanchard's cricket frog, *Acris crepitans Blanchardi* (Hylidae), from eastern Nebraska: morphology, phylogeny, and critical comments on amphibian *Myxidium* taxonomy. J. Parasitol. 92: 611-619.

Jurine L. L. 1825: Histoire des poissons du Lac Léman. Mém. Soc. Phys. Hist. Nat. , Genève, 3.

Kallert D. M. and El-Matbouli M. 2008: Differences in viability and reactivity of actinospores of three myxozoan species upon ageing. Folia Parasitol. 55: 105-110.

Kallert D. M., El-Matbouli M. and Haas W. 2005: Polar filament discharge of *Myxobolus cerebralis* actinospores is triggered by combined non-specific mechanical and chemical cues. Parasitol. 131: 609-616.

Kallert D. M., Ponader S., Eszterbauer E., El-Matbouli M. and Haas W. 2007: Myxozoan transmission via actinospores: new insights into mechanisms and adaptations for host invasion. Parasitol. 134: 1741-1750.

Kaltner H., Stippl M., Knaus M. and El-Matbouli M. 2007: Characterization of glycans in the developmental stages of *Myxobolus cerebralis* (Myxozoa), the causative agent of whirling disease. J. Fish Dis. 30: 637-647.

Karagouni E., Athanassopoulou F., Tsagozis P., Ralli E., Moustakareas T., Lytra K. and Dotsika E. 2005: The impact of a successful anti-myxosporean treatment on the

phagocyte functions of juvenile and adult *Sparus aurata* L. Int. J. Immunopathol. Pharmacol. 18: 121-132.

Katayama T., Wada H., Furuya H., Satoh N. and Yamamoto M. 1995: Phylogenetic position of the dicyemid Mesozoa inferred from 18S rDNA sequences. Bio. Bull. 189: 81-90.

Kent M. L., Andree K. B., Bartholomew J. L., El-Matbouli M., Desser S. S., Devlin R. A., Feist S. W., Hedrick R. P., Hoffmann R. W., Khattri J., Hallett S. L., Lester R. J. G., Longshaw M., Palenzeula O., Siddall M. E. and Xiao C. 2001: Recent advances in our knowledge of the Myxozoa. J. Eukaryot. Microbiol. 48: 395-413.

Kent M. L. and Hedrick R. P. 1986: Development of the PKX myxosporean in rainbow trout *Salmo gairdneri*. Dis. Aquat. Org. 1: 169-182.

Kent M. L., Hervio D. M. L., Docker M. F. and Devlin R. H. 1996: Taxonomy studies and diagnostic tests for myxosporean and microsporidian pathogens of salmonid fishes utilising ribosomal DNA sequence. J. Eukaryot. Microbiol. 43: 98-99.

Kent M. and Lom J. 1999: Can a new species of Myxozoa be described based solely on their actinosporean stage? Parasitol. Today 15: 472-473.

Kent M. L., Margolis L., Whitaker D. J., Hoskins G. E. and McDonald T. E. 1994: Review of Myxosporidia of importance in salmonid fisheries and aquaculture in British Columbia. Folia Parasitol. 41: 27-37.

Kim J., Kim W. and Cunningham C. W. 1999: A new perspective on lower metazoan relationships from 18S rDNA sequences. Mol. Biol. Evol. 16: 423-427.

Koehler A., Romans P., Desser S. and Ringuette M. 2004: Encapsulation of *Myxobolus pendula* (Myxosporidia) by epithelioid cells of its cyprinid host *Semotilus atromaculatus*. J. Parasitol. 90: 1401-1405.

Køie M. 2002: Spirorchid and serpulid polychaetes are candidates as invertebrate hosts for Myxozoa. Folia Parasitol. 49: 160-162.

Køie M., Karlsbakk E. and Nylund A. 2007: A new genus *Gadimyxa* with three new species (Myxozoa, Parvicapsulidae) parasitic in marine fish (Gadidae) and the two-host life cycle of *Gadimyxa atlantica* n. sp. J. Parasitol. 93: 1459-1467.

Køie M., Karlsbakk E. and Nylund A. 2008: The marine herring myxozoan *Ceratomyxa auerbachii* (Myxozoa: Ceratomyxidae) uses *Chone infundibuliformis* (Annelida: Polychaeta: Sabellidae) as invertebrate host. Folia Parasitol. 55: 100-104.

Køie M., Whipps C. M. and Kent M. L. 2004: *Ellipsomyxa gobii* (Myxozoa: Ceratomyxidae) in the common goby *pomatoschistus microps* (Teleostei: Gobiidae) uses *Nereis* spp. (Annelida: Polychaeta) as invertebrate hosts. Folia Parasitol. 51: 14-18.

Kovaleva A. A. 1988: Suborder Sphaeromyxina (Myxosporea, Bivalvulida), its structure and place in myxosporidian classification. Zool. Zh. 67: 1616-1620.

Le Morvan C., Troutaud D. and Deschaux P. 1998: Differential effects of temperature on specific and nonspecific immune defences in fish. J. Exp. Biol. 201: 165-168.

Lester R. J., Hallett S. L., El-Matbouli M. and Canning E. 1999: Can a new species of Myxozoa be described based solely on their actinosporean stage? Reply. Parasitol. Today 15: 508.

Lom J. 1969: Notes on the ultrastructure and sporoblast development in fish parasitizing myxosporidian of the genus *Sphaeromyxa*. Z. Zellforsch. 97: 416-437.

Lom J. 1987: Myxosporea: a new look at long-known parasites of fish. Parasitol. Today 3: 327-332.

Lom J. and Dyková I. 1992: Protozoan Parasites of Fishes. *Developments in Aquaculture and Fisheries Science* 26, Elsevier Amsterdam, 315 pp.

Lom J. and Dyková I. 1993: Scanning electron microscopic revision of common species of the genus *Chloromyxum* (Myxozoa: Myxosporea) infecting European freshwater fishes. Folia Parasitol. 40: 161-174.

Lom J. and Dyková I. 2006: Myxozoan genera: definition and notes on taxonomy, life cycle terminology and pathogenic species. Folia Parasitol. 53: 1-36.

Lom J. and Hoffman G. L. 2003: Morphology of the spores of *Myxosoma cerebralis* (Hofer, 1903) and *M. cartilaginis* (Hoffman, Putz, and Dunbar, 1965). J. Parasitol. 89: 653-657.

Lom J., McGeorge J., Feist S. W., Morris D. and Adams A. 1997: Guidelines for the uniform characterisation of the actinosporean stages of parasites of the phylum Myxozoa. Dis. Aquat. Org. 30: 1-9.

Lom J. and Noble E. R. 1984: Revised classification of the class Myxosporea Bütschli, 1881. Folia Parasitol. 31: 193-205.

Lom J. and Puytorac P. 1965: Studies on the myxosporidian ultrastructure and polar capsule development. Protistol. 1: 53-65.

Lom J. and Vávra J. 1965: Notes on the morphogenesis of the polar filament in *Heneguya* (Protozoa, Cnidosporidia). Acta Protozool. 3: 57-60.

Markiw M. E. 1989a: Portals of entry for salmonid whirling disease in rainbow trout. Dis. Aquat. Org. 6: 7-10.

Markiw M. E. 1989b: Salmonid whirling disease: myxosporean and actinosporean stages cross-react in direct fluorescent antibody test. J. Fish Dis. 12: 137-141.

Markiw M. E. 1992a: Experimentally induced whirling disease. II. Determination of longevity of the infective triactinomyxon stage of *Myxobolus cerebralis* by vital staining. J. Aquat. Anim. Health 4: 44-47.

Markiw M. E. 1992b: Salmonid whirling disease. Fish and Wildlife Leaflet 17. Washington DC: US Department of Interior, Fish and Wildlife Service p. 1-11.

Markiw M. E. and Wolf K. 1974: *Myxosoma cerebralis*: isolation and concentration from fish skeletal elements — sequential enzymatic digestions and purification by differential centrifugation. J. Fish. Res. Board Can. 31: 15-20.

Masoumian M., Baska F. and Molnár K. 1994: Description of *Myxobolus karuni* sp. n. and

Myxobolus persicus sp. n. (Myxosporea, Myxozoa) from *Barbus grypus* of the River Karun, Iran. Parasit. Hung. 27: 21-26.

Matos E., Tajdari J. and Azevedo C. 2005: Ultrstructural studies of *Henneguya rhamdia* n. sp. (Myxozoa) a parasite from the Amazon teleost fish, *Rhamdia quelen* (Pimelodidae). J. Eukaryot. Microbiol. 52: 532-537.

Matus D. Q., Pang K., Marlow H., Dunn C. W., Thomsen G. H. and Martindale M. Q. 2006: Molecular evidence for deep evolutionary roots of bilaterality in animal development. Proc. Nat. Acad. Sci. USA 103: 11195-11200.

McArthur C. P. and Sengupta S. 1982: Antigenic mimicry of eel tissues by a myxosporidian parasite? Z. Parasitenkd. 66: 249-255.

McGeorge J., Sommerville C. and Wootten R. 1997: Studies of actinosporean myxozoan stages parasitic in oligochaetes from the sediments of a hatchery where Atlantic salmon harbor *Sphaerospora truttae* infection. Dis. Aquat. Org. 30: 107-119.

McGurk C., Morris D. J. and Adams A. 2006: Sequential development of *Buddenbrockia plumatellae* (Myxozoa: Malacosporea) within *Plumatella repens* (Bryozoa: Phylactolaemata). Dis. Aquat. Org. 73: 159-169.

Meaders M. D. and Hendrickson G. L. 2009: Chronological development of *Ceratomyxa shasta* in the polychaete host, *Manayunkia speciosa*. J. Parasitol. 95: 1397-1407.

Molnár K. 1988: Further evidence that C blood protozoa of the common carp are stages of *Sphaerospora renicola* Dyková et Lom, 1982. Bull. Eur. Ass. Fish Pathol. 8: 3-4.

Molnár K. 1991: Comments on the host, organ and tissue specificity of fish myxosporeans and on the types of their intrapiscine development. Hung. Soc. Parasitol. 27: 5-20.

Molnár K. 1998: Taxonomic problems, seasonality and histopathology of *Henneguya creplini* (Myxosporea) infection of the pikeperch *Stizostedion lucioperca* in Lake Balaton. Folia Parasitol. 45: 261-269.

- Molnár K. 2002: Redescription and histopathology of *Myxobolus cyprinicola* Reuss, 1906, an intestinal parasite of the common carp (*Cyprinus carpio* L.). *Acta Protozool.* 41: 279-283.
- Molnár K. and Kovács-Gayer E. 1986: Experimental induction of *Sphaerospora renicola* (Myxosporea) infection in common carp (*Cyprinus carpio*) by transmission of SB- protozoans. *J. Appl. Ichthyol.* 2: 86-94.
- Molnár K. and Székely C. 1999: *Myxobolus* infection of the gills of common bream (*Abramis brama* L.) in Lake Balaton and in the Kis-Balaton reservoir, Hungary. *Acta Vet. Hung.* 47: 419-432.
- Molnár K. and Székely C. 2003: Infection in the fins of the goldfish *Carassius auratus* caused by *Myxobolus diversus* (Myxosporea). *Folia Parasitol.* 50: 29-34.
- Moncada L. I., López M. C., Murcia M. I., Nicholls S., León F., Guío O. L. and Corredor A. 2001: *Myxobolus* sp., another opportunistic parasite in immunosuppressed patients? *J. Clin. Microbiol.* 39: 1938-1940.
- Monteiro A. S., Okamura B. and Holland P. W. H. 2002: Orphan worm finds a home: *Buddenbrockia* is a myxozoan. *Mol. Biol. Evol.* 19: 968-971.
- Moran J. D. W., Margolis L., Webster J. M. and Kent M. L. 1999a: Development of *Kudoa thyrsites* (Myxozoa: Myxosporea) in netpen-reared Atlantic salmon determined by light microscopy and a polymerase chain reaction test. *Dis. Aquat. Org.* 37: 185-193.
- Moran J. D. W., Whitaker D. J. and Kent M. L. 1999b: Natural and laboratory transmission of the marine myxozoan parasite *Kudoa thyrsites* to Atlantic salmon. *J. Aquat. Anim. Health* 11: 110-115.
- Morris D. J. 2010: Cell formation by myxozoan species is not explained by dogma. *Proc. R. Soc. Lond. B Biol. Sci.* 277: 2565-2570.
- Morris D. J. and Adams A. 2007: Sacculogenesis of *Buddenbrockia plumatellae* (Myxozoa) within the invertebrate host *Plumatella repens* (Bryozoa) with comments on the evolutionary relationships of the Myxozoa. *Int. J. Parasitol.* 37: 1163-1171.

Morris D. J., Adams A. and Richards R. H. 2000: *In situ* hybridization identifies the gill as a portal of entry for PKX (Phylum Myxozoa), the causative agent of proliferative kidney disease in salmonids. *Parasitol. Res.* 86: 950-956.

Morris D. J., Morris D. C. and Adams A. 2002: Development and release of a malacosporean (Myxozoa) from *Plumatella repens* (Bryozoa: Phylactolaemata). *Folia Parasitol.* 49: 25-34.

Müller J. 1841: Über Psorospermien. *Arch. Anat. Physiol. Wissensch. Med.* 5: 477-496.

Muñoz P., Cuesta A., Athanassopoulou F., Golomazou H., Crespo S., Padrós F., Sitjà-Bobadilla A., Albiñana G., Esteban M. A., Alvarez-Pellitero P. and Meseguer J. 2007: Sharpsnout sea bream (*Diplodus puntazzo*) humoral immune response against the parasite *Enteromyxum leei* (Myxozoa). *Fish Shellfish Immunol.* 23: 636-645.

Muñoz P., Palenzuela O., Alvarez-Pellitero P. and Sitjà-Bobadilla A. 1999a: Comparative studies on carbohydrates of several myxosporean parasites of fish using lectin histochemical methods. *Folia Parasitol.* 46: 241-247.

Muñoz P., Palenzuela O., Sitjà-Bobadilla A. and Alvarez-Pellitero P. 1999b: Immunohistochemical reactivity of polyclonal antibodies against *Sphaerospora testicularis* and *Ceratomyxa labracis* (Myxosporea: Bivalvulida), with other myxosporean parasites. *Int. J. Parasitol.* 29: 521-525.

Muñoz P., Sitjà-Bobadilla A. and Alvarez-Pellitero P. 2000: Cellular and humoral immune response of European sea bass (*Dicentrarchus labrax* L.) (Teleostei: Serranidae) immunized with *Sphaerospora dicentrarchi* (Myxosporea: Bivalvulida). *Parasitology* 120: 465-477.

Noble E. R. 1944: Life cycles in the Myxosporidia. *Q. Rev. Biol.* 19: 213-235.

O'Grodnick J. J. 1979: Susceptibility of various salmonids to whirling disease (*Myxosoma cerebralis*). *Trans. Am. Fish. Soc.* 108: 187-190.

Okamura B., Curry A., Wood T. S. and Canning E. U. 2002: Ultrastructure of *Buddenbrockia* identifies it as a myxozoan and verifies the bilaterian origin of the Myxozoa. *Parasitology* 124: 215-223.

Oumouna M., Hallett S. L., Hoffmann R. W. and El-Matbouli M. 2003: Seasonal occurrence of actinosporeans (Myxozoa) and oligochaetes (Annelida) at a trout hatchery in Bavaria, Germany. *Parasitol. Res.* 89: 170-184.

Özer A. and Wootten R. 2002: Biological characteristics of some actinosporeans. *J. Natur. Hist.* 36: 2199-2209.

Özer A., Wootten R. and Shinn A. P. 2002: Survey of actinosporean types (Myxozoa) belonging to seven collective groups found in a freshwater salmon farm in Northern Scotland. *Folia Parasitol.* 49: 189-210.

Padrós F., Palenzuela O., Hispano C., Tosas O., Zarza C., Crespo S. and Alvarez-Pellitero P. 2001: *Myxidium leei* (Myxozoa) infections in aquarium-reared Mediterranean fish species. *Dis. Aquat. Org.* 47: 57-62.

Palenzuela O., Redondo M. J. and Alvarez-Pellitero P. 2002: Description of *Enteromyxum scopthalmi* gen. nov., sp. nov. (Myxozoa), an intestinal parasite of turbot (*Scophthalmus maximus* L.) using morphological and ribosomal RNA sequence data. *Parasitology* 124: 369-379.

Palenzuela O., Trobridge G. and Bartholomew J. L. 1999: Development of a polymerase chain reaction diagnostic assay for *Ceratomyxa shasta*, a myxosporean parasite of salmonid fish. *Dis. Aquat. Org.* 36: 45-51.

Pampoulie C., Marques A., Rosecchi E., Bouchereau J. L. and Crivelli A. J. 2001: Long-term monitoring on the occurrence of a myxosporean parasite *Kudoa camarguensis* (Myxosporean) on the common goby (Teleostei, Pisces) *Pomatoschistus microps*. *Dis. Aquat. Org.* 45: 69-71.

Pantin C. F. A. 1942: The excitation of nematocysts. *J. Exp. Biol.* 19: 294-310.

Pickens P. E. 1988: Systems that control the burrowing behavior of a sea anemone. J. Exp. Biol. 135: 133-164.

Pronin N. M., Fleischer G. W., Baldanova D. R. and Pronina S. V. 1997: Parasites of the recently established round goby (*Neogobius melanostomus*) and tubenose goby (*Proterorhinus marmoratus*) (Cottidae) from the St. Clair River and Lake St. Clair, Michigan, USA. Folia Parasitol. 44: 1-6.

Prunescu C.-C., Prunescu P., Pucek Z. and Lom J. 2007: The first finding of myxosporean development from plasmodia to spores in terrestrial mammals: *Soricimyxum fegati* gen. et sp. n. (Myxozoa) from *Sorex araneus* (Soricomorpha). Folia Parasitol. 54: 159-164.

Quiroga M. I., Redondo M. J., Sitjà-Bobadilla A., Palenzuela O., Riaza A., Macías A., Vázquez S., Perez A., Nieto J. M. and Alvarez-Pellitero P. 2006: Risk factors associated with *Enteromyxum scophthalmi* (Myxozoa) infection in cultured turbot, *Scophthalmus maximus* (L.). Parasitology 133: 433-442.

Raikova E. V. 2008: Cytomorphological peculiarities of *Polypodium Hydriforme* (Cnidaria). J. Mar. Biol. Assoc. U. K. 88: 1695-1702.

Rajulu G. S. and Radha R. S. P. 1966: A myxosporidian parasite *Symmetrula cochinealis* gen. et sp. nov. from an invertebrate host *Dactylopius indicus* (a cochineal bug). Ann. Mg. Nat. Hist. Ser. 13. 9: 167-171.

Rangel L. F., Cech G., Székely C. and Santos M. J. 2011: A new actinospore type Unicapsulactinomyxon (Myxozoa), infecting the marine polychaete, *Diopatra neapolitana* (Polychaeta: Onuphidae) in the Aveiro estuary (Portugal). Parasitology 24: 1-15.

Rangel L. F., Santos M. J., Cech G. and Székely C. 2009: Morphology, molecular data, and development of *Zschokkella mugilis* (Myxosporea, Bivalvulida) in a polychaete alternate host, *Nereis diversicolor*. J. Parasitol. 95: 561-569.

Redondo M. J., Palenzuela O. and Alvarez-Pellitero P. 2003: *In vitro* studies on viability and proliferation of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of cultured turbot *Scophthalmus maximus*. Dis. Aquat. Org. 55: 133-144.

Redondo M. J., Palenzuela O. and Alvarez-Pellitero P. 2004: Studies on transmission and life cycle of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of turbot *Scophthalmus maximus*. Folia Parasitol. 51: 188-198.

Redondo M. J., Palenzuela O., Riaza A., Macías A. and Alvarez-Pellitero P. 2002: Experimental transmission of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of turbot *Scophthalmus maximus*. J. Parasitol. 88: 482-488.

Reimschuessel R., Gieseke C. M., Driscoll C., Baya A., Kane A. S., Blazer V. S., Evans J. J., Kent M. L., Moran J. D. W. and Poynton S. L. 2003: Myxosporean plasmodial infection associated with ulcerative lesions in young-of-the-year Atlantic menhaden in a tributary of the Chesapeake Bay, and possible links to *Kudoa clupeiidae*. Dis. Aquat. Org. 53: 143-166.

Reite O. B. 2005: The rodlet cells of teleostean fish: their potential role in host defence in relation to the role of mast cells/eosinophilic granule cells. Fish Shellfish Immunol. 19: 253-267.

Rose J. D., Marrs G. S., Lewis C. and Schisler G. 2000: Whirling disease behavior and its relation to pathology of brain stem and spinal cord in rainbow trout. J. Aquat. Anim. Health 12: 107-118.

Roubal F. R. 1994: Infection of the kidney of *Acanthopagrus australis* (Pisces: Sparidae) with *Sphaerospora* sp. (Myxosporea), *Proserhynchus* sp. (Digenea), and cysts of unknown origin. Dis. Aquat. Org. 20: 83-93.

Roubal F. R., Hallett S. L. and Lester R. J. G. 1997: First record of triactinomyxon actinosporean in marine oligochaete. Bull. Eur. Ass. Fish Pathol. 17: 83-85.

Sanderson M. J. and Shaffer H. B. 2002: Troubleshooting molecular phylogenetic analysis. Annu. Rev. Ecol. Syst. 33: 49-72.

Schisler G. J., Myklebust K. A. and Hedrick R. P. 2006: Inheritance of *Myxobolus cerebralis* resistance among F₁-generation crosses of whirling disease resistance and susceptible rainbow trout strains. J. Aquat. Anim. Health 18: 109-115.

Severin V. I. C. and El-Matbouli M. 2007: Relative quantification of immune-regulatory genes in two rainbow trout strains, *Oncorhynchus mykiss*, after exposure to *Myxobolus cerebralis*, the causative agent of whirling disease. *Parasitol. Res.* 101: 1019-1027.

Siddall M. E., Martin D. S., Bridge D., Desser S. S. and Cone D. K. 1995: The demise of a phylum of protists: phylogeny of Myxozoa and other parasitic Cnidaria. *J. Parasitol.* 81: 961-967.

Siddall M. E. and Whiting M. F. 1999: Long-branch abstractions. *Cladistics* 15: 9-24.

Sitjà-Bobadilla A. 2008: Fish immune response to Myxozoan parasites. *Parasite* 15: 420-425.

Sitjà-Bobadilla A. and Alvarez-Pellitero P. 1993a: Pathologic effects of *Sphaerospora dicentrarchi* Sitjà-Bobadilla and Alvarez-Pellitero, 1992 and *S. testicularis* Sitjà-Bobadilla and Alvarez-Pellitero, 1990 (Myxosporea: Bivalvulida) parasitic in the Mediterranean sea bass *Dicentrarchus labrax* L. (Teleostei: Serranidae) and the cell-mediated immune reaction: a light and electron microscopy study. *Parasitol. Res.* 79: 119-129.

Sitjà-Bobadilla A. and Alvarez-Pellitero P. 1993b: *Zschokkella mugilis* n. sp. (Myxosporea: Bivalvulida) from mullets (Teleostei: Mugilidae) of Mediterranean waters: light and electron microscopic description. *J. Eukaryot. Microbiol.* 40: 755-764.

Sitjà-Bobadilla A. and Alvarez-Pellitero P. 2001: *Leptotheca sparidarum* n. sp. (Myxosporea: Bivalvulida), a parasite from cultured common dentex (*Dentex dentex* L.) and gilthead sea bream (*Sparus aurata* L.) (Teleostei: Sparidae). *J. Eukaryot. Microbiol.* 48: 627-639.

Sitjà-Bobadilla A., Palenzuela O., Riaza A., Macías M. A. and Alvarez-Pellitero P. 2007: Protective acquired immunity to *Enteromyxum scophthalmi* (Myxozoa) is related to specific antibodies in *Psetta maxima* (L.) (Teleostei). *Scand. J. Immunol.* 66: 26-34.

Sitjà-Bobadilla A., Redondo M. J., Bermúdez R., Palenzuela O., Ferreira I., Riaza A., Quiroga I., Nieto J. M. and Alvarez-Pellitero P. 2006: Innate and adaptive immune responses of turbot, *Scophthalmus maximus* (L.), following experimental infection with *Enteromyxum scophthalmi* (Myxosporea: Myxozoa). *Fish Shellfish Immunol.* 21: 485-500.

Sitjà-Bobadilla A., Redondo M. J., Macías M. A., Ferreiro I., Ríaza A. and Álvarez-Pellitero P. 2004: Development of immunohistochemistry and enzyme-linked immunosorbent assays for the detection of circulating antibodies against *Enteromyxum scophthalmi* (Myxozoa) in turbot (*Scophthalmus maximus* L.). Fish Shellfish Immunol. 17: 335-345.

Smothers J. F., von Dohlen C. D., Smith L. H. and Spall R. D. 1994: Molecular evidence that the myxozoan protists are metazoans. Science 265: 1719-1721.

Sogin M. L., Gunderson J. H., Elwood H. J., Alonso R. A. and Peattie D. A. 1989: Phylogenetic meaning of the Kingdom concept: an unusual ribosomal RNA from *Giardia lamblia*. Science 243: 75-77.

Sugiyama A., Yokoyama H. and Ogawa K. 1999: Epizootiological investigation on kudoosis amami caused by *Kudoa amamiensis* (Multivalvulida: Myxozoa) in Okinawa prefecture, Japan. Fish Pathol. 34: 39- 43.

Swearer S. E. and Robertson D. R. 1999: Life history, pathology, and description of *Kudoa ovivora* n. sp. (Myxozoa, Myxosporea): and ovarian parasite of Caribbean labroid fishes. J. Parasitol. 85: 337-353.

Székel C., El-Mansy A., Molnár K. and Baska F. 1998: Development of *Thelohanellus hovorkai* and *Thelohanellus nikolskii* (Myxosporea: Myxozoa) in oligochaete alternate hosts. Fish Pathol. 33: 107-114.

Velasco G. M., Rodero M., Zapatero L. and Cuéllar C. 2002: Humoral immune responses induced by *Kudoa* sp. (Myxosporea: Multivalvulida) antigens in BALB/c mice. Mem. Inst. Oswaldo Cruz, Rio de Janeiro. 97: 1091-1095.

Vossbrinck C. R., Maddox J. V., Friedman S., Debrunner-Vossbrinck B. A. and Woese C. R. 1987: Ribosomal RNA sequence suggests microsporidia are extremely ancient eukaryotes. Nature 326: 411-414.

Wagner E. J. 2002: Whirling disease prevention, control, and management: a review. Am. Fish. Soc. Sym. 29: 217-225.

Wagner E., Arndt R., Brough M. and Roberts D. W. 2002a: Comparison of susceptibility of five cutthroat trout strains to *Myxobolus cerebralis* infection. J. Aquat. Anim. Health 14: 84-91.

Wagner E. J., Cannon Q., Smith M., Hillyard R. and Arndt R. 2002b: Extrusion of polar filaments of the *Myxobolus cerebralis* triactinomyxon by salts, electricity, and other organs. Am. Fish. Soc. Sym. 29: 61-76.

Wagner E. J., Smith M., Arndt R. and Roberts D. W. 2003: Physical and chemical effects on viability of the *Myxobolus cerebralis* triactinomyxon. Dis. Aquat. Org. 53: 133-142.

Wang P. C., Huang J., Tsai M. A., Cheng S. Y., Tsai S. S., Chen S. D., Chen S. P., Chiu S. H., Liaw L. L., Chang L. T. and Chen S. C. 2005: Systemic infection of *Kudoa lutjanus* n. sp. (Myxozoa: Myxosporidia) in red snapper *Lutjanus erythropterus* from Taiwan. Dis. Aquat. Org. 67: 115-124.

Weidner E. and Overstreet R. M. 1979: Sporogenesis of the myxosporidan with motile spores. Cell Tissue Res. 201: 331-342.

Westfall J. A. 2004: Neural pathways and innervation of cnidocytes in tentacles of sea anemones. Hydrobiologia 530/531: 117-121.

Whipps C. M., Grossel G., Adlard R. D., Yokoyama H., Bryant M. S., Munday B. L. and Kent M. L. 2004: Phylogeny of the multivalvulidae (Myxozoa: Myxosporidia) based on comparative ribosomal DNA sequence analysis. J. Parasitol. 90: 618-622.

Willis A. G. 1949: On the vegetative forms and life history of *Chloromyxum thyrssites* Gilchrist and its doubtful systematic position. Aust. J. Sci. Res. 2: 379-398.

Wise D. J., Griffin M. J., Terhune J. S., Pote L. M. and Khoo L. H. 2008: Induction and evaluation of proliferative gill disease in channel catfish fingerlings. J. Aquat. Anim. Health 20: 236-244.

Wolf K. and Markiw M. E. 1984: Biology contravenes taxonomy in the Myxozoa: new discoveries show alternation of invertebrate and vertebrate hosts. Science 225: 1449-1452.

Wolf K. and Markiw M. E. 1985: Salmonid whirling disease. Fish Disease Leaflet 69. Washington DC: US Department of Interior, Fish and Wildlife Service p. 1-12.

Wolf K., Markiw M.E. and Hiltunen J. K. 1986: Salmonid whirling disease: *Tubifex tubifex* (Müller) identified as the essential oligochaete in the protozoan life cycle. J. Fish Dis. 9: 83-85.

Xiao C. and Desser S. S. 2000: Molecular characterization of myxozoan parasites from Lake Sasajewun, Algonquin Par, Ontario, by riboprinting. J. Eukaryot. Microbiol. 47: 85-89.

Yasuda H., Ooyama T., Iwata K., Tun T., Yokoyama H. and Ogawa K. 2002: Fish-to-fish transmission of *Myxidium* spp. (Myxozoa) in cultured tiger puffer suffering from emaciation disease. Fish Pathol. 37: 29-33.

Yokoyama H., Danjo T., Ogawa K. and Wakabayashi H. 1997: A vital staining technique with fluorescein diacetate (FDA) and propidium iodide (PI) for the determination of viability of myxosporean and actinosporean spores. J. Fish Dis. 20: 281-286.

Yokoyama H., Inoue D., Sugiyama A. and Wakabayashi H. 2000: Polymerase chain reaction and indirect fluorescent antibody technique for the detection of *Kudoa amamiensis* (Multivalvulida: Myxozoa) in yellowtail *Seriola quinqueradiata*. Fish Pathol. 35: 157-162.

Yokoyama H. and Masuda K. 2001: *Kudoa* sp. (Myxozoa) causing a post-mortem myoliquefaction of North-Pacific giant octopus *Paroctopus dofleini* (Cephalopoda: Octopodidae). Bull. Eur. Ass. Fish Pathol. 21: 266-268.

Yokoyama H., Ogawa K. and Wakabayashi H. 1993: Some biological characteristics of actinosporeans from the oligochaete *Branchiura sowerbyi*. Dis. Aquat. Org. 17: 223-228.

Yokoyama H., Ogawa K. and Wakabayashi H. 1995: Chemoresponse of actinosporean spores of *Myxobolus cultus* to skin mucus of goldfish *Carassius auratus*. Dis. Aquat. Org. 21: 7-11.

Yokoyama H. and Urawa S. 1997: Fluorescent labeling of actinospores for determining the portals of entry into fish. *Dis. Aquat. Org.* 30: 165-169.

Zrzavý J. 2001: The interrelationships of metazoan parasites: a review of phylum- and higher-level hypotheses from recent morphological and molecular phylogenetic analyses. *Folia Parasitol.* 48: 81-103.

Zrzavý J. and Hypša V. 2003: Myxozoa, *Polypodium*, and the origin of the Bilateria: the phylogenetic position of “Endocnidozoa” in light of the rediscovery of *Buddenbrockia*. *Cladistics* 19: 164-169.

Zrzavý J., Mihulka S., Kepka P., Bezděc A. and Tietz D. 1998: Phylogeny of the Metazoa based on morphological and 18S ribosomal DNA evidence. *Cladistics* 14: 249-285.

CHAPTER 2

Description of *Triangulamyxa psittaca* sp. nov. (Myxozoa: Myxosporea), a new parasite in the urinary bladder of *Colomesus psittacus* (Teleostei) from the Amazon River, with emphasis on the ultrastructure of plasmodial stages

Acta Protozoologica 50: (*In press*)

Description of *Triangulamyxa psittaca* sp. nov. (Myxozoa: Myxosporea), a new parasite in the urinary bladder of *Colomesus psittacus* (Teleostei) from the Amazon River, with emphasis on the ultrastructure of plasmodial stages

Sónia ROCHA¹, Graça CASAL^{1,2}, Patrícia MATOS³, Edilson MATOS⁴, Mohamed DKHIL^{5,6}, Carlos AZEVEDO^{1,5,*}

¹Department of Cell Biology, Institute of Biomedical Sciences (ICBAS/UP), and Laboratory of Pathology, Centre for Marine and Environmental Research (CIIMAR/UP), University of Porto, Portugal; ²Departamento de Ciências, Instituto Superior de Ciências da Saúde, Gandra, Portugal; ³Edilson Matos Research Laboratory, Federal University of Pará, Belém, Brazil; ⁴Carlos Azevedo Research Laboratory, Federal Rural University of Amazonia, Belém, Brazil; ⁵Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia; ⁶Department of Zoology and Entomology, Faculty of Science, Helwan University, Egypt

Running head: Ultrastructure of the plasmodium of *Triangulamyxa psittaca* sp. nov.

*Address for correspondence: Carlos Azevedo, Department of Cell Biology, Institute of Biomedical Sciences, University of Porto, Lg. Prof. Abel Salazar no. 2, 4099-003 Porto, Portugal; E-mail: azevedoc@icbas.up.pt

Summary. A fish-infecting myxosporean was found in the urinary bladder of the teleostean *Colomesus psittacus*, collected from the Amazon River, Brazil. Specimens were sampled in three different periods: May and June, with water temperature ranging from 18-23 °C; August, with water temperature ranging from 24-28 °C; and November and December, with water temperature ranging from 29-32 °C. Upon observation, several fish displayed abnormal behaviour, consisting of erratic movements, and mortality was recorded among them. Necropsy of all sampled fishes revealed hypertrophy of the urinary bladder only among specimens previously displaying the irregular behaviour. Microscopic analysis of this organ confirmed the parasitic infection, resulting in the observation of spores floating free in the urine, and numerous plasmodia attached to the epithelium of the urinary bladder. Light and ultrastructural studies allowed recognition of the spores and plasmodia morphological characteristics. Coelozoic plasmodia were polysporic with varying organizational structure, according to the sampling period. Spores were equilaterally triangular with rounded ends in valvar view, measuring $8.8 \pm 0.4 \mu\text{m}$ ($n = 30$) in length and $8.4 \pm 0.5 \mu\text{m}$ ($n = 30$) in width, and displaying a ridge surface pattern. Two

polar capsules were observed in the anterior end of the spores, measuring 3.1-3.2 μm in diameter. The spores were morphologically identified as belonging to the recently described genus *Triangulamyxa*. Further observation and comparison to the morphological features described for *Triangulamyxa amazonica*, the only other species within this genus, allowed us to conclude our parasite as a new species, herein named *Triangulamyxa psittaca* sp. nov. from the Amazon River, Brazil. Also, three different stages were distinguished in the plasmodium evolution, based on the observed morphological features at the three sampling periods. Fish sampled during May and June displayed small plasmodia (up to ~15-20 μm long), containing early stages of sporogenic development. Fish sampled during November and December presented larger plasmodia (up to ~850 μm long), which appeared flattened against and lining the urinary bladder epithelial cells and contained the later stages of sporogenic development, including some mature spores. Fish sampled during August presented plasmodia displaying intermediate morphological features between those observed in infected fish from the other sampling periods. Several immature and mature spores were among the different developmental stages. The parasite-host interface evolution is described throughout the different observed stages, emphasizing the formation of septate junctions. Considering several previous reports, as well as the different environmental conditions during the sampling periods, the plasmodium development here described appears to be influenced by environmental factors, namely water temperature.

Key words: Ultrastructure, plasmodia, myxosporean, *Triangulamyxa psittaca* sp. nov., parasite, urinary bladder, freshwater fish, *Colomesus psittacus*.

INTRODUCTION

The Class Myxosporea Bütschli, 1881 of the Phylum Myxozoa Grassé, 1970 is an assemblage of more than 2180 parasite species (Lom and Dyková 2006), with new species being frequently added. Widely distributed, this class contains the causative agents of some of the most severe and expanding parasitic diseases for marine and freshwater fish (Kent *et al.* 2001). Notwithstanding the effort made in improving the description of myxosporean species, little knowledge is available from these parasites infecting South American freshwater fishes, including those from Brazil. The few existing reports are based only on light microscopy and diagrammatic drawings of spores (Lutz 1889, Cunha and Fonseca 1917, Nemeczek 1926, Penido 1927, Pinto 1928, Guimarães 1931, Walliker 1969, Kent and Hoffman 1984, Gioia and Cordeiro 1996, Molnár *et al.* 1998, Cellere *et al.* 2002), and lack the useful description of the parasites development,

namely the extrasporogonic stages (Molnár and Békési 1993, Molnár *et al.* 1998, Azevedo *et al.* 2002, 2005, Casal *et al.* 2002, 2003, Cellere *et al.* 2002, Vita *et al.* 2003, Adriano *et al.* 2009a, 2009b). Recently, the employment of more effective and reliable microscopic procedures, lead to the establishment of a new myxosporean taxon from this geographical area. Based on morphological and ultrastructural comparative features, the genus *Triangulamyxa* was created within the family Ortholineidae Lom and Noble, 1984, upon the report of the then new species *Triangulamyxa amazonica*, from the intestine of the freshwater teleostean *Sphoeroides testudineus* (Azevedo *et al.* 2005). Spores of *Triangulamyxa* are equilaterally triangular in valvar view with rounded ends and ridged surface. Two subspherical polar capsules are present in the anterior portion of the spores, and the sporoplasm is binucleate. Coelozoic in freshwater fishes, plasmodia are polysporic and display a variable number of spores and other developmental stages that are found floating free in the lumen or attached to the epithelium of the hosts organ, trough numerous peripheral extensions (Lom and Dyková 1992, Azevedo *et al.* 2005).

Reports of myxosporean species inhabiting tropical regions have concluded some environmental conditions as important factors influencing the parasites development (Booker and Current 1981, Haaparanta *et al.* 1994, Molnár 1998, Canning *et al.* 1999, Molnár and Székely 1999). However, few of those references relate to species infecting the Brazilian fish fauna (Azevedo *et al.* 2005, 2009a, 2009b, 2011a, 2011b, Adriano *et al.* 2009a, 2009b, Casal *et al.* 2009).

Amongst the documented species, there is no report of a myxosporean parasitizing fish from Brazil or other South American countries with similar plasmodium development and consequent hypertrophy of the host organ, as described in the present work.

MATERIALS AND METHODS

Thirty five specimens (23 females and 12 males) of the freshwater fish *Colomesus psittacus* Schneider, 1801 (Teleostei, Tetraodontidae) (common Brazilian name: Baiacú), were collected in the low Amazon River, near the city of Cametá (02° 14' S/ 49° 30' W) in the State of Pará, Brazil. Sampling occurred in three different periods. The first sampling period occurred during the months of May and June; the second during August; and the third during the months of November and December. Upon arrival to the laboratory, fish were kept alive for 5 days for observation, in aquaria using water collected from the capture site at the same temperature range as in the original site. All the specimens were necropsied, and only samples of the urinary bladder (UB) and urine were taken for parasitological evaluation, because of its abnormal appearance. No other organs were examined and no bacteriological analyses were performed.

Smears of small fragments from the UB and urine were examined by light microscopy (LM), including differential interference contrast (DIC) optics. For transmission electron microscopy (TEM), small fragments of epithelial tissue from the parasitized UB were fixed in 5% glutaraldehyde buffered in 0.2 M sodium cacodylate (pH 7.2) at 4 °C for 20 h, washed overnight in the same buffer at the same temperature, and post-fixed in 2% OsO₄ buffered with the same solution for 3 h, again at the same temperature. The biological material was then dehydrated in an ascending ethanol series followed by propylene oxide, and embedded in Epon. Semithin sections were stained with methylene blue–Azur II for LM, and ultrathin sections were double contrasted with uranyl acetate and lead citrate and observed and photographed in a JEOL 100 CXII TEM operated at 60 kV.

RESULTS

During the period of observation, several fish displayed abnormal behaviour consisting of erratic movements, and mortality was recorded among them. All the animals displaying these movements, upon necropsy, exhibited an outstanding macroscopic hypertrophy of the UB, and resulted to be infected by a myxosporean parasite. Microscopic observation revealed the presence of myxosporean spores (Fig. 1) floating free in the UB fluid in the samples taken in August and November-December. No bacteria were observed in the fluid. The histological study revealed no hyperplasia of the UB and the presence of plasmodia attached to the epithelium in the three sampling periods (Figs. 2-16). Table 1 shows prevalence of infection and the type of plasmodium found in each period, as described below.

Sampling period	Water temperature range (°C)	Sample size (n)	Prevalence of infection (%)	Parasite stages
May-June	18-23	14	35.7	P-1
August	24-28	4	50	P-2,S
November-December	29-32	17	64.7	P-3, S

Table 1. Sampling details and prevalence of infection of *Triangulamyxa psittaca* sp. nov. in *Colomesus psittacus*. See the text for the description of the three stages of the plasmodium (P); S= spores.

Spores observed at DIC clearly displayed an equilaterally triangular shape with rounded ends in valvar view, two polar capsules and ornamental ridges in the valves (Fig. 1, 15). According to these morphological characteristics of the spores, the myxosporean was ascribed to the recently described genus *Triangulamyxa*. Morphological and ultrastructural comparison with the only species thus far belonging to the genus, *Triangulamyxa*

amazonica, allow us to determine the current species as a new one, *Triangulamyxa psittaca* sp. nov.

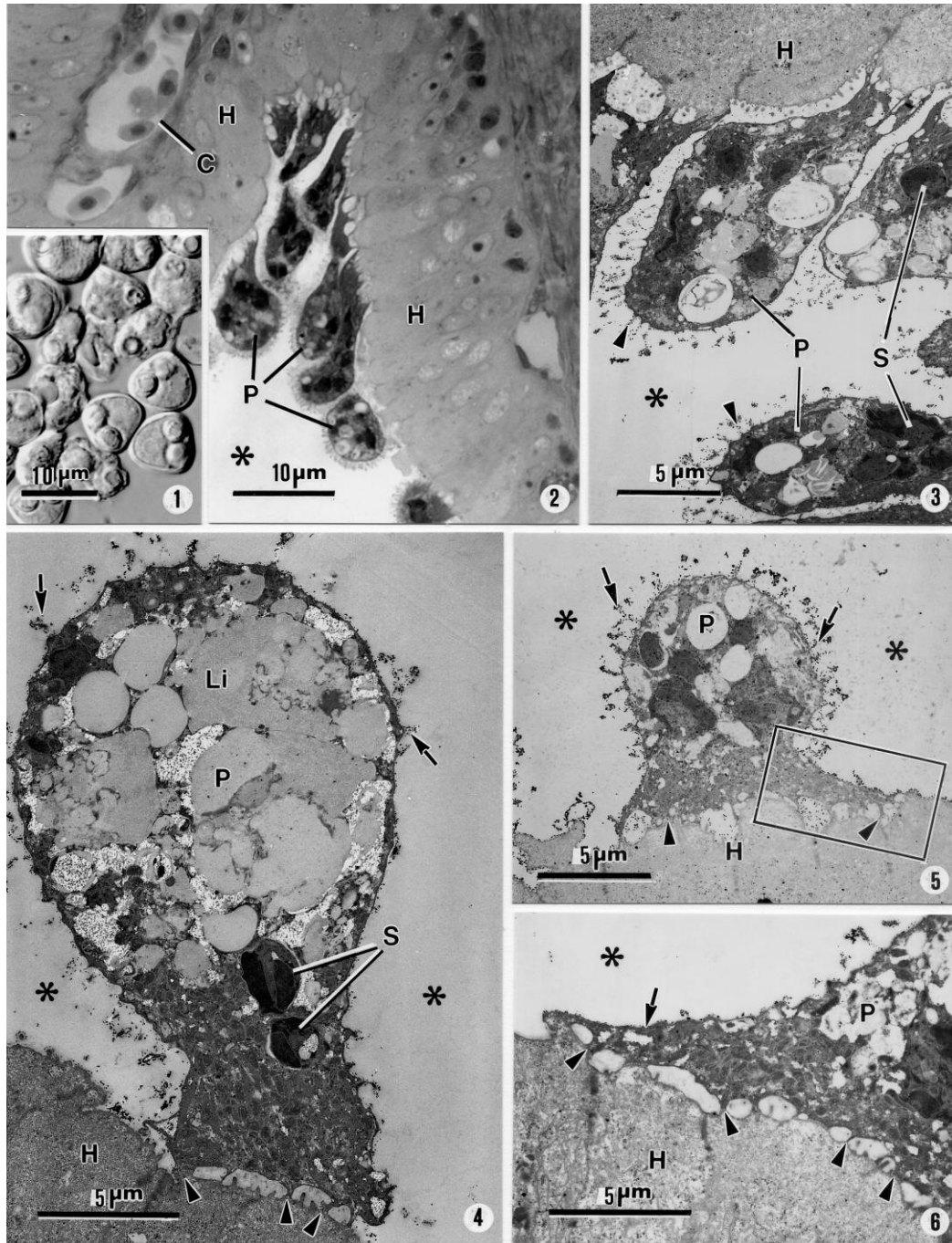
Three plasmodial developmental stages are described, based on the observation of several morphological and ultrastructural differences between plasmodia collected from the three sampling periods. Due to a scarcity of observations relating to sporogenesis, we lack a better description of the different sequential stages occurring during this process. Nevertheless, we could observe the existence of polysporic development; since pansporoblasts encased more than two sporoblasts within the pericyte envelop (Fig. 14).

Stage 1 of the plasmodium

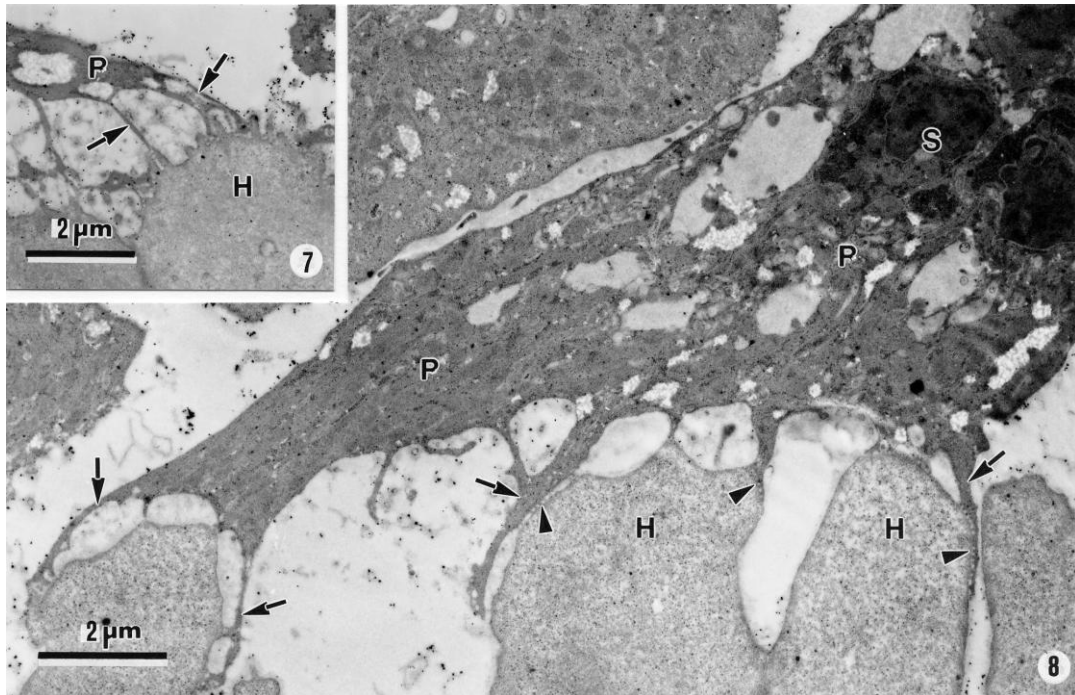
The stage 1 of the plasmodium corresponds to the observations from infected hosts sampled during May and June, when water temperature was lower. In this stage, plasmodia measured up to 15-20 μm in length and up to 8 - 12 μm in width (Figs 2-5), being smaller than the plasmodia found in hosts captured during the other sampling periods. Plasmodia contained early stages of sporogenic development and were pyriform-like shaped, tapering basally to contact the host tissue. The basal outline was irregular due to the presence of 7 to 12 pseudopodia, some of which in close contact with the UB epithelium (Figs 2-5). The apical surface of the plasmodia also presented numerous fine hair-like pseudopodia projecting towards the UB lumen (Figs 2-6). Dense structures later identified as immature spores, some unidentified developmental stages and several large lipid droplets were observed in the cytoplasm (Figs 3-5). The nucleus was hardly seen among the different cytoplasmatic structures observed. Some capillaries located among the UB epithelium appeared compressed (Fig. 2). Although the described characteristics were consistent for plasmodia in this stage, TEM allowed the observation of slightly different developmental aspects (Figs 3-6). In some sections, plasmodia appeared more rounded in shape, with only a small basal portion contacting the UB epithelium (Figs 3, 4), whereas in others it was possible to observe a marked lateral growth of the basal pseudopodia, augmenting the contact surface with the UB epithelium (Figs 5, 6). Septate junctions were frequently observed between the pseudopodia and the host epithelium (Fig. 6).

Stage 2 of the plasmodium

The stage 2 of the plasmodium corresponds to the observations from infected hosts sampled during August. In this stage, plasmodia presented transitional morphologic aspects between those described from hosts sampled during the other two periods. Plasmodia appeared to be flattening, displaying a larger contact surface with the UB epithelium than the plasmodia observed in stage 1 (Figs 7, 8).



Figs 1-6. Micrographs of fresh smears and histological sections of *Triangulamyxa psittaca* sp. nov., from the urinary bladder of *Colomesus psittacus* sampled during May and June. **1** - Free fresh mature spores observed at DIC optics. **2** - Semithin section showing several plasmodia in stage 1, located in the lumen and attached to the host epithelium. A capillary (C) is located near the base of the epithelium. **3** - TEM micrograph of plasmodia from stage 1, attached to the epithelium and containing some spores and numerous fine hair-like pseudopodia (arrowheads) projected into the lumen. **4** - Ultrastructural detail of a the stage 1 of the plasmodium showing sectioned spores and some large lipid droplets, as well as numerous peripheral fine hair-like pseudopodia (arrows) projected into the lumen. The tapering basal portion of the plasmodium is attached to the host epithelium by some pseudopodia (arrowheads). **5** - Stage 1 plasmodium showing extending basal region in closed attachment (arrowheads) with the epithelium. The periphery of the plasmodium in contact with the lumen has numerous fine hair-like pseudopodia (arrows). The box highlights an area similar to the one detailed in figure 6. **6** - Ultrastructural detail of a stage 1 plasmodium, showing the basal extending pseudopodia (arrow), closely contacting the epithelium. Some of the contact zones correspond to septate junctions (arrowheads). H= host epithelium, P= plasmodium, S= spore, Li = lipids, *= lumen of the urinary bladder.

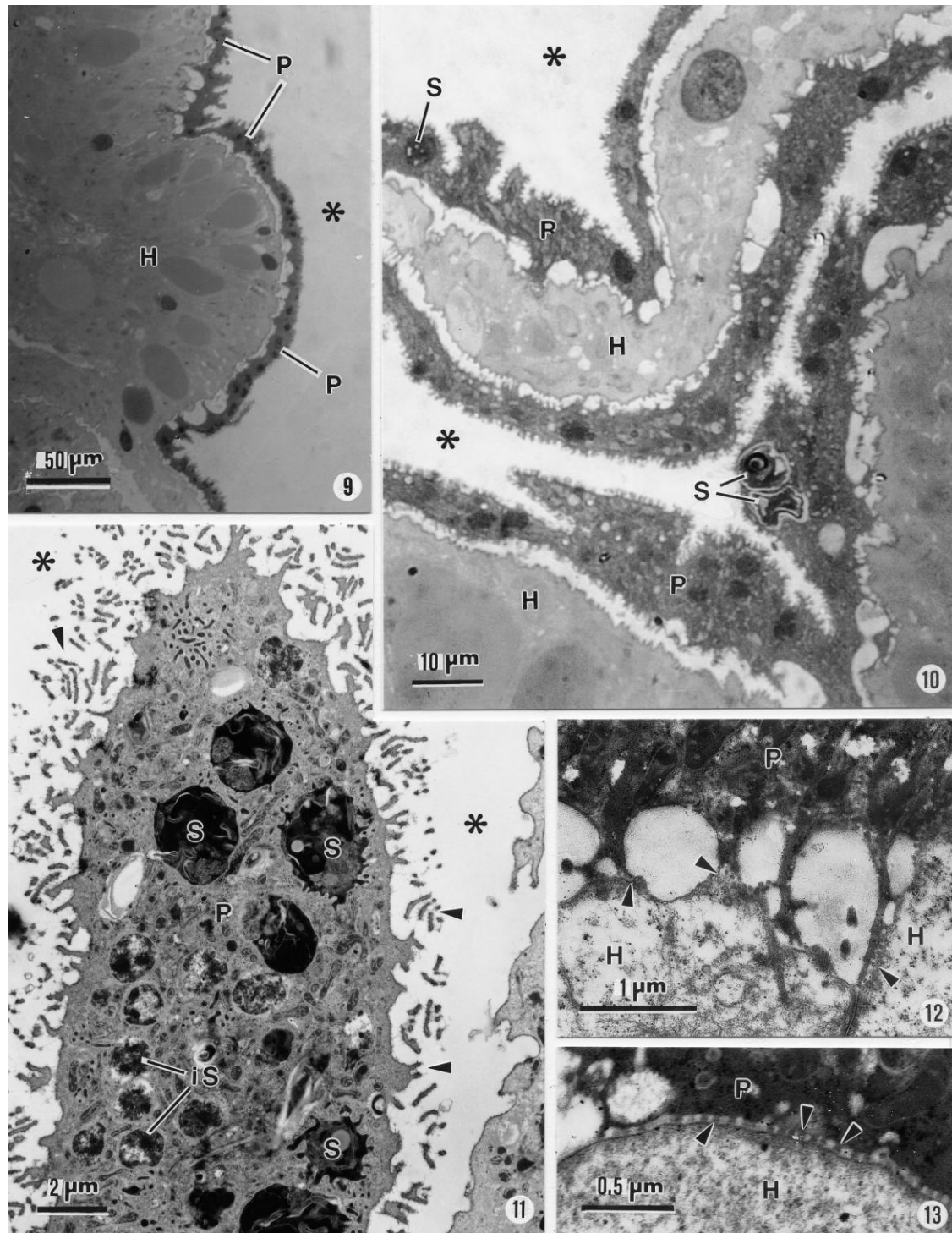


Figs 7, 8. TEM micrographs of the stage 2 of the plasmodium of *Triangulamyxa psittaca* sp. nov., from the urinary bladder of *Colomesus psittacus*, sampled during August. **7** - Part of the basal region of a plasmodium showing several types of pseudopodia (arrows) contacting the epithelium; some of which form septate junctions in the contact zone. **8** - Detailed ultrastructural aspect of a plasmodium contact zone between the pseudopodia (arrows) and the host epithelium. Some zones of contact form septate junctions (arrowheads). H= host epithelium, P= plasmodium, S= spore.

Pseudopodia were also longer and thinner, attaining up to $\sim 10 \mu\text{m}$ in length, and were ramified and anastomosed (Figs 7, 8). In some sections, septate junctions were observed in the contact zone with the epithelial cells of the host (Fig. 7, 8).

Stage 3 of the plasmodium

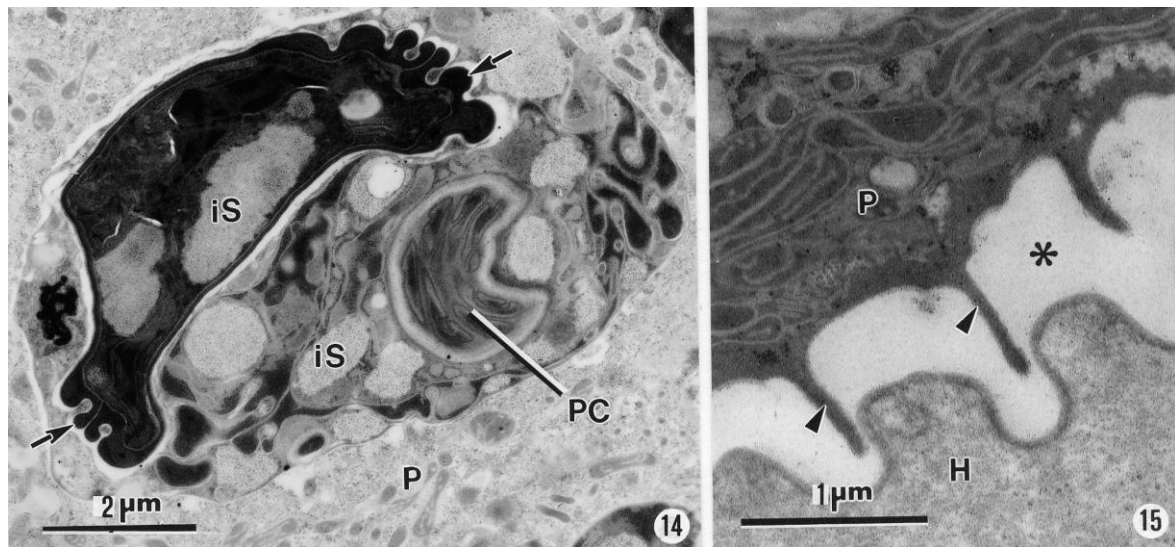
The stage 3 of the plasmodium corresponds to the observations from infected hosts sampled during November and December, when water temperature is higher. The observed plasmodia were much larger in size, if compared to the measurements obtained in stage 1 and stage 2, measuring up to $\sim 850 \mu\text{m}$ in length and $10 - 20 \mu\text{m}$ in thickness (Figs 9, 10). Plasmodia appeared elongated as if flattened against the UB epithelial cells, forming a thin irregular layer over the simple columnar UB epithelium (Figs 9, 10). The irregularity of the layer was due to the presence of space ridges in the host tissue (Fig. 10). The parasite-host interface was maintained through numerous cytoplasmatic bridges and pseudopodia between the plasmodia and the epithelial cells (Figs 9-11), and presented a plasmalemma reinforced by a homogenous dense layer (Fig. 15). In stage 3, several pseudopodia, including anastomosed pseudopodia, projected towards the UB lumen (Figs 10-12), instead of the fine hair-like pseudopodia observed in the other stages. Some sections displayed yet another type of liaison between the pseudopodia membranes and the UB epithelium membrane, as they formed septate junctions



Figs 9-13. Light and TEM micrographs of the stage 3 of the plasmodium of *Triangulamyxa psittaca* sp. nov., from the urinary bladder of *Colomesus psittacus* sampled during the months of November and December. **9** - Semithin section of the simple columnar epithelium showing a large and flattened plasmodium covering its entire surface and projecting into the lumen. **10** - Semithin section showing a plasmodium covering the entire surface of the epithelium. **11** - TEM micrograph showing a plasmodium with several immature and mature spores. The periphery of the plasmodium shows numerous sections of ramified and anastomosed pseudopodia (arrowheads) projected into the lumen. **12** - Details of the different forms of contact between the pseudopodia of the plasmodium and the cytoplasmic membrane of the urinary bladder epithelium; some of which form septate junctions (arrowheads). **13** - Detail of the contact zone between the membrane of the plasmodium and the membrane of the epithelial cell, forming a large septate junction (arrowheads). H= host epithelium, P= plasmodium, S= spore, iS= immature spore, *= lumen of the urinary bladder.

constituted by parallel rows and corresponding to a regular periodicity of junctional proteins (Figs 12, 13). The plasmodia in this stage also contained a variable number of spores (up to ~54 were observed in sequential semithin sections) randomly distributed in the cytoplasm and displaying apparent lysed aspects (Figs 11, 14); thus suggesting a polysporic pansporoblast origin.

The schematic drawing (Fig. 16) represents the morphological evolution of the plasmodia according to our observations during the three sampling periods.



Figs 14, 15. TEM micrographs of the stage 3 of the plasmodium of *Triangulamyxa psittaca* sp. nov., from the urinary bladder of *Colomesus psittacus* sampled during the months of November and December. **14** - Two juxtaposed immature spores within the plasmodium. A sectioned polar capsule and the primordial wall ridges (arrows) can be seen. **15** - Detail of the plasmodium periphery showing the pseudopodia (arrowheads) interdigitated with the epithelial cells. H= host epithelium, P= plasmodium, iS= immature spore, PC = polar capsule, *= lumen of the urinary bladder.

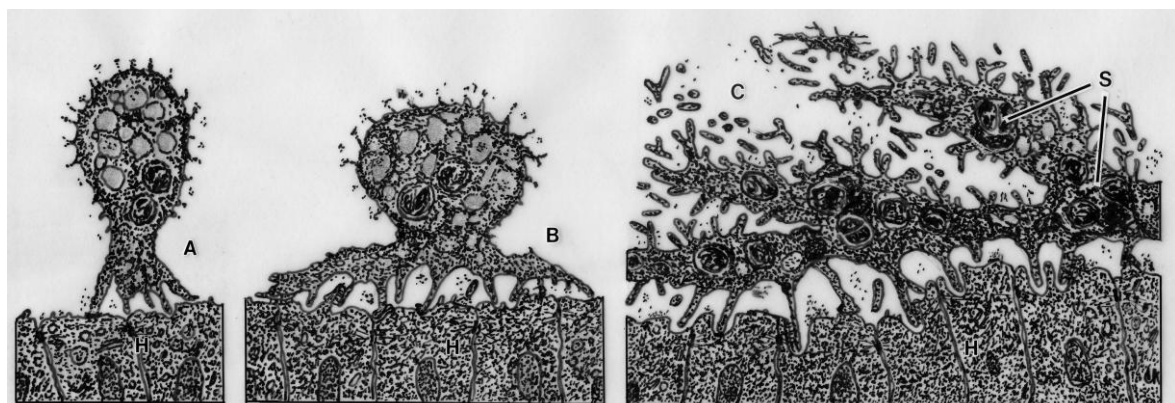


Fig 16. Schematic drawing of the sequential developmental evolution of the plasmodium of *Triangulamyxa psittaca* sp. nov. adhered to the urinary bladder of *Colomesus psittacus*, according to the three sampling periods. (A) Stage 1 plasmodium displaying rounded shape and tapering basally to contact the host epithelium through the establishment of some pseudopodia; (B) Stage 2 plasmodium displaying a larger contact surface with the host epithelium and several more pseudopodia; (C) Stage 3 plasmodium flattened against the host epithelium and displaying a much developed contact zone. The spores contained within pansporoblasts present polysporic development. H= host epithelium, Sp=spore.

***Triangulamyxa psittaca* sp. nov. (Figs 1-16)**

Type host: *Colomesus psittacus* Schneider, 1801 (Teleostei, Tetraodontidae).

Host size: Average of 30 cm in total length.

Type locality: Amazon estuarine region, near the city of Cametá (02° 14' S/ 49° 30' W) in the State of Pará, Brazil.

Site of infection: urinary bladder and urine.

Prevalence: 18 of 35 fishes sampled during the three sampling periods were parasitized (51.4%) with no observed difference in prevalence between sexes.

Type specimens: One slide of semithin sections containing mature spores and plasmodia displaying other developmental stages of the hapantotype was deposited in the Myxozoa Type Slide Collection at the “Instituto Nacional de Pesquisa da Amazônia – INPA”, Manaus, Brazil, under “INPA” no. 010/ 11.

Etymology: The specific name is derived from the scientific name of the host species.

Description of the spores: For the description of spores, light microscopy (including DIC) (Figs 1, 2, 9, 10), TEM (Figs 3-8, 11-15) and a schematic drawing (Fig. 16) were used. Spores appeared equilaterally triangular with rounded ends in valvar view (Fig. 1), and measured $8.8 \pm 0.4 \mu\text{m}$ ($n=30$) in length and $8.4 \pm 0.5 \mu\text{m}$ ($n=30$) in width. The spores' wall was comprised of two valves uniting together along a straight sutural line, and displayed a surface ridged pattern. Within the spores, two polar capsules measuring 3.1-3.2 μm in diameter were observed located in the anterior end, and lacking intercapsular space between them.

DISCUSSION

The main criterion used for the description and determination of new myxosporean species is morphology, including spores and polar capsules measurements and features (Lom and Hoffman 2003, Lom and Dyková 2006). Nevertheless, the distinct ultrastructural aspects displayed by the vegetative stages are most useful upon classification, since these structures provide valuable information concerning the parasites development (Lom and Noble 1984, Lom and Dyková 1992, 1993, 2006, Canning *et al.* 1999, Lom and Hoffman 2003). Specificity for host species and site of infection are also considered when describing a new species (Lom and Noble 1984, Lom and Dyková 1992, 2006).

Analysis of morphological features, such as spore shape, wall structure and ridges organization, determined our parasite as belonging to the recently described genus *Triangulamyxa*, within the family Ortholineidae (Lom and Noble 1984). Further ultrastructural comparison to *Triangulamyxa amazonica* (Azevedo *et al.* 2005), thus far the only species established in this genus, revealed several specific differences in both

spores and plasmodia. The spores, equilaterally triangular with rounded ends in valvar view, differed in dimension, as well as in several other aspects. *Triangulamyxa amazonica* spores were smaller (~8.5 µm long and ~7.6 µm wide), with ridged surface walls and a slightly curved sutural line. The spores observed in our study measured ~8.8 µm long and ~8.4 µm wide and the sutural line was straight. *Triangulamyxa amazonica* polar capsules were also smaller (2.5-2.8 µm in diameter) and separated by an intercapsular space (~1.3 µm), when compared to the polar capsules observed in our parasite, measuring 3.1-3.2 µm in diameter and lacking the intercapsular space between them. The apparent lysed aspect of the spores in TEM observations did not allow recognition of the number of polar filament coils in our species. Congruent with the spores morphology, the development of the plasmodium also displays several morphological and ultrastructural differences between this two species, namely in shape, dimensions and internal organization. The plasmodium described for *Triangulamyxa amazonica* was smaller, contained variable number of spores (up to ~18 were described) and often appeared free in the intestinal lumen or contacting the epithelial cells of the intestinal tract trough the insertion of fine adhesion processes. The contact surface corresponded to a small portion covered by numerous microvilli and displaying hemidesmosome-like structures. Also, the plasmodium structure was similar in all the observed developmental stages (Azevedo *et al.* 2005). The plasmodium observed in our studies varied greatly in structure throughout the developmental stages, always contacting the epithelium of the UB and containing a higher number of spores (up to ~54 in semithin sections). During the earliest stages of development, correspondent to the stages 1 and 2, the plasmodium was smaller (up to ~15-20 µm long) and pyriform-like, tapering basely to contact the epithelial cells trough the establishment of pseudopodia. In the later stage of development, correspondent to stage 3, the plasmodium appeared flattened, thus forming a layer that nearly covered the entire epithelium, and consequently attaining larger dimensions (up to ~850 µm long). Again, pseudopodia were observed in the parasite-host interface of the plasmodium in stage 3. In our species, septate junctions were present in all the observed stages, but were more evident in stage 2 and stage 3 sections. Host species and site of infection were also different for our species and *Triangulamyxa amazonica*. Although both species are coelozoic in freshwater fish species, *Triangulamyxa amazonica* infects the intestinal tract of *Sphoeroides testudineus* (Azevedo *et al.* 2005), while our parasite infects the UB of *Colomesus psittacus*. Based in all these morphological differences, as well as in the specificity for site of infection and host species, the myxosporean here described represents a new species of the genus *Triangulamyxa*, thus named *Triangulamyxa psittaca* sp. nov.

Despite the existence of some reports relating to the presence and description of coelozoic myxosporeans infecting Brazilian fishes, none refers to the ultrastructural evolution of the plasmodium (Molnár *et al.* 1998, Adriano *et al.* 2002, 2009a, 2009b, Azevedo *et al.* 2005, 2009a, 2009b, 2011a). In fact, this report is the first ultrastructural study of a plasmodial development occurring in a coelozoic myxosporean species collected from South America. During sporogenesis, the major occurring events in the plasmodium development are both an increase of size and the occurrence of pronounced surface alterations (Lom and Dyková 1995, 1996, Canning *et al.* 1999). Surface alterations are characterized by the development of pinocytic channels into the histozoic plasmodium ectoplasm (Current 1979, Current *et al.* 1979, Cho *et al.* 2004, Azevedo *et al.* 2011a), and the differentiation of peripheral extensions in the coelozoic plasmodium (Sitjà-Bobadilla and Alvarez-Pellitero 1993, 2001, Canning *et al.* 1999). Considering previous reports, the myxosporean parasite here described possesses a most interesting plasmodial development. The marked morphological differences between the three developmental stages described in this study, in which an increase of size and the formation of a large stratified layer nearly covering the entire UB epithelium occur, represent the most relevant ultrastructural comparative feature relating to other species. The majority of previously reported studies describe species with plasmodium ultrastructural aspects completely unlike the ones observed in our study (Sitjà-Bobadilla and Alvarez-Pellitero 1993, 2001, Lom and Dyková 1995, Canning *et al.* 1999, Cho *et al.* 2004, Azevedo *et al.* 2005, Adriano *et al.* 2006). Lom *et al.* (1986) described similar ultrastructural aspects to those found in our stage 3 of the plasmodium, for the trophozoites of *Hoferellus gilsoni*, observed in the urinary tract of the European eel *Anguilla anguilla*. *Hoferellus gilsoni* also displayed elongated plasmodia firmly attached to the UB epithelium, but the adherence zones lacked pseudopodia in the parasite-host interface. Instead, evaginations of the host cell were drawn into corresponding invaginations of the parasite surface, sometimes forming desmosome-like junctions; and thus compensating the absence of cellular peripheral extensions and pinocytic activity for nutritional intake. On the contrary, similarly to several other coelozoic myxosporeans, *Triangulamyxa psittaca* sp. nov. plasmodium displays several peripheral extensions in the interface zone with the host epithelial cells, which are usually associated with the intensification of trophic functions (Lom 1969, Current *et al.* 1979, Sitjà-Bobadilla and Alvarez-Pellitero 1993, 2001, Lom and Dyková 1996, Canning *et al.* 1999).

It comes as no surprise that environmental factors influence the myxosporean life cycle, and that specific physical, chemical and biological conditions are necessary for the parasite successful development. We suggest water temperature as the influencing factor

in our study, since this physical parameter has been correlated with the development of vegetative stages in several myxosporean species. Temperature appears to influence the shape of the plasmodia, the type and extension of attachment to the host cells and the development of the parasite-host interface (Booker and Current 1981, Haaparanta *et al.* 1994, Molnár and Székely 1999). In our observations, plasmodia evolved markedly increasing its dimensions between the samples collected in May and June and those collected in November and December. In the natural environment, one of the most preponderant changing variables between these time periods is water temperature range. As the fish were kept in water brought from the original site and maintained at the same range of water temperature verified during sampling, plasmodium evolution appears influenced by this physical parameter, attaining its more advanced evolutionary forms when water temperature increases, as described for other species (Molnár and Székely 1999, Viozzi and Flores 2003). However, some myxosporean species are reported to develop mature plasmodia when the water temperature range is lower (Molnár 1998). Several studies consider that the basic structure and sequential stages of plasmodial development is similar between different myxosporean species (Lom and Dyková 1992, Canning *et al.* 1999). When analysing our results and considering the abundance of myxosporean species that parasitize fishes inhabiting the tropical regions, we speculate that the plasmodial development here described may not be unique. Further studies on Brazilian myxosporean species may still be surprising when it comes to increasing the available knowledge referring to the development of vegetative stages. Also, studies should consider the possible influence of other environmental factors of this region in the parasitic development. The abnormal behaviour and mortality displayed by the infected sampled fishes leads us to suspect that *Triangulamyxa psittaca* sp. nov. is a pathogen. Nevertheless, our observations are not statistically significant to infer such a conclusion, as we aimed only at the ultrastructural description of this new parasite. Further studies should be performed in order to elucidate this observation.

Acknowledgements. This study was partially supported by Engº António de Almeida Foundation (Porto-Portugal), “CNPq” and “CAPES” (Brazil). We would like to thank Newton de Souza, technician of UFF (Oriximiná, Brazil) for the help in collecting the fish samples, and João and Joana Carvalheiro (ICBAS/UP) for the iconographic work. We would also like to thank the anonymous reviewers for the precious help provided in improving our manuscript. The performed methodology complies with the current laws of both countries.

REFERENCES

- Adriano E. A., Arana S., Alves A. L., Silva M. R. M., Ceccarelli P. S., Henrique-Silva F., Maia A. A. M. (2009a) *Myxobolus cordeiroi* n. sp., a parasite of *Zungaro jahu* (Siluriformes: Pimelodiade) from Brazilian Pantanal: Morphology, phylogeny and histopathology. *Vet. Parasitol.* **162**: 221-229
- Adriano E. A., Arana S., Carriero M. M., Naldoni J., Ceccarelli P. S., Maia A. A. M. (2009b) Light, electron microscopy and histopathology of *Myxobolus salminus* n. sp., a parasite of *Salminus brasiliensis* from the Brazilian Pantanal. *Vet. Parasitol.* **165**: 25-29
- Adriano E. A., Arana S., Ceccarelli P. S., Cordeiro N. S. (2002) Light and scanning electron microscopy of *Myxobolus porofilus* sp. n. (Myxosporea: Myxobolidae) infecting the visceral cavity of *Prochilodus lineatus* (Pisces: Characiformes: Prochilodontidae) cultivated in Brazil. *Folia Parasitol.* **49**: 259-262
- Adriano E. A., Arana S., Cordeiro N. S. (2006) *Myxobolus cuneus* n. sp. (Myxoporea) infecting the connective tissue of *Piaractus mesopotamius* (Pisces: Characidae) in Brazil: histopathology and ultrastructure. *J. Soc. Franc Parasitol.* **13**: 137-142
- Azevedo C., Casal G., Garcia P., Matos P., Teles-Grilo L., Matos E. (2009a) Ultrastructural and phylogenetic data of *Chloromyxum riorajum* sp. nov. (Myxozoa), a parasite of the stingray *Rioraja agassizii* in Southern Brazil. *Dis. Aquat. Org.* **85**: 41-51
- Azevedo C., Casal G., Marques D., Silva E., Matos E. (2011a) Ultrastructure of *Myxobolus brycon* n. sp. (Phylum Myxozoa), parasite of the piraputanga fish *Brycon hilarii* (Teleostei) from Pantanal (Brazil). *J. Eukaryot. Microbiol.* **58**: 88-93
- Azevedo C., Casal G., Mendonça I., Matos E. (2009b) Fine structure of *Henneguya hemiodopsis* sp. n. (Myxozoa), a parasite of the gills of the Brazilian teleostean fish *Hemiodopsis microlepes* (Hemiodontidae). *Mem. Inst. Oswaldo Cruz, Rio de Janeiro* **104**: 975-979
- Azevedo C., Corral L., Matos E. (2002) *Myxobolus desaequalis* n. sp. (Myxozoa. Myxosporea), parasite of the Amazonian freshwater fish, *Apteronotus albifrons* (Teleostei, Apteronotidae). *J. Eukaryot. Microbiol.* **49**: 485-488
- Azevedo C., Corral L., Matos E. (2005) Ultrastructure of *Triangulamyxa amazonica* n. gen. and n. sp. (Myxozoa, Myxosporea), a parasite of the Amazonian freshwater fish, *Sphoeroides testudineus* (Teleostei, Tetraodontidae). *Europ. J. Protistol.* **41**: 57-63
- Azevedo C., Ribeiro M., Clemente S. S. C., Casal G., Lopes L., Matos P., Al -Quraishy S. A., Matos E. (2011b) Light and ultrastructural description of *Meglitschia mylei* n. sp. (Myxozoa) from *Myleus rubripinnis* (Teleostei: Serrasalminidae) in the Amazon river system. *J. Eukaryot. Microbiol.* (In press)

- Booker O. J., Current W. L. (1981) *Myxobilatus mictospora* (Kudo, 1920) (Myxozoa: Myxosporidia) in the largemouth bass (*Micropterus salmoides* Lacépède): plasmodium morphology and fine structure. *J. Parasitol.* **67**: 859-865
- Canning E. U., Curry A., Anderson C. L., Okamura B. (1999) Ultrastructure of *Myxidium trachinorum* sp. nov. from the gallbladder of the lesser weever fish *Echiichthys vipera*. *Parasitol. Res.* **85**: 910-919
- Casal G., Garcia P., Matos P., Monteiro E., Matos E., Azevedo C. (2009) Fine structure of *Chloromyxum menticirrhoi* n. sp. (Myxozoa) infecting the urinary bladder of the marine teleost *Menticirrhus americanus* (Sciaenidae) in Southern Brazil. *Europ. J. Protistol.* **45**: 139-146
- Casal G., Matos E., Azevedo C. (2002) Ultrastructural data on the spore of *Myxobolus maculatus* n. sp. (Phylum Myxozoa), parasite from the Amazonian fish *Metynnis maculatus* (Teleostei). *Dis. Aquat. Org.* **51**: 107-112
- Casal G., Matos E., Azevedo C. (2003) Light and electron microscopic study of the myxosporean, *Henneguya friderici* n. sp. from the Amazonian teleostean fish, *Leporinus friderici*. *Parasitology* **126**: 313-319
- Cellere E. F., Cordeiro N. S., Adriano E. A. (2002) *Myxobolus absonus* sp. n. (Myxozoa: Myxosporidia) parasiting *Pimelodus maculatus* (Siluriformes: Pimelodidae), a South American freshwater fish. *Mem. Inst. Oswaldo Cruz, Rio de Janeiro* **97**: 79-80
- Cho J. B., Kwon S. R., Kim S. K., Nam Y. K., Kim K. H. (2004) Ultrastructure and development of *Ceratomyxa protopsettae* Fujita, 1923 (Myxosporidia) in the gallbladder of cultured olive flounder, *Paralichthys olivaceus*. *Acta Protozool.* **43**: 241-250
- Cunha A. M., Fonseca O. (1917) Sobre os myxosporídios dos peixes do Brasil. *Brazil-Médico.* **31**: 321 (in Portuguese)
- Current W. L. (1979) *Henneguya adiposa* Minchew (Myxosporidia) in the channel catfish: Ultrastructure of the plasmodium wall and sporogenesis. *J. Protozool.* **26**: 209-217
- Current W. L., Janovy Jr. J., Knight S. A. (1979) *Myxosoma funduli* Kudo (Myxosporidia) in *Fundulus kansae*: Ultrastructure of plasmodium wall and of sporogenesis. *J. Protozool.* **26**: 574-583
- Gioia I., Cordeiro N. S. (1996) Brazilian myxosporidians' check-list (Myxozoa). *Acta Protozool.* **35**: 137-149
- Guimarães J. R. A. (1931) Myxosporídeos da ictiofauna brasileira. Thesis, Faculdade de Medicina, São Paulo, Brasil, pp. 1-50 (in Portuguese)

- Haaparanta A., Valtonen E. T., Hoffmann R. W. (1994) Pathogenecity and seasonal occurrence of *Henneguya creplini* (Protozoa, Myxosporea) on the gills of perch *Perca fluviatilis* in central Finland. *Dis. Aquat. Org.* **20**: 15-22
- Kent M. L., Andree K. B., Bartholomew J. L., El-Matbouli M., Dessler S. S., Devlin R. H., Feist S. W., Hedrick R. P., Hoffmann R. W., Khattra J., Hallett S. L., Lester R. J. G., Longshaw M., Palenzuela O., Siddall M. E., Xiao C. (2001) Recent advances in our knowledge of the Myxozoa. *J. Eukaryot. Microbiol.* **48**: 395-413
- Kent M. L., Hoffman G. L. (1984) Two new species of Myxozoa, *Myxobolus inaequus* sp. n. and *Henneguya theca* sp. n. from the brain of a South American knife fish, *Eigemannia virescens* (V.). *J. Protozool.* **31**: 91-94
- Lom J. (1969) Notes on the ultrastructure and sporoblast development in fish parasitizing myxosporidian of the genus *Sphaeromyxa*. *Z. Zellforsch.* **97**: 416-437
- Lom J., Dyková I. (1992) Protozoan Parasites of Fishes. Developments in Aquaculture and Fisheries Science 26. Elsevier, Amsterdam, Netherlands, pp. 159-235
- Lom J., Dyková I. (1993) Scanning electron microscopic revision of common species of the genus *Chloromyxum* (Myxozoa: Myxosporea) infecting European freshwater fishes. *Folia Parasitol.* **40**: 161-174
- Lom J., Dyková I. (1995) New species of the genera *Zschokkella* and *Ortholinea* (Myxozoa) from the Southeast Asian teleost fish, *Tetraodon fluviatilis*. *Folia Parasitol.* **42**: 161-168
- Lom J., Dyková I. (1996) Notes on the ultrastructure of two myxoporean (Myxozoa) species, *Zschokkella pleomorpha* and *Ortholinea fluviatilis*. *Folia Parasitol.* **43**: 189-202
- Lom J., Dyková I. (2006) Myxozoan genera: definition and notes on taxonomy, life-cycle terminology and pathogenic species. *Folia Parasitol.* **53**: 1-36
- Lom J., Hoffman G. L. (2003) Morphology of the spores of *Myxosoma cerebralis* (Hofer, 1903) and *M. cartilaginis* (Hoffman, Putz, and Dunbar, 1965). *J. Parasitol.* **89**: 653-657
- Lom J., Molnár K., Dyková I. (1986) *Hoferellus gilsoni* (Debaisieux, 1925) comb. n. (Myxozoa, Myxosporea): redescription and mode of attachment to the epithelium of the urinary bladder of its host, the European eel. *Protistology* **4**: 405-413
- Lom J., Noble E. R. (1984) Revised classification of the Class Myxosporea Bütschli, 1881. *Folia Parasitol.* **31**: 193-205
- Lutz A. (1889) Ueber ein *Myxosporidium* ans der Gallenblase brasilianischer. *Centralbl. Bakt. u Parasit.* **5**: 84-88

- Molnár K. (1998) Taxonomic problems, seasonality and histopathology of *Henneguya creplini* (Myxosporea) infection of the pikeperch *Stizostedion lucioperca* in Lake Balaton. *Folia Parasitol.* **45**: 261-269
- Molnár K., Békési L. (1993) Description of a new *Myxobolus* species, *M. colossomatis* n. sp. from the teleost *Colossoma macropomum* of the Amazon River basin. *J. Appl. Ichthyol.* **9**: 57-63
- Molnár K., Ranzani-Paiva M. J., Eiras J. C., Rodrigues E. L. (1998) *Myxobolus macroplasmodialis* sp. n. (Myxozoa: Myxosporea), a parasite of the abdominal cavity of the characid teleost, *Salminus maxillosus*, in Brazil. *Acta Protozool.* **37**: 241-245
- Molnár K., Székely Cs. (1999) *Myxobolus* infection of the gills of common bream (*Abramis brama* L.) in Lake Balaton and in the Kis-Balaton reservoir, Hungary. *Acta Vet. Hung.* **47**: 419-432
- Nemeczek A. (1926) Beitrage zur Kenntnis der Myxosporidien fauna Brasiliens. *Arch. Protist.* **54**: 137-149
- Penido J. C. N. (1927) Quelques nouvelles Myxosporidies parasites de poissons d'eau douce du Brésil. *C. R. S. Brésil Biol.* **97**: 850-852
- Pinto C. (1928) Mixosporideos e outros protozoários intestinais de peixes observados na América do Sul. *Arch. Inst. Biol. S. Paulo.* **1**: 101-126 (in Portuguese)
- Stijà-Bobadilla A., Alvarez-Pellitero P. (1993) *Zschokkella mugilis* n. sp. (Myxoporea: Bivalvulida) from mullets (Teleostei: Mugilidae) of Mediterranean waters: light and electron microscopic description. *J. Eukaryot. Microbiol.* **40**: 755-764
- Stijà-Bobadilla A., Alvarez-Pellitero P. (2001) *Leptotheca sparidarum* n. sp. (Myxosporea: Bivalvulida), a parasite from cultured common dentex (*Dentex dentex* L.) and gilthead sea bream (*Sparus aurata* L.) (Teleostei: Sparidae). *J. Eukaryot. Microbiol.* **48**: 627-639
- Viozzi G. P., Flores V. R. (2003) *Myxidium biliare* sp. n. (Myxozoa) from gall bladder of *Galaxias maculatus* (Osmeriformes. Galaxiidae) in Patagonia (Argentina). *Folia Parasitol.* **50**: 190-194
- Vita P., Corral L., Matos E., Azevedo C. (2003) Ultrastructural aspects of the myxosporean *Henneguya astyanax* n. sp. (Myxozoa: Myxobolidae), a parasite of the Amazonian teleost *Astyanax keithi* (Characidae). *Dis. Aquat. Org.* **53**: 55-60
- Walliker D. (1969) Myxosporidea of some Brazilian freshwater fishes. *J. Parasitol.* **55**: 942-948

CHAPTER 3

Morphological and molecular characterization of *Chloromyxum clavatum* n. sp. (Myxozoa: Myxosporea), infecting the gall bladder of *Raja clavata* (Chondrichthyes), from the Portuguese Atlantic coast

Journal of Parasitology (*Submitted*)

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *CHLOROMYXUM CLAVATUM* N. SP. (MYXOZOA: MYXOSPOREA), INFECTING THE GALL BLADDER OF *RAJA CLAVATA* (CHONDRICHTHYES), FROM THE PORTUGUESE ATLANTIC COAST

S. Rocha*, G. Casal*†, S. Al-Quraishy‡, and C. Azevedo*‡

†Laboratory of Sciences, High Institute of Health Sciences (CESPU), Gandra, Portugal; and Laboratory of Pathology, Centre for Marine and Environmental Research (CIIMAR), Porto, Portugal.

‡Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia.

Running Head: ROCHA ET AL.- CHARACTERIZATION OF *C. CLAVATUM* N. SP.

*Department of Cell Biology, Institute of Biomedical Sciences (ICBAS/UP) and Laboratory of Pathology, Centre for Marine and Environmental Research (CIIMAR/UP), University of Porto, Lg. Abel Salazar no. 2, P-4099-003 Porto, Portugal. *E-mail adress:* azevedoc@icbas.up.pt

ABSTRACT: A new myxosporean species infecting the cartilaginous fish *Raja clavata* Linnaeus, 1758 collected from the Northwest Atlantic coast of Portugal is described based on microscopic and molecular procedures. Plasmodia and mature spores were found floating free in the fish gall bladder. Spherical to subspherical spores with a pointed anterior end measured $14.4 \pm 0.5 \mu\text{m}$ ($n = 25$) in length, $11.9 \pm 0.5 \mu\text{m}$ ($n = 25$) in width and $9.4 \pm 0.5 \mu\text{m}$ ($n = 15$) in thickness. The spore's wall was composed of two equally sized valves, each displaying 6-8 elevated surface ridges and boring a bundle of several tapering caudal filaments attached to its basal portion. Spores contained 4 pyriform equal-sized polar capsules ($5.5 \pm 0.4 \mu\text{m} \times 2.9 \pm 0.5 \mu\text{m}$) ($n = 25$), each possessing an obliquely arranged isofilar polar filament coiled in 7-8 coils. Morphological data, host specificity and tissue tropism identify this parasite as belonging to the genus *Chloromyxum* Mingazzini, 1890. Molecular analysis of the SSU rDNA gene, including maximum parsimony, determines our parasite as a new species, herein named *Chloromyxum clavatum* n. sp., together with *Chloromyxum leydigi* and *Chloromyxum riorajum* forming a clade positioned at the basis of the freshwater clade. This result further supports the existence of a correlation between tissue tropism and myxosporean phylogeny, while constituting an exception to the major division of the class Myxosporea into the freshwater and marine clades.

INTRODUCTION

The genus *Chloromyxum* Mingazzini, 1890 is the fourth larger within class Myxosporea Bütschli, 1882, comprising about 119 documented nominal species (Lom and Dyková, 2006; Jirkù et al., 2011). Parasites of this genus are mainly coelozoic in the urinary tract and gall bladder of freshwater and marine fish (Fiala and Dyková, 2004; Lom and Dyková, 2006; Azevedo et al., 2009; Casal et al., 2009), and less frequently in non-fish hosts (Upton et al., 1995; Duncan et al., 2004; Jirkù et al., 2006, 2011). Most descriptions from fish hosts are made from freshwater species rather than marine species; and correspond mainly to observations in Osteichthyes, with far less species reported from Chondrichthyes (Lom and Dyková, 2006).

Chloromyxum species have spherical or slightly elongated spores with four equal or unequal polar capsules at the apex and a single uninucleate or binucleate sporoplasm. The spore's wall surface may be straight or irregular with ridges. The pattern formed by the ridges is often very similar between distinct species and, therefore, constitutes an important character for the description of individual species. Small plasmodia are monosporic and medium sized plasmodia are polysporic. However, morphological aspects and host preference vary according to the environment inhabited by the host species (Lom and Dyková, 1992, 1993, 2006; Fiala and Dyková, 2004). In the case of marine fish hosts, *Chloromyxum* spp. infect only Chondrichthyes, and present oval spores with an attenuated apex and a posterior bunch of filamentous appendages (Pinto, 1928; Kuznetsova, 1977; Gioia and Cordeiro, 1996; Azevedo et al., 2009). The intricate and variable morphological features displayed by these species warrant proper determination of new species through the association of comprehensive morphological data and molecular analysis (Andree et al., 1999; Kent et al., 2001; Lom and Dyková, 2006). Light microscopy (LM) alone is insufficient for the taxonomic determination of myxosporean species and results in poor and unreliable descriptions, as well as phylogenetic confusions. The combined use of several microscopic procedures, including LM, transmission electron microscopy (TEM) and scanning electron microscopy (SEM), allows the acquirement of more accurate morphological terms of comparison, namely specific ultrastructural aspects such as the pattern formed by the ridges in the spore surface (Lom and Dyková, 1992, 1993; Jirkù et al., 2011). To culminate, the relatively recently applied molecular based taxonomic studies provide the most reliable analysis for phylogenetic position, and therefore should always be performed (Andree et al., 1999; Kent et al., 2001; Jirkù et al., 2006, 2011; Lom and Dyková, 2006).

In the case of *Chloromyxum* spp., GenBank provides information on the SSU rDNA gene of only 10 *Chloromyxum* species: 7 from freshwater fish hosts (Fiala and Dyková, 2004;

Holzer et al., 2004; Hallett et al., 2006; Bartošová and Fiala, 2011), 2 from marine fish hosts (Fiala and Dyková, 2004; Fiala, 2006; Azevedo et al., 2009) and 1 parasitizing a batrachian amphibian (Jirků et al., 2011). The molecular information broadly regarding Myxosporea demonstrates not only that phylogeny is not always consistent with morphological aspects, but also reveals the existence of two main clades dividing freshwater and marine species (Kent et al., 2000, 2001; Fiala, 2006; Bartošová et al., 2009). Myxosporean parasites infecting anadromous hosts, as well as some species belonging to the genera *Chloromyxum*, *Henneguya*, *Ceratomyxa*, *Myxobolus*, *Parvicapsula* and *Sphaeromyxa* constitute an exception to the division (Diamant et al., 2004; Fiala and Dyková, 2004; Fiala, 2006; Azevedo et al., 2009). This molecular based division in two clades appears to be supported by the evolution of morphological characters between marine and freshwater species, including those observed for *Chloromyxum* spp. (Fiala and Dyková, 2004; Fiala and Bartošová, 2010). Molecular studies also suggest that myxosporeans group more according to tissue tropism, rather than spore morphology (Andree et al., 1999; Eszterbauer, 2004; Holzer et al., 2004, 2006; Fiala, 2006; Bartošová et al., 2009; Fiala and Bartošová, 2010; Jirků et al., 2011). Despite the pronounced need for more accurate molecular information, and subsequent comparison to morphological differentiation, data on the SSU rDNA gene of the Myxosporea, and more specifically of the genus *Chloromyxum*, remains very poor (Fiala and Dyková, 2004; Fiala and Bartošová, 2010). Although two new marine species of the genus *Chloromyxum* were recently described (Azevedo et al., 2009; Casal et al., 2009), the need for molecular characterization is even more pronounced for marine species. The present study provides morphological, ultrastructural and molecular characterization for the parasitic stages found in the gall bladder of the stingray *Raja clavata* Linnaeus, 1758, a cartilaginous fish captured from the northwest Portuguese Atlantic coast, and aims to increase the non-conflictuous available knowledge relating to this genus.

MATERIAL AND METHODS

Fish, location of infection, and prevalence

A total of 29 adult specimens of the marine stingray, *Raja clavata* Linnaeus, 1758 (Chondrichthyes, Rajiidae) (Portuguese common name “Raia Lenga”) were collected during the time period between November 2010 and July 2011, in the Portuguese Northwest Atlantic coast (39° 34′ N/ 48° 25′ W), near the city of Porto, Portugal. All specimens underwent gender determination, followed by necropsy and macroscopic observation of the gills, muscles, liver, intestine and gall bladder. Preliminary microscopic analysis of these organs and tissues revealed the gall bladder as the sole infected organ.

The bile of the parasitized fishes was collected and prepared for observation by LM, including Nomarski differential interference contrast (DIC) optics, TEM and SEM, as well as for molecular biology procedures. Free spores were measured with an ocular micrometer adapted to the photomicroscope.

Transmission electron microscopy

For TEM, free spores and plasmodia isolated from the bile were fixed in 5% glutaraldehyde buffered in 0.2 M sodium cacodylate (pH 7.4) at 4 C for 24 h, washed overnight in buffer at the same temperature, and post-fixed in 2% osmium tetroxide with the same buffer for 24 h at 4 C. Dehydration in an ascending ethanol series and propylene oxide, was followed by inclusion in EPON. Semi-thin sections were stained with methylene blue-Azure II for LM. Ultrathin sections were double-stained with uranyl acetate and lead citrate, observed and photographed using a JEOL 100CXII TEM (JEOL, Ltd., Tokyo, Japan) operated at 60 kV.

Scanning electron microscopy

For SEM, free spores and plasmodia isolated from the bile were fixed in 5% glutaraldehyde buffered in 0.2 M sodium cacodylate (pH 7.4) at 4 C for 24 h, washed in the same buffer at the same temperature, dehydrated in an ascending ethanol series, critical point dried, coated with a gold-palladium alloy (60%) and observed and photographed in a JSM-6301F SEM (JEOL, Ltd., Tokyo, Japan) operated at 15 kV.

DNA isolation and PCR amplification

Free spores and plasmodia obtained from the bile were fixed and preserved in 80% ethanol at 4 C before genomic DNA extraction, which was performed using a GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma, St. Louis, Missouri), following the manufacturer's instructions for animal tissue. The DNA was stored in 50 µl of TE buffer at -20 C until further use. Amplification of the SSU rDNA gene was achieved by using both universal and specific primers: the 5'-end with the primers 18e/MyxospecR, the central region of the gene with the primers MyxospecF/ChloromyxR1, and the 3'-end with the primers ChloromyxF1/18r (Table I). PCR was carried out in 50-µl reactions using 10 pmol of each primer, 10 nmol of each dNTP, 2.5 mM MgCl₂, 5 µl 10× *Taq* polymerase buffer, 1.5 units *Taq* DNA polymerase (Finnzymes Products), and 3 µl of the genomic DNA. The reactions were run on a Hybaid PxE Thermocycler (Thermo Electron Corporation, Milford, Massachusetts) with initial denaturation at 95 C for 3 minutes, followed by 35 cycles of 94 C for 45 seconds, 53 C for 45 seconds and 72 C for 90 seconds. The final elongation step was performed at 72 C for 7 minutes. Five-microliter aliquots of the PCR products were electrophoresed through a 1% agarose 1× tris-acetate-EDTA buffer (TAE) gel stained with ethidium bromide.

TABLE I. Polymerase chain reaction primers used for amplification and sequencing of the small subunit ribosomal DNA of *Chloromyxum clavatum* n. sp.

Name	Sequence (5'-3')	Position	Pared with	Source
18e	CTG GTT GAT CCT GCC AGT	1	MyxospecR, 18r	Hillis and Dixon, 1991
MyxospecF	TTC TGC CCT ATC AAC TTG TTG	312	ChloromyxR1	Fiala 2006
ChloromyxF1	CTT AAA GGA ATT GAC GGA AGG	1209	18r	Azevedo et al., 2009
MyxospecR	CAA CAA GTT GAT AGG GCA GAA	332	18e	Azevedo et al., 2009
ChloromyxR1	CCT TCC GTC AAT TCC TTT AAG	1229	MyxospecF	Azevedo et al., 2009
18r	CTA CGG AAA CCT TGT TAC G	1832	18e, ChloromyxF1	Whipps et al., 2003

DNA sequencing

The PCR products for the SSU rDNA gene with an approximate size of 300 bp (18e/MyxospecR), 900 bp (MyxospecF/ChloromyxR1) and 600 bp (ChloromyxF1/18r) were sequenced directly. The sequencing reactions were performed using a BigDye Terminator v1.1 kit (Applied Biosystems, Carlsbad, California) and were run on an ABI3700 DNA analyzer (Perkin-Elmer, Applied Biosystems, Stabvida, Co., Oeiras, Portugal).

Distance and phylogenetic analysis

To evaluate the phylogenetic relationship of *Chloromyxum clavatum* n. sp. to other myxosporean species, 38 SSU rDNA sequences from Genbank were obtained and analyzed (Table II). The alignment was performed with ClustalW in MEGA 5 software (Tamura et al., 2011) with an opening gap penalty of 10 and a gap extension of 4 for both pairwise and multiple alignments. Subsequent phylogenetic and molecular evolutionary analyses were conducted using MEGA 5, with the 38 rDNA sequences for myxosporidian species and the outgroup species selected. Distance estimation was carried out using the Kimura-2 parameters model distance matrix for transitions and transversions. For the phylogenetic tree reconstruction, maximum parsimony analysis was conducted using the close neighbour interchange heuristic option with a search factor of 2 and random initial trees addition of 2,000 replicates. Bootstrap values were assessed over 100 replicates.

TABLE II. Sampling for the molecular analysis, including organ of infection and accession numbers from GenBank.

Myxosporidian species	Organ of infection	Accession number
<i>Chloromyxum auratum</i>	Gall bladder	AY971521
<i>Chloromyxum careni</i>	Kidney	HM641794
<i>Chloromyxum cristatum</i>	Gall bladder	GU471261
<i>Chloromyxum cyprini</i>	Gall bladder	AY604198
<i>Chloromyxum legeri</i>	Gall bladder	AY604197
<i>Chloromyxum leydigi</i>	Gall bladder	AY604199
<i>Chloromyxum leydigi</i>	Gall bladder	DQ377710
<i>Chloromyxum fluvatile</i>	Gall bladder	GU471265
<i>Chloromyxum riorajum</i>	Gall bladder	FJ624481
<i>Chloromyxum trijugum</i>	Gall bladder	AY954689
<i>Chloromyxum truttae</i>	Gall bladder	AJ581916
<i>Chloromyxum schurovi</i>	Kidney	AJ581917
<i>Henneguya salminicola</i>	Muscle	AF031411
<i>Henneguya zschokkei</i>	Muscle	AF378344
<i>Hoferellus gilsoni</i>	Urinary bladder	AJ582062
<i>Henneguya</i> sp. IF-2006	Peritoneum	DQ377706
<i>Myxidium anatum</i>	Bile ducts	EF602629
<i>Myxidium chelonarum</i>	Gall bladder	DQ377694
<i>Myxidium cuneiforme</i>	Gall bladder	DQ377709
<i>Myxidium giardi</i>	Kidney	AJ582213
<i>Myxidium hardella</i>	Kidney	AY688957
<i>Myxidium lieberkuehni</i>	Kidney	X76638
<i>Myxidium melleni</i>	Gall bladder	DQ003031
<i>Myxidium scripta</i>	Kidney	DQ851568
<i>Myxobilatus gasterostei</i>	Kidney	AY495703
<i>Myxobolus cerebrales</i>	Cartilage	EF370481
<i>Myxobolus longisporus</i>	Gills	AY364637
<i>Myxobolus osburni</i>	Pancreas	AF378338
<i>Sphaeromyxa longa</i>	Gall bladder	DQ377691
<i>Sphaerospora molnari</i>	Gills	AF378345
<i>Sphaerospora oncorhynchi</i>	Kidney	AF201373
<i>Sphaeromyxa zaharoni</i>	Gall bladder	AY538662
<i>Sphaerospora</i> sp.	Kidney	AY735411
<i>Soricimyxum fegati</i>	Liver	EU232760
<i>Thelohanellus hovorkai</i>	Connective tissue	DQ231155
<i>Zschokkella nova</i>	Gall bladder	DQ377688
<i>Zschokkella parasiluri</i>	Gall bladder	DQ377689
<i>Zschokkella</i> sp.	Urinary bladder	AJ581918
<i>Enteromyxum leei</i>	Intestine	AF411334
<i>Kudoa thyrsites</i>	Muscle	AY941819

DESCRIPTION***Chloromyxum clavatum* n. sp.**

(Figs. 1-4)

Diagnosis: The collected and analyzed stingrays did not present external symptoms of infection or disease. Upon necropsy, the macroscopic observation of several internal organs and tissues showed hypertrophy of the gall bladder in several specimens. Microscopic analysis of those same organs and tissues confirmed the parasitic infection only for the hypertrophied gall bladders, which contained brownish colored bile. Several mature spores, with spherical to subspherical shape (Figs. 1A, B), as well as irregularly rounded plasmodia (Fig. 1C), were observed floating free in the bile of the infected gall bladders.

Description of the plasmodia and spores: Monosporic plasmodia measured on average up to ~ 32 µm and presented a slightly irregular cell membrane, due to the presence of few peripheral projections (Fig. 1C, E). Some early sporogonic developmental stages, namely generative cells, surrounded by numerous mitochondria, Golgi complexes, lipidic droplets and

greenish reserve bodies (Fig. 1C-E) were observed within these vegetative structures. Most generative cells presented a well-defined nucleus and nucleolus (Fig. 1D, E). Plasmodia containing the more advanced stages of sporogonic development were not

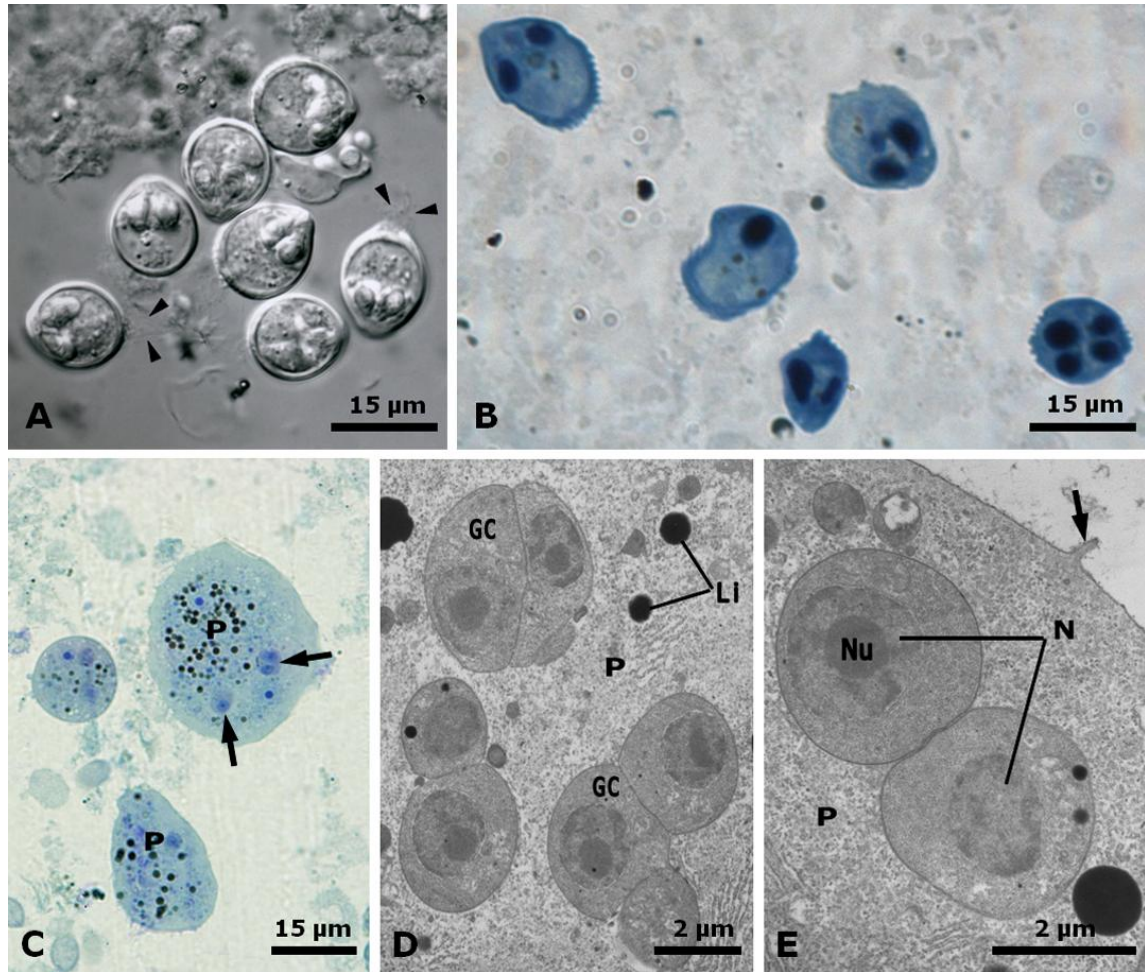


FIGURE 1. Light and transmission electron micrographs of *Chloromyxum clavatum* n. sp., found infecting the gall bladder of the cartilaginous fish *Raja clavata*. **A.** Free fresh mature spores observed in DIC optics, displaying spherical to subspherical shape and the presence of tapering caudal filamentous projections (arrowheads). **B.** Semi-thin section of several spores in longitudinal and transversal view. **C.** Semi-thin section displaying small and slightly irregular rounded plasmodia (P), which contain early developmental stages (arrowheads). **D.** Ultrathin section of a plasmodium (P) displaying growing and proliferative generative cells (GC) surrounded by several lipidic droplets (Li). **E.** Detailed aspect of a plasmodium (P) showing two generative cells, each displaying its nucleus (N). The nucleolus (Nu) is also visible in one of them. In the plasmodium membrane, a singular peripheral projection is observed (arrow).

observed. Mature spores appeared only floating free in the bile and were spherical to subspherical with a pointed anterior end, and measured $14.4 \pm 0.5 \mu\text{m}$ ($n = 25$) in length, $11.9 \pm 0.5 \mu\text{m}$ ($n = 25$) in width and $9.4 \pm 0.5 \mu\text{m}$ ($n = 15$) in thickness (Figs. 1A, B). The spores wall measured about 190 nm and was composed of two equally sized valves (Figs. 2A, B), adhering together along a prominent sutural line (Figs. 2A, E). Some DIC and TEM observations showed the existence of surface ridges projecting from the posterior end of the spores (Figs. 1A, 2A, F). SEM allowed the recognition of their pattern. Each valve possessed six to eight elevated surface ridges located in its posterior half, parallel to the basal portion of the sutural line (Figs. 3A-C) and coalescing towards the apical pole (Fig. 3B).

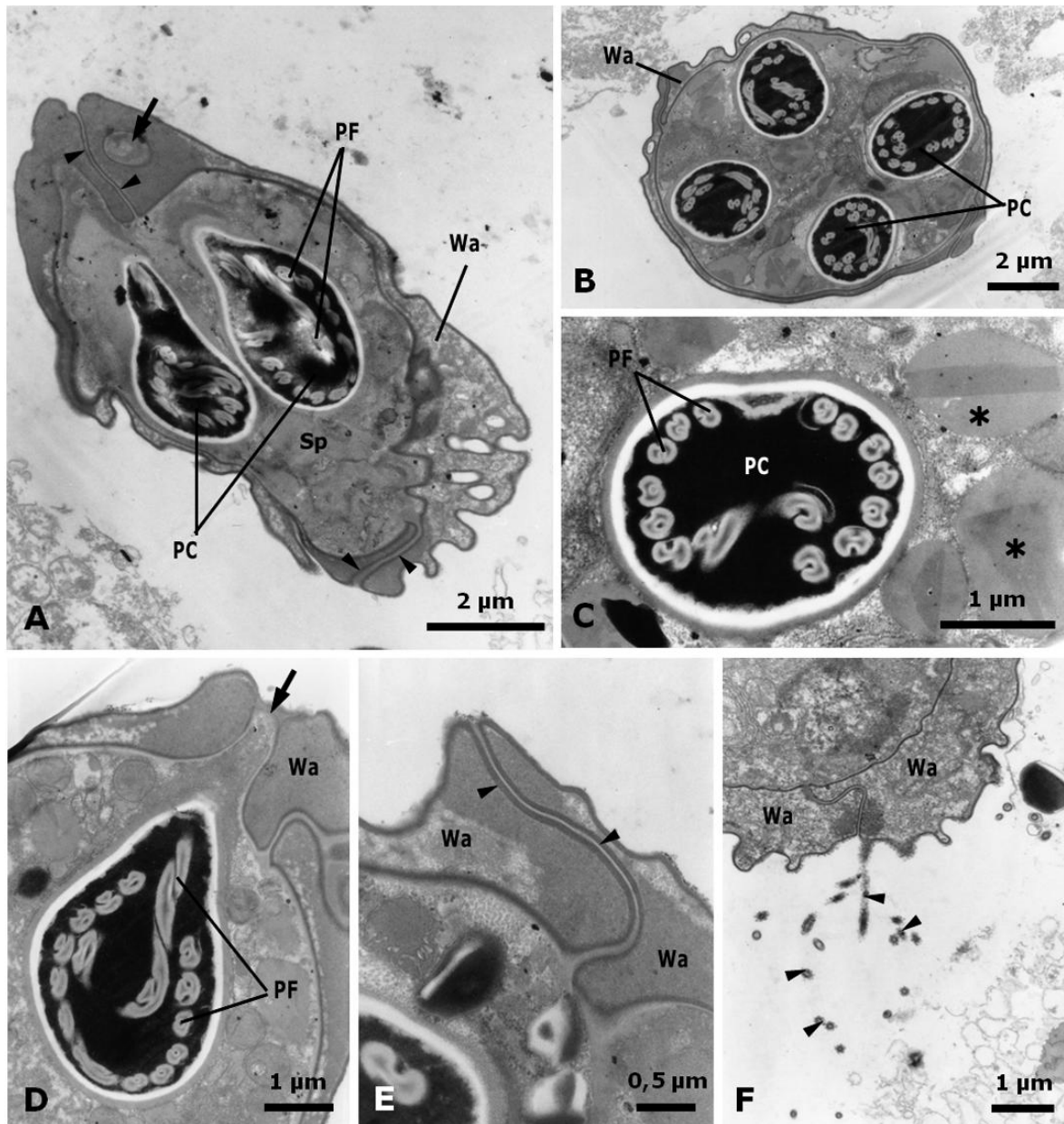


FIGURE 2. Transmission electron micrographs of *Chloromyxum clavatum* n. sp., found infecting the gall bladder of the cartilaginous fish *Raja clavata*. **A**. Longitudinal section of a spore showing several structural aspects, such as the spore wall (Wa), polar capsules (PC) and sporoplasm (Sp). The spore wall appears comprised of two valves joined along a prominent sutural line (arrowheads). Each polar capsule contains a coiled polar filament (PF). In the anterior end of the spore, the apical end of a polar capsule can be observed (arrow). **B**. Transverse section of a spore allowing the visualization of the four polar capsules (PC) at the same level, as well as the spore wall (Wa). **C**. Polar capsule (PC) in transverse section, showing the number of coils of the polar filament (PF). Close to the polar capsule, paracrystalline structures are contained within vesicles (*). **D**. Polar capsule in longitudinal section, displaying the oblique arrangement of the polar filament (PF). The apical end of the polar capsule (arrow) is depicted in the anterior portion of the spore wall (Wa). **E**. Detailed aspect of the two valves constituting the spore wall (Wa) and adhering together along a prominent sutural line (arrowheads). **F**. Oblique and transverse sections of several caudal filaments (arrowheads), one of which is attached to the spore wall (Wa).

In the same manner, the bundle of tapering caudal filamentous projections attached to the basal portion of each valve and perceptible in the ultrathin sections (Fig. 2F), were much

more detailed in SEM (Figs. 3B, C). The precise number and length of caudal filaments could not be determined since these structures were highly intertwined. However, it is most probable that they possess variable length. The projections were constituted of the same material as the valves, a continuous layer of external and internal dense material delimiting a middle lighter area (Figs. 2E, F), and originated from the sutural line and the more basal ridge of the 2 valves (Fig. 3A). Some caudal filaments appeared anastomosed, forming conical valvar insertions (Fig. 3C).

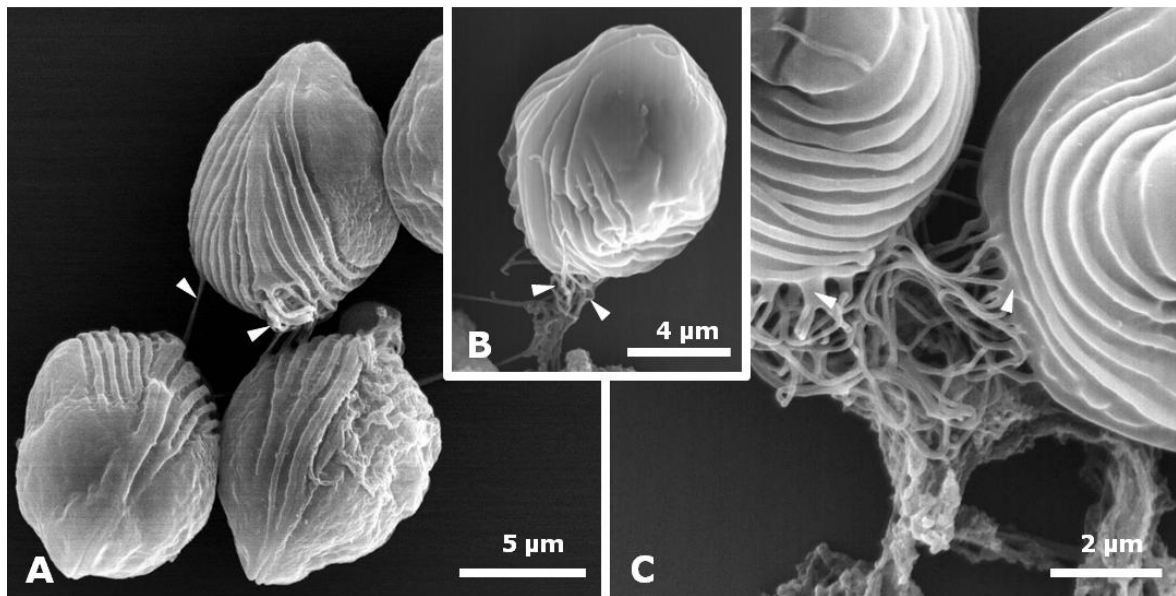


FIGURE 3. Scanning electron micrographs of *Chloromyxum clavatum* n. sp., found infecting the gall bladder of the cartilaginous fish *Raja clavata*. **A.** Spores showing the surface pattern formed by the ridges located in the basal portion of each valve, as well as caudal filaments (arrowheads) projecting from the posterior end of the spores, more precisely from the more basal ridge and sutural line of each valve. **B.** Singular spore showing a bundle of caudal filaments (arrowheads) projecting from its posterior end. **C.** Detailed aspect of the caudal filaments, some of which appear anastomosed (arrowheads) close to the valvar insertion.

Four pyriform equal-sized polar capsules were observed at the same level within the spores and measured 5.5 ± 0.4 ($n = 25$) in length and 2.9 ± 0.5 μm ($n = 25$) in diameter (Figs. 2A-D). Each polar capsule possessed an isofilar polar filament obliquely arranged and forming an angle of about 70° to its longitudinal axis. Seven to eight coils were observed (Figs. 2C, D), as the polar filament formed a loop along the inner wall of the polar capsule, before starting to coil from the apical to the basal portion (Fig. 2D). Pore-like shaped structures, consistent with the apical end of the polar capsules, were visible in the anterior portion of the spore's wall (Figs. 2A, D). Paracrystallin structures, possibly of protein nature, were observed within vesicles that appeared close to the polar capsules (Fig. 2C). The hardly visible sporoplasm was irregular in shape and displayed two nuclei randomly positioned in its matrix (Fig. 2A). The cytoplasm contained numerous

mitochondria, lipid globules, small vesicles and some sporoplasmosomes. The spore morphology of our parasite is represented in a schematic drawing (Fig. 4A, B), providing a better perception of our overall morphological and ultrastructural observations.

Molecular characterization and phylogeny: The amplified sequences were assembled and the resulting consensus DNA sequence of the SSU rDNA gene, which was 1844 bp in length, was deposited in GenBank (Accession number #####). A total of 38 SSU rDNA sequences, including those with the higher BLAST scores, were aligned with the SSU rDNA sequence obtained for *Chloromyxum clavatum* n. sp. The resulting alignment consisted of 1740 positions after trimming the 3'-end (870 ambiguously aligned positions were excluded).

TABLE III. Comparison of some small subunit ribosomal DNA sequences: percentage of identity (top diagonal) and pairwise distance (bottom diagonal) obtained by Kimura-2 parameter analysis.

	<i>C. clavatum</i>	<i>C. leydigii</i> 1	<i>C. leydigii</i> 2	<i>C. riorajum</i>	<i>C. cristatum</i>	<i>C. auratum</i>	<i>C. cyprini</i>	<i>C. fluviatile</i>	<i>C. truttae</i>	<i>C. trijugum</i>	<i>C. careni</i>	<i>C. legeri</i>	<i>C. schurovi</i>
<i>Chloromyxum clavatum</i> (#####)	-	99.0	98.8	96.5	82.2	82.0	82.0	81.7	80.9	80.6	78.5	77.8	77.5
<i>Chloromyxum leydigii</i> 1 (DQ377710)	0.010	-	99.6	96.7	83.0	82.7	82.7	82.2	81.4	81.1	79.3	79.3	78.1
<i>Chloromyxum leydigii</i> 2 (AY604199)	0.012	0.004	-	96.7	83.0	82.7	82.7	82.2	81.1	80.9	79.6	78.0	78.1
<i>Chloromyxum riorajum</i> (FJ624481)	0.035	0.033	0.033	-	82.2	82.0	82.0	81.5	80.9	80.6	78.2	78.1	77.8
<i>Chloromyxum cristatum</i> (GU471261)	0.178	0.170	0.170	0.178	-	99.8	99.8	98.6	98.6	90.4	87.7	83.1	87.5
<i>Chloromyxum auratum</i> (AY971521)	0.180	0.173	0.173	0.180	0.002	-	99.6	98.4	95.4	90.1	87.7	83.1	87.5
<i>Chloromyxum cyprini</i> (AY604198)	0.180	0.173	0.173	0.180	0.002	0.004	-	98.4	95.4	90.1	87.5	82.8	87.3
<i>Chloromyxum fluviatile</i> (GU471265)	0.183	0.178	0.178	0.185	0.014	0.016	0.016	-	96.1	90.1	87.5	83.6	87.5
<i>Chloromyxum truttae</i> (AJ581916)	0.191	0.186	0.189	0.191	0.044	0.046	0.046	0.039	-	89.0	86.7	83.9	87.3
<i>Chloromyxum trijugum</i> (AY954689)	0.194	0.189	0.191	0.194	0.096	0.099	0.099	0.099	0.110	-	86.5	82.8	85.9
<i>Chloromyxum careni</i> (HM641794)	0.215	0.207	0.204	0.218	0.123	0.123	0.125	0.125	0.133	0.135	-	82.4	85.5
<i>Chloromyxum legeri</i> (AY604197)	0.222	0.207	0.220	0.219	0.169	0.169	0.172	0.164	0.161	0.172	0.176	-	82.8
<i>Chloromyxum schurovi</i> (AJ581917)	0.225	0.219	0.219	0.222	0.125	0.125	0.127	0.125	0.127	0.141	0.145	0.172	-

Pairwise comparisons among the SSU rDNA sequences showed maximal similarity between all *Chloromyxum* species: *C. leydigi* (99.0 and 98.8%), *C. riorajum* (96.5%), *C. auratum* (82.0%), *C. cyprini* (82.0%), *C. cristatum* (82.2%), *C. truttae* (80.9%), *C. trijugum* (80.6%), *C. schurovi* (77.5%), *C. legeri* (77.8%), *C. fluviatile* (81.7%) and *C. careni* (78.5%) (Table III). Maximum parsimony analysis of the SSU rDNA gene sequences places *Chloromyxum clavatum* n. sp. clustering in the clade comprising its most closely related species: *C. leydigi* (AY604199, DQ377710) and *C. riorajum* (FJ624481) with a 100% bootstrap support.

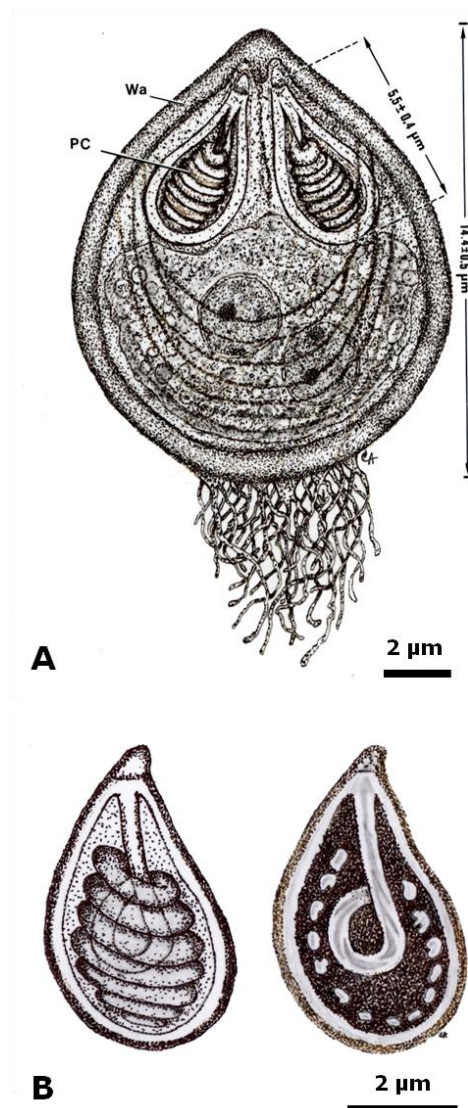


FIGURE 4. Schematic drawing of *Chloromyxum clavatum* n. sp., found infecting the gall bladder of the cartilaginous fish *Raja clavata*. **A.** Spore in longitudinal section showing the internal and external organization described in the text. **B.** Detailed longitudinal sections of the polar capsule, depicting the arrangement of the polar filament.

Taxonomic summary

Type-host: *Raja clavata* Linnaeus, 1758 (Chondrichthyes: Rajidae), with a total length range between 51-100 cm.

Type-locality: Northwest Portuguese Atlantic coast (39° 34' N/48° 25' W), near the city of Porto, Portugal.

Site of infection: Plasmodia and mature spores were found floating free in the gall bladder.

Prevalence of infection: Fourteen of 29 fishes (48.3%), in a host sample containing a greater number of females (11/20 females; 3/9 males).

Type-material: One glass slide with semi-thin sections displaying mature spores and the earliest stages of plasmodial development of the hapantotype was deposited in the International Protozoan Type Slides Collection at the Smithsonian Institute, Washington, D.C., under #####.

Etymology: The specific epithet "*clavatum*" derives from the specific epithet of the host species, *Raja clavata*.

Remarks

The overall morphological characters displayed by the spores and plasmodia here observed, as well as host species and tissue tropism, identifies our parasite as belonging to the genus

Chloromyxum, according to the classification proposed by Lom and Dyková (2006). Similarly to other *Chloromyxum* species reported from marine cartilaginous fish, the parasite infecting *Raja clavata* was coelozoic in the gall bladder, with plasmodia and mature spores floating free in the bile (Pinto, 1928; Jameson, 1929; Kuznetsova, 1977; Kovaljova, 1988; Gioia and Cordeiro, 1996; Azevedo et al., 2009). Morphological comparison to those same species allowed the recognition and differentiation of some specific aspects referring to the spores shape and structure. Compared to the observations of our parasite: *C. leydigi* spores and polar capsules are significantly smaller; *C. ovatum* spores display different surface ridges pattern; *C. transversocostatum* spores are unique in displaying transversal concentric surface ridges; and *C. riorajum* has only 3 to 4 (rarely 5) elevated ridges projecting from the posterior end of the spore and no anastomosed caudal filaments (Kudo, 1919; Pinto, 1928; Jameson, 1929; Kuznetsova, 1977; Lom and Dyková, 1992; Gioia and Cordeiro, 1996; Azevedo et al., 2009). Other four species belonging to the genus *Chloromyxum* were reported from cartilaginous fishes of the Atlantic coast of Africa (Kovaljova, 1988). However, *C. dogieli*, *C. lissosporum*, *C. schulmani* and *C. striatellus* are described based only in diagrammatic drawings that account for spores dimension and surface ridges pattern, but make no reference to the number or organization of the caudal filaments, which appear to be different from ours. Other marine species of the genus *Chloromyxum*, include *C. sphyrnae* from the Brazilian Atlantic coast (Gioia and Cordeiro, 1996), *Chloromyxum* sp. from the urinary bladder of flatfish *Paralichthys adspersus* from the Northern Pacific coast of Chile (Oliva et al., 1996), and *C. menticirrho* infecting the urinary bladder of *Menticirrhus americanus* from the South Atlantic coast of Brazil (Casal et al., 2009). The description made for the two first species is once more very limited, based only on LM and diagrammatic drawings; the description of *C. menticirrho* is based on TEM and SEM observations that demonstrate the lack of caudal filaments attached to the spores' wall. Comprising all of these comparative morphological and ultrastructural features, namely spore and polar capsules dimensions, arrangement and number of coils of the polar filaments, and the unique organization of the caudal filaments, not forgetting specificity for host and tissue tropism, we further suggest our parasite as a new species within the genus *Chloromyxum*, herein named *Chloromyxum clavatum*.

Despite the use of specific morphological characters reliable for the description of individual species, classification by microscopic analysis is insufficient and a molecular approach is most advised, especially in broad and polyphyletic taxa, such as the genus *Chloromyxum* (Lom and Dyková, 1993, 2006; Andree et al., 1999; Kent et al., 2001; Fiala and Dyková, 2004). The SSU rDNA gene has been proved as a reliable marker for discerning the

correct species evolution and phylogeny of the Myxosporea (Bartošová et al., 2009; Fiala and Bartošová, 2010). However, GenBank provides SSU rDNA information for only 9 *Chloromyxum* species infecting fishes: *C. leydigi* (AY604199) from the gall bladder of *Torpedo marmorata* captured in the Mediterranean Sea (Fiala and Dyková, 2004); *C. leydigi* (DQ377710) from the gall bladder of *Centroscymnus coelolepsis* of the North Atlantic (Fiala, 2006); *C. auratum* (AY971521) and *C. trijugum* (AY954689) from the gall bladder of *Carassius auratus* and *Pomoxis nigromaculatus*, respectively and from the United States (Hallett et al., 2006); *C. cyprini* (AY604198) and *C. legeri* (AY604197) from the gall bladder of *Hypophthalmichthys molitrix* and *Cyprinus carpio*, respectively and from the Czech Republic (Fiala and Dyková, 2004); *C. truttae* (AJ581916) from the gall bladder and *C. schurovi* (AJ581917) from the kidney of *Salmo salar* in Scotland (Holzer et al., 2004); *C. riorajum* (FJ624481) from the gall bladder of the stingray *Rioraja agassizii* in the South Atlantic coast of Brazil (Azevedo et al., 2009); *C. fluviatile* (GU471262) from the gall bladder of *Hypophthalmichthys molitrix*, and *C. cristatum* (GU471261) from the gall bladder of *Hypophthalmichthys molitrix* and *Ctenopharyngodon idella*, both from the Czech Republic (Bartošová and Fiala, 2011). *C. careni* (HM641794) SSU rDNA is also sequenced from the kidney of *Megophrys nasuta*, the Malayan horned frog from Indonesia (Jirků et al., 2011). Among the available sequences, only *C. leydigi* from both *Torpedo marmorata* and *Centroscymnus coelolepsis*, and *C. riorajum* infect the gall bladder of marine cartilaginous fish. The remaining available sequences refer to freshwater species, with *C. cyprini* and *C. cristatum* being synonyms (Bartošová and Fiala, 2011). The combined analysis of these sequences along with the one obtained for *Chloromyxum clavatum* n. sp. shows the phylogenetic relationships for maximum parsimony in concordance with previously published cladograms (Fiala and Dyková, 2004; Fiala, 2006; Holzer et al., 2006; Azevedo et al., 2009; Bartošová and Fiala, 2011; Jirků et al., 2011). As expected, bootstrap value is higher between our sequence and the sequences from *Chloromyxum* species also described from marine cartilaginous fish. The results from the pairwise sequence analysis also present higher similarity between these species.

DISCUSSION

In spite of the great number of described myxosporean species, only a few have been reported from marine fish hosts, probably reflecting the general lack of studies concerning the marine environment. Also, most reports were made early on and therefore lack the employment of more sophisticated methods to species classification and description (Pinto, 1928; Jameson, 1929; Kuznetsova, 1977; Kovaljova, 1988; Gioia and Cordeiro, 1996).

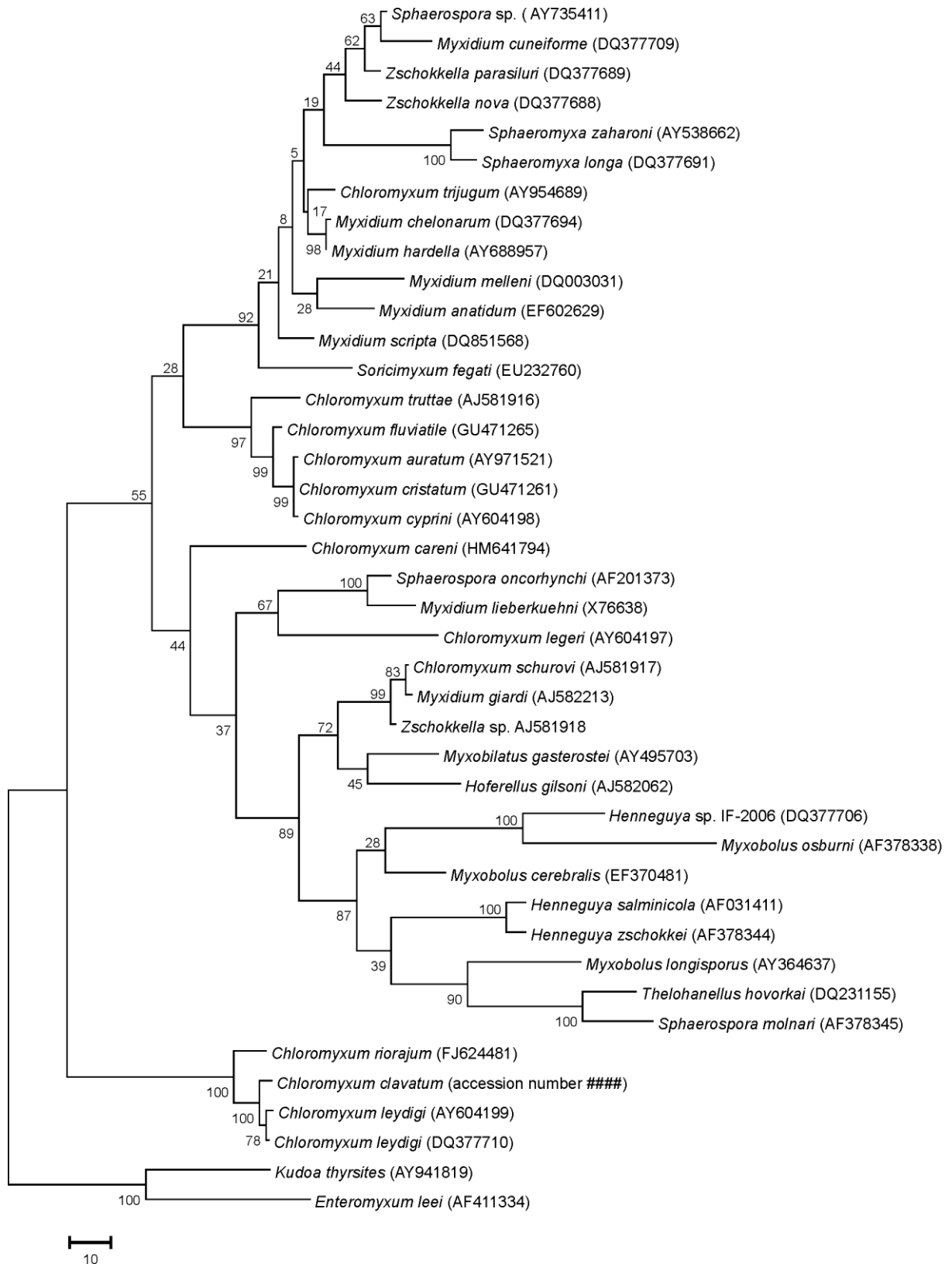


FIGURE 5. Maximum parsimony tree for the small subunit ribosomal DNA sequences of *Chloromyxum clavatum* n. sp. and other selected myxosporean species. The numbers on the branches are bootstrap confidence levels on 100 replicates. GenBank accession numbers in parentheses after the species name; scale is given under the tree.

The use of comparative morphological, ultrastructural and molecular data allows the frequent erection of new species. In fact, two new species of the genus *Chloromyxum* were recently described from marine species of the Brazilian Atlantic coast (Azevedo et al., 2009; Casal et al., 2009). Our study presents a new *Chloromyxum* species found infecting a marine cartilaginous fish of the Northwest Portuguese Atlantic coast. The employment of combined microscopic procedures, including LM (DIC), TEM and SEM, provided many morphological and ultrastructural comparative features. Structural comparison to other species recognized our parasite as a new species and further noticed that only the *Chloromyxum* species inhabiting the marine environment display attached caudal filaments to the posterior end of the spore's wall. Contradictorily, caudal appendages are thought to be characters typical of freshwater myxosporean species. Similarly, surface ridges are also considered a freshwater feature, and yet most reports concerning marine *Chloromyxum* species refer to their presence (Pinto, 1928; Jameson, 1929; Kuznetsova, 1977; Kovaljova, 1988; Gioia and Cordeiro, 1996; Azevedo et al., 2009; Casal et al., 2009). Recent studies consider that these features evolved in the ancestor of the freshwater lineage after the separation between the marine and freshwater lineages (Fiala and Bartošová, 2010). Considering that *C. leydigi*, *C. riorajum* and *C. clavatum* n. sp. together form a clade located at the basis of the freshwater clade, these species most probably represent the link between the freshwater and marine clades, having kept morphological characteristics that later became typical of freshwater myxosporeans. Nevertheless, further molecular analysis is warranted in order to assess character evolution (Fiala and Dyková, 2004; Fiala, 2006; Holzer et al., 2006; Bartošová et al., 2009; Fiala and Bartošová, 2010).

Most myxosporean genera are poly/paraphyletic, including the genus *Chloromyxum* (Kent et al., 2011). The data nowadays available in GenBank concords with this affirmation since most *Chloromyxum* sequences appear as paraphyletic groups, with the exception of the freshwater species *C. auratum*, *C. cyprini*, *C. cristatum* and *C. truttae*, as well as the marine species infecting cartilaginous fishes. Furthermore, molecular data divides Myxosporea into two main clades: freshwater and marine species. This division may result from the unique presence of several insertions in the V7 region of the SSU rDNA gene of freshwater myxosporeans, which have longer sequences than marine species (Kent et al., 2001; Fiala and Dyková, 2004; Fiala, 2006). The length differences caused by insertions in the variable regions are also true for the LSU rDNA gene (Bartošová et al., 2009). Consistent with previous results, the marine species *Chloromyxum clavatum* n. sp., *C. leydigi* and *C. riorajum* lack insertions in the V7 region of the SSU rDNA gene. However, they constitute an exception to the myxosporean division into the marine and freshwater

clades, since they cluster together to form a clade positioned at the basis of the freshwater clade. Again, the most plausible explanation for this phylogenetic positioning concludes *C. leydigi*, *C. riorajum* and *C. clavatum* n. sp. as representative of the adaptation of a common myxosporean ancestor from the marine to the freshwater environment (Fiala and Dyková, 2004; Fiala, 2006; Holzer et al., 2006), resulting in the formation of a direct marine lineage closely related to the first myxosporean and a parallel freshwater lineage (Fiala and Bartošová, 2010).

Maximum parsimony trees have also shown that most myxosporean taxa, including the genus *Chloromyxum*, cluster according to their host tissue tropism, with well-defined gall bladder infecting clades for both freshwater and marine clades (Holzer et al., 2004, 2006; Fiala, 2006; Bartošová et al., 2009; Fiala and Bartošová, 2010; Jirků et al., 2011). *C. clavatum* n. sp. is no exception and as expected clusters within the clade containing the other sequenced parasites of this genus infecting the gall bladder of marine cartilaginous fish species: *C. leydigi* and *C. riorajum*. Together, they form a clade with a stronger phylogenetic relationship towards freshwater myxosporean species infecting the gall bladder than to myxosporean histozoic species. As a consequence, *C. clavatum* appears more closely related not only to freshwater *Chloromyxum* species infecting the gall bladder but also to several freshwater species of other genera with the same tissue tropism, opposed to the *Chloromyxum* species that cluster with freshwater histozoic species, namely *C. schurovi*, *C. legeri* and *C. careni*.

Despite the increasing number of available SSU rDNA sequences, the proportion of sequenced species is still much reduced, especially for marine species. Hopefully, the acquisition of further molecular data will allow the discernment of several incongruences regarding taxonomic and phylogenetic issues. For now, our study provides yet another valuable data for future references.

ACKNOWLEDGMENTS

This work was partially supported by Eng.º A. Almeida Foundation (Porto, Portugal). We would like to thank the technical assistance of Joana and João Carvalheiro (ICBAS/UP), as well as Dr. Fernanda Castilho, Mr. Emanuel Pombal and Mr. Paulo Castro (IPIMAR-Matosinhos, Portugal) for providing the biological material and facilities for preliminary analysis of specimens. This work complies with the current laws of our country.

LITERATURE CITED

- Andree, K. B., C. Székely, K. Molnár, S. J. Gresoviac, and R. P. Hedrick. 1999. Relationships among members of the genus *Myxobolus* (Myxozoa: Bivalvidae) based on small subunit ribosomal DNA sequences. *Journal of Parasitology* **85**: 68-74.
- Azevedo, C., G. Casal, P. Garcia, P. Matos, L. Teles-Grilo, L., and E. Matos. 2009. Ultrastructural and phylogenetic data of *Chloromyxum riorajum* sp. nov. (Myxozoa), a parasite of the stingray *Rioraja agassizii* in Southern Brazil. *Diseases of Aquatic Organisms* **85**: 41-51.
- Bartošová, P., and I. Fiala. 2011. Molecular evidence for the existence of cryptic species assemblages of several myxosporeans (Myxozoa). *Parasitology Research* **108**: 573-583.
- Bartošová, P., I. Fiala, and V. Hypša. 2009. Concatenated SSU and LSU rDNA data confirm the main evolutionary trends within myxosporeans (Myxozoa: Myxosporea) and provide an effective tool for their molecular phylogenetics. *Molecular Phylogenetics and Evolution* **53**: 81-93.
- Casal, G., P. Garcia, P. Matos, E. Monteiro, E. Matos, and C. Azevedo. 2009. Fine structure of *Chloromyxum menticirrhoi* n. sp. (Myxozoa) infecting the urinary bladder of the marine teleost *Menticirrhus americanus* (Sciaenidae) in Southern Brazil. *European Journal of Protistology* **45**: 139-146.
- Diamant, A., C. M. Whipps, and M. L. Kent. 2004. A new species of *Sphaeromyxa* (Myxosporea: Sphaeromyxina: Sphaeromyxidae) in devil firefish, *Pterois miles* (Scorpanidae), from the northern Red Sea: morphology, ultrastructure, and phylogeny. *Journal of Parasitology* **90**: 1434-1442.
- Duncan, A. E., M. M. Garner, J. L. Bartholomew, T. A. Reichard, and R. W. Nordhausen. 2004. Renal myxosporidiasis in Asian horned frog (*Megophrys nasuta*). *Journal of Zoo and Wildlife Medicine* **35**: 381-386.
- Eszterbauer, E. 2004. Genetic relationship among gill-infecting *Myxobolus* species (Myxosporea) of cyprinids: molecular evidence of importance of tissue-specificity. *Disease of Aquatic Organisms* **58**: 35-40.

Fiala, I. 2006. The phylogeny of Myxosporea (Myxozoa) based on small subunit ribosomal RNA gene analysis. *International Journal of Parasitology* **36**: 1521-1534.

Fiala, I., and P. Bartošová. 2010. History of myxozoan character evolution on the basis of rDNA and EF-2 data. *BMC Evolutionary Biology* **10**: 228.

Fiala, I., and I. Dyková. 2004. The phylogeny of marine and freshwater species of the genus *Chloromyxum* Mingazzini, 1890 (Myxosporea: Bivalvulida) based on small subunit ribosomal RNA gene sequences. *Folia Parasitologica* **51**: 211-214.

Gioia, I., and N. S. Cordeiro. 1996. Brazilian myxosporidians' check-list (Myxozoa). *Acta Protozoologica* **35**: 137-149.

Hallett, S. L., S. D. Atkinson, R. A. Holt, C. R. Banner, and J. L. Bartholomew. 2006. A new myxozoan from feral goldfish (*Carassius auratus*). *Journal of Parasitology* **92**: 357-363.

Hillis, D. M., and M. T. Dixon. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *The Quarterly Review of Biology* **66**: 411-453.

Holzer, A. S., C. Sommerville, and R. Wootten. 2004. Molecular relationships and phylogeny in a community of myxosporeans and actinosporeans based on their 18S rDNA sequences. *International Journal of Parasitology* **34**: 1099-1111.

Holzer A. S., C. Sommerville, and R. Wootten. 2006. Molecular identity, phylogeny and life cycle of *Chloromyxum schurovi* Shul'man and Ieshko 2003. *Parasitology Research* **99**: 90-96.

Jameson, A. P. 1929. Myxosporidia from Californian fishes. *Journal of Parasitology* **16**: 59-68.

Jirků, M., P. Bartošová, A. Kodádková, and F. Mutschmann. 2011. Another chloromyxid lineage: molecular phylogeny and redescription of *Chloromyxum careni* from the Asian horned frog *Megophrys nasuta*. *Journal of Eukaryotic Microbiology* **58**: 50-59.

- Jirků, M., M. G. Bolek, C. M. Whipps, J. Janovy, M. L. Kent, and D. Modrý. 2006. A new species of *Myxidium* (Myxosporea: Myxidiidae), from the western chorus frog, *Pseudacris triseriata triseriata*, and Blanchard's cricket frog, *Acris crepitans Blanchardi* (Hylidae), from eastern Nebraska: morphology, phylogeny, and critical comments on amphibian *Myxidium* taxonomy. *Journal of Parasitology* **92**: 611-619.
- Kent, M. L., K. B. Andree, J. L. Bartholomew, M. El-Matbouli, S. S. Desser, R. H. Devlin, S. W. Feist, R. P. Hedrick, R. W. Hoffmann, J. Khattra, S. L. Hallett, R. J. G. Lester, M. Longshaw, O. Palenzeula, M. E. Siddall, and C. Xiao. 2001. Recent advances in our knowledge of the Myxozoa. *Journal of Eukaryotic Microbiology* **48**: 395-413.
- Kent, M. L., J. Khattra, R. P. Hedrick, and R. H. Devlin. 2000. *Tetracapsula renicola* n. sp. (Myxozoa: Saccosporidae); the PKX myxozoan – the cause of proliferative kidney disease of salmonid fishes. *Journal of Parasitology* **86**: 103-111.
- Kovaljova, A. A. 1988. Myxosporidia of the genus *Chloromyxum* (Cnidospora, Myxosporea) of cartilaginous fish from the Atlantic coast of Africa. *Parazitologiya* **22**: 384-388. (In Russian)
- Kudo, R. R. 1919. Studies on Myxosporidia. A synopsis of genera and species of Myxosporidia. *Illinois Biological Monographs* **5**: 241-503.
- Kuznetsova, I. G. 1977. Myxosporidians of Chondrostei from the Patagonian shelf. *Parazitologiya* **11**: 74-77. (In Russian)
- Lom, J., and I. Dyková. 1992. Myxosporidia (Phylum Myxozoa). *In* *Protozoan Parasites of Fishes*, vol. 26, *Developments in Aquaculture and Fisheries Science*, Elsevier, Amsterdam, The Netherlands, p. 159-235.
- Lom, J., and I. Dyková. 1993. Scanning electron microscopic revision of common species of the genus *Chloromyxum* (Myxozoa: Myxosporea) infecting European freshwater fishes. *Folia Parasitologica* **40**: 161-174.
- Lom, J., and I. Dyková. 2006. Myxozoan genera: definition and notes on taxonomy, life-cycle terminology and pathogenic species. *Folia Parasitologica* **43**: 1-36.

Oliva, M. E., R. E. Castro, and R. Burgos. 1996. Parasites of the Flatfish *Paralichthys adspersus* (Steindachner, 1867) (Pleuronectiformes) from Northern Chile. *Memórias do Instituto Oswaldo Cruz, Rio de Janeiro* **91**: 301-306.

Pinto, C. 1928. Mixosporídeos e outros protozoários intestinais de peixes observados na América do Sul. *Arquivos do Instituto Biológico, São Paulo* **1**: 101-126. (In Portuguese)

Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology Evolution* **28**: 2731-2739.

Upton, S. J., C. T. McAllister, and S. E. Trauth. 1995. A new species of *Chloromyxum* (Myxozoa, Chloromyxidae) from the gall bladder of *Eurycea* spp. (Caudata, Plethodontidae) in North America. *Journal of Wildlife Diseases* **31**: 394-396.

Whipps, C. M., R. D. Adlard, M. S. Bryant, R. J. G. Lester, V. Findlay, and M. L. Kent. 2003. First report of three *Kudoa* species from Eastern Australia: *Kudoa thyrsites* from Mahi mahi (*Coryphaena hippurus*), *Kudoa amamiensis* and *Kudoa minithyrsites* sp. nov. from sweeper (*Pempheris ypsilychnus*). *Journal of Eukaryotic Microbiology* **50**: 215-219.

CHAPTER 4

**Morphological and Ultrastructural Re-description of
Chloromyxum leydigi (Myxozoa: Myxosporea) from the Gall
Bladder of the Marine Cartilaginous fish *Torpedo marmorata*
(Chondrichthyes: Torpedinidae) from the Portuguese Atlantic
coast**

Folia Parasitologica (*Unsubmitted*)

Morphological and Ultrastructural Re-description of *Chloromyxum leydigi* (Myxozoa: Myxosporea) from the Gall Bladder of the Marine Cartilaginous fish *Torpedo marmorata* (Chondrichthyes: Torpedinidae) from the Portuguese Atlantic coast

Sónia Rocha¹, Graça Casal^{2,3} and Carlos Azevedo^{1, 2, 4}

¹Department of Cell Biology, Institute of Biomedical Sciences (ICBAS), University of Porto, 4099-003 Porto, Portugal

²Laboratory of Pathology, Interdisciplinary Centre for Marine and Environmental Research (CIIMAR), University of Porto, 4050-123 Porto, Portugal

³Laboratory of Sciences, High Institute of Health Sciences (CESPU), 4585-116 Gandra, Portugal

⁴Zoology Department, College of Science, King Saud University, 11451Riyhad, Saudi Arabia

Running header: Ultrastructural re-description of *Chloromyxum leydigi*

Key words: Myxosporea, *Chloromyxum leydigi*, gall bladder, cartilaginous fish, *Torpedo marmorata*, ultrastructure

Address for correspondence: C. Azevedo, Department of Cell Biology, Institute of Biomedical Sciences (ICBAS/UP), University of Porto, Largo A. Salazar no. 2, P- 4099-003 Porto, Portugal. Phone: ++351 222 062 200; Fax: ++351 222 062 232/33; E-mail address: azevedoc@icbas.up.pt

Abstract: A myxosporean of the genus *Chloromyxum* Mingazzini, 1890 was found infecting the gall bladder of the cartilaginous fish *Torpedo marmorata* Risso (Torpedinidae), collected from the Portuguese Atlantic coast. Plasmodia and mature spores were observed floating free in the bile. Plasmodia were polysporic and highly polymorphic in shape and organization. Several different stages of the sporogenic development were observed, allowing recognition of the main events occurring in the parasite development. Free unfixed mature spores were spherical to subspherical with an anterior pointed end, and measured on average $12.3 \pm 0.5 \mu\text{m}$ ($n = 20$) in length and $9.0 \pm 0.5 \mu\text{m}$ ($n = 20$) in width. The spore's wall was composed of two asymmetric valves united along an S-shaped sutural line. Each valve displayed 4-5 elevated surface ridges. A

bundle of 40-50 extended tapering caudal filaments appeared attached to the basal portion of the valves. Four pyriform equal-sized polar capsules ($5.3\ \mu\text{m} \times 3.2\ \mu\text{m}$) ($n = 15$) were observed at the anterior end of the spores, each containing a polar filament coiled in 8-9 (rarely 10) turns along its inner wall. Based on spore morphology, tissue tropism and host species, our parasite was identified as *Chloromyxum leydigi* Mingazzini, 1890, the type-species of the genus *Chloromyxum*. Since its discovery, this species has been dubiously reported from several cartilaginous hosts, namely due to the poor description of its features. The present study provides a complete morphological and ultrastructural re-description of the plasmodia and spores of *Chloromyxum leydigi*, based on light microscopy, transmission and scanning electron microscopy. Furthermore, the abundance of different sporogenic stages in our overall observations allows the portrayal of the sequential development of the plasmodia and spores.

INTRODUCTION

The class Myxosporea Bütschli, 1882 of the phylum Myxozoa Grassé, 1970 assembles more than 2180 species in about 60 genera (Lom and Dyková 2006), widely distributed in different geographic areas (Casal et al. 2009). Amongst them, the genus *Chloromyxum* Mingazzini, 1890 is the fourth larger, containing about 119 documented nominal species (Lom and Dyková 2006, Jirkù et al. 2011), most of which are coelozoic in the urinary tract and gall bladder of both freshwater and marine fishes (Fiala and Dyková 2004, Azevedo et al. 2009, Casal et al. 2009), and less frequently in non-fish hosts (Upton et al. 1995, Duncan et al. 2004, Jirkù et al. 2006, 2011). The majority of reports relating to this genus refer to freshwater species infecting Osteichthyes, comparing to the few reports that concern marine species (Lom and Dyková 2006). In the marine environment, *Chloromyxum* species appear to infect only Chondrichthyes (Pinto 1928, Kuznetsova 1977, Gioia and Cordeiro 1996, Azevedo et al. 2009), thus revealing differential host preference according to the habitat. The morphological features displayed by the spores also diverge between the freshwater and marine environment. *Chloromyxum* species infecting marine cartilaginous fish hosts commonly possess oval spores with an attenuated apex and a posterior bundle of filamentous appendages (Pinto 1928, Kuznetsova 1977, Kovaljova 1988, Gioia and Cordeiro 1996, Azevedo et al. 2009). Considering that this genus is polyphyletic (Kent et al. 2001), it is not hard to understand the difficulties inherent to the morphological and taxonomical description of its species. Nowadays, the employment of several new technologies ease this task, but for many years reports were solely based on scarce light microscopy (LM) observations and line schematic drawings (Pinto 1928, Jameson 1929, Kuznetsova 1977, Mitchell et al. 1980,

Kovaljova 1988, Gioia and Cordeiro 1996), leading to weak and vague descriptions that in most cases ultimately became the motive of many taxonomic and phylogenetic confusions (Lom and Dyková 1992, 1993, 2006). Such is the case of *Chloromyxum leydigi* Mingazzini, 1890, the type-species of the genus *Chloromyxum* and the object of many morphological reports that add to an unclear description (Kudo 1919, Pinto 1928, Gioia and Cordeiro 1996). In order to elucidate such misconceptions, the application of combined microscopical technologies is warranted. Although LM provides valuable information, only transmission electron microscopy (TEM) allows the recognition of specific ultrastructural features, and scanning electron microscopy (SEM) permits the observation of specific surface patterns and characters (Lom and Dyková 1993, 2006, Jirků et al. 2011). Recurring to all the previously mentioned technologies, the present study provides an accurate morphological and ultrastructural re-description of *Chloromyxum leydigi*, collected from the gall bladder of the cartilaginous fish *Torpedo marmorata* Risso, captured from the northwest Portuguese Atlantic coast.

MATERIALS AND METHODS

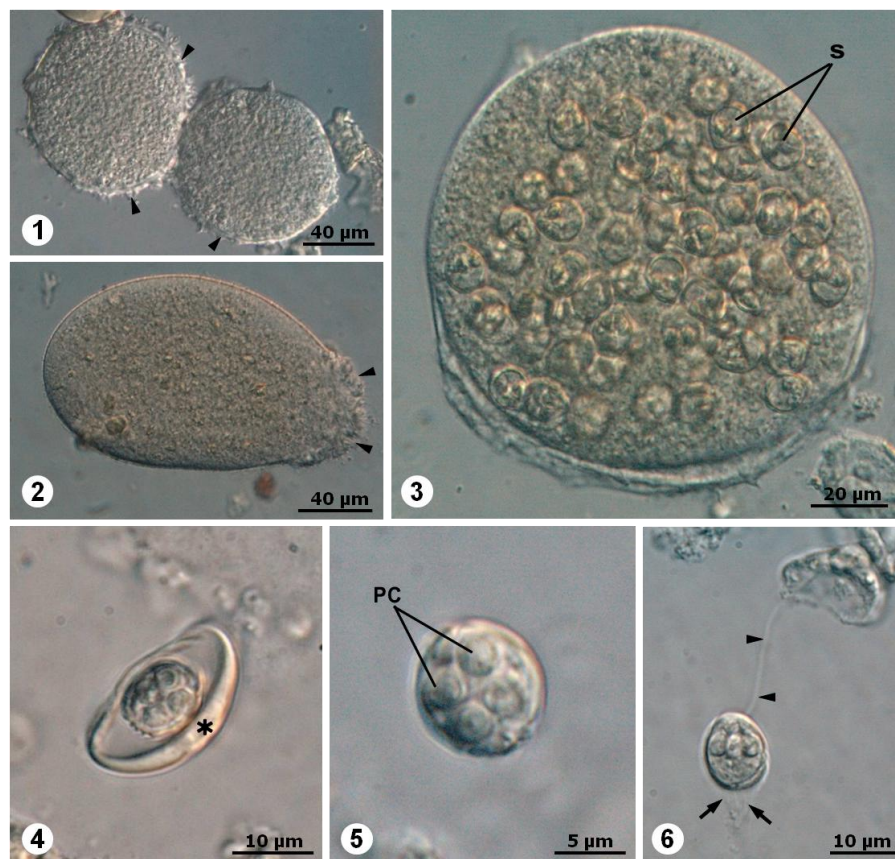
Twelve specimens of the marine spotted torpedo, *Torpedo marmorata* Risso (Chondrichthyes, Torpedinidae), were sampled between November 2010 and October 2011 from the Portuguese Northwest Atlantic coast (41° 25' N/ 08° 46' W), near the city of Póvoa do Varzim, Portugal. Upon necropsy, several organs and tissues, including muscles, gills, liver, intestine and gall bladder, were macroscopically analyzed. Preliminary microscopic analysis of these same organs and tissues revealed the gall bladder as the only infected organ. Unfixed plasmodia and mature spores were observed using Normarski differential interference contrast (DIC) optics, and measured with an ocular micrometer adapted to the photomicroscope. For TEM, the parasitized bile was fixed in 5% glutaraldehyde buffered in 0.2 M sodium cacodylate (pH 7.4) for 24 h, washed overnight in buffer, and post-fixed in 2 % osmium tetroxide with the same buffer for 3 h. During these procedures, the samples were kept at 4°C. After dehydration in an ascending ethanol series ending in propylene oxide, the samples were embedded in EPON. Semi-thin sections for LM observations were stained with methylene blue-Azure II. Ultrathin sections were double-stained with uranyl acetate and lead citrate, observed and photographed using a JEOL 100CXII TEM, operated at 60 kV. For SEM, the parasitized bile was fixed in 5% glutaraldehyde buffered in 0.2 M sodium cacodylate (pH 7.4) for 20 h at 4°C, washed in the same buffer at the same temperature, dehydrated in an ascending ethanol series and critical point dried. The samples were then coated with a gold-

palladium alloy (60%) and observed and photographed in a JSM-6301F SEM, operated at 15 kV.

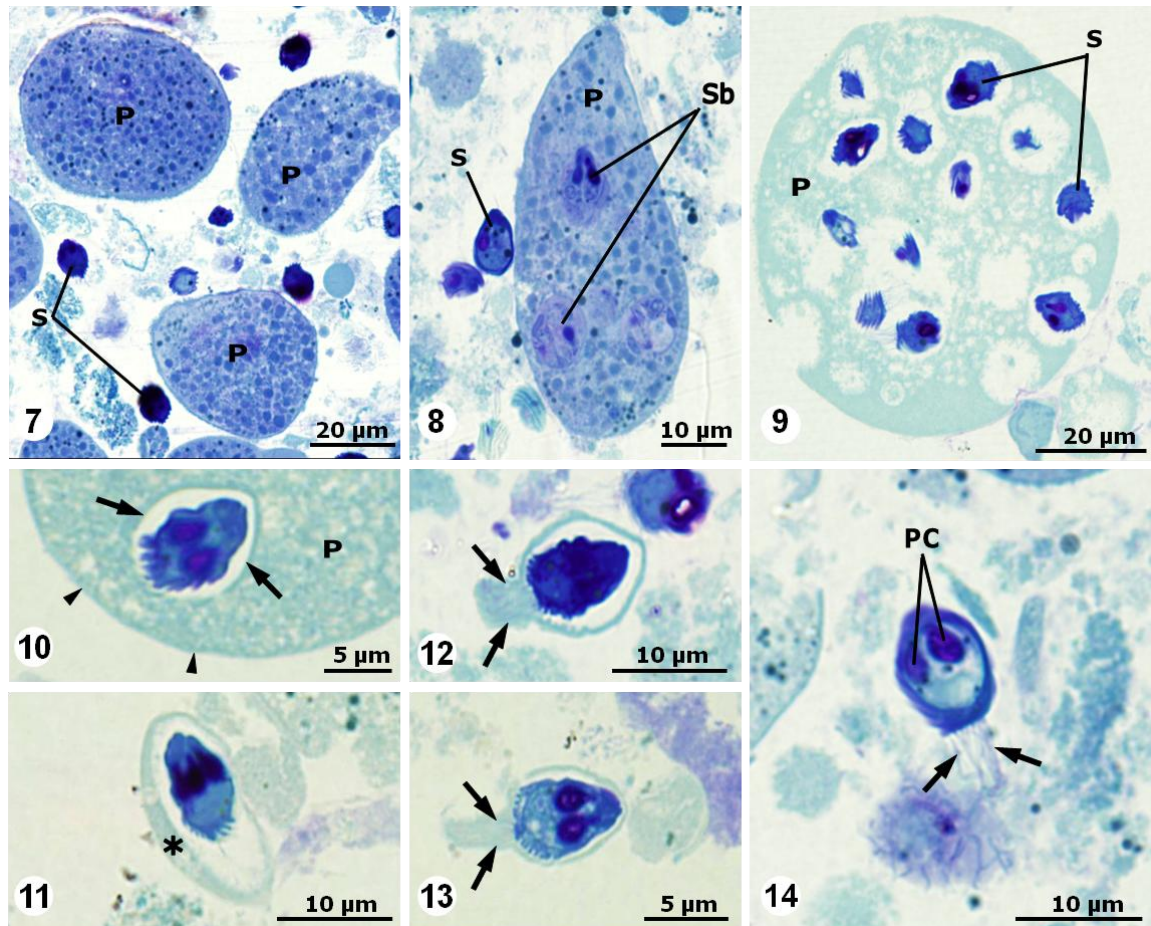
RESULTS

The collected and analyzed stingrays did not present macroscopic signs of infection. Necropsy revealed some specimens possessing hypertrophic gall bladders, which contained brownish colored bile. Preliminary microscopic observation of the several analyzed organs and tissues determined the presence of parasitic infection only in the gall bladders that appeared hypertrophied. Prevalence of infection was calculated at about 42% (5 infected in 12 examined fishes).

LM observations, including DIC optics, allowed the observation of several developmental vegetative stages and mature spores floating free in the bile of the infected specimens (Figs. 1-14).



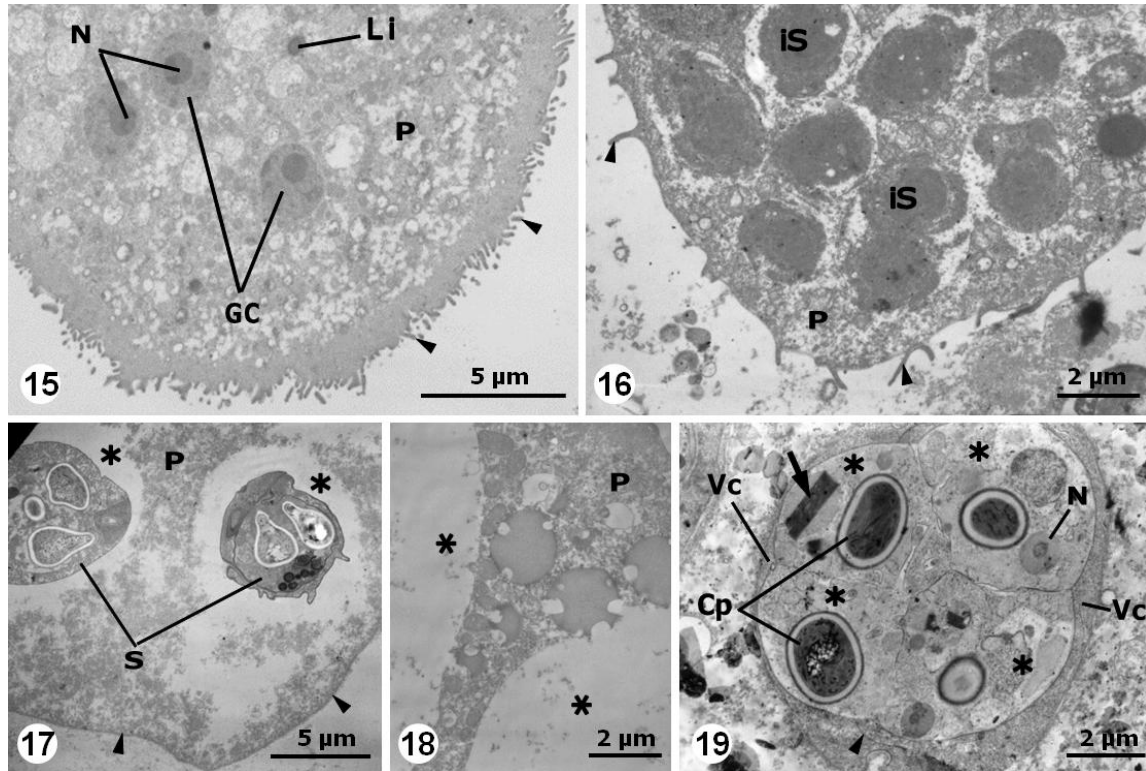
Figs. 1-6. Plasmodia and spores of *Chloromyxum leydigi* collected from the gall bladder of *Torpedo marmorata*, as observed using Normarski differential interference contrast. **Figs. 1, 2.** Polymorphic plasmodia displaying highly irregular cell membranes, due to the presence of numerous peripheral projections (arrowheads). **Fig. 3.** Round-shaped plasmodium containing numerous mature spores (S). **Fig. 4.** Spore enclosed in a crescent-shaped structure (*). **Fig. 5.** Four polar capsules (PC) are observed within a spore. **Fig. 6.** Spore allowing the visualization of its general features, including a bundle of caudal filaments (arrows) attached to the posterior end of the wall. Notice the extruded polar filament (arrowheads).



Figs. 7-14. Light microscopy micrographs of *Chloromyxum leydigi* collected from the gall bladder of *Torpedo marmorata*, showing semi-thin sections of several stages of the sporogonic development. **Fig. 7.** Several polymorphic plasmodia (P) corresponding to the earliest stages of sporogonic development, as well as mature spores (S), were observed in the bile. **Fig. 8.** Plasmodium (P) displaying some developing sporoblasts (Sb). A mature spore (S) is located nearby. **Fig. 9.** Round-shaped plasmodium (P) containing several mature spores (S). **Fig. 10.** Detailed aspect of the slightly more dense cytoplasmic material (arrows) surrounding the spore located within a mature plasmodium (P). The latter lacks peripheral projections in its cellular membrane (arrowheads). **Fig. 11.** Spore enclosed in a crescent-shaped structure (*). **Fig. 12, 13.** Detailed aspect showing the spores caudal filaments tightly fitted in the crescent-shaped structure (arrows). **Fig. 14.** Free mature spore presenting two of the four polar capsules (PC), as well as a bundle of caudal filaments (arrows) attached to the posterior end of its wall.

Plasmodia presented polysporic development and were highly polymorphic, appearing spherical, oval or very irregular (Figs. 1, 2, 3, 7, 8, 9). Dimensions varied greatly, namely as a result of the polymorphic nature of these structures. The cellular membrane organization varied according to the developmental stage, appearing highly irregular (Figs. 1, 2, 7, 8), or almost smooth (Figs. 3, 9, 10). LM, including DIC, and TEM observations allowed the observation of several stages of the parasite sporogenesis and, consequently, the recognition of its sequential development in all the mentioned microscopic technologies (Figs. 1-23). In the earliest stages of sporogonic development, plasmodia contained several generative cells surrounded by lipidic globules and other

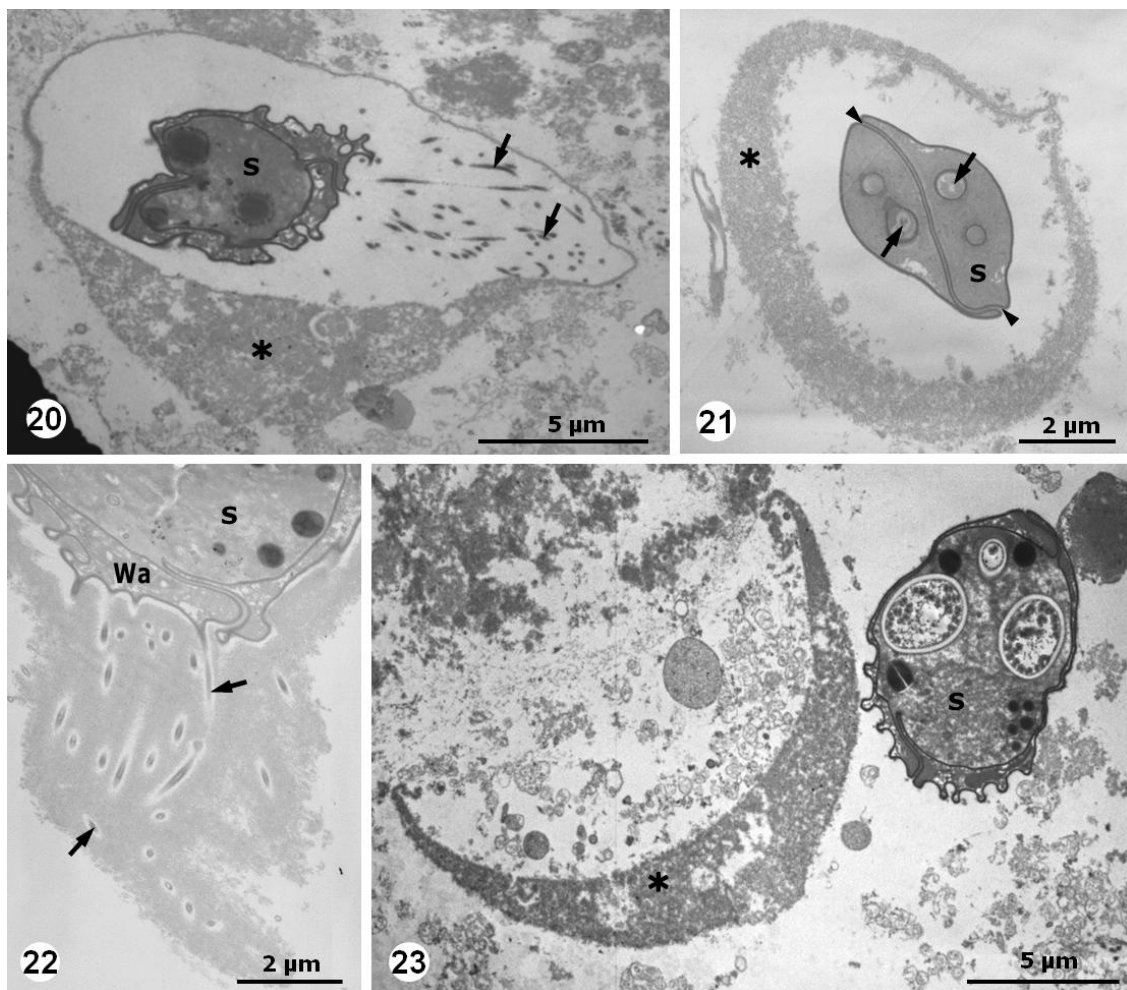
reserve bodies, and were profusely covered by microvillousities (Figs. 7, 15), some of which anastomosed (Fig. 15). In the later stages of sporogenic development, plasmodia displaying immature spores presented less microvillousities in comparison to the earlier stages (Figs. 8, 16), and plasmodia containing mature spores assumed a completely rounded shape with no peripheral projections and poor constituted cytoplasm (Figs. 9, 17).



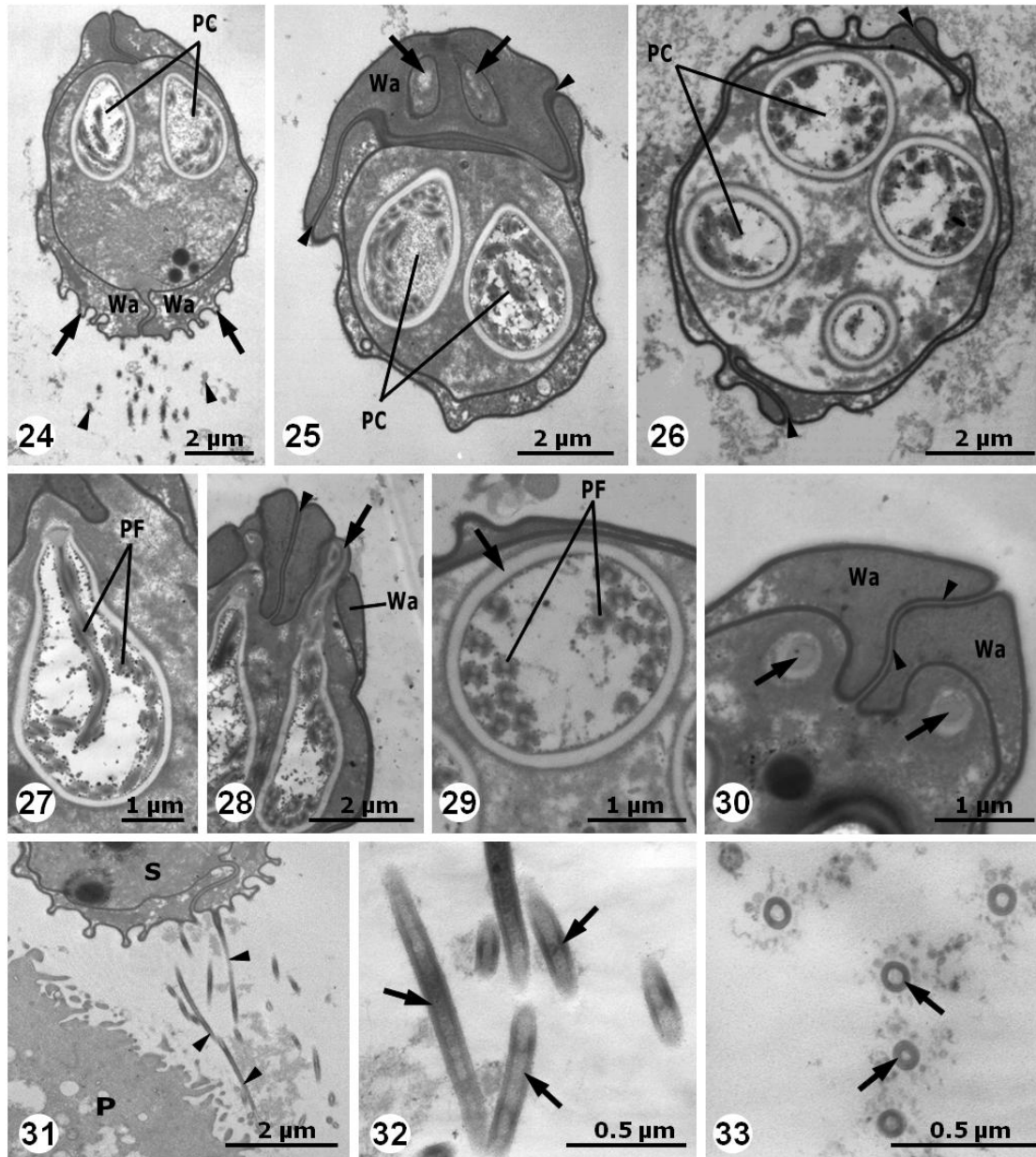
Figs. 15-19. Transmission electron micrographs of *Chloromyxum leydigi* collected from the gall bladder of *Torpedo marmorata*, emphasizing some stages of the sporogenic development. **Fig. 15.** Plasmodium (P) displaying the structural organization of early sporogenesis. Generative cells (GC) with well-defined nucleus (N), as well as lipidic globules (Li), can be observed in the cytoplasm. The cellular membrane is completely covered by numerous microvillousities (arrowheads). **Fig. 16.** Several immature spores (IS) are observed within a plasmodium (P) presenting few microvillousities (arrowheads) along its cellular membrane. **Fig. 17.** Plasmodium (P) displaying two mature spores (S) located in vacuole-like structures (*). The cellular membrane shows no peripheral projections (arrowheads). **Fig. 18.** Detailed aspect of the cytoplasmic area surrounding the vacuole-like structures (*) that encloses the spores within the plasmodium (P). **Fig. 19.** Sporoblast showing two valvogenic cells (Vc) adhering together along a sutural line (arrowhead) and surrounding four capsulogenic cells (*), each containing a developing capsular primordium (Cp). The nucleus (N) remains until capsulogenesis is completed. Paracrystallin structures enclosed within vesicles (arrow) are also present.

Immature spores were recognized in early sporoblasts by their valvogenic and capsulogenic cells (Fig. 8). The two valvogenic cells were positioned externally and surrounded the remaining sporogenic cells before adhering together along a sutural line. Four capsulogenic cells were observed, each containing a capsular primordium, a nucleus and some paracrystallin structures, possibly of protein nature, enclosed within

vesicles. In the later stages of capsulogenesis, the structural organization of the polar capsules, including the arrangement of the polar filament could be observed. The nuclei of these cells persisted until completion of the capsulogenesis (Fig. 19). Mature spores were also observed within the plasmodia. Each spore appeared as if enclosed in a vacuole-like structure (Figs. 9, 17), surrounded by a cytoplasmic material slightly denser than the remaining cytoplasm of the plasmodium (Figs. 10, 18). Upon disintegration of the plasmodium, mature spores were released enclosed in a crescent-shaped structure (Figs. 4, 11, 12, 13, 20, 21). In some LM and TEM observations, the spore's caudal filaments appeared tightly fitted into these structures (Figs. 12, 13, 22). Spores were later liberated from these crescent-shaped structures, which then appeared empty (Fig. 23).

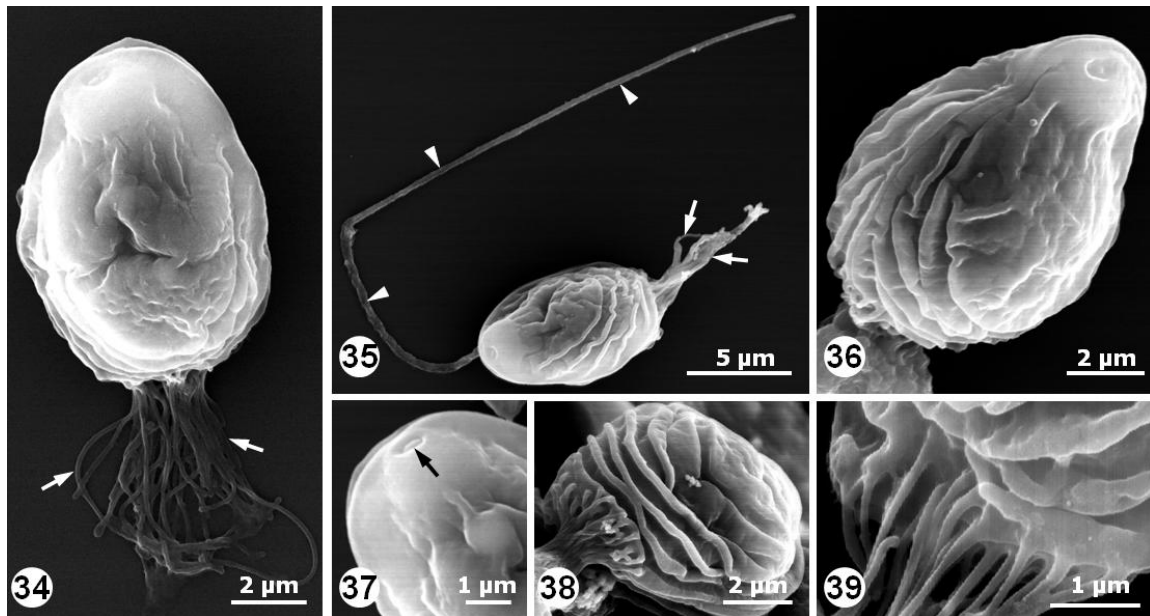


Figs. 20-23. Transmission electron micrographs of *Chloromyxum leydigi* collected from the gall bladder of *Torpedo marmorata*, emphasizing some stages of the sporogenic development. **Fig. 20.** Oblique section of a spore (S) enclosed in a crescent-shaped structure (*) and showing the caudal filaments (arrows) extended along the posterior end of that same structure. **Fig. 21.** Transverse section of the anterior end of the crescent-shaped structure (*) allowing the recognition of the four pore-like structures (arrows) located in the apical end of the spore (S), close to the sutural line (arrowheads). **Fig. 22.** Detailed aspect of a spore (S) showing the caudal filaments (arrows) projecting from the surface wall (Wa), tightly fitted into the material comprising the crescent-shaped structure. **Fig. 23.** Free mature spore (S) close to an empty crescent-shaped structure (*) and some lysed material.



Figs. 24-33. Transmission electron micrographs of *Chloromyxum leydigi* collected from the gall bladder of *Torpedo marmorata*, illustrating the specific morphological features of the spores. **Fig. 24.** Longitudinal section of a spore in lateral valvar view, presenting the two valves in the spore wall (Wa), two of the four polar capsules (PC) and the caudal filaments (arrowheads) attached to the posterior end. Notice the pattern formed by the surface ridges (arrows). **Fig. 25.** Longitudinal section of a spore in frontal valvar view, in which two pore-like structures (arrows) are observed located in the anterior end of the spore wall (Wa) and close the sutural line (arrowheads). These structures correspond to the apical end of the polar capsules (PC). **Fig. 26.** Slightly oblique transverse section of a spore, allowing the visualization of the four polar capsules (PC) and the two shell valves united along an S-shaped sutural line (arrowheads). **Fig. 27.** Polar capsule in longitudinal section, showing the polar filament (PF) coiled along its inner wall. **Fig. 28.** Polar capsule initiating extrusion of its polar filament through the pore-like structure (arrow) located at the anterior end of the spore wall (Wa), close to the sutural line (arrowhead). **Fig. 29.** Transverse section of a polar capsule showing the double-layer wall (arrow) and the polar filament (PF). **Fig. 30.** Detailed aspect of the apical end of the spore wall (Wa), displaying the S-shaped sutural line (arrowheads) and the pore-like structures (arrows). **Fig. 31.** Oblique and longitudinal sections of the caudal filaments (arrowheads) attached to the posterior end of the spore (S). A plasmodium (P) is located nearby. **Fig. 32.** Longitudinal section detailing the aspect of the caudal filaments (arrows). **Fig. 33.** Transverse section of the caudal filaments (arrows) allowing recognition of their average diameter.

Mature spores floating free in the bile were spherical to subspherical with a pointed anterior end and measured $12.3 \pm 0.5 \mu\text{m}$ ($n = 20$) in length and $9.0 \pm 0.5 \mu\text{m}$ ($n = 20$) in width (Figs. 6, 14). The spore's wall was composed of two asymmetric shell valves adhering together along an S-shaped sutural line (Figs. 25, 26, 30). Each valve displayed 4-5 elevated surface ridges located in the spore posterior half, parallel to the basal portion of the sutural line and coalescing towards the apical pole (Figs. 36, 38). A bundle of 40 to 50 tapering caudal filamentous projections appeared attached to the basal portion of the shell valves (Figs. 24, 31, 34, 38, 39), more specifically to the basal ridge and sutural line (Fig. 38). The caudal filaments measured on average $6.5 \pm 0.5 \mu\text{m}$ ($n = 5$) in length and $\sim 0.1 \mu\text{m}$ ($n = 10$) in diameter, and were constituted of the same material composing the shell valves: a continuous layer of external and internal dense material delimiting a middle lighter area (Figs. 32, 33).

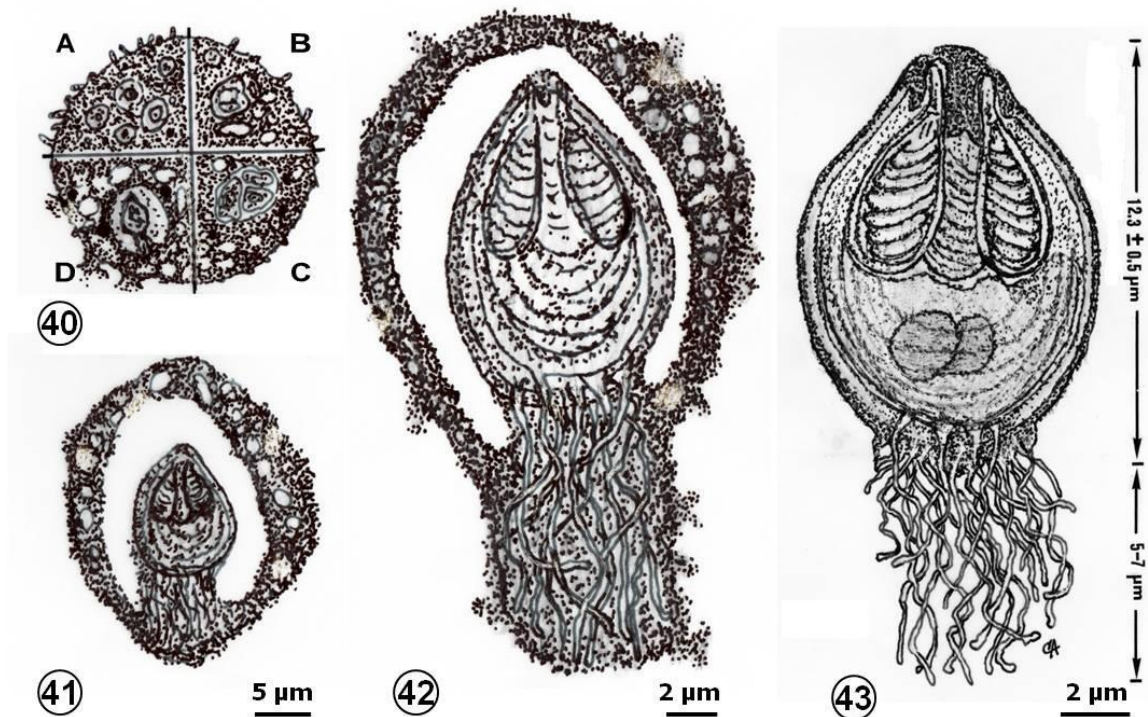


Figs. 34-39. Scanning electron micrographs of *Chloromyxum leydigii* collected from the gall bladder of *Torpedo marmorata*. **Fig. 34.** Spore showing the bundle of caudal filaments (arrows) attached to its posterior end. **Fig. 35.** Extruded polar filament (arrowheads) projecting from the pore-like structure located at the apical end of the spore wall. The bundle of caudal filaments (arrows) is visible in the posterior end of the spore. **Fig. 36.** Spore allowing the recognition of the surface pattern formed by the ridges positioned in the basal portion of each valve. **Fig. 37.** Detailed aspect of one of the four pore-like structures (arrow) located at the apical end of the spores wall, and trough which the extrusion of the polar filaments is made possible. **Fig. 38.** Posterior end of a spore showing the surface ridges and the caudal filaments attached to the sutural line and more basal ridge of each valve. **Fig. 39.** Detailed aspect of the caudal filaments, which are observed attached trough singular insertions.

Four pyriform equal-sized polar capsules measuring $5.3 \mu\text{m}$ ($n = 15$) in length and $3.2 \mu\text{m}$ ($n = 15$) in diameter were observed at the same level in the anterior end of the spores (Figs. 24-29). Each polar capsule possessed an isofilar polar filament obliquely arranged to its longitudinal axis (Fig. 27). The polar filament coiled along the inner wall of the polar

capsules in 8-9 (rarely 10) turns (Fig. 29) and, when extruded, measured 35 μm on average (Fig. 6, 35). In the anterior portion of the spore's wall, the pore-like structures corresponding to the apical end of the polar capsules were visible (Figs. 25, 28, 30, 37). In the few TEM observations allowing recognition of the sporoplasm, this structure was irregular in shape and displayed two nuclei randomly positioned in its matrix. The cytoplasm contained numerous mitochondria, lipid globules, small vesicles and some sporoplasmosomes.

Our overall morphological and ultrastructural observations are represented in a series of schematic drawings (Fig. 40, 41, 42, 43), depicting the main steps of the parasite sporogenic development, as well as the internal and external structural organization of the mature spores, with special focus on the arrangement of the polar capsules, the pattern of the surface ridges and the caudal bundle of filaments.



Figs. 40-43. Schematic drawings displaying the sequential sporogenic developmental aspects of *Chloromyxum leydigi* collected from the gall bladder of *Torpedo marmorata*, including the structure of mature spores, according to our observations. **Fig. 40.** Highly polymorphic plasmodium depicting some generative cells located in the cytoplasm and numerous microvillousities in the cellular membrane (A); Plasmodium displaying the growth and proliferation of generative cells and few microvillousities in its cellular membrane (B); Plasmodium containing developing sporoblasts (C); Plasmodium containing mature spores enclosed in vacuole-like structures and starting to disintegrate (D). **Fig. 41.** Detailed aspect of the spore enclosed in a vacuole-like structure inside the plasmodium. **Fig. 42.** Spore enclosed in a crescent-shaped structure, after the disintegration of the plasmodium. Notice the caudal filaments tightly fitted in that structure. **Fig. 43.** Longitudinal aspect of a mature spore in frontal valvar view, detailing the overall structural organization of the polar capsules, the pattern formed by the surface ridges and the bundle of caudal filaments.

DISCUSSION

For the proper and accurate description of myxosporean specific morphological characters, the employment of several focused microscopic procedures is required. As those methodologies were not always available and failed to be performed, many species warrant the elucidative and conclusive re-description of its features. This is a task that, in some cases, will undoubtedly lead to taxonomical revision (Lom and Dyková 1993, 2006). Several *Chloromyxum* species have been described from the gall bladder of marine cartilaginous fish. However, most reports lack reliable specific data for taxonomic and comparative purposes (Cunha and Fonseca 1918, Pinto 1928, Jameson 1929, Kuznetsova 1977, Mitchell et al. 1980, Kovaljova 1988, Gioia and Cordeiro 1996). Fortunately, the use of new technologies has become somewhat of a common practice, thus making the re-description of established species as frequent as the erection of new ones (Baska 1993, Lom and Dyková 1993, Ali 1998, Fiala and Dyková 2004, Holzer et al. 2006, Abdel-Baki 2007). In the present study, the combined use of LM, TEM and SEM microscopy allowed the complete re-description of a well-known myxosporean parasite. The general morphological and ultrastructural characters displayed by the spores and plasmodia sampled from the infected specimens of *Torpedo marmorata*, identify this species as belonging to the genus *Chloromyxum* of the family Chloromyxidae Thélohan, 1982 of the phylum Myxozoa, according to the taxonomical key published by Lom and Dyková (2006). More profound comparison to the specific ultrastructural characters of other marine *Chloromyxum* spp., further indicates our parasite as the type-species of this genus: *Chloromyxum leydigi* (Kovaljova 1988, Kuznetsova 1997, Azevedo et al. 2009, Casal et al. 2009). Upon comparison, *Chloromyxum ovatum* Jameson, 1921 and *Chloromyxum transversocostatum* Kuznetsova, 1977 display different surface ridges pattern (Kuznetsova 1977); *Chloromyxum dogieli* Kovaljova, 1988, *Chloromyxum lissosporum* Kovaljova, 1988, *Chloromyxum schulmani* Kovaljova, 1988 and *Chloromyxum striatellus* Kovaljova, 1988 description makes no reference to the organization of caudal filaments, but they appear to be very different (Kovaljova 1988); *Chloromyxum menticirrhi* Casal, Garcia, Matos, Monteiro, Matos et Azevedo, 2009 spores displayed 37-45 surface ridges in each shell valve and no caudal filaments (Casal et al. 2009); and *Chloromyxum riorajum* Azevedo, Casal, Garcia, Matos, Teles-Grilo et Matos, 2009 spores possess two equal-sized shell valves with 3-4 ridges each, as well as 6 (rarely 7) coils of the polar filament (Azevedo et al. 2009). Other reports of marine *Chloromyxum* species simply do not provide features for morphological comparison (Cunha and Fonseca 1918, Gioia and Cordeiro 1996, Oliva et al. 1996). The host species and tissue tropism of our parasite are also consistent with this classification, since *C.*

leydigi has been previously found infecting the gall bladder of *Torpedo marmorata*, amongst several other host species. In fact, *C. leydigi* was originally described from the gall bladder of numerous species of sharks and skates belonging to the genera *Mustellus*, *Galeus*, *Raja*, *Scyllium*, *Squatina*, *Torpedo* and *Trygon* (Kudo 1919, Pinto 1928, Lom and Dyková 1992, 2006, Gioia and Cordeiro 1996). Although broad host specificity constitutes a documented feature of several myxosporean species (Hoffman et al. 1965; Diamant et al. 2006, Jirků et al. 2006), it is more likely that *C. leydigi* has become an assemblage of several species that infect the gall bladder of Chondrichthyes (Jameson 1929, Kuznetsova 1977, Lom and Dyková 1992, 1993, 2006). Consequently, many morphological features are consistent between the numerous different reports, but several others remain ambiguous (Kudo 1919, Lom and Dyková 1992). According to the revision of myxosporean species made by Kudo (1919), all the previous reports of *C. leydigi* referred to the presence of polymorphic plasmodia with polysporic development and highly irregular cell membranes, due to the presence of many peripheral projections. The true disparities among reports concern the overwhelming differences found between spore measurements and the lack of conclusive comparative morphological characters (Kudo 1919, Pinto 1928, Lom and Dyková 1992, Gioia and Cordeiro 1996). Therefore it is not unreasonable to affirm that in these circumstances, *C. leydigi* constituted nothing but a name attributed to every individual species found infecting the gall bladder of the usual suspects. Finally, after many contradictory reports, this species gained molecular identity through sequencing of the SSU rDNA gene of the parasitic specimens sampled from the gall bladder of *Torpedo marmorata* captured from the Mediterranean Sea (Fiala and Dyková 2004), and later from the gall bladder of *Centroscymnus coelolepis* Barbosa du Bocage & de Brito Capelo captured from the North Atlantic (Fiala 2006). However, those studies disregarded morphological characterization as it was not their aim, contributing only with one scaled line drawing from *C. leydigi* in *Torpedo marmorata* (Fiala and Dyková 2004) and one DIC micrograph from *C. leydigi* in *Centroscymnus coelolepis* (Fiala 2006). Hence, ours is the first study providing conclusive LM, TEM and SEM observations for the vegetative stages and spores of *C. leydigi*. Although the spores and polar capsules in our study are smaller and the valves display less surface ridges than those considered *sensu stricto* for *C. leydigi* by Lom and Dyková (1992, 2006), they concur with the measurements and surface ridges pattern given by Fiala and Dyková (2004) for *C. leydigi* infecting *Torpedo marmorata*. Curiously, they appear to be slightly smaller than the few spores depicted in the DIC micrograph provided by Fiala (2006) for *C. leydigi* infecting *Centroscymnus coelolepis*.

Although often neglected in the description of myxosporeans, sporogenic development may provide valuable supplementary information for the distinction of individual species. During sporogenesis, plasmodia undergo many morphological and ultrastructural alterations, which vary slightly according to the parasite tissue tropism. In coelozoic species such as *C. leydigi*, plasmodial development is usually associated with an increase of dimension and the occurrence of significant surface modifications through the differentiation of peripheral projections (Lom 1969, Lom et al. 1986, Lom and Dyková 1995, 1996, Canning et al. 1999, Rocha et al. 2011), which are most probably related to nutritional intake and trophic functions (Lom 1969, Sitjà-Bobadilla and Alvarez-Pellitero 1993, 2001, Lom and Dyková 1996, Canning et al. 1999, Rocha et al. 2011). These alterations are in concordance with those observed for *C. leydigi*. Plasmodia corresponding to the earliest stages of development were highly polymorphic and the cell membrane saturated with peripheral projections. As the plasmodia evolved, the number of peripheral projections strongly diminished, until these structures were ultimately absent in the round-shaped plasmodia containing mature spores. A possible explanation for this type of plasmodial morphological evolution derives from the presumption of existing different nutritional necessities, according to the developmental stage (Lom 1969, Sitjà-Bobadilla and Alvarez-Pellitero 1993, 2001, Lom and Dyková 1996, Canning et al. 1999, Rocha et al. 2011). The fact that mature spores are released enclosed in crescent-shaped structures reminiscing from the plasmodium cytoplasm may possibly be explained by a remaining necessity for protection, even after the complete disintegration of the plasmodium. However, further microscopic analyses of those structures are necessary to assess the liability of our conjecture. Although they are apparently constituted of the same material composing the plasmodium, these structures assume a consistent crescent-like shape, indicating that their constituents might be more resistant than the remaining cytoplasm of the vegetative structure. The possibility of them actually corresponding to degenerating cells was assessed, but no cellular membranes or cytoplasmatic divisions were observed between the spores contained within the plasmodia. The mature spores are ultimately released from the crescent-shaped structures that are then observed floating empty in the bile. This occurrence in *C. leydigi* sporogenic development is rather unique, and appears to constitute a single case among *Chloromyxum* species, thus representing a specific character for morphological diagnosis.

Notwithstanding the amount of descriptive information that the association of several microscopic analyses allowed us to obtain, this study is still lacking the employment of molecular analysis. Data on the SSU rDNA gene would ultimately confirm our results, thus clarifying all misconceptions surrounding the morphological and ultrastructural aspects of

this species. This would be of great importance, not only because microscopic diagnosis would be simplified, but also because *C. leydigi* is phylogenetically positioned in a clade situated at the basis of the freshwater clade and is believed to represent the link between freshwater and marine species (Fiala and Dyková 2004, Fiala 2006, Holzer et al. 2006). Therefore, morphological data concerning this species, as well as others clustering in the same clade, such as *C. riorajum*, may allow recognition of the evolutionary history of specific characters. Furthermore, although molecular information demonstrates that phylogeny may not always be consistent with morphological features (Kent et al. 2001, Fiala 2006), recognition of the morphological diversity among myxosporean species is essential for the comprehension of the parasite systematic and the evolutionary tracing of host specificity, tissue tropism and host-parasite interaction (Fiala and Bartošová 2010, Bartošová and Fiala 2011).

Acknowledgments. This work was partially supported by Eng.^o A. Almeida Foundation (Porto, Portugal). The authors wish to thank the investigator Miguel Cordeiro, as well as the fishermen from Póvoa do Varzim, for providing the specimens for analysis. This work complies with the current laws of our country.

REFERENCES

- Abdel-Baki A.S. 2007: *Chloromyxum alii* sp. n. (Myxozoa: Myxosporea) infecting the gallbladder of African butter catfish *Schilbe mystus* (Linnaeus 1758) from the River Nile, Egypt: light and scanning electron microscopy. Acta Protozool. 46: 263-267.
- Ali M.A. 1998: Light and scanning electron microscopy of *Chloromyxum vanasi* sp. n. (Myxozoa: Myxosporea) infecting gallbladder of the Nile catfish *Bagrus bayad* (Forsk., 1775) (Teleostei: Bagridae). Acta Protozool. 37: 57-61.
- Azevedo C., Casal G., Garcia P., Matos P., Teles-Grilo L., Matos E. 2009: Ultrastructural and phylogenetic data of *Chloromyxum riorajum* sp. nov. (Myxozoa), a parasite of the stingray *Rioraja agassizii* in Southern Brazil. Dis. Aquat. Org. 85: 41-51.
- Bartošová P., Fiala I. 2011: Molecular evidence for the existence of cryptic species assemblages of several myxosporeans (Myxozoa). Parasitol. Res. 108: 573-583.

Baska F. 1993. Light and electron microscopic studies on the development of *Sphaerospora colomani* Baska, 1990 and *Chloromyxum inexpectatum* Baska, 1990. Acta Vet. Hung. 41: 59-72.

Canning E.U., Curry A., Anderson C.L., Okamura B. 1999: Ultrastructure of *Myxidium trachinorum* sp. nov. from the gallbladder of the lesser weever fish *Echiichthys vipera*. Parasitol. Res. 85: 910-919.

Casal G., Garcia P., Matos P., Monteiro E., Matos E., Azevedo C. 2009: Fine structure of *Chloromyxum menticirrho* n. sp. (Myxozoa) infecting the urinary bladder of the marine teleost *Menticirrhus americanus* (Sciaenidae) in Southern Brazil. Europ. J. Protistol. 45: 139-146.

Cunha A. M., Fonseca C. 1918: Sobre os mixosporídios dos peixes brasileiros. Bras. Med. 32: 393.

Diamant A., Ram S., Paperna I. 2006: Experimental transmission of *Enteromyxum leei* to freshwater fish. Dis. Aquat. Org. 72: 171-178.

Duncan A.E., Garner M.M., Bartholomew J.L., Reichard T.A., Nordhausen R.W. 2004: Renal myxosporidiasis in Asian horned frog (*Megophrys nasuta*). J. Zoo Wildl. Med. 35: 381-386.

Fiala I. 2006: The phylogeny of Myxosporea (Myxozoa) based on small subunit ribosomal RNA gene analysis. Int. J. Parasitol. 36: 1521-1534.

Fiala I., Dyková I. 2004: The phylogeny of marine and freshwater species of the genus *Chloromyxum* Mingazzini, 1890 (Myxosporea: Bivalvulida) based on small subunit ribosomal RNA gene sequences. Folia Parasitol. 51: 211-214.

Fiala I., Bartošová P. 2010: History of myxozoan character evolution on the basis of rDNA and EF-2 data. BMC Evol. Biol. 10: 228.

Gioia I., Cordeiro N.S. 1996: Brazilian myxosporidians' check-list (Myxozoa). Acta Protozool. 35: 137-149.

Hoffman G.L., Putz R.E., Dunbar C.E. 1965: Studies on *Myxosoma cartilaginis* n. sp. (Protozoa: Myxosporidea) of centrarchid fish and a synopsis of the *Myxosoma* of North American freshwater fishes. J. Protozool. 12: 319-332.

Holzer A.S., Sommerville C., Wootten R. 2006: Molecular identity, phylogeny and life cycle of *Chloromyxum schurovi* Shul'man and Ieshko, 2003. Parasitol. Res. 99: 90-96.

Jameson A.P. 1929: Myxosporidia from Californian fishes. J. Parasitol. 16: 59-68.

Jirků M., Bartošová P., Kodádková A., Mutschmann F. 2011: Another chloromyxid lineage: molecular phylogeny and redescription of *Chloromyxum careni* from the Asian horned frog *Megophrys nasuta*. J. Eukaryot. Microbiol. 58: 50-59.

Jirků M., Bolek M.G., Whipps C.M., Janovy J., Kent M.L., Modrý D. 2006: A new species of *Myxidium* (Myxosporaea: Myxidiidae), from the western chorus frog, *Pseudacris triseriata triseriata*, and Blanchard's cricket frog, *Acris crepitans blanchardi* (Hylidae), from eastern Nebraska: morphology, phylogeny, and critical comments on amphibian *Myxidium* taxonomy. J. Parasitol. 92: 611-619.

Kent M.L., Andree K.B., Bartholomew J.L., El-Matbouli M., Dessler S.S., Devlin R.A., Feist S.W., Hedrick R.P., Hoffmann R.W., Khattra J., Hallett S.L., Lester R.J.G., Longshaw M., Palenzeula O., Siddall M.E., Xiao C. 2001: Recent advances in our knowledge of the Myxozoa. J. Eukaryot. Microbiol. 48: 395-413.

Kovaljova A.A. 1988: Myxosporidia of the genus *Chloromyxum* (Cnidospora, Myxosporaea) of cartilaginous fish from the Atlantic coast of Africa. Parasitologiya 22: 384-388. (In Russian.)

Kudo R.R. 1919: Studies on Myxosporidia. A synopsis on genera and species of Myxosporidia. III. Biol. Monogr. 5: 1-265.

Kuznetsova I.G. 1977: Myxosporidians of Chondrostei from the Patagonian shelf. Parazitologiya 11: 74-77. (In Russian.)

Lom J. 1969: Notes on the ultrastructure and sporoblast development in fish parasitizing myxosporidian of the genus *Sphaeromyxa*. Z. Zellforsch. 97: 416-437.

Lom J., Dyková I. 1992: Protozoan Parasites of Fishes. Developments in Aquaculture and Fisheries Science. Vol. 26. Elsevier, Amsterdam, 315 pp.

Lom J., Dyková I. 1993: Scanning electron microscopic revision of common species of the genus *Chloromyxum* (Myxozoa: Myxosporidia) infecting European freshwater fishes. *Folia Parasitol.* 40: 161-174.

Lom J., Dyková I. 1995: New species of the genera *Zschokkella* and *Ortholinea* (Myxozoa) from the Southeast Asian teleost fish, *Tetraodon fluviatilis*. *Folia Parasitol.* 42: 161-168.

Lom J., Dyková I. 1996: Notes on the ultrastructure of two myxosporidian (Myxozoa) species, *Zschokkella pleomorpha* and *Ortholinea fluviatilis*. *Folia Parasitol.* 43: 189-202.

Lom J., Dyková I. 2006: Myxozoan genera: definition and notes on taxonomy, life-cycle terminology and pathogenic species. *Folia Parasitol.* 43: 1-36.

Lom J., Molnár K., Dyková I. 1986: *Hoferellus gilsoni* (Debaisieux, 1925) comb. n. (Myxozoa, Myxosporidia): redescription and mode of attachment to the epithelium of the urinary bladder of its host, the European eel. *Protistology* 4: 405-413.

Mitchell L.G., Listebarger J.K., Bailey W.C. 1980: Epizootiology and histopathology of *Chloromyxum trijugum* (Myxosporidia: Myxosporidia) in centrarchid fishes from Iowa. *J. Wildl. Dis.* 16: 233-236.

Oliva M.E., Castro R.E., Burgos R. 1996: Parasites of the Flatfish *Paralichthys adspersus* (Steindachner, 1867) (Pleuronectiformes) from Northern Chile. *Mem. Inst. Oswaldo Cruz, Rio de Janeiro* 91: 301-306.

Pinto C. 1928: Mixosporídeos e outros protozoários intestinais de peixes observados na América do Sul. *Arch. Inst. Biol. S. Paulo* 1: 101-126.

Rocha S., Casal G., Matos P., Matos E., Dkhil M. and Azevedo C. 2011: Description of *Triangulamyxa psittaca* sp. nov. (Myxozoa: Myxosporidia), a new parasite in the urinary

bladder of *Colomesus psittacus* (Teleostei) from the Amazon River, with emphasis on the ultrastructure of plasmodial stages. *Acta Protozool.* 50: (*In press*)

Stijà-Bobadilla A., Alvarez-Pellitero P. 1993: *Zschokkella mugilis* n. sp. (Myxoporea: Bivalvulida) from mullets (Teleostei: Mugilidae) of Mediterranean waters: light and electron microscopic description. *J. Eukaryot. Microbiol.* 40: 755-764.

Stijà-Bobadilla A., Alvarez-Pellitero P. 2001: *Leptotheca sparidarum* n. sp. (Myxosporea: Bivalvulida), a parasite from cultured common dentex (*Dentex dentex* L.) and gilthead sea bream (*Sparus aurata* L.) (Teleostei: Sparidae). *J. Eukaryot. Microbiol.* 48: 627-639.

Upton S.J., McAllister C.T., Trauth S.E. 1995: A new species of *Chloromyxum* (Myxozoa, Chloromyxidae) from the gall bladder of *Eurycea* spp. (Caudata, Plethodontidae) in North America. *J. Wildl. Dis.* 31: 394-396.

CHAPTER 5

Final Remarks

5.1. General Discussion

The growing amount of knowledge concerning myxozoan parasites, and more specifically myxosporean species, clearly indicates a necessity for major taxonomic revisions to its genera and the establishment of far more reliable classification criteria. The more or less frequent arise of new information uncovers the outstanding lack of consensus in the classification of myxosporean species and brings forth many questions relating to their origin, evolution and life cycle. In order to satisfy the demand for more accurate descriptions and taxonomic identification, detailed morphological features are being combined with molecular data for the establishment of new species and the revision of established ones (Andree et al. 1999; Lom and Dyková 2006). Tissue tropism has also acquired a stronger impact in the classification of myxosporeans, since several studies actually report taxa to cluster more by development and tissue location than by spore morphology (Lom and Dyková 1992, 2006; Kent et al. 2001; Bahri et al. 2003; Eszterbauer 2004; Bartošová et al. 2009). On the contrary, classification based upon host specificity is very ambiguous, as most species display broad host specificity both in the actinosporean and the myxosporean stage (Hoffman et al. 1965; O'Grodnick 1979; Sitjà-Bobadilla and Alvarez-Pellitero 1993; El-Mansy and Molnár 1997; Diamant et al. 2006; Fiala 2006; Jirků et al. 2006).

The comprehensive association of several classification features and the employment of more advanced techniques to morphological and molecular characters are currently allowing the clarification of several phylogenetic relationships among myxosporean organisms. As a result, the volume of molecular data grows exponentially, and morphological information is refined by the usage of ultrastructural and biochemical methods (Andree et al. 1999; Kent et al. 2001; Zrzavý 2001). Nevertheless, the available molecular data base is still very limited and warrants the establishment of new DNA sequences, not only from the SSU rDNA gene, but also from the LSU rDNA gene and other molecular markers (Bartošová et al. 2009; Fiala and Bartošová 2010). Furthermore, considering that only a small fraction of all existing myxosporean species is known, some surprises in our understanding of their evolution, development and life cycles may be expected. Great caution must be exerted when interpreting the results of the different combined classification features (Lom and Dyková 2006).

The data presented in this thesis increases the available knowledge concerning the diversity and biological characteristics of the myxosporean stages of two different genera: *Triangulamyxa* and *Chloromyxum*. Both these genera present clear difficulties to the description and classification of its species. The first because it constitutes a recently described genus with only one unsequenced species, and therefore lacks wide terms of

comparison; and the second because it is a polyphyletic genus and the fourth larger within the class Myxosporea (Fiala and Dyková 2004; Azevedo et al. 2005; Lom and Dyková 2006; Bartošová and Fiala 2011). An effort to combine as many different classification characters as possible was made. Spore morphology, which remains the main criterion for the characterization of myxosporeans, is depicted using several microscopic methodologies. LM, namely DIC, is used to provide an overall view of these structures; TEM to understand its ultrastructure; and SEM to better recognize its shape, the external patterns in the surface wall and, whenever present, the number and organization of the caudal appendages (Lom and Dyková 1992, 1993, 2006; Lom and Hoffman 2003). In fact, for both the description of *Chloromyxum clavatum* n. sp. and the re-description of *Chloromyxum leydigi*, SEM was indeed a fundamental tool, allowing the clear observation of the ridges pattern on their surface and the analysis of their caudal appendages, which constitute important morphological characters to the differentiation of individual species within this genus (Lom and Dyková 1993, 2006; Jirků et al. 2011). Unfortunately, it was not possible to perform SEM for *Triangulamyxa psittaca* n. sp., due to the lack of biological material. The host species found infected by this parasite, *Colomesus psittacus*, inhabits the Amazon River, which difficults new samplings due to the rare periodicity of expeditions.

Although often disregarded by authors, the description of the morphological aspects and the ultrastructural development of vegetative stages can also be most valuable for classification purposes, and proved so in the case of the new species *Triangulamyxa psittaca* n. sp. and in the re-description of *Chloromyxum leydigi*. Consequently, this trend is slowly shifting and vegetative stages begin to be perceived as structures that may provide valuable information, namely referring to sporogenesis (Lom and Noble 1984; Lom and Dyková 1992, 1993, 2006; Lom and Hoffman 2003). The specific developmental characters displayed throughout sporogenesis also reveal the fascinating adaptation mechanisms adopted by the parasite in order to endure and surpass the varying conditions that the host body may present. In both *Triangulamyxa psittaca* n. sp. and *Chloromyxum leydigi*, the morphological differentiation displayed throughout the sporogenic development appears related to nutrient-intake. Nevertheless, other factors may be involved and further result from the indirect action of environmental conditions. The effects of environmental factors and host species in the development and morphology of spores remain unclear, and thus warrant further evaluation (Molnár 1991; Andree et al. 1999).

Since classification based solely on morphological features has been shown to reduce myxosporean diversity (Bartošová and Fiala 2011), this thesis also aimed to provide new

molecular information for this class. However, this was only possible for *Chloromyxum clavatum* n. sp., again due to the lack of biological material infected by *Triangulamyxa psittaca* n. sp. and *Chloromyxum leydigi*. *Triangulamyxa psittaca* n. sp. for the same reasons explained above and *Chloromyxum leydigi* as a result of the apparent degeneration of the sampled DNA. New samples are needed in order to give continuity to this study but, due to weather conditions, among other factors, the acquisition of new specimens for parasitic diagnosis and sampling was not possible. Hopefully, the attainment of new biological material will allow molecular characterization of both species, which would result in distinct advantages. Sequencing of the SSU rDNA gene of *Triangulamyxa psittaca* n. sp. would add molecular data to a genus yet deprived of this type of knowledge, thus instituting a basis for more reliable comparisons. Sequencing of the SSU rDNA gene of *Chloromyxum leydigi* would ultimately confirm its re-description, improving diagnosis. Also, the latter constitutes a most interesting exception to the division of myxosporean species in the freshwater and marine clades, together with *Chloromyxum riorajum* and now *Chloromyxum clavatum* n. sp. forming a clade located at the basis of the freshwater lineage. This clade appears to represent the evolutionary link between the marine and freshwater clades (Fiala and Dyková 2004; Fiala 2006; Holzer et al. 2006; Azevedo et al. 2009). But only the further acquisition of new molecular data concerning both new and established myxosporean species infecting marine cartilaginous fish will be able to assess the veracity of this assumption.

The results presented in this thesis concur that only the combination of different updated methodologies focused to the interpretation of parasitological data, namely for the class Myxosporea, is able to attain the astonishing diversity displayed by microparasites and provide new perspectives and conclusive information for their intriguing phylogenetic relationships, life cycles and biological interactions.

5.2. General Conclusion

As all scientific work never truly ends, this thesis allows the discrimination of future tasks and perspectives, some more immediate than others. Several subjects remain undetermined or ambiguous for the class Myxosporea. Is the life cycle with alternate hosts common for all species? What specific mechanisms mediate transmission and host specificity? How deep is the impact of environmental factors? These are just some questions among many others. But, in order for them to be effectively assessed, a more immediate necessity rises. Myxosporea warrants establishment of better classification standards and terms of comparison for both new and determined genera and species

(Lom and Dyková 2006; Fiala and Bartošová 2010). First time descriptions and re-descriptions should be as accurate as possible, and therefore include the morphological and ultrastructural description of both spores and vegetative stages, as well as the molecular characterization of the species (Azevedo et al. 2009; Jirků et al. 2011). When not possible, a redescription featuring the missing data is required. Also, if possible, it would be most interesting to couple the myxosporean stage to the corresponding actinosporean stage, since most myxosporean species have their complete life cycle unresolved (Lom and Dyková 2006). These are tasks that will, undoubtedly, take much time and result in significant modifications to our view on myxosporean taxonomy and phylogeny. A continuous effort must be made in order to resolve these topics and truly demonstrate the amazing diversity of biological characters displayed by these parasites.

References

- Andree K. B., Székely C., Molnár K., Gresoviac S. J. and Hedrick R. P. 1999: Relationships among members of the genus *Myxobolus* (Myxozoa: Bilvalvidae) based on small subunit ribosomal DNA sequences. *J. Parasitol.* 85: 68-74.
- Azevedo C., Casal G., Garcia P., Matos P., Teles-Grilo L. and Matos E. 2009: Ultrastructural and phylogenetic data of *Chloromyxum riorajum* sp. nov. (Myxozoa), a parasite of the stingray *Rioraja agassizii* in Southern Brazil. *Dis. Aquat. Org.* 85: 41-51.
- Azevedo C., Corral L. and Matos E. 2005: Ultrastructure of *Triangulamyxa amazonica* n. gen. and n. sp. (Myxozoa, Myxosporea), a parasite of the Amazonian freshwater fish, *Sphoeroides testudineus* (Teleostei, Tetraodontidae). *Eur. J. Protistol.* 41: 57-63.
- Bahri S., Andree K. B. and Hedrick R. P. 2003: Morphological and phylogenetic studies of marine *Myxobolus* spp. from mullet in Ichkeul Lake, Tunisia. *J. Eukaryot. Microbiol.* 50: 463-470.
- Bartošová P. and Fiala I. 2011: Molecular evidence for the existence of cryptic species assemblages of several myxosporeans (Myxozoa). *Parasitol. Res.* 108: 573-583.
- Bartošová P., Fiala I. and Hypša V. 2009: Concatenated SSU and LSU rDNA data confirm the main evolutionary trends within myxosporeans (Myxozoa: Myxosporea) and provide an effective tool for their molecular phylogenetics. *Mol. Phylog. Evol.* 53: 81-93.

Diamant A., Ram S. and Paperna I. 2006: Experimental transmission of *Enteromyxum leei* to freshwater fish. Dis. Aquat. Org. 72: 171-178.

El-Mansy A. and Molnár K. 1997: Development of *Myxobolus hungaricus* (Myxosporea: Myxobolidae) in oligochaete alternate hosts. Dis. Aquat. Org. 31: 227-232.

Eszterbauer E. 2004: Genetic relationship among gill-infecting *Myxobolus* species (Myxosporea) of cyprinids: molecular evidence of importance of tissue-specificity. Dis. Aquat. Org. 58: 35-40.

Fiala I. 2006: The phylogeny of Myxosporea (Myxozoa) based on small subunit ribosomal RNA gene analysis. Int. J. Parasitol. 36: 1521-1534.

Fiala I. and Bartošová P. 2010: History of myxozoan character evolution on the basis of rDNA and EF-2 data. BMC Evol. Biol. 10: 228.

Fiala I. and Dyková I. 2004: The phylogeny of marine and freshwater species of the genus *Chloromyxum* Mingazzini, 1890 (Myxosporea: Bivalvulida) based on small subunit ribosomal RNA gene sequences. Folia Parasitol. 51: 211-214.

Hoffman G. L., Putz R. E. and Dunbar C. E. 1965: Studies on *Myxosoma cartilaginis* n. sp. (Protozoa: Myxosporidea) of centrarchid fish and a synopsis of the *Myxosoma* of North American freshwater fishes. J. Protozool. 12: 319-332.

Holzer A. S., Sommerville C. and Wootten R. 2006: Molecular identity, phylogeny and life cycle of *Chloromyxum schurovi* Shul'man and Leshko, 2003. Parasitol. Res. 99: 90-96.

Jirků M., Bartošová P., Kodádková A., Mutschmann F. 2011: Another chloromyxid lineage: molecular phylogeny and redescription of *Chloromyxum careni* from the Asian horned frog *Megophrys nasuta*. J. Eukaryot. Microbiol. 58: 50-59.

Jirků M., Bolek M. G., Whipps C. M., Janovy J., Kent M. L. and Modrý D. 2006: A new species of *Myxidium* (Myxosporea: Myxidiidae), from the western chorus frog, *Pseudacris triseriata triseriata*, and Blanchard's cricket frog, *Acris crepitans blanchardi* (Hylidae), from eastern Nebraska: morphology, phylogeny, and critical comments on amphibian *Myxidium* taxonomy. J. Parasitol. 92: 611-619.

Kent M. L., Andree K. B., Bartholomew J. L., El-Matbouli M., Dessler S. S., Devlin R. A., Feist S. W., Hedrick R. P., Hoffmann R. W., Khattra J., Hallett S. L., Lester R. J. G., Longshaw M., Palenzeula O., Siddall M. E. and Xiao C. 2001: Recent advances in our knowledge of the Myxozoa. *J. Eukaryot. Microbiol.* 48: 395-413.

Lom J. and Dyková I. 1992: Protozoan Parasites of Fishes. *Developments in Aquaculture and Fisheries Science* 26, Elsevier Amsterdam, 315 pp.

Lom J. and Dyková I. 1993: Scanning electron microscopic revision of common species of the genus *Chloromyxum* (Myxozoa: Myxosporea) infecting European freshwater fishes. *Folia Parasitol.* 40: 161-174.

Lom J. and Dyková I. 2006: Myxozoan genera: definition and notes on taxonomy, life cycle terminology and pathogenic species. *Folia Parasitol.* 53: 1-36.

Lom J. and Hoffman G. L. 2003: Morphology of the spores of *Myxosoma cerebralis* (Hofer, 1903) and *M. cartilaginis* (Hoffman, Putz, and Dunbar, 1965). *J. Parasitol.* 89: 653-657.

Lom J. and Noble E. R. 1984: Revised classification of the class Myxosporea Bütschli, 1881. *Folia Parasitol.* 31: 193-205.

Molnár K. 1991: Comments on the host, organ and tissue specificity of fish myxosporeans and on the types of their intrapiscine development. *Hung. Soc. Parasitol.* 27: 5-20.

O'Grodnick J. J. 1979: Susceptibility of various salmonids to whirling disease (*Myxosoma cerebralis*). *Trans. Am. Fish. Soc.* 108: 187-190.

Sitjà-Bobadilla A. and Alvarez-Pellitero P. 1993: *Zschokkella mugilis* n. sp. (Myxosporea: Bivalvulida) from mullets (Teleostei: Mugilidae) of Mediterranean waters: light and electron microscopic description. *J. Eukaryot. Microbiol.* 40: 755-764.

Zrzavý J. 2001: The interrelationships of metazoan parasites: a review of phylum- and higher-level hypotheses from recent morphological and molecular phylogenetic analyses. *Folia Parasitol.* 48: 81-103.