Beth Okamura · Alexander Gruhl
Jerri L. Bartholomew *Editors*

Myxozoan Evolution, Ecology and Development

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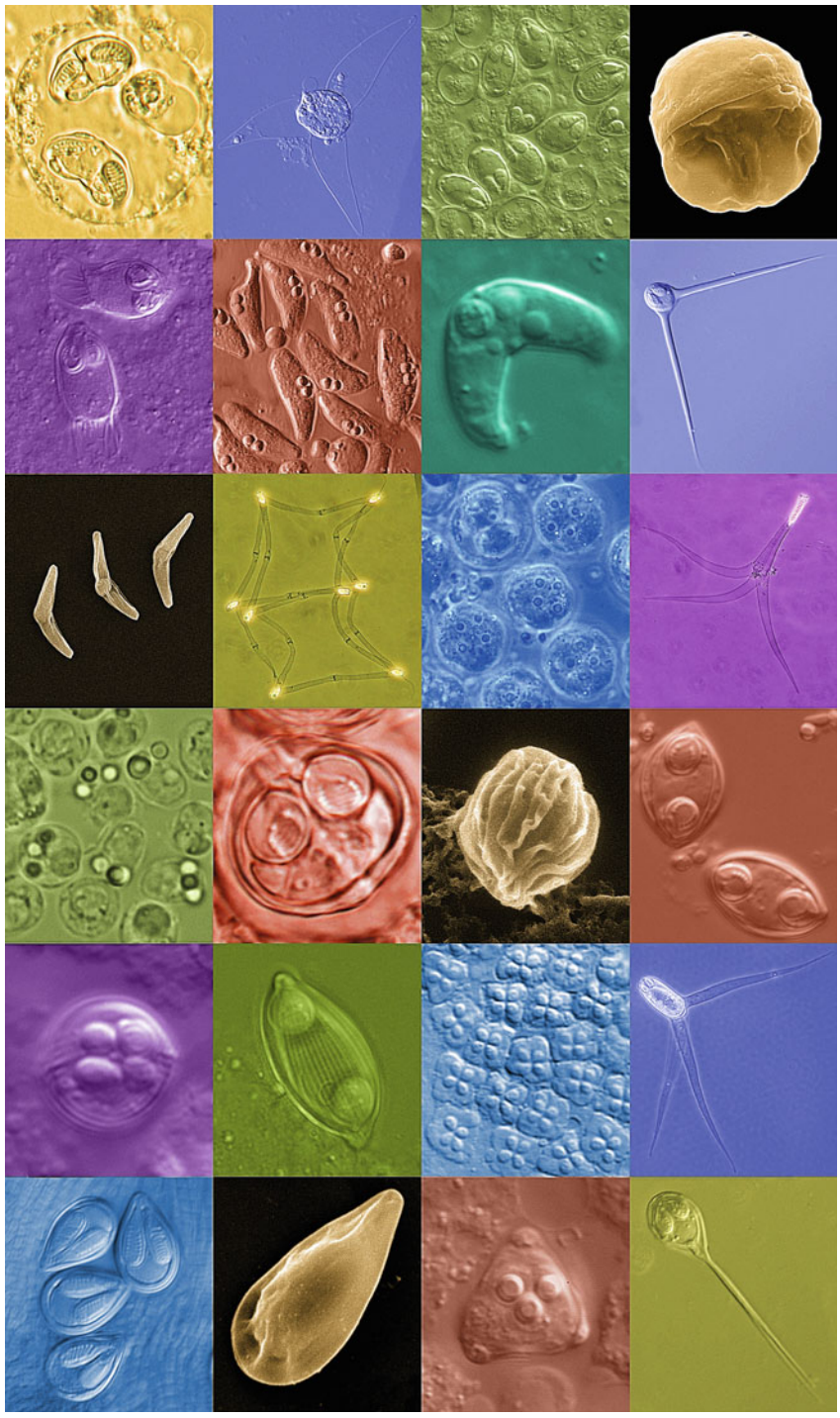

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We dedicate this book to Elizabeth Canning, Iva Dyková and Jiří Lom. Their curiosity and insights have enabled many important advances in understanding myxozoan development and diversity and in revising the taxonomy and systematics of this most fascinating group.

Preface

The genesis of this book lies in research by the lead editor (BO) on the dispersal and metapopulation biology of the freshwater bryozoan, *Cristatella mucedo*. This research, which entailed routine screening of bryozoan colonies for signs of sexual reproduction, led to startling observations of strange globular sacs tumbling within the capacious body cavity of *C. mucedo* colonies collected from sites in the Thames Valley of southern England. These sacs were clearly not of bryozoan origin, and a determination to resolve their mysterious nature led to collaborative work with Liz Canning. This fruitful collaboration resulted in the recognition that those strange sacs were myxozoans and the subsequent description of a previously unrecognised clade of myxozoans—the Malacosporea. The unanticipated discovery of malacosporeans highlights how at the end of the twentieth century, unrecognised biodiversity remains even in a highly populated, developed country whose fauna and flora are more comprehensively documented than in any other. It also emphasises how unexpected outcomes of both fundamental and practical significance can arise when studying what many would regard as obscure organisms (freshwater bryozoans). Thus, following their serendipitous discovery, malacosporeans have played a central role in confirming the longstanding speculation that myxozoans are an endoparasitic radiation of cnidarians. In addition, the causative agent of a devastating disease of salmonid fish was finally identified, the enigmatic nature of a bizarre worm-like parasite of bryozoans (*Buddenbrockia plumatellae*) was resolved when it was revealed to belong to the Malacosporea, and the loss of primitive features during myxozoan evolution has been revealed (muscles and epithelia are lacking in the highly derived Myxosporea).

As a result of the above developments myxozoans are now of specific interest for understanding evolutionary trajectories of early diverging metazoans. They are also of practical interest as causative agents of fish diseases that threaten wild populations and impact aquaculture and fisheries worldwide. Changing environmental conditions are exacerbating some of these diseases while introductions effected by human activities may contribute to disease spread. It is therefore now timely to synthesise the diverse literature on the biology of myxozoans in the context of understanding them as a radiation of endoparasitic cnidarians with complex life cycles that involve invertebrate and vertebrate hosts. Thus, when the lead editor was contacted by Springer Publishers about producing such a book, she readily accepted.

The ambition to develop an edited volume that incorporates chapters broadly representative of myxozoan research led to the assembly of an editorial team with sufficient multidisciplinary expertise to commission such chapters (and sometimes to contribute as authors to them). The result is the first comprehensive book on myxozoans. Our book provides up-to-date cover of subjects ranging from myxozoan ecology, evolution and developmental biology through to immunology, risk assessment, disease mitigation and predicting the impacts of climate change on disease. We are gratified that nearly all of the myxozoan researchers contacted were keen to contribute and, consequently, our authors are based in academic and government agencies in 13 countries in Europe, North America and Asia. This has enabled us to achieve our original ambition—to provide extensive cover of myxozoan research today and to characterise the functional biology of these endoparasitic cnidarians. The synthesis of knowledge provided by the various chapters in our book will be of fundamental interest to invertebrate zoologists, evolutionary biologists, developmental biologists, ecologists and parasitologists and of practical interest to fisheries and conservation biologists. Our chapters also identify gaps in knowledge which are highlighted as key questions for future research. We hope these questions may inspire the next generation of myxozoan researchers to contribute in new ways.

We are grateful to Stephen Atkinson for designing and putting together our book's lovely frontispiece which illustrates a diversity of myxozoan spore stages. The images are described below as species (where known), spore stage and image credit: Ariadna Sitjà-Bobadilla (ASB), Stephen Atkinson (SA), Chris Whipps (CW), Hanna Hartikainen (HH).

Top row: *Enteromyxum leei* myxospores (ASB); aurantiactinomyxon-type actinospore (SA); *Myxobolus notropis* myxospores (SA); *Sphaerospora dicentrarchi* myxospore (ASB)
Second row: *Hoferellus carassii* myxospores (SA); *Ceratomyxa sparusaurati* myxospores (ASB); *Ceratonova gasterostea* myxospore (SA); *Dicauda atherinoidi* myxospore (SA)
Third row: *Sphaeromyxa kenti* myxospores (CW); *Chloromyxum auratum* antonactinomyxon-type actinospore (SA); *Tetracapsuloides bryosalmonae* malacospores (HH); *Myxobolus cerebralis* triactinomyxon-type actinospore (SA)
Fourth row: tetractinomyxon-type actinospores (SA); *Sphaerospora sparis* myxospore (ASB); *Chloromyxum auratum* myxospore (SA); *Myxidium* sp. myxospores (SA)
Fifth row: *Chloromyxum* sp. myxospore (SA); *Myxidium anatidum* myxospore (SA); *Kudoa inornata* myxospores (SA); echinactinomyxon-type actinospore (SA)
Bottom row: *Myxobolus insidiosus* myxospores (SA); *Myxobolus* sp. myxospore (CW); *Ceratonova shasta* tetractinomyxon-type actinospore (SA); *Henneguya zschokkei* myxospore (SA)

Each of our chapters received two reviews (one from a contributing author to another book chapter, one from someone with more general expertise in the subject area) and further extensive evaluations by the editors. Such appraisal has helped to improve the quality and cover of our chapters. We are therefore grateful to the following who have variously acted as reviewers for one or more of our chapters: Pilar Alvarez-Pellitero, Stephen Atkinson, Vickie Blazer, Isaure de Buron, Allen Collins, Arik Diamant, Iva Dyková, Daphne Fautin, Ivan Fiala, Stephen Feist, Bart Gorglione, Sascha Hallett, Peter Hammond, Ashlie Hartigan, Hanna Hartikainen, Jason Holland, Jukka Jokela, Simon Jones, Egil Karlsbakk, Thomas Lang, Tim Littlewood, Christine Moffitt, David Morris, Oswaldo Palenzuela, Ed Peeler, Russell Perry, Maria Santos, Andrew Shinn, Ariadna Sitjà-Bobadilla, Andrea Waeschenbach, Thomas Wahli, Chris Whipps, Geert Wiegertjes and Hiroshi Yokoyama.

We are deeply indebted to all of our authors whose contributions have made our book possible and who bore with us during the various stages of chapter development. They responded to our numerous editorial requests in a helpful and accommodating fashion even when they did not have the overview of all of the developing chapters that was available to us. We hope that they will be happy with the outcome and gratified by the synergism arising from their collective contributions.

Finally, we thank Annette Schneider from Springer Publishers who originally contacted the lead editor regarding her multifaceted research on bryozoans and myxozoans and to enquire about publishing a book that would integrate the evolution of highly specific body-plans, life cycles and host-parasite interactions. The resulting book is clearly broader than this and we are grateful to Annette for encouraging us to drive its subsequent development forwards. We also thank Andrea Schlitzberger who later took over as the Springer editor and has provided valuable feedback especially on production matters.

Beth Okamura
Alexander Gruhl
Jerri L. Bartholomew

Contents

An Introduction to Myxozoan Evolution, Ecology and Development

1

Beth Okamura, Alexander Gruhl, and Jerri L. Bartholomew

Abstract

Cnidarians are familiar invertebrates that are widely recognised as typical representatives of marine and freshwater environments. Yet, until recently, all cnidarians were regarded as free-living animals. It is now clear, however, that a clade of cnidarians diverged in ancient times to become endoparasites that today comprise the Myxozoa—common and occasionally highly problematic parasites of fish known since the 1800s. This chapter introduces our volume that focuses on the evolution, ecology and development of myxozoans in light of their cnidarian origin and comprises the first comprehensive book on the group. In this introductory chapter we briefly describe myxozoan biology and highlight milestones in myxozoan research. We then summarise our contributing chapters that review and synthesise recent research and which collectively provide insights on: myxozoan origins, evolutionary trends and diversification, development and life cycles, interactions with hosts, and disease ecology. Key areas for future research commonly identified in our chapters include: improved knowledge of myxozoan diversity, resolution of life cycles and the implications of environmental change for disease risk. We explore how new technologies (particularly, environmental DNA and-omics approaches) will contribute to understanding these and other issues, such as identifying and linking myxozoan developmental stages with those of free-living cnidarians and identifying virulence factors and other adaptations to parasitism. We conclude that myxozoans now merit broad recognition as a clade exhibiting comparable patterns of species richness

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and host exploitation to those of macroparasites (cestodes and even trematodes) but which has converged on strategies of microparasites (parasitic protists) for host exploitation.

Keywords
Cnidarians · Myxozoan diversity · Myxozoan biology · Disease impacts · Life cycle · Malacosporea · Myxosporea · History of myxozoan research · Macroparasites · Microparasites · Future myxozoan studies

1.1 Introduction

Animals belonging to the Phylum Cnidaria are widely recognised for their global ecological and economic importance and their contributions to quality of life. For instance, the Great Barrier Reef is the world's largest living structure and can be seen from outer space (Australian Government Great Barrier Reef Marine Park Authority 2011). The impressive diversity of organisms found here is dependent on the corals that collectively create the reef framework. Worldwide, coral reef ecosystems are home to a quarter to a third of all described marine species, although even this may be an underestimate (Plaisance et al. 2011), and they provide ecosystem services for tourism, fisheries and coastline protection. Meanwhile, ocean acidification threatens coral reef integrity (Hoegh-Guldberg et al. 2007) and spectacular jellyfish blooms may be increasing in frequency and extent as a result of environmental degradation, thus creating alarming 'oceans of slime' (Schrope 2012; Condon et al. 2013). Members of the Phylum Cnidaria are also important model organisms for fundamental research. In particular, *Hydra* is the model organism used for understanding processes of regeneration and morphogenesis (Chapman et al. 2010) and of non-senescence (Boehm et al. 2013) while the sea anemone, *Nematostella vectensis*, is used as a model for early diverging metazoans in evolutionary developmental biology and comparative genomics (Darling et al. 2005; Putnam et al. 2007). Finally, cnidarians have a long and venerable fossil record which reveals many reef building taxa distinct from the scleractinian corals that dominate in warm, low latitude seas of today (Clarkson 1986). Yet all these cnidarians are free-living animals.

It is now clear that at some distant time in an ancient environment a clade of cnidarians diverged from their free-living cnidarian ancestors ultimately to become parasitic. During the process of divergence these cnidarians became miniaturised, morphologically simplified and evolved a complex parasitic life cycle. Today these animals comprise the Myxozoa. Although some early researchers had suggested a cnidarian affinity of myxozoans, extreme morphological and molecular divergence has precluded determining their status with certainty until very recently. This book is inspired by the recognition that myxozoans are a radiation of endoparasitic cnidarians that are of considerable ecological, economic and even medical concern as well as being of great evolutionary interest.

It is increasingly apparent that myxozoans are widespread, diverse and important components of ecosystems. With some 2,200 species currently described (Lom and Dyková 2006), they represent around 18 % of cnidarian species diversity as it is currently known (Fig. 1.1). Indeed, there are many more species of myxozoans than scyphozoans (n = 228)—a surprising statistic given the endoparasitic nature of the former and the distinctiveness of the latter. This picture may be somewhat inaccurate (for instance potentially not accounting for convergence or cryptic speciation) but we predict this will not grossly influence the order of magnitude difference in diversity between the two clades.

Certain myxozoans cause emerging diseases that are linked with environmental change. These emerging diseases impact wild populations of

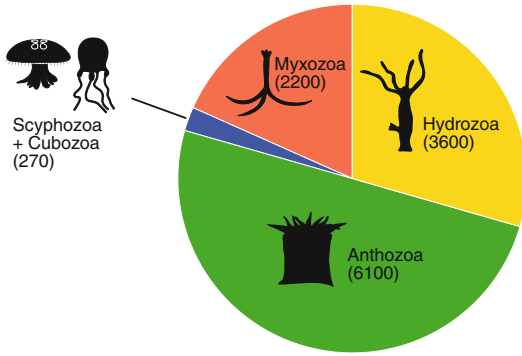

Fig. 1.1 Pie-chart showing the proportions (and numbers) of described species within the Myxozoa and other cnidarian clades. Cubozoa has been pooled with Scyphozoa due to the low number of species (42) in this group. Data for the number of described species from Zhang (2011)

some of our most iconic fish, such as salmon and trout (causing, for instance, whirling disease and proliferative kidney disease). Myxozoan infections can also result in substantial economic loss to aquaculture and fisheries, impacting the health of fish in farms and hatcheries and reducing the marketability of wild fish. Human health can be challenged by myxozoans when raw infected fish are consumed by immunocompromised individuals. Taxonomic publications that continue to describe new myxozoan species and to recognise species complexes (including cryptic species) provide evidence that myxozoan species diversity is probably greatly underestimated. Indeed, patterns of myxozoan infections in South American fish led Naldoni et al. (2011) to speculate that there may be two novel myxozoan species per freshwater fish species in the Neotropics, suggesting a total of 16,000 species of myxozoans in this biogeographic region alone. While extrapolating upwards from local patterns is of course prone to error, such speculations arise for a reason. It is therefore possible to conjecture that the total number of species of endoparasitic cnidarians may compete with or even exceed the number of species of their free-living counterparts. Finally, myxozoans also provide fundamental insights into the evolution of early diverging metazoans. In particular, they illustrate how relatively simple diploblastic organisms have evolved to become endoparasites that engage in sophisticated interactions with their hosts. These interactions have increased over time as myxozoans evolved complex life cycles from parasites that initially infected a single host. Diversification has enabled myxozoans to exploit freshwater, marine and terrestrial hosts and to expand beyond their original vertebrate host range (fish) to incorporate amphibian, reptile, waterfowl and even small mammal hosts.

Our ambition is to bring this previously orphan group of endoparasites into the cnidarian fold by reviewing various facets of the biology of this fascinating radiation. Thus, we present a series of chapters that summarise and synthesise recent progress in understanding the evolution, ecology and development of the Myxozoa and, where possible, derive new interpretations of myxozoan features in light of a descent from free-living cnidarian ancestors. By way of introduction we present a brief account of myxozoan biology and highlight milestones in understanding the group. These historical research advances provide the context for current myxozoan research which, at least in part, is variously conveyed by the broad cover of our chapters. We then outline the nature of our specific chapter contributions, consider how collectively they present an overview of recent advances in myxozoan research and suggest future research directions.

1.2 Myxozoan Biology

The Myxozoa are endoparasites characterised, in general, by a two host life cycle which typically involves invertebrates and vertebrates as definitive and intermediate hosts, respectively. There are two clades presently recognised: the Malacosporea and the Myxosporea. The former is a very small clade (4 described species) that has retained primitive features (e.g. epithelia and muscles). Malacosporeans develop as inactive sacs or active myxoworms in their definitive hosts—freshwater bryozoans. Myxosporeans have undergone substantial radiation (2,180

described species; Lom and Dyková 2006) and are characterised by derived features (e.g. lack of tissues, complex spores). They utilise annelids as definitive hosts. Intermediate hosts of myxosporeans include amphibians and homeotherms (waterfowl and shrews) in addition to fish. Fish are the only known intermediate hosts for malacosporeans.

All myxozoans achieve transmission to new hosts by multicellular spores comprised of external valve cells that enclose infectious amoeboid cells (sporoplasms) and cells bearing polar capsules. The latter are intracellular organelles with eversible filaments that attach to host surfaces. Polar capsules are homologous to nematocysts (see Chap. 3) that are used for prey capture and defense in free-living cnidarians. After the attachment of spores, sporoplasms (or their secondary cells—sporoplasm germ cells) invade hosts to achieve infection. Malacosporean spores are simple and degrade relatively quickly. Myxosporean spores produced in fish hosts (myxospores) possess thickened walls and can remain infectious to annelids for months to years while those produced in annelid hosts (actinospores) are relatively short-lived and morphologically complex with inflatable caudal appendices for buoyancy.

Malacosporean sporogonic (spore-producing) stages develop as multicellular sacs or worms in freshwater bryozoans and as unicellular pseudoplasmodia in fish hosts. Myxosporean sporogonic stages develop as pansporocysts with an outer cellular wall in annelids and as simple membrane-bound structures such as plasmodia (containing many nuclei) or pseudoplasmodia (containing one nucleus) in fish hosts. In both malacosporeans and myxosporeans, presporogonic stages may proliferate as single cells before reaching sites where sporogonic stages develop.

Many myxozoan infections are innocuous (not readily apparent) and have little impact on fish hosts (Shul'man 1990; Lom and Dyková 1992). However, certain myxozoans cause severe diseases with economic impacts on fisheries and aquaculture (e.g. Pote et al. 2000; Diamant et al. 1994; Kent et al. 1994b; Hallett and Bartholomew 2011) at least one of which is linked with environmental change (Okamura et al. 2011). Myxozoans have evolved to infect virtually all fish tissues and exhibit a degree of host and tissue specificity. Infection development in invertebrates remains poorly investigated, in part because few myxozoan infections in invertebrate hosts are identified and, unlike fish, invertebrates have little direct commercial or recreational value. Exceptions are those myxozoans associated with severe diseases in economically important fish. Invertebrate-myxozoan interactions in these cases have received relatively intense study.

Myxozoans particularly exemplify morphological simplification associated with parasitism and this has, in part, contributed to the long history of debate over their higher level affinities. Although some tissues are retained in malacosporeans, features associated with a digestive tract and a nervous system are lacking. Cilia and centrioles are also absent. Furthermore, distinguishing gametes is challenging as is the identification of embryonic and larval stages.

1.3 History of Study: Milestones in Understanding the Myxozoa

Myxozoans were first encountered by Jurine in 1825 who found cysts in the musculature of the whitefish, *Coregonus fera*, in Lake Geneva (Jurine 1825). It is quite likely that the first myxozoan reported in the literature is now extinct, as *C. fera* has not been observed since 1920 (Freyhof and Kottelat 2008). Subsequent early research on myxozoans is reviewed by Shul'man (1990). Here we summarise highlights of this research up through the first half of the last century referring the reader to Shul'man (1990) for specific references. In the 1840s Müller provided the first details on myxosporean spores, calling them 'psorosperms', and Dujardin concluded that spores (psorosperms) are produced within the plasmodia. Lieberkühn's publications in the 1850s describe how an amoeboid embryo (sporoplasm) emerges from spores and the development of plasmodia while, in the 1860s, Balbiani described polar capsules and the

discharge of polar filaments. In the 1880s Bütschli undertook more extensive studies to describe plasmodial stages as multinucleated pansporoblasts, the development of spores, the discharge of polar filaments and the role of spores in transmission. In the 1890s Thélohan classified the myxozoans as Myxosporidia using spore characters and established many genera. He also studied how spores achieve transmission, the subsequent development of stages within fish hosts and noted the multicellular nature of spores and the formation of polar filaments. In the late 1800s and early 1900s, Cohn, Laveran and Mesnil, and Doflein highlighted the occurrence and importance of asexual multiplication in infections. The first monograph on Myxosporidia was published in 1910 by Auerbach who also described many new species and conducted infection studies. Kudo's 1919 monograph was highly valuable for subsequent researchers, providing information on all the species known. The multicellular nature of spores led to early proposals that myxozoans should not be classified as Protozoa but as Metazoa (e.g. Štolc 1899; Emery 1909; Ikeda 1912). In 1938, Weill pointed out the similarity between polar capsules and nematocysts (Weill 1938).

Ultrastructural investigations enabled new insights on myxozoan functional biology and cytological features. The technological advances of transmission electron microscopy allowed study of spore structure, polar capsules and polar filaments and of sporogony and polar capsule formation (e.g. Lom 1969; Lom and de Puytorac 1965; Lom and Vavra 1965). Further ultrastructural studies documented developmental stages and endogeny (characterising enclosed secondary and tertiary cells) while others focused on features of multinucleate plasmodia such as microvilli, pinocytotic vesicles, Golgi complexes, mitochondria and other constituents. Many of these investigations involved joint publications by Lom and Dyková and the reader is referred to their important review (Lom and Dyková 1992) as well as their comparative study of actinosporeans and myxosporeans (Lom and Dyková 1997) for further details and references. In parallel with insights gained through ultrastructural studies there was growing documentation of the histopathology of myxozoan diseases in fish (e.g. Molnár 1982) along with experimental studies on pathogenicity and transmission. The reader is referred to Feist and Longshaw (2006) and to Chaps. 8, 14 and 15 of this volume for further discussion and associated references.

Breakthroughs in understanding fundamental aspects of myxozoan life histories, diversity and evolution have been achieved over the past 35 years. In the 1980s Markiw and Wolf united the actinosporeans and myxosporeans by demonstrating these were stages in a common life cycle (Markiw and Wolf 1983; Wolf and Markiw 1984). Suppression of the class Actinosporea was thus proposed (Corliss 1985; Kent et al. 1994a). Documentation of meiosis in actinosporean and malacosporean developmental stages identified annelids (Marquès 1987; El-Matbouli and Hoffman 1998) and bryozoans (Canning et al. 2002) as definitive hosts. The metazoan-nature of myxozoans was confirmed through molecular (Smothers et al. 1994; Schlegel et al. 1996) and ultrastructural (Desser et al. 1983) evidence, while support for Weill's (1938) proposed affinity of myxozoans with cnidarians was provided by combined molecular and morphological analysis (Siddall et al. 1995). In the 1990s the first malacosporeans were discovered as odd sac-shaped myxozoans freely floating in the body cavity of freshwater bryozoan hosts (Canning et al. 1996; Okamura 1996; see also Preface) and a few years later a close relative was linked with salmonid proliferative kidney disease (Anderson et al. 1999; Feist et al. 2001) demonstrating that malacosporeans also possess a complex life cycle. Finally, the enigmatic, worm-like *Buddenbrockia* (described in 1910 as an endoparasite of freshwater bryozoans; Schröder 1910) was shown to be a myxozoan on the basis of possessing polar capsules (Okamura et al. 2002) and through molecular sequence data (Monteiro et al. 2002). Further developments in molecular biology (gaining sequence data for expressed genes) provided evidence that *Buddenbrockia,* and hence the Myxozoa, group within the Cnidaria with strongest support as a sister taxon to the Medusozoa (Jiménez-Guri et al. 2007)

(see Chap. 2 for further cover). This assignment has subsequently been upheld by independent studies based on cnidarian-specific genes (Holland et al. 2011; Shpirer and Chang 2014) and phylogenomic analyses of two myxosporeans (Nesnidal et al. 2013; Feng et al. 2014), thus finally resolving the longstanding debate about the nature of myxozoans. A recent publications has proposed myxozoans as a subphylum within Cnidaria (Collins 2009) and myxozoans are now presented in invertebrate zoology textbook chapters on cnidarians (e.g. Ruppert et al. 2004; Brusca and Brusca 2003; Pechenik 2009).

1.4 Recent Progress in Understanding Myxozoan Evolution, Ecology and Development

The chapters in this book present an overview of recent advances in myxozoan research and have been contributed by researchers with diverse backgrounds. Thus, our authors are based in academic and government agencies and their expertise ranges from evolutionary biology, ecology, invertebrate zoology, parasitology, developmental biology, immunology, pathology, epidemiology, disease risk and modelling. Below we outline how our chapters contribute to summarising our understanding of myxozoans according to several general areas.

1.4.1 Origins, Evolutionary Trends and Diversification

Although the cnidarian affinity of myxozoans is now broadly supported many aspects of myxozoan evolution remain obscure. How did endoparasitism evolve and how might cnidarian traits have promoted an endoparasitic lifestyle? Who are the closest cnidarian relatives and how have myxozoans radiated to exploit a variety of hosts? Can we discern any evolutionary trends within the Myxozoa and how can we practically recognise species given the extreme morphological simplification that characterises the group? Our first six contributed chapters focus on these and other issues.

In Chap. 2 Okamura and Gruhl review the history of the placement of Myxozoa based on morphological and molecular evidence that was insufficient to robustly support a higher level affinity and how further collective support now establishes a cnidarian status. They then evaluate possible pathways in the evolution of the complex myxozoan life cycle. They first consider how a free-living myxozoan precursor may have evolved to become endoparasitic through close association with ancestral hosts. Such associations may have involved precursors feeding on hosts, surviving predation, or using hosts to reduce environmental variability or to achieve dispersal. Current patterns of host exploitation suggest that ancestral hosts may have been freshwater bryozoans, annelids or fish, or precursors to these groups living in freshwater or marine environments. Critical examination of the likelihood of various scenarios suggests that freshwater bryozoans or fish may have acted as ancestral hosts of adult or larval stages, respectively. Relevant arguments for this include the occurrence of meiosis in invertebrate hosts, the more derived nature of myxozoans that infect annelids and that fish are hosts for most members of all major myxozoan clades. Okamura and Gruhl then discuss how new hosts may have been adopted subsequently, resulting in complex life cycles. The more likely scenarios include fish being acquired as secondary hosts of endoparasites of freshwater bryozoans (or their precursors) or bryozoans being acquired as secondary hosts of fish, the latter entailing a switch to using invertebrates as definitive hosts. Annelids may have been acquired as secondary hosts if parasites in bryozoans or fish evolved to infect worms. The former scenario would imply the subsequent divergence of the Malacosporea and Myxosporea and transfer of adult development from bryozoan to annelid hosts.

By comparing and contrasting myxozoan and cnidarian features, Okamura et al. explore traits that may have facilitated the transition to an

endoparasitic lifestyle in Chap. 3. Cnidarian features identified as particularly relevant include their extensive capacity for regeneration, transdifferentiation, and dormancy as well as a clear propensity to produce novel stages with such capabilities. Myxozoans similarly can undergo dormancy, for instance as myxosporean spores or as covert infections in dormant stages of malacosporeans in bryozoan hosts. They subsequently regenerate from the dormant state and undergo development to trophic stages. The diploblastic condition may also have predisposed cnidarians to endoparasitism since this body plan is based on resource capture and uptake across extensive external and internal epithelial layers. The cell-within-cell development that is a hallmark of myxozoans has also been noted during cnidarian development, and both malacosporeans and cnidarians achieve asexual reproduction via fission and budding. The authors point out that the cnidarian tendency for shortening the life cycle obscures equating stages of the complex parasitic life cycle of myxozoans with the benthic polyp and planktonic medusa stages of cnidarians. For instance, *Hydra* has lost the medusa stage and sexual reproduction now occurs in the polyp. The knotty issue of sexual reproduction in myxosporeans is also tackled. Thus, the standard view of myxosporean development implies either the production of multicellular chimaeras with genetically distinct cells cooperating to produce gametes, or exclusive selfing in a group that has undergone extensive radiation. These issues do not apply in a more recent interpretation of myxosporean development. The chapter concludes by examining the homologies between polar capsules and nematocysts and their divergence in structure according to function. This leads to the identification of similar apical plug-like structures that close the undischarged capsules in both the myxozoans and medusozoans. The absence of this structure in anthozoans provides further support for a myxozoan/medusozoan affinity.

Comprising 18 % of cnidarian species diversity, myxozoans demonstrate a considerable (and underestimated) radiation of endoparasites. In Chap. 4 Fiala et al. compare and contrast radiations of the species-poor malacosporeans and the species-rich myxosporeans and examine reasons for disparity in the diversification of the two groups. They present the most up-to-date analysis of malacosporean diversity by undertaking a combined analysis of two SSU rDNA datasets that reveals new lineages and demonstrates, as previously (Hartikainen et al. 2014b), that transitions between vermiform and sac-like stages have occurred repeatedly across the phylogeny. Although malacosporean diversity is no doubt currently underestimated (e.g. because infections may be undetected and many regions are unsampled) it is anticipated that low species diversity truly characterises the Malacosporea. Factors that may have driven the radiation of the diverse Myxosporea include invasions of freshwater and marine habitats, tissue tropism, the acquisition of hardened spores, the great variety of definitive worm hosts potentially available for exploitation, and the incorporation of additional vertebrate host groups. This radiation has been accompanied by a diversification in spore types that may be linked with increasing the probability of transmission (e.g. by prolonging suspension in the water column or achieving dormancy) while the convergent acquisition of spore morphotypes may relate to inhabiting particular host environments. Factors that may have contributed to lack of diversification in the depauperate malacosporeans include short-lived, simple spores (that are so far indistinguishable amongst taxa), a low diversity of definitive bryozoan hosts and reduced uptake and excretion across epithelia. Both malacosporeans and myxosporeans exhibit morphological simplification and reductions in body size. In malacosporeans this is exemplified by loss of musculature which is present in worm-like stages and absent in smaller sacs. The myxosporeans have entirely lost tissues and their trophic stages consist of tiny plasmodial or pseudoplasmodial stages.

Traditional myxozoan classification is challenged by convergence in spore morphologies but it is clear that characters such as host preference, tissue specificity, traits of developmental stages and DNA sequence data can be incorporated to improve classification. Fiala et al. present a revised myxozoan classification and an updated

taxonomic key in Chap. 5. They recommend a combination of morphological, biological and molecular data for characterising lineages at various levels (e.g. species, genus) and to develop phylogenetically informative taxonomies. The fast-evolving SSU rDNA has proved to be useful for discriminating species in molecular phylogenies and can be informative at higher levels. However, problems in alignment and in differential rates of evolution within different portions of the SSU gene as well as variation in rates of evolution of the gene between different myxozoan taxa can lead to problems of long branch attraction. The few other molecular markers currently available for phylogenetic analysis have provided some independent support for higher level inferences based on SSU rDNA, but these are generally less informative and new markers are required. Reviewing the molecular phylogenetic relationships resolved so far within the major myxozoan clades (based on SSU rDNA data) enables Fiala et al. to identify where additional taxon sampling may improve our understanding of myxozoan evolution and to suggest possible cases for taxon suppression or splitting.

Atkinson et al. provide explicit consideration of best approaches for characterising myxozoan species in Chap. 6. As concluded by Fiala et al. in Chap. 5, they suggest that a combination of both molecular and morphological characters is required for characterising species. However, obtaining reliable data can be problematic. For instance, researchers need to be aware of plasticity in spore morphologies, taking into account natural morphological and morphometric variation in spores within and between hosts. It is also important to consider, when using tissue specificity as a taxonomic criterion, whether parasites may be present incidentally and how individual host susceptibility may influence infection sites. Potential problems with DNA sequence data include the presence of mixed infections and PCR bias. The use of general primers can also lead to amplification of host DNA, however myxozoan genomes (see Chap. 2) should now greatly reduce attributing host sequences to those of myxozoans. Complementary to Chap. 4's focus on identifying novel diversity at higher taxonomic levels, Atkinson et al. review genetic evidence for novel diversity at the species level (e.g. strains and cryptic species). They anticipate that the number of recognised myxozoan species will continue to expand as new species complexes and cryptic species are encountered and described.

While fish are intermediate hosts for the vast majority of myxozoans, certain myxosporeans have adopted other vertebrate hosts such as amphibians and reptiles. In addition, there is now strong evidence that some myxosporeans have made the transition to exploiting warm-blooded hosts, including waterfowl and small mammals. Hallett et al. review myxozoan infections in homeotherms in Chap. 7, and describe how the presence of mature spores in individuals of three shrew species sampled in Europe and of seven duck species sampled in North America suggest that these homeotherms are true hosts. So far two myxosporean species belonging to separate genera appear to exploit ducks and shrews but undersampling suggests that further diversity can be expected. Accidental infections of homeotherms by myxozoans may also occur (e.g. possibly in moles). The consumption of infected raw fish by humans can result in damage to intestinal cells due to activation of myxospores or to immune reactions. Myxospores have also been detected in human faeces when infected fish are consumed. There is no evidence, however, that any myxozoan life cycle actually involves a human host. The relatively restricted number of lineages of myxosporeans infecting homeotherms (e.g. in comparison to lineages infecting amphibians) may reflect body temperature as a barrier to host switching or limited opportunities for transmission. An alternative explanation is biased sampling. Hallett et al. speculate that homeotherms may have been acquired as hosts if parasites of fish or amphibians switched to utilising birds and then some of these parasites subsequently adopted mammal hosts—a scenario supported by the close phylogenetic relationship between parasites of shrews and ducks. Many fascinating issues remain to be resolved regarding myxosporeans in homeotherms, including the

nature of their life cycles and transmission pathways and whether myxozoans have evolved to exploit the many diverse vertebrates that utilise aquatic habitats.

1.4.2 Aspects of Development and Resolution of Life Cycles

The extreme morphological simplification of the Myxozoa is epitomised by the evolution of plasmodia and pseudoplasmodia and the associated vital functions conducted at the cellular level. Nevertheless, a tissue level of development is retained in malacosporeans and, on close inspection, is expressed in the multicellular spores of myxosporeans. These insights are only gained by examining and understanding development that characterises different stages in the life cycle of myxozoans. The resolution of myxozoan life cycles is therefore critical—it enables discovering fundamental aspects of myxozoan development and illustrates the diversity of hosts that myxozoans have evolved to exploit.

In Chap. 8 Feist et al. describe the fundamental cellular processes that underlie development, sporogony and motility in myxozoans. Endogeny, the development of internal, secondary cells within an outer, primary cell is a common phenomenon in many myxozoan life cycle stages. This cell-within-cell arrangement also occurs in certain protistan taxa, which provided support for the previously hypothesised protistan affinities of myxozoans. Endogeny is achieved by the engulfment of one mitotic daughter cell by the other, as occurs during the formation of malacospores and actinospores. It may also arise by the formation of an internal cell membrane from endoplasmic reticulum to enclose an endomitotically formed nucleus. Cellular processes associated with development are described, including meiosis (which occurs in the definitive hosts of both malacosporeans and myxosporeans), the fate of the gametes, the nature of presporogonic stages, and how these transform into sporogonic stages. Patterns of sporogony (spore formation) differ between taxa and life cycle phases, but detailed chronological documentation of the process is only available for a few myxozoan species. During sporogony cells differentiate into the spore cell types (sporoplasms, valve cells, capsulogenic cells). The assembly of the spores requires cellular interactions, including motility, aggregation and the formation of cell-cell junctions. Active motility is a key cellular process that enables host invasion, migration through host tissues, and immune system evasion. Three main modes of motility occur in myxozoans: amoeboid movement, twitching of presporogonic stages in certain species, and muscle-mediated movements in myxoworms. The authors review the cellular basis for these types of motility and identify F-actin as the most important cytoskeletal element. Motility based on cilia and flagella is entirely lacking in myxozoans. Many details of myxozoan motility remain to be clarified.

In the course of the evolution of endoparasitism many morphological, cytological and developmental traits that potentially link myxozoans with their free-living cnidarian relatives and with metazoans in general have become obscured, hindering the identification of homologous features. Comparisons are further impeded by the adoption of a non-standard terminology for myxozoan features, mainly influenced by their long classification as protists. The aim of Chap. 9 by Gruhl and Okamura is to describe the architecture and development of myxozoans from a comparative zoological perspective. Myxozoans were long viewed to lack any tissue-level of development. However, the discovery of malacosporean stages in freshwater bryozoans revealed recognisable tissues in the form of epithelial sheets and musculature. A detailed review of myxozoan cell-junction types and their distribution, and of the occurrence and nature of extracellular matrix enables the identification of vestiges of epithelia in many myxozoan stages. For instance, cell junctions characteristic for epithelia, cell polarity, and extracellular matrix are also found in pansporocysts and spores. Specialised tissue types, such as resorptive and secretory tissues and musculature are described,

along with evidence for nervous tissue based on the identification of components of neurotransmission pathways from transcriptomes of *Buddenbrockia* myxoworms. Finally, the scattered literature on myxozoan gametogenesis and development is reviewed. This especially raises questions concerning the identity of germ layers and the presence of blastula and gastrula stages.

Our current understanding of myxozoan development and life cycles is poor and mostly derives from laboratory studies. Information on complete life cycles is available for only some 50 myxozoan species. Eszterbauer et al. tabulate the vertebrate and invertebrate hosts affiliated in these life cycles in Chap. 10 and provide descriptions of the laboratory techniques used for examining myxozoan life cycles and development. Details of how to establish and maintain laboratory stocks of particular invertebrate hosts are described. It is to be hoped that such methods will be amenable, or they may be tweaked, for the successful maintenance of other invertebrate hosts when these become known. Issues to consider when conducting experimental transmission studies are stressed, such as infectious dose and temperature. Life cycles that have been well characterised through laboratory investigations involve only a small number of myxozoans that cause economically important diseases of fish and these are reviewed. Unusual aspects of certain myxozoan life cycles are also highlighted including fish-to-fish transmission in the genus *Enteromyxum* and vertical transmission that is achieved in clonally reproducing bryozoan hosts and in fissiparous oligochaetes. The development of in vivo culturing would enable further more sophisticated studies of myxozoan life cycles, however, despite some attempts, sustained in vivo culturing has yet to be achieved.

1.4.3 Host-Parasite Interactions

Host-parasite interactions involve the complex interplay of adaptations and counter-adaptations that arise as a result of parasites exploiting hosts and minimising the effects of host defences and hosts responding to limit such exploitation. These interactions can be evident by examining fluctuations in host and parasite populations, and how environmental variation influences the development of parasites and hosts. The mechanisms of interactions involve host recognition, host invasion and development within hosts and the concomitant immune or behavioural responses of hosts to resist or tolerate infection. Specificity of infection to particular hosts or to particular sites within hosts is an outcome of the ongoing interplay between parasites and hosts.

Chapters 11 and 12 examine myxozoan interactions with invertebrate hosts. Hartikainen and Okamura describe how interactions of malacosporeans are closely linked with the clonal life history of their bryozoan hosts. Most insights have been gained from the well-studied malacosporean/bryozoan system involving *Tetracapsuloides bryosalmonae* and *Fredericella sultana*, as *T. bryosalmonae* is the causative agent of salmonid proliferative kidney disease (PKD). Laboratory and field studies indicate that malacosporeans cycle between covert infections (single cells associated with the host body wall) and overt infections (spore-filled sacs) and that cycling is dictated by host condition. Thus, energetically demanding overt infections develop when bryozoans are in good condition and growing rapidly. When conditions for growth become poor, covert infections predominate. Overt infections are virulent, causing temporary castration of their colonial hosts and reducing growth. In contrast, covert infections are avirulent and persist throughout the year. Malacosporeans exploit clonal reproduction of their hosts by achieving vertical transmission to bryozoan colonies that develop from infected dormant propagules (statoblasts) and that are produced from colony fission and fragmentation. Such vertical transmission likely contributes to high infection prevalences and may facilitate dispersal. These various insights suggest transmission-virulence trade-offs that result in intermediate virulence levels in bryozoan hosts and which maximise horizontal transmission to fish without killing their bryozoan hosts.

However, when host mortality is imminent, malacosporeans may undergo a terminal effort to achieve horizontal transmission. While it is widely appreciated that parasites can regulate host populations, host condition-dependent developmental cycling in malacosporeans may downplay such regulation. In particular, when bryozoans would be most vulnerable to regulation, malacosporeans will tend to revert to non-virulent stages. There is some evidence for adaptation of malacosporeans in the form of strain variation and host specificity but there is much scope for examination of adaptation to local host clones, particularly given the capacity for long term persistence of malacosporeans within bryozoan populations. Current evidence suggests that environmental change is influencing bryozoan-malacosporean interactions. Increasing temperatures and eutrophication may, in particular, drive overt infection development resulting in fish disease outbreaks.

Myxospores are released into the environment in order to achieve transmission to annelid hosts. Once transmission is achieved myxosporeans must then develop within their hosts in order to produce actinospores for transmission to fish. Alexander et al. review how myxosporeans deal with this set of sequential filters by examining traits that may influence success at each stage during the actinospore phase of development. Because all known routes of annelid infection involve ingestion of myxospores, they consider how spores may be adapted to increase the likelihood of ingestion by deposit- and suspension-feeding annelids, the environments in which transmission is likely to be maximised and how host trophic ecology may have driven adaptations for transmission by ingestion. Factors that may influence host invasion are poorly understood but there is limited evidence that host susceptibility, immune responses and myxospore dose influence invasion success. Development within annelids can involve targeting particular tissues and this may be associated with release of actinospores by egestion (for intestinal infections), through pores in the host body wall (for infections in the coelom) or directly through the body wall (for infections in the body wall). Myxosporean infections can affect annelid hosts by reducing fecundity and causing tissue damage. Interestingly there is evidence for increased growth and respiration in infected worms. This suggests that myxosporeans may manipulate their hosts, causing energy to be reallocated to produce greater returns (actinospore output) from larger hosts by diverting energy normally targeted to host reproduction. Malacosporeans manipulate hosts causing larger zooids within bryozoan colonies (Chap. 11, Hartikainen et al. 2013), thus manipulation of invertebrate host phenotypes to increase parasite resources may be a common myxozoan strategy for maximising transmission to fish.

The next four chapters in our book examine aspects of fish host-myxozoan interactions. The steps and mechanisms required for successful transmission of myxozoans to their fish hosts are described by Kallert et al. in Chap. 13. The authors start by reviewing the functional morphology of actinospores. They describe how actinospores vary across species in terms of shape, presence of appendages, number of sporozoites and polar capsules, potentially reflecting adaptations to certain habitats or host species. Spore infectivity depends on spore durability and conditions of storage and is reflected in both the viability of sporoplasm primary cells and functional polar capsule discharge. The invasion process is initiated by polar capsule discharge, which is triggered by chemical and potentially also mechanical host cues. Spores anchored to host tissue by their polar filament then release sporoplasms that penetrate into host tissue. It is still under debate which step in the invasion process facilitates host-specificity and whether host-specificity is the result of specific host-recognition or reflects differential survival of parasites in susceptible versus non-susceptible hosts. Successful survival in susceptible hosts may rely on the ability to evade the host immune system. The potential to invade multiple host species may have contributed to myxozoan radiations. Successful experimental transmission is an important component of many laboratory-based studies, and critically depends on the collection of functional spores and the maintenance of spore infectivity.

The authors summarise recommended procedures for harvesting and handling actinospores.

Development of disease control methods (e.g. treatments or vaccines) in order to reduce economic losses in aquaculture caused by myxozoans requires knowledge of fish immune responses and how myxozoans, in turn, respond to these. In Chap. 14 Sitjà-Bobadilla et al. review the processes triggered by myxozoan parasites that can lead to their elimination and the subsequent development of resistance in the host. Innate immune responses are non-specific and based on cells such as lymphocytes, granulocytes, phagocytes, natural killer cells, or on humoral factors, such as peroxidases, lysozyme, or complement. Host tissues can also encapsulate parasite stages. Adaptive immune responses in the form of specific antibodies against myxozoans occur, but seem to be less pronounced as compared to infections with other parasites. A promising approach to understand the immune responses is to analyse changes in immune gene expression in response to infection. Sitjà-Bobadilla et al. review such data for three well studied myxozoan infections (*Tetracapsuloides bryosalmonae*, *Enteromyxum leei*, and *Ceratomyxa shasta*) and also describe how environmental factors may alter immune responses resulting in changes in disease severity and outcome. Parasites have, correspondingly, evolved various mechanisms for evading host immune responses. This may be accomplished by a preference for body regions where the immune system is less active (in immunologically privileged sites), concealment within host cells, the modification of antigens and immunosuppression. Knowledge of fish immune responses is relevant for several strategies for disease control. For instance, breeding can aim to increase the natural resistance of cultured fish strains, vaccination may promote acquired resistance, immunotherapies may alleviate symptoms caused by chronic or excessive immune responses, and immunostimulants may increase survival of parasitised fish.

Although it is widely appreciated that many myxozoans cause asymptomatic infections in fish there is increasing evidence that both biotic and abiotic factors can influence pathogenicity and these are reviewed by Schmidt-Posthaus and Wahli in Chap. 15. Environmental factors that can influence disease severity include temperature and eutrophication both of which are of concern in view of pervasive environmental degradation and global warming. Temperature is widely demonstrated to influence parasite development and pathogenicity in general and this chapter reviews the evidence for and potential mechanisms underlying the influence of temperature on whirling disease caused by *Myxobolus cerebralis* in the USA, proliferative kidney disease caused by *Tetracapsuloides bryosalmonae* in Europe and the United Kingdom, ceratomyxosis caused by *Ceratonova shasta* (formerly *Ceratomyxa shasta*) in the Pacific northwest, and enteromyxosis caused by *Enteromyxum* spp. in the Mediterranean. Biotic factors that contribute to pathogenicity can include host identity and coevolutionary history with myxozoans and there is considerable evidence of a genetic basis for resistance to disease. Other host-related factors associated with disease development include age, size and nutritional state. Ultimately disease development may reflect the influence of multiple factors but the extent to which environmental effects on disease development reflect variation in parasite development or host susceptibility is poorly understood.

Among the defining characteristics of myxozoans is their host and tissue specificity. In Chap. 16 Molnár and Eszterbauer define and review these traits and provide numerous examples of site selection in tissues. The host specificity of most myxozoan species is narrow, with the parasites able to develop only in a single or closely related host species. However, some myxozoans (typically marine species) have broad host ranges. Most myxozoans start their development in blood vessels but further development and sporulation typically occurs in a specific tissue. For example, presporogonic stages of *Sphaerospora dykovae* occur in the blood, but sporogony occurs in the lumen of kidney tubules. Infections of the gill are common and plasmodia may develop in or within lamellae, in gill filaments and inside the gill arch. In contrast to the well-defined locations of infection in the gill,

species with a tropism for connective tissue may be found in different organs. A number of myxozoans infect fish ovaries and a few infect testes and some of these may affect the reproductive success of their hosts. Finally, the authors review these traits in light of molecular data. They demonstrate how host relatedness and tissue tropism are relevant for myxozoan taxonomy and phylogenetics and highlight the importance of molecular tools to investigate host ranges and to differentiate morphologically similar species that reside in the same host.

1.4.4 Disease Ecology

Myxozoan infections in fish can cause economic loss to both aquaculture and wild fisheries and challenge the conservation of threatened wild fish populations. These adverse effects have prompted researchers to understand how myxozoans impact fish populations, what factors influence the development and spread of disease and may therefore represent disease risks, and how disease can be predicted and mitigated. The last five chapters of our book focus on various aspects of disease ecology in relation to these issues.

Epidemiological studies are critical for understanding disease in aquaculture and wild fish populations; however, long-term and consistently collected data are lacking. In Chap. 17, Fontes et al. discuss how epidemiological data can be collected and then compare what is known about seven important myxozoan species: *Myxobolus cerebralis, Ceratonova shasta, Tetracapsuloides bryosalmonae, Henneguya ictaluri, Enteromyxum leei, Kudoa thyrsites* and *Parvicapsula pseudobranchicola*. Methods for collecting data on the prevalence and severity of disease vary and consistency is essential for long-term comparisons. Not surprisingly, there is more known about the first four species, which cause disease in cultured salmonids and catfish in fresh water. Data are lacking for disease and mortality in wild populations, while estimates of mortalities in fish farms, when available, are highly variable and difficult to compare because of different data collection methods. The lack of information on the life cycles of marine myxozoans is a barrier to understanding disease characteristics, such as transmission. All but one of the species reviewed requires an invertebrate host for transmission. Fish-to-fish transmission in *E. leei* presents exceptional challenges for control and poses a serious threat to aquaculture and conservation. *T. bryosalmonae* is unique amongst the well-studied species reviewed in this chapter, in undergoing vertical transmission in the invertebrate host. It therefore presents similar control challenges to those posed by *E. leei*. Examination of factors that affect disease outbreaks shows that temperature has the clearest effect, and this is seen in the seasonality of infections. However other factors, such as host diet, salinity and water flow are also likely to influence transmission and disease. The authors also review available treatment and control strategies and discuss how epidemiological tools can be used to collect data on disease epizootics and to gain new insights on disease transmission.

Myxozoan distributions result from ongoing dispersal and establishment in previously inhabited and occasionally new environments. Today such dispersal may be facilitated by increased connectivity and reduced transportation times associated with global trade, environmental alteration and recreational activities. In Chap. 18 Hallett et al. review the considerable evidence that a number of myxozoans have been disseminated in modern times through such human activities, cropping up as exotic species that can cause emerging diseases. They begin by outlining myxozoan traits that may promote or impede dispersal and establishment. Despite the potential limitations of complex life cycles (requiring both invertebrate and vertebrate hosts to be present) myxozoans have become established in novel environments. This may reflect widespread distributions of particular hosts (e.g. the oligochaete worm, *Tubifex tubifex,* for *Myxobolus cerebralis*), the suitability of a broad range of hosts (e.g. *Kudoa thyrsites* can infect many fish species) or the ability to undergo vertical transmission and long term persistence in clonal invertebrate hosts following dispersal (e.g. *Tetracapsuloides*

bryosalmonae in bryozoan hosts). The outcomes of introductions of myxozoan-infected hosts into novel habitats are: infection of native hosts with exotic parasites, infection of exotic hosts with native parasites (original infection lost, host then picks up native parasites, potentially causing 'spillback' into native hosts) and loss of parasites from exotic hosts (enemy release hypothesis). Myxozoans provide evidence for all three scenarios. There are numerous potential natural and anthropogenic mechanisms of dispersal and these are usefully reviewed along with associated evidence for the dissemination of myxozoans. These mechanisms of dispersal include: natural movements of hosts and predators, activities associated with aquaculture and mariculture, commercial and recreational fishing, the aquarium trade, and shipping. Hallett et al. then describe problematic myxozoans and examples of emerging diseases, many of which arise from dispersal and establishment in new environments and impact both farmed and wild fish populations. In view of the substantial potential for further emerging disease outbreaks due to increased connectivity and shorter transport times, methods are described to identify and limit introductions, including baseline monitoring, diagnostic tools, risk assessments, management strategies, education and legislation.

Shifts in future temperature and precipitation patterns will have profound effects on myxozoan-host interactions and on the dynamics of disease. In Chap. 19, Ray et al. present an overview of myxozoan disease dynamics in the context of climate change and illustrate how changes might be predicted through a case study. Challenges in predicting disease effects under future conditions are numerous and start with the high variability in the climate prediction models themselves. How organisms will respond to changing conditions is equally complicated, especially for myxozoans with complex life cycles. The authors review how parameters that will be affected by climate change (water temperature, precipitation and discharge, nutrients) are expected to influence interactions between aquatic myxozoans and their hosts. Changes in water temperatures will almost certainly cause seasonal shifts in parasite life cycles and range changes as water temperatures either become permissive or limiting. Precipitation and discharge effects are likely to be as important but harder to predict. In some systems changes in precipitation form (snow to rain) will affect habitat stability and may directly and indirectly affect transmission. Changes in water quality and nutrients are expected to be important, primarily through effects on invertebrate hosts. The authors then describe a combination of models to predict the dynamics of *C. shasta* in the Klamath River basin, California USA under several future climate scenarios. They use predictions from existing climate models as inputs to determine future water temperature and discharge at locations in the river where there are long-term data on parasite density, invertebrate host density and fish infection prevalence. Models predicting invertebrate host (polychaete) presence (as a function of discharge and longevity) of free-living infectious spore stages (as a function of temperature) feed into an epidemiological model. The latter predicts changes in population size of infected fish and polychaetes and of the two free-living spore stages. Despite some clear limitations, the model predicts that for the majority of future climate scenarios the projected numbers of infected salmon will be similar to those in a year when high salmon mortality was sustained. The great influence of high winter discharge on disease dynamics predicted by the model suggests that discharge could be manipulated to manage disease in the Klamath River.

Central to controlling myxozoan disease is the development of tools that examine how these parasites are introduced and the consequences of these introductions. In Chap. 20, Bartholomew and Kerans review the components of a risk assessment, discuss specific data requirements relevant to myxozoans and provide examples of how risk assessments have been applied. In the first step of a risk assessment (release assessment) data requirements are similar to epidemiological studies and include frequency and severity of infections in source populations, how the parasite is dispersed and the frequency of events that might result in introduction. In the

second step (exposure assessment), factors that affect the ability of the parasite to establish and proliferate at the introduction site are considered. The third step (consequence assessment) is determined by management aims with consequences likely to be highest for parasites that affect economically important fish species. Each step involves uncertainty, and sources of this uncertainty are discussed. Risk assessments for myxozoans have been developed for various reasons. Commodity import risk assessments primarily involve assessing risks of myxozoan introduction in products intended for human consumption. These have generally considered introductions to be a low risk, with the exception of *M. cerebralis* which resulted in stringent precautions that have affected trade. Risk assessments have also been applied to identify myxozoan risks for new aquaculture species such as bluefin tuna in Tasmania. Risks to wild fish have focused primarily on *M. cerebralis* in North America with risk assessments including a variety of quantitative and qualitative approaches to address concerns about introducing and managing this parasite. Additionally, risk assessments are increasingly being used to examine the potential for pathogen exchange between aquacultured and wild fish (e.g. transmission of *Enteromyxum leei* in Mediterranean aquaculture). The authors point out that, in addition to identifying potential introduction routes, risk assessments can identify data gaps that can be used to guide future research. Reducing uncertainty of risk assessments will require data on geographic distribution, infection prevalence in fish hosts, and knowledge of complete life cycles.

Disease in wild populations is difficult to measure and to control. There are few myxozoans for which there are sufficient data to demonstrate impacts on wild fish populations, and these impacts are likely to vary annually and between locations. In Chap. 21, Jones et al. review evidence for host population-level impacts of four myxozoans (*M. cerebralis*, *T. bryosalmonae* and *C. shasta* in salmonids, and *M. honghuensis* in gibel carp) and how the diseases they cause may be mitigated. Each myxozoan has been associated with declines in wild fish populations, but characterising their impacts is challenged by absence of long-term data on population structure, fish abundance, disease prevalence and severity, and on factors that contribute to disease (susceptibility, environmental factors, etc.). Attempts to decrease disease effects include the development and application of methods for detecting the parasite in hosts and in water. Strategies most effective in mitigating whirling disease caused by *M. cerebralis* include changes in stocking protocols (using older or more resistant fish species or strains). Other strategies include treating effluent from affected trout production facilities, prohibiting the use of trout as baitfish, habitat manipulation to reduce temperature and sediment, and providing information to anglers regarding disinfection of gear and boats. Similar strategies for stocking restrictions and habitat manipulation have been proposed for the control of proliferative kidney disease (PKD) caused by *T. bryosalmonae*. The impacts of *C. shasta* have been most extensively studied in the Klamath River, CA USA, and here long-term monitoring of parasite densities in water and infection in fish has characterised a threshold parasite density that can be tied to management of ceratomyxosis (to decrease invertebrate host habitat and reduce spore transmission rates by altering flows using water in reservoirs). Declines in wild gibel carp are not as clearly linked to disease caused by *M. honghuensis*, but this is an area of active research. Strategies for disease control include reducing disease in cultured populations.

1.5 Recommendations for Future Studies

All contributing authors were asked to provide a list of questions for future study arising from issues that had received attention in their chapters. We hope these questions, which appear at the end of each chapter (apart from this one), may inspire future research programmes—perhaps especially for myxozoan researchers at the beginning of their careers. While many key questions were unique, there was also some

overlap. Table 1.1 outlines topics that were addressed by three or more key questions and lists those questions. This information encapsulates how the myxozoan research community (as represented by our authors) has collectively but independently highlighted topics that especially require further research. Of course there will be biases in formulating these topics. For instance, the data are not independent as many authors were involved with more than one chapter. There are also more chapters in certain areas than others due to the historical development of research. Given these caveats, there was a considerable convergence of questions relating to myxozoan diversity, the resolution of myxozoan life cycles and understanding transmission. Other areas commonly identified were: gaining insights on the importance of co-infection on disease dynamics and characterising how environmental change may influence disease development and risk. The focus thus ranges from understanding fundamental aspects of myxozoan biology to practical issues associated with global change biology.

We expect that many of these questions will be facilitated by recent developments. For

Table 1.1 Questions common to particular topics that appeared as 'Key questions for future research' in various book chapters

Topic	Questions
Characterising myxozoan diversity	To what degree is the diversity of malacosporean and myxosporeans underestimated (Chap. 4)?
	How will myxozoan diversity change as we begin to study them further in unsampled regions of the world (Chap. 7)?
	How diverse are myxozoans that exploit hosts other than fish (Chap. 4)?
	What is the diversity of polar capsules (Chap. 3)?
	What is the diversity of cell junctions (Chap. 9)?
Life cycle resolution	Are there other invertebrate taxa that act as primary hosts (Chap. 2)?
	What is the life cycle of myxozoans infecting terrestrial hosts (Chap. 7)?
	Do species of *Enteromyxum* incorporate an invertebrate host in their life cycles (Chap. 10)?
	What are the invertebrate hosts of marine myxozoans associated with important fish diseases (*Enteromyxum leei*, *Kudoa thyrsites*, *Parvicapsula pseudobranchicola*) (Chap. 17)?
	What are the life cycles of myxozoans that may be threats or may currently cause emerging diseases (Chap. 18)?
Transmission	Is transmission possible between small mammals (Chap. 7)?
	Can myxozoan infections in fish be achieved by trophic transmission (Chap. 10)?
	How widespread is fish-to-fish transmission (Chap. 10)?
	Are actinospores with one discharged polar filament infective (Chap. 13)?
Co-infection	How do multiple infections influence infection dynamics in bryozoan hosts (Chap. 11)?
	How is pathogenicity influenced by co-infection with other parasites and pathogens (Chap. 15)?
	Does tissue specificity reflect competitive interactions and niche differentiation amongst myxozoans (Chap. 16)?
Disease risk and environmental change	How do multiple sources of environmental variation influence disease development (Chap. 15)?
	How will changing environmental conditions resulting from global warming and anthropogenic modifications affect current risk assessments (Chap. 20)?
	How do environmental variables drive development in invertebrate hosts, infectivity to fish hosts and fish host susceptibility (Chap. 21)?

instance, studies based on environmental DNA (eDNA) have greatly expanded the diversity of endoparasitic protistan lineages (Hartikainen et al. 2014a, c). Understanding myxozoan diversity may similarly be aided by eDNA approaches in view of the inconspicuous nature of most myxozoan infections. Detection of novel lineages in environmental samples (e.g. water, faeces, sediments) will immediately contribute to species richness and other measures of diversity. Searches for potential hosts carrying infections may then be undertaken in order to link parasites with sequence data and to conduct standard parasitological studies to describe new taxa. Resolving myxozoan life cycles may also be aided by eDNA studies because sequence data for both primary and secondary hosts may be present within the same samples. Studies such as these may lead to the proper incorporation of myxozoans into food webs, demonstrating their importance to ecosystem function. If conducted over appropriate spatial scales, eDNA studies may also provide insights into patterns of myxozoan diversity within and across ecosystems. Finally, eDNA studies may inform on responses to environmental change. For instance, myxozoan responses to warming waters or to eutrophication may be examined by qPCR studies to examine abundances of free-living myxozoan stages (spores) in water samples across environmental gradients. Similar studies may be conducted across geographic scales in relation to myxozoan dispersal, disease emergence, risk analysis and environmental change.

Answers to other key questions raised by our authors will be facilitated by insights gained through genomic, transcriptomic, targeted gene-expression and proteomic studies. We are no doubt on the brink of gaining many fundamental insights on the biology of myxozoans as a result of second-generation-sequencing technologies that expedite research on non-model organisms. For instance, high-throughput marker discovery, such as restriction-site-associated DNA sequencing (RAD-seq), and tests for functional changes in gene expression through transcriptome profiling (RNA-seq) could be achieved by genotyping multiple individuals at thousands of random and functionally expressed loci (Davey et al. 2011). Such approaches enable exploring associations between genes and phenotype and the identification of genomic regions of interest. Dual RNA-seq of myxozoans and their hosts could detect simultaneous changes in gene expression over the course of infection (e.g. Westermann et al. 2012). Genomic approaches should also enable the discovery of phylogenetically informative genes to identify the cnidarian sister group and resolve the longstanding question about the relationship between *Polypodium hydriforme* and myxozoans. As a result of these technologies we may soon be able to: properly identify and characterise gametogenesis and embryogenesis in myxozoans, determine whether actinospore and myxospore development is controlled by common gene repertoires, and resolve whether myxozoans express recognisable germ layers. We may also undertake comparative studies on: axial patterning and the development of symmetry in myxoworms and free-living cnidarians (for instance in comparison to the development of biradial symmetry in *Hydra*; Watanabe et al. 2014), the genetic basis of morphological simplification within and between malacosporeans and myxosporeans, and the evolution of trait loss. Knowledge of transcriptomes combined with proteomic approaches can be used to identify the proteins involved in the key process of attachment to hosts by undertaking mass spectrometry to characterise the proteome of polar capsules as has been done for nematocysts of *Hydra* (Balasubramanian et al. 2012). Finally, insights gained through second-generation sequencing technologies may lead to practical applications, such as the development of vaccines based on the identification of virulence factors that myxozoans employ to exploit their hosts.

1.6 Conclusion

Myxozoans now merit broad recognition as a highly successful adaptive radiation of endoparasitic cnidarians with complex life cycles. This radiation is characterised by relatively high species richness and the invasion of a diversity of hosts in freshwater, marine and terrestrial

habitats. These attributes equate the myxozoan radiation with the well known radiations of endoparasitic platyhelminths—the cestodes (with 3,400 described species) and perhaps even the trematodes (with 11,000 described species), which comprise some 17 and 55 % of platyhelminth species diversity, respectively (based on data in Ruppert et al. 2004). Myxozoans, however, have evolved to become 'microparasites', a term used to denote parasites that multiply within their hosts. Such multiplication within hosts may be facilitated by the pervasive miniaturisation that characterises myxozoans. All other metazoan endoparasites are macroparasites (e.g. trematodes, cestodes, nematodes cirripedes, acanthocephalans). They do not multiply within their hosts and do not exhibit a general trend of miniaturisation. It is notable that multiplication within hosts is a strategy shared by myxozoans and protists. These points demonstrate how myxozoans have taken a unique route and illustrate how early branching metazoans with a diploblast condition have adopted an endoparasitic lifestyle that can be compared and contrasted with transitions to parasitism by the morphologically complex bilaterian metazoans and also by unicellular organisms. We are fortunate that malacosporeans and myxosporeans demonstrate progressions in body plan simplification and that the presence of both today enables us to examine how myxozoans have evolved endoparasitic strategies that are comparable to those of parasitic protists. Illuminating the evolution of body plans in free-living animals is arguably more challenging because forms that may have exhibited informative traits are extinct, leaving only tantalising fossils that may be difficult to interpret.

Recognising the key innovations and underlying mechanisms that have driven evolutionary transitions in the Myxozoa and incorporating myxozoans as integral components of ecosystems with functional roles in our changing world remain grand challenges for future study.

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Part I

Origins, Evolutionary Trends and Diversification

Myxozoan Affinities and Route to Endoparasitism

2

Beth Okamura and Alexander Gruhl

Abstract

There is now strong evidence that myxozoans have evolved from free-living cnidarians but until recently their higher level relationships have been the subject of considerable controversy. This chapter reviews the morphological and molecular evidence that has contributed to problems in placement and how further collective support has finally resolved their cnidarian affinity. We then consider the inherently difficult but fascinating topic of how myxozoans may have evolved as endoparasitic cnidarians. We first explore how a close association of free-living precursors could have led to the evolution of myxozoans with simple life cycles and the nature of the first myxozoan hosts. We propose that either freshwater bryozoans or fish (or their precursors) were ancestral hosts (in view of the more derived nature of myxozoans that infect annelids and the fact that fish are hosts for most members of all major myxozoan clades) and suggest that the morphological complexity of myxozoans in freshwater bryozoans renders a scenario of fish as first hosts less likely. We then discuss how new hosts may have been adopted subsequently, resulting in the complex life cycles involving invertebrate and vertebrate hosts that now characterise all myxozoans. Cnidarian traits, including life cycle plasticity and a capacity to evolve novel propagative stages, ultimately support many different scenarios regarding the route to endoparasitism.

Keywords

Long branch attraction · Morphological simplification · Phylogeny · Polar capsules · SSU rDNA · Minicollagen · Mitochondrial genes · Cnidaria · *Buddenbrockia* · *Polypodium hydriforme* · Host acquisition · Life cycle evolution · Undiscovered diversity

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2.1 Introduction

The affinities of some metazoans have been exceedingly difficult to ascertain (Conway Morris 1991). For extant taxa this is largely because comparisons of species may have to bridge enormous time spans of independent evolution (Jenner and Littlewood 2008). Signs of ancestry may therefore have vanished because of extensive modification or loss of characters. In addition, convergent evolution may obscure phylogenetic signal. Thus, in practice, extensive morphological modifications may preclude straightforward comparisons with potential relatives for some problematic metazoan taxa while high levels of molecular divergence and associated long branch attraction may artificially infer close relationships. Prominent examples include certain sessile taxa (e.g. bryozoans and brachiopods), tiny and possibly miniaturised taxa (e.g. tardigrades and acoels) and parasitic taxa (e.g. pentastomids and strepsipterans) (Jenner and Littlewood 2008; McKenna and Farrell 2010).

A combination of features has rendered placement of the Myxozoa especially challenging. Apparently rapid molecular evolution has resulted in accumulation of homoplastic characters causing long branch attraction that has proven to be highly problematic for placement within the Metazoa (e.g. Hanelt et al. 1996; Zrzavý et al. 1998; Kim et al. 1999; Evans et al. 2010). In addition, myxozoans present the most extreme case of morphological simplification associated with parasitism. Thus, not only do they lack features associated with organs, such as a digestive tract and a nervous system, but cilia and centrioles are also absent as are recognisable gametes, embryonic and larval stages (Lom 1990; Canning et al. 2000; Canning and Okamura 2004). As a counter example, the development of cypris larvae enabled the parasite, *Sacculina*, to be recognised as a highly modified barnacle in the nineteenth century, although the specific affinities with barnacles and other cirripede crustaceans are not fully resolved (Høeg 1992). In this chapter we review the history of and difficulties in determining the higher level relationships of the enigmatic Myxozoa and how their status as cnidarians has finally been confirmed on the basis of morphological and molecular data. We then explore the potential origins of parasitism including the nature of the first myxozoan hosts and how subsequent hosts may have been incorporated.

2.2 History of Higher Level Relationships

2.2.1 Discovery of Myxozoans and Placement with Protists

Myxozoans were discovered in the first half of the nineteenth century by Jurine (1825) and were assigned to the Sporozoa by Bütschli (1882). For a long time the Sporozoa comprised a diverse group of organisms regarded as unicellular, spore-forming parasites of animals including coccidians, gregarines, haemosporidians, *Plasmodium* spp. and piroplasms [today classified as Apicomplexa (Cavalier-Smith 1998)] along with the Microsporidia [now associated with Cryptomycota in the Kingdom Fungi (Hirt et al. 1999; James et al. 2013)] and the Myxosporida (now the Myxozoa). Myxozoan characters such as absence of centrioles and cryptomitosis and the presence of tubular (rather than plate-like) mitochondrial cristae in some taxa (Marquès 1987; Lom and Dyková 1997) were suggestive of a protistan nature. However, it is increasingly apparent that some of these features are variable (e.g. mitochondrial cristae, closed vs. open mitosis; Canning et al. 2000; Redondo et al. 2003).

2.2.2 Recognition as Multicellular Animals

Recognition of the multicellular nature of myxozoan spores led Štolc (1899) to propose that myxozoans should be included with Metazoa. This conclusion was echoed by others (e.g. Emery 1909; Ikeda 1912) and gained additional

support when Weill (1938) noted the similarity between the eversible, intracellular organelles present in both groups—polar capsules in myxozoans and nematocysts in cnidarians. Weill (1938) suggested that myxozoans are indeed cnidarians and further alluded to their potential similarity to the parasitic larval stages of *Polypodium hydriforme*, a cnidarian affiliated with the Narcomedusae and now placed by some in its own group, the Polypodiozoa (Raikova 1988; Bouillon et al. 2004). These similarities were re-emphasised by Lom (1990) and Siddall et al. (1995) (see below).

Myxozoa were accorded the status of a phylum within the Metazoa by Grassé (1970) as proposed previously by Grell (1956) and Lom (1969). This status was subsequently independently confirmed by molecular sequence data when Smothers et al. (1994) demonstrated that myxozoans grouped with bilateral animals on the basis of SSU rDNA and were possibly a sister group to the nematodes. This conclusion was similarly reached by Schlegel et al. (1996) who also analysed SSU rDNA. However, Siddall et al. (1995) concluded that myxozoans grouped within the Cnidaria as sister to *Polypodium hydriforme* on the basis of combined analyses of SSU rDNA and morphological data. At the time, morphological features that supported a metazoan nature included septate and adherens-type cell junctions, structural and functional differentiation of cells and separation of somatic and germ cells (Siddall et al. 1995; Lom and Dyková 1997). However, the separation of somatic and germ cells is not a clear-cut metazoan character as it has evolved several times in different lineages of multicellular organisms (and bacteria: Oliveiro and Katz 2014), and is also implemented in varying degrees in metazoans (Grosberg and Strathmann 2007). Further purported metazoan features used in phylogenetic analyses (the presence of collagen and acetyl-choline/cholinesterase activity; Siddall et al. 1995) were pointed out to be questionable (Lom and Dyková 1997; Canning and Okamura 2004). The putative protistan-like features of myxozoans (lack of centrioles, cryptomitosis and tubular mitochondrial cristae) have also been demonstrated more broadly. Centrioles have been shown to be absent in planarians apart from in terminally differentiating ciliated cells (Azimzadeh et al. 2012) while structures resembling microtubule organising centres have been observed in *Enteromyxum scophthalmi* (Redondo et al. 2003). Furthermore, it is now evident that mitochondrial cristae can assume many different shapes (Griparic and van der Bliek 2001) often reflecting differences in biochemistry even within the same tissues (Riva et al. 2005) and tubular cristae are widely distributed across the eukaryotes (Cavalier-Smith 1993; Seravin 1993) including in free-living cnidarians (e.g. Gray et al. 2009).

Despite the relatively early recognition of their multicellularity, textbooks largely continued to classify myxozoans as protists (e.g. Hyman 1940; Kudo 1966; Margulis and Schwartz 1998; Lom 1990) until very recently (e.g. Ruppert et al. 2004; Brusca and Brusca 2003; Pechenik 2009). This may, in part, reflect a propensity to adhere to the prior, entrenched classification, particularly in view of the conflicting evidence over the specific metazoan affinities of myxozoans. The extent that misclassification can muddy the taxonomic waters is exemplified by the 'honorary' inclusion of myxozoan papers in, for instance, the annual conferences of the British Section of the Society of Protozoologists (now the British Society for Protist Biology).

2.2.3 Clarification of Myxozoan Life Cycles and Diversity

The complex parasitic life cycles of myxozoans were not appreciated until the causative agent of salmonid whirling disease, *Myxobolus cerebralis*, was shown to incorporate tubificid worm hosts in a common life cycle (Markiw and Wolf 1983; Wolf and Markiw 1984). Only then was it recognised that actinospores and myxospores represented two different spore types produced within a common life cycle, thus uniting Actinosporea and Myxosporea within a single class (the Myxosporea). Previous to this work, actinospore- and myxospore-producing taxa were classified separately. For instance, an early

classification placed the Myxozoa (myxozoans producing myxospores) with Microsporidia and Actinosporea (myxozoans producing actinospores) in the class Cnidosporidia Doflein, 1901. The discovery of life cycle complexity simultaneously reduced the diversity of myxozoan species and opened the stage for exploring which actinospores and myxospores are involved in a common life cycle.

2.2.4 Recognition of the Malacosporea and Inclusion of *Buddenbrockia*

The most significant developments in understanding the higher level relationships of the Myxozoa have arisen from the discovery of myxozoans parasitic in freshwater bryozoans followed by the inclusion of the bizarre, worm-like endoparasite of freshwater bryozoans, *Buddenbrockia plumatellae*, as a myxozoan. This evidence was based on both molecular and morphological features (see Chap. 4 for further discussion). Thus, SSU rDNA analyses demonstrated a close relationship of *Buddenbrockia plumatellae* to *Tetracapsuloides bryosalmonae* (the causative agent of Proliferative Kidney Disease) (Monteiro et al. 2002) and hence an association with the Malacosporea, an early diverging clade of myxozoans so far associated with freshwater environments, utilising freshwater bryozoans and fish as hosts (Anderson et al. 1999; Canning et al. 2000). Meanwhile, an ultrastructural study simultaneously revealed that diagnostic polar capsules were present in the body wall of *Buddenbrockia* (Okamura et al. 2002). This study confirmed the early light-microscopy observations by Schröder (1910) that *Buddenbrockia* possesses a vermiform body plan with four sets of longitudinal muscles but lacks a digestive tract and anterior/posterior differentiation. Okamura et al. (2002) suggested these muscles were congruent to the four longitudinal muscles of nematodes and noted the presence of a basal lamina. These two complementary studies based on molecular and morphological characters thus provided the first insights into the body plan of a myxozoan that may have retained more in the way of ancestral features than observed in any other taxon.

2.3 Evaluation of Current Phylogenetic Evidence

Myxozoa are generally viewed as a monophyletic group in almost all recent studies. However, due to the paucity of phylogenetically informative morphological characters, apparently high rates of sequence evolution, and problems in resolving basal metazoan relationships (see e.g. Dohrmann and Wörheide 2013) support for the position of Myxozoa within the animal kingdom in most studies has remained moderate. Furthermore, the conflicting hypotheses of Myxozoa as sister-group to the Bilateria and as ingroup of the Cnidaria receive support from different sets of phylogenetic data and analytical approaches. Other related phylogenetic questions include the exact position of the Myxozoa within the Cnidaria and relationships of myxozoans to the endoparasite *Polypodium*. In the following sections we review the hypotheses and problems for interpreting the phylogenetic relationships of the Myxozoa that result from various data sets and show how the collective evidence now strongly supports their cnidarian nature (Fig. 2.1).

2.3.1 Morphological Evidence

The general body architecture of myxozoans offers few clues regarding their phylogenetic affinities. As mentioned earlier the presence of cell junctions clearly identifies myxozoans as metazoans. Epithelial characteristics in the stages of malacosporeans infecting invertebrates further support inclusion in the Eumetazoa (Ctenophora, Cnidaria, Bilateria). Other crucial apomorphic features (e.g. nerve cells) are lacking in myxozoans. The recognition of an independent, mesodermal-like muscle layer in *Buddenbrockia* first appeared to link myxozoans to bilaterians. However, three dimensional reconstruction of the

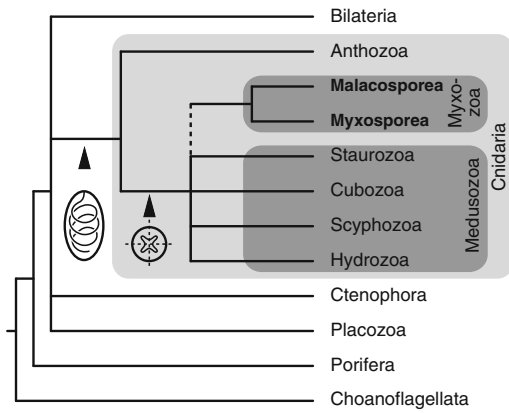

Fig. 2.1 Consensus tree of basal metazoan relationships and proposed position of Myxozoa as supported by phylogenomic studies (see text). Further support is gained from morphological characters: nematocysts (included in figure), with the inclusion of polar capsules constitute an apomorphic character of cnidarians; tetraradial body symmetry (included in figure) is a shared apomorphic feature of medusozoans and myxozoans

muscle architecture demonstrates a tetraradial symmetry (Gruhl and Okamura 2012), a unique trait of Medusozoa, the cnidarian taxon comprising Hydrozoa, Cubozoa and Scyphozoa. Also, contrary to traditional views, mesodermal-like musculature may occur in cnidarians as well (Seipel and Schmid 2006; Technau and Scholz 2003; but see Burton 2008), and may therefore be equally consistent with a cnidarian affinity. Characters of embryonic development such as cleavage patterns, modes of gastrulation and cell lineages, have proven useful to resolve some metazoan relationships (e.g. Valentine 1997; Nielsen 2012). However, with no regular pattern of cleavage, gastrulation or germ layer specification, myxozoan development currently does not offer any clear links with other metazoan phyla.

The most informative ubiquitous morphological character is the polar capsule, an intracellular organelle found in all myxozoan spores (Weill 1938; Lom 1990; Canning and Okamura 2004). The concordance to cnidarian nematocysts is striking and includes ultrastructural features (e.g. capsule wall and inverted tubule), formation (Golgi-secretory pathway, tubule invagination), and molecular architecture (proteins involved in capsule walls; see below and Chap. 3 for further discussion). Homology of the two structures appears highly likely, but of course this inference assumes that the nematocyst is a bona fide apomorphy of Cnidaria. For instance, nematocyst-like elements in a small number of unicellular eukaryotes have led to the hypothesis that nematocysts are a general eukaryote feature and have been lost repeatedly in various lineages (Özbek et al. 2009). Although this cannot be completely discounted it seems unlikely given that, in addition to crucial differences in the fine structure of these various organelles, there is not a single known case of nematocyst loss in the entire Cnidaria. It has also been hypothesised that nematocysts originated as endosymbionts derived from free-living unicellular organisms either once or multiple times during eukaryote evolution (Shostak 1993). This theory is now mostly refuted by: (a) the fact that, unlike other endosymbiotic organelles like mitochondria or plastids, nematocysts and polar capsules lack genetic material, and; (b) proteins involved in nematocyst formation appear to be unique, bearing little relationship with other eukaryote proteins (Balasubramanian et al. 2012). Horizontal transfer of nematocysts, as seen in the cleptocnidae of e.g. nudibranch molluscs (Edmunds 1966) and some ctenophores (Carré et al. 1989), is also highly unlikely as in all known cases these have to be acquired anew in each individual since, due to lack of genetic material, they do not reproduce.

In summary, both morphological and molecular and data on polar capsules now provide convincing support for Myxozoa belonging to Cnidaria (Fig. 2.1). Chapter 3 describes how polar capsules may also be phylogenetically informative with regard to their position within Cnidaria by pointing out similarities of the lid-like apical plugs that seal polar capsules and medusozoan nematocysts. The absence of apical plugs in nematocysts of anthozoans adds further support for a myxozoan-medusozoan affinity. However, the evolution of nematocysts has not been fully resolved (David et al. 2008; Fautin 2009) and convergence cannot be entirely discounted. The relative simplicity of polar capsules

may reflect their modified function of host attachment (see Chap. 13) and whether this is primary or due to secondary reduction remains obscure.

2.3.2 Molecular Evidence

2.3.2.1 RDNA Data Sets

The SSU ribosomal RNA gene was the first molecular marker widely applied to resolve metazoan relationships (Field et al. 1988). Myxozoans were firstly included in a metazoan-wide data set in a study by Smothers et al. (1994) who found good support for a position within the Bilateria, as sister-group to nematodes. However, exclusion of fast-evolving nematode sequences removed Nematoda from its basal bilaterian position and demonstrated major changes among bilaterian relationships (e.g. Aguinaldo et al. 1997). In addition, studies on lower metazoan groups (e.g. Hanelt et al. 1996; Pawlowski et al. 1996; Winnepenninckx et al. 1998) demonstrated the importance of long branch attraction in impeding phylogenetic resolution of the Myxozoa (see Canning and Okamura 2004, for review). Analyses of SSU rDNA sequences were also demonstrating variable phylogenetic placement for myxozoans depending, for instance, on the incorporation of *Polypodium hydriforme* and the analyses of partial versus full length sequences (see Canning and Okamura 2004, for review). Kim et al. (1999) attempted to control for long branch attraction by analysing only full (or near full) length SSU rDNA sequences obtained for the species with the shortest branch to the ancestral node in each monophyletic lineage but their results were relatively inconclusive. Thus, using distance analyses weak support was obtained for a sister taxon relationship between myxozoans and *Polypodium hydriforme* and they did not fall within the Cnidaria. However, maximum likelihood analyses identified Myxozoa as sister to the triploblasts while *Polypodium hydriforme* was unresolved within the diploblasts.

More recently, Evans et al. (2010) examined the effects of missing data, model choice and inference methods in placing highly divergent taxa and confirmed the two relatively stable placements that previous researchers had found for myxozoans, with Cnidaria or Bilateria, based on various types of analyses of SSU rDNA and LSU rDNA sequences. The analyses by Evans et al. (2010) thus exemplified the importance of careful model selection, taxon and data sampling, and in-depth data exploration when investigating the phylogenetic placement of highly divergent taxa such as the Myxozoa.

2.3.2.2 Protein Coding Genes, Expressed Sequence Tags, and Genomic and Transcriptomic Data Sets

The conflicting conclusions and generally weak support gained by phylogenetic analyses based on nuclear rDNA data suggested that information of another type was required to gain meaningful insights into myxozoan origins. The identification of central class Hox genes in myxozoans was therefore of great interest since the absence of these genes in cnidarians implied a bilaterian affinity for the Myxozoa (Anderson et al. 1998). However, these genes were later shown to be host contaminants (Jiménez-Guri et al. 2007). Success in developing suitable markers for phylogenetic studies has clearly been highly problematic due to the extreme divergence of myxozoan genes which precludes the use of universal primers for obtaining sequence data.

More recent technological innovations that generate data on multiple gene loci, such as large scale sequencing of expressed sequence tags (ESTs), transcriptomes or genomes, have overcome the limitations of earlier phylogenetic analyses based on only a single or a few genes. The breakthrough in understanding myxozoan higher level phylogeny came when Jiménez-Guri et al. (2007) were able to construct an EST library for *Buddenbrockia*. This enabled a phylogenomic investigation based on 50 protein coding genes which provided evidence that *Buddenbrockia* groups within the Cnidaria and, with strongest support, as sister to the Medusozoa (Jiménez-Guri et al. 2007). Subsequent

genomic and transcriptomic studies have consistently provided further confirmation of the cnidarian nature of myxozoans. Thus, Nesnidal et al. (2013) came to similar conclusions based on phylogenomic analysis of 128 protein-coding genes identified by whole genome shotgun sequencing of the myxosporean, *Myxobolus cerebralis* (the causative agent of whirling disease). In addition to confirming the cnidarian status of the Myxozoa and the sister group relationship between the Myxozoa and Medusozoa, they also explicitly tested the effects of missing data and showed that these cannot explain the placement of Myxozoa within the Cnidaria as posited by Evans et al. (2010). Most recently, Feng et al. (2014) obtained genomic and transcriptomic data from the myxosporean *Thelohanellus kitauei* and gained strong support for Myxozoa as sister to the Medusozoa by analysing a subset (86 genes) of the 128 genes analysed by Nesnidal et al. (2013).

2.3.2.3 Taxonomically Restricted Genes (Minicollagens)

Taxonomically restricted genes (TRGs) represent another useful and independent source of data relevant for evaluating myxozoan affinities. TRGs may be identified in the genomes and transcriptomes of a wide range of organisms and will contribute to the high percentage (~20–50 %) of genes with no detectable homologies to proteins in public databases and which are thus referred to as "orphan" or "novel" genes (Khalturin et al. 2009). In cnidarians certain genes involved in nematocyst formation are regarded as TRGs (Milde et al. 2009) since nematocysts are unique to cnidarians and genes coding for proteins specifically involved in nematocysts (e.g. minicollagens, NOWA) have not been found in bilaterian, sponge or protist genomes sequenced to date (David et al. 2008; Khalturin et al. 2009) (although sequence data are lacking for protists with similar organelles).

Holland et al. (2011) sequenced and characterised a minicollagen in the malacosporean, *Tetracapsuloides bryosalmonae*, demonstrating the presence of a gene homologous to those encoding for nematocyst proteins in the Myxozoa. This minicollagen protein has now been localised to polar capsules (Gruhl et al. in prep.). Feng et al. (2014) have identified two further minicollagens in the myxosporean, *Thelohanellus kitauei*, which were distinct from that identified in *Tetracapsuloides bryosalmonae*. The three myxozoan minicollagens identified so far cluster with minicollagens of medusozoans but because taxon sampling is very poor these results should be viewed with caution. Further minicollagens have been detected in original EST and in new transcriptomic libraries for both malacosporeans and myxosporeans (Holland et al. unpub. data; Gruhl et al. unpub. data) and Shpirer and Chang (2014) report three minicollagens and three nematogalectins in genomic and transcriptomic libraries of *Kudoa iwatai*, *Enteromyxum leei* and *Sphaeromyxa zaharoni*. Nematogalactins represent a further family of cnidarian-specific genes (Hwang et al. 2010).

TRGs offer a promising alternative to support relationships where conflicting evidence or low support obscures resolution of problematic taxa. The power of TRGs is limited by the comprehensiveness of the reference data used for identification, but as the number of sequences deposited in public databases increases, precision is likely to increase as well.

2.3.2.4 Mitochondrial Genes and Genomes

Mitochondrial gene sequences and especially the arrangement of genes in the mitochondrial DNA molecule are important data that are largely independent of nuclear DNA evolution. In addition, the aberrant evolution of mitochondrial genomes within the Cnidaria is now coming to light. The Medusozoa in particular do not retain the archetypal circular mitochondrial chromosome typical of animals, but possess linear chromosomes and in some groups these are fragmented into more than one chromosome (see Kayal et al. 2012 for review). Further unique features of at least some medusozoan mitogenomes include the telomeres forming inverted repeats and duplicate genes and pseudogenes in

the subtelomeric regions as revealed in some species of *Hydra*. Comparative mitogenome architecture, gene arrangements and telomere sequences may therefore be relevant for understanding the cnidarian affinities of the Myxozoa. However, until recently difficulties imposed by extreme molecular divergence precluded the development of mitochondrial markers by a number of groups including our own. Indeed, consistent lack of success in amplifying mitochondrial sequence data from myxozoans despite persistent efforts led authors of a conference abstract to claim that myxozoans are amitochondriate (Wood et al. 2002)! This claim is of course countered by the numerous mitochondria evident in ultrastructural studies.

Data on myxozoan mitochondrial sequences have now been obtained by several groups. In a recent poster abstract Fiala et al. (2013) reported sequences of the 12S, NADH and COX1 genes from six myxosporean species and additionally retrieved a partial mitochondrial genome sequence for *Polypodium.* Preliminary analyses support a medusozoan affinity for Myxozoa and suggest that *Polypodium* is distinct and closely related to the Narcomedusae. In another recent poster abstract Yahalomi et al. (2013) present mitochondrial sequences from genomic data for three myxosporeans and found that myxozoans, like some medusozoans, are characterised by fragmented mitogenomes that have unusually high rates of sequence evolution and are comprised of several linear chromosomes. Preliminary data suggest that malacosporean mitogenomes may be similarly comprised of fragmented, linear molecules (Hartikainen and Okamura, unpub. data).

2.4 Myxozoans and *Polypodium*: Close Relatives or Independently-Evolved Lineages?

It is of interest to further explore the possibility that *Polypodium* and myxozoans are indeed sister taxa with a common origin supporting assignment to the clade Endocnidozoa (Zrzavý and Hypša 2003). Molecular phylogenetic analyses based on SSU data have often concluded that both *Polypodium* and myxozoans are sister to bilaterians (e.g. Cavalier-Smith et al. 1996; Pawlowski et al. 1996; Winnipenninckx et al. 1998; Kim et al. 1999). However, as discussed above, myxozoans are now confirmed as cnidarians. For *Polypodium* there is now also tentative support based on broad SSU sampling of the Cnidaria that *Polypodium* is a cnidarian (Evans et al. 2009). However, if myxozoans are included in these comprehensive analyses, cnidarian affinities of both *Polypodium hydriforme* and myxozoans disappear (Evans et al. 2008, 2009), highlighting the generic problem of long branch attraction based on SSU data. New evidence based on analyses of 128 genes presented by Rubinstein et al. (2013) indicates *Polypodium* and myxozoans cluster together and form the sister clade to the Medusozoa. However, because the study is ongoing and results may be altered with further cnidarian sampling it is relevant to undertake comparison of other features.

Polypodium is an intracellular parasite infecting the eggs of primitive freshwater bony fish (sturgeon and paddlefish). It undergoes an extraordinary although simple life cycle with a larval stage infecting fish eggs as intracellular parasites and an adult, free-living stage that produces gametophores which infect fish upon contact (Raikova 1994). The life cycle entails development as a so-called planuliform larva with the gastrodermis located externally within a fish egg. The larva is enclosed by a cell called the trophamnion which is likely to have a protective and nutritive role (Raikova 1994). The larval form inverts prior to host spawning to produce a free-living stolon comprised of a chain of tentaculate units with the gastrodermis situated internally. At spawning the fish egg membranes are disrupted to release the stolon which subsequently fragments into tentaculate individuals that actively feed, walk on their tentacles and produce infectious gametophores as a result of sexual reproduction. Gametophores containing binucleate cells have been observed to infect larval fish. Nothing is known about how fish eggs eventually become infected. Infection of

larval fish implies that prolonged periods of arrested development may be required since the time of first spawning may be up to 16 years (see Raikova 1994 for review).

Some basic features present in *Polypodium* that are absent in myxozoans include centrioles, flagellated gastrodermal cells, a cnidocil (a cilium-derived structure associated with nematocysts), gonads and a network of nerve fibres underlying the epidermis (see Raikova 2008 for review). Features shared by *Polypodium* and myxozoans include parasitism of fish, infection via nematocysts, a similar type of nematocyst (putatively atrichous isorhiza), longitudinally arranged and mesodermal-like muscle cells, cell-within-cell stages (see Chap. 8 for review of endogeny processes in myxozoans) and mitochondria with tubular cristae (Raikova 2008). Many of these shared features are, however, also found in other cnidarians. For instance, atrichous isorhizas are broadly distributed and mesodermal-like muscle arrangements characterise many cnidarians (reviewed in Seipel and Schmidt 2006). Furthermore, cell-within-cell stages have been observed during development of the trachylinid *Pegantha smaragdina* which Bigelow (1909) described as parasitizing the parent (which lacks gonads) by developing within the gelatinous matrix close to the gastric cavity. During this development a nurse cell surrounds an embryonic cell that then subsequently divides while continuing to be enclosed within the nurse cell (Bigelow 1909). We have already reviewed how the shape of mitochondrial cristae is highly variable and should no longer be considered a character of phylogenetic significance.

At present, both molecules and other traits provide conflicting evidence. Myxozoans and *Polypodium* may represent independently-derived endoparasitic lineages or they may represent sister cnidarian taxa that have undergone extensive divergence following their separation. If they are sister taxa this could imply that primitive bony fish (Order Acipenseriformes) were ancestral hosts to myxozoans in freshwater environments since both *Polypodium* and malacosporeans (which retain primitive morphologies) both exploit freshwater hosts. Ancestral marine fish hosts are less likely in view of the combination of primitive and simple features demonstrated in malacosporeans (see later discussion), the apparent restriction of malacosporeans to freshwater environments (see later discussion) and phylogenetic analyses of character evolution that identify freshwater fish as primitive hosts (Fiala and Bartošová 2010). In turn, myxozoans may have diverged to exploit a diversity of fish and to subsequently evolve a complex parasitic life cycle with adult stages exploiting invertebrate hosts. The alternative possibility, that a complex parasitic life cycle was ancestral to both myxozoans and *Polypodium*, would imply the unlikely event of *Polypodium* regaining free-living adult stages. The possibility that myxozoans and *Polypodium* may represent two independent transitions to endoparasitism within the Cnidaria is supported by the independent evolution of anthozoan and narcomedusan species with larval stages that develop parasitically in the gastrovascular cavities of other cnidarians (Spaulding 1972; Pagès et al. 2007) or in the stomach of a ctenophore (Bumann and Puls 1996).

2.5 New Markers, New Methods

As outlined above for myxozoans and as is also the case for several other problematic taxa in the tree of life, there are still numerous sources of error that prevent us from deciphering true phylogenetic relationships. General strategies to improve support include firstly an increase of both the number of taxa and the number of characters used (Philippe and Telford 2006). However, this is not always easily achievable, for example when the organisms are rare, difficult to sample, or highly divergent. Also, an uncritical use of more characters does not necessarily lead to higher support values, but can instead introduce further new sources of error (e.g. Nosenko et al. 2013). Systemic errors can only be reduced by optimising the algorithms and models used for phylogenetic analysis (e.g. Philippe and Roure 2011; Struck 2013).

A different approach is to use more complex characters as these are less likely to be

homoplastic. Such characters can be morphological as well as non-sequence genomic characters. Examples of the latter that have been used to resolve phylogenies include rare genomic changes (Rokas and Holland 2000), near-intron-pairs (Lehmann et al. 2013; Hill et al. 2013), microRNAs (Wheeler et al. 2009), retroposons (Suh et al. 2011), and protein indels (Gupta 2001). Mitochondrial genome structure data such as linearity versus circularity are also very informative in cnidarians (Bridge et al. 1992) and are now promising to add further resolution to the position of myxozoans (see earlier section).

Finally, in myxozoans as well as in many other understudied taxa, morphological and developmental studies are extremely valuable, because many traits may be unrecognised or have been characterised for only a few representatives. Such poor cover may potentially bias inferences of body plan evolution and development.

2.6 Evolution of Parasitism: From Free-Living Cnidarians to Endoparasites

After a long period of controversy, the cnidarian affinity of myxozoans appears at last to be clear. We are therefore now able to interpret the Myxozoa within the context of their cnidarian nature and the evolution of endoparasitism from free-living ancestors. In this section we explore the evolution of their complex parasitic life cycles, the nature of the first myxozoan hosts and the incorporation of new hosts. Chapter 3 considers the extent that ancestral cnidarian features may be reflected in their subsequent evolution and life histories as well as unique traits that may have enabled their radiation as endoparasites, the latter is also considered further in Chap. 4.

2.6.1 Evolution of a Complex Parasitic Life Cycle

Present knowledge indicates that all myxozoans incorporate invertebrate and vertebrate hosts in a complex life cycle. A marine origin was inferred by Shul'man (1990) who suggested that the common ancestor was a coelozoic parasite of the gall bladder and urinary bladder of actinopterygian teleosts (ray fins) but this was proposed before invertebrate hosts and the malacosporeans were recognised. On the basis of current evidence a freshwater origin is also conceivable. This is implied by phylogenetic analyses of character evolution based on stages in fish (Fiala and Bartošová 2010) and the primitive characters of malacosporeans along with their simple spores (see below). It would also be supported if *Polypodium* is confirmed as sister to the Myxozoa. The evolution of the complex myxozoan life cycle would have first involved a transition from a free-living lifestyle to a parasitic form that exploited a single host. Such a transition would be preceded by the two organisms coming into contact for some time. Pre-adaptations of parasite precursors will then have enabled initial stages of host exploitation when greater fitness was attained by maintaining the association (Poulin 2007). Routes to parasitism may involve parasite precursors feeding on hosts, utilising hosts for dispersal (via phoresy) or to reduce environmental variability, and survival following predation (see Poulin 2007; Schmid-Hempel 2011 for further review and examples).

We note that the number of species observed in the present day is not informative about how old lineages may be and thus cannot provide potential insights about original hosts or habitats. For instance, relatively depauperate clades may once have been more speciose. In addition, speciose clades may have arisen by adaptive radiation enabled by the evolution of a key trait. Chapter 4 explores how such adaptive radiation may have resulted in the highly speciose myxosporeans. Other parasitic taxa are similarly characterised by depauperate clades sister to highly speciose clades with derived characters. For instance, the Aspidogastrea comprises four families and some 80 species and is sister to the Digenea which is composed of 100 families and >10,000 species) (Cribb et al. 2003). Another example is the Cyclophyllidea, which is the most highly derived

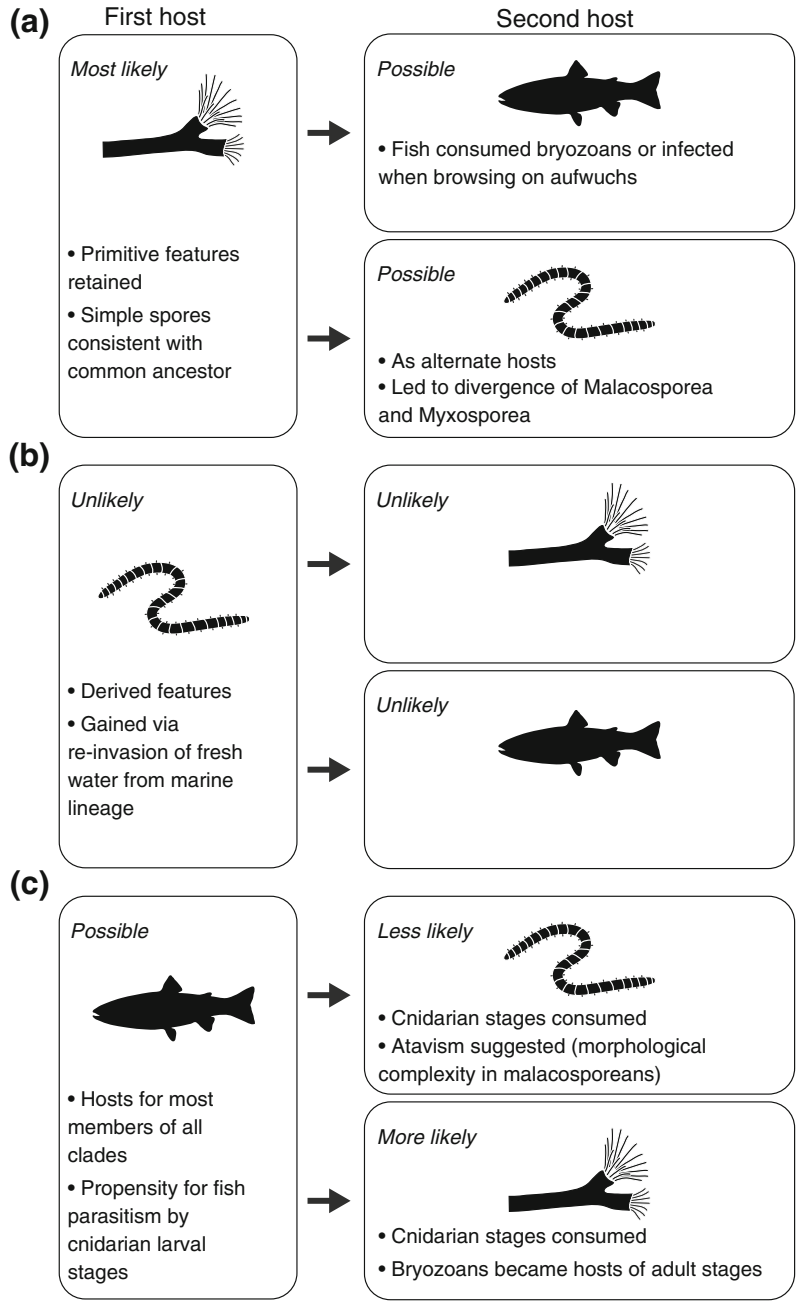

Fig. 2.2 The main hypothetical scenarios for the origin and evolution of myxozoan life cycles (see text for further details, including use of precursors to host groups, as well as consideration of further scenarios). **a** Freshwater bryozoans as first host with subsequent acquisition of fish as secondary host. Switch to annelids, initially as secondary and then as alternate hosts could have led to divergence of Malacosporea and Myxosporea. **b** Annelids as first hosts. This scenario is unlikely given that myxozoans infecting annelids are derived. **c** Fish (or early fish-like vertebrates) as first hosts with later addition of bryozoans or annelids as second host. Subsequent switch of sexual phase to the invertebrate host

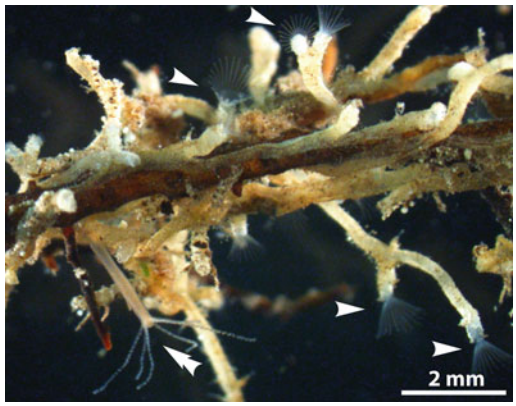

Fig. 2.3 *Hydra* sp. (*double arrowhead*) on the phylactolaemate bryozoan *Fredericella* sp. Several tentacular crowns (lophophores) of the bryozoan are extended for suspension feeding (*arrowheads*)

Eucestode order and contains 380–400 genera, while the other, less derived orders of eucestodes contain 1–66 genera (Brabec 2012).

The current topology of myxozoan phylogeny with a basal split into the subtaxa Malacosporea and Myxosporea enables us to identify characters that are shared by both groups as ancestral for Myxozoa. However, this is not possible for traits that show different character states between the two groups and are also absent in any potential myxozoan outgroup. Since the ancestral myxozoan was a free-living cnidarian we cannot determine whether the malacosporean condition (bryozoan host) or the myxosporean condition (annelid host) is plesiomorphic. One way to approach this dilemma is therefore to develop scenarios (Fig. 2.2) that can be examined for plausibility based on the evolution of other characters or on ecological or functional considerations. Below we use this approach to consider how endoparasitism and life cycle complexity may have arisen in the Myxozoa. The main scenarios for patterns of host acquisition are outlined in Fig. 2.2. The relationship between myxozoan life cycles and the complex life cycles of free-living cnidarians is explored in Chap. 3.

2.6.1.1 Invertebrates as First Hosts

The retention of primitive features (tissues, muscle blocks, tetraradial symmetry) in freshwater bryozoan hosts suggests that myxozoans may have evolved from a cnidarian ancestor in freshwater environments with bryozoans or their precursors acting as ancestral hosts of adult stages (since meiosis occurs in invertebrate hosts) (Fig. 2.2a). An alternate scenario is that mxyozoans evolved to become parasites of marine precursors that subsequently invaded freshwater environments to diversify as freshwater bryozoans. The relatively simple, soft-walled spores produced by malacosporeans in both bryozoan and fish hosts are similar to the simple spores identified as present in the common ancestor identified by phylogenetic analyses of character evolution based on stages in vertebrate hosts and which was inferred to have exploited freshwater hosts (Fiala and Bartošová 2010).

Relatively few cnidarians occur in freshwaters and current evidence suggests that all (except possibly *Polypodium*) are hydrozoans. Hydrozoans exhibit several instances of independent evolution to inhabit freshwaters (Jankowski et al. 2008), and *Hydra* is often found attached to bryozoan colony surfaces (Fig. 2.3) perhaps reflecting an association with future hosts by an ancestral hydrozoan form which preceded a transition to parasitism (Poulin 2007). Notably, the suspension feeding activity of bryozoans may predispose them to ingest a wide range of potential food items, including propagative stages of hydrozoans such as eggs, planulae, or small regressed stages (see Chap. 3 for review of the latter) that could potentially invade bryozoan tissues. Furthermore, the ability of cnidarians to absorb dissolved organic compounds through the integument (e.g. Ferguson 1982; Grover et al. 2008) and the incomplete digestion and even survival of organisms consumed by freshwater bryozoans (Hyman 1959; Raddum and Johnsen 1983; Okamura pers. obs.) may both be significant processes that enabled the invasion and development within the tissues of bryozoans (or their precursors) by ingested stages. Acanthocephalans provide evidence for such a postulated transition to parasitism with the stem species inferred to have lived epizootically on a marine arthropod ancestor prior to invading the host body cavity to establish an endoparasitic life

cycle. The present-day close association of the free-living *Seison nebaliae* (the sister taxon to acanthocephalans) with marine arthropods, may reflect such a situation (Herlyn et al. 2003).

It is also possible that myxozoans evolved from a cnidarian ancestor that became an endoparasite of annelid worms (Kent et al. 2001). Thus, myxozoans may have originated in the marine environment where annelids are a diverse and old group. Alternatively, freshwater oligochaetes could have been first hosts. However, we view either scenario of annelids as first hosts (Fig. 2.2b) as less likely than that of ancestral bryozoan hosts (Fig. 2.2a) since myxosporeans are highly derived. Furthermore, myxozoans infecting freshwater worms appear to have arisen via re-invasion of freshwater environments from marine myxosporean lineages (Kent et al. 2001; Fiala and Bartošová 2010). This suggests that if freshwater oligochaetes were first hosts these lineages have vanished without trace whilst malacosporeans have remained in freshwater environments exploiting a host group of low diversity (freshwater bryozoans) relative to the diversity of freshwater oligochaetes. Finally, it should be mentioned that myxozoans could have originated in other invertebrate hosts that remain undetected either because of extinction or lack of sampling.

2.6.1.2 Fish as First Hosts

An alternative scenario (Fig. 2.2c) is that fish (or early fish-like vertebrates, see below) were original hosts for stages of myxozoans that are likely to have developed in renal tissues (Kent et al. 2001; Fiala and Bartošová 2010). The absence of meiosis in myxozoans in fish hosts suggests that fish may have supported the development of larval myxozoans. The adoption of fish as hosts may have occurred in freshwater or marine environments. Invertebrates would subsequently have been incorporated as hosts for adult stages in a two-host life cycle. Support for this scenario is that fish are hosts for most members of all major clades of myxozoans (malacosporeans and the freshwater and marine clades of myxosporeans) (Kent et al. 2001; see also Chap. 4) and the propensity of other cnidarians to evolve parasitic larval stages (Spaulding 1972; Bumann and Puls 1996; Pagès et al. 2007). Additional support would be gained if *Polypodium* (which exploits fish hosts as larval stages) and myxozoans were determined to be sister taxa. The free-living adult stage may then, by association, have evolved endoparasitism using invertebrates as definitive hosts.

At present it is unclear whether myxozoans are sister to or were derived within the Medusozoa. This has implications for interpreting the nature of myxozoans under a scenario of utilising fish as first hosts. Analyses of mitochondrial protein-coding genes and the fossil record suggest that medusozoans diverged prior to the Cambrian (Park et al. 2012). The first cartilaginous and bony fish appear in the Devonian and Silurian, respectively. A sister-group relationship of myxozoans and medusozoans would therefore imply that myxozoans either must have been free-living for many millions of years prior to the utilisation of such fish as first hosts, or have used other hosts before switching to such fish. If, however, myxozoans were derived within Medusozoa then the period of time that myxozoan precursors were free-living prior to parasitising fish may have been greatly reduced, and the postulation of an additional host-switch becomes unnecessary. Similarly, the period of time may have been reduced if precursors to cartilaginous or bony fish served as first hosts. This would be supported if agnathans (hagfish and lampreys) turned out to be regular hosts of myxozoans, because the origin of vertebrates dates well back into the Ediacaran and agnathans diverged from gnathostomes in the Cambrian (Donoghue and Keating 2014). Thus far, only isolated findings of myxozoans in lampreys have been reported (Mori et al. 2000).

Larval stages of myxozoans may originally have been transmitted to fish by direct contact with the adult form, perhaps via a stage analogous or homologous to the gametophores of *Polypodium*. Alternatively, spores may have evolved as larval stages adapted to attach to fish hosts to enable phoresy (like glochidium larvae of some freshwater bivalves). This may have

been particularly advantageous in freshwater environments, providing a means of colonising habitats otherwise precluded or retention within favourable adult habitats rather than being swept downstream to unsuitable sites. It is difficult to envision how tiny larval stages released into a three-dimensional watery world would have achieved the close association with fish hosts required for spores to evolve in the first place unless release was somehow triggered by proximity of fish. The firing of polar filaments from polar capsules by exposure to fish mucous or mechanical contact of spores (see Chap. 13 for further discussion) may illustrate how such transmission may have been achieved.

The other possibility, that myxozoans first exploited fish hosts as adult stages, would entail a subsequent transition to using invertebrates as definitive hosts. The apparent flexibility of cnidarian life cycles (see Chap. 3) suggests a precedent for this, but as developed below, this scenario implies unlikely evolutionary events associated with the incorporation of secondary invertebrate hosts.

2.6.2 Incorporation of New Hosts

Complex life cycles have evolved independently in several groups of parasites with the drivers of host expansion likely to reflect historical events that affected parasite transmission or survival of the host (Poulin 2007). An increase in life cycle complexity may be explained, for instance, if parasites evolve to exploit predators or prey of the first host thereby enabling higher growth and fecundity or higher transmission rates (e.g. Choisy et al. 2003; Parker et al. 2003). This could be achieved by upward incorporation—when original hosts are frequently ingested and become intermediate hosts (Parker et al. 2003). Such upward incorporation could be driven by increased parasite fecundity in larger predator hosts with selection for delayed maturity and enhanced reproduction in this larger host. For example, upward incorporation appears to have occurred when the ancestor of acanthocephalans, an endoparasite of a marine arthropod, incorporated a vertebrate predator as a second host (Near et al. 1998; Herlyn et al. 2003). Complex parasitic life cycles may also be achieved by downward incorporation—when prey of the original host frequently ingest parasite propagules and become intermediate hosts. This may enhance transmission to the original host which then becomes the definitive host (Parker et al. 2003). Platyhelminthes appear to present an example of downward incorporation with the lineage ancestral to digeneans and cestodes becoming parasitic in vertebrates (Littlewood et al. 1999) and the subsequent addition of invertebrate hosts in each group (see Poulin 2007; Schmid-Hempel 2011 for review).

Additional hosts may also be incorporated in parasite life cycles if there is an increased probability of finding a sexual partner (Brown et al. 2001). Note that this assumes there are selective benefits of cross-fertilization which may not be the case for myxosporeans (see Chap. 3) but could apply to malacosporeans (see below). Finally, additional hosts may serve to transfer infectious stages from one host to the next. However, such paratenic hosts have no effect on completion of the parasite's life cycle. Below we consider how the complex myxozoan life cycles observed today may have evolved by expansion from various potential hosts. When this occurred is obscure—myxozoans may have remained endoparasites with a simple life cycle for millions of years. Our discussion focuses on how complex myxozoan life cycles may have arisen via expansion from precursors that exploited either invertebrate or vertebrate hosts. The subsequent adoption of a new host via host switching by myxozoans demonstrates a capacity for host substitution. For instance, myxosporeans have replaced fish with amphibian hosts on at least three times independently (see Chaps. 4 and 7 for further discussion).

2.6.2.1 Expansion from Bryozoans?

If freshwater bryozoans or their precursors were first hosts (Fig. 2.2a), fish may have been incorporated as secondary hosts by direct ingestion of infected bryozoans or of myxozoan

spores. So far there is no evidence for trophic transmission from invertebrate hosts to fish but the presence of bryozoan dormant stages (statoblasts) in fish guts in the present day (e.g. Dendy 1963; Applegate 1966) and demonstration that statoblasts carry myxozoan infections (Hill and Okamura 2007; Abd-Elfattah et al. 2014) suggests that trophic transmission may have been possible. On the other hand, ingestion of myxozoan spores might easily occur during predation and browsing of invertebrates commonly associated with and concentrated in dense stands of freshwater bryozoans (Bushnell and Rao 1979; Okamura pers. obs.). Utilisation of fish hosts may have enabled persistence during adverse conditions or amplifying transmission as a result of the greater biomass and longevity of fish. However, if the invertebrate hosts were highly clonal, as in present-day bryozoans, these advantages may not have pertained. For instance, malacosporeans infect dormant asexual bryozoan propagules (statoblasts) that enable survival during adverse conditions (Hill and Okamura 2007; Abd-Elfattah et al. 2014). In addition, extensive clonal growth in freshwater bryozoans combined with vertical transmission of infection in clonal fragments and propagules (see Chap. 11) could amplify parasite biomass and transmission to levels equal to if not greater than those achieved by exploiting fish. Alternatively or additionally, infection of fish may have enabled retention of larval stages within suitable habitats rather than being swept downstream or it may have enhanced outcrossing (Rauch et al. 2005) if infectious spores released by fish are more likely to be genetically distinct than those produced by parasites in highly clonal local bryozoan populations. For instance, fish movements may result in exposure to infection from multiple sources while extensive vertical transmission of parasites in bryozoans may amplify the biomass of only a single or a few parasite genotypes in local bryozoan populations.

An alternative or perhaps additional possibility is that annelid worms were incorporated as alternate hosts via ingestion of spores released from bryozoans or direct consumption of infected bryozoans. This may have been facilitated by an association of oligochaetes with freshwater bryozoans—something commonly observed for a variety of species in the present-day (Okamura, pers. obs.). Indeed, oligochaetes are occasionally encountered that have ingested statoblasts (Okamura, pers. obs.) perhaps exemplifying the potential consumption of infected statoblasts by annelids that eventually were incorporated as hosts. This scenario would imply that life cycles subsequently evolved along different trajectories leading to the divergent Malacosporea and Myxosporea along with the capacity for adult development to be transferred from bryozoan to annelid hosts. The most parsimonious interpretation is that fish hosts were incorporated prior to the malacosporean/myxosporean split.

2.6.2.2 Expansion from Annelids?

Fish may have been incorporated as secondary hosts of myxozoans developing in annelid worms (Fig. 2.2b) by trophic transmission, with predation of infected annelids selecting for parasites with the ability to survive passage through fish and use of the gut as the primordial entry portal. This is supported by the common inclusion of annelids in fish diets. However, as outlined earlier, the derived nature of myxosporeans suggests this is unlikely to have involved expansion to fish hosts from basal myxozoans that infected annelids. A recent molecular phylogenetic analysis has identified *Bipteria* sp. to comprise the earliest diverging myxosporean branch of the derived marine lineage (Kodádková et al. 2014). Exploitation of the holocephalan fish, *Chimaera monstrosa*, as the vertebrate host by *Bipteria* sp. suggests that fish may have been incorporated as hosts as early as the Silurian when the oldest living group of jawed vertebrates (the cartilaginous fishes comprising the chimaeras, sharks, skates and rays) diverged from a common ancestor of bony vertebrates. It is possible that holocephalans were incorporated by myxozoans that infected annelids or even marine

precursors to freshwater bryozoans. It is, however, also possible that holocephalans were adopted as hosts more recently. In this respect it may be significant that the early branching malacosporeans in the present day do not infect particularly ancient fish.

Finally, if annelids were incorporated as alternate hosts to bryozoans (see above), it is possible that fish may already have acted as hosts in myxozoan life cycles prior to the addition of annelid hosts. Another scenario, in parallel with that outlined in the previous section, is that freshwater bryozoans were incorporated as alternate hosts via ingestion of spores released from annelid hosts. This would then entail subsequent evolution leading to the divergence of Malacosporea and Myxosporea and adult development transferred from annelid to bryozoan hosts. As argued previously, it is more likely that fish hosts were incorporated prior to the malacosporean/myxosporean split.

2.6.2.3 Expansion from Fish?

If fish were acquired as hosts of larval stages (Fig. 2.2c) trophic transmission of adult stages released from fish would seem difficult to achieve given the suspension-feeding and scavenging activities of bryozoans and annelids. However, the propensity of cnidarians to release small propagative stages by budding processes (see Chap. 3) may have enabled such transmission. Alternatively, if fish were acquired as hosts of adult stages (see above) trophic transmission via the ingestion (either of spores or of stages in which spores were present; see Chap. 3 for further discussion of myxozoan life cycle stages) by bryozoans and worms would be feasible. This scenario would require a switch to using invertebrates as definitive hosts. Since cnidarian life cycles demonstrate considerable plasticity, for instance with transition of sexual reproduction from medusa to polyp stages (e.g. in *Hydra*), the scenario is not entirely unfeasible (see Chap. 3 for further discussion). Nevertheless, the scenario does not readily explain why morphological complexity would subsequently characterise the adult malacosporean stages in bryozoans. Such complexity is rather suggestive of atavism, with complex worm-like stages re-evolving in malacosporeans, unless of course early fish hosts harboured morphologically complex myxozoan parasites. Finally, cnidarians [e.g. *Polypodium*, anthozoans and narcomedusae (Bumann and Puls 1996; Spaulding 1972; Pagès et al. 2007)] demonstrate a proclivity to evolve parasitic larval rather than adult stages.

2.6.3 Undiscovered Diversity and Hosts

The diversity of myxosporeans described in fish and the discovery of new hosts for malacosporeans (see Chap. 4) provide evidence that myxozoan host ranges in these two invertebrate groups will continue to expand as further life cycles are resolved and new material encountered. Several other groups of invertebrates may have also been incorporated in myxozoan life cycles, such as octopus (Yokoyama and Masuda 2001) (see also Lom and Dyková 2006). However, because myxozoan infections are generally innocuous many are probably very often overlooked. The demonstration of extensive covert infections by malacosporeans in freshwater bryozoans (see Chap. 11) also suggests that many myxozoans may be unrecognised if they occur for prolonged periods of time as single cells associated with host tissues. The recent astonishing expansion in the diversity of Haplosporidia and Mikrocytida via both environmental DNA detection and sampling of invertebrate hosts (Hartikainen et al. 2014a, b) provides evidence that the diversities of innocuous, endoparasitic microbial taxa, such as Myxozoa, are likely to be greatly underestimated. Thus, it is possible that myxozoans have evolved to exploit a much broader range of invertebrate host groups than is currently evident.

Marine counterparts of freshwater bryozoans could be considered as likely candidate hosts for malacosporeans. However, we are unaware of any convincing observations in the literature of malacosporeans in marine bryozoans (Classes Gymnolaemata and Stenolaemata) nor have any

been found by specifically sampling marine bryozoans (Okamura, unpub. data on gymnolaemates). A number of early bryozoan researchers noted 'vermiform' and other apparently parasitic bodies in marine bryozoans. Some of these were likely to have been bryozoan organ systems (e.g. paired vestibular glands) including various specifically-located vermiform bodies described by Hastings (1943). Others apparently occurred collectively within a common matrix (e.g. Waters 1912). However, myxozoan stages are directly exposed to the host body cavity fluids. The description of cilia on one such stage is also inconsistent (Waters 1912). Some of the vermiform bodies observed by Hastings (1943) were examined by authorities who could not confirm their identity without fresh, properly fixed material although one authority suggested they could be Protozoa. The recent discovery of orthonectids endoparasitic in marine bryozoans suggests a possible identity for some of the vermiform bodies that have been observed in bryozoans (Hochberg and Kruse 2009).

The apparent absence of malacosporeans in marine bryozoans could of course reflect low or patchy infection prevalence, and relatively little research focusing on what are regarded as 'minor phyla' such as the Bryozoa. Nevertheless, we believe that marine bryozoans are unlikely hosts because the presence of walls between constituent zooids in colonies results in a very small space in which sacs or worms could develop—the body cavity of a single zooid. In phylactolaemates (freshwater bryozoans) the lack of walls between constituent zooids in colonies produces a voluminous, colony-wide, fluid-filled body cavity that supports the proliferation of numerous sacs and worms whose maximum dimensions range from 0.3 (for sacs) to 3 mm (for worms). Even at maturity these malacosporean stages are bathed by host fluids and undergo active movements (worms) or are passively circulated (sacs) within the common, colony-wide body cavity. In contrast, a single *Buddenbrockia* worm or a couple of sacs would completely pack the volume of a single zooid of a marine bryozoan, entailing difficulty in spore release and probably in nutrient uptake. We further suggest that the size constraints associated with developing in marine bryozoan zooids would drastically reduce transmission due to extremely low concentrations of spores released in marine environments from small stages. More plausible candidates for marine invertebrate malacosporean hosts might therefore be found among groups that exhibit similar features, i.e. deposit or suspension feeding life styles allowing contact with infectious spores in combination with large body cavities providing space and nutrients for parasite trophic stages. These would include potential relatives of bryozoans such as phoronids or brachiopods, but also invertebrates such as echinoderms, hemichordates or molluscs.

2.7 Conclusions

The cnidarian nature of myxozoans is increasingly supported by evidence from multiple independent sources. However, both morphological and molecular markers appear to be highly divergent in myxozoans and thus currently do not provide confidence in a more precise phylogenetic hypothesis regarding their closest cnidarian relatives. This picture is likely to be resolved in the near future as many studies are now focussing on these questions by searching for new phylogenetically informative characters.

The evolution of the complex myxozoan life cycle is a fascinating but inherently difficult topic to evaluate. The close association of a free-living precursor with what would become the first myxozoan hosts would have led to myxozoans with simple life cycles. The occurrence of meiosis in invertebrate hosts, the more derived nature of myxozoans that infect annelids and the fact that fish are hosts for most members of all major myxozoan clades suggest that either freshwater bryozoans or fish (or their precursors) acted as such ancestral hosts. The morphological complexity of malacosporeans in freshwater bryozoans renders a scenario of fish as first hosts perhaps less probable. However, cnidarian characters that likely pre-adapted them to endoparasitism, including life cycle plasticity and a

capacity to evolve novel propagative stages, can also be invoked to explain many alternative scenarios for the evolution of complex parasitic life cycles.

The diversity of myxozoans as currently recognised is no doubt underestimated due to the biased focus on a narrow range of economically-important hosts. Further research on myxozoan diversity and life cycles will enable greater insights into the phylogeny and evolution of this group.

2.8 Key Questions for Future Study

- Which (cnidarian) group is sister to myxozoans?
- Are there other invertebrate taxa that act as primary hosts?
- Have *Polypodium* and Myxozoa independently evolved endoparasitism?
- How might we gain insights into when endoparasitism evolved and what hosts were first acquired?

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Cnidarian Origins of the Myxozoa

3

Beth Okamura, Alexander Gruhl, and Abigail J. Reft

Abstract

Now that we have strong evidence for the phylogenetic placement of Myxozoa within the Cnidaria it is of great interest to explore their evolutionary history. In particular, what cnidarian features may have facilitated the transition to an endoparasitic lifestyle and can we identify a potential cnidarian sister group? In this chapter we summarise evidence for characters linking myxozoans to cnidarians and identify cnidarian traits that may have promoted endoparasitism including: their diploblastic condition, their capacity for regeneration, transdifferentiation, and dormancy, the production of novel propagative stages, cell-within-cell development, and asexual reproduction. Equating the basic cnidarian life cycle (benthic polyps and planktonic medusae) with the complex myxozoan life cycle is problematic because of great plasticity in cnidarian development, which can entail the loss of stages and associated transfer of function. The sexual phase of myxozoans involves the production of isogametes but divergent views on their subsequent fusion lead to questions about whether sexual reproduction involves selfing or outcrossing and if it may result in the development of multicellular chimaeras. The apical structures of myxozoan polar capsules closely resemble those of medusozoan but not those of anthozoan nematocysts, thus supporting a medusozoan affinity for Myxozoa.

Keywords

Life cycles · Sexual reproduction · Body plan complexity · Dormancy and regeneration · Polar capsules · Nematocysts · *Polypodium*

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3.1 Introduction

Myxozoans are a radiation of endoparasites that are derived from free-living ancestors. As discussed in Chap. 2 it is now clear that this divergence occurred within the Cnidaria and led to the evolution of complex life cycles, using invertebrate and vertebrate hosts. In this chapter we explore how we may understand the transition to endoparasitism by myxozoans in view of their cnidarian origins. The myxozoans at present are characterised by two clades. The more derived myxosporeans are highly speciose and utilise annelids as invertebrate hosts. In contrast, the malacosporeans comprise only a few described species and use freshwater bryozoans as invertebrate hosts (see Chap. 4 for review of myxozoan radiations). The retention of morphological features, such as recognisable epithelial layers (in all malacosporeans) and tetradially organised muscles (in the active, wormlike malacosporean, *Buddenbrockia*) (see Chap. 9), suggests that malacosporeans are primitive (see Chaps. 2 and 9 for further discussion). Our discussion will therefore often tend towards comparisons of malacosporean and cnidarian features. Chapters 4 and 9 explore more specifically how key features of myxozoans have diverged between and within the two clades. A further potentially significant consideration is the occurrence of meiosis in stages infecting invertebrate hosts in both Malacosporea and Myxosporea (see below for details). This, by definition, identifies invertebrates as final or determinate hosts for both groups and may signify how adult stages in myxozoans and cnidarians are equated. Given this background information, we begin our discussion by comparing and contrasting the life cycles of myxozoans and cnidarians (Fig. 3.1).

3.2 Comparative Life Cycles

3.2.1 Myxozoans

Ultrastructural evidence of meiosis in the form of paired homologous chromosomes and synaptonemal complexes of myxozoan sporogonic stages in annelids (Marquès 1987; El-Matbouli and Hoffmann 1998; Lom and Dyková 1997) and bryozoans (Canning et al. 2000, 2002, 2007; Tops et al. 2005) identifies invertebrates as final hosts. A recognisable gamete stage is absent and infection of both vertebrate and invertebrate hosts is achieved by amoeboid sporoplasms that are released from multicellular spores. Spores that develop in bryozoan hosts are produced within sacs or worms in the coelomic cavity. The most comprehensive series of observations from early to late stages of malacosporean development in bryozoans (Fig. 3.1a) derives from ultrastructural studies of the sac-forming *Buddenbrockia allmani* (Canning et al. 2007) and ultrastructural and confocal studies of the vermiform *Buddenbrockia plumatellae* (Canning et al. 2002, 2008; Gruhl and Okamura 2012). Cells associated in small groups comprise early developmental stages. The development of cell junctions then resolves an external layer of mural cells enclosing a loose collection of inner cells that then undergo proliferation. It is currently unclear whether all of these internally proliferating cells eventually undergo meiosis or if some only undergo mitosis (Canning et al. 2007). Spore formation appears to involve the aggregation of cells but whether these all derive from a single sporogonic cell or by aggregation of previously determined cell types remains unknown (Canning et al. 2007). One hypothesis is that diploid cells differentiate into the valve and capsulogenic cells of spores while haploid cells become sporoplasms, a scenario supported by the presence of differentiated capsulogenic cells at an early stage and the discarding of valve cells upon infection (Canning et al. 2007). In fish hosts malacosporeans undergo initial proliferation of presporogonic stages in blood and kidney interstitium prior to spore development in kidney tubules (Fig. 3.1a). Spores develop intracellularly within pseudoplasmodia that produce a single spore at a time (Kent et al. 2000).

Spores in annelid hosts are produced in pansporocysts (Fig. 3.1b) in various tissues following invasion by sporoplasms. Prior to pansporocyst development, sporoplasm nuclei multiply to form multinucleate cells that divide

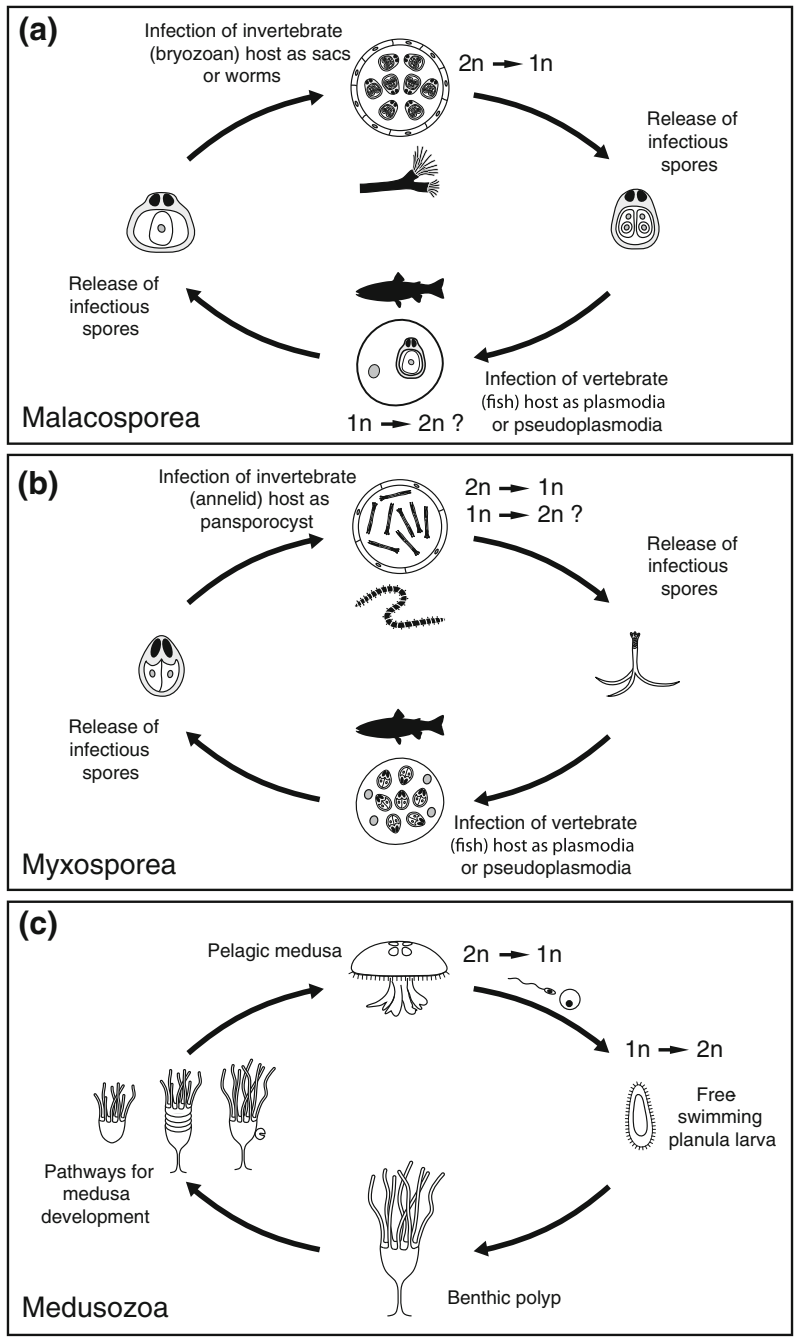


Fig. 3.1 Myxozoan and medusozoan life cycles. **a** Malacosporean life cycle. Trophic stages (sacs or worms) in invertebrate (bryozoan) final host produce spores containing haploid sporoplasms, which invade vertebrate intermediate host. Fusion between sporoplasm cells is hypothesised to take place within the intermediate host, resulting in endogenous plasmodia or pseudoplasmodia which produce spores (fishmalacospores) with a single diploid sporoplasm and two polar caspules. **b** Myxosporean life cycle. Trophic stages (pansporocysts) in invertebrate (annelid) final hosts produce actinospores. This process involves meiosis and subsequent self-fertilisation resulting in diploid sporoplasm cells, which invade the vertebrate intermediate host. Endogenous plasmodia or pseudoplasmodia produce myxospores with diploid sporoplasm(s) that invade the final host. **c** Medusozoan life cycle. Pelagic medusae undergo sexual reproduction resulting in free-swimming ciliated planula larvae. The latter settle and metamorphose into primary polyps. These in turn form medusae either by metamorphosis or asexually via fission or budding

by plasmotomy to numerous uninucleate stages. Further bouts of multinucleate and uninucleate stages may proceed via nuclear division and plasmotomy within the gut epithelium. Spores develop within the space enclosed by the pansporocyst wall which consists of 2–8 cells interconnected by cell-cell junctions. Various fish tissues and internal spaces may support spore development following proliferation of presporogonic stages. Spores develop within simple membrane-bound structures, including pansporoblasts (produced by engulfment of the spore-forming cell by another cell), plasmodia (containing many nuclei) or pseudoplasmodia (containing one nucleus) (for review see Canning and Okamura 2004; Feist and Longshaw 2006).

Malacosporean sporoplasms that infect vertebrate hosts appear to be haploid and how diploidy is achieved remains a mystery (discussed further below). In contrast, parallel myxosporean stages infecting fish are inferred to be diploid as a result of autogamy via fusion of haploid α- and β-generative cells produced during spore formation (El-Matbouli and Hoffmann 1998; see Sect. 3.2.3.1 and Chap. 8 for further discussion of meiosis and when fusion occurs). Myxozoans proliferate in their hosts asexually by cell division (including endogeny; see Chap. 8) and fission (see below). As outlined above, myxosporeans also exhibit plasmotomy to produce uninucleate cells from multinucleate plasmodia. In addition, uninucleate cells can undergo fusion to produce binucleate stages (see Feist and Longshaw 2006 for review).

3.2.2 Cnidarians

Cnidarians are renowned for their diversity of forms which may often be expressed within the same life cycle. Although a clear outgroup of Cnidaria is unknown, the ancestral life cycle implied by the anthozoans and other basal metazoans such as sponges and ctenophores is likely to have involved the release of gametes from polyps with fertilisation occurring in the water column to produce swimming larvae (planulae) that develop directly into new polyps. Brooding occurs in some cnidarians—eggs are fertilised by broadcast sperm and the developing embryo is retained in the maternal organism and released at a later stage of development. Anthozoans demonstrate a simple life cycle involving a benthic adult stage comprised of one or more polyps which often undergo extensive asexual reproduction to form aggregations of genetically-identical individual polyps or physiologically integrated colonies. The Medusozoa demonstrate relatively more complex life cycles (Fig. 3.1c), typically involving an alternation between benthic polyp forms and planktonic medusae forms. Sexual reproduction generally occurs in medusae which release gametes. Planula larvae develop after external fertilization and these then metamorphose to a primary polyp. At a later stage polyps form medusae either by asexual reproduction, or, in cubozoans, by metamorphosis. However, the life cycles of many medusozoans have been shortened. For instance, some Hydrozoa and Scyphozoa lack polyp stages while in the majority of hydrozoans (A. Collins, pers. comm.), including the model animal *Hydra,* the medusa has been lost and sexual reproduction occurs in the polyp stage. These life cycle modifications demonstrate plasticity in the expression of sexual reproduction which may be transferred from medusa to polyp stages or vice versa. Cnidarians are mostly gonochoric although some anthozoans, hydrozoans and scyphozoans are hermaphroditic (Fautin 2002; Bouillon et al. 2004; Morandini and Marques 2010).

The great variety of developmental pathways in cnidarians may be explained by freedom from anatomical and physiological constraints (Fautin 1991). For instance, because cnidarians lack secondary sexual characters such as intromittent organs and true gonads, selective pressures on developmental pathways can directly influence e.g. the timing, quality, quantity or retention of gametes which are simply derived by cells that originate either from the endoderm (Anthozoa, Scyphozoa, Cubozoa) or the ectoderm (Hydrozoa). In other animals, complex anatomical traits that might be required for certain sexual modes may be so costly or inflexible as to prohibit their

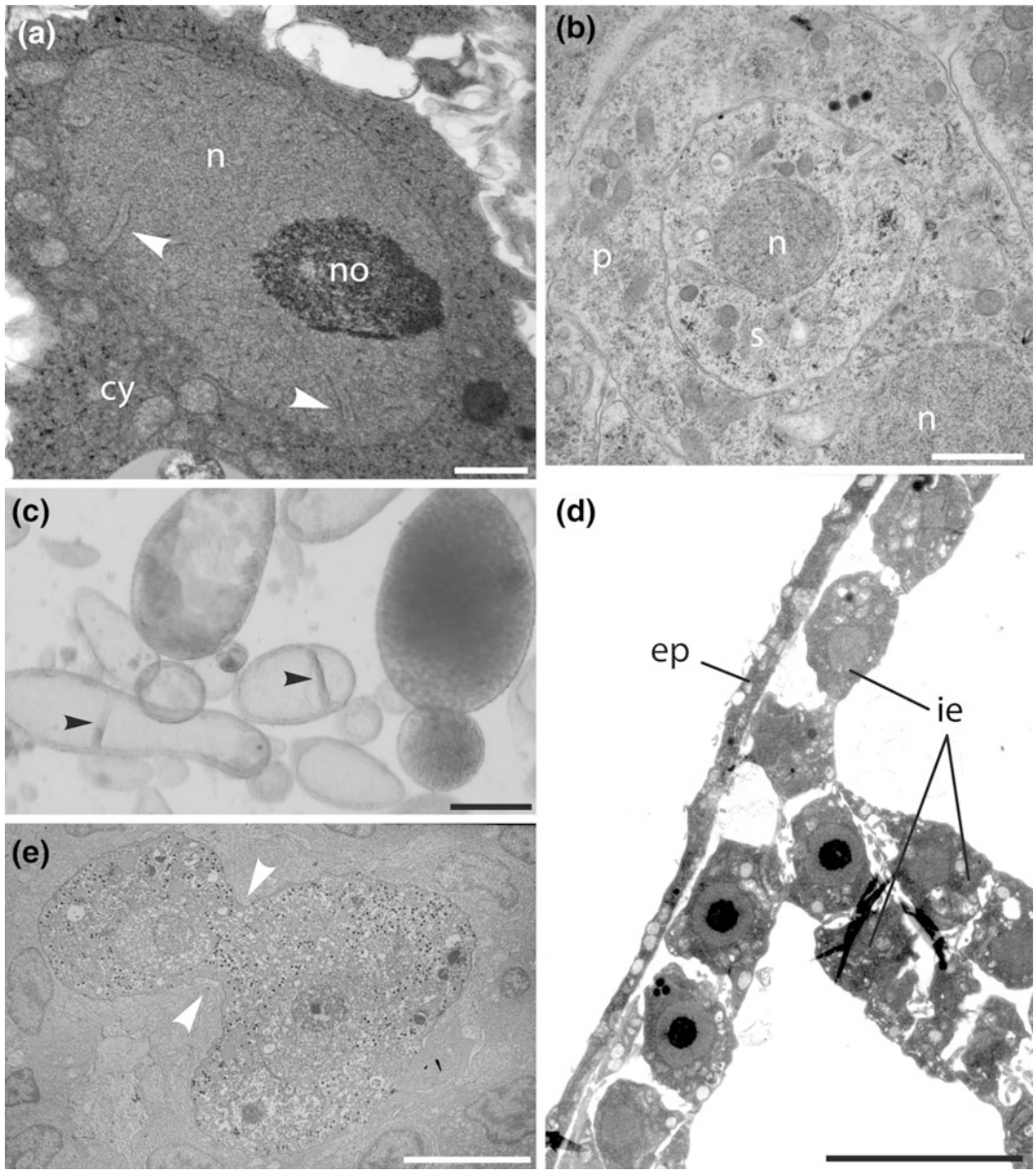


Fig. 3.2 Reproductive features in myxozoans. **a** Sporogony in malacosporean invertebrate stages involves meiosis as indicated by synaptonemal complexes (*arrowheads*) in cells proliferating from outer wall, *scale bar* 1 μm, photo by Alan Curry. **b** Endogenic sporoplasm in spore of *Buddenbrockia plumatellae* worm consisting of primary cell (*p*) and secondary cell (*s*), *scale bar* 1 μm, photo by Alan Curry. **c** Stereomicroscopic image of *B. plumatellae* sacs in the bryozoan host *Cristatella mucedo*. Note developing walls (*arrowheads*) within some sacs indicative of fission, *scale bar* 200 μm. **d** TEM image of *B. plumatellae* sac developing in *C. mucedo* showing the developing wall is comprised of layers of inner epithelium (*ie*), *scale bar* 20 μm. **e** TEM image of potential plasmotomy in early stage of *Tetracapsuloides bryosalmonae* in fish kidney, photo by Stephen Feist, *scale bar* 5 μm. *c* cytoplasm, *ep* epidermis, *n* nucleus, *no* nucleolus

further modification. The relatively simple nature of cnidarians may therefore enable evolutionary plasticity in development.

The diversity of cnidarian life cycles is further complicated by the widespread occurrence of various forms of asexual reproduction, morphological transformations and the production of novel stages. For instance, some polyps and medusae produce small, non-feeding stages called frustrules that fall to the bottom, then bud, creep away and transform into polyps (e.g. Carré and Carré 1990). Frustrules are produced asexually, adding a further dimension to complex reproductive cycles, and appear to act as dormant stages that enable persistence during adverse conditions (Carré and Carré 1990). Some scyphozoans produce podocysts, cyst-like dormant stages beneath the pedal disk that excyst small polyps (Arai 2009). Some hydrozoans demonstrate a capacity for medusae to transform into polyps (Bavestrello et al. 1992; Piraino et al. 1996; Kubota 2011), and in some anthozoans tentacle buds or fragments can develop to planula-like larvae (Miller and Grange 1997) or to new polyps (Pearse 2001). Finally, medusae of several hydrozoan species can bud new medusae from the manubrium, tentacular bulbs, radial canals, exumbrellar rim, marginal canal, subumbrellar rim, or gonads (see Boero et al. 2002 and references therein).

3.2.3 Modes of Reproduction

3.2.3.1 Sexual Reproduction

The haploid nature of malacosporean sporoplasms and of generative cells in actinospores (see below) identify these stages as equivalent to gametes. Gametes of free-living cnidarians are typically released in the water column where they are fertilised to produce planula larvae that then metamorphose to polyps. However, union of myxosporean gametes is somewhat mysterious. To appreciate just why requires us to consider their inferred developmental patterns.

Ultrastructural studies of malacosporeans demonstrate meiosis during early stages of the proliferation of cells within sacs and worms (Fig. 3.2a, Canning et al. 2000, 2002, 2007; Tops et al. 2005). The two sporoplasms that are eventually enclosed within spores are assumed to be haploid (Canning et al. 2000). If so, fusion must occur at some point between early infection of fish and early infection of invertebrate hosts. This scenario would support the hypothesis that the incorporation of fish as hosts enhances the probability of outcrossing (Brown et al. 2001; Rauch et al. 2005) by sampling, amplifying and spreading parasite clonal genotypes over space and time as a result of the comparatively large size, extensive movement and longevity of fish hosts (see Chap. 2). On the other hand, outcrossing may be more likely in bryozoans. In particular, cryptic myxozoan stages may persist indefinitely in long-lived, clonally reproducing bryozoan hosts (see Chap. 11) while host suspension feeding samples the environment to capture small particles. These two processes may provide a greater opportunity for outcrossing in invertebrate hosts.

Ultrastructural studies of myxosporeans document the presence of binucleate cells during early stages of development of myxosporeans in annelid hosts, including *Myxobolus cerebralis* (El-Matbouli and Hoffmann 1998), *Aurantiactinomyxon* sp. 1 and *Myxobolus cultus* (Lom et al. 1997). It has been proposed that these binucleate stages may arise by fusion of uninucleate stages that are produced by schizogony (El-Matbouli and Hoffmann 1998). Recent evidence suggests these uninucleate stages are microsporidians (Morris 2012; Morris and Freeman 2010) (and possibly the occasional binucleate stage with nuclei out of the plane of section). The nuclei in these binucleate cells are inferred to have predetermined development, one involving the development of somatic cells, the other of generative cells. An early developmental stage is proposed to then arise via fusion of two binucleate cells to form a stage with four nuclei. Plasmotomy (subdivision of these multinucleate forms) then produces a four cell stage (El-Matbouli and Hoffmann 1998). Two of the cells within this four cell stage envelop the two generative cells—the so-called α and β cells (see

Chap. 8). This complex of cells (now regarded as an early pansporocyst) undergoes further cell divisions via mitosis and then meiosis occurs exclusively in α and β cells resulting in eight cells of each type (El-Matbouli and Hoffmann 1998). It is proposed that fusion then occurs between α and β cells (now gametocytes) to produce eight zygotes, each giving rise, by mitotic divisions, to one spore consisting entirely of diploid cells (El-Matbouli and Hoffmann 1998; Rangel et al. 2011). The relative similarity in size and shape of the α and β cells is indicative of isogamy. The presence of small cells suggested to be polar bodies and of synaptonemal complexes and paired homologous chromosomes observed via ultrastructural studies of several species provides broad confirmation that meiosis occurs in developing actinospores (Marquès 1987; Lom et al. 1997; El-Matbouli and Hoffmann 1998; Rangel et al. 2011).

An alternate view of development (Morris 2012) proposes that the early binucleate stages are not produced by fusion of uninucleate stages but originate via mitosis from binucleate sporoplasms that infect worms. Further variation in actinosporean development includes meiosis only in β cells, a lack of fusion between α and β cells and the production of haploid sporoplasms. As in malacosporeans, diploidy may thus be achieved by fusion of haploid β cells or their subsequent derivatives in fish or during early stages of development in invertebrate hosts. Morris (2012) suggests this occurs during the formation of the tetranucleate stage in worm hosts; thus, myxospore sporoblasts and all generative nuclei of the vertebrate phase would be haploid. Chapter 8 provides further cover of the details of meiosis and fusion according to various models.

The above developmental scenarios inferred for myxosporeans have great implications. If binucleate cells develop by plasmogamy of genetically distinct uninucleate cells arising from schizogony (as would be possible in the original model if there are multiple infections in hosts) it would involve subsequent cooperative development of genetically mosaic hybrid stages. These stages would form multicellular pansporocysts in which gametes representative of both uninucleate lineages are eventually produced and undergo fusion. Chimaeric coral colonies may also develop via fusion of kin in species that brood their larvae and following gregarious settlement of related planula larvae in *Acropora millipora,* which broadcasts gametes (Puill-Stephan et al. 2012 and references therein). Such capacity for chimaeric development demonstrates that cooperation, delayed allorecognition, or both may underlie genetic mosaicism in free-living cnidarians. Sexual reproduction in such chimaeric corals would generally be achieved when polyps in colonies free spawn gametes or brood larvae fertilised by allosperm. All polyps in a chimaeric colony would theoretically have this capacity (barring intracolonial competition selecting against certain genetic lineages). However, as noted, such chimaeras arise when closely-related individuals fuse to become a physiologically integrated colony. In myxozoans, the envelopment of generative nuclei by somatic nuclei deriving from two binucleate cells would ensure joint rewards, but fertilisation is determined prior to gamete development. How relatedness would influence this process is an important and unresolved question.

Another scenario is that outcrossing may be precluded in myxosoporean life cycles if haploid α and β cells that share a common origin undergo fusion during the development of actinospores (e.g. if binucleate cell populations arise via schizogony and subsequent mitosis). Meiosis will generate some genetic variation of gametes as a result of independent assortment of chromosomes and cross-over, but the association of a sexual system based on selfing with the radiation of such a large group of animals is unprecedented although it would appear to characterise the dicyemid mesozoans, a small group of endoparasites of octopus (Furuya and Tsuneki 2007; Czaker 2011). Furthermore, if outcrossing is achieved by malacosporeans but not myxosporeans, then cross fertilisation must be inferred to be inconsequential with regard to driving myxozoan diversity. In short, if outcrossing is truly absent in myxosporeans this suggests that meiotic "reshuffling of the same cards in the deck"

along with the occasional generation of beneficial mutations and the purging of deleterious mutations via inbreeding (selfing) (Crnokrak and Barrett 2002) has been sufficient to support this radiation. Since germ lines are not sequestered in cnidarians (including myxozoans) this might increase the potential for beneficial mutations to be present in germ cells arising from somatic cell precursors.

A scenario of complete reliance on selfing in the Myxosporea is incompatible with a large body of theoretical and empirical evidence that loss of genetic variation, inbreeding depression and genetic degradation (via deleterious mutations) are the primary reasons that selfing may not persist as a long term strategy (e.g. Takabayashi and Morrell 2001; Morran et al. 2009). The scenario is perhaps particularly unanticipated for a radiation of parasites. Arms races that reflect the ability of parasites to keep one step ahead of host defences and of subsequent host counter adaptations are hallmarks of host-parasite interactions. In these systems the reciprocal adaptations that characterise hosts and parasites are typically linked with the generation of genetic variation through sexual reproduction achieved by outcrossing in both antagonists (or in bacteria via transformation, transduction, conjugation and horizontal gene transfer). Indeed, a recent study has demonstrated that outcrossing nematode populations adapted to changes in bacterial pathogens while selfing populations went extinct because adaptive counter-responses were absent (Morran et al. 2011). Such empirical evidence in combination with theory indicates that sexual reproduction may be maintained by the coevolutionary interactions of antagonists that favour the long-term maintenance of outcrossing relative to selfing (Agrawal and Lively 2001) or asexual reproduction (Jaenike 1978; Hamilton 1980).

Cnidarians demonstrate some common features of sexual reproduction as described above for myxozoans. Because hermaphroditism is relatively unusual, selfing may often be constrained. We are unaware of any reports of obligately selfing cnidarians, but selfing appears to be possible across the phylum, being observed in some anthozoans (e.g. Hinsch and Moore 1992; Schlesinger et al. 2010; Woolsey 2012), siphonophores (Sherlock and Robison 2000) and hydrozoans (Mali et al. 2011). This can involve selfing of male and female gametes derived from separate male or female polyps of the same colony (e.g. Mali et al. 2011), selfing between sexually plastic anemones deriving from common clonal lineages (i.e. genetically identical polyps that develop as males, females or hermaphrodites) (Schlesinger et al. 2010) and selfing in hermaphroditic colonies (Heyward and Babcock 1986).

Like other metazoans, cnidarians are characterised by anisogamy—the production of relatively few large eggs and of numerous tiny sperm. The selection pressures for the evolution of anisogamy from the primitive isogamous condition have been the focus of extensive theoretical investigation and remain conjectural (see Billiard et al. 2011 for review). Hypotheses for dimorphism in animal gamete sizes include conflict amongst gamete producers (Parker et al. 1972), an adaptation that increases gamete encounter rates (e.g. Levitan 1993) and preventing nuclear-cytoplasmic conflict through uniparental inheritance of organelles (Hurst and Hamilton 1992). However all three explanations are based on outcrossing systems. If myxozoan gametes self-fertilise and are produced in close proximity where gamete encounter rate is irrelevant, then many of the selective pressures are absent and perhaps it is not surprising that myxozoans would be essentially isogamous. Dicyemids may present a parallel system of reversion to isogamy. Their gametes are also produced in close proximity within an internal axial cell and are believed to reproduce via self-fertilisation (Furuya and Tsuneki 2007; Czaker 2011). Selfing in these systems supports the hypothesis that meiosis is primarily an adaptation for DNA repair (Michod et al. 2008; Bernstein et al. 2011) rather than for the generation of genetic variation at least in the short term.

If myxosporean development proceeds as proposed by Morris (2012) then the above scenarios of selfing or genetic mosaicism are avoided. It is therefore of great interest to resolve the

nature of binucleate cells observed during early development and where in the myxosporean life cycle diploidy is achieved.

3.2.3.2 Asexual Reproduction

Myxozoans undergo extensive asexual reproduction during schizogony or the proliferative stage of the life cycle. Single-cell stages of both malacosporeans and myxosporeans replicate via binary fission prior to the development of sporogonic stages (see Chap. 8 and above). Endogeny represents a form of internal budding in myxozoan cells and occurs in both presporogonic and sporogonic phases of the life cycle (Fig. 3.2b, see Chap. 8). Other vegetative processes of sporogonic stages include apparent fission and budding in malacosporeans. The former is witnessed by the development of internal walls in sacs (Fig. 3.2c, d) and may be homologous to transverse fission—a mode of reproduction that occurs in the three major cnidarian classes but is most common in Scyphozoa. Budding is suggested by the development of protuberances on sacs in bryozoan hosts (Okamura 1996; McGurk et al. 2006) and on proliferative stages in fish (Fig. 3.2e). Myxosporeans undergo proliferation when plasmotomy (Lom 1990; Shul'man 1990) results in the production of both external and internal buds, the latter being liberated when the maternal plasmodium splits up. Another mode of asexual reproduction comparable to budding occurs in *Buddenbrockia*, when worms grow from early developmental stages embedded in the host extracellular matrix (Canning et al. 2008; Gruhl and Okamura 2012). After detachment of the worm some of the embedded tissue is thought to remain as a covert infection, enabling the further development of worm-like stages (Gruhl and Okamura 2012).

Cnidarian life cycles are characterised by a capacity for extensive asexual proliferation. Budding, transverse fission (e.g. strobilation), longitudinal fission and fragmentation are well known examples (Ruppert et al. 2004) and these occur across the free-living cnidarians. Less widely appreciated forms include pedal laceration in anemones, planuoid buds that fall off of scyphozoans, and even swallowing and incubation of autotomized tentacles that subsequently form new anemones (see Fautin 2002 for review). Such vegetative growth is intimately linked to regeneration processes (see below).

3.2.3.3 Regeneration, Transdifferentiation and Dormancy

Dormancy followed by regeneration in myxosporeans is demonstrated by spores protected by hardened walls. Experimental studies demonstrate that myxospores of *Myxobolus cerebralis* can remain in a dormant state for at least 3–5 months without loss of infectivity to worms (El-Matbouli and Hoffmann 1991). Although the soft, unprotected spores of malacosporeans are short-lived (loosing infectivity within 24 h; de Kinkelin et al. 2002), malacosporeans undergo dormancy during infection by cryptic stages of bryozoan resting bodies (statoblasts). Statoblasts are dormant during winter months and hatch when favourable conditions return, enabling the formation of a small colony (see Chap. 11). Malacosporean infections in statoblasts have been demonstrated for *T. bryosalmonae* (Abd-Elfattah et al. 2014) and *Buddenbrockia allmani* (Hill and Okamura 2007) and represent a form of vertical transmission (see Chap. 11). Longitudinal studies demonstrate that infections arising in colonies that develop from infected statoblasts are, in turn, transmitted to fish (Abd-Elfattah et al. 2014), providing evidence that dormancy in statoblasts is effective.

Free-living cnidarians demonstrate an immense capacity for regeneration. Often this is associated with dormancy and it may sometimes involve transdifferentiation of tissues. For instance, planulae or polyps may shrink and persist in states with low metabolic costs as e.g. cysts and dormant hydrorhizae. This process enables survival during stressful periods and is then followed by cell proliferation and morphogenesis when favourable conditions return (Boero et al. 1992). Recurrent cycles of these regenerative processes are observed in polyps without any sign of senescence (Piraino et al.

2004; Bosch 2008). In addition, polyp stages can be reformed from regressed tissues of sexual stages (medusae) via reverse ontogeny in hydrozoans of the genus *Turritopsis*. This reverse transformation is achieved by the proliferation of I-cells (interstitial stem cells) and directly by cell transdifferentiation processes (Piraino et al. 2004; Bosch 2008). In the hydrozoan, *Sarsia tubulosa*, medusa buds have also been observed to transform back into polyp buds when exposed to different temperatures (Werner 1963).

3.2.4 Life Cycle Stages

Myxozoans exhibit a complex parasitic life cycle involving an alternation of distinct stages between two hosts mediated by transmission via infectious spores (Fig. 3.1a, b). The free-living medusozoans are characterised by an alternation of generations as benthic polyp and pelagic medusa stages with planulae metamorphosing into polyps which in turn form medusae (Fig. 3.1c). The complex parasitic life cycle of myxozoans and the alternation of generations in free-living medusozoans pose the question of whether and how these life histories may be equated in the two groups.

Myxozoa appears to be sister to or to group within the Medusozoa. One interpretation is therefore that myxozoans in invertebrate hosts may correspond to medusae since both are sexual stages. The interpretation would also be supported if the locomotory ability of *Buddenbrockia* in bryozoan hosts is homologous to that of swimming medusae. This scenario would imply that sporoplasms in spores that develop in invertebrate hosts are homologous to either gametes (if, in malacosporeans sporoplasms are haploid: see above) or that spores enclose zygotes (in myxosporeans sporoplasms fuse and are diploid; see above). In turn, spores released from fish may be homologous to polyp stages. However, as highlighted above, shortening of free-living cnidarian life cycles may result in secondary transfer of sexual reproduction to the polyp stage, as in *Hydra*. On the basis of this consideration alone, we are therefore at present left with little further insight regarding whether the medusa or polyp stages may equate to myxozoan stages that infect invertebrate and vertebrate hosts nor how myxozoan spores may equate to cnidarian stages. We cannot even discount the possibility that spores developing in invertebrate and vertebrate hosts could, for instance, represent medusa and polyp stages, respectively. Such a scenario would imply that only a fleeting proportion of time in the myxozoan life cycle is devoted to the predominant life history stages in medusozoans. This is not entirely unprecedented since medusa stages have been lost in many hydrozoans. It would also imply, on the one hand, considerable progenesis with sexual reproduction occuring in early stages infecting invertebrate hosts prior to the production and release of medusoid spores while a pelagic phase would be associated with polyps before colonisation of fish hosts. Again, hydrozoans provide some parallels with progenesis enabling sexual reproduction in hydrozoans that have lost medusa stages (Boero et al. 1992).

On the other hand, if myxozoans are sister to medusozoans their life cycle may have diverged from that of anthozoans. Under this scenario myxozoan stages in invertebrates may represent polyps that release gametes (e.g. in malacosporean spores) or zygotes (e.g. in myxosporean spores) that then undergo extensive development as a novel, pre-adult cnidarian stage that evolved to exploit fish hosts. Spores released from fish might simply represent specialised totipotent stages akin to e.g. frustrules that are capable of developing to adult stages.

We note that evidence based on molecular phylogenetic analyses of LSU and SSU data have suggested that the stalked 'upside-down jellyfish' (the Stauromedusae) may be sister to all of Medusozoa (Collins et al. 2006). The lack of a pelagic medusa stage in Stauromedusae implies that this stage may have evolved subsequently in medusozoans. If myxozoans and stauromedusans are sister taxa then drawing homologies of myxozoan stages with pelagic medusae may be inappropriate. However, recent mitogenome

analyses have upheld the more traditional view that Stauromedusae are derived within the Medusozoa (Kayal et al. 2013).

Finally, in view of the unclear relationships of myxozoans to the parasitic cnidarian *Polypodium* (see Chap. 2), another interpretation is that myxozoan stages that develop in fish may represent larvae, as in *Polypodium*. Since *Polypodium* has a free-living adult stage, this may then imply that the homologous adult stage in myxozoans became parasitic in invertebrates (see also discussion in Chap. 2). This explanation implies no particular homology between myxozoan stages that develop in different hosts and medusozoan or anthozoan benthic and pelagic stages. The great plasticity in cnidarian life cycles, including the capacity to generate novel forms is equally consistent with this scenario.

3.3 Morphological Simplification

It has been widely observed that parasites, and most especially endoparasites, often exhibit simpler morphologies than their closest free-living relatives. However, while morphological simplification is observed in some parasitic taxa it does not characterise others (Poulin 2007). In addition, while particular morphologies may be reduced, morphological innovations such as sensory receptors and complex attachment organs are often observed (Brooks and McLennan 1993). Several drivers of morphological simplification in parasites can be hypothesised. For example, complexity may be costly. Because parasites live in patchy, discontinuous environments (hosts) they may have to allocate disproportionately more resources to reproduction than to non-reproductive structures. Furthermore, host immune responses may require parasites to allocate resources to avoidance strategies. Thus, morphologies no longer required for functions such as digestion or locomotion may be selected against. Alternatively, parasite morphologies may reflect the physical requirements of host environments. For instance, endoparasites often live in confined spaces such as body cavities, blood vessels, and even between or within cells of certain tissues. Morphological simplification via loss of appendages or by miniaturisation may represent adaptations to host environmental niches comparable to those characterising interstitial meiofauna.

Myxozoans present the most extreme example of overall morphological simplification associated with endoparasitism. Although the precise sister taxon is not yet resolved, the last common ancestor of the Myxozoa was a free-living animal closely related to or grouping within the Medusozoa. The body plan of this animal would have included two epithelial tissue layers (an epidermis and gastrodermis), an intermediate extracellular matrix layer (mesoglea), differentiated cell types (e.g. gland cells, ciliated cells), a common opening serving as a mouth/anus, a digestive tract (gastrovascular cavity), a nervous system with sensory cells, nematocysts, musculature and anisogamy. Many of these characters have been partially or fully reduced in the Myxozoa, and most particularly in the Myxosporea which have retained only nematocysts (polar capsules).

Within the Myxozoa several trajectories of morphological simplification are evident. These include: (1) trophic stages in fish are simpler than those in invertebrates (2) malacosporean invertebrate stages are more complex than those of myxosporeans (3) histozooic and intracellular stages are simpler than coelozoic fish stages (see Feist and Longshaw 2006 and Chaps. 3, 9 and 16 for further discussion). Although, myxozoan spores have increased in size and morphological complexity (see Chap. 4), morphological simplification is a major form of innovation and adaptation to the environment of particular parasite life cycle stages and has enabled myxozoans to evolve as endoparasites and to exploit new host resources.

3.4 A Cnidarian Predisposition for Endoparasitism?

Certain cnidarian features may have predisposed the myxozoan precursor to evolve an endoparasitic lifestyle. In particular, the overall

diploblastic body plan and the associated lack of organ development have prompted invertebrate zoologists to characterise cnidarians as 'epithelial organisms' (Ruppert et al. 2004). Essentially cnidarians utilise extensive external and internal epithelial layers that enhance resource capture and uptake by their large collective surface areas. In addition, cnidarians are able to absorb dissolved amino acids across these epithelial surfaces (Ferguson 1982; Grover et al. 2008). These combined features of large areas for resource capture and an inherent capacity for uptake of dissolved organic material may have been key cnidarian pre-adaptations that readily supported endoparasitism.

In addition, many cnidarians have evolved intimate relationships with endosymbionts based on nutritional uptake. Such endosymbionts have been acquired repeatedly and independently (e.g. in some anemones, corals, green *Hydra*, and stalked jellyfish) demonstrating the facility with which cnidarians may evolve intimate relationships with other organisms. This facility is also illustrated by the common association of hydrozoans with bryozoans, demonstrating a propensity for forming the kind of commensal relationships that may eventually lead to the evolution of parasitism (Poulin 2007). For instance, *Hydra* is often encountered on the surfaces of freshwater bryozoans (e.g. see Fig. 2.3 in Chap. 2) and hydroid colonies symbiotic with marine bryozoans are observed in both present-day settings (e.g. Boero and Hewitt 1992) and in ancient environments as fossils (McKinney 2009).

The development of cells within cells by phagocytosis and endogeny-like processes is particularly prominent in myxozoans (see Chap. 8). In free-living cnidarians such processes result in e.g. nurse cells within oocytes and the incorporation of algal symbionts (see Morris 2012 for further discussion). The cell-within-cell organisation is also achieved by the larval parasitic stage of *Polypodium hydriforme* in which a haploid cell is contained within a nurse cell (Raikova 2008).

Finally, as discussed above, the cnidarian capacity for regeneration and dormancy combined with their plasticity in producing novel stages may have predisposed the myxozoan precursor for survival within hosts. Indeed these traits may have been fundamental to initial stages of host invasion if dormant stages, for instance, are not targeted by host immune systems.

3.5 Polar Capsules and Nematocysts

Polar capsules are intracellular organelles of spores diagnostic of all myxozoans known to date and exhibit extensive similarities to cnidarian cnidae, which include nematocysts, spirocysts and ptychocysts. The latter two forms are structurally and chemically distinct from nematocysts. Nematocysts are common to all cnidarian subtaxa and their particular similarities to polar capsules provide compelling evidence for cnidarian affinities of Myxozoa (e.g. Weill 1934; Siddall et al. 1995). Comprehensive reviews of polar capsules are provided by Canning and Okamura (2004) and Cannon and Wagner (2003).

3.5.1 Structure, Development, and Function

Mature polar capsules are easily recognisable by light microscopy as micrometre-sized, spherical to ovoid, and sometimes elongate vesicles situated terminally and in close proximity to the spore surface within capsulogenic cells (Figs. 3.3a–g, 3.4a–c, 3.5a). The latter are specific cell types that are connected to overlying valve cells by cell-cell junctions (Fig. 3.5a). There is always one polar capsule per capsulogenic cell. Similarly, nematocysts (Figs. 3.3h–l, 3.4f–j and 3.5b–d) are formed by a specific cell type, the nematocyte, and each cell only produces one capsule (Moebius 1866; Weill 1934). The number of capsulogenic cells per spore typically varies from 1 to 4, but can be up to 15 in some species (Lom and Dyková 2006). The only case known so far of polar capsules occurring outside the spore stage is in *Buddenbrockia* worms from the freshwater bryozoan host *Hyalinella punctata*. In these

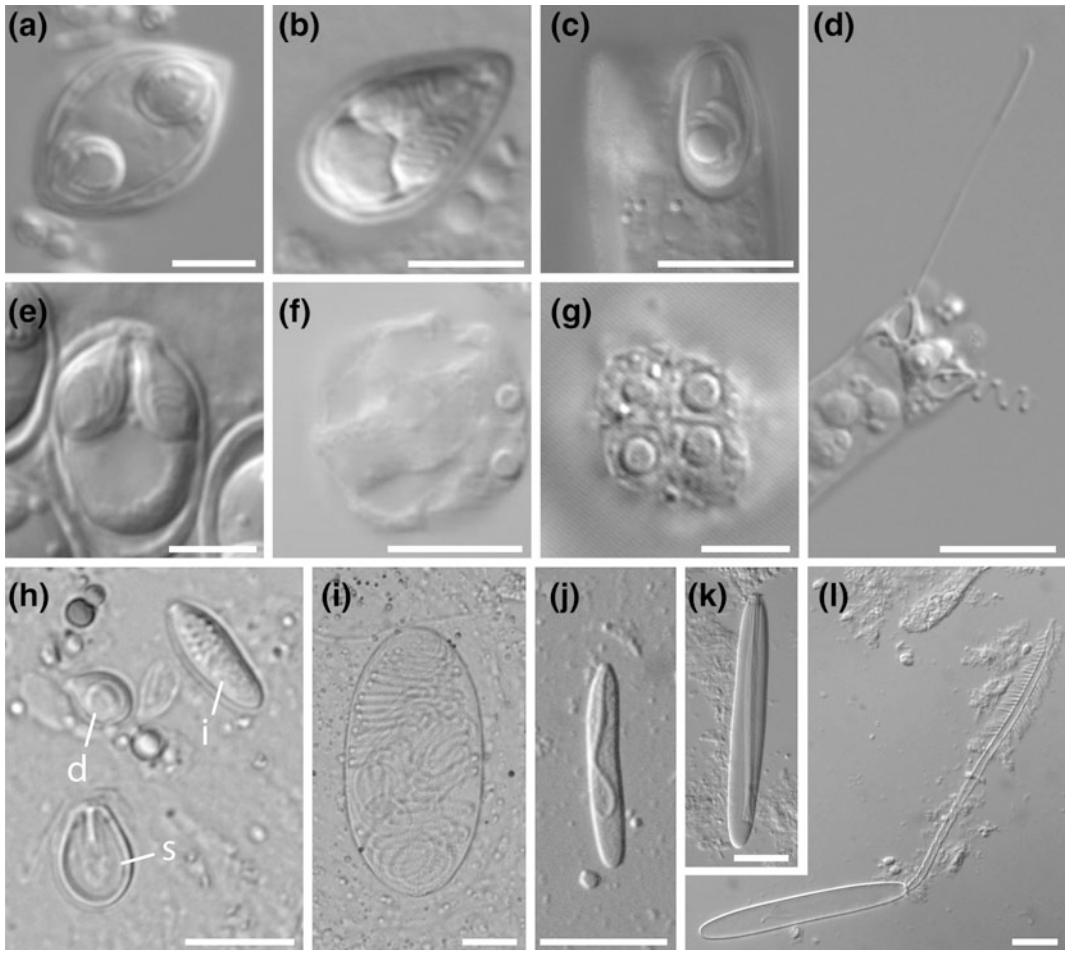
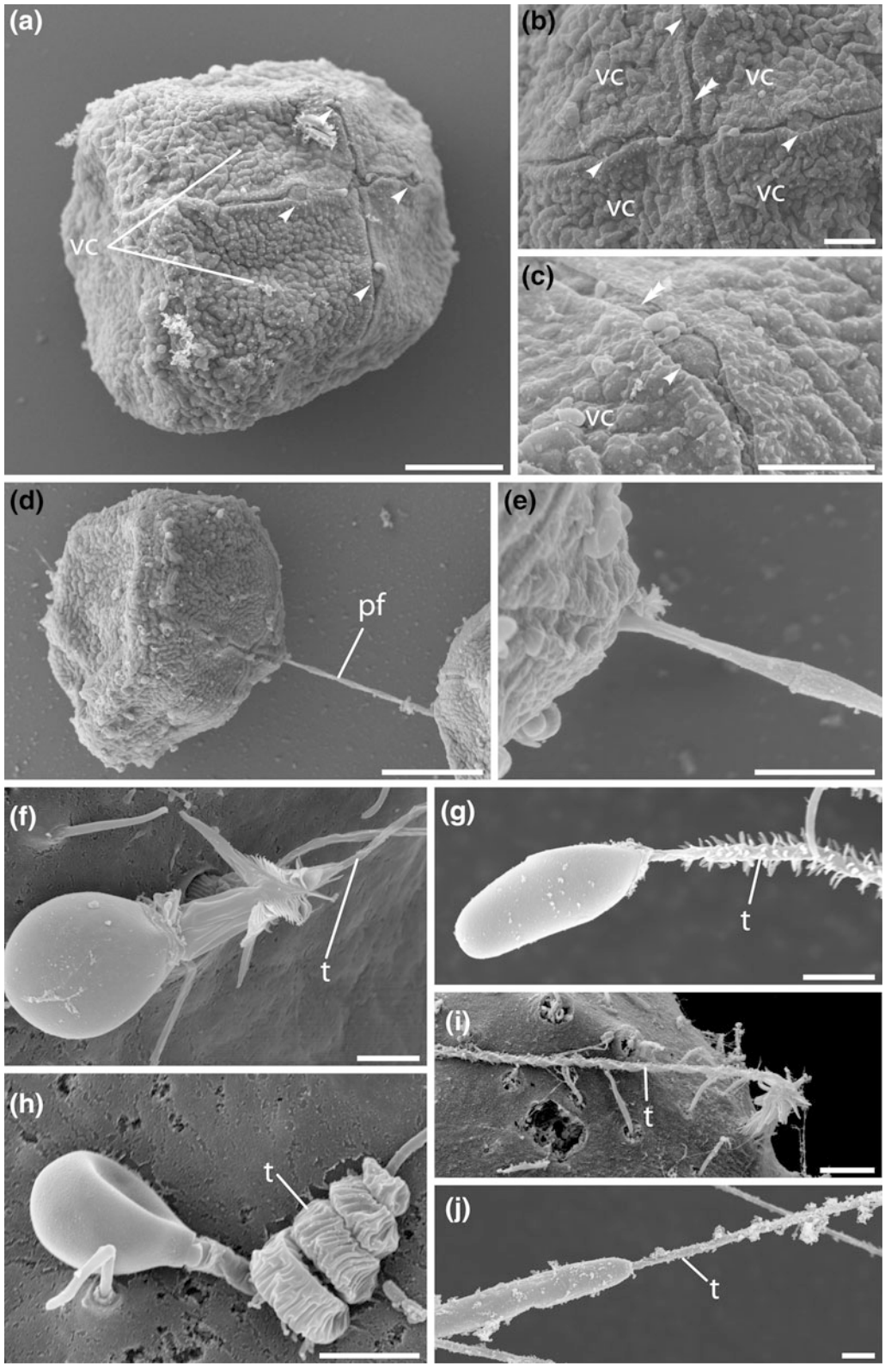


Fig. 3.3 Diversity of polar capsules and nematocysts. **a**–**e** myxosporean polar capsules, photos by Stephen Atkinson. **a** Myxospore of *Myxidium* sp. ex Sculpin (*Cottus* sp.), *scale bar* 5 µm. **b** Myxospore of *Myxobolus* sp. ex Oregon Chub (*Oregonichthys crameri*), *scale bar* 5 µm. **c** Raabeia type actinospore ex *Limnodrilus hoffmeisteri*, *scale bar* 5 µm. **d** same as in c, discharged capsule, *scale bar* 10 µm. **e** Myxospore of *Myxobolus* sp. ex Blue Chub (*Gilia coerula*), *scale bar* 5 µm. **f** *Tetracapsuloides bryosalmonae* (Malacosporea) spore released from bryozoan, lateral view, *scale bar* 10 µm. **g** apical view, *scale bar* 5 µm. **h**–**k** Nematocysts, *scale bars* 10 µm. **h** Stenotele (*s*), isorhiza (*i*), and desmoneme (*d*) from *Hydra vulgaris* (Medusozoa, Hydrozoa). **i** Holotrichous isorhiza from *Protopalythoa mutuki* (Anthozoa, Hexacorallia, Zoantharia). **j** Basitrichous isorhiza *Urticina felia* Anthozoa, Hexacorallia, Actiniaria). **k** Amastigophore from *Sagartia elegans* (Anthozoa, Hexacorallia, Actiniaria). **l** Discharged amastigophore from *S. elegans*

worms, polar capsules develop not only in spores (Gruhl and Okamura pers. obs.) but also in the mural (epidermal) cell layer (Okamura et al. 2002).

The general structure of polar capsules is very consistent (Fig. 3.5a): they are bound by a single membrane underlain by a bilayered wall with an electron dense outer and an electron lucent inner layer (Figs. 3.5a and 3.6a). The inner compartment is filled by a matrix that, depending on developmental stage and fixation/preparation, presents different stages of precipitation—from homogeneous to granulated and from electron-dense to electron lucent. Likewise, the nematocyst wall is bilayered (Figs. 3.5b, h and 3.6b) enclosing a matrix that varies in appearance


(a)
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◀ **Fig. 3.4** Comparison of myxozoan polar capsules and nematocysts, scanning electron micrographs. **a–e** Spores of *Tetracapsuloides bryosalmonae* (Malacosporea). **a** Whole mount, lateral/apical view. The four capsulogenic cells are almost completely covered by the neighbouring valve cells (*vc*). Polar capsule apical structures (plugs) visible through the uncovered surface (*arrowheads*), *scale bar* 3 µm. **b**, **c** Detailed views. Apical surface of capsulogenic cells visible in cleft between valve cells (*double arrowheads*), *scale bars* 1 µm. **d** Spore with discharged polar capsule, *scale bar* 5 µm. **e** Detail of everted polar filament (*pf*), *scale bar* 1 µm. **f–j** Cnidarian nematocysts, *scale bars* 3 µm. **f** Discharged stenotele from *Hydra vulgaris* (Medusozoa, Hydrozoa). **g** Discharged isorhiza from *H. vulgaris*. **h** Discharged desmoneme from *Hydra vulgaris*. **i** Discharged eurytele still imbedded in the surface tissue of *Cordylophora caspia* (Medusozoa, Hydrozoa). **j** Discharged holotrichous isorhiza from *Bunodosoma cavernata* (Anthozoa, Hexacorallia, Actiniaria). *t* tubule

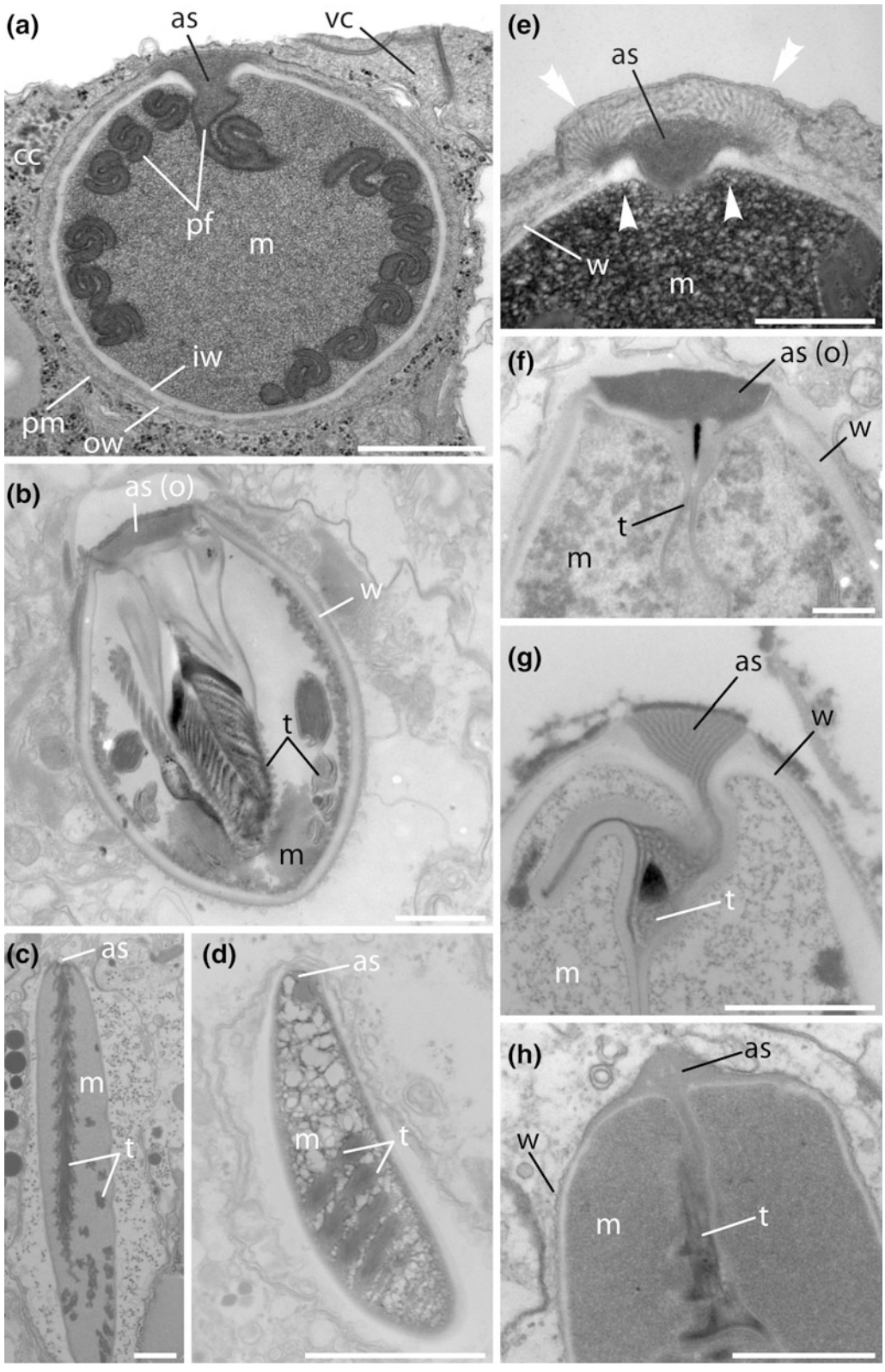
(Fig. 3.5b–d, f, h, Mariscal 1974). At the apical pole of the polar capsule, the wall is interrupted and a membrane-bound tubule, the polar filament, is invaginated and lies in several coils in the inner capsule compartment (Fig. 3.5a). The membrane of the tubule usually bears a conspicuous dark glycocalyx-like layer. Cross-sections of the coiled tubule appear collapsed, especially in mature capsules, with an S-like (Figs. 3.5a and3.6a) or triradiate (e.g. Casal et al. 2007) profile. In nematocysts of both anthozoans (Westfall 1965) and medusozoans (Hessinger and Ford 1988), portions of the inverted tubule often have a triradiate profile (Fig. 3.6b).

Both polar capsules and nematocysts bear conspicuous elements, referred to as apical structures that seal the capsule at the site where the tubule is invaginated, presumably preventing precocious discharge. The myxozoan apical structure (Fig. 3.4c) consists of an electron-dense core and a striated peripheral region (Fig. 3.5e). The basal part of the apical structure is surrounded by a ridge formed by the capsule wall. The overall shape of the apical structure varies between myxozoan taxa, from lenticular to conical. The capsulogenic cells are covered by the neighbouring valve cells apart from at a narrow region of the apical membrane where they are exposed to the exterior and the apical structures are visible (Figs. 3.4 a–c and3.5a, e). Medusozoan nematocysts possess very similar apical structures called opercula (Fig. 3.5b, f, g; see Reft and Daly 2012), whereas anthozoan apical structures resemble caps (Fig. 3.5h) or triangular flaps (Reft and Daly 2012).

Capsulogenesis involves the formation of one large intracellular membrane-bound vesicle that grows in size, apparently by fusion with smaller vesicles. The capsule primordium has been inconsistently described in the literature to originate from either rough or smooth endoplasmic reticulum or Golgi apparatus (summarised in Lom and Dyková 1997). At some stage a cone-shape extrusion occurs at one end of the vesicle with a recognisable concentration of peripheral microtubules. This extrusion grows into a long external tubule (Fig. 3.6e) that can be coiled around the capsule primordium. In nematocysts, the capsule always forms in a post-Golgi vesicle deriving from such fusion (Fig. 3.6g; Özbek 2011). The external tubule initially develops outside the capsule (Fig. 3.6f) before being inverted and coiled in the capsule (see Fig. 3.1a in Beckmann and Özbek 2012 for summary).

Exposure to specific chemical and mechanical environmental stimuli (Cannon and Wagner 2003; Kallert et al. 2005, 2010, 2011) causes eversion of the polar filament. In parallel with nematocysts, eversion is presumably mediated by both high pressure in the capsule matrix and elastic force provided by the protein scaffold of the capsule wall (minicollagens and associated other proteins; see Chap. 2). Unlike many cnidarian nematocysts the filament of polar capsules does not penetrate the host tissue nor inject venoms or digestive enzymes. Instead it functions to contact and anchor the spore to the next host in the life cycle, a task that may be achieved by a simple tubule that lacks spines. Spirocysts, ptychocysts and some forms of nematocysts are non-penetrating (Weill 1934; Mariscal 1974), and several types are involved in adhesion and locomotion rather than penetration (see Mariscal 1974 for a summary).

Although stimulation of nematocyst discharge from surrounding nerve and sensory cells has been shown (see Holstein 2012), it is mainly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◀ **Fig. 3.5** Comparison of myxozoan polar capsules and nematocysts, transmission electron micrographs. **a**–**d** *scale bars* 2 μm. **a** Polar capsule of undescribed malacosporean myxozoan (closely related to *Tetracapsuloides bryosalmonae*), longitudinal section showing capsule membrane (*pm*), inner and outer walls of capsule (*ow*, *iw*), matrix (*m*), inverted polar filament (*pf*) and site of tubule invagination with apical structure (*as*), valve cell (*vc*). Photo by Alan Curry. **b** Longitudinal section of a stenotele nematocyst from *Ectopleura larynx* (Medusozoa, Hydrozoa) showing the capsule wall (*w*), matrix, inverted tubule (*t*) and the apical structure (*as*), operculum (*o*). **c** Longitudinal section of a *b*-mastigophore nematocyst of *Ceriantheopsis americanus* (Anthozoa, Hexacorallia, Ceriantharia) showing the matrix, inverted tubule, apical structure. **d** Longitudinal section of *Phenganax parrini* (Anthozoa, Octocorallia) nematocyst. **e**–**h** Apical structures in polar capsules and nematocysts, *scale bars* 1 μm. **e** Apical structure in malacosporean myxozoan showing continuity with inner wall (*arrowheads*), dense central matrix and peripheral striated region (*double arrowheads*). Photo by Alan Curry. **f** Operculum of *Ectopleura larynx* nematocyst. **g** Striated operculum of *Haliclystus borealis* (Medusozoa, Staurozoa) nematocyst. **h** Apical cap of *p*-mastigophore from *Stomphia coccinea* (Anthozoa, Hexacorallia, Actiniaria)

triggered by sensory structures of the nematocytes (the cells bearing nematocysts) themselves. These can be chemoreceptors or a very characteristic type of mechanoreceptor, the cnidocil (Holstein and Hausmann 1988). Cnidocils are absent in capsulogenic cells and nerve and sensory cells have never been detected in myxozoans, but both chemical and mechanical stimuli have been shown to trigger discharge (see Chap. 13 and Cannon and Wagner 2003). Notably, nematocysts can also discharge without cellular input (Aerne et al. 1991).

3.5.2 Homology of Nematocysts and Polar Capsules

The similarities between polar capsules and cnidarian nematocysts led early researchers to suggest a potential myxozoan-cnidarian relationship. However, extrusible organelles of comparable structure are also found in various unicellular eukaryote lineages (e.g. Hausmann 1978, 2002; Westfall et al. 1983). In fact, some studies have hypothesized an evolutionary relationship between protist extrusomes (extrusive, membrane-bound organelles) and nematocysts, and have even suggested an endosymbiotic origin for both these organelles (Shostak and Kolluri 1995; Hwang et al. 2008; Holstein 2012). If cnidarians acquired nematocysts via lateral gene (or organelle) transfer from protist lineages, a similar origin could be hypothesized for polar capsules. Thus, the presence of similar structures in myxozoans and cnidarians may not be a synapomorphy.

However, the above hypotheses ignore considerable evidence that nematocysts and polar capsules share similarities not shared by other extrusomes. In both polar capsules and nematocysts the extrusive portion develops external to the capsule, and later invaginates (Desser et al. 1983; Westfall 1966; Siddall et al. 1995; Özbek 2011). After invagination, an apical structure develops to seal the capsule. This developmental sequence is unique to polar capsules and nematocysts. In other extrusomes, the extrusive element develops inside the capsule concurrently with the apical sealing structure (Hausmann 1978; Westfall et al. 1983; Mikrjukov 1995; Hoppenrath et al. 2010). Furthermore, even the extrusome hypothesized to be most like nematocysts (the "nematocysts" of *Polykrikos*; Hoppenrath et al. 2010), displays a complex, multipart operculum, a distinct anterior and posterior chamber in the capsule, and a simple coiled tubule (Westfall et al. 1983; Hwang et al. 2008; Hoppenrath et al. 2010). In contrast, nematocysts and polar capsules exhibit a single apical structure, capsules with only one chamber, and a more complex coiled extrusive element (tubules and polar filaments) associated with a tri- or biradiate profile (e.g. Chapman and Tilney 1959a, b; Westfall 1965, 1966; Mariscal 1974; Desser et al. 1983; Westfall et al. 1983; Siddall et al. 1995). Finally, in *Polykrikos*, the nematocyst is always associated with another extrusome, the taeniocyst, with which it forms the taeniocyst-nematocyst complex (Westfall 1983; Hoppenrath et al. 2010).

Characterisation of molecular constituents now provides very good evidence that nematocysts

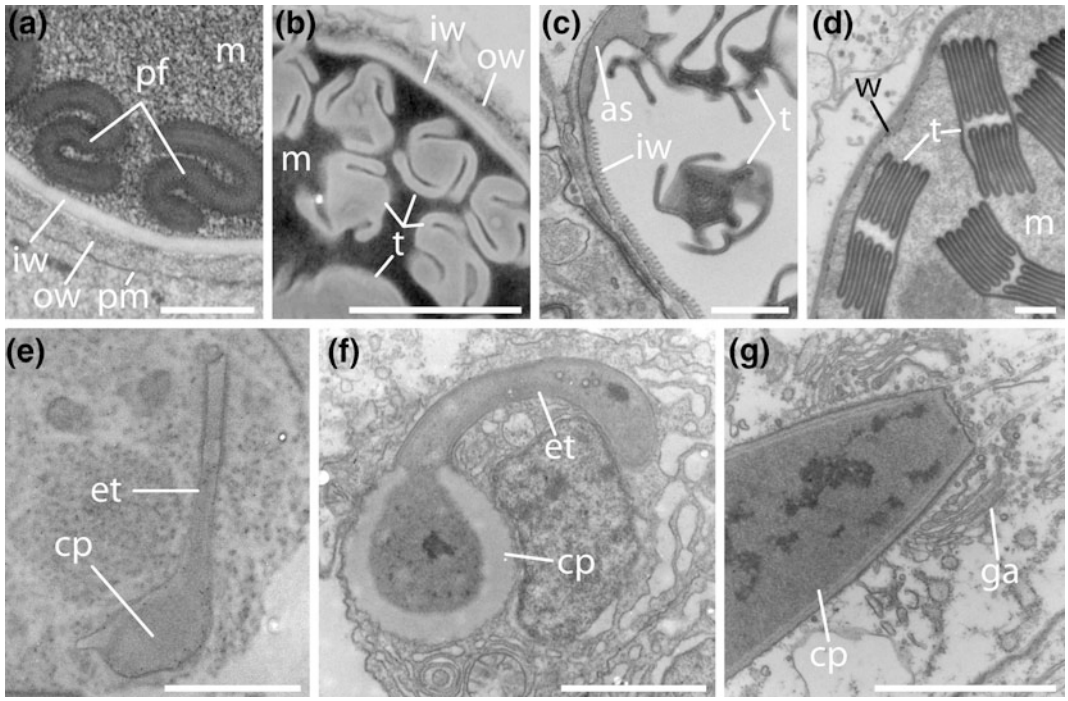

Fig. 3.6 **a–d** Cross sections of inverted tubules, *scale bars* 500 nm. **a** Malacosporean myxozoan polar capsule. Photo by Alan Curry. **b** Cross section of the inverted tubule of a nematocyst from *Metridium senile* (Anthozoa, Hexacorallia, Actiniaria) showing the three part profile. **c** Cross section of the apical end of a spirocyst from *Protopalythoa mutuki* (Anthozoa, Hexacorallia, Zoantharia) showing the inverted tubule, the apical cap, and the characteristic serrated inner wall **d** Cross section of ptychocyst from *Ceriantheopsis americanus* (Anthozoa, Hexacorallia, Ceriantharia) showing the capsule wall, matrix, and the multilobed profile of the inverted tubule. **e–f** Developing capsules/nematocysts, *scale bars* 2 μm. **e** Developing polar capsules of *Tetracapsuloides bryosalmonae* showing capsule primordium (*cp*) and external tubule (*et*). **f** Developing nematocyst from *Ectopleura larynx* (Medusozoa, Hydrozoa) showing the capsule primordium and external tubule. **g** Developing nematocyst capsule from *Stomphia coccinea* (Anthozoa, Hexacorallia, Actiniaria) where the end grows from addition of vesicles originating from the Golgi apparatus (*ga*). *as* apical structure, *iw* inner wall, *m* matrix, *ow* outer wall, *pm* polar capsule membrane

and polar capsules are unique, homologous structures since minicollagens, NOWA and other cnidarian-specific proteins contribute as structural proteins to the wall and the filament armature and are regarded as taxonomically restricted genes (David et al. 2008; Milde et al. 2009; Özbek 2011). One of these, a minicollagen, has been demonstrated in the malacosporean *Tetracapsuloides bryosalmonae* (Holland et al. 2011) and others have more recently been characterised (see Chap. 2). None of the protist genomes for which we currently have full sequence data contain any evidence of homologues of nematocyst-specific proteins (Holstein 2012). Furthermore, antiserum produced against *Myxobolus pendula* spores showed immunoreactivity with extruded anthozoan and hydrozoan tubules (Ringuette et al. 2011). Taken together, the developmental, ultrastructural, and molecular data strongly indicate that nematocysts and polar capsules are homologous structures that are independently derived and unrelated to the extrusomes of protists.

3.5.3 Affinities of Polar Capsules with Nematocyst Types

Given the homology of nematocysts and polar capsules it is of interest to explore whether we can identify any specific affinities of polar

capsules with certain types of nematocysts. Nematocysts demonstrate a diversity of form and function and are commonly used for phylogenetic and taxonomic purposes. Around 30 nematocyst types are distinguished morphologically (Weill 1934; Mariscal 1974; Fautin 2009) with the highest diversity in hydrozoans and the lowest in anthozoans. Nematocyst types are largely distinguished by tubule morphology, e.g. whether the tubule is uniform in diameter (isorhiza/haploneme versus anisorhiza/heteroneme) and the presence and location of spines (atrichous, holotrichous, basitrichous).

The simple function of attachment may preclude the diversification of polar capsules into an array of distinct forms. Indeed, using nematocyst classification schemes (e.g. Fautin 2009), myxozoan polar capsules would be classified generally as "atrichous isorhizae" because of the lack of spines and the uniform diameter of the tubule. However, polar capsule diversity may be unappreciated since there are likely to be many undescribed myxozoan species (e.g. see Chap. 6) and stages that develop in invertebrate hosts are poorly known. The evolutionary relationships between holotrichous and atrichous isorhizae are currently unclear and both may have evolved independently on multiple occasions within Cnidaria (Fautin 2009), thus, homologies of myxozoan polar capsules with atrichous isorhizae remain obscure. Immunohistochemical experiments have shown antiserum, raised against myxozoan polar capsules, to react exclusively with *Nematostella* b-mastigophores and with *Hydra* isorhizas, despite the presence many other nematocyst types in *Nematostella* and *Hydra* (Ringuette et al. 2011).

One phylogenetically informative character may be the apical structure of nematocysts and polar capsules. Like myxozoans, medusozoan nematocysts bear a plug-like structure (known as an operculum in medusozoans) at the apex (site of tubule invagination). The operculum closes the undischarged capsule like a lid and, in the medusozoans, has a hinge at its base. Anthozoan nematocysts lack an operculum and are closed by an apical cap formed by an electron dense material which is distinct from both the capsule and tubule walls, or, in the case of Actinaria, by three flaps formed from extentions of the capsule wall. Although not investigated in detail, the plug-like structure of polar capsules might correspond to the medusozoan operculum since the presence of striations and a rim between the capsule wall and the operculum matrix characterises both myxozoans (Fig. 3.5e and Canning and Okamura 2004) and medusozoans (Fig. 3.5g and Reft and Daly 2012). The characteristic hinge of the nematocyst operculum (Reft and Daly 2012) has not been demonstrated in polar capsules but this tiny structure is difficult to observe and may therefore have been overlooked in myxozoans.

Although there are some differences in the morphology of polar capsules and medusozoan nematocysts, these two structures are more similar to each other than are some other nematocyst forms. For instance there are much larger differences in capsule and tubule structure among nematocysts, spirocysts and ptychocysts of hexacorallian anthozoans (Fig. 3.6c, d, Mariscal 1984; Reft and Daly 2012). Furthermore, while elongate nematocysts occur in all cnidarian subtaxa, spherical nematocysts are restricted to medusozoans (Reft, pers. obs). Polar capsules of the primitive malacosporeans are likewise predominantly spherical.

3.6 Cnidarian Sister-Group?

As reviewed in Chap. 2, molecular data including DNA sequence data, the presence of taxonomically restricted genes, and mitochondrial genes and genomes consistently provide high support for a medusozoan affinity of myxozoans, and particularly as a sister group to medusozoans. However, a major obstacle for further phylogenetic inference is that myxozoans are characterised by accelerated rates of sequence evolution and, as discussed above, have undergone massive morphological simplification, restricting the number of characters in common with other cnidarian groups. Thus, an inferred position at the base of medusozoans may simply reflect a lack of phylogenetically informative characters

(both morphological and molecular) for revealing a closer relationship to any medusozoan ingroup. Tetraradial symmetry, similarity of polar capsules with medusozoan nematocysts, and (perhaps) life cycle complexity all place myxozoans near medusozoans but fail to provider higher resolution.

Current molecular clock estimates within Cnidaria date the last common ancestor of all medusozoans at ~570 Ma (Park et al. 2012), thus, by implication potentially rendering myxozoans an extremely old group, predating the occurrence of their present-day host taxa (Actinopterygii, Annelida, Bryozoa). More recent derivation of myxozoans within the Medusozoa would therefore be more consistent with exploitation of these hosts (see Chap. 2 for exploration of the first myxozoan hosts). Given the occurrence of hydrozoans in fresh water and the inferred freshwater origin for the Myxozoa (see Chap. 2) derivation of myxozoans within Hydrozoa would seem feasible. Hydrozoans also appear to exhibit the highest plasticity in life cycles among cnidarians including morphological transformations, specialised dormant stages, progenesis and transverse fission all of which may have supported the remarkable transition to endoparasitism exhibited by myxozoans. Chapter 2 further considers the potential candidates for myxozoan affinities including potential links with *Hydra* (which is often associated with freshwater bryozoan hosts) and as a sister taxon to *Polypodium*.

3.7 Conclusions

Cnidarians may be particularly suited to evolve an endoparasitic lifestyle because their simple diploblastic condition is based on the development of extensive epithelial layers that mediate processes such as active uptake and excretion. Other cnidarian features that may have been preadaptive include capacities for regeneration, transdifferentiation, dormancy and the production of novel propagative stages. Cnidarian origins may be reflected by myxozoan features such as cell-within-cell development (a hallmark of myxozoans and noted during cnidarian development) and asexual reproduction via fission and budding (in vegetative stages of malacosporeans). The great plasticity of cnidarian life cycles, however, obscures equating stages of the complex life cycle of myxozoans with the benthic polyp and planktonic medusa stages of free-living cnidarians. It is of great interest to evaluate divergent interpretations about processes of gamete production and fusion in myxosporeans as these suggest scenarios of exclusive selfing, the possibility of outcrossing and the development of multicellular chimaeras in the myxozoan clade characterised by extensive radiation. Nematocysts have clearly been co-opted for parasitism, enabling attachment of infectious spores to hosts. Our chapter provides evidence for a homology of the apical plug-like structures that seal both myxozoan polar capsules and medusozoan nematocysts, providing further support for affinities of the Myxozoa with the Medusozoa.

3.8 Key Questions for Future Studies

- When does fusion of haploid stages occur in myxozoan life cycles?
- Are binucleate cells the first myxosporean stages in annelid hosts?
- Are myxozoans characterised by inbreeding?
- Can gene expression/functional studies inform on how cnidarian medusa and polyp stages equate to stages within myxozoan life cycles?
- What is the mechanism that causes polar capsules to discharge and does the apical structure of polar capsules have a hinge?
- Do polar capsules demonstrate a greater array of morphologies than currently appreciated?

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Adaptive Radiation and Evolution Within the Myxozoa

4

Ivan Fiala, Pavla Bartošová-Sojková, Beth Okamura, and Hanna Hartikainen

Abstract

Myxozoans are endoparasites characterized by extensive morphological simplification and complex life cycles. Their definitive hosts are bryozoans—in the case of the more primitive and species-poor Malacosporea, or annelids—in the case of the more derived and speciose Myxosporea. This chapter reviews patterns of adaptive radiation within the Myxozoa and explores the drivers that may have promoted evolutionary change. Topics covered include: multiple transitions between worm-like and sac-like forms in the Malacosporea; undersampling that likely limits our appreciation of malacosporean diversity; and multiple shifts between marine and freshwater environments in the Myxosporea. We also examine morphological simplification that is observed in stages that produce spores and associated changes in the size of these sporogonic stages. This contrasts with the evolution of morphological complexity in spores. Features proposed to be involved in diversification include the acquisition of hardened spores and plasmodia, a high diversity of definitive hosts and invasion of novel hosts and host environments in the

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Myxosporea. The evolution towards higher complexity in spores can, in some cases, be linked with increasing transmission success. Convergence in spore form suggests that certain morphologies are highly adaptive. The significance of many features of spores, however, remains poorly known.

Keywords
Malacosporean diversification · Myxosporean diversification · Evolutionary transitions · Taxon sampling · Morphological simplification · Spore complexity

4.1 Introduction

The evolutionary trajectories of endoparasites are greatly influenced by interactions with their hosts. Thus, endoparasite diversification can be expected to reflect processes such as co-speciation and host switching along with the evolution of host specificity. In addition, parasites must achieve transmission to new hosts, a process that typically requires persistence outside their hosts. Finally, parasites with complex life cycles, such as myxozoans, require the ability to exploit distinctly different hosts. Drivers of diversification and evolution within the Myxozoa will therefore include biotic factors associated with host exploitation and abiotic factors associated with the environment of their free-living spores.

This chapter expands on these themes by focusing on patterns of evolution within the Myxozoa. We begin by comparing and contrasting diversification of the more primitive malacosporeans and more derived myxosporeans. We then explore why the malacosporeans have remained relatively species poor while the myxosporeans have undergone extensive diversification. Finally, we consider more specific adaptations displayed particularly by the myxosporeans that have enabled this group to exploit a variety of hosts and tissues and to survive in the environment when switching hosts. We refer readers to Chap. 2 for discussion of the evolution of parasitism, including discussion of the first myxozoan hosts and how myxozoan life cycles may have expanded to incorporate intermediate hosts. Chapter 5 reviews myxozoan taxonomy and systematics and discusses phylogenetic relationships within the Myxozoa.

4.2 The Malacosporean and Myxosporean Radiations

Although myxozoans have only recently been understood to be cnidarians (see Chap. 2), myxosporeans infecting fish have been recognized since the first half of the 19th century (Jurine 1825) and have had a relatively long period of study (Lom and Dyková 2006). Actinosporean stages of the Myxosporea had been described by the end of 19th century (Štolc 1899) and were regarded as a distinct group of endoparasites of annelids until it became clear that they share a common life cycle with fish-infecting counterparts (Wolf and Markiw 1984). Today there are some 2,200 myxosporean species (Lom and Dyková 2006). In contrast, the Malacosporea was described at the beginning of the 21st century as an early-diverging clade of myxozoans based on the distinctive features of sac-forming parasites of freshwater bryozoans (Canning et al. 2000). The enigmatic vermiform endoparasite of freshwater bryozoans, *Buddenbrockia plumatellae*, described in 1910 (Schröder 1910), was finally affiliated with the Malacosporea in 2002 (Monteiro et al. 2002; Okamura et al. 2002). As we show in this chapter there is currently evidence for some 16 malacosporean species, three of which have so far been described. The traditional taxonomy of myxozoans is based largely on spore morphologies and morphometrics but it is increasingly clear that molecular data are also

required for species discrimination due to convergence of spore morphotypes (see Sect. 4.4 and Chaps. 5 and 6). Below we review how molecular data combined with other data such as malacosporean body plans, patterns of host utilization and myxosporean infection sites are expanding our general understanding of myxozoan diversity.

4.2.1 Malacosporean Diversification

Unlike in myxosporeans, malacosporean diversity has been revealed by the discovery of stages in invertebrate hosts. These stages occur as sacs in *Tetracapsuloides bryosalmonae* (Canning et al. 2000) and *Buddenbrockia allmani* (Canning et al. 2007), and as worm-like stages (myxoworms; Canning et al. 2008) in *Buddenbrockia plumatellae* (Okamura et al. 2002). A striking result is that in some cases the sac-forming and vermiform malacosporeans are characterized by extremely low molecular sequence divergence (Monteiro et al. 2002). Indeed, this led Canning et al. (2002) to synonymise the sac-forming parasite of the gelatinous bryozoan, *Cristatella mucedo* (originally described as *Tetracapsula bryozoides*; Canning et al. 1996) with *Buddenbrockia plumatellae*, a myxoworm infecting tubular, branching species of *Plumatella* and *Hyalinella*. The two forms were proposed to represent alternate morphologies that developed in different bryozoan hosts (Canning et al. 2000). However, subsequent molecular phylogenetic studies (Tops et al. 2005; Jiménez-Guri et al. 2007; Bartošová-Sojková et al. 2014; Hartikainen et al. 2014) consistently separate these forms suggesting that the malacosporean infecting *Cristatella mucedo* is indeed a distinct species as originally described (Canning et al. 1996).

Recent molecular and morphological analyses show that the malacosporean clade includes parasites that develop as myxoworms (*Buddenbrockia plumatellae* and four undescribed species; Tops et al. 2005; Hartikainen et al. 2014), spherical sacs (*Tetracapsuloides bryosalmonae, Buddenbrockia allmani*, two undescribed *Tetracapsuloides* and *Buddenbrockia* species), oblong sacs (‘*Buddenbrockia plumatellae*’ in *C. mucedo*; see above), and elongate sacs with lobes (Hartikainen et al. 2014). Notably, sacs and myxoworms occur across the phylogeny and morphological transitions between sac- and worm-like forms have occurred repeatedly (Hartikainen et al. 2014). Further studies have discovered novel malacosporean diversity by detecting infections in fish kidney (Bartošová-Sojková et al. 2014). However, whether these novel isolates from fish kidneys identify true fish hosts requires confirmation of spore development and, ideally, demonstration of transmission back to bryozoans. Here, we combine the datasets from these two studies to present the most comprehensive analysis of malacosporean SSU rDNA data to date (Fig. 4.1). In addition to the three malacosporean species recognized before 2013, this analysis reveals 13 new malacosporean lineages representing new species or even genera (see Chap. 5). The analysis also shows, as previously demonstrated (Hartikainen et al. 2014), that sacs and myxoworms occur across the phylogeny.

Despite problems with taxon undersampling, the molecular phylogeny reveals several potentially notable patterns. These include the apparent association of species in the *Buddenbrockia* clade with single bryozoan hosts compared with the diversity of bryozoan hosts utilized by the species of the *Tetracapsuloides* clade (Fig. 4.1). This suggests that these clades may be characterized by specific versus generalist bryozoan host exploitation strategies. Similarly, some malacosporean infections are associated with a broad fish host range. Thus, some are detected in kidney tissues of both cypriniform and perciform fish (i.e. Malacosporea sp. A and the vermiform *Buddenbrockia plumatellae*), while others have been detected only in kidneys of fish belonging to a single family (Fig. 4.1). The degree of host specificity that malacosporeans exhibit at either invertebrate or fish host level requires further investigation and understanding of malacosporean life cycles. The current molecular phylogeny also demonstrates the utilization of fredericellids as bryozoan hosts in the two earliest diverging lineages (Malacosporea sp. E and F) as well as in the early diverging lineage of the *Buddenbrockia* clade

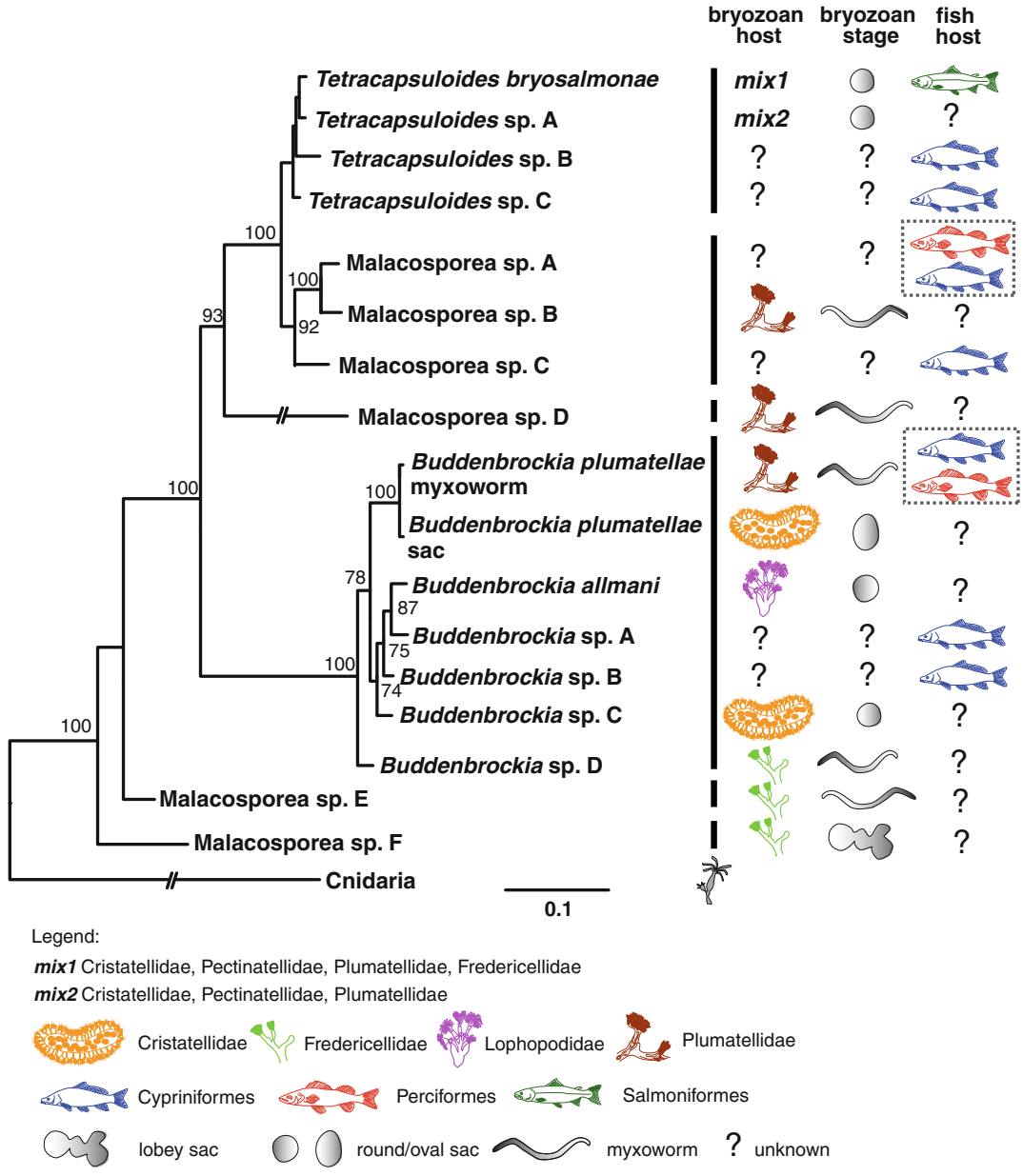

Fig. 4.1 The phylogeny of the Malacosporea based on the maximum likelihood analysis of SSU rDNA data. Bootstrap supports with >50 % shown at nodes. Cnidaria and Malacosporea sp. D branches shortened to 50 % of original length. *Tetracapsuloides* sp. A = *Tetracapsuloides* sp. 1 in Bartošová-Sojková et al. (2014) (BS) and *Tetracapsuloides* spp. in Hartikainen et al. (2014) (H); *Tetracapsuloides* sp. B = *Tetracapsuloides* sp. 4 in BS; *Tetracapsuloides* sp. C = *Tetracapsuloides* sp. 5 in BS; Malacosporea sp. A = *Tetracapsuloides* sp. 3 in BS; Malacosporea sp. B = novel lineage 3 in H; Malacosporea sp. C = *Tetracapsuloides* sp. 2 in BS; Malacosporea sp. D = novel malacosporean lineage in BS and *Buddenbrockia* sp. 4 in H; *Buddenbrockia* sp. A = *Buddenbrockia* sp. 3 in BS; *Buddenbrockia* sp. B = *Buddenbrockia* sp. 2 in BS and *Buddenbrockia* sp. 3 in H; *Buddenbrockia* sp. C = *Buddenbrockia* sp. 1 in H; *Buddenbrockia* sp. D = *Buddenbrockia* sp. 1 in BS and *Buddenbrockia* sp. 2 in H; Malacosporea sp. E = novel lineage 2 in H; Malacosporea sp. F = novel lineage 1 in H

(*Buddenbrockia* sp. D). This suggests that derived freshwater bryozoans (Okuyama et al. 2006) may have served as the first invertebrate hosts of malacosporeans which co-diversified with their hosts. An undescribed lobey sac-like species (Malacosporea sp. F) with an unknown fish host is predicted at the base of the phylogeny (Fig. 4.1). Further sampling is clearly required to substantiate or refute these patterns and predictions.

In view of poor taxon sampling, we can certainly expect malacosporean diversity to be greater than the 16 species revealed so far. The southern hemisphere is particularly undersampled with only one myxoworm found in freshwater bryozoans in Borneo (Hartikainen et al. 2014; Fig. 4.1). Vast regions of the northern hemisphere also remain unsampled. Indeed, early reports of vermiform parasites in freshwater bryozoans, identified at the time as *Buddenbrockia plumatellae,* in Brazil (Marcus 1941), Japan (Oda 1980) and Turkestan (present day Kyrgyzstan; Braem 1911) provide evidence for a global distribution of malacosporeans, many of which are likely to represent new species. The detection of novel malacosporean species with our limited sampling largely within Europe (Bartošová-Sojková et al. 2014; Hartikainen et al. 2014) suggests that greater diversity may even be expected in relatively well-sampled regions. Furthermore, as discussed in Chap. 2 we cannot discount the possibility that there may be undetected malacosporeans in marine habitats in suspension or deposit-feeding marine hosts, such as phoronids or brachiopods, whose body cavities could support the development of the relatively large spore-forming sacs and myxoworms. Hastings (1943) commented on the vermiform bodies found in some marine bryozoan zooids, and although no illustrations are provided, the descriptions do not match closely with myxoworms as seen in freshwater bryozoans. Chapter 2 reviews further studies suggesting the presence of worm-like endoparasites within marine bryozoans, none of which appear to be myxozoans. Marine bryozoans, seem unlikely hosts in view of their compartmentalized and miniaturized zooids that offer little space for parasites to develop.

There are several explanations for the lack of detection of malacosporeans. First, infections in freshwater bryozoans remain covert for much of the year with parasites occurring as cryptic, single cell stages within host tissues that cannot be detected unless polymerase chain reaction (PCR) or histology is conducted (see Chap. 11). Second, infections may be asymptomatic, with infected fish exhibiting no external signs of disease or compromised health. Finally, presporogonic and sporogonic stages in fish (single cells in blood and kidney interstitium and very small pseudoplasmodia in kidney tubules, respectively) may be easily overlooked or not recognised.

4.2.2 Myxosporean Diversification

Reconstruction of myxosporean evolution reveals three well supported lineages: a marine lineage that utilises polychaetes as final hosts, a freshwater lineage that utilises oligochaetes as invertebrate hosts and a lineage comprised of sphaerosporids whose invertebrate hosts remain unknown (Fig. 4.2; Fiala and Bartošová 2010; Bartošová et al. 2013). The position of the sphaerosporid lineage is unclear: it may be sister to all myxosporeans, to the freshwater lineage or to the marine lineage (Holzer et al. 2007; Jirků et al. 2007; Bartošová et al. 2009, 2013; Karlsbakk and Køie 2009). The marine and freshwater myxosporean lineages contain a relatively large number of species and a variety of myxospore morphotypes (see Sect. 4.4.1). In contrast, uniform myxospore morphology of an inferred ancestral sphaerosporid morphotype (Fiala and Bartošová 2010) characterises the sphaerosporid lineage. The number of species in the sphaerosporid lineage is expected to be relatively high due to the high number of nominal *Sphaerospora* species that lack molecular data (Bartošová et al. 2013).

Molecular phylogenetic analyses suggest some correspondence between the main myxosporean lineages with fish host environment and also demonstrate that myxosporeans have shifted between hosts occupying freshwater and marine environments on multiple occasions (e.g.

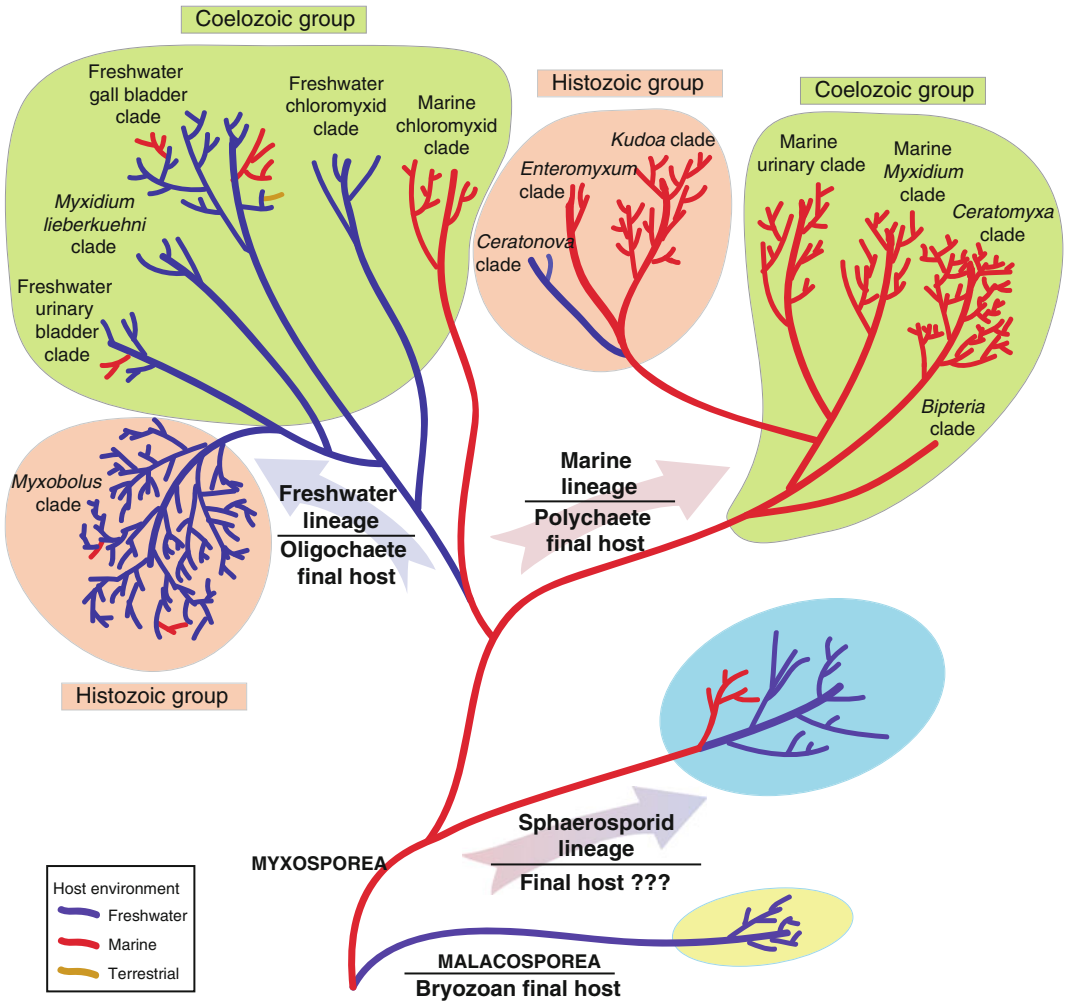

Fig. 4.2 Summary of hypothetical evolution of the Myxozoa inferred from molecular data based on studies of Fiala and Bartošová (2010), Fiala et al. (submitted), Kodádková et al. (submitted)

Kent et al. 2001; Fiala and Bartošová 2010). Figure 4.2 provides an up-to-date summary of hypothetical evolutionary trends of myxosporeans as revealed by mapping host and environmental characters onto molecular phylogenetic data (phylogenies based on those in: Fiala 2006; Fiala and Bartošová 2010; Jirků et al. 2011; Gleeson and Adlard 2012; Bartošová et al. 2013; Fiala et al. 2014, submitted; Kodádková et al. 2015). The early-diverging Malacosporea radiated in freshwaters while early-diverging myxosporeans (the sphaerosporid lineage and the marine chloromyxid and *Bipteria* clades) inhabited the marine environment. Myxosporeans then radiated into many species that comprise the large marine lineage which utilises polychaete worm hosts. One clade of the marine lineage, which currently contains two *Ceratonova* spp., has invaded freshwaters, perhaps via stickleback hosts (Fiala et al., submitted). Myxosporeans in the freshwater lineage use oligochaetes as invertebrate hosts, and we can infer that the ancestor of this lineage invaded freshwaters after the split from the marine chloromyxid clade. Reinvasion of the marine environment has happened several times independently by taxa in this lineage.

Examples include the large clade of typical marine *Sphaeromyxa* species (Kristmundsson and Freeman 2013), two marine *Myxidium* species (Fiala 2006; Kalavati et al. 2013), several *Myxobolus* and *Henneguya* spp. from marine or brackish fish (e.g. Li et al. 2012; Carriero et al. 2013), the marine zschokkellids (Heiniger and Adlard 2014), and the marine *Ortholinea* spp. (Karlsbakk and Køie 2011). Moreover, freshwater myxosporeans have invaded the terrestrial environment with *Soricimyxum fegati* infecting shrews (Prunescu et al. 2007; Dyková et al. 2007, 2011).

Whilst the major myxosporean lineages follow the freshwater-marine and invertebrate host separation (Kent et al. 2001; Holzer et al. 2007), divergences within these lineages appear to be related to tissue tropism in the intermediate vertebrate host (Eszterbauer 2004; Holzer et al. 2004, see also Chap. 16). For instance, clades within both the freshwater and marine lineages contain species that are exclusively coelozoic (infecting the gall bladder or the urinary bladder and kidney tubules) or histozoic (infecting muscles or other tissues) (Fig. 4.2). Unlike myxospore morphology, the site of infection can be linked with myxosporean phylogenies (see later discussion and Chap. 5). For example, species classified as belonging to the genus *Zschokkella* clearly cluster in molecular phylogenies according to their site of infection (gall or urinary bladder) irrespective of their classification according to myxospore morphology. There are no strong phylogenetic affinities within particular myxosporean clades, however some myxosporean radiations have occurred within host families and genera e.g. in the *Ceratomyxa* and *Myxobolus* clade (Gunter et al. 2009; Carriero et al. 2013).

4.2.3 Drivers of Radiations

Although myxozoan diversity is, in general, underestimated, myxosporeans are clearly more speciose than malacosporeans. There are several key factors that may have enabled myxosporeans to establish themselves in new environments and to subsequently radiate. These include: (1) the acquisition of hardened, environmentally-resistant spore valves (soft valves characterise malacosporean spores; Canning and Okamura 2004); (2) reduced rates of uptake and excretion across epithelia present in malacosporean stages in invertebrate hosts; (3) the acquisition of plasmodia that may be better suited to sporulation in organs and tissues of diverse fish hosts and, in some cases, retaining spores until host death (malacosporeans are limited to sporulation in renal tubules of certain hosts and release spores from living hosts via urine); (4) a high diversity of primary hosts which promotes diversification i.e. 3,500 species of oligochaetes and 8,000 species of polychaetes (Ruppert et al. 2004) versus 94 species of freshwater bryozoans (Massard and Geimer 2008); (5) incorporation of additional vertebrate host groups by myxosporeans (to date malacosporeans are only known to infect fish).

4.2.4 Incorporation of Novel Hosts

The common ancestor of the freshwater and marine myxosporeans may have exploited cartilaginous fish as the first vertebrate hosts. This inference is supported by utilisation of a chimaera (*Chimaera monstrosa*) by the early diverging *Bipteria vetusta* (Kodádková et al. 2015) and the utilisation of elasmobranchs by the early diverging marine *Ceratomyxa* clade (which is sister to other members of the marine myxosporean lineage) and the marine chloromyxids (which form a sister group to the freshwater clade) (Fig. 4.2).

There are several cases where myxosporeans adopted vertebrate hosts other than fish and elasmobranchs. Eiras (2005) reported 15 myxosporean species belonging to 6 genera that infect amphibians. Since that time at least six other amphibian-infecting species have been described and the genus *Cystodiscus*, whose members also infect amphibians, has been resurrected (Hartigan et al. 2011). Switching to amphibian hosts has occurred at least three times independently (Kodádková et al. 2015): once in the sphaerosporid lineage (Jirků et al. 2007; Bartošová et al. 2013) and two times in the freshwater myxosporean lineage—once in the *Cystodiscus* clade (Hartigan et al. 2011) and once

in a single species, *Chloromyxum careni* (Jirků et al. 2011). Although only a small number of myxosporean species that infect Amphibia has been described so far, myxosporeans appear to exploit a broad range of amphibian species. For example, *Myxidium serotoninum* is recorded from 37 amphibian species (Eiras 2005). At present it is unclear whether amphibian-infecting myxosporeans are truly generalist parasites or whether they may represent cryptic species assemblages. Also, no complete life cycles are known and potential invertebrate hosts and transmission pathways remain a mystery. Nevertheless, since relatively little research has been conducted on myxozoans parasitic in amphibians, their diversity is likely to be underestimated. This may change as conservation biologists attempt to understand drivers of global declines in amphibian populations (Hartigan et al. 2013).

There are four described myxosporean species from aquatic reptiles (Eiras 2005). Like the amphibian-infecting myxosporeans, at least some of these may be generalists or they may represent cryptic species assemblages. Thus, Johnson (1969) found *Myxidium chelonarum* in 14 of the 21 North American turtle species. Only a single myxosporean species has been recorded so far from birds and is described in ducks (*Myxidium anatidum*; Bartholomew et al. 2008). Similarly, a single species has been encountered in mammals and infects three species of shrews (Prunescu et al. 2007; Dyková et al. 2007, 2011). Myxozoan-like developmental stages, causing xenomas, have been detected in the brain of the mole *Talpa europaea* (Friedrich et al. 2000). However, no spores that would enable parasite identification, were observed. Despite the fact that Myxozoa are not human pathogens the consumption of raw fish meat with myxozoan infection is associated with diarrhoea and *Kudoa septempunctata* was identified as the etiological agent (Kawai et al. 2012). The pathogenicity of *K. septempunctata* was demonstrated in an in vitro experiment on human intestinal cells, which were rapidly invaded by sporoplasms (Ohnishi et al. 2013). Notably, all of these myxosporeans recorded in reptile, bird and mammal hosts appear to have originated independently within the gall bladder clade of the freshwater lineage. Since many myxozoan infections are innocuous there is a reasonable possibility that these myxosporeans are diverse and widespread endoparasites of a variety of vertebrate hosts and are therefore extremely undersampled. In all cases the invertebrate hosts remain unknown (see Chap. 7 for further discussion of myxozoans infecting homeotherms).

Finally, we note that there are several reports of myxozoans in invertebrate hosts other than worms and freshwater bryozoans. For instance, a species of *Kudoa* has been discovered in muscles of giant octopus and produces spores in these molluscan hosts (Yokoyama and Masuda 2001) and a species of *Myxidium* has been described which is capable of infecting and producing spores in monogenean parasites of fish gills (Freeman and Shinn 2011). Observations of myxozoan infections in other gill monogeneans (reviewed in Freeman and Shinn 2011) suggest that hyperparasitism of fish parasites may be an overlooked strategy of myxozoans. However, the dynamics of such infections require further investigation, for instance to determine if monogeneans acquire myxozoan infections through infected fish or vice versa. Early reports of parasites inferred to be myxozoans include *Chloromyxum diploxys* in the lepidopteran *Tortrix viridana* (Thélohan 1895), but inferences based on early studies that lack molecular or ultrastructural confirmation should be viewed with some caution. As argued above for vertebrate hosts, the possibility that myxozoans exploit a diversity of invertebrate hosts remains unclear and merits further investigation. For instance, exploitation of shrews as hosts suggests the possibility that myxosporeans may have radiated to exploit terrestrial oligochaetes. Infections may then be transmitted when vertebrates consume earthworms (see Chap. 7).

4.3 Morphological Simplification and Changes in Body Size

As discussed in Chap. 2, myxozoans demonstrate the most extreme example of morphological simplification relative to their ancestors in any

group of parasites—a trait commonly but not universally associated with parasitism (Poulin 2007). The great reduction in body size that characterises myxozoans is likely to be adaptive for living within restricted host environments, much as occurs in meiofaunal organisms that live interstitially between sand grains (e.g. as in meiofaunal sea anemones; Giere 2009). However, once the plasmodial level of organisation was obtained, 'body size' in some cases has also subsequently increased. Below we examine more specifically the patterns of morphological simplification and variation in body size in the two myxozoan clades.

4.3.1 Malacosporeans: From Worms to Sacs

The *Buddenbrockia* myxoworm displays tetraradial symmetry, characterized by four blocks of longitudinal muscles that are enclosed by external and internal epithelial layers during pre-sporogonic stages of development (Canning et al. 2002; Okamura et al. 2002). The chiral pattern of muscle fibre orientation in *Buddenbrockia* and the connecting cells that are anchored to the extracellular matrix and link muscle blocks are novel myxozoan features that result in helical swimming (Gruhl and Okamura 2012). The sac-forming malacosporeans demonstrate morphological simplification, as they lack muscles and connecting cells as well as the internal epithelial layer that develops in the pre-sporogonic stages of myxoworms. Mature *Buddenbrockia* myxoworms are larger (up to 3.7 mm in length and 100 μm in width; Canning and Okamura 2004) than mature sacs whose longest dimensions are 350, 300 and 700 μm in *Tetracapsuloides bryosalmonae* (Canning et al. 2000), *Buddenbrockia allmani* (Canning et al. 2007) and '*Tetracapsula bryozoides*' (Canning et al. 1996), respectively.

Molecular phylogenetic analyses indicate a striking pattern of repeated transitions between vermiform and sac-like taxa within the Malacosporea (Hartikainen et al. 2014). At present this has apparently occurred at least in: the lineage leading to the *Tetracapsuloides* clade; the *Buddenbrockia plumatellae* clade, and the lineage leading to the clade containing *Buddenbrockia allmani* and three novel *Buddenbrockia* species (species A, B, C) (Fig. 4.1). The low levels of genetic divergence between myxoworms and sacs (Tops et al. 2005; Hartikainen et al. 2014; Fig. 4.1) suggests that the evolution of morphologically simplified sacs may be achieved readily, possibly by modifications of regulatory gene networks, the drivers of which are unclear, but may be associated with e.g. host switching.

4.3.2 Myxosporeans: From Coelozoic to Histozoic Forms

Reductions in body size and complexity reach an extreme level in the Myxosporea, which have entirely lost tissues (but see Chap. 9 regarding apparent tissue loss) and consist of tiny stages comprised of only a few cells that then develop into the sporogonic plasmodial and pseudoplasmodial (both spore-producing) stages. Myxosporeans of the sphaerosporid lineage and myxosporeans of the marine and freshwater lineages associated with early splits in molecular phylogenies are coelozoic and occur in the cavities of organs in fish hosts (Fig. 4.2; Fiala and Bartošová 2010; Bartošová et al. 2013). Coelozoic plasmodia of the early-diverging sphaerosporids are usually very small (10–20 μm) and are mono- or disporic (producing one or two spores), exceptionally tetrasporic (Jirků et al. 2007). Plasmodia of intermediate size (e.g. tens to hundreds of micrometres) may be mono-, di- or polysporic and are produced by coelozoic myxosporeans of both marine and freshwater lineages (e.g. *Ceratomyxa*, *Chloromyxum,* and *Parvicapsula*). Some coelozoic plasmodia can be large (up to several millimetres) (e.g. *Sphaeromyxa*; Kristmundsson and Freeman 2013, *Myxidium* from amphibians; Jirků et al. 2006). Species that infect tissues as histozoic forms evolved from coelozoic species independently in both freshwater and marine lineages. Plasmodia of histozoic myxosporeans often grow to enormous size (up to several millimetres). These large plasmodia can be encased within a fibroblast envelope and are visible as large

cysts in infected tissues (e.g. *Myxobolus*, *Henneguya* and *Kudoa*).

4.4 Diversification of Spores

In this section we review how spores have diversified to display a variety of morphologies, the considerable plasticity of these morphologies, and how spores may be adapted to their environments. Apart from the presence of one versus two sporoplasms and two versus four polar capsules in spores that develop in fish (Hedrick et al. 2004; Morris and Adams 2008) and bryozoan (Canning et al. 2000) hosts, respectively, the soft-bodied spores produced by malacosporeans so far investigated are morphologically indistinguishable. In addition, only a few malacosporean species have been described. Our discussion therefore focuses on spores produced by myxosporeans.

4.4.1 Myxosporean Spore Morphotypes, Drivers of Diversification and Plasticity in Form

Before it was demonstrated that myxozoans are characterised by a complex two-host life cycle (Wolf and Markiw 1984), actinospores and myxospores were regarded as belonging to independent groups of parasites (Actinosporea and Myxosporea). This classification was based on the exploitation of invertebrate and vertebrate hosts and by the morphologically distinct actinospores and myxospores that are produced in these invertebrate and vertebrate hosts, respectively. In the typically triradiate actinospores, valve cells inflate osmotically upon release into the environment producing caudal processes that diverge in different directions. These processes likely reduce sinking rates. Actinospores possess three polar capsules and numerous sporoplasms in a region anterior to the caudal processes. In myxospores the valve cells are hardened and joined by a conspicuous suture. One to four polar capsules and one or two sporoplasms are generally produced in myxospores (Lom and Dyková 2006). The taxonomy of both 'groups' was largely based on variation in spore morphology. As a result, genera or collective groups (morphotypes) of myxosporeans and actinosporeans were recognised (Lom and Dyková 2006). Despite the fact that only a small fraction of myxosporean life cycles (see Chap. 10) has been resolved, it is now clear that several myxospore morphotypes share the same actinospore morphotype (e.g. in *Ceratomyxa auerbachi*, *Ceratonova shasta* (syn. *Ceratomyxa shasta*), *Gadimyxa atlantica*, *Parvicapsula minibicornis,* and *Ellipsomyxa gobii*; Fig. 4.3). This suggests that myxospores may have undergone greater morphological differentiation than actinospores, although further sampling of actinospores is required to confirm this speculation.

The production of morphologically distinct actinospores and myxospores within the same life cycle demonstrates considerable plasticity in spore design and may be related to maximising transmission from fish to invertebrate hosts (myxospores) and from invertebrate to vertebrate hosts (actinospores). Furthermore, it may be inappropriate to equate actinospores and myxospores as homologous stages that are reiterated within a life cycle. For instance, since myxozoans are cnidarians, the two spore types could reflect highly modified medusa and polyp stages and the sporogonic stages that produce them may represent specialised propagative forms such as frustrules (see Chap. 3). Unravelling the molecular basis for the striking morphological variation displayed by myxospores and actinospores is of great interest and could be achieved by transcriptomic studies to identify variation in gene expression repertoires.

Myxospore morphotypes are distinguished by e.g. the number and shape of spore valves, the shape, position and number of polar capsules, the relative position of the suture line and polar capsules, the presence of surface ridges and appendages, and the number of polar filament coils (Feist and Longshaw 2006; Lom and Dyková 2006). Figure 4.4 shows the main myxospore morphotypes that are associated with the majority of myxosporean diversity (i.e. those produced in species of *Myxobolus*, *Henneguya*, *Ceratomyxa*, *Myxidium*, *Zschokkella*, *Chloromyxum*, *Sphaerospora*,

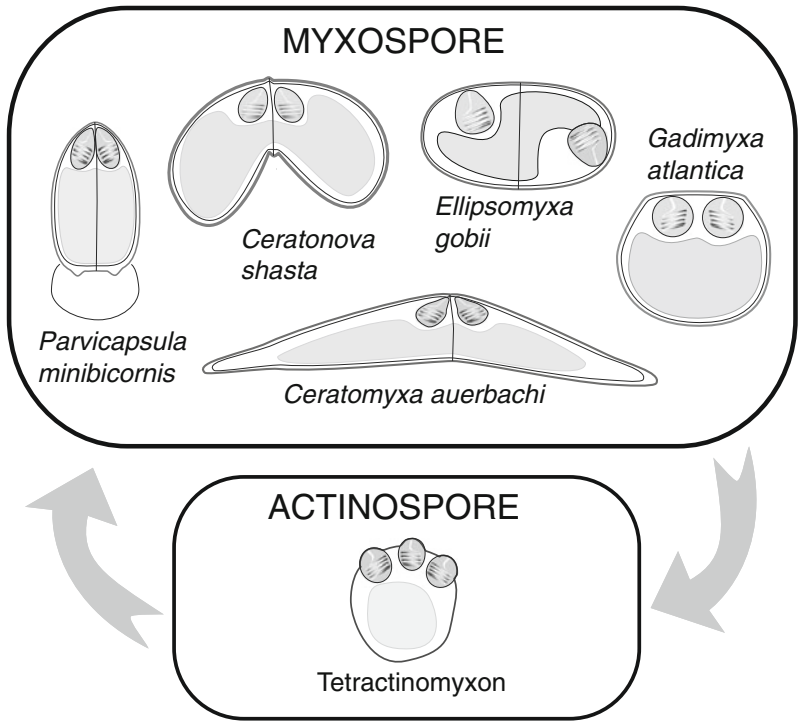

Fig. 4.3 Example of myxosporean species characterised by different myxospore morphotypes but the same actinospore morphotype

Kudoa, *Thelohanellus* and *Sphaeromyxa*) and thus can be considered as most evolutionarily successful. The remaining morphotypes are associated with some 255 species in about 50 genera (Lom and Dyková 2006). These rare morphotypes represent only 10 % of all described myxosporeans but they illustrate the broad range of myxospore morphologies that have evolved (detailed description of all myxozoan genera/morphotypes is provided in the taxonomic key of Chap. 5).

The most common myxospore morphotype is that of *Myxobolus,* a genus which has diversified to more than 800 species histozoic in fish (Liu et al. 2013). Many myxospore morphotypes associated with other tissue-dwelling genera appear to be modifications of this relatively simple morphotype, varying in only minor ways (e.g. loss of one polar capsule, development of spore caudal appendages) (i.e. *Henneguya*, *Hennegoides*, *Unicauda*, *Dicauda*, *Tetraauronema*, *Thelohanellus*, *Neothelohanellus* and *Phlogospora*). The *Myxobolus* morphotype and its variations are thus associated with over 1,100 species—some 50 % of myxosporean species described to date (Lom and Dyková 2006; Liu et al. 2013). The success of the *Myxobolus* morphotype may relate to the lateral flattening of spores that enabled invasion of tissues from precursors that lived in organ cavities and then subsequently radiated to exploit a range of niches offered by different tissues. According to Shulman (1964), tissue-dwelling myxozoans experience mechanical pressures that favour flattened spores (as e.g. in *Myxobolus*) or spores of decreased size and which incorporate strengthening features (e.g. multiple valve cells forming an arch in e.g. *Kudoa*). Such designs were proposed to avoid premature opening of shell valves. However, mechanical pressures in tissues versus organ cavities may not be sufficiently different to drive such variation in form. This is because tissues are comprised of cells and water contributes 70 % to total cell weight. Although water will contribute to a greater percentage of the fluid in organ cavities the pressures experienced in tissues versus organ cavities

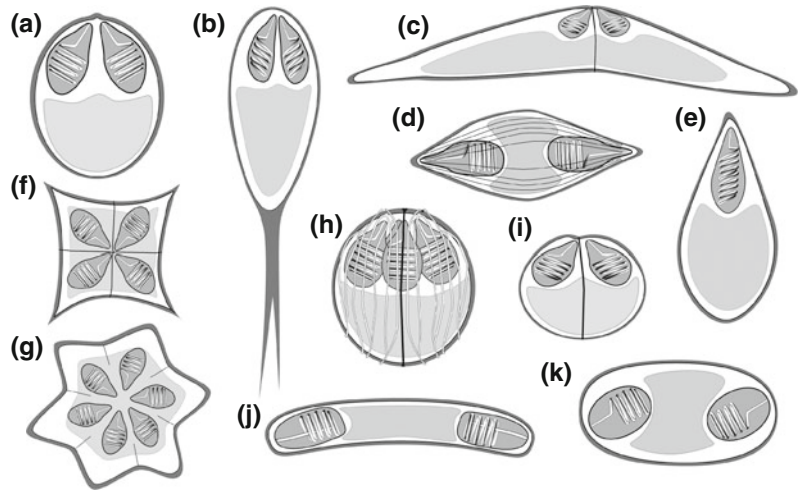

Fig. 4.4 Representatives of genera of major myxospore morphotypes. **a** *Myxobolus*, **b** *Henneguya*, **c** *Ceratomyxa*, **d** *Myxidium*, **e** *Thelohanellus*, **f** *Kudoa* (four valves), **g** *Kudoa* (six valves), **h** *Chloromyxum*, **i** *Sphaerospora*, **j** *Sphaeromyxa*, **k** *Zschokkella*

must be quite similar. The significance of morphologies of spores in tissue-dwelling species may relate more to the maintenance of spore integrity during release from decaying histozoic environments (when fish hosts die) and subsequent spore survival in sediments prior to ingestion by worms.

A typical example of convergence in myxospore morphotypes is exhibited by the myxospores of *Myxidium* and *Zschokkella*, which are both characterised by polar capsules situated at opposite ends of an elongate myxospore (Fig. 4.4d, k). Species of these genera, which parasitise the gall and urinary bladder of marine and freshwater fish, have evolved similar myxospore morphotypes in freshwater and marine lineages several times independently. Perhaps there is some aspect of their convergent myxospore morphologies that suits development in cavity organs although what this is remains obscure. The *Chloromyxum* morphotype represents another case of remarkable convergent evolution with evolutionary reconstruction suggesting multiple origins of this morphotype (Fiala and Bartošová 2010). The success of the *Chloromyxum* morphotype may derive from the development of a large number of polar capsules that may facilitate attachment of spores to hosts and thus enhance transmission.

Many convergent events have been suggested for the *Sphaerospora* myxospore morphotype since distant positions of *Sphaerospora* spp. in molecular phylogenies were indicative of extensive polyphyly (Fiala and Bartošová 2010). However, it has subsequently been determined that PCR amplification of SSU rDNA of a group of sphaerosporids with long inserts (Jirků et al. 2007; Holzer et al. 2007; Bartošová et al. 2013) is problematic. This has led to erroneous results for PCR of samples with mixed myxozoan infections, which, in turn, led to misinterpretations of sphaerosporid evolutionary history. Corrected and additional molecular data have revealed *Sphaerospora* as separate myxosporean evolutionary lineage (Bartošová et al. 2013; Eszterbauer et al. 2013; Holzer et al. 2013). However, there still appear to be a few cases of convergent evolution of myxosporeans with *Sphaerospora* myxospore morphotypes. These include similar spores of *S. testicularis* (which clusters in the marine urinary clade; Bartošová et al. 2011) and of *S. dicentrarchi* (which clusters within multivalvulids; Kent and Palenzuela 2001). A convergent origin of the latter would have entailed loss of the multivalvulid character in some kudoid ancestor giving rise to the sphaerosporid morphotype of extant *S. dicentrarchi*.

Above we have explored how myxosporeans exhibit plasticity in spore morphologies in terms of producing highly distinct spore types at different stages in the life cycle (actinospores and myxospores) and in terms of convergence of spore morphologies to similar myxospore morphotypes. Thus, there appears to be considerable flexibility in the development of spores resulting in morphological variation, which is likely to have some functional significance (see Sect. 4.4.2). Furthermore, it is apparent that morphological change may evolve within very short evolutionary timescales since closely related species can demonstrate substantial variation in spore morphologies. For example, each of the closely related species in the freshwater urinary bladder clade (*Acauda*, *Chloromyxum*, *Hoferellus*, *Myxidium*, *Myxobilatus*, *Ortholinea* and *Zschokkella*) represents highly distinct spore morphotype (Fiala 2006; Karlsbakk and Køie 2011; Whipps 2011) and similar levels of variation amongst myxospore morphologies characterise species in the marine urinary clade (Bartošová et al. 2011; Kodádková et al. 2014). This is in contrast to rather morphologically uniform myxospores produced in the *Ceratomyxa*, *Kudoa* and *Sphaerospora* clades. Further research is required to examine why morphological variation in myxospores may occur in some myxosporeans but not in others.

4.4.2 Morphological Adaptations of Myxosporean Spores as Free-Living Stages

As parasites with complex life cycles, myxozoans have not only evolved to exploit two hosts but during transmission they must be adapted to abiotic factors that spores experience when exposed to the external environment. For instance, hardening of myxospore valves is associated with dormancy. Thus, frog sphaerosporids produce robust myxospores that may have evolved for protection during the period of frog hibernation (Jirků et al. 2007) and the myxospores of *M. cerebralis* may remain viable for many years before they are ingested by oligochaetes (Halliday 1976). As mentioned above, the caudal processes of actinospores that inflate upon release from annelid hosts almost certainly provide a large surface area that prolongs the period of time that spores remain in the water column to enhance transmission to fish. Shulman (1964) suggested that characters that influence spore sinking rates may be some of the most important adaptive features of myxospores, acting similarly to the caudal processes and anchor-like structures of actinospores. Thus, surface ridges and projections such as tails, posterior or lateral protuberances, or bumps increase the surface area of myxospores and may reduce sinking rates thereby enabling the spores to disperse longer distances. Evidence that such features have evolved several times independently (e.g. multiple origins of caudal appendages of *Henneguya* spores) supports the hypothesis that they play an important functional role. Particularly notable surface elaborations include the membranaceous veils on myxospores of deep sea ceratomyxids (e.g. *Palliatus*, *Myxodavisia*; Fiala et al., submitted), and the keel-like or wing-like extensions of five rare myxospore morphotypes produced by *Bipteria*, *Neobipteria*, *Noblea*, *Paramyxoproteus* and *Schulmania* (see review of Lom and Dyková 2006) which may serve as floats for better dispersal.

4.5 Conclusions

Myxozoans adapted to the parasitic way of life by evolving complex two-host life cycles, simplifying their morphology and introducing evolutionary novelties, such as hardened spores and using polar filaments to attach to hosts. During their evolution from cnidarian ancestors, myxozoans fundamentally transformed from highly organised multicellular organisms to very simple myxoworms and sacs, in the case of malacosporeans, and to even simpler plasmodial forms, in the case of myxosporeans. However, in parallel with this reduction in body complexity, myxosporeans have evolved different types of plasmodia and myxospore morphotypes. Myxosporeans appear to have first invaded fish body cavities and later adapted to exploit host tissues. The remarkable

variability of myxospore morphotypes, reflects adaptations to achieve transmission in different host (biotic) and abiotic environments. Further research on myxozoan phylogeny will enable more detailed reconstruction of myxozoan evolution, allowing further inference of some of the key drivers of the adaptive radiation of these extraordinary endoparasitic cnidarians.

4.6 Key Questions for Future Study

- To what degree is the diversity of malacosporeans and myxosporeans underestimated?
- How will genetic data for unsequenced morphotypes influence molecular phylogenies and our understanding of myxozoan radiations?
- How diverse are myxozoans that exploit hosts other than fish?
- Are there special physiological adaptations that enable myxozoans to develop in homeothermic hosts (e.g. birds, shrews)?
- Is the development of actinospores and myxospores controlled by a common gene expression repertoire?
- How can the extensive occurrence of convergence in spore morphologies be explained?

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Classification and Phylogenetics of Myxozoa

5

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Abstract

Myxozoans evolved as an endoparasitic radiation of cnidarians exploiting invertebrate and vertebrate (primarily fish) hosts in freshwater and marine environments. Currently, there are some 2,200 nominal species classified to 64 genera and 17 families. These groupings are mainly based on spore morphology. This chapter provides an updated spore-based taxonomic key that takes into account the recent recognition of the cnidarian origin of myxozoans as well as important revisions at generic, family and suborder levels over the last decade. A list of generic synonyms is also reviewed here. Myxozoan molecular phylogenies largely disagree with traditional spore-based classification systems, probably due to extreme plasticity in myxospore morphologies that has resulted in extensive convergence. Molecular phylogenies of myxozoans (based on all existing SSU sequences and those available for species with known actinospore-producing stages) resolve the malacosporeans, the freshwater myxosporeans, the marine myxosporeans and the sphaerosporid lineages. Within these clades species can be resolved according to definitive host type and, partially, according to host environment. Numerous exceptions are indicative of several recursions of species into freshwater or marine environments. Further resolution within clades identifies groups of species

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according to tissue tropism in their vertebrate hosts. We suggest future studies of myxozoan systematics to address persisting taxonomic and phylogenetic discrepancies and make recommendations for describing taxa in the absence of sequence data or when sequence and morphological data are incongruent.

Keywords
Taxonomy · Classification · Myxosporea · Actinosporea · Spore · Phylogeny

5.1 Introduction

Myxozoan evolution and radiation has led to a diverse range of morphologically distinct parasite species with complex life cycles, exploiting primarily aquatic vertebrate and invertebrate hosts the world over. Appropriate species descriptions and classification schemes are critical for the characterization and organization of known species. The main criterion for myxozoan classification is spore morphology; however, molecular markers reveal unexpected myxosporean relationships and show discrepancies between spore-based classifications and phylogenies based on gene sequences (see Chap. 6).

In this chapter we present a revised myxozoan classification with an updated taxonomic key. Our key accounts for the recent recognition of the cnidarian origin of myxozoans and the establishment and demise of several myxosporean genera, families and suborders. We summarise phylogenetic trends associated with the main lineages and relationships within particular clades. We explore the discrepancies between spore-based classification and molecular phylogeny and focus on phylogenetic markers and their use in analysing myxozoan phylogeny. We conclude by suggesting how both spore morphology and DNA sequence data may be used in nomenclature.

5.2 Taxonomy of the Myxozoa

Classification of the Myxozoa has a long history with many twists and turns. The first myxosporean species was reported by Jurine (1825); however, myxosporean taxonomy was founded when Bütschli (1881) established the phylum Myxosporidia within the Sporozoa. Subsequent species descriptions led to the establishment of many new myxosporean taxa, culminating in the currently recognized 64 genera (Table 5.1) in 17 families (see Sect. 5.2.2). During the 20th century, several major taxonomic revisions resulted as the number of nominal species grew. The first myxosporean classification systems were proposed by Kudo (1933) and Tripathi (1948). Shul'man (1959, 1966) built on these, and, with some modifications, this is largely the scheme that remains today. A major modification took place with the discovery that a myxozoan life cycle contains both actinospore and myxospore phases (Wolf and Markiw 1984; see Chap. 6). At the time, the phylum Myxozoa Grassé, 1970 was divided into the classes Myxosporea Bütschli 1881 (fish parasites, myxospores) and Actinosporea Noble, 1980 (worm parasites, actinospores), but with the knowledge that these classes actually represent morphologically distinct phases of single species, the Actinosporea was suppressed by Kent et al. (1994). The last thorough revision of myxosporean taxonomy was conducted by Lom and Noble (1984) who modified Shul'man's system following their re-evaluation of taxonomic characters, which led to a re-arrangement of taxa within the class Myxosporea. This system has remained moderately stable at the higher taxonomic ranks, with the notable exception of the class Malacosporea containing the order Malacovalvulida, which was erected to accommodate the genera *Buddenbrockia* and *Tetracapsuloides,* parasites of bryozoans and fish (Canning et al. 2000).

Table 5.1 The list of the 64 currently recognised myxozoan genera with corresponding type species

Genus	Type species	Genus	Type species
Acauda	***A. hoffmani*** Whipps, 2011	*Neobipteria*	*N. macrouri* Kovaleva, Gaevskaya et Krasin, 1986
Agarella	*A. gracilis* Dunkerly, 1915	*Neohenneguya*	*N. tetraradiata* Tripathi, 1953
Alatospora	*A. samaroidea* Shulman, Kovaleva et Dubina, 1979	*Neomyxobolus*	*N. ophiocephalus* Chen et Hsieh, 1960
Auerbachia	*A. anomala* Meglitsch, 1968	*Neoparvicapsula*	*N. ovalis* Kovaleva, Gaevskaya et Shulman, 1982
Bipteria	*B. admiranda* Kovaleva, Zubchenko et Krasin, 1983	*Neothelohanellus*	*N. catlae* Das et Haldar, 1986
Buddenbrockia	***B. plumatellae*** Schröder, 1910	*Noblea*	*N. admiranda* Kovaleva (1989)
Cardimyxobolus	*C. leshanensis* Ma, Dong et Wang, 1982	*Octospina*	*O. tongrensis* Hsieh et Xiao, 1993
Caudomyxum	*C. nanum* Bauer, 1948	*Ortholinea*	*O. divergens* (Thélohan, 1895)
Ceratomyxa	*C. sphaerulosa* Thélohan, 1892	*Palliatus*	*P. mirabilis* Shulman, Kovaleva et Dubina, 1979
Ceratonova	***C. shasta*** Atkinson, Foott, et Bartholomew, 2014	*Paramyxoproteus*	*P. reinhardti* Wierzbicka, 1986
Chloromyxum	***C. leydigi*** Mingazzini, 1890	*Parvicapsula*	***P. asymmetrica*** Shulman, 1953
Coccomyxa	*C. morovi* Léger et Hesse, 1907	*Phlogospora*	*P. mysti* Qadri, 1962
Cystodiscus	***C. immersus*** Lutz, 1889	*Pseudalatospora*	*P. scombri* Kovaleva et Gaevskaya, 1983
Dicauda	*D. atherinoidi* Hoffman et Walker, 1978	*Renispora*	*R. simae* Kalavati, Longshaw et MacKenzie, 1996
Ellipsomyxa	***E. gobii*** Køie, 2003	*Schulmania*	*S. ovale* Kovaleva, Zubchenko et Krasin, 1983
Enteromyxum	***E. scophthalmi*** Palenzuela, Redondo et Alvarez-Pellitero, 2002	*Sigmomyxa*	***S. sphaerica*** (Thélohan, 1892)
Fabespora	*F. nana* Naidenova et Zaika, 1969	*Sinuolinea*	***S. dimorpha*** (Davis, 1916)
Gadimyxa	***G. atlantica*** Køie, Karlsbakk et Nylund, 2007	*Soricimyxum*	***S. fegati*** Prunescu, Prunescu, Pucek et Lom, 2007
Globospora	*G. sphaerica* (Evdokimova 1973)	*Sphaeromyxa*	***S. balbianii*** Thélohan, 1892
Hennegoides	*H. longitudinalis* Lom, Tonguthai et Dyková, 1991	*Sphaerospora*	***S. elegans*** Thélohan, 1892
Henneguya	***H. psorospermica*** Thélohan, 1892	*Spirosuturia*	*S. carassii* Chen et Hsieh, 1984
Hoferellus	*H. cyprini* (Doflein 1898)	*Tetracapsuloides*	***T. bryosalmonae*** (Canning, Curry, Feist, Longshaw et Okamura, 1999)
Kentmoseria	*K. alata* (Kent et Moser, 1990)	*Tetrauronema*	*T. macropodus* Wu, Wang et Jiang, 1988
Kudoa	***K. clupeidae*** (Hahn, 1917)	*Thelohanellus*	***T. hovorkai*** Akhmerov, 1960
Laterocaudata	*L. mastacembala* Chen et Hsieh, 1984	*Triangula*	*T. yangkiangensis* Chen et Hsieh, 1984
Latyspora	***L. scomberomori*** Bartošová, Freeman, Yokoyama, Caffara et Fiala, 2011	*Trigonosporus*	*T. acanthogobii* Hoshina, 1952

(continued)

Table 5.1 (continued)

Genus	Type species	Genus	Type species
Meglitschia	*M. insolita* (Meglitsch, 1960)	*Trilospora*	*T. californica* Noble, 1939
Myxidium	***M. lieberkuehni*** Bütschli, 1882	*Trilosporoides*	*T. platessae* Køie, 2005
Myxobilatus	***M. gasterostei*** (Parisi, 1912)	*Unicapsula*	*U. muscularis* Davis, 1924
Myxobolus	***M. muelleri*** Bütschli, 1882	*Unicauda*	*U. clavicauda* (Kudo, 1934)
Myxodavisia	*M. spinosa* (Davis, 1917)	*Wardia*	*W. ovinocua* Kudo, 1919
Myxoproteus	*M. ambiguus* (Thélohan, 1895)	*Zschokkella*	***Z. hildae*** Auerbach, 1910

Bold type species with SSU sequence available in NCBI database

5.2.1 Main Criteria for Myxozoan Classification

Classification of the Myxozoa is based almost exclusively on spore morphology (see Chaps. 4 and 6). In the class Myxosporea, myxospore morphology has been the main criterion for classification of species since the first systems were established (Kudo 1933; Tripathi 1948; Shul'man 1966). The myxospore is a unique structure possessing many characters important for classification. Spores are composed of shell valves (joined by a suture) that enclose one or more sporoplasms and one or more polar capsules (Lom and Dyková 1992). The number and configuration of shell valves and the number and arrangement of polar capsules in relation to the sutural plane are used to characterise orders and suborders. Additional features, such as details of polar filaments, presence or absence of caudal appendages, and form of the sutural line are informative at the family and generic level. Myxosporean species-level classification is based on spore and polar capsule dimensions and other fine details of myxospore structure (described by Lom and Arthur 1989), such as the number of turns of the polar filament, the presence of ribs, ridges, and striations on the spore valves, presence or absence of a mucous envelope, and the numbers of sporoplasms and their nuclei.

Other features besides myxospore structure and morphology also characterise individual taxa at the species level. For example, Lom and Arthur (1989) recommended important features to be provided for the proper description of myxosporean species if possible. These include a reliable host identification, information about the habitat of hosts, as much information as possible about the vegetative stages (e.g. site of infection and the shape, size, and structure of vegetative stages), characteristics of both extrasporogonic and sporogonic stages (plasmodia and pseudoplasmodia), and whether the latter stages are mono-, di- or polysporous.

Although myxospores possess a number of taxonomically informative morphological features, several genera demonstrate similar morphological characteristics. For instance, this is evident for myxospores produced by *Myxidium*, *Zschokkella*, *Elipsomyxa* and *Sigmomyxa* and for those produced by *Ceratomyxa* and *Sphaerospora*. These similarities hinder correct designation of existing as well as new species. However, the main problem of the myxospore morphology-based taxonomy is that it is inconsistent with phylogenies based on molecular markers (see Sect. 5.2.5 for more details).

In Malacosporea, the worm-like (myxoworm) and sac-like trophic stages present the main diagnostic characters (Canning et al. 2000, 2002). Malacospores developing in *Tetracapsuloides* and *Buddenbrockia* spp. sacs and myxoworms in bryozoan hosts do not possess distinctive diagnostic characters for classification. Moreover, fish malacospores, assumed to be homologous to myxosporean myxospores, are rarely seen in fish and therefore cannot provide reliable criteria for identification. The few morphological features of malacosporean species combined with DNA sequence data can reveal hidden malacosporean diversity in bryozoan hosts (Hartikainen et al. 2014). Molecular data have also revealed new malacosporean lineages in fish hosts (Bartošová-Sojková et al. 2014).

However, due to the lack of morphological data, the newly discovered malacosporean lineages, which most probably represent novel species or even genera, remained undescribed. Although the International Code of Zoological Nomenclature (ICZN) allows naming new species based on molecular data alone, it is clearly preferable to incorporate relevant morphological or biological characters (e.g. host species and habitat, sequential development in the host, tissue specificity, etc.).

Following the discovery of myxozoan life cycle complexity, Kent et al. (1994) proposed that new myxosporean species should not be described based solely on actinospore forms but on the myxospore stages. Similar approaches are used in naming other parasites with complex life cycles. For instance, in digeneans, adult worms are required for species description, and cercarial and metacercarial stages that have not been linked with adult stages are categorized in collective groups. Similarly, myxozoan genera formerly found in the "Actinosporea" are assigned to collective groups (Lom et al. 1997). Thus, actinosporeans that are not identified as life cycle stages of a described species are given vernacular names, e.g. 'Unicapsulactinomyxon type 1 of Rangel et al. (2011)'.

5.2.2 Classification of the Myxozoa

As described in Chap. 2 it is now clear that myxozoans are cnidarians. Moreover, myxozoan taxonomy, especially in the last decade, has faced important changes at generic, family and suborder levels, thus meriting a new classification within Cnidaria with a revision of the spore-based taxonomic system up to the genus level as shown below:

Phylum **Cnidaria**
Unranked subphylum **Myxozoa**
Class **Malacosporea**
Order **Malacovalvulida**
Family **Saccosporidae**: *Buddenbrockia*, *Tetracapsuloides*
Class **Myxosporea**
Order **Bivalvulida**
Suborder **Variisporina**
Family **Sphaeromyxidae**: *Sphaeromyxa*
Family **Myxidiidae**: *Myxidium*, *Zschokkella*, *Enteromyxum*, *Sigmomyxa*, *Soricimyxum*, *Cystodiscus*
Family **Ortholineidae**: *Ortholinea*, *Neomyxobolus*, *Cardimyxobolus*, *Triangula*, *Kentmoseria*
Family **Sinuolineidae**: *Sinuolinea*, *Myxodavisia*, *Myxoproteus*, *Bipteria*, *Paramyxoproteus*, *Neobipteria*, *Schulmania*, *Noblea*, *Latyspora*
Family **Fabesporidae**: *Fabespora*
Family **Ceratomyxidae**: *Ceratomyxa*, *Meglitschia*, *Ellipsomyxa*, *Ceratonova*
Family **Sphaerosporidae**: *Sphaerospora*, *Wardia*, *Palliatus*
Family **Myxobilatidae**: *Myxobilatus*, *Acauda, Hoferellus*
Family **Chloromyxidae**: *Chloromyxum*, *Caudomyxum*, *Agarella*
Family **Coccomyxidae**: *Coccomyxa*, *Auerbachia*, *Globospora*
Family **Alatosporidae**: *Alatospora*, *Pseudalatospora*, *Renispora*
Family **Parvicapsulidae**: *Parvicapsula*, *Neoparvicapsula*, *Gadimyxa*
Suborder **Platysporina**
Family **Myxobolidae**: *Myxobolus*, *Spirosuturia*, *Unicauda*, *Dicauda*, *Phlogospora, Laterocaudata*, *Henneguya*, *Hennegoides*, *Tetrauronema*, *Thelohanellus*, *Neothelohanellus*, *Neohenneguya*, *Trigonosporus*
Order **Multivalvulida**
Family **Trilosporidae**: *Trilospora*, *Unicapsula*
Family **Kudoidae**: *Kudoa*
Family **Spinavaculidae**: *Octospina*
Incertae sedis in Multivalvulida: *Trilosporoides*

5.2.3 Taxonomic Key of the Myxozoa

The last taxonomic key of myxozoans was published more than 20 years ago (Lom and Dyková 1992). Since then many important taxonomic changes have occurred, including the establishment, demise, or synonymization of several genera, families and suborders. We therefore

present a new taxonomic key reflecting these revisions (Table 5.2). As in previous keys, the present key is mostly based on the morphology of myxozoan spores and also on the morphology of their trophic stages (i.e. plasmodia, pseudoplasmodia, sacs and myxoworms), tissue specificity and host group preference. We also present a list of important synonyms of myxozoan genera (Table 5.3).

5.2.4 Phylogenetic Markers and Their Use in Myxozoan Phylogeny

Myxozoans represent an ancient cnidarian lineage derived from a common ancestor early in cnidarian evolution (see Chaps. 2 and 3). Conservative phylogenetic markers are therefore required to reconstruct myxozoan diversification that likely started hundreds of millions of years ago. The small subunit ribosomal RNA gene (SSU) is a universal gene found in all life on Earth and has been used successfully in higher level phylogenetic analyses of many eukaryotic taxa (reviewed in Avise 2004). The first myxosporean SSU sequences were used to ascertain the position of myxozoans in the eukaryotic tree of life (Smothers et al. 1994; Siddall et al. 1995; Schlegel et al. 1996). Since then, myxozoan SSU sequences have been used regularly to clarify the relationships among myxozoan species (e.g. Hervio et al. 1997; Andree et al. 1999b). Although not without limitations, this marker has repeatedly proved to be sufficiently informative to estimate the phylogenetic relationships among myxozoan species (e.g. Kent et al. 2001; Holzer et al. 2004; Fiala 2006; Burger and Adlard 2011; Bartošová et al. 2013; Bartošová-Sojková et al. 2014; Hartikainen et al. 2014).

In addition to being a universal genetic marker, the SSU is useful for myxozoan phylogenetics because its heterogeneity (conserved and variable regions) facilitates discrimination at different taxonomic levels. The conserved regions allow the development of general primers and alignment of DNA sequences, while the variable regions inform on diversification. The high substitution rates of myxosporean SSU sequences, especially in their variable regions, indicate rapid evolution of the myxozoan SSU gene (Kent et al. 1996; Saulnier et al. 1999; Evans et al. 2010) and myxozoans in general (changes are accumulating twice as fast as in Radiata; Cavalier-Smith et al. 1996). Extensive sequence variation of the myxosporean SSU is reflected in different SSU sequence lengths between marine and freshwater species (1,500–1,800 bp vs. >2,000 bp) (Fiala and Dyková 2004; Fiala 2006) and the long variable regions with numerous nucleotide insertions present in myxosporeans of the *Sphaerospora* sensu stricto clade which have one of the longest SSUs (>3.7 kb) of all eukaryotes (Jirků et al. 2007; Holzer et al. 2007, 2013a, b; Bartošová et al. 2013; Eszterbauer et al. 2013). Similar length heterogeneity is observed in the neighbouring large subunit ribosomal RNA(LSU) gene between myxosporean lineages (Bartošová et al. 2009, 2013; see Chap. 6).

Such variation in molecular markers can be problematic for DNA sequence alignment, upon which estimates of molecular phylogeny are based. Variable regions are often excluded from analyses of distantly related myxozoan species because of unclear homology. This removal of characters leads to a loss of information and, ultimately, to insufficient resolution of some nodes. Some authors have proposed the use of SSU secondary structure characteristics to more accurately identify homologous sites and to optimize alignment procedures (Palenzuela et al. 2002; Holzer et al. 2007). In the main marine myxosporean subgroups, fast evolution of myxozoan SSU sequences along with long divergence times, are probably the main causes for lack of resolution and node instability (Fiala et al. 2014). Moreover, different lengths of myxosporean SSU sequences can cause difficulties when creating multiple sequence alignments, because long expansion segments are associated with misalignment. It is not only the general "fast-clock" character of the myxozoan SSU but also

Table 5.2 Updated key for determination of myxozoan genera

Myxozoa			
		Spores with soft (unhardened) shell valves	Malacosporea
		Spores with hardened shell valves	Myxosporea
Malacosporea			
		Sac- or worm-like trophic stages, the latter with triploblast organization, sacs usually irregularly shaped, elongate, ellipsoid or constricted	*Buddenbrockia*
		Sac-like trophic stages usually of regular spherical shape formed by a simple layer of cells	*Tetracapsuloides*
Myxosporea			
1	a	Mature spore contains only one PC	2
	b	Two PCs per spore (one of them may be much smaller than the other one)	7
	c	Three PCs per spore	26
	d	Four or more PCs per spore	27
2	a	Spores pyriform, drop-like or elongate-ellipsoidal	3
	b	Spores more or less spherical, with the sutural line indistinct	6
	c	Spores club-like with a broad anterior part and a narrow caudal part	*Auerbachia*
3	a	The sutural line of the two shell valves sigmoidal	*Coccomyxa*
	b	The sutural line of the two shell valves straight	4
4	a	Spores without a bifurcated caudal process	5
	b	Spores with a bifurcated caudal process	*Phlogospora*
5	a	Spores with PC discharging apically and axially	*Thelohanellus*
	b	Spores with PC discharging subapically and to the side	*Neothelohanellus*
6	a	Spores with three shell valves (two small ones and one large) difficult to discern under the light microscope, exclusively histozoic	*Unicapsula*
	b	Spores with two shell valves adhering along a delicate sinuous sutural line, coelozoic	*Globospora*
7	a	PCs located each separately in the ends of spore	8
	b	PCs not terminally in the opposing ends, but also not close to each other	10
	c	PCs close to each other	16
8	a	Polar filament within the PC thin and spirally wound tube of about the same thickness all along its length	9
	b	Polar filament strongly tapering from its base to the tip, not forming a regular coil within the PC, being rather folded upon itself several times	*Sphaeromyxa*
9	a	Spores fusiform, straight or slightly crescent or sigmoid shaped with more or less pointed ends, usually pyriform PCs, mostly coelozoic in fishes and reptiles	*Myxidium*
	b	Spores usually ellipsoidal, slightly bent or semicircular in valvular view, with rounded or bluntly pointed ends and almost spherical PCs, mostly coelozoic in fishes and reptiles	*Zschokkella*
	c	Spores ellipsoid in outline with thin-walled protrusions associated with PC tips, coelozoic in gall bladder of marine fishes	*Sigmomyxa*
	d	Spores slightly crescent shaped, very large elongated PCs tapering to the end, histozoic in epithelia of digestive tract of fishes	*Enteromyxum*
	e	Spores ovoid with rounded ends, in liver bile ducts and parenchyma of terrestrial mammals	*Soricimyxum*
	f	Spores oval or ellipsoidal with raised sutural ridge between the valves, disc-like plasmodia with spore development in gall bladders of amphibians	*Cystodiscus*

(continued)

Table 5.2 (continued)

10	a	Elongated spores with central, transversal sutural line, PCs near opposite ends discharging laterally	*Fabespora*
	b	Spores thin-walled ellipsoid, sutural line straight, central, perpendicular to PCs or forming an acute angle to thickness axis or sinuous	*Ellipsomyxa*
	c	Rounded, ovoid, triangular or elongated spores with PCs set widely apart in the sutural plane, sutural line straight	11
	d	Spherical, rounded or pyramidal spores having PCs in a plane perpendicular to the sutural line which is mostly sinuous	12
	e	Inversely pyramidal spores with PCs in a plane obliquely to the sutural line, a stiff keel-like membrane running meridionally on the surface of each valve	*Paramyxoproteus*
11	a	Spores spherical or subspherical, in marine fishes	*Ortholinea*
	b	Spores ovoid, flattened parallel to the sutural plane, in freshwater fishes	*Neomyxobolus*
	c	Spores ovoid with sutural markings along the posterior border, spindle shaped in sutural view, in freshwater fishes	*Cardimyxobolus*
	d	Spores triangular with rounded corners, flattened parallel to sutural plane, without projections	*Triangula*
	e	Spores elongated, wider anteriorly than posteriorly, flattened parallel to sutural plane, with pointed projections extending backwards	*Kentmoseria*
12	a	Shell valves without projections	13
	b	Shell valves with various projections	14
13	a	Spores spherical or subspherical	*Sinuolinea*
	b	Spores inversely pyramidal or triangular and with rounded outlines	*Myxoproteus*
14	a	Spores spherical or ovoid, each shell valve bearing a hollow, usually horn-like projection approximately at its center	*Myxodavisia*
	b	Spores inversely pyramidal with tapering end extending backwards, a wing-like projection attached at the anterior surface of each valve	*Bipteria*
	c	In addition to valvular projections, a stiff, keel-like membrane runs sideways along the sutural line	15
15	a	Wing-like projections like in *Bipteria* and additional keel-like extensions running meridionally along the sutural line	*Neobipteria*
	b	Four longitudinal stiff keel-like valvular projections, two running along the sutural line and additional two run meridionally along midline of the valves	*Schulmania*
	c	Valvular projections as thickenings covering spore apex and slightly raised from its surface and two keel-like membranes along the sutural line	*Noblea*
16	a	Spores elongated, asymmetrical, curved and very thin-walled	*Parvicapsula*
	b	Spores essentially (with the exception of *Hennegoides*) bilaterally symmetrical	17
17	a	Two spherical PCs positioned in tandem and at a distance from the anterior end, two fine projections at both spore ends of the spindle shaped spore	*Neohenneguya*
	b	PCs located anteriorly and set obliquely to the sinuous sutural line	*Latyspora*
	c	PCs set in a plane perpendicular to the sutural line	18
	d	PCs in the apex of the spore set in the sutural plane	22
18	a	Large spherical PCs in the center of oval spore in valvular view and with triangular shape in sutural view	*Wardia*
	b	PCs more or less close to the anterior apex of the spore	19
19	a	Spores with no projections or veils	20
	b	Spores with projections or veils	21

(continued)

Table 5.2 (continued)

20	a	Spores spherical, subspherical or slightly elongated in the direction perpendicular to the sutural plane, mostly coelozoic in the excretory system of fishes and amphibians	*Sphaerospora*
	b	Spores generally elongate, crescent-shaped or arcuate, occasionally subspherical or oval, coelozoic in gall bladder of marine, rarely freshwater fishes	*Ceratomyxa*
	c	Spores V-shaped with PCs lying almost axially in each valve	*Meglitschia*
	d	Spores crescent-shaped, histozoic in intestine of freshwater and anadromous fishes	*Ceratonova*
	e	Spores pyriform or mitre-like with ridged valves	*Acauda*
21	a	Subspherical spores enveloped in a large membranaceous veil	*Palliatus*
	b	Reniform spores with laterally extending adhering all around the valves	*Renispora*
	c	Triangular spores laterally drawn into wing-like membranous projections	*Alatospora*
	d	Triangular spores with projections doubled to form parachute-like pockets	*Pseudalatospora*
	e	Spores spindle-shaped, with a pair of long posterior projections	*Myxobilatus*
	f	Spores pointed, mitre-like or rounded in valvular view with numerous stiff filaments at posterior end	*Hoferellus*
22	a	Spores without projections	23
	b	Spores with projections	24
23	a	The sutural line straight	25
	b	The sutural line strongly sinuous	*Spirosuturia*
24	a	A single caudal projection	*Unicauda*
	b	Two caudal, often slightly divergent projections	*Henneguya*
	c	Two caudal projections, spore asymmetric	*Hennegoides*
	d	Two caudal projections extending in opposite directions	*Dicauda*
	e	Four posterolateral projections	*Tetrauronema*
	f	Two projections extending laterally from one side of the posterior end of the spore	*Laterocaudata*
	g	The valves of broadly triangular spores drawn into filamentous processes on each side, each pair connected by a filament	*Trigonosporus*
25	a	Spores with ellipsoidal, ovoid or rounded shape	*Myxobolus*
	b	Two morphological forms of spores, wide, semicircular and thick-walled, sub (spherical) in valvular view	*Gadimyxa*
26	a	Spores with three shell valves, appearing as triradiate star with rounded ends from apical view, PCs discharging apically	*Trilospora*
	b	Spore with conical posterior end and PCs situated peripherally and discharging laterally	*Trilosporoides*
27	a	Four or more shell valves	29
	b	Two shell valves	28
28	a	Spores spherical	*Chloromyxum*
	b	Spores almost spherical, with one or two caudal projections	*Caudomyxum*
	c	Spores spindle-shaped, with two caudal projections	*Agarella*
	d	Spores elongated, asymmetrical, curved and thin-walled	*Neoparvicapsula*
29	a	Spores stout spindle-shaped with sutural ridge extending both spore ends as a spine, coelozoic in freshwater fishes	*Octospina*
	b	Spores stellate, quadrate, subspherical to ovoid in apical view, histozoic in marine fishes	*Kudoa*

PC polar capsule

Table 5.3 Important generic synonyms

Correct genus name	Synonym	Type species	Reference for synonymy
Buddenbrockia	*Tetracapsula* Canning, Okamura et Curry, 1996	*T. bryozoides* Canning, Okamura et Curry, 1996	Canning et al. (2002)
Tetracapsuloides			
Ceratomyxa in GB	*Leptotheca* Thélohan, 1895	*L. agilis* Thélohan, 1892	Gunter and Adlard (2010)
Sphaerospora in ES			
Hoferellus	*Hoferia* Doflein, 1898	*H. cyprini* Doflein, 1898	Mercier (1908), Shul'man (1966)
	Mitraspora Fujita, 1912	*M. cyprini* Fujita, 1912	Lom (1986), Kovács-Gayer et al. (1987)
Kentmoseria	*Paraortholinea* Kovaleva in Shulman, Donets et Kovaleva, 1997	*P. alata* Kovaleva, 1997	Lom and Dyková (1995)
Kudoa	*Neochloromyxum* Matsumoto, 1954	*N. cruciformum* Matsumoto, 1954	Shul'man (1966)
	Tetraspina Xie et Chen, 1988	*T. decapterus* Xie et Chen, 1988	Lom and Dyková (2006)
	Pentacapsula Naidenova et Zaika, 1970	*P. shulmani* Naidenova et Zaika, 1970	Whipps et al. (2004)
	Hexacapsula Arai et Matsumoto, 1953	*H. neothunni* Arai et Matsumoto, 1953	
	Septemcapsula Hsieh et Chen, 1984	*S. yasunagai* Hsieh et Chen, 1984	
Myxobolus	*Disparospora* Akhmerov, 1954	*D. pavlovskii* Akhmerov, 1954	Shul'man (1966), Landsberg and Lom (1991)
	Facieplatycauda Wyatt, 1979	*F. pratti* Wyatt, 1979	Landsberg and Lom (1991)
	Gyrospora Qadri, 1962	*G. crucifila* Qadri, 1962	Landsberg and Lom (1991)
	Lentospora Plehn 1905	*L. cerebralis* (Hofer) Plehn, 1903	Kudo (1933)
	Myxosoma Thélohan, 1892	*M. dujardini* Thélohan 1892	Lom and Noble (1984), Landsberg and Lom (1991)
	Rudicapsula Kalavati et Narasimhamurti, 1984	*R. esomi* Kalavati et Narasimhamurti, 1984	Landsberg and Lom (1991)
Myxodavisia	*Davisia* Laird, 1953	*D. diplocrepis* Laird, 1953	Zhao et al. (2008)
Myxoproteus	*Conispora* Sankurathri, 1977	*C. renalis* Sankurathri 1977	Kovaleva et al. (1983)
Neothelohanellus	*Lomosporus* Gupta and Khera, 1988	*L. indicus* Gupta et Khera, 1988	Lom and Dyková (2006)
Sphaerospora	*Podospora* Chen and Hsieh, 1984	*P. hypophthalmichthydis* Chen et Hsieh, 1984	Arthur and Lom (1985)
	Polysporoplasma Sitjà-Bobadilla et Alvarez-Pellitero, 1995	*P. sparis* Sitjà-Bobadilla et Alvarez-Pellitero, 1995	Bartošová et al. (2013)

(continued)

Table 5.3 (continued)

Correct genus name	Synonym	Type species	Reference for synonymy
Unicapsula	*Pileispora* Naidjenova and Zaika, 1970	*P. galeata* Naidjenova and Zaika, 1970	Lom and Noble (1984), Lom and Dyková (2006)
	Parapileispora Naidjenova and Zaika, 1970	*P. pyramidata* Naidjenova and Zaika, 1970	Lom and Noble (1984), Lom and Dyková (2006)
Zschokkella	*Parazschokkella* Kalavati and Narasimhamurti 1987	*P. auerbachi* (Weill, 1929)	Lom and Dyková (2006)
Correct genus name	Most probably a synonym[a]	Type species	Reference for synonymy
Bipteria	*Paramyxoproteus* Wierzbicka, 1986	*P. reinhardti* Wierzbicka, 1986	Kovaleva (1989), Lom and Dyková (2006)
Coccomyxa	*Thelohanelloides* Sarkar, 2009	*T. bengalensis* Sarkar, 2009	This work
Thelohanellus	*Neothelohanellus* Das et Haldar, 1986	*N. catlae* Das et Haldar, 1986	Lom and Dyková (2006)
Triangula	*Triangulamyxa* Azevedo, Corral et Matos, 2005	*T. amazonica* Azevedo, Corral et Matos, 2005	Lom and Dyková (2006)

In cases where the original publication with the synonymy of a particular genus was not found, we refer to the review works of Lom and Noble (1984) and Lom and Dyková (2006)
GB gall bladder-infecting species, *ES* excretory system-infecting species, [a] indicates generic status warrants further investigation due to poor genus description or lack of sufficient differences from a morphologically similar genus

the presence of "super" fast evolving sequences that may impede correct inferences of phylogenetic relationships based on molecular phylogenetic analyses. A number of long-branching (rapidly evolving) species, mainly in the *Ceratomyxa* clade and the marine urinary clade, may particularly influence the resulting tree topology (Bartošová et al. 2011; Fiala et al. 2014). The phylogenetic position of these taxa can thus be influenced by long-branch attraction (LBA), the most common phylogenetic artefact (e.g. Anderson and Swofford 2004).

The LSU is another marker that possesses conserved and variable regions and is used for myxozoan phylogenetics. The LSU was first used to better assess the relationships of multivalvulidan species (Whipps et al. 2004a). It has also been used as a marker supporting the SSU-based phylogenies (Whipps and Kent 2006) and more often in combined analyses together with the SSU (Whipps et al. 2004a; Bartošová et al. 2009, 2013). The LSU has been shown to be more informative for myxosporean phylogeny than SSU (Bartošová et al. 2009). However, the high number of myxosporean SSU records in NCBI database still makes the SSU the first choice of marker when analysing myxozoan phylogeny.

Ribosomal DNA (rDNA)-based phylogenies can suffer from concerted evolution of both the SSU and LSU (Hillis and Davis 1988), and are not entirely independent as they are separated by only several a few hundred base pairs. As such, other markers have been sought to test rDNA-based phylogenies. These have largely supported existing estimates of phylogeny. For example, the gene for heat shock protein 70 showed the same pattern as SSU in intraspecies relationships between regional populations of *Kudoa thyrsites* (Whipps and Kent 2006). Phylogenies based on the elongation factor 2 (EF2) gene were found to agree with those based on rDNA (Fiala and Bartošová 2010; Bartošová et al. 2013; Bartošová-Sojková et al. 2014). Although EF2 is much less informative than rDNA markers (Fiala et al. 2014), it was the first non-ribosomal marker used for higher-level phylogeny of the Myxozoa and is consistent with the results of rDNA-based phylogeny (see Chap. 6).

Universality of the SSU renders this marker useful to reveal the relationships even among very closely related species. However, SSU is usually insufficient to distinguish differences at intraspecies level. To overcome this limitation, the internal transcribed spacer region 1 (ITS1) has been successfully used. The ITS1 is a more rapidly evolving phylogenetic marker than SSU and, because it is adjacent to the SSU, designing primers to obtain ITS1 sequence is not overly challenging. Identity of ITS1 between American and European *Myxobolus cerebralis* isolates revealed a recent introduction of this parasite followed by dispersal via anthropogenic means (Andree et al. 1999a; Whipps et al. 2004b). ITS1 is also a relevant marker for large scale phylogeographical studies of various myxozoan species, e.g. *Kudoa thyrsites* (Whipps and Kent 2006) and *Tetracapsuloides bryosalmonae* (Henderson and Okamura 2004), although, it may sometimes be problematic for characterising strain variation due to extensive intragenomic variation (Henderson and Okamura 2004). At the species level, ITS1 was examined in populations in different hosts of *Ceratonova shasta* (syn. *Ceratomyxa shasta*; Atkinson and Bartholomew 2010). In addition, ITS1 together with ITS2 were used to unmask cryptic species of amphibian myxosporeans (Hartigan et al. 2011; see Chap. 6).

5.2.5 Discrepancies Between Morphology-Based Classification and Molecular Phylogeny

The first comprehensive phylogenetic analyses using SSU sequences conducted by Kent et al. (2001) revealed inconsistencies between DNA-based phylogenies and existing spore-based classification schemes. Four of the five genera studied (*Myxobolus*, *Henneguya*, *Sphaerospora* and *Myxidium*) were poly- or paraphyletic. *Kudoa* was the only clearly monophyletic genus. Similar discrepancies were revealed as additional DNA sequences became available. For example, *Kudoa* became polyphyletic when *Sphaerospora dicentrarchi* was placed within Kudoidae based on SSU sequence data (Kent and Palenzuela 2001). Moreover, the polyphyly of genera *Henneguya*, *Sphaerospora*, *Myxidium*, *Zschokkella*, and *Chloromyxum* was confirmed in a comprehensive analysis based on more than 130 sequenced species (Fiala 2006). In addition, SSU sequences supported evidence for paraphyly of the genera *Myxobolus*, *Kudoa* and *Ceratomyxa* (Diamant et al. 2005; Fiala 2006). For some groups, however, molecular phylogenies based on SSU data were consistent with traditional, morphology-based taxonomies, suggesting some morphological characters are indeed taxonomically informative, e.g. in *Parvicapsula* (Nylund et al. 2005), *Enteromyxum* (Palenzuela et al. 2002) and *Sphaeromyxa* (Fiala 2006).

The first revision of myxozoan taxonomy based on molecular phylogenetics came only 8 years after the first myxozoan DNA sequence was published by Smothers et al. (1994). Specifically, Palenzuela et al. (2002) proposed the transfer of formerly described *Myxidium leei* to the newly erected genus *Enteromyxum*. Later, Whipps et al. (2004a) proposed a taxonomic redescription of the Multivalvulida to maintain an evolutionarily consistent taxonomic scheme. They suppressed three families and synonymized three genera with the genus *Kudoa* in the Kudoidae. The definition of the genus *Sphaerospora* was amended to include specific inserts in the *Sphaerospora* SSU gene as a defining character (Jirků et al. 2007). The family Myxobilatidae was resurrected to encompass genera *Myxobilatus*, *Hoferellus,* and *Acauda* that were formerly ranked in different families in the taxonomic system (Whipps 2011). Likewise, the Coccomyxidae was re-established based in part on phylogenetic analyses that revealed the group to be monophyletic (Heiniger et al. 2011). The genus *Polysporoplasma* was supressed due to the clustering of its type species within the *Sphaerospora* sensu stricto clade, which includes members with very similar morphology (Bartošová et al. 2013; Table 5.3). Each of these examples represents the incremental improvement of myxosporean classification schemes that molecular data have largely driven. As sampling and sequencing become more common and encompass more species and genera, additional

revisions can be proposed and carefully evaluated by the research community.

Molecular data allow hypotheses based on morphology to be tested and provide powerful independent evidence relevant for phylogenetically informative classifications. The genus *Myxidium,* for example, is paraphyletic and difficult to distinguish from *Zschokkella* species (Heiniger and Adlard 2014). However, using molecular data as a guiding tool and assessing distinctive morphological features within lineages has allowed the description of the genus *Enteromyxum* (Palenzuela et al. 2002) and the revival of *Cystodiscus* (Hartigan et al. 2012) from species once considered as *Myxidium*. In another example, Whipps (2011) described *Acauda* to accommodate species within the Myxobilatidae with spores that lack caudal appendages. Although the genetic relationships to sister taxa *Myxobilatus* and *Hoferellus* were close, assigning the tail-less species to either existing genus would have been arbitrary, given the lack of comprehensive data, and would have led to subsequent instability. Thus, a new genus was carefully described and future studies may suggest the synonymy of some or all of these genera. Until then, there are clear morphological differences between them. Sometimes a clear case is not made for discriminating taxa. For example, the description of *Sigmomyxa* does not include an explanation of how this taxon is distinguished from other related genera such as *Ellipsomyxa* and *Myxidium* (Karlsbakk and Køie 2012).

Other examples of progressive changes to myxozoan taxonomy and nomenclature are informative and provide useful guidelines for how to deal with future taxonomic issues when similar situations arise. For instance, the polyphyly of *Sphaerospora* is now well recognized and several clarifications have been made between this and other genera using molecular phylogenies reassigning other species to and from this group (Jirků et al. 2007; Gunter and Adlard 2010). A recent study demonstrates that an asymmetric approach is sometimes warranted. Bartošová et al. (2011) described a new genus, *Latyspora*, for a new *Sphaerospora*-like species and argued that assignment remain the same for an existing *Sphaerospora* species until further data can be collected. Former genera and families of marine multivalvulidan species were synonymized or suppressed once molecular phylogenies revealed they were nested within the Kudoidae (Whipps et al. 2004a). In contrast, the distinctiveness of a lineage of *Coccomyxa* and *Auerbachia* species revealed by molecular phylogenetic analysis prompted Heiniger et al. (2011) to revive the family Coccomyxidae. Similarly, Whipps (2011) proposed that the family Myxobilatidae be revived based on molecular data. Kristmundsson and Freeman (2013) proposed suppression of an entire suborder, Sphaeromyxina, based on genetic data. These various examples demonstrate that molecular data are likely to be necessary for myxozoan taxonomic revisions and many species descriptions and should certainly be obtained for discriminating or describing myxozoans related to these problematic taxa. Ultimately, clear justification must be made for any proposed taxonomy, and those that are lacking justification only serve to further obscure myxozoan taxonomy and systematics.

Important changes in the myxozoan taxonomy over the last decade (2004–2014) can be summarized as follows:

- **Demise of suborder**: **Sphaeromyxina** (family Sphaeromyxidae placed in Variisporina, Kristmundsson and Freeman 2013)
- **Demise of families**: **Auerbachiidae** (=Coccomyxidae; Heiniger et al. 2011); **Pentacapsulidae**, **Hexacapsulidae**, **Septemcapsulidae** (=Kudoidae; Whipps et al. 2004a); **Polysporoplasmidae** (=Sphaerosporidae; Bartošová et al. 2013)
- **Demise of genera**: ***Leptotheca*** (synonymized with *Ceratomyxa* and *Sphaerospora*; Gunter and Adlard 2010); ***Pentacapsula***, ***Hexacapsula*** and ***Septemcapsula*** (synonymized with *Kudoa*; Whipps et al. 2004a); ***Polysporoplasma*** (synonymized with *Sphaerospora*; Bartošová et al. 2013)
- **Resurrection of families**: **Coccomyxidae** (Heiniger et al. 2011); **Myxobilatidae** (Whipps 2011)

- **Resurrection of genus**: ***Cystodiscus*** (Hartigan et al. 2012)
- **New genus descriptions**: ***Acauda*** (Whipps 2011); ***Ceratonova*** (Atkinson et al. 2014); ***Gadimyxa*** (Køie et al. 2007); ***Latyspora*** (Bartošová et al. 2011); ***Sigmomyxa*** (Karlsbakk and Køie 2012); ***Soricimyxum*** (Prunescu et al. 2007)
- **Replacement of a preoccupied taxon**: ***Myxodavisia*** replaced *Davisia* (Zhao et al. 2008)

5.3 Phylogenetic Relationships Within the Myxozoa

5.3.1 Major Myxozoan Lineages

The first branching myxozoan lineage was probably the one leading to extant malacosporean species, based on the SSU analyses and on their primitive morphological features (Monteiro et al. 2002; see also Chaps. 2 and 4). After the separation of the Malacosporea, the *Sphaerospora* sensu stricto clade (Jirků et al. 2007) split from the rest of the Myxosporea which then underwent extensive radiation as the marine and freshwater lineages (Kent et al. 2001; Kent and Palenzuela 2001; Fiala 2006; Sitjà-Bobadilla and Palenzuela 2013; Fig. 5.1; see also Chap. 4).

5.3.2 The Malacosporea

In this section, we review the SSU-based phylogeny of the Malacosporea which summarizes the data from the most recent studies focused on the diversity of malacosporeans in their bryozoan and fish hosts (Bartošová-Sojková et al. 2014; Hartikainen et al. 2014). The malacosporean lineage comprises species with life cycles in fish and bryozoan hosts and which demonstrate worm- and sac-like morphologies in the latter. The whole clade is sister to myxosporeans (Fig. 5.1) and considered by some authors to be a primitive myxozoan clade (Anderson et al. 1999; and see Chap. 2). Malacosporeans are currently split into six main sublineages: *Buddenbrockia*, *Tetracapsuloides*, Malacosporea sp. A–C, Malacosporea sp. D, Malacosporea sp. E, and Malacosporea sp. F (see Fig. 4.1 in Chap. 4). No strict separation of malacosporean clades is apparent according to host species, host habitat, morphology of the bryozoan-infecting stages, and biogeography (Bartošová-Sojková et al. 2014). There is some evidence that these clades are characterized by specialist versus generalist host exploitation strategies (see Chap. 4). Myxoworms (vermiform stages) may have been lost or gained several times within the Malacosporea (Hartikainen et al. 2014). The earliest branch of malacosporeans presently known is represented by Malacosporea sp. F, which is a non-motile, irregularly shaped, elongate parasite of bryozoans belonging to the genus *Fredericella* (Hartikainen et al. 2014; Chap. 4). The series of bulges or lobes that characterise this malacosporean is so far unique across malacosporeans. The Malacosporea sp. E sublineage is represented by a vermiform and motile parasite of fredericellids. The other malacosporean clades comprise a mixture of myxoworms and sac-like parasites of bryozoans and have also been linked with fish hosts (Fig. 4.1 in Chap. 4). Based on the molecular, morphological, and biological data, the two closely related clades of sac and vermiform *Buddenbrockia plumatellae* appear to be separate species (Jiménez-Guri et al. 2007; Bartošová-Sojková et al. 2014; Hartikainen et al. 2014).

5.3.3 The Marine Myxosporean Lineage

The marine myxosporean lineage contains species with life cycles in the marine environment that use a polychaete definitive host (Kent et al. 2001; Holzer et al. 2007; Sitjà-Bobadilla and Palenzuela 2013). The marine myxosporean lineage branches into seven well-supported sublineages (Fig. 5.1). The early diverging *Bipteria* sublineage is represented by a gall bladder parasite of rabbit fish (*Chimaera monstrosa*). The *Ceratomyxa* and the marine gall bladder clades include species infecting the gall bladder and the

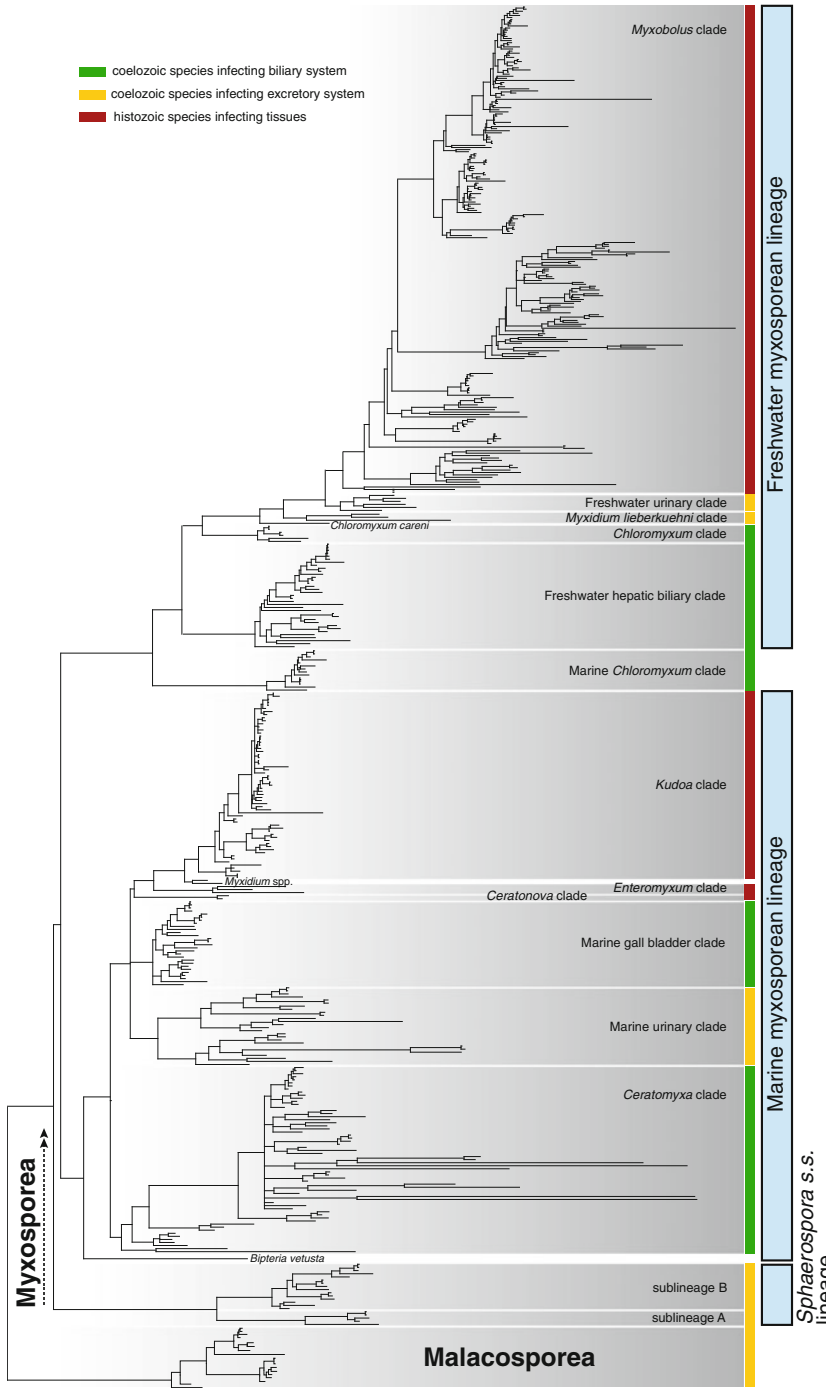


Fig. 5.1 Maximum likelihood phylogenetic tree based on SSU rDNA sequences of all myxozoan species available in NCBI database (September 2014). Main myxozoan phylogenetic lineages and clades are *highlighted*

marine urinary clade encompasses species from the host excretory system; *Kudoa*, *Enteromyxum* and *Ceratonova* clades group histozoic species (Fiala 2006; Fiala et al. 2014; Kodádková et al. 2015). The mutual relationships of these clades are not entirely resolved. Recent phylogenetic analyses (Fiala et al. 2014; Kodádková et al. 2015) with all available sequences of members of the marine myxosporean lineage suggest that following the split of the *Bipteria* sublineage, the first basal branch of the marine lineage is the *Ceratomyxa* clade. The same analyses indicated that the *Enteromyxum* and *Kudoa* clades were closely related and the most derived groups in the marine lineage. Positions of the marine urinary clade, the marine gall bladder clade and the *Ceratonova* clade are very unstable and their relationships are highly dependent on the phylogenetic method and marker used (Fiala and Bartošová 2010; Fiala et al. 2014).

Topological tests of SSU and LSU data by Fiala et al. (2014) suggested that the histozoic species (*Kudoa* + *Enteromyxum* + *Ceratonova*) were closely related, a relationship also supported by the phylogenetic analysis of Freeman et al. (2008) and the evolutionary reconstruction of Fiala and Bartošová (2010). Other phylogenetic analyses revealed the *Ceratonova* clade (former lineage of the single species, *Ceratomyxa shasta*) to be closely related to *Parvicapsula* spp. (Heiniger et al. 2008; Køie et al. 2008; Gunter and Adlard 2009), with both comprising a sister lineage to all marine groups with the exception of the marine gall bladder clade (Jirků et al. 2007). Yet other analyses identified the *Ceratonova*/*Parvicapsula* clade as sister to the marine gall bladder clade (Fiala and Dyková 2004; Jirků et al. 2006). However, none of these studies were focused on the phylogenetic position of *C. shasta*. The latter was addressed in a comprehensive phylogenetic analyses of all *Ceratomyxa* spp. which placed *C. shasta* as sister lineage to the *Ceratomyxa* clade (Gunter and Adlard 2008; Gunter et al. 2009). A close relationship of *C. shasta* and *Ceratomyxa* spp. was not supported in the analyses of Fiala et al. (2014).

The *Ceratomyxa* clade is a taxon-rich group consisting almost exclusively of *Ceratomyxa* species. It is the most intensively studied taxon, from the phylogenetic point of view, within the marine myxosporean lineage (e.g. Gunter et al. 2009; Gleeson and Adlard 2011). There are more than 70 sequenced *Ceratomyxa* species, which corresponds to about 30 % of all described nominal *Ceratomyxa* species. The majority of species for which DNA sequences are available originated from fish from Australian waters in the Pacific Ocean, so there is room for increased geographic sampling. Phylogenetic analysis of *Ceratomyxa* species has identified five well-supported subclades (Fiala et al. 2014). One subclade is a group of closely related basal ceratomyxids from elasmobranchs (Gleeson and Adlard 2011). The discovery of their phylogenetic position supported the hypothesis that the genus *Ceratomyxa* is divided into two separate elasmobranch- and teleost-infecting groups (Gunter et al. 2009). However, two sequences of *Ceratomyxa* spp. from Teleostei cluster as the most early diverging *Ceratomyxa* clade in an analysis (Fiala et al. 2014), which suggests that elasmobranch *Ceratomyxa* spp. branch within the teleost-infecting *Ceratomyxa* species. The largest ceratomyxid group is characterized by generally unresolved relationships at many nodes (Gunter et al. 2009). Several long-branching *Ceratomyxa* species are placed within this clade indicating accelerated evolution of some ceratomyxids relative to their congeners. A non-ceratomyxid species (*Palliatus indecorus*) currently clusters inside the *Ceratomyxa* clade thus disrupting the monophyly of the genus (Fiala 2006). Its taxonomic reassignment is, however, expected (Fiala et al. 2014).

The marine gall bladder clade contains the remainder of the non-ceratomyxid gall bladder infecting species in the marine clade. The term "marine gall bladder clade" was established in Fiala (2006) for the gall bladder-infecting clade containing several *Myxidium* species. Currently, representatives from seven genera cluster in this clade and only two of them are monophyletic (*Coccomyxa* and *Ellipsomyxa*) (Heiniger et al. 2011). *Sigmomyxa sphaerica* and *Sinuolinea phyllopteryxa* are single representatives of their genera in this clade. *Sinuolinea* is known to be

polyphyletic because its type species clusters in the marine urinary clade (Dyková et al. 2013). The cluster of coccomyxids contains the monophyletic *Coccomyxa*, and the genus *Auerbachia* which is paraphyletic (Heiniger et al. 2011). These two genera form a common distinct group of species having a single polar capsule and infecting gall bladder. The species-rich and highly polyphyletic genera *Myxidium* and *Zschokkella* also have representatives in the marine gall bladder clade, although, their type species are placed outside this clade.

Almost all myxosporeans infecting urinary bladder of marine fish cluster in the marine urinary clade with a few exceptions that appear in the myxosporean freshwater lineage (see Sect. 5.3.4). The marine urinary clade splits into two well-resolved subclades: the *Zschokkella* and the *Parvicapsula* one. The *Zschokkella* subclade includes the type species *Z. hildae* and several other marine *Zschokkella* species. However, the *Zschokkella* subclade is polyphyletic as it also includes several *Sinuolinea* species with the type species *S. dimorpha* branching with *Latyspora scomberomori* (Dyková et al. 2013; Kodádková et al. 2014). The only sequenced member of the genus *Schulmania* is also placed within the *Zschokkella* subclade (Kodádková et al. 2014). The *Parvicapsula* subclade comprises *Parvicapsula* species, but monophyly of this genus is disrupted by the inclusion of *Gadimyxa* species and *Sphaerospora testicularis* (Køie et al. 2007; Bartošová et al. 2011; Kodádková et al. 2014).

Marine histozoic myxosporeans cluster in three well-defined clades. Two of these clades are species-poor, each containing only species of either of the two monophyletic genera *Enteromyxum* and *Ceratonova* (Atkinson et al. 2014). The third histozoic group is the *Kudoa* clade that comprises all multivalvulidan *Kudoa* spp. The genus *Kudoa* is not monophyletic because *Sphaerospora dicentrarchi* and an unnamed *Sphaerospora* species branch within the *Kudoa* clade (Kent and Palenzuela 2001; Diamant et al. 2005; Fiala 2006). At the base of the *Kudoa* clade are the three-valved *Unicapsula* species (Whipps et al. 2004a). The two-valved *Myxidium*-like parasites of monogeneans and fish are sister to the *Kudoa* + *Unicapsula* group (Freeman and Shinn 2011; Freeman et al. 2014). This branching supports the idea that valve number changed from two, to three, to four and more in the evolution of the multivalvulidans (see Chap. 4).

5.3.4 The Freshwater Myxosporean Lineage

The freshwater myxosporean lineage encompasses species using oligochaetes as definitive hosts and developing mostly in the freshwater environment (Kent et al. 2001; Holzer et al. 2007; Sitjà-Bobadilla and Palenzuela 2013). However, there is a growing number of exceptions to the strict freshwater-marine separation. Several lineages whose ancestors invaded the marine environment now occur in the freshwater myxosporean lineage, i.e. sphaeromyxids, two related marine *Myxidium* species, brackish/marine *Myxobolus*, marine *Zschokkella* and *Orthobolinea* species (see Chap. 4). The well-supported marine elasmobranch-infecting *Chloromyxum* clade clusters as sister to the freshwater myxosporean lineage (Fiala and Dyková 2004; Fiala 2006; Azevedo et al. 2009; Gleeson and Adlard 2012). The freshwater myxosporean lineage splits into five clades (Fig. 5.1): the hepatic biliary clade, the freshwater *Chloromyxum* clade, the *Myxidium lieberkuehni* clade, the *Myxobolus* clade and the freshwater urinary clade (freshwater UB clade of Fiala 2006).

The freshwater hepatic biliary clade includes two groups of myxosporeans infecting marine fish: (1) sphaeromyxids (basal marine taxa are *Myxidium coryphaenoidium* and *Myxidium baueri*) and (2) *Zschokkella* species. The monophyletic *Sphaeromyxa* subclade contains the "*balbianii*" and "*incurvata*" lineages distinguished according to spore morphology (Karlsbakk et al. 2013; Kristmundsson and Freeman 2013). *Myxobolus spirosulcatus* is an exceptional myxobolid clustering outside the large *Myxobolus* clade close to three marine *Zschokkella* spp. (Yokoyama et al. 2010). The freshwater hepatic biliary clade is unique within the Myxosporea as members of this clade also infect non-fish

vertebrate host groups, such as amphibians, reptiles, birds and mammals (Dyková et al. 2007; Bartholomew et al. 2008; Roberts et al. 2008; Hartigan et al. 2012) (see Chap. 7). Myxosporeans infecting these non-fish hosts are scattered within the fish-infecting taxa of the freshwater hepatic biliary clade and are not closely related. The exceptions are amphibian-infecting *Cystodiscus* spp., which form a monophyletic group, and two closely related turtle-infecting *Myxidium* spp. (Hartigan et al. 2012). Both the diversity of taxonomically distinct hosts and the large number of genera make this clade of particular interest. The relationships among most of the taxa within the clade are not well resolved (e.g. Kristmundsson and Freeman 2013).

The freshwater *Chloromyxum* clade sensu Jirků et al. (2011) includes *Chloromyxum* species from freshwater teleosts (Bartošová and Fiala 2011). *Chloromyxum careni* from the renal system of amphibians represents either an independent lineage within the freshwater myxosporean diversification (Jirků et al. 2011) or it clusters with the *Myxidium lieberkuehni* clade (Rocha et al. 2013). The *M. lieberkuehni* clade consists of species infecting the urinary tract of freshwater fish as well as the freshwater urinary clade which is sister to the most diverse group of freshwater myxosporeans (the *Myxobolus* clade).

The freshwater urinary clade revealed by molecular phylogenetic analyses is a striking example of a taxonomically heterogeneous group of myxosporeans that are inferred to be phylogenetically closely related based on SSU sequence data. It consists of myxosporeans classified on the basis of morphological characters into seven different genera: *Acauda*, *Chloromyxum*, *Hoferellus*, *Myxidium*, *Myxobilatus*, *Ortholinea* and *Zschokkella* (Karlsbakk and Køie 2011; Whipps 2011). Ortholineids parasitize the urinary system of marine fish and as such represent an example where marine species cluster within a freshwater lineage.

The *Myxobolus* clade is the largest lineage within the myxozoan phylogenetic tree. It mostly includes species of the taxon-rich genera *Myxobolus* and *Henneguya*. The tree topology suggests that taxonomic characters defining platysporinid genera are not congruent with an evolutionary scheme based on DNA sequence data (see Chap. 4 for more discussion on the evolutionary trends within Platysporina). *Henneguya* is polyphyletic with species clustering at many different positions within the *Myxobolus* clade (Carriero et al. 2013). Other platysporinids with available molecular data in the NCBI database (*Cardimyxobolus*, *Hennegoides*, *Thelohanellus*, and *Unicauda*) are similarly scattered within the *Myxobolus* clade (Li and Sato 2014; Shin et al. 2014).

5.3.5 The Sphaerosporid Lineage

The *Sphaerospora* sensu stricto clade contains mostly coelozoic myxosporeans that infect the urinary tracts of marine and freshwater fish and amphibians and which typically possess long inserts in their rRNA genes (Jirků et al. 2007). The clade includes *S. elegans* (the type species of the genus) and other typical *Sphaerospora* species. It also contains species previously placed in other genera (*S. formosa* syn. *Bipteria formosa*, *S. fugu* syn. *Leptotheca fugu* and *S. sparis* syn. *Polysporoplasma sparis*). The clade is a strongly supported monophyletic lineage that usually clusters as sister to the freshwater and marine myxosporean clades (Bartošová et al. 2013).

Two sphaerosporid lineages (Fig. 5.1) share specific morphological, biological and sequence traits. Lineage A taxa (marine *Sphaerospora* spp.) have a single binucleate sporoplasm and shorter AT-rich rDNA inserts. Lineage B includes freshwater/brackish *Sphaerospora* spp. + marine/brackish *Sphaerospora* spp. (previously *Polysporoplasma* spp.) and is characterized by 2–12 uninucleate sporoplasms and longer GC-rich rDNA inserts. Lineage B has four subclades that correlate with host group and habitat (i.e. closely-related species infecting mugilids, cyprinids, salmonids or amphibians (Bartošová et al. 2013; Eszterbauer et al. 2013; Holzer et al. 2013a, b). *Sphaerospora fugu* and *S. molnari* are the only histozoic species (Lom et al. 1983; Tun et al. 2000) of the *Sphaerospora* sensu stricto clade. They cluster separately in Lineage

A (*S. fugu*) and Lineage B (*S. molnari*), indicating that a histozoic way of life has been converged on at least twice in the sphaerosporid lineage (Bartošová et al. 2013; Eszterbauer et al. 2013; Holzer et al. 2013a, b).

5.3.6 Actinospores in the Myxosporean Phylogenetic Tree

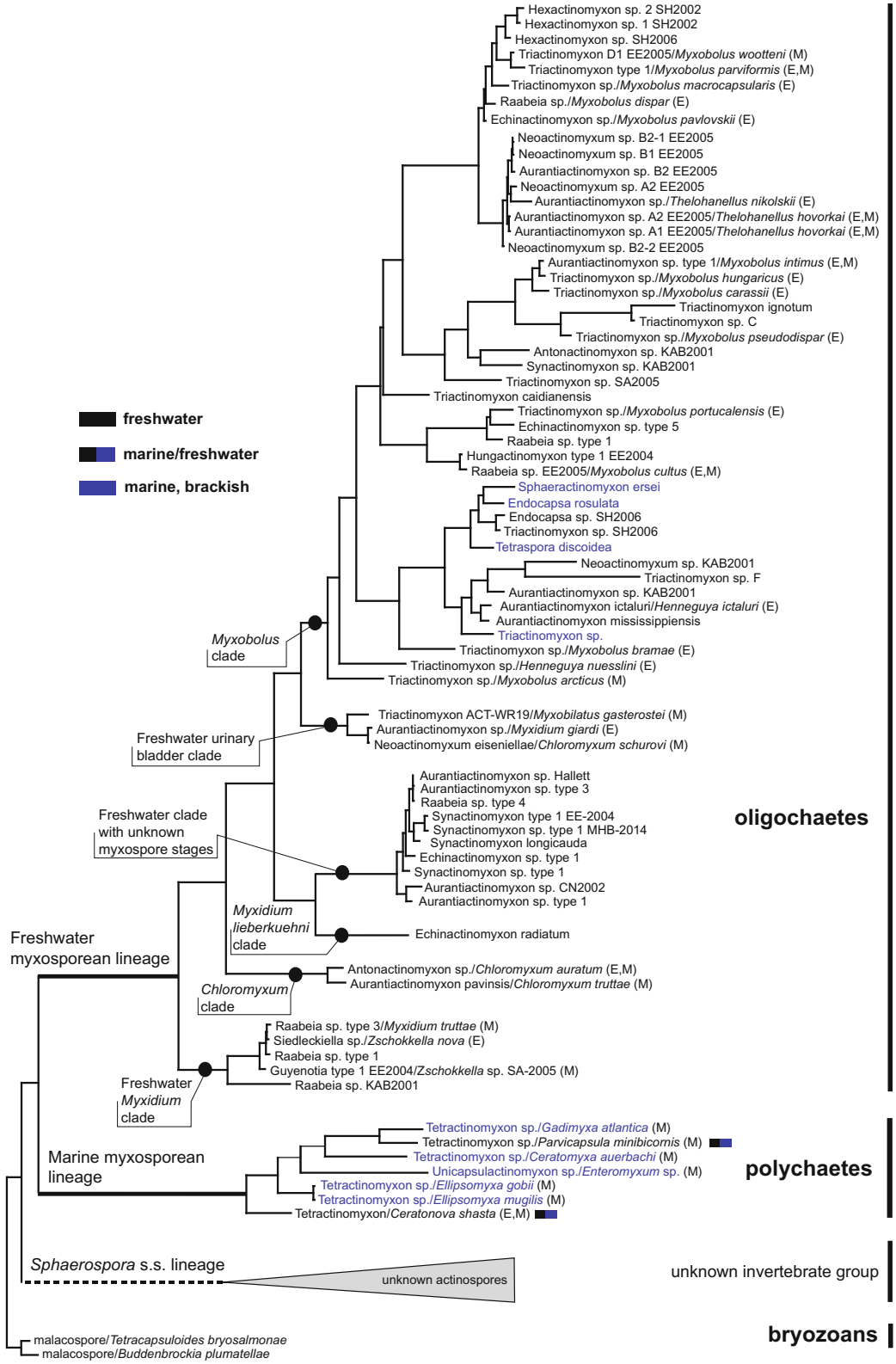
The vast majority of myxozoan life cycles are unknown and most sequences available in the NCBI database belong to their myxospore stages. In very few cases are sequences of actinospore-producing counterparts (henceforth called actinospore stages) linked with those producing myxospores. Figure 5.2 summarises myxosporean phylogenetic relationships of species with known actinospore stages. The *Myxobolus* clade encompasses the majority of actinospore stages available in the NCBI database representing a wide range of actinospore morphotypes (antonactinomyxon, auractinomyxon, echinactinomyxon, endocapsa, hexactinomyxon, hungactinomyxons, neoactinomyxum, raabeia, tetraspora, triactinomyxon, sphaeractinomyxon, and synactinomyxon) from oligochaetes in freshwater and marine environments (Hallett et al. 1999; Kent et al. 2001; Holzer et al. 2004; Eszterbauer et al. 2006; Székely et al. 2007). The freshwater urinary bladder clade contains stages of *Myxobilatus, Myxidium*, and *Chloromyxum* species from freshwater oligochaetes, and is characterized by three actinospore morphotypes (aurantiactinomyxon, neoactinomyxum and triactinomyxon) (Holzer et al. 2006). The *Myxidium lieberkuehni* clade is represented by a single actinospore morphotype (echinactinomyxon) from a freshwater oligochaete. Its myxosporean counterpart is unknown. The freshwater *Chloromyxum* clade includes antonactinomyxon and aurantiactinomyxon actinospores of *Chloromyxum* spp. from freshwater oligochaetes (Holzer et al. 2004). The freshwater *Myxidium* clade includes the actinospore morphotypes (guyenotia, raabeia, and siedleckiella) of *Zschokkella* and *Myxidium* spp. from freshwater oligochaetes (Kent et al. 2001; Eszterbauer et al. 2006). In addition, there is a well-defined lineage containing ten actinospore sequences representing four morphotypes (aurantiactinomyxon, echinactinomyxon, raabeia, and synactinomyxon) from freshwater annelids. Myxospores have not been identified and this lineage represents a novel clade within the freshwater myxosporean lineage.

The marine myxosporean lineage so far includes only two actinospore morphotypes, both from polychaetes: (i) tetractinomyxon of *Ceratonova*, *Ceratomyxa*, *Ellipsomyxa*, *Gadimyxa*, *Parvicapsula*, and *Sigmomyxa*, and (ii) unicapsulactinomyxon of *Enteromyxum* (Køie et al. 2004; Rangel et al. 2011).

To investigate the evolution of the Myxosporea, it would be of great interest to explore the morphology and definitive hosts of actinospore morphotypes of myxosporeans from vertebrate host groups other than fish (i.e. *Soricimyxum fegati* from shrews, *Myxidium* species from waterfowl and many myxosporeans infecting amphibians and reptiles) as well as to investigate the actinospore counterparts of species from marine environment that cluster within the freshwater lineage (i.e. *Sphaeromyxa* and several *Ortholinea*, *Zschokkella* and *Myxidium* species). The morphology of actinospore stages of the *Sphaerospora* sensu stricto lineage remains unknown (as do the definitive hosts) (see Chap. 10 for more details about myxosporean life cycle).

5.4 Conclusions and Future Recommendations

Although the SSU is an excellent marker that works well at different taxonomic levels, it has certain limitations and weaknesses in phylogenetic analyses (see Sect. 5.2.4). Other markers, such as LSU (Bartošová et al. 2009) and EF2 (Fiala and Bartošová 2010) add additional molecular characters, but many nodes in phylogenetic trees based on these markers remain unresolved or poorly supported. Therefore, new phylogenetic markers are needed to explore


freshwater
marine/freshwater
marine, brackish
Hexactinomyxon sp. 2 SH2002
Hexactinomyxon sp. 1 SH2002
Hexactinomyxon sp. SH2006
Triactinomyxon D1 EE2005/Myxobolus wootteni (M)
Triactinomyxon type 1/Myxobolus parviformis (E,M)
Triactinomyxon sp./Myxobolus macrocapsularis (E)
Raabeia sp./Myxobolus dispar (E)
Echinactinomyxon sp./Myxobolus pavlovskii (E)
Neoactinomyxum sp. B2-1 EE2005
Neoactinomyxum sp. B1 EE2005
Aurantiactinomyxon sp. B2 EE2005
Neoactinomyxum sp. A2 EE2005
Aurantiactinomyxon sp./Thelohanellus nikolskii (E)
Aurantiactinomyxon sp. A2 EE2005/Thelohanellus hovorkai (E,M)
Aurantiactinomyxon sp. A1 EE2005/Thelohanellus hovorkai (E,M)
Neoactinomyxum sp. B2-2 EE2005
Aurantiactinomyxon sp. type 1/Myxobolus intimus (E,M)
Triactinomyxon sp./Myxobolus hungaricus (E)
Triactinomyxon sp./Myxobolus carassii (E)
Triactinomyxon ignotum
Triactinomyxon sp. C
Triactinomyxon sp./Myxobolus pseudodispar (E)
Antonactinomyxon sp. KAB2001
Synactinomyxon sp. KAB2001
Triactinomyxon sp. SA2005
Triactinomyxon caidianensis
Triactinomyxon sp./Myxobolus portucalensis (E)
Echinactinomyxon sp. type 5
Raabeia sp. type 1
Hungactinomyxon type 1 EE2004
Raabeia sp. EE2005/Myxobolus cultus (E,M)
Sphaeractinomyxon ersei
Endocapsa rosulata
Endocapsa sp. SH2006
Triactinomyxon sp. SH2006
Tetraspora discoidea
Neoactinomyxum sp. KAB2001
Triactinomyxon sp. F
Aurantiactinomyxon sp. KAB2001
Aurantiactinomyxon ictaluri/Henneguya ictaluri (E)
Aurantiactinomyxon mississippiensis
Triactinomyxon sp.
Triactinomyxon sp./Myxobolus bramae (E)
Triactinomyxon sp./Henneguya nuesslini (E)
Triactinomyxon sp./Myxobolus arcticus (M)
Myxobolus clade
Triactinomyxon ACT-WR19/Myxobilatus gasterostei (M)
Aurantiactinomyxon sp./Myxidium giardi (E)
Neoactinomyxum eiseniellae/Chloromyxum schurovi (M)
Freshwater urinary bladder clade
Aurantiactinomyxon sp. Hallett
Aurantiactinomyxon sp. type 3
Raabeia sp. type 4
Synactinomyxon type 1 EE-2004
Synactinomyxon sp. type 1 MHB-2014
Synactinomyxon longicauda
Echinactinomyxon sp. type 1
Synactinomyxon sp. type 1
Aurantiactinomyxon sp. CN2002
Aurantiactinomyxon sp. type 1
Freshwater clade with unknown myxospore stages
oligochaetes
Myxidium lieberkuehni clade
Echinactinomyxon radiatum
Freshwater myxosporean lineage
Antonactinomyxon sp./Chloromyxum auratum (E,M)
Aurantiactinomyxon pavinsis/Chloromyxum truttae (M)
Chloromyxum clade
Raabeia sp. type 3/Myxidium truttae (M)
Siedleckiella sp./Zschokkella nova (E)
Raabeia sp. type 1
Guyenotia type 1 EE2004/Zschokkella sp. SA-2005 (M)
Raabeia sp. KAB2001
Freshwater Myxidium clade
Tetractinomyxon sp./Gadimyxa atlantica (M)
Tetractinomyxon sp./Parvicapsula minibicornis (M)
Tetractinomyxon sp./Ceratomyxa auerbachi (M)
Unicapsulactinomyxon sp./Enteromyxum sp. (M)
Tetractinomyxon sp./Ellipsomyxa gobii (M)
Tetractinomyxon sp./Ellipsomyxa mugilis (M)
Tetractinomyxon/Ceratonova shasta (E,M)
Marine myxosporean lineage
polychaetes
Sphaerospora s.s. lineage
unknown actinospores
unknown invertebrate group
malacospore/Tetracapsuloides bryosalmonae
malacospore/Buddenbrockia plumatellae
bryozoans

◀ **Fig. 5.2** SSU rDNA maximum likelihood tree with the focus on the phylogenetic relationships of actinospore morphotypes of myxosporean life cycle inferred from the molecular and/or experimental data. In the case of complete life cycle, the actinospore name follows the myxosporean species name. *E* life cycle proven by experimental methods, *M* life cycle proven by molecular methods. Final host groups are designated by *vertical lines*

certain myxozoan relationships. One promising marker is mitochondrial 12S rDNA which appears to be highly informative and to provide good nodal resolution (Fiala et al. 2013).

The diversity of myxozoan spore shapes and structures has enabled the creation of a sophisticated system of myxozoan classification and nomenclature. However, the molecular phylogenetics era has introduced a wealth of molecular characters, allowing for independent estimates of phylogeny that can be compared with traditional classifications. Such comparisons have revealed a general incongruence of morphological and molecular characters. Because only one synapomorphic morphological character has been found (i.e. polar filament organisation of *Sphaeromyxa* species; Fiala and Bartošová 2010), a whole evidence approach that combines morphological, biological (host tissue location, morphology of sporogonic and other developmental stages) and molecular characters should be applied wherever possible for species descriptions and the development and revision of myxozoan taxonomy and systematics (Lom and Dyková 2006; Holzer et al. 2010).

Continued exploration of myxozoan phylogenetic relationships to reveal the evolution of their morphological and ecological characters is essential to provide additional information for future taxonomic revisions that may help to solve persisting taxonomic discrepancies. Moreover, other morphological and ecological characters should be investigated to seek additional informative features for phylogenetic inference. Incongruent results of cladistic analyses based on the morphological characters of myxospore and actinospore stages (Xiao and Desser 2000) suggest that myxozoan systematics based only on myxospores may result in incorrect groupings. It was suggested that a more stable and predictive classification of myxozoans should be based on combined total evidence for both life-cycle stages (Xiao and Desser 2000). This, however, is impractical with our current knowledge of myxozoan life cycles, given that only a small fraction are known. Another feature of potential taxonomic value is information about myxozoan development. For example, five distinct sequences of sporogonial development have been shown to be congruent with five myxozoan clades (Morris and Adams 2008).

Our current understanding of myxozoan diversity and systematics is constrained in several ways. Additional taxon sampling is needed for certain genera, for which only a handful of species have been sequenced, e.g. *Myxidium*, *Zschokkella*, *Chloromyxum*, *Sphaeromyxa* and *Thelohanellus*. Moreover, we lack molecular data for many genera (including *Myxodavisia*, *Myxoproteus*, *Fabespora*, *Dicauda*). To date, DNA sequence data are available for 23 of the 64 myxozoan type species (Table 5.1). Some genera were described from remote locales or less abundant fishes, making sample collection difficult. Attempts should be made to gain sequences representative of these described types. In addition, wherever possible, molecular data for new type species and genera should be available before taxonomic changes are made (Fiala 2006; Lom and Dyková 2006; Holzer et al. 2010). Revisions are required to deal with the existing polyphyletic and paraphyletic genera. It is also important to evaluate the distinction between genera established on small differences (such as *Myxidium* and *Zschokkella* or *Myxobolus* and *Henneguya*) and whether certain genera should be suppressed. In other cases, while some existing genera may best be split, as was done for the genus *Leptotheca* and *Tetracapsula* (Canning et al. 2002; Gunter and Adlard 2010; Table 5.3).

Future taxonomic changes should follow general rules to avoid chaotic changes in myxozoan systematics. The discrepancies between genetic relationships and spore morphology-based classification raise an important issue of how to deal with assigning new species to a

taxon if molecular data are missing or if they disagree with morphology. The traditional scheme takes distinct spore types and assigns them to new genera or possibly families. When a phylogenetic relationship cannot be estimated, this phenotype-based approach is the most conservative because creation of a new genus does not require an existing generic description to be modified. If it is determined subsequently by genetic analysis that the new genus is nested within an existing one, then a simple synonymization can be made. Of course, in all cases, the greatest amount of evidence possible should be presented in order to make a strong case for describing new genera or assigning a morphologically distinct species to an existing genus. In some cases the species may correspond morphologically to a described genus, but by molecular phylogeny cluster elsewhere than the type species. In such cases, it is recommended that the myxozoan should be named as genus + species name + sensu lato, or alternatively, genus name + -like organism. This approach is more appropriate than erection of a new genus with uncertain taxonomic future. The critical distinction here is that if there is no morphological distinction from an existing genus, given the data in hand, the description of the genus becomes ambiguous. However, there may be even subtle morphological characters that can delineate these phylogenetically distinct lineages (Bartošová et al. 2011). Thus, if a myxozoan species morphologically does not correspond to some already described genus and does not cluster by molecular phylogeny inside some well defined monophyletic genus, it is recommended that a clear argument be made to establish a new genus.

5.5 Key Questions for Future Studies

- Is it possible to create a new classification of the Myxozoa that accounts for insights gained from molecular phylogenies along with morphological and other biological data?
- Will new DNA sequence data alter our general understanding of myxozoan phylogenetic relationships and taxonomy?
- Can we resolve the phylogenetic relationships of currently unstable clades in the marine myxosporean lineage?
- How many myxozoan genes are required to gain a phylogeny that reflects general patterns of evolution (not the evolution of specific genes)?

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Approaches for Characterising Myxozoan Species

6

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Abstract

Myxozoan species and genera have been defined traditionally using morphological characteristics of spores and developmental stages, host preference and tissue specificity. Use of morphology is, however, limited in some taxa by a lack of reliable characters or ambiguities in their origin. For instance, morphological variation can characterise spores of closely-related species while similar spore morphologies in distantly-related species reflect convergence. Therefore, morphological traits are accompanied routinely by DNA sequences in the identification of myxozoan species. DNA sequencing methods have inherent limitations, too, which include co-amplification of host, inability to distinguish mixed infections and PCR and primer biases for some taxa over others. By combining several approaches, myxozoan researchers are revealing novel diversity and demonstrating that strains and morphologically cryptic species characterise many taxa. Extensive geographic sampling of economically significant myxozoans, including *Myxobolus cerebralis*, *Kudoa thyrsites*, *Ceratonova shasta* and *Tetracapsuloides bryosalmonae*, has demonstrated

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intraspecific genetic variation, strains linked with geographic localities, and species complexes. Application of new approaches to myxozoan classification is expected to facilitate reassessment of existing taxa, discovery of new taxa and further resolution of cryptic species.

Keywords
Genotype · Cryptic species · Species complex · Morphology · Molecular markers · SSU

6.1 Introduction

Allocation of organisms into hierarchical groupings is central to biology. This process involves examination and characterisation of individuals using one or more accepted diagnostic methods to assign them to taxa. Frequently however, no one method can be applied across all taxa, and different methods of categorisation can yield different results, especially at the genus and species levels of taxonomic ranking (see Mayden 1997). For instance, while two or more species may be indistinguishable using morphological criteria, molecular sequence information may enable discrimination. In such cases, they are referred to as "cryptic species" (Pfenninger and Schwenk 2007; Pérez-Ponce de León and Nadler 2010). Typically, recognition of cryptic species occurs when new information demonstrates that multiple species characterise what once was believed to be a single species. For parasites, such information may include unique DNA sequences, or consistent differences in host preference or tissue tropism (e.g. Nolan and Cribb 2005; Martinsen et al. 2006). Above the species level, closely related species may form species complexes, while below the species level, organisms may be discriminated if they can be assigned to e.g. strains, genotypes or populations. In this chapter we review how myxozoan species have been characterised and the problems that have arisen in species discrimination. We then explore cases of cryptic speciation, evidence for species complexes and examples of strain-level discrimination within myxozoan species. Our review demonstrates how the unprecedented loss of morphology resulting from adaptations to an endoparasitic lifestyle (see Chap. 3) combined with considerable plasticity and convergence in spore morphology pose challenges for discriminating myxozoan species.

6.2 Use of Morphology for Species Discrimination

For much of the 20th century, myxozoan researchers described species of Myxosporea and Actinosporea using morphology of mature spore stages (Shul'man 1966). The former were characterised based on myxospores, which develop in vertebrate hosts, and the latter on actinospores, produced in invertebrate hosts. This practice was modified after Wolf and Markiw (1984) demonstrated that myxospores and actinospores are conspecific myxosporean stages that develop in alternating hosts. Thus, class Actinosporea was suppressed, and myxosporean genera and species are now described from myxospore stages, while actinospores are described as collective groups and types (Kent et al. 2001; Lom and Dyková 2006).

Morphological characters including symmetry, polar capsule orientation and surface features are used traditionally to classify myxospores to species level (Fig. 6.1) (Lom and Dyková 2006; Chap. 5). Use of spore morphology, however, has its limitations. For instance, a lack of suitable characters can be problematic for distinguishing morphologically simple myxospores, such as *Myxobolus* species, which can lead to taxonomic ambiguities. This has impelled researchers to incorporate morphology of vegetative stages (pseudoplasmodia, plasmodia, sporoblasts) (e.g.

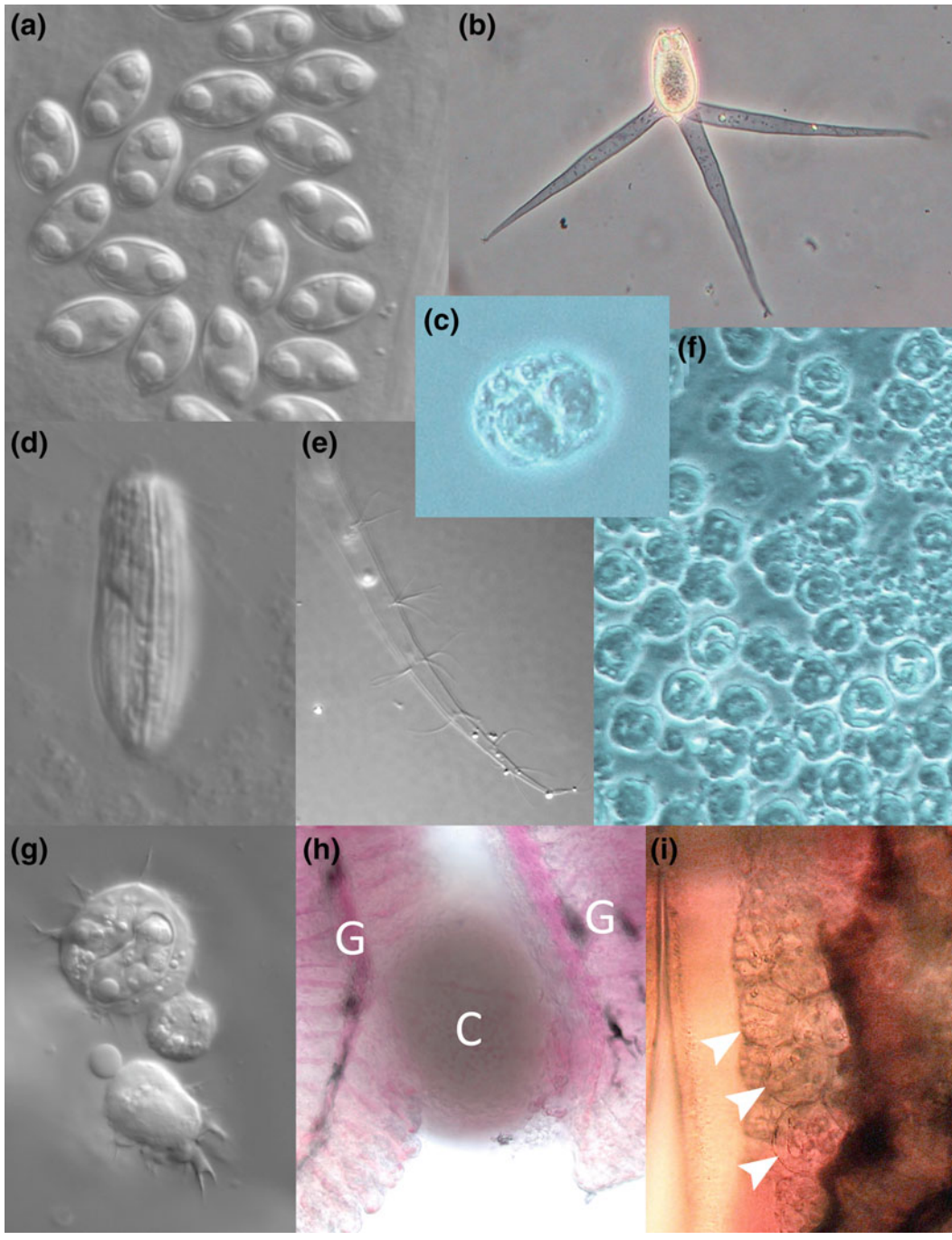

Fig. 6.1 Examples of myxozoan morphological and developmental characters used to define species. Overall spore morphology: **a** *Myxidium* myxospores, **b** Echinactinomyxon type actinospore, **c** *Tetracapsuloides* malacospore; Other morphological characters: **d** *Acauda* surface ridges, **e** Triactinomyxon type actinospore with valve cell projections; Vegetative stages: **f** ruptured sac of *Tetracapsuloides*, **g** filiform pseudopodia in *Ceratonova*; Site of development in host: **h** *Myxobolus* pseudocyst (*C*) in epithelium of fish gills (*G*), **i** pansporocysts (*arrowed*) of Raabeia type in coelom of tubificid oligochaete worm

Fig. 6.1f, g) and tissue specificity (e.g. Fig. 6.1h, i) (see Sect. 6.3.2) in species definitions (Kent et al. 2001; Lom and Dyková 2006). A lack of discriminatory spore characters is especially problematic in the class Malacosporea (e.g. Fig. 6.1c), where species descriptions are augmented with other morphological criteria such as spore sac size and shape, and host preference (Canning and Okamura 2004; Bartošová-Sojková et al. 2014; Hartikainen et al. 2014).

Below we elaborate more specifically on how morphological variation can result in character ambiguity and the challenges this poses for species discrimination in various myxozoan taxa.

6.2.1 Ambiguity of Characters

Fiala and Bartošová (2010) examined character evolution across many myxozoan genera, including *Sphaerospora*, *Sphaeromyxa*, *Myxobolus*, *Henneguya*, *Kudoa*, *Chloromyxum* and *Myxidium*. They concluded that only a few morphological characters corresponded with the molecular phylogeny derived from SSU data. They attribute discrepancies between molecular phylogenies and morphology-based taxonomy to plasticity in myxospore morphology. Such plasticity is evident from several studies that demonstrate that differences in spore morphology may not be associated with significant genetic divergence. For example, caudal appendages have been used traditionally to distinguish *Henneguya* from *Myxobolus*, however, molecular phylogenetic analyses show *Henneguya* species do not form a single clade but are scattered within the larger *Myxobolus* clade (Kent et al. 2001; Fiala 2006; Liu et al. 2010). Similarly, the split of *Myxodavisia* from *Ceratomyxa* based on the presence of valve cell projections is not supported by DNA data (Fiala et al. 2014). Molecular data also do not support discrimination of *Polysporoplasma* from *Sphaerospora* based on the number of sporoplasms (Bartošová et al. 2013). Furthermore, spore characters that provide reliable information for one genus may not be useful for another. For example, the number of myxospore polar capsules was used to distinguish *Pentacapsula*, *Hexacapsula* and *Septemcapsula* as distinct genera, but these taxa cluster phylogenetically as the single genus *Kudoa* using SSU sequence data (Whipps et al. 2004b). In contrast, the number of polar capsules remains a fundamental character for defining *Thelohanellus* (one capsule) from *Myxobolus* (two capsules). Hence, caution is needed when using only spore morphology for taxon discrimination.

Plasticity in spore morphology can be expected to result in convergence of spore morphotypes (Fiala and Bartošová 2010), with particular characters arising independently in different phylogenetic groups. Examples of convergence include polar capsules situated at opposite ends of elongated myxospores of *Zschokkella* and *Myxidium*, multiplication of polar capsules in *Chloromyxum* and *Kudoa*, and the presence of surface ridges on myxospores of *Myxidium*, *Zschokkella*, *Chloromyxum* etc. (see Chap. 4 for expanded discussion of these examples).

A further problem of using spore morphology in species discrimination is the high level of natural morphological and morphometric variation in spores, both within and between hosts. Spores change in shape and size as they mature, as exemplified in studies of *Sphaerospora angulata* and *S. dykovae* (syn. *S. renicola*) (Holzer et al. 2013) and *Zschokkella pleomorpha* (Lom and Dyková 1995). Hence, as myxozoan infections typically feature asynchronous spore development, a given sample will include a range of spore ages and thus morphologies. Recently, Principal Component Analysis (PCA) has been employed to control for spore variation. This approach reduces measurements of spore features to sets of uncorrelated variables (components) and plots pairs of these against each other in two-dimensional space. PCA has been used to distinguish species of *Auerbachia* (Heiniger et al. 2011), *Coccomyxa* (Heiniger et al. 2011), *Kudoa* (Burger and Adlard 2010) and *Sphaerospora* (Holzer et al. 2013). Heiniger and Adlard (2013) used a combination of morphological (spore variation assessed with PCA), biological (host, locality)

and sequence (SSU, LSU) characters to identify ceratomyxids from cardinal fishes (Apogonidae). Their data show significant genetic and biological differences but minimal morphological variation amongst spores, thus providing evidence for cryptic species of *Ceratomyxa*.

6.3 Other Non-DNA Based Characters for Discriminating Species

6.3.1 Host Specificity

Most myxozoan species appear to infect a single host family or species, thus host identity is an important character for distinguishing myxozoans (Molnár 1994; see Chap. 16). However, host data should be used in combination with other data, as at least some myxozoans appear to infect a broad range of hosts (e.g. *Sphaeromyxa balbianii*, Lom 2004; *Kudoa thyrsites*, Whipps and Kent 2006; *Enteromyxum leei*, Sitjà-Bobadilla and Palenzuela 2012). A combined approach supported the validity of *Chloromyxum legeri* from common carp (*Cyprinus carpio*) as a species distinct from *C. fluviatile* from silver carp (*Hypophthalmichthys molitrix*) (Bartošová and Fiala 2011). The two myxozoans were characterised by different host preferences and substantial SSU sequence variation (up to 22.3 %). In contrast, synonymy of *C. cyprini* with *C. cristatum* isolates (from grass carp, *Ctenopharyngodon idella*, and silver carp, respectively) was confirmed by showing that the different host-specific isolates had low genetic variation in the SSU (0.56 %) and LSU (up to 0.4 %) (Bartošová and Fiala 2011). *Chloromyxum cristatum* is therefore regarded as having low host specificity (see also Sect. 6.6.2).

6.3.2 Tissue Specificity

Many myxozoans have a preference for particular sites for spore development within hosts (e.g. Eszterbauer 2004; Holzer et al. 2004; see also Chap. 16). This 'tissue tropism' has been used to support transfer of some members of the genus *Leptotheca* to *Ceratomyxa* (gall bladder) and others to *Sphaerospora* (excretory system) (Gunter and Adlard 2010). However, when distinguishing myxozoan species by their tissue tropism it is important to confirm that the parasite is targeting particular host tissues and not present incidentally as a result of: non-selective invasion of an atypical fish host (Holzer et al. 2013), host immune processes (e.g. spores sequestered in kidney or spleen melanomacrophage centres), rupture of cysts and physical spread of spores throughout the body cavity or tissues, or consumption of myxozoan-infected prey items (e.g. *Henneguya* in humans; see Chap. 7). Furthermore, tissue specificity may not apply or may be difficult to infer in some cases. For instance, some myxozoans may develop in multiple tissues in more susceptible hosts (e.g. *Ceratonova shasta*, Hurst and Bartholomew 2012).

Tissue tropism has been used in combination with spore morphology and DNA analyses to resolve cryptic myxozoan species. For instance, *Myxobolus insidiosus* was described from cysts in skeletal muscle of Chinook salmon (*Oncorhynchus tschawytscha*), coho salmon (*Oncorhynchus kisutch*), rainbow trout (*O. mykiss*) and cutthroat trout (*O. clarki*) (Amandi et al. 1985). Ferguson et al. (2008) examined histological sections from various salmonids and observed *M. insidiosus*-like myxospores confined to either nerves within the skeletal muscle or to the muscle itself. Spores that matched the description of *M. insidiosus* were found only in skeletal muscles of Chinook and coho salmon. Therefore, a new species, *Myxobolus fryeri*, was described from peripheral nerves of coho salmon, and rainbow and cutthroat trout. Although the SSU sequence variation between *M. fryeri* and *M. insidiosus* was relatively low (0.5 %), a diagnosis of separate species was supported by distinct tissue tropisms and statistically significant differences in spore length. Similar evidence was used to identify other cryptic *Myxobolus* species. For instance, *Myxobolus intramusculi* was distinguished from *M. procerus* based on

tissue tropism in trout-perch (*Percopsis omiscomaycus*) (intramuscular versus in connective tissue), and significant differences in myxospore lengths but low SSU variation (~2 %) (Easy et al. 2005). Molnár et al. (2010) used fine-scale tissue tropism to characterise morphologically similar *Myxobolus* species from gills of roach (*Rutilus rutilus*). They observed that *M. sommervillae* developed in blood vessels whereas *M. rutili* development was extravascular in the gill filament. SSU variation of >10 % provided further discrimination of the two species. These examples illustrate the importance of identifying the exact site of infection, which can even relate to specific parts of a tissue or organ, as this may be an essential character for recognising myxozoan species.

6.4 DNA Sequence Data

Unlike phenotype, the fidelity of a myxosporean's DNA is maintained throughout its life cycle. DNA analysis therefore can be used to identify and compare myxosporean DNA from different sources, which include hosts (developmental and cryptic stages in vertebrates and invertebrates), parasite spores (released or purified from hosts), environmental samples (mud, water, etc.), and samples from different geographic regions. Molecular methods are especially useful for identifying morphologically different yet conspecific spore stages and thus inferring multi-host life cycles (see Chap. 10). Identification of novel myxozoans using DNA is becoming easier with the increasing number of myxozoan sequences in the NCBI Nucleotide database.

6.4.1 Ribosomal RNA Gene Sequences

By far the most myxozoan data available in the NCBI database exist for nuclear ribosomal RNA gene sequences. The SSU in particular has become an integral part of myxozoan species descriptions and phylogenetic studies (e.g. Kent et al. 2001; Eszterbauer 2004; Fiala and Dyková 2004; Fiala 2006; Burger et al. 2007). As with most eukaryotes, the ribosomal RNA gene array in myxozoans typically comprises 2 longer subunits, the SSU (1.8–2.0 kb) and the LSU (3.5–3.9 kb), separated by shorter regions (ITS-1, 5.8S, ITS-2). A notable exception is the myxozoan *Sphaerospora sensu stricto* clade, whose members have characteristically longer ribosomal genes: SSU up to 3.7 kb and LSU up to 6.3 kb (Bartošová et al. 2013). Much like bacterial SSU sequences, myxozoan SSU and LSU have well-defined, alternating conserved and hyper-variable regions (Nelles et al. 1984). Conserved regions facilitate primer design and sequence alignment (e.g. Holzer et al. 2007) and comparisons of variable regions provide insights on genetic variation. Interspecific SSU variation in the Myxosporea is typically >2 % (Fiala 2006) but within some genera, e.g. *Myxobolus*, interspecific SSU differences can be as low as 0.2 % (Ferguson et al. 2008; see Sect. 6.4.2). Interspecific SSU variation in the Malacosporea is >1 % (Bartošová-Sojková et al. 2014; Hartikainen et al. 2014), although this figure may change as more malacosporeans are sequenced. Intraspecific SSU differences typically range from 0–3.6 % (Schlegel et al. 1996; Andree et al. 1999; Ferguson et al. 2008). The LSU is 2–3 times longer than the SSU but has been shown to have a similar level of interspecific variation and thus provides similar taxonomic information (e.g. Bartošová et al. 2009, 2013; Burger and Adlard 2011).

The substantial variation in levels of interspecific and intraspecific divergence based on SSU sequence data can result in overlap in ranges of variation between taxa, which may confound species identification. The variation also suggests that there has been disparity in rates of molecular evolution within the Myxozoa.

6.4.2 Other Molecular Markers

Markers such as the ITS can be used to test for the presence of cryptic species if these are suggested by differences in some other character (i.e. morphology, host, tissue, geography). ITS-1

sequences are more variable than SSU and LSU, and have been used to discriminate between myxozoan subpopulations from different geographic areas or hosts, for example *M. cerebralis* (Whipps et al. 2004a), *C. shasta* (Atkinson and Bartholomew 2010a) and *Cystodiscus* spp. (Hartigan et al. 2012). In some species, the ITS-1 may have high levels of intragenomic variation (e.g. kudoids: Whipps and Kent 2006), which must be taken into account when using the ITS for phylogeographic studies.

The use of several other genes for discriminating myxozoans at various levels has been investigated to a limited extent. Loci include HSP70 (*Kudoa thyrsites*, Whipps and Kent 2006; *Kudoa dianae* and *Myxidium* spp., Bartošová 2010; *C. shasta*, Atkinson unpublished; malacosporeans, Hartikainen unpublished); EF-2 (*Kudoa* spp., *Henneguya* spp., *Myxidium* spp., *Zschokkella* spp. and others, Fiala and Bartošová 2010; Bartošová et al. 2013); and several proteases (*K. thyrsites*, Funk et al. 2008; *M. cerebralis*, Kelley et al. 2004). Jiménez-Guri et al. (2007a) found several protein-coding genes (*rad51*, *AdoMet*, *rpl27e* and *rpl23a*) discriminated between worm-like and sac-like malacosporeans (*Buddenbrockia plumatellae* and *Tetracapsuloides bryosalmonae*, respectively). Some preliminary myxozoan mitochondrial 12S and COX-1 sequences have been reported (Fiala et al. 2013). There is presently low taxonomic coverage across the Myxozoa at these other loci compared with the nuclear ribosomal RNA genes, hence they are not yet applicable broadly for species discrimination or examination of divergence within species. As myxozoan whole genome sequences become available over the next 5–10 years, further markers for species discrimination are likely to be identified.

6.5 Problems with DNA Sequence Data

Inferences based on DNA sequence data for parasite identification can be confounded by various factors. Below we elaborate on how amplification of host DNA, mixed infections and PCR bias can impede accurate myxozoan identification.

6.5.1 Inadvertent PCR Amplification of Host DNA

Co-amplification of parasite and host or other contaminating organisms is possible and can be especially problematic when general PCR primers are used. For example, "myxozoan" HOX genes (Anderson et al. 1998) were revealed later to be of host origin (Jiménez-Guri et al. 2007b). Problems in interpretation can arise also when the range of variation at a particular locus is not well understood. For instance, unresolved co-amplification of host and parasite tissue led to a chimeric sequence for *Sinuolinea* sp. (NCBI: AF378346; Kent et al. 2001) in which the first 450 bp represented the fish host (Holzer et al. 2010a). Most chimeric or otherwise ambiguous sequences have now been either re-sequenced or removed from the NCBI database.

6.5.2 Mixed Infections

Mixed myxozoan infections are common in fish hosts. For instance, *Sphaerospora testicularis* and *Sphaerospora dicentrarchi* in seabass (*Dicentrarchus labrax*; Fioravanti et al. 2004), *Zschokkella hildae* and *Gadimyxa atlantica* in Atlantic cod (*Gadus morhua*; Holzer et al. 2010b), *Sphaerospora* spp. with other myxosporean species in cyprinids (Bartošová et al. 2013; Holzer et al. 2013), and *Chloromyxum* and *Zschokkella* spp. in cyprinids (Bartošová and Fiala 2011). The presence of multiple myxozoans in a particular DNA sample can lead to amplification of non-target spores or cryptic stages (Bartošová et al. 2013). Extrasporogonic myxozoan stages in blood can be particularly widespread within the host, not just at the site of sporogenesis, and may confound interpretations of infections (Holzer et al. 2013). Subsampling small portions of heavily-infected tissue or cysts should improve

the quality of sequenced material and exclude contamination e.g. from spores consumed incidentally with food. Sampling replicates (several infected hosts) would also increase confidence in sequence identity. In situ hybridisation probes can then be used to confirm the identity and location of myxozoans within a host (e.g. Eszterbauer 2011; Eszterbauer et al. 2013).

6.5.3 PCR Bias

PCR or primer bias towards some myxozoan taxa over others can lead to amplification of non-target myxozoans. This may particularly compromise the identification of cryptic mixed infections. For example, *Myxobolus* sp. was misidentified as *Sphaerospora molnari* in goldfish (*Carassius auratus*; Kent et al. 2001) and as *S. dykovae* in common carp (*Cyprinus carpio*; Eszterbauer and Székely 2004), *Myxidium/Zschokkella* spp. were misidentified as *S. angulata* in goldfish (Eszterbauer and Székely 2004) and as *S. oncorhynchi* (Kent et al. 1998) and *S. elwhaiensis* in salmonids (Jones et al. 2011). These misidentifications have been clarified by more recent re-sequencing (Eszterbauer et al. 2006, 2013; Bartošová and Fiala 2011; Eszterbauer 2011; Bartošová et al. 2013; Holzer et al. 2013; Bartošová-Sojková unpub. data). The atypically long, divergent SSU sequences of *Sphaerospora sensu stricto* spp. renders their detection especially problematic as they are prone to low or no amplification when other myxozoan DNA is present and general primers are used (Bartošová et al. 2013).

The likelihood of amplifying the 'correct' myxozoan can be improved by trialling a range of myxozoan general primers or established genus-specific primers (e.g. Hallett and Diamant 2001; Kent et al. 2001; Whipps et al. 2004b; Bartošová et al. 2013; Hartikainen et al. 2014). Decreasing costs of DNA sequencing should enable metagenomic approaches, which are free of many PCR-related biases.

6.6 Intraspecific Variation and Species Complexes

6.6.1 Intraspecific Genetic Variation

DNA sequence data (see Sects. 6.1, 6.2) have been used to map patterns of intraspecific variation and to gain insights on dispersal and evolutionary history, particularly in the context of invasive species and emerging diseases (see Chap. 18). Most population studies have been constrained to economically important myxozoans with wide geographic distributions, several examples of which are described below.

Myxobolus cerebralis, the causative agent of whirling disease in salmonid fishes, is perhaps the most widely studied pathogenic myxozoan. The parasite is of European origin, but has been introduced to at least 26 countries by human activities (Hoffman 1970; Bartholomew and Reno 2002). Andree et al. (1997) and Whipps et al. (2004a) demonstrated low levels of genetic variation between *M. cerebralis* from Germany and the United States, using SSU and ITS-1 sequences, respectively. *M. cerebralis* isolates from a broader geographic range and a larger number of hosts revealed greater variation in the ITS-1 than found previously, and demonstrated that representatives from West Virginia, USA were genetically distinct (Lodh et al. 2012). The authors concluded that there may have been multiple introductions of *M. cerebralis* to the USA.

Similar phylogeographic studies have been undertaken on the causative agent of salmonid proliferative kidney disease, the malacosporean *T. bryosalmonae*. Genetically distinct parasite populations were identified in European and North America based on variation in SSU and ITS-1 (Okamura et al. 2001; Henderson and Okamura 2004; Tops et al. 2005). The greater genetic variation among North American isolates suggests a North American origin for this species (Henderson and Okamura 2004). High levels of divergence of most European isolates suggests an introduction to Europe that pre-dates aquaculture,

but isolates from Italy and France that group within the North American clade are evidence for further dispersal events. Thus, multiple introductions may have contributed to the genetic diversity observed within Europe (see Chap. 18). Whether the isolates from Europe and North America are representative of a single species with a broad distribution rather than a species complex is unclear, but could be tested by cross-infection studies with rainbow (native North American fish hosts) and brown trout (native European fish hosts) using North American and European isolates of the parasite (see also Chap. 11).

Ceratonova shasta (*syn. Ceratomyxa shasta*; Atkinson et al. 2014), a virulent intestinal pathogen of multiple species of salmon and trout in the Pacific Northwest (Noble 1950), has intraspecific genetic variation associated with river basins and vertebrate host species. Initially, variations in the ITS-1 region were linked with differences in severity of infection and mortality between sympatric (i.e. native to the basin) *O. tshawytscha* and allopatric (i.e. out-of-basin) *O. mykiss*, which had been exposed experimentally to the parasite in the Klamath River (Oregon/California; Atkinson and Bartholomew 2010a). Additional sampling of *C. shasta* from multiple salmon and trout species revealed four principal ITS-1 genotypes (O, I, II, III; Atkinson and Bartholomew 2010a, b) that have marked host preferences and different virulence in each fish host. All genotypes appear to infect a single invertebrate host—a new species of freshwater polychaete belonging to the genus *Manayunkia* (Atkinson, in prep.). It is now clear that the genotypes are present in river basins from California to British Columbia (Stinson 2013) and can occur in sympatry, which indicates little geographical structure in the parasite population. These sympatric distributions of genotypes illustrate the profound isolating effects of utilising different vertebrate hosts and suggest that parasites and hosts may have co-evolved for a sufficient period to modify host-parasite interactions and influence virulence.

6.6.2 Species Complexes

Several myxozoan taxa are now regarded as species complexes. Species complexes may arise when closely related populations diverge sufficiently to develop into distinct entities, for instance when populations become established in different regions or hosts. Studies to date suggest that species complexes often may be comprised of cryptic species.

"*K. thyrsites*" infects multiple marine fish species across a broad geographic distribution and appears to represent a species complex. Variation in myxospore morphology between regional representatives of *K. thyrsites* is inconsistent. Molecular phylogenetic analyses based on four genetic markers (HSP70, SSU, LSU, ITS-1), however, revealed geographically distant isolates associated uniquely with Japan, Australia, eastern Pacific, and eastern Atlantic. Within these regions there is little additional genetic structure, which suggests high local gene flow and low host specificity (Whipps and Kent 2006).

Chloromyxum leydigi has been recorded from the gall bladder of 21 elasmobranch species in multiple genera (Rocha et al. 2014, and summarised in Gleeson and Adlard 2012). Most of these records pre-date PCR and identification relied on spore morphology and site of infection. The ambiguity of some characters, together with the atypically broad host range suggests that *C. leydigi* is a species complex, as shown for other *Chloromyxum* spp. (Bartošová and Fiala 2011). The diagnosis of *C. leydigi* as a species complex is supported by recent genetic data from *Chloromyxum* spp. with similar morphology to *C. leydigi*, from a range of hosts (Gleeson and Adlard 2012; Rocha et al. 2014).

Similarly, "*Parvicapsula minibicornis*" may represent a species complex. The parasite infects kidneys of salmon and trout in the Pacific Northwest of North America, with disease severity and presentation varying among salmon species and geographical localities (Jones et al. 2003).

Parasite SSU sequences are >98.9 % similar across different hosts and localities (Atkinson et al. 2011). However, sufficient sequence variation exists to define 15 genetic types, many of which exist in sympatry and some of which correlate with fish host species or sample locality. These data indicate that the *P. minibicornis* species complex is structured by both geography and salmonid host species.

We expect that future work will reveal many more examples of intraspecific variation, cryptic speciation and species complexes within the Myxozoa, particularly within genera that have few morphological characters (e.g. *Myxobolus*) or where morphological characters used in traditional taxonomy (especially spore characters) have high intraspecific variation (e.g. *Ceratomyxa*, *Ceratonova*, *Kudoa*). We also anticipate that some future examples of intraspecific variation will reflect historic transport of myxozoans to new geographic regions.

6.7 Conclusions

The evolutionary switch made by once free-living cnidarians when adapting to an endoparasitic existence involved profound morphological simplification. The loss of these characters, along with intraspecific plasticity of spore form and interspecific convergence of features creates challenges for myxozoan taxonomists to discriminate species accurately. The most robust species descriptions integrate multiple characters, including morphology, ultrastructure, tissue tropism, life-cycle, host factors and SSU sequence data. Application of new approaches and technologies will continue to facilitate reassessment (including re-description) of existing taxa, discovery of new taxa, identification of species complexes, and resolution of cryptic species (e.g. Bartošová-Sojková et al. 2014; Hartikainen et al. 2014). The discovery of novel SSU sequences in environmental samples is expected to reveal new myxozoan diversity (e.g. Hartikainen et al. in prep). We predict that DNA sequencing will continue to reveal that many myxozoans, especially those presently regarded as having broad geographic and host ranges, are complexes of cryptic species. Hence, as with many other taxa (Blaxter 2003), myxozoan diversity is presently underestimated.

6.8 Key Questions for Future Studies

- Are there better markers than the SSU for species resolution?
- Do some myxozoan groups have a higher propensity for cryptic speciation?
- What are the adaptive values of convergent characters?
- How can we best develop species databases to facilitate classification and recognition of myxozoan taxa?

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Myxozoans Exploiting Homeotherms

7

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Abstract

Discoveries published in 2007 and 2008 expanded the known host range of myxozoans beyond poikilotherms to include mammals and birds. Here we review records of myxozoans from small terrestrial mammals, waterfowl and those associated with humans, and augment them with data from our ongoing studies. True myxosporean infections—those with active parasite development and sporogenesis—have been recorded for *Soricimyxum* spp. in central European shrews and *Myxidium* spp. in North American waterfowl. In all cases, bile ducts within the liver were the nidal tissue and complete life cycles are unknown. Incidental myxosporean infections—the presence of myxospores without parasite development—have been observed in humans, usually in association with the ingestion of infected fish. Clinical presentations of these cases range from no disease (e.g. *Henneguya* spp.), allergic responses (*Kudoa* sp.) or acute gastroenteritis (*Kudoa septempunctata*). Phylogenetically, myxosporean parasites of homeotherms cluster closely with *Myxidium* and *Cystodiscus* species known to infect other terrestrial vertebrates (reptiles and amphibians), which suggests a single evolutionary expansion from an aquatic *Myxidium*-clade ancestor to semi-aquatic and terrestrial hosts and environments. Given the diversity of potential mammalian and avian hosts, we expect additional myxosporean parasites

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to be discovered with further scrutiny of these homeotherms, especially in sparsely surveyed regions including Africa and South America.

Keywords
Homeotherms · Small mammals · Waterfowl · Humans · *Soricimyxum* · *Myxidium* · *Kudoa*

7.1 Introduction

For the first 150 years after their discovery, myxozoans were regarded exclusively as parasites of poikilothermic vertebrates and aquatic invertebrates. The overwhelming majority, some 2,000 species, have been described from fishes in marine, brackish and freshwater habitats from all continents except Antarctica. Myxosporeans in non-fish vertebrate hosts have been known for almost as long as those in fishes but account for <5 % of records, with some 50 species from chelonid reptiles and amphibians (Eiras 2005; Garner et al. 2005; Jirkù et al. 2006) (Fig. 7.1).

At the turn of the 21st century, the known host range of Myxozoa was broadened to include terrestrial homeotherms. These records from small mammals and birds are limited in both geographic and taxonomic diversity: two myxosporean species are known from three species of shrew in Europe (Prunescu et al. 2007; Dyková et al. 2007; Székely et al. 2011) (Table 7.1) and at least two species of myxosporeans are known from six species of waterfowl from North America (Bartholomew et al. 2008; Atkinson, unpublished data) (Table 7.2). The presence in small mammals and birds of a range of developmental stages, from presporogonic through to mature myxospores, is indicative of true host status rather than of a vector or incidental occurrence. The latter describes the nature of infections in humans, in which only myxospores have been observed in association with consumption of infected fish.

Herein, we review records of myxozoans from small terrestrial mammals, waterfowl and those associated with humans. We discuss the phylogenetic context of myxosporeans in homeotherms, possible sources of sample bias, factors that could influence host range expansion into homeotherms, possible modes of transmission and conclude with key questions for future studies.

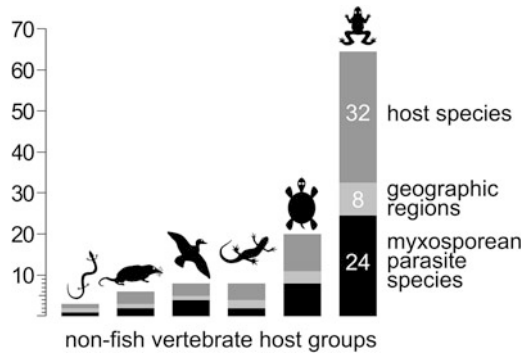

Fig. 7.1 Myxosporean parasitism in non-fish vertebrates, from, *right-to-left*: frogs/toads, turtles/tortoises, newts/salamanders, waterfowl, small mammals, primitive caudates (*Proteus*). The number of host species, the number of geographic regions from which they have been recorded (North America, South America, Africa, Europe, North Asia, South Asia (India), Southeast Asia, Australia) and the number of recorded myxosporean parasite species are indicated

7.2 Small Mammals

The first observation of a "myxozoan" developing in a host other than an aquatic poikilotherm was of myxosporean-like presporogonic stages (cells within cells) in the brains of wild Austrian moles (*Talpa europaea*; Friedrich et al. 2000). Xenomic (hypertrophic growth of an invaded host cell) pericytes of brain capillaries were found in 55 % of 55 moles, collected in 1982–1985 and 1994. Although this high prevalence is inconsistent with merely accidental infection, the lack of mature spores confounds definitive identification of the parasite and no DNA sequence data exist for comparison. Similar xenoparasitic complexes, again containing a

Table 7.1 Terrestrial mammalian hosts of myxozoans

Host common name	Host scientific name	Myxozoan	Reference
Common shrew	*Sorex araneus*	*Soricimyxum fegati*	Prunescu et al. (2007), Dyková et al. (2007, 2011), Székely et al. (2011)
Pygmy shrew	*Sorex minutus*	*Soricimyxum fegati*	Dyková et al. (2011)
		Soricimyxum minuti	Székely et al. (2011)
Lesser white-toothed shrew	*Crocidura suaveolens*	*Soricimyxum fegati*	Dyková et al. (2011)

Two myxosporean species infect European shrews

Table 7.2 Avian hosts of myxozoans, all of which are waterfowl from North American localities

Host common name	Host scientific name	Myxozoan *Myxidium* spp.	USA locality	Source
Pekin duck*	*Anas platyrhynchos*	*M. anatidum*	Georgia	Wild
Mallard duck*	*Anas platyrhynchos*	*Myxidium* sp. 2	California	Wild
Baikal teal*	*Anas formosa*	*Myxidium* sp. 2	Texas	Captive
Wood duck*	*Aix sponsa*	*Myxidium* sp. 2	California	Wild
African yellow-billed duck	*Anas undulata undulata*	*Myxidium* sp.	California	Captive
African yellow-billed duck	*Anas undulata undulata*	*Myxidium* sp.	California	Captive
Mallard duck	*Anas platyrhynchos*	*Myxidium* sp.	California	Wild
Mallard duck	*Anas platyrhynchos*	*Myxidium* sp.	California	Wild
Smew	*Mergus albellus*	*Myxidium* sp.	California	Captive
Cape teal	*Anas capensis*	*Myxidium* sp.	Florida	Captive

Spore morphologies from histology or TEM images show infections are characteristic of genus *Myxidium* (Bartholomew et al. 2008)
SSU sequence data from four birds (*) indicate that at least two myxosporean species are present (Atkinson unpublished data)

range of parasite developmental stages excluding mature spores, were observed subsequently in European shrews with overt myxosporean infections (Dyková et al. 2011).

Common shrews (*Sorex araneus*) collected in Poland from 2001–2005 were infected with a novel species of an undoubtedly myxosporean parasite *Soricimyxum fegati*, which was observed in livers of 41 % of 46 shrews (Prunescu et al. 2007). Both developmental stages and mature spores were documented, which indicated that myxosporeans can infect homeothermic, wholly terrestrial hosts. *Soricimyxum fegati* was found subsequently in common shrews (42 % of 24; 52 % of 98) in both the Czech Republic (Dyková et al. 2007, 2011) and Hungary (36 % of 21; Székely et al. 2011) (Fig. 7.2) and in pygmy shrews *Sorex minutus* (20 % of 70) and the lesser white-toothed shrew *Crocidura suaveolens* (10 % of 10) in the Czech Republic (Dyková et al. 2011). A second species, *Soricimyxum minuti*, was discovered in the pygmy shrew (33 % of 3) in Hungary (Székely et al. 2011).

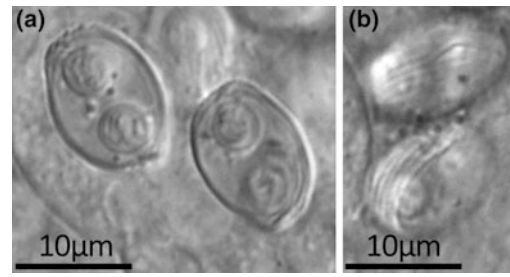

Fig. 7.2 *Soricimyxum fegati* myxospores from bile ducts of a common shrew (*Sorex araneus*). **a** Brightfield showing spore internal details, and **b** valve cell surface ridges and thickened suture

Myxospores of *S. fegati* develop asynchronously as disporic pansporocysts in polysporic plasmodia in the hepatic bile ducts and

parenchyma. Plasmodia in both locations elicit a vigorous inflammatory host response (Prunescu et al. 2007; Dyková et al. 2011). Proliferative stages cause hepatic lesions during their migration towards the lumina of bile ducts; lesions are similar in the different shrew species (Dyková et al. 2011). In 11 % of cases, covering all three species, xenoma-like formations (XLFs) containing presporogonic stages damaged the blood vessels in other organs, particularly the myocardium (Dyková et al. 2011). The role of XLFs in the myxozoan developmental cycle is unclear and atypical in fish infections (e.g. *Myxidium lieberkuehni* in renal corpuscles of pike, *Esox lucius*; Lom et al. 1989). Xenomas are associated most commonly with microsporidian infections, in which all developmental stages are present including mature spores (Lom and Dyková 2005). In the few shrew xenomal infections, no mature spores were visible (Dyková et al. 2011), which lends some weight to the original assignation of mole xenomas as myxozoan infections. In the absence of spores, the parasites within the shrew XLF's were identified by DNA sequencing (Dyková et al. 2011); technology not readily available at the time of the mole report.

Myxospores of the closely-related shrew parasite, *Soricimyxum minuti*, also develop within plasmodia in the bile ducts (Székely et al. 2011). This species is indistinguishable phenotypically from *S. fegati*, but differs by 4 % in its SSU gene sequence. Given the lack of clear phenotypic differences between these *Soricimyxum* species, taxonomists should remain vigilant for cryptic species when describing further occurrences of these homeotherm parasites.

Although myxosporean parasites occur at relatively high prevalence in European shrews, a limited survey in North America of East coast shrews ($N = 28$ *Sorex* sp.) and West coast shrews ($N = 3$ *Sorex* sp.) and voles ($N = 6$ *Microtus canacaudis*) did not reveal any visible myxosporean infections (Atkinson unpublished data). Molecular analyses and more extensive host sampling are needed to better assess myxosporean presence and diversity in small North American mammals.

7.3 Waterfowl

Routine post-mortem histological examinations of waterfowl in zoological collections first revealed myxozoan parasitism of these hosts in 1994 (Bartholomew et al. 2008). Over the 12 years that followed, myxozoans were found in the lumina of bile ducts of ten North American ducks of six different species (Anseriformes: Anatidae), which included free-flying native and captive exotic individuals (Table 7.2; Bartholomew et al. 2008). The myxozoans were associated with mild to severe pericholangeal hepatitis but were not the primary cause of death, although severe infections likely contributed to overall poor health. Polysporic plasmodia and in most cases free, mature spores were observed in the lumen of afferent bile ducts. Granulomas were present at foci of destroyed tissue, and severe inflammatory lesions centered on ruptured ducts extruded myxospores into the hepatic parenchyma. DNA sequenced from frozen or formalin-fixed material has shown at least two species of *Myxidium* are present: *M. anatidum* (Fig. 7.3; Bartholomew et al. 2008) and "*Myxidium* sp. 2" (Table 7.2; Atkinson, unpublished data). The SSU sequences of the two species vary by 5 % over ~1,100 bp. Both were detected in mallard/Pekin ducks (*Anas platyrhynchos*) and *Myxidium* sp. 2 was found also in a Baikal teal and a wood duck.

Myxozoan-infected waterfowl are distributed widely in North America, but there are no records of myxozoans in birds from any other continent. Examination of gall bladders and ureters from 23 waterfowl (including nine ducks and four swans) and other birds from Hungary did not reveal any myxozoan infections (Székely, unpublished data). However, the prevalence and diversity of bird myxozoans is difficult to determine, as all records are from non-randomly sampled bird mortalities and not from systematic parasitological surveys of larger waterfowl populations. Given the broad distribution of cases spatially and temporally in North America, myxozoan infections in waterfowl may be more common than currently documented.

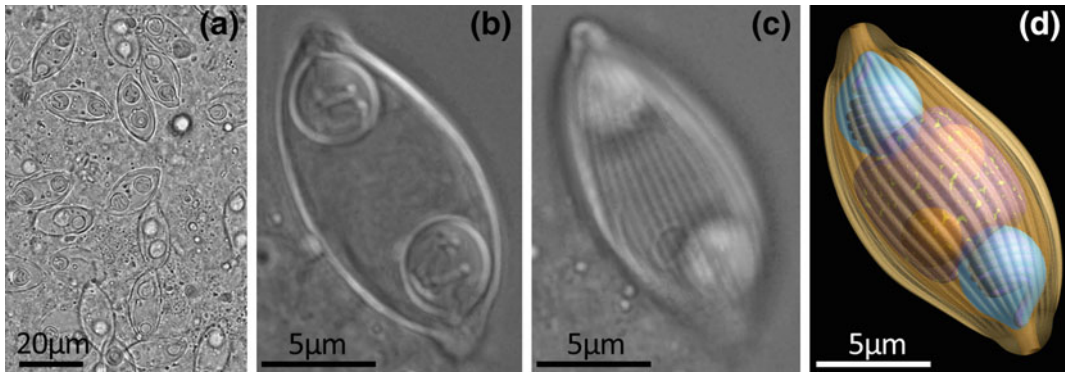

Fig. 7.3 Myxospores of *Myxidium anatidum* from the hepatic bile ducts of a Pekin duck (*Anas platyrhynchos*). **a** Spores viewed under bright field. (**b–c)** Nomarski interference contrast. **b** Polar capsules containing coiled polar filaments at either end of the elongate spore. **c** Spore surface ridges. **d** Composite graphic highlighting spore features including the binucleate sporoplasm

7.4 Humans

Humans are the only large mammal from which myxosporeans have been recorded, though none were viable infections (i.e. neither parasite proliferation nor spore development was observed). Myxospores have been detected in faeces of patients presenting abdominal pain and/or diarrhea, and from immunocompromised individuals. The first report of myxospores in humans was of the salmonid fish parasite *Henneguya salminicola* in stool samples of two Canadian patients with diarrhea; one sample was misidentified initially due to its resemblance to human spermatozoa (McClelland et al. 1997). The myxospores were likely ingested as part of infected fish flesh and passed through the gut undigested. These aberrant parasites were not considered responsible for illness in either patient. This study prompted a brief report of occasional cases of the closely related myxosporean *Henneguya zschokkei* in faeces of Swedish patients (Lebbad and Willcox 1998). There was no mention of symptoms or indication that the myxospores were a disease agent. Again, the myxospores were linked to consumption of fish, most often the salmonid whitefish (*Coregonus lavaretus*).

Three independent cases are reported from Australian patients whose faecal samples contained myxospores of *Myxobolus plectroplites*, known from the freshwater fish *Plectroplites ambiguus* (syn. *Macquaria ambigua*; Boreham et al. 1998). The patients had consumed cooked infected fish and *M. plectroplites* cysts were found in the remaining frozen fillets. The myxospores had passed unchanged through the alimentary tract and were considered unrelated to clinical symptoms of abdominal pain and/or diarrhea as other enteric pathogens were present in two of the patients. Myxospores of a different *Myxobolus* sp. were observed in stool samples, along with other pathogens, from an immunosuppressed patient (Moncada et al. 2001). Again, the myxospores were considered to be incidental and not associated directly with the abdominal symptoms.

Most recently, a myxosporean has been linked directly to outbreaks of food poisoning in Japan. Infections were connected with consumption of raw olive flounder (*Paralichthys olivaceus*) imported from South Korea where it is grown in aquaculture (Kawai et al. 2012; Iwashita et al. 2013). Since 2003, an average of 100 incidents have occurred each year, reaching 158 in 2010 (Kawai et al. 2012) and food poisoning from olive flounder is now considered a major public health concern (Harada et al. 2012b). An extensive epidemiological analysis identified *Kudoa septempunctata* as the culprit; no alternative causative agents such as bacteria or viruses, or bacterial and other toxins were detected (Kawai et al. 2012). Before it was linked to human

illness, *Kudoa septempunctata* had been reported from the muscle of aquacultured olive flounder (Matsukane et al. 2010). The parasite spreads throughout the trunk muscle of the fish and has a range of stages present simultaneously within plasmodia in the myofibres (Iijima et al. 2012; Ohnishi et al. 2013b).

In 1–20 h after consumption of raw, infected olive flounder, humans develop self-limiting diarrhea and emesis (Kawai et al. 2012; Iwashita et al. 2013). The parasite induces a similar reaction in non-human mammals fed myxospores experimentally. Suckling mice had watery stools and an elevated fluid accumulation ratio after intragastric inoculation with a spore suspension, and house musk shrews exhibited vomiting after being fed fish slices spiked with myxospores (Kawai et al. 2012). In vitro inoculation of Caco-2 human intestinal cells with *K. septempunctata* myxospores revealed that the parasite's sporoplasm became active, emerged from the spore valves and invaded the intestinal cells, severely damaging them and compromising cell monolayer integrity (Ohnishi et al. 2013a). Similar assessments with *Myxobolus honghuensis*, common in an important Chinese food fish, allogynogenetic gibel carp (*Carassius auratus gibelio*), showed no adverse effects in suckling mice (Guo et al. 2014), indicating that toxicity to mammal cells is myxozoan species specific.

Identification of the causative agent of the food poisoning led to the development of several molecular assays to facilitate rapid detection: a PCR assay to differentiate *K. septempunctata* from other olive flounder *Kudoa* species (Grabner et al. 2012), two qPCR assays to detect the parasite in fish samples (Harada et al. 2012a; Iijima et al. 2012), and modification of the Harada qPCR to detect the parasite in patient clinical samples (faeces and vomitus) (Harada et al. 2012b). Ohnishi et al. (2013b) caution that since toxicity is provoked only by the myxospore stage, molecular assays that inherently quantify all genetically identical cells will overestimate the dose.

Although sushi and sashimi are popular in other countries, illness has been reported solely from Japan and in connection only with *K. septempunctata.* Flounder is the only known food fish infected with this parasite (Ohnishi et al. 2013a), but many *Kudoa* species occur in other marine food fishes (e.g. *Kudoa thyrsites* in Atlantic salmon; *Kudoa crumena* in yellowfin tuna; *Kudoa inornata* in spotted sea trout; *Kudoa islandica* in spotted wolffish), but these myxosporeans do not appear to illicit illness in humans nor do they adversely affect the health of the fish host. They can have economic impacts however, as some cause post-mortem myoliquefaction, which affects marketability of the fillets.

A third category of myxosporean associations with humans concerns apparent allergic reactions, again to consumption of fish infected with a *Kudoa* species. Infected Chilean hake (*Merluccius gayi gayi*) imported into Spain were linked with 4/15 patients with gastroallergic and/or allergic symptoms and who were positive to a *Kudoa*-pseudocyst skin prick test (Martínez de Velasco et al. 2008). The parasite material used in the skin tests was processed (frozen and homogenised) but uncooked. Whether the hake was consumed raw or cooked was not disclosed in the publication, but this species is usually eaten cooked (Gema Alama-Bermejo, pers. comm.). The elevated humoral response (IgG1 and IgE antibodies) of BALB/c mice immunised with pseudocyst extracts suggested that some components of the parasite could be allergenic and thus result in immunopathological effects in humans (Martínez de Velasco et al. 2002; Martínez de Velasco and Cuéllar 2003). This was followed up with a survey of the seroprevalence of anti-*Kudoa* sp. antibodies in human sera that supported an association between ingestion of *Kudoa* sp. and the allergic reaction (Martínez de Velasco et al. 2007).

In summary, no known myxosporean life cycle involves a human host. Developmental stages have never been observed, which indicates presence of myxospores is incidental from consumption of infected food, rather than the humans being natural hosts. The occurrence of *Myxobolus* and *Henneguya* myxospores in patients was coincidental and not related to symptoms, although reaction to these species by humans has not been investigated. Heat-treated (cooked) myxospores of both species passed intact through the human digestive tract.

Untreated myxospores of *Myxobolus cerebralis* retain both morphology and viability following passage through the gut of piscivorous fishes and birds (e.g. El-Matbouli and Hoffmann 1991). However, most myxospores were digested and none remained viable after passage of this species through mice (El-Matbouli et al. 2005). The spore activation exhibited by *Kudoa septempunctata* in the human gut is unique, and restricted to consumption of raw infected fish. Myxospores of *K. septempunctata* can be inactivated in 3–4 h at −15 to −20 °C, and after 5 min at 75 °C (Iwashita et al. 2013), and thus cooked infected fish should not pose a human health risk. The allergic reactions elicited by another species of *Kudoa* (see above) suggest that ingestion of these parasites can result in immunopathology.

7.5 Phylogenetic Context of Myxosporeans in Homeotherms

Figure 7.4 illustrates the positions of myxosporean parasites with non-fish vertebrate hosts in relation to a myxosporean phylogenetic tree (synthesised from Fiala 2006; Hartigan et al. 2012; Fig. 5.11 in Whipps 2013). DNA data are not yet available for many of the species isolated from homeotherms, so we placed them on the tree in clades with matching myxospore morphology. Figure 7.4 illustrates two primary patterns of species distribution: myxospores from frogs and toads belong to at least seven genera and are distributed widely across the tree, whereas myxospores from homeotherms and reptiles are from five genera and are restricted to only a few clades.

7.6 Diversity and Evolution of Homeotherm-Infecting Myxozoans

Myxosporean records from homeotherms cover limited host and geographic ranges: the two *Soricimyxum* species from mammals are recorded only from shrews in Europe and the two *Myxidium* species from birds are known only from waterfowl

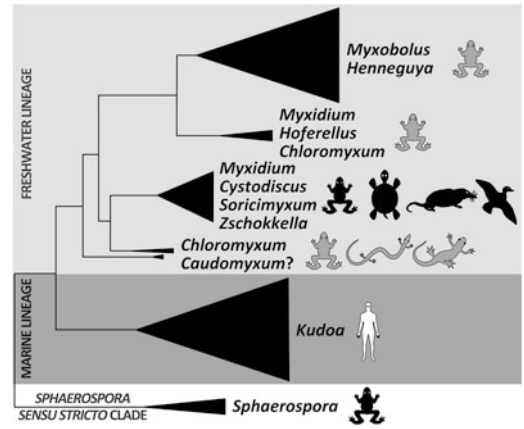

Fig. 7.4 Non-fish vertebrate hosts of myxosporeans shown relative to a generalized myxosporean phylogeny. *Dark* coloured icons represent hosts for which parasite DNA data are known. *Grey* icons indicate no parasite DNA data are available; hosts have been placed based on myxospore morphology. *White* icon (human) indicates incidental host. Host groups: frogs/toads, turtles/tortoises, small mammals, waterfowl, primitive caudate (*Proteus*), newts/salamanders, humans. [Tree topology synthesised from Fiala (2006), Hartigan et al. (2012) and Fig. 5.11 in Whipps (2013)]

in North America. All of these reports are from the last 15 years. This is in stark contrast with records of >2,200 myxosporean species in fishes and >50 in reptiles and amphibians, spanning more than 150 years and from most continents. But is the apparent paucity of taxa from homeothermic hosts an artifact of sample bias or does it reflect actual limited diversity due to barriers to myxosporean exploitation of warm-blooded hosts?

Fewer opportunities exist to reveal hosts in wild homeotherm populations, because surveys of wild populations of small mammals and birds are less frequent and involve fewer animals than fish surveys. In all hosts, disease caused by myxosporean infection is rare, so pathological examinations of mammals and birds typically reveal myxosporeans as incidental findings only, with other pathogens associated with disease or mortality, e.g. botulism in waterfowl. Another source of sample bias could be the failure to recognise myxosporean infections, because of either a lack of diagnostic stages, particularly mature spores, or unfamiliarity with the appearance of myxozoan infections by the examiner.

If the few records of myxosporeans in homeotherms are an accurate indication of diversity, then this suggests fundamental barriers exist to myxosporean exploitation of avian and mammalian hosts. These barriers include the relatively high body temperature of homeotherms, the natural host specificity of myxozoans, and physical restrictions which both limit opportunities for contact of aquatic myxosporeans with potential semi-aquatic or terrestrial hosts, and require unique solutions to problems of myxozoan transmission in semi-aquatic and terrestrial environments.

Previously, we considered body temperature of the host a barrier to host switching from fish to birds (Bartholomew et al. 2008), as most myxozoans have poikilothermic hosts with body temperatures within a few degrees of ambient, and typically much less than the body temperatures of warm blooded vertebrates (>35 °C). However, many myxozoan fish hosts exist in habitats where temperatures approach the body temperatures of homeotherms, especially species from tropical and subtropical regions. Common carp (*Cyprinus carpio*), which inhabit ponds in southern United States, can tolerate water temperatures up to 35.7 °C (McLarney 1998 in Ficke et al. 2007) and are host for a number of myxozoans including *Myxidium* species. Therefore, survival of some fish myxozoans at homeothermic body temperatures is fundamentally feasible and fish-to-homeotherm switching events are more likely to occur in warmer environments, where thermophilic myxosporeans are more prevalent. Indeed, the known cases of myxosporean infection in waterfowl are from warmer areas.

The presence of myxosporean infections in shrews in the relatively cool environment of central Europe, may be the result of a homeotherm-homeotherm switching event—with a fish-to-migratory bird jump in tropical areas and subsequent bird-to-mammal transfer in temperate climates. This hypothesis is supported by the close phylogenetic relationship between *Soricimyxum* in shrews in Europe and *Myxidium* in migratory waterfowl in North America, which are sister taxa in the biliary *Myxidium* clade defined by Fiala (2006) (Hartigan et al. 2012; Fig. 7.3). This clade has a wide variety of non-fish vertebrate hosts, including reptiles and amphibians and the only myxozoans known from birds and mammals. This diversity of non-fish hosts suggests a single host switch from a common *Myxidium*-like ancestor in a fish to an amphibian, followed by radiation into semi-aquatic and terrestrial poikilothermic and homeothermic hosts.

At this early stage of homeotherm myxozoan research, it is unclear which fundamental characteristics of these *Myxidium*-clade myxosporeans enabled them to exploit this variety of non-fish hosts and what adaptations to their life cycles and transmission modes have allowed them to be successful in semi-aquatic and terrestrial environments.

Most myxozoan species infect a single family or species of fish (or annelid). This fundamental characteristic reduces the probability of successful cross-species infections. No myxozoan species from a non-fish vertebrate is known also from sympatric fish hosts, which suggests fish to non-fish host switches are infrequent, and genetic divergence after a host switch is rapid.

No life cycle is known from myxozoans that infect homeotherms, though they are most likely analogous to life cycles known throughout the Myxosporea (see Chap. 10), which involve an obligate, aquatic, annelid definitive host and two morphologically distinct waterborne spore stages. Transmission via waterborne spores may be the largest barrier to myxosporean host range expansion into semi-aquatic and terrestrial homeothermic hosts. Frogs and toads have aquatic larvae morphologically similar and sympatric with fish populations and are exposed naturally to fish-infecting waterborne actinospore stages, thus the diversity of myxosporeans in frogs and toads reflects these natural opportunities for cross-infection in the aquatic environment. Actinospores would infect the mammal or bird host through the thin epithelia of mouth, eyes or throat, like the epidermal surfaces of fish (El-Matbouli et al. 1999). Reinfection of the aquatic annelid host would occur via myxospores expelled from the vertebrate host by defecation or urination into water.

Adaptations would be required for transmission in a terrestrial setting. Alternation of invertebrate and vertebrate hosts and spore stages could still occur if the spore stages remained viable either within the host (i.e. not released) or within faeces in moist terrestrial habitats such as leaf litter or below ground. The parasite would utilize trophic transmission—spores would enter the host via direct consumption of infectious material, either in faeces or invertebrate prey items. The diet of *Sorex araneus* consists of a wide variety of invertebrates, dominated by earthworms, molluscs, beetles and spiders (Churchfield et al. 2012). Although the alternate stage of *Soricimyxum* is thought to involve a terrestrial annelid (Dyková et al. 2011), other invertebrates may be involved as *Sorex minutus* typically eat spiders not earthworms (Butterfield et al. 1981). This completely non-aquatic pathway of myxosporean transmission would require evolution of the parasite to exploit both non-aquatic vertebrate and invertebrate hosts. The inherent difficulty or low probability of these multiple adaptations may be the reason few myxozoans are known from terrestrial mammals.

An alternate parasite transmission strategy is direct vertebrate-to-vertebrate passage. Parasites could be transmitted through ingestion of myxospores shed from infected individuals, or from consumption of host tissues that contain spores or parasite proliferative stages. Direct fish-to-fish transmission has been shown to occur via developmental stages (but not spores) of several members of Family Myxidiidae: *Enteromyxum leei*, *Enteromyxum scophthalmi* and *Enteromyxum fugi* (Diamant 1997; Redondo et al. 2002; Yasuda et al. 2002). Direct transmission permits spread of infection in host populations in the absence of the alternate host. This does not eliminate the possibility that these myxosporeans could utilise an invertebrate host for amphimixis, which would maintain genetic diversity, as myxosporean sexual reproduction is known to occur only in the invertebrate host.

If the observed paucity of myxosporean parasites of homeotherms is due to a relative lack of sampling of non-fish versus fish hosts then many more taxa remain to be discovered in these hosts. The myxosporean pattern of infecting vertebrate hosts that are associated intimately with water (fish, frogs, turtles, ducks) suggests that myxozoan infections might occur in many animals that utilise aquatic habitats, including mammals such as cetaceans (dolphins and whales), carnivores (otters), monotremes (platypus) and rodents (beavers, nutria). The diversity of potential mammalian and avian hosts suggests many more myxosporean parasites remain to be discovered with further scrutiny of these homeotherms, especially in Africa and South America where relatively few myxosporeans have yet been described.

7.7 Key Questions for Future Studies

- Is direct transmission possible between small mammals, and does this explain the observed high infection rates in shrews?
- What is the 'terrestrial' life cycle? Are earthworms involved, or a different invertebrate?
- Could sampling shrew faeces be used to assess parasite geographic range?
- How do waterfowl become infected: trophic transmission via consumption of aquatic invertebrates or direct contact of actinospores with epithelial surfaces?
- What is unique about *Kudoa septempunctata* that it is the only known myxosporean whose myxospores cause gastroenteritis in humans?
- There are whole continents (Africa, South America) with rich non-fish vertebrate faunae —will the diversity of potential hosts be reflected in the discovery of novel myxozoans?

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Part II
Development and Life Cycles

Cellular Processes in Myxozoans

8

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Abstract

This chapter describes general cellular processes associated with myxozoan development and includes the first review of motility at the cellular level. We consider modes of cell proliferation by endogeny and division by mitosis and meiosis. We also describe cellular processes associated with sporogony, including the formation of plasmodia and spores. We review cellular motility by amoeboid movement in host invasion and 'dancing' or twitching of presporogonic proliferative stages in some myxosporeans. The key role of F-actin and myosin in cellular motility is highlighted.

Keywords

Parasite division · Endogeny · Plasmodia · Sporogenesis · Motility

8.1 Introduction

The cellular processes associated with myxozoan development vary considerably. These can include a proliferative phase soon after entering the host, characteristic cell-in-cell development and budding. There is also a sexual phase in invertebrate hosts. Interactions between cells lead to the formation of different types of spores (myxospores, actinospores and malacospores). Cellular processes also enable motility for invasion, migration through host tissues and immune system evasion. This chapter synthesizes our current knowledge of these various cellular processes.

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8.2 Cellular Processes During Myxozoan Development

The vertebrate phase of myxozoan life cycles is dominated by stages that are composed of a single cell, containing one to several hundred cells within its cytoplasm. The outer cell is referred to as the primary cell and the internal

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cells as secondary cells (Lom and Dyková 2006). The primary cell is often multinucleated, and the number of secondary cells depends on developmental stage and species. Within the secondary cells, tertiary cells have been reported and, more rarely, quaternary cells (Yokoyama et al. 1990) (Fig. 8.1a). Prior to sporogony (i.e. when the internal cells develop into spores) the parasite is referred to as presporogonic. When the parasite enters sporogony it is referred to as either a plasmodium or a pseudoplasmodium (Lom et al. 1982). The secondary cells that form spores are called generative cells. These differentiate and divide into the distinct cell types of the spore.

8.2.1 Endogenous Budding

The appearance of internal cells occurs via a process referred to as endogeny (Lom and Dyková 2006). Endogeny has been described to occur by endogenous budding in much of the myxozoan literature relating to cellular processes. Endogenous budding is viewed to involve the partitioning of the outer cell by endoplasmic reticulum to form the internal cells in a process similar to that described for protistan species of *Paramyxa* and *Paramarteilia* (Davis 1916; Lom et al. 1982). Such a partitioning process has, however, never been confirmed (Morris 2010).

8.2.2 Endogeny and Mitosis

Endogeny of *Tetracapsuloides bryosalmonae* occurs through the engulfment of one cell by another. This has been reported in ultrastructural studies for the bicellular sporoplasms contained within the malacospore, and the tertiary cell in presporogonic stages entering sporogony within the fish host (Feist and Bucke 1987; Clifton-Hadley and Feist 1989; Morris and Adams 2007, 2008). Subsequently, the internal cells divide by an open mitosis (Morris and Adams 2008). Division of the presporogonic primary cell through cytokinesis has been reported for *T. bryosalmonae*, indicating that this cell also divides by mitosis (Morris and Adams 2008). Open mitosis also occurs within the bryozoan sac stages of a *Buddenbrockia* sp., but a closed mitosis has been reported in the mural cells of *B. plumatellae* (see Canning et al. 2002, 2008).

Evidence for endogenous budding in myxosporeans has been sought through examining the origin of the actinospore sporoplasm. Like malacosporean species, endogeny has only been reported as a result of one cell engulfing another (Morris 2010; Rangel et al. 2012) (Fig. 8.1b). However, it is noted that after engulfment, the boundaries of internal cells became indistinct when viewed by electron microscopy, before reappearing later in sporoplasm development (Morris 2012). This suggests that although a process reminiscent of endogenous budding does occur within Myxozoa, it is likely to represent a feeding stage whereby cytoplasm/nutrients are transferred between extant cells, rather than the formation of totally new cells (Morris 2012). In contrast to malacosporeans, the mitosis in myxosporeans has only been described as closed (Lom and Dyková 2006). This difference is intriguing, but since only a small number of cases of mitosis have been reported, it is difficult to assess whether this is a reliable characteristic to differentiate the two groups. The lack of centrioles is a notable feature of all myxozoan cell divisions.

8.2.3 Meiosis

Meiosis has been reported in the invertebrate phase of development for both the Malacosporea and the Myxosporea (Janisewska 1957; Canning et al. 2000). The few observations made of meiosis in the vertebrate phase are thought to be erroneous (Uspenskaya 1982). Observations of meiosis have reported the ejection of polar bodies and the formation of synaptonemal complexes (Janisewska 1957; Ormières and Frézil 1969; Marquès 1986; El-Matbouli and Hoffmann 1998; Canning et al. 2000). Meiosis of both myxosporeans and malacosporeans occurs within flexible, fluid filled stages (Janisewska 1957; Canning et al. 2000). In the Myxosporea, these stages are referred to as pansporocysts. In the Malacosporea meiosis occurs in sacs or

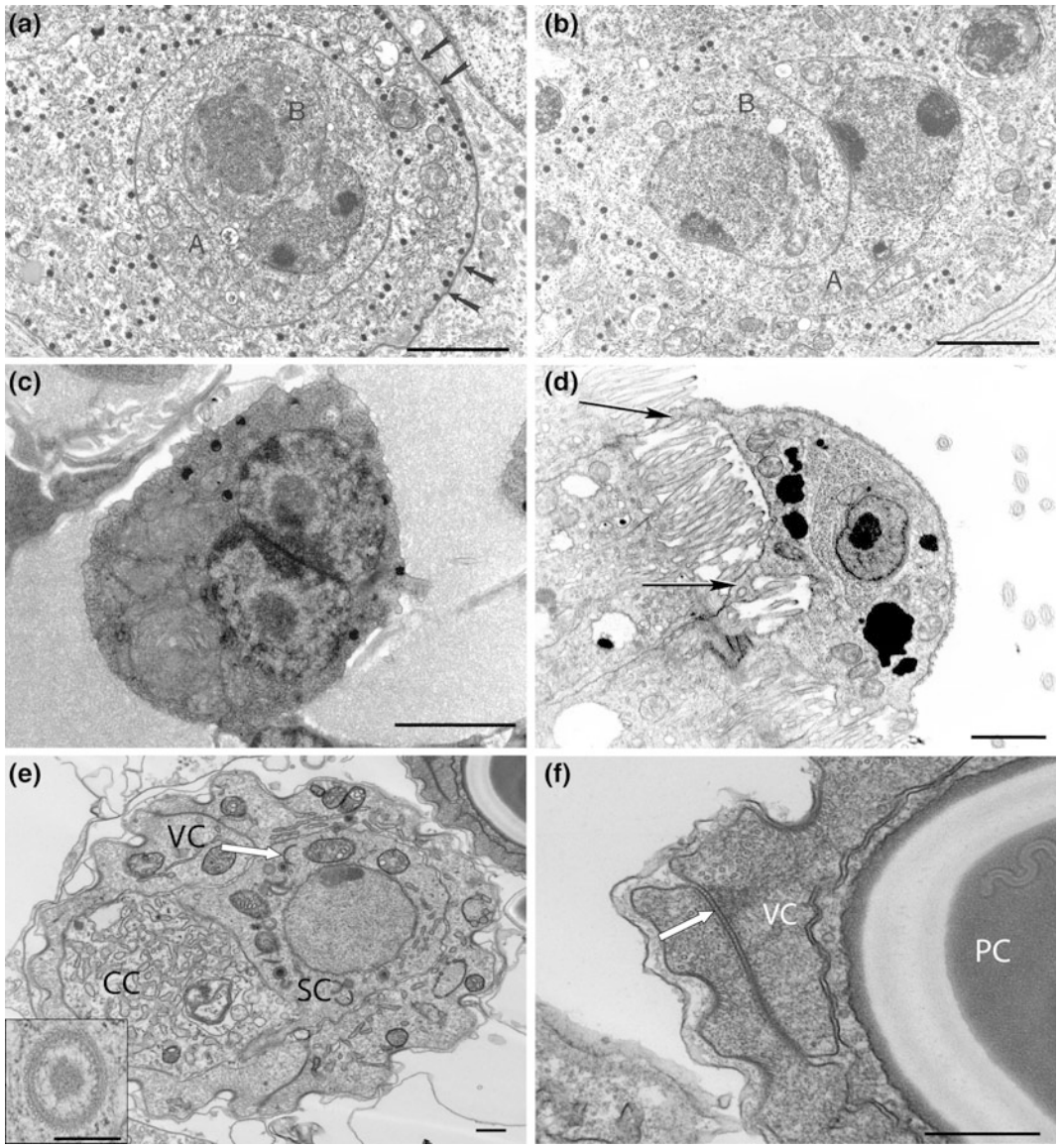

Fig. 8.1 **a** *Tetracapsuloides bryosalmonae* parasite in rainbow trout kidney. Primary cell (*arrowed*) with a secondary cell (pericyte) (*A*) containing a fully enveloped tertiary cell (*B*). Bar = 2 µm. **b** Process of secondary cell engulfment by the pericyte (*A*) to form a tertiary cell (*B*). The nucleus of the pericyte is characterized by the presence of two prominent nucleoli (see also Fig. 8.1). Bar = 2 µm. **c** Binucleate proliferative stage of Aurantiactinomyxon type actinospore in the peri-intestinal vessel of *Tubifex*. Note orientation of sporoplasmosome to cell surface. Bar = 2 µm. **d** Section of *Myxidium lieberkuehni* plasmodium attached to the renal tubule epithelium of pike (*Esox lucius*) by thin intercellular pseudopodia. Bar = 2 µm. **e** Ultrastructure of *Sphaeromyxa* sp. sporoblast, showing the association of valvogenic (*VC*), capsulogenic cells (*CC*) with conspicuous network of distended endoplasmic reticulum, sporoplasmogenic cell (*SC*) with characteristic arcuate sporoplasmosomes present in the sporoplasm cell cytoplasm (*arrow*) and pseudopodia extending between the capsulogenic and valvogenic cells within the plasmodium. Mitochondria with plate-like cristae are prominent in each cell type. Bar: 500 nm. *Inset* Transverse section showing supporting microtubular framework around a developing polar tube of *Myxobolus cotti*. Bar = 200 nm. **f** Septate junction between two valvogenic cells (*VC*) of a *Sphaeromyxa* sp. spore (*white arrow*). An almost mature polar capsule (*PC*) with a partially coiled polar filament is also present in the section. Bar: 500 nm

myxoworms (worm-like stages) (Lom and Dyková 2006). There are considerable differences in the formation and organisation of the structures that support meiosis as described below.

The origin of the myxosporean pansporocyst is a tetranucleate stage (El-Matbouli and Hoffmann 1998; Morris 2012) which undergoes plasmotomy to form four cells. Two of these cells form the delimiting envelope cells of the pansporocyst and the other two become the internal cells (El-Matbouli and Hoffmann 1998). The internal cells are deemed to be different from one another and form two populations based on size. These are called α (small) cells and β (large) cells (Janisewska 1957). At this stage, immediately prior to sporogenesis, there are 8 flattened envelope cells, 8α cells and 8β cells. The standard view is that the α and β cells are both gametic and are produced by meiosis. This meiosis is considered one step, producing 16 polar bodies, one for each of the α and β cells (Marquès 1982; El-Matbouli and Hoffman 1998). The resulting cells subsequently undergo fusion to form diploid zygotes (El-Matbouli and Hoffman 1998; Rangel et al. 2012). Marquès (1986) suggested that if meiosis were a two step process, the α and β cells could eject a total of 16 polar bodies before cell fusion. These could rapidly degrade thus a further 16 polar bodies could be ejected immediately after fusion. It is documented that mature pansporocysts contain reduced numbers of polar bodies and so this rapid removal is feasible. However, the fusing of α and β cells was challenged by Morris (2012), who suggested a two step meiosis for only one of the α and β cell types. This alternate scenario means that the cells observed represent eight diploid and eight haploid cells, while still accounting for the 16 polar bodies observed. Morris (2012) speculated that the somewhat larger β cells are gametic and haploid and that the α cells are somatic and diploid. Instead of fusing, each α cell would engulf one β cell to result in the eight sporoblasts. This theory for myxosporean development would indicate that the restoration of ploidy occurs prior to pansporocyst formation in the invertebrate host but this has not been confirmed. Studies using either light or electron microscopy have yet to be conclusive regarding these matters. Light microscopy studies are hampered by resolution and the very similar staining characteristics of the α and β cell types, while electron microscopy only allows for the observation of very thin sections taken through relatively large 3D structures. This means that important details are often missed, and therefore interpretation of processes is more difficult. Both types of study are limited by the need to identify cell types by physical size when the cells involved are actively dividing and provide only snapshots of dynamic processes. Studies across animal species indicate a diversity of roles and fates for polar bodies (Schmerler and Wessel 2011). By using polar body counts to infer meiotic processes for the Myxozoa, assumptions have been made regarding their degradation rates, possible reassimilation, fusion, generation and function that may not be warranted.

In Malacosporea, the gametic cells have been suggested to derive directly from stellate cells that initially occupy the lumen of the spore sac stage in *T. bryosalmonae* (Canning et al. 2000). The observation of stellate cells much earlier in development than gametic cells in *Buddenbrockia plumatellae*, however, challenges this view (McGurk et al. 2006). In all studies the gametic cells are nevertheless considered to have originally derived from the wall of the spore sac (Canning et al. 2000, 2002; McGurk et al. 2006). Meiosis of malacosporeans occurs prior to sporogony and sporoplasms are considered to be haploid (Canning et al. 2000). Fertilisation has not been identified, but is considered most likely to occur upon entry to a subsequent host (Canning et al. 2000; Canning and Okamura 2004).

8.2.4 Nature of Presporogonic Cellular Stages

The presporogonic stages of myxosporeans in invertebrate hosts are binucleate stages (Fig. 8.1c) (El-Matbouli and Hoffman 1998). These have been inferred to develop following an early schizogonic phase that releases unicellular cells which then fuse to form the binucleate cells (El-Matbouli and Hoffman 1998). Morris and Freeman (2010) provide evidence that these

schizogonic stages are derived from a microsporidian co-infection, and proposed that the binucleate stages arise directly from the binucleate sporoplasm of the myxospore. This is supported by the lack of observations of schizogonic stages in other studies. The binucleate cells appear amoeboid and can have sporoplasmosomes within them. The nuclei may be observed tightly abutted together. These presporogonic stages fuse to form the tetranucleate stage that precedes the pansporocyst.

Within the vertebrate host presporogonic myxosporean stages are derived from the actinospore sporoplasm. They are multicellular and composed of a primary cell containing secondary cells. Presporogonic stages may persist and continue replicating in fish after other stages have started to form spores. These stages have sometimes been referred to as extrasporogonic and can occur in tissues and organs that differ in location from the final site of sporogony (Lom and Dyková 2006). These stages have been reported as replicating through division or schizogony (Yokoyama et al. 1990; El-Matbouli et al. 1995; Bjork and Bartholomew 2010).

Amoeboid presporogonic stages of malacosporeans in bryozoan hosts have been described for both *Buddenbrockia* spp. and *T. bryosalmonae* (Canning et al. 2002, 2008; McGurk et al. 2006; Morris and Adams 2007). These amoeboid stages are uninucleate, and the cells divide and migrate through the host. Eventually they form the sac or worm-like stages. Studies on presporogonic stages of Malacosporea in fish hosts are restricted to *T. bryosalmonae*. Here the presporogonic stage is morphologically identical to the sporoplasm within the malacospore, consisting of a primary cell and a single secondary cell within it (Grabner and El-Matbouli 2008; Morris and Adams 2008). This stage undergoes a massive replication through division, rather than schizogony (Morris and Adams 2008).

For most species of Myxozoa the presporogonic stages are unknown because the life-cycles of most species have not been identified. Furthermore, as the majority of myxozoan descriptions are from fish exposed to unfiltered, environmental water sources, multiple infections may confound the identification of specific stages. A partial solution to this is the use of molecular probes to identify parasites in tissue sections. In situ hybridization using species specific probes has been successfully adopted in several studies to characterise the development of myxozoans, including those involving multiple co-infections (Bjork and Bartholomew 2010; Morris et al. 2000a; Grabner and El-Matbouli 2008; Holzer et al. 2003, 2006, 2010). Follow up ultrastructural examination is recommended where possible (e.g. Grabner and El-Matbouli 2008).

8.3 Cellular Processes Associated with Sporogony

8.3.1 Formation of Plasmodia

Plasmodial stages of the myxosporeans *Sphaerospora truttae*, *Ceratomyxa puntazzi*, and *Myxobolus cerebralis* are produced within the presporogonic stages, as secondary/tertiary cell complexes. These are released through the disintegration of the presporogonic stage at the target organ where sporogony occurs (El-Matbouli et al. 1995; Holzer et al. 2003; Alama-Bermejo et al. 2012). For other species, sporogony occurs within pansporoblasts formed inside the presporogonic stage (Sect. 8.3.4). For the myxosporean *Ceratomyxa shasta*, release from the presporogonic stages occurs within the gill, and the secondary/tertiary cell stages migrate, via the blood system, to the final site of sporogony in the intestine, where further replication may occur (Yamamoto and Sanders 1979; Bjork and Bartholomew 2010). For the malacosporean, *T. bryosalmonae*, the secondary/tertiary cells are retained within the presporogonic stage as it migrates to the site of sporogony in the kidney tubule lumens. Once in the tubules this stage directly enters sporogony (there is no release from the surrounding proliferative stage) (Morris and Adams 2008). It has been speculated that the tertiary cell heralded the transition of a presporogonic stage to a sporogonic stage for all myxozoan species (Morris and Adams 2008), but it is now clear that this is not the case (Bjork and Bartholomew 2010).

8.3.2 Cellular Processes in Plasmodia and Pseudoplasmodia

Plasmodial stages are classified into two types. Plasmodia are multinucleate, produce many spores within them (polysporic) and can become very large (>2 cm). Pseudoplasmodia are smaller, uni-nucleate and their internal cells produce one (monosporic) or two (disporic) spores. The peripheral region of plasmodia generally contains nuclei and replicating cells, while the centre of the plasmodia contains those stages associated with sporogony. A single study has reported the presence of a specialized scavenger cell, termed a lobocyte, that wanders through the parasite plasmodium ingesting necrotic stages (Grassé and Lavette 1978). The plasmodium not only represents a sporogonic structure, it is also an active feeding phase as pinocytosis, and microvilli have been repeatedly reported (e.g. Current et al. 1979; Stehr and Whitaker 1986; Azevedo et al. 2013). Indeed, in many species the periphery of plasmodia is clearly distinct and shows high levels of pinocytotic activity (see also Chap. 9 for discussion of resorptive processes).

There is no obvious demarcation of the cytoplasm in pseudoplasmodia. The surface of the pseudoplasmodia, however, can extend to form fine cytoplasmic anchoring structures (Fig. 8.1d). These structures are often recorded in coelozoic species, where they can act as holdfasts on epithelial tissues (Morris and Adams 2008; McGeorge et al. 1994; Canning et al. 1999). Pseudoplasmodial stages have also been noted to possess a limited motility (Alama-Bermejo et al. 2012) and this may help to explain the widespread tissue distribution associated with the pseudoplasmodia of species such as *Sphaerospora molnari* (see Lom et al. 1983b). A further notable difference between pseudoplasmodia and plasmodia, apart from their size, is that during sporogony in pseudoplasmodia pansporoblast formation is absent.

8.3.3 Actinospore Formation

The myxosporean whose actinospore development has been studied in most detail is an Aurantiactinomyxon-type from the oligochaete *Tubifex tubifex* (see Morris 2010, 2012; Morris and Freeman 2010). The following description of cellular events is largely based on this work.

After meiosis, the first stage of sporogenesis is the surrounding of a β cell by one or more α cells (see Sect. 8.2.4 regarding different views on ploidy of these cells). The haploid cell (henceforth referred to as a germ cell), and the surrounding cells, that form the spore body and sporoplasm primary cell, are the sporogonic cells. The whole structure is now referred to as a sporoblast. Usually eight sporoblasts form within each pansporocyst, although four have been reported (Hallett and Lester 1999).

The sporogonic cells divide and differentiate. Those cells surrounding the germ cells fuse to form the sporoplasm, while the other cells develop into the valvogenic and capsulogenic cells that make the spore body. This occurs by an open mitosis to produce six cells, that eventually arrange themselves so that three cells (sporoplasmogenic) surround the germ cells, and three (valvocapsulogenic) are at one pole. The fluid from the vacuole is removed and the three surrounding sporogonic cells fuse, to form the sporoplasm. The remaining sporogonic cells remain attached to the sporoplasm and are now termed valvocapsulogenic cells. The valvocapsulogenic cells divide once more to form three valvogenic cells and three capsulogenic cells, while the germ cells within the sporoplasm can undergo repeated divisions.

During sporoblast development, the sporoplasm migrates around the pansporocyst, and has notable interactions with the envelope cells, presumably to aid nutrient uptake. The valvogenic cells become extended and the cytoplasm reduced so that these cells eventually resemble two opposing membranes separated by a thin

space. They can also produce extensions that form the caudal processes of the mature valve cells. (Lom and Dyková 1997; Morris 2012). The capsulogenic cells form polar capsules within them and become attached to the valvogenic cells by septate junctions (see Chap. 9).

Towards the end of sporogony, the sporoplasm becomes increasingly filled with membrane bound bodies, the origin of which is unclear. These bodies have been referred to as sporoplasmosomes, although they appear distinct from the organelles observed in presporogonic stages (Lom and Dyková 1997). As these changes occur the sporoplasm migrates into the spore body, which is now composed of the three, very reduced valvogenic cells (now valve cells) and the capsulogenic cells containing mature polar capsules. The valve cells then close around the sporoplasm to produce the mature spore. The pansporocyst migrates across tissues in the oligochaete to the lumen of the intestine, where it is released. Upon expulsion from the worm, the pansporocyst breaks open which releases the actinospores. The caudal processes of the valve cells then inflate in the water to produce floats.

Myxozoan sporogony in polychaete hosts has not been examined in the same detail as oligochaete hosts. After the formation of the sporoblast there are differences between the processes in the two host groups. Sporoblasts in polychaetes initially resemble those within oligochaetes, with one cell (analogous to the germ cell in oligochaete infecting myxosporeans) totally surrounded by a group of dividing cells (sporogonic cells) that go on to form the polar capsules and valve cells (Rangel et al. 2011, 2012). Sporoplasm development is different. Another cell whose origin is unclear (possibly derived from one of the surrounding sporogonic cells, rather than through germ cell mitosis), appears in the vacuole alongside the germ cell. This cell represents a sporoplasmogenic cell, and engulfs or fuses with the germ cell, creating the sporoplasm which is often reported as binucleate (e.g. Barthlomew et al. 1997; Koie et al. 2004). The sporogonic cells become valvocapsulogenic and divide to form valvogenic and capsulogenic cells (Rangel et al. 2012).

8.3.4 Myxospore Formation

Myxospores are formed within plasmodia or pseudoplasmodia in a variety of ways. They are comprised of between two and seven valves and between one and seven polar capsules and contain usually one or two sporoplasm cells (Lom and Dykovà 1992) with up to 12 in the genus *Polysporoplasma* (see Sitjà-Bobadilla and Alvarez-Pellitero 1995). In most species forming large polysporic plasmodia containing many generative cells, sporogony is initiated by the envelopment of one generative cell by another (termed the pericyte) to form a pansporoblast. Within this structure the repeated mitotic division of the enveloped cell produces sufficient cells to construct individual myxospores. However, spore formation is not always achieved in this manner and pansporoblast formation has not consistently been observed. In *Myxidium zealandicum* (see Hulbert et al. 1977) and in *Myxidium gadi* spores can be formed from clusters of generative cells without pericyte formation (Feist 1995).

Differentiation to form valvogenic, capsulogenic and sporoplasmogenic cells is synchronous within the pansporoblast and is discernable based on ultrastructural features of the constituent cells and their spatial relationship (Fig. 8.1e). The cells all remain in variable degrees of contact during sporogenesis but it is not known whether there is any exchange of material between them. Valvogenic cells become attenuated to surround the sporoplasm(s) and capsulogenic cells and gradually loose their nuclei and cellular organelles as they mature. A septate junction forms between valvogenic cells (Fig. 8.1f) during maturation of the spore. This forms the conspicuous suture line(s) between shell valves (numbers dependent on species) that have been used as a morphological feature for taxonomic purposes.

Sporoplasmogenic cells are uni- or bi-nucleate depending on the genus and are often characterised by the presence of intracellular structures of unknown function, the sporoplasmosomes (Lom et al. 1989). These have variable morphology and are often membrane bound and contain electron lucent material. Their function

has not been established, but in *T. bryosalmonae* they can be observed within the vertebrate proliferative phases and in sporoplasms (see next section), where they orientate to the plasma membrane (Feist 1997; Morris et al. 2000b; Canning and Okamura 2004). They have also been noted to do this in the binucleate cells of actinospore development (Morris and Freeman 2010). It can be speculated that a secretory role seems likely, either to assist in host invasion or as a mechanism to avoid host immune response. Alternatively, a role in extracellular digestion may be possible.

Capsulogenic cells are often characterised by the presence of large amounts of distended endoplasmic reticulum present in the cytoplasm. From an early stage in their development the capsular primordium and attached external tube can be observed. The latter is destined to become internalised inside the capsular primordium to form the polar capsule. In tangential or transverse section the supporting microtubular network is visible during maturation (Fig. 8.1e inset). Eventually, as the capsulogenic cell reaches maturity (with a fully formed polar capsule containing the coiled polar filament) the cytoplasm and nucleus atrophy. The polar filaments are sealed with a plug-like structure and are positioned at locations along the sutural line (exit pores). The polar filament is extruded through the pores prior to separation of the shell valves and release of the infective sporoplasm. Many myxospores have characteristic ornamentation comprised of ridges, bristles, pits, mucous envelopes or a variety of valve cell extensions. These characters are thought to have roles in environmental distribution and transmission (see Chaps. 4 and 13 for further discussion), but this has not been confirmed.

8.3.5 Malacospore Formation in Bryozoans

Malacospores are derived from 'type B cells' forming the inner layer of the malacosporean sacs. Type B cells divide by binary fission (Canning and Okamura 2004) and by meiosis to form clusters of fourteen cells destined to differentiate into the constituent components of mature malacospores (Canning et al. 2000, 2002). However, in *Buddenbrockia allmani* there is uncertainty whether spores are formed by aggregation of sporogonic cells or arise from division of a single cell (Canning et al. 2007). Spores are approximately 19 μm in diameter. McGurk et al. (2005) observed eight valve cells enclosing two putatively haploid sporoplasm cells (each of which contain a secondary cell) and four capsulogenic cells each containing a spherical polar capsule with internalised coiled polar filament. Subsequent observations have, suggested that ten valve cells can also occur in malacospores produced in bryozoan hosts (A. Gruhl, pers. obs.). The valve cells do not form hard shell valves typical of most myxosporean species, rather they retain their cytoplasm and cellular organelles.

During early sporogenesis, capsulogenic cells are not closely associated with each other but are easily identified by the presence of a capsular primordium appearing initially as a spherical or sub-spherical structure containing lightly granular material. External tube formation has been observed (Tops et al. 2005; Canning et al. 2007; Morris and Adams 2007) and may occur rapidly (Canning et al. 1996) with internalisation into the capsular primordium to form the mature polar capsule and coiled polar filament with a plug and cap arrangement at the exit point of the capsule. As capsulogenic cells mature the polar capsules become pyriform and migrate to their final apical positions in the mature spore in a cruciate arrangement (McGurk et al. 2005). The two sporoplasm cells, which each contain a secondary cell, are closely associated with the neighbouring sporoplasm and valve cells, forming pseudopodia that are insinuated in the spaces between them. Characteristic sporoplasmosomes ($\sim$100–150 nm in diameter) with a bar-like electron lucent region are formed within the sporoplasm primary cell cytoplasm during the latter stages of spore formation. Sporoplasmosomes are not present in the secondary cells. During early spore assembly, valvogenic cells migrate to encircle the sporoplasm cells and capsulogenic cells but leave a space from which polar filaments are extruded in the mature spore.

Valve cells are connected to capsulogenic cells and to each other via outer adherens and inner septate junctions. Mature spores do not show external ornamentation as exhibited in many myxosporean species.

8.3.6 Malacospore Formation in Fish

Spore formation in fish hosts has been reported in *T. bryosalmonae* (Kent and Hedrick 1986; Clifton-Hadley and Feist 1989; Morris and Adams 2008). Presporogonic stages with tertiary cells migrate to the lumen of the kidney tubule. Here the primary cell transforms into the pseudoplasmodium, stops producing sporoplasmosomes and develops holdfast structures that interact with the brush border. The generative cells are initially composed of one secondary cell and a secondary/tertiary cell doublet which are precursors to the two capsulogenic, two valvogenic cells and the single sporoplasm that compose the mature malacospore (Hedrick et al. 2004; Morris and Adams 2008). How the cells of the mature malacospore develop from the initial cells is unknown.

8.4 Myxozoan Motility Modes

Motility is the spontaneous, self-generated movement of a living organism (Risler 2009). In metazoans motility can be achieved at the whole organism level by the coordinated contraction of muscle systems. Motility is also achieved by protists as they swim, crawl and contract as free living organisms or as parasites within eukaryotes. Such cell motility reflects the movement of cellular components mediated by the cytoskeleton, an ancient machinery that is functionally conserved from protozoans to vertebrates (Pollard and Borisy 2003).

Myxozoans, like cnidarians in general, demonstrate motility at the cell and, in some cases, at the whole organism level. Motility has variously been observed in different stages of myxozoan life cycles. To date three distinct modes of motility are recognised in myxozoans: amoeboid motility, "dancing" or twitching, and muscular movement.

Amoeboid movement is the most commonly reported motility mode. After stimulation of the spore, the infective amoeba-like sporoplasm is released and attaches to and penetrates the host epithelium (El-Matbouli et al. 1995; El-Matbouli and Hoffman 1998; Kallert et al. 2007; Bjork and Bartholomew 2010; Grabner and El-Matbouli 2010; Ohnishi et al. 2013). Amoeboid movement is undertaken by sporoplasms during host invasion (e.g. Pote and Waterstrat 1993; El-Matbouli et al. 1995; Bjork and Bartholomew 2010; Grabner and El-Matbouli 2010), migration within the host (e.g. El-Matbouli et al. 1995; Bjork and Bartholomew 2010), and proliferation and early sporogony (Noble 1941; Meglitsch 1960; Sitjà-Bobadilla et al. 1995; Cho et al. 2004; Grabner and El-Matbouli 2010; Alama-Bermejo et al. 2012). Amoeboid motility (or cellular crawling) is based on membrane protrusion at the leading edge of the cell and is often accompanied by the production of three dimensional projections or pseudopodia (Fig. 8.2a, b). This movement is generally directional. The sporoplasm shows amoeboid motility, protruding and retracting with the production of pseudopodia (Pote and Waterstrat 1993; Grabner and El-Matbouli 2010). This movement is enabled by F-actin and β-tubulin in the cytoplasm (Uspenskaya and Raikova 2004; Ohnishi et al. 2013). There is also evidence that related contractile proteins may be involved in movements of sporoplasms as there is an increase in their expression in activated sporoplasms in actinospores (Eszterbauer et al. 2009). The sporoplasm can penetrate intercellularly or intracellularly (Bjork and Bartholomew 2010; Ohnishi et al. 2013).

Although it is generally accepted that pseudopodia in eukaryotic cells are involved in endocytosis, the filopodia in *C. puntazzi* are involved in the active locomotion of presporogonic and sporogonic stages in the bile (Alama-Bermejo et al. 2012). F-actin rich pseudopodia are present exclusively at one pole of the primary cell, whereas the posterior pole represents a long and rigid cytoplasmic extension. These filipodia are classified as filigranous pseudopodia and their mobility serves to move the parasites in one direction. To achieve this movement, the

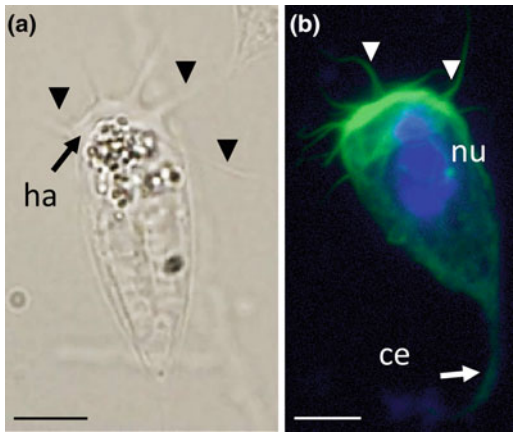

Fig. 8.2 Amoeboid motility in *Ceratomyxa puntazzi* in the bile of *Diplodus puntazzo*. **a** Pyriform stage showing abundant and large filopodia (*arrow heads*) in the hyaline area (*ha*) at the rounded end (light microscopy); **b** Pyriform stage with abundant filopodia at *the anterior edge*, where F-actin (phalloidin, *green stain*) is accumulated, and rigid cytoplasmic extension (*CE*) at the posterior end. Four nuclei (*Nu*) are visible (DAPI stain) (confocal laser scanning microscopy). *Scale bars* A = 10 μm; B = 4 μm (reproduced from Alama-Bermejo et al. 2012)

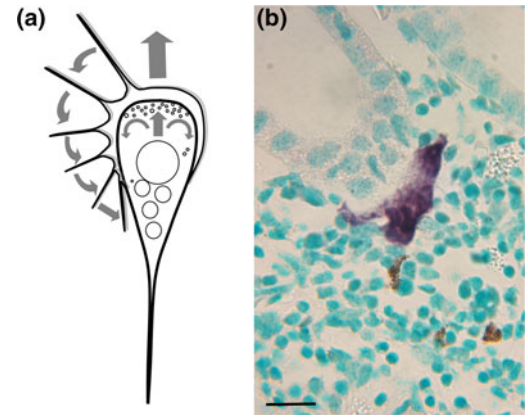

Fig. 8.3 **a** Amoeboid motility in *Ceratomyxa puntazzi* in the bile of *Diplodus puntazzo*. Schematic drawing of the locomotion of an active pyriform stage. Projection of filopodia from the anterior, median part radially to most posterior part of the hyaline area, allowing active parasite movement. **b** Intercellular multicellular stage of *Sphaerospora truttae* in the kidney interstitium, forming a protrusion (pseudopodium) while trying to penetrate the epithelium of the renal tubule, its site of spore development in Atlantic salmon (in situ hybridization, parasite stages in *purple*, background *green-blue*; *Scale bar* 10 μm. Figure 8.3a reproduced from Alama-Bermejo et al. (2012)

filopodia are projected forward and then posterolaterally backward, just like the arms of a swimmer. Thereafter the filopodia are reabsorbed in the posterior part of the parasite (see videos in Alama-Bermejo et al. 2012; Fig. 8.3a). Motility in a viscous medium, like the bile, is unlikely to target effect translocation but may help to retain proliferating stages within the lumen of the bile. Less mobile stages, such as mature spores, tend to settle and to be flushed out of the gall bladder (Alama-Bermejo et al. 2012).

Multicellular stages of the genus *Sphaerospora* demonstrate a unique rapid twitching or dancing behaviour. First described as unidentified blood objects (UBOs; Csaba 1976), these multicellular stages occur in the blood of common carp and other fish hosts (Csaba 1976; Molnár 1980a, b; Kovács-Gayer et al. 1982; Lom et al. 1983a, b; Grupcheva et al. 1985; Lom et al. 1985; Baska and Molnár 1988; Supamattaya et al. 1993). This twitching behaviour arises from a mobile fold of plasmalemma that functions like an undulating membrane (Lom et al. 1983a) and results in non-directional movement.

Myxozoan spores are typically physiologically inactive, usually relatively rigid and immobile. They are passively transmitted to the next host. One report, however, describes motility in myxozoan spores. The spores of *Fabespora vermicola* show short bursts of undulation of their valves believed to be initiated by 7 nm microfilaments associated with the sutures (Weidner and Overstreet 1979). This may aid in the liberation of the spores from the host tissue.

The worm-like members of the early diverging malacosporeans (see Chap. 4) are capable of helical swimming which results from four muscle blocks, each containing obliquely oriented muscle cells (Gruhl and Okamura 2012; see Chap. 9 for further discussion). The vast majority of myxozoans, however, including the sac-like malacosporeans and the more derived myxosporeans, have lost this musculature and thus depend entirely on simple, cellular motility for movement. Below we review what is known the importance of cellular motility in myxozoan development and life cycles and the underlying mechanisms.

8.4.1 Functions of Cellular Motility

Complex migration patterns with portals of entry different from the target tissue and several distinct developmental stages in different tissues are common in the Myxozoa. Migration of myxozoans within their hosts can be active by amoeboid crawling, or passively, such as by transport in the blood prior to amoeboid penetration into the target organ/tissue (El-Matbouli et al. 1995; Holzer et al. 2003; Bjork and Bartholomew 2010). *M. cerebralis*, one of the most intensively studied myxozoans, uses exclusively amoeboid migration as it penetrates the epidermis of rainbow trout, burrows through the connective tissue, and travels along the peripheral nerves to the cartilage (El-Matbouli et al. 1995, 1999; Hedrick et al. 1998). Migratory intercellular stages exhibit pseudopodia (El-Matbouli et al. 1995) that probably drag the stages forward through the host cell mass. The crawling strategy is considerably slower than passive transport via blood as used by e.g. *Sphaerospora truttae* or *C. shasta* to reach their target organs within 3 days (Holzer et al. 2003; Bjork and Bartholomew 2010). *M. cerebralis* takes 21 days to reach the cartilage (El-Matbouli et al. 1995). Following passive transport in blood, amoeboid migration is still often necessary to reach the site of sporogenesis. For instance, it enables the penetration of capillaries and intercellular migration to kidney tubules in *S. truttae* and *T. bryosalmonae* (Holzer et al. 2003; Morris and Adams 2008; Fig. 8.3b).

When a proliferative stage reaches the site of sporogenesis, an active swimming behaviour may be required. For instance, abundant pseudopodia occur in coelozoic myxozoans inhabiting the gall bladder, the renal tubules or the urinary bladder (Noble 1941; Sitjà-Bobadilla and Alvarez-Pellitero 1993; Lom and Dyková 1996; Morrison et al. 1996; Canning et al. 1999; Cho et al. 2004; Azevedo et al. 2005; Morris and Adams 2008).

8.4.2 The Basis for Cellular Motility

The process of cellular motility requires the integration and coordination of complex biochemical and biomechanical signals and relies on key molecular components (Pollard and Borisy 2003). There are two basic molecular systems responsible for cellular motility in a wide range of cell types. One system involves filamentous polymers of the globular protein actin, often in combination with myosin. The other involves hollow, tube-shaped polymers of the globular protein tubulin, known as microtubules. Associated with both actin filaments and microtubules are accessory enzymes that convert the chemical energy stored in adenosine triphosphate (ATP) into mechanical energy, and thus motility.

Microtubules are the major components of cilia and flagella and promote a rapid movement based on cellular appendages. In all eukaryotic cells these appendages occur in a 9 + 2 arrangement of bundles of doublet microtubules. Though microtubules are present in the cytoplasm of myxozoans (*e.g.* Uspenskaya and Raikova 2004; Lom and Dyková 2006; Casal et al. 2007) they do not form complex cilia or flagella and likely serve cytoplasmatic organelle transport and positioning as well as karyokinesis (Lom and Dyková 1992). Thus only the actomyosin machinery can be related to cellular motility in myxozoans.

Actins are an ancient and evolutionarily conserved family of proteins which are involved in cell morphology, motility and division (Steinmetz et al. 2012). These proteins have the ability to assemble and disassemble between monomeric (G-actin) and filamentous (F-actin), with the polymerization process being assisted by actin related proteins (ARP) (Schafer and Schroer 1999; Lodish et al. 2000; Goodson and Hawse 2002).

Myosins are actin motor proteins, enzymes that specifically bind to actin filaments to drive movement along the filaments. Actin and myosin systems can be found in muscles but also in motile non-muscle cells. Phalloidin-staining showed that amoeboid motility in the myxozoan *C. puntazzi* relies on the extension of the leading edge of the multicellular parasite by polymerization of actin, which causes a pressure in the cell cortex and thus extends the cell membrane (Alama-Bermejo et al. 2012). High concentrations of F-actin are present in the ectoplasm, located at the anterior pole or the direction pole of the parasite (Fig. 8.2b; Alama-Bermejo et al. 2012). F-actin

also promotes the rapid creation and reabsorption of pseudopodia in this area and is pivotal for amoeboid motility in myxozoans. Amoeboid invasion of host cells by the sporoplasm of *Kudoa septempunctata* is also promoted by F-actin. Actin filaments at the leading edge of the sporoplasm are involved in penetrating the cell (Ohnishi et al. 2013). The inhibition of actin polymerization (by the specific F-actin inhibitor cytochalasin) demonstrated the importance of actin for cell invasion (sporoplasms lacking actin were unable to invade host cells) (Ohnishi et al. 2013). It is likely that myosin is also involved in the amoeboid motility of myxo- and actinospore sporoplasms. This was shown by a suppression substractive hybridisation (SSH) study of *M. cerebralis*. During host penetration by the actinosporean sporoplasms of *M. cerebralis* there was a significantly higher expression of transcripts of an ARP3 homolog and a myosin regulatory light chain (Eszterbauer et al. 2009). The cellular actomyosin system thus appears to be fundamental for host cell invasion.

The motility promoting proteins causing the "dancing" behaviour of sphaerosporid blood stages have not yet been investigated. Microscopical and ultrastructural studies, however, confirm that they lack a microtubule based apparatus in the form of flagella and cilia (Lom and Dyková 1992; Supamattaya et al. 1993). It is likely that the actomyosin machinery is also responsible for the rapid twitching of these stages.

Molecular data on motility promoting proteins available to date include: β-actin sequences for 10 myxozoans belonging to the Myxosporea [*M. cerebralis* (NCBI Accession number AY156508), *M. insidiosus* (AY157008), *M. spinacurvata* (AY157009), *M. muelleri* (AY157010), *M. exiguus* (AY157011), *M. episquamalis* (AY157012), *M. pavlovskii* (AY157013), *Thelohanellus nikolskii* (AY157014), *Kudoa ovivora* (AY157015) and *C. shasta* (AY157016)] (Kelley et al. 2004). Three different copies of β-actin are consistently present in the Myxozoa (Kelly et al. 2004). Eszterbauer et al. (2009) published the mRNA sequences of an actin-related protein 3 (EU595011) and of the myosin heavy chain 11 isoform (EU595012). These proteins were preferentially expressed in sporoplasms of *M. cerebralis* actinospores after mucous activation when compared with inactivated spores (Eszterbauer et al. 2009). Further anatomical and gene expression studies on contractile proteins in myxozoans are needed to promote understanding of the organization and function of promoter proteins in parasite motility. These proteins may also be good candidates for analysing the phylogenetic affinities of myxozoans with different groups of cnidarians and bilaterians (Steinmetz et al. 2012).

8.5 Conclusions

Cellular processes involved in development have been highly modified in myxozoans, presumably in response to their endoparasitic life styles. For example, myxozoan developmental stages, such as multinucleate plasmodia in vertebrate hosts) and proliferating cells in invertebrate hosts, have converged with protists in sustaining extensive interactions with their environment at the cellular level. Many aspects of cellular processes require further research and clarification, including details of meiosis across a broad range of taxa and documentation of fusion. The loss of tissues in myxosporeans and in sac-like malacosporeans has meant that motility must be achieved at the cellular level in the vast majority of myxozoans. Observations of twitching and dancing in myxosporean stages may demonstrate solutions to this.

8.6 Key Questions for Future Studies

- What is the chronology of cytoplasmic processes in actinospore development?
- What is the function of sporoplasmosomes and are there parallels with similar organelles in other parasite groups such as the haplosporidians?
- What is the role of promoter proteins (i.e. actin, myosin) in myxozoan motility?
- Are promoter proteins (i.e. actin, myosin) good candidates for understanding the evolution of malacosporeans and myxosporeans from cnidarians and bilaterians?
- When is diploidy achieved in the life cycle?

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Tissue Characteristics and Development in Myxozoa

9

Alexander Gruhl and Beth Okamura

Abstract

For most of the time that they have been recognised, myxozoans were viewed to lack any tissue-level of development. However, the discovery of malacosporean stages in freshwater bryozoans revealed recognisable tissues in the form of epithelial sheets and musculature. In this chapter we compare and contrast myxozoan tissues with those of other metazoans and review the scattered literature on myxozoan development in order to explore differences and similarities to normal development in cnidarians and bilaterians. Malacosporean trophic stages possess a bona fide epithelium (including a basal lamina). Close inspection, however, demonstrates that some epithelial features are found in all myxozoan spores (cell-junctions) and even in syncytial plasmodial stages of myxosporeans (polarity, directed transport). Vestiges of tissue-level traits of their free-living ancestors can therefore be observed in all myxozoans. Resorptive and secretory tissues in myxozoans and muscle tissues in malacosporeans are evaluated with respect to typical cnidarian or bilaterian tissues. Elements of neurotransmission pathways identified in a transcriptomic survey suggest that muscle activity in myxoworms is coordinated by nervous signal transduction. Nerve cells may therefore be highly reduced and have not been recognised in structural investigations so far. Gametogenesis and embryogenesis in myxozoans have clearly been highly modified but remain poorly understood. Outstanding issues that remain to be resolved include the identification and formation of blastula and gastrula stages and the orientation of the ectoderm and endoderm (gastrodermis) of myxozoans.

Keywords

Epithelium · Mesenchyme · Germ layer · Gastrulation · Development · Intestinal tract · Nervous system · Musculature · Gametes · Cell-junctions · Extracellular matrix · Body-plan

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9.1 Introduction

In the process of adapting to their endoparasitic lifestyles, myxozoans have undergone extreme morphological simplification to exploit their hosts and have evolved novel forms to achieve transmission (spores). This evolutionary trajectory greatly obscures many developmental features that myxozoans should have in common with their free-living cnidarian relatives. Indeed, for most of the time that they have been recognised, myxozoans were viewed to entirely lack a tissue-level body organisation. However, discoveries of malacosporean stages in freshwater bryozoans have enabled the demonstration of bona fide tissues in the form of epidermal sheets and musculature. In this chapter we examine how the development of these tissues and also of early stages in malacosporeans may equate to parallel developmental processes in typical cnidarians and bilaterians. We similarly explore whether the highly derived myxosporeans demonstrate parallel traits, and whether evidence for tissue characteristics has been overlooked.

We begin by describing key features of tissues with particular focus on the underlying extracellular matrix (ECM) associated with epithelia and the various types of junctions that join cells in epithelial layers. This background enables us to explore the types of cell junctions that characterise myxozoans and evidence for ECM. We then describe the specialised tissue types that develop in myxozoans and how these tissues compare with those in free-living cnidarians and bilaterians. Finally, we turn our attention to gametogenesis and embryogenesis in myxozoans. We review the scattered literature on myxozoan development and explore differences and similarities to embryonic development in cnidarians and bilaterians.

9.2 Epithelial and Mesenchymal Tissues

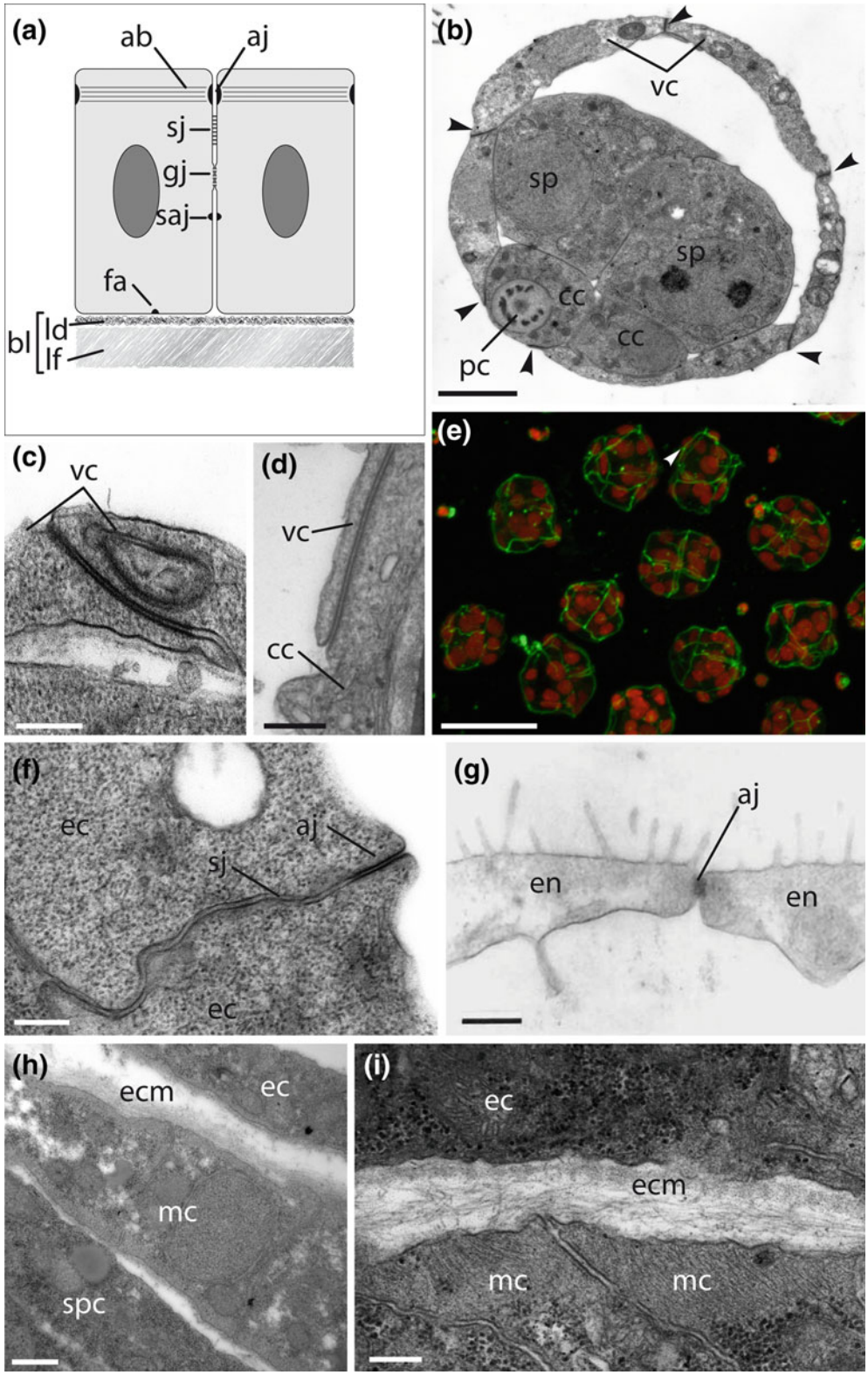
Metazoan tissues can be broadly classified into epithelial and mesenchymal tissues. Epithelia are sheet-like tissues consisting of polarised cells that are attached to a basal lamina and interlinked by a characteristic set of cell-cell junctions: apical adherens junctions and occluding junctions (Fig. 9.1a, Tyler 2003). Epithelia are an evolutionary novelty of Eumetazoa (comprising Cnidaria, Ctenophora, Bilateria, and potentially, Placozoa) and play important roles in the development and establishment of adult morphologies. Epithelia also act as diffusion barriers, sealing the body against the exterior or delimiting inner body compartments. The polarity of epithelial cells (in terms of cytoplasmic and membrane composition) enables directed uptake, secretion, or transport of substances (Cereijido et al. 2004).

In typical eumetazoan development, cleavages result in a blastula stage bound by an epithelium that becomes segregated into endodermal and ectodermal domains during gastrulation. Bilaterian gastrulation typically involves formation of a third germ layer, the mesoderm, which gives rise to a range of adult epithelial and mesenchymal tissues (Technau and Scholz 2003). Mesenchymes generally arise from epithelia by a process called epithelial-mesenchymal transition and are constituted of unpolarised cells that are usually completely embedded in extracellular matrix (ECM), e.g. as in vertebrate cartilage or other connective tissues (Shook and Keller 2003). Only a few mesenchymal cells are present in adult cnidarians whose morphologies are largely composed of endoderm and ectoderm.

Due to the highly derived nature of most myxozoans, epithelial and mesenchymal tissues are not readily recognisable in adult morphology nor during development. Thus, to enable a comparison of myxozoan tissues and developmental processes with those of cnidarians and other animal groups it is essential to properly assess the types of tissues that develop in myxozoans. In the following sections we review the extent that important defining features of tissues, such as cell-cell junctions and ECM, are present in myxozoans.

9.3 Cell-Cell Junctions

Metazoan cell-cell junctions are classified into three main functional groups (Schmidt-Rhaesa 2007): (1) adherens junctions, which provide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◀**Fig. 9.1** Metazoan and myxozoan epithelial characteristics and cell junctions. **a** Schematic drawing of general metazoan cell junctions and ECM components. **b–d** TEM images of junctions in malacosporean spores. **b** Overview horizontal section. **c** Junction between valve cells. **d** Junction between capsulogenic and valve cell. **e** Confocal image of *Tetracapsuloides bryosalmonae* spores stained against f-actin (*green*) and DNA (*red*) revealing actin belts in cell junctions. **f** Junctions in malacosporean epidermis. **g** Junction between *Sphaeractinomyxon ersei* pansporocyst envelope cells. **h**, **i** TEM images showing extracellular matrix beneath epidermal layer in *Buddenbrockia plumatellae*. Photos **c**, **d**, **h**, **i** Alan Curry, **b**, **f** reproduced from Canning et al. (2007) with permission from John Wiley and Sons. **g** Reproduced from Hallett et al. (1998), with permission from John Wiley and Sons. *ab* apical actin belt, *aj* apical adherens junction, *bl* basal lamina, *cc* capsulogenic cell, *ecm* extracellular matrix, *en* pansporocyst envelope cell, *ep* epidermis, *fa* focal adhesion, *gj* gap junction, *ld* lamina densa, *lf* lamina fibroreticularis, *pc* polar capsule, *saj* spot adherens junction, *sj* septate junction, *sp* sporoplasm, *spc* sporogonic cell, *vc* valve cell. *Scale bars* **b** 2 μm, **c** 200 nm, **d** 500 nm, **e** 20 μm, **f** 200 nm, **g** 400 nm, **h** 500 nm, **i** 200 nm

mechanical coupling of neighbouring cells, (2) occluding junctions, which seal the intercellular space and act as diffusion barriers, and (3) communicative junctions, which enable exchange of small molecules carrying electrical or chemical signals between cells (Table 9.1; Fig. 9.1a).

Adherens junctions involve the adhesive transmembrane protein, E-cadherin, associated with α- and β-catenin on the cytoplasmic side and can be organised either as spot adherens junctions, or, exclusively in epithelia, as apical adherens junctions (also termed apical belt desmosome or zonula adherens). In transmission electron microscopy (TEM), adherens junctions are characterised by electron dense concentrations on the cytoplasmic sides and uniformly opposed membranes, 14–22 nm apart. The intercellular space is electron lucent. Apical adherens junctions encircle epithelial cells near the apical region, and are connected on the cytoplasmic side to the actin/myosin cytoskeleton to form a continuous apical actin/myosin network. This provides both stability and contractability, enabling morphogenetic movements, such as bending and invagination of epithelial sheets during embryonic development (Pilot and Lecuit 2005).

Occluding junctions are only found in epithelial tissues. In invertebrates these are represented by septate junctions, which are situated basal to the apical adherens junction. Vertebrate epithelia have tight junctions apical to the apical adherens junction. Septate junctions utilise the transmembrane protein neurexin IV and have a characteristic ultrastructural appearance; opposing membranes are connected by electron dense bars, giving the whole structure a ladder-like appearance in cross section. Specific types of septate junctions in invertebrates seem to bear some phylogenetic signal (Green and Bergquist 1982).

Gap junctions are communicative junctions known from all eumetazoans except anthozoans, in which they may have been lost secondarily. Gap junctions are organised as fields of transmembrane channels formed by the proteins innexin and connexin in invertebrates and vertebrates, respectively. Innexins and connexins are very similar in 3D-structure, but differ in their amino

Table 9.1 General and specific types of cell junctions and their patterns of occurrence in the metazoa

Types of cell junctions			Occurrence in metazoa
Cell-cell junctions	Adherens junctions	Apical adherens junction	Eumetazoan epithelia
		Spot adherens junction	Metazoan mesenchyme and epithelia
	Occluding junctions	Septate junction	Eumetazoan epithelia (except vertebrates)
		Tight junction	Vertebrate epithelia
	Communicative junctions	Gap junction	Eumetazoa
		Cytoplasmic bridge	Metazoa?
		Tunneling nanotubes	Metazoa?
Cell-matrix junctions		Hemidesmosome	Vertebrate mesenchyme and epithelia
		Focal adhesion	Invertebrate mesenchyme and epithelia

acid sequences (Magie and Martindale 2008). Gap junctions are difficult to identify in TEM images and are mostly demonstrated experimentally, e.g. by dye coupling. Although gap junctions primarily occur and are most easily recognisable in nervous tissue (as electrical synapses) they are a common feature of epithelial cells, facilitating for example, epithelial conduction or exchange of small molecules. They appear, ultrastructurally, as areas where membranes are separated by a gap of only 2–3 nm. Other communicative junctions include tunneling nanotubes and cytoplasmic bridges, both of which enable exchange of larger molecules (Bloemendal and Kück 2013; Davis and Sowinski 2008). Open cytoplasmic bridges between cells serve as communicative junctions and are common in the animal kingdom, but are usually restricted to certain cell types.

Given the proposed cnidarian affinity of myxozoans (see Chaps. 2 and 3), all ancestral eumetazoan types of cell junctions are to be expected in myxozoan tissues. It is, however, difficult to identify characteristic cell junction types in myxozoans because they are often atypical or inconspicuous. The presence of cell junctions is nevertheless significant for interpreting myxozoan tissue types, morphology and development.

9.3.1 Junctions Between Myxozoan Cells

The identification of types of cell junctions in the myxozoan literature is inconsistent reflecting both the aberrant structure of some junctions and the classification of myxozoans as protists for so long. For instance, both apical adherens junctions and septate junctions in myxozoans have been referred to as gap junctions—a term reserved for a type of metazoan communicative junction. Unless true gap junctions are discovered, this term should be avoided when referring to cell junctions in myxozoans. Similarly, myxozoan adherens junctions have been referred to as tight junctions, but these only occur in vertebrates.

Cell-cell junctions have been described extensively in ultrastructural studies of sporoblasts and spores. Most researchers conclude that junctions occur between valve cells, between valve and capsulogenic cells, and between capsulogenic cells. Junctions between valve cells have been reported to differ slightly from those between valve and capsulogenic cells and those between capsulogenic cells. Only a few studies report junctions between sporoplasm and valve cells (Alvarez-Pellitero et al. 2002; Rangel et al. 2012) or between sporoplasm and capsulogenic cells (Feist 1995).

Septate junctions have been observed in spores of many myxosporean species and several good images of their characteristic ladder-like structure have been provided (e.g., Fig. 21 in Cuadrado et al. 2008; Fig. 28 in Canning et al. 1996; Fig. 22 in Feist 1995). In most cases the septate junctions are extensive, occurring along almost the entire region where the membranes of the neighbouring cells are apposed. Visualising the transmembrane components of septate junctions is possible with freeze-fracture preparations, as shown by Desportes-Livage and Nicolas (1990). These myxozoan septate junctions exhibit highest similarity to the double septum septate junctions found in cnidarians (Green and Flower 1980), chaetognaths, echinoderms and hemichordates (Green and Bergquist 1982). There appears to be variation in features of septate junctions depending on their location. For instance, in many myxozoan spores the cell membranes between adjacent valve cells in the region of sutures show perfect parallel alignment, indicative of a proper junction, but the characteristic horizontal bars in the intercellular space are lacking (or are not visible). These junctions have therefore misleadingly been termed gap junctions. Feist (1995) noted that in *Myxidium gadi* the typical ladder-like pattern becomes less conspicuous in mature spores because the secreted surface layer hinders penetration of fixatives. Thus, a variable degree of fixation is one possible explanation for the inconsistent observations of typical septate junctions.

Apical adherens junctions occur as part of the complex junction between cells of the external wall (valve cells, capsulogenic cells) of malacopsorean spores. These junctions consist of: an inner, either septate or gap area, and the outer

adherens-like zone (Fig. 9.1b–d; Canning et al. 1996, 2000; Morris and Adams 2008) with dense submembrane areas. The presence of apical actin belts surrounding both valve and capsulogenic cells in spores of *Tetracapsuloides bryosalmonae* as revealed by f-actin staining (Fig. 9.1.e) confirms the bona fide nature of these apical adherens junctions. In myxosporean spores, adherens type junctions are mostly described from sporogonic stages rather than mature spores (Morris 2010; Lom and Dyková 1992; Morrison et al. 1996). Adherens junctions may be less pronounced in mature myxosporean spores because the valves are hard and microtubules (running parallel to the suture) and fibrillar material (reminiscent of striated ciliary rootlets) may provide mechanical support (e.g. Figs. 24, 25 in Desser et al. 1983; Fig. 1E in Casal et al. 2003; Figs. 13, 18, 46 in Sitjà-Bobadilla and Alvarez-Pellitero 1995). Perhaps the mechanisms that release sporoplasms differ for myxsoporeans and malacosporeans. In the latter, apical contraction of the soft valve cells may rupture the spore enabling sporoplasm release—a process dependent on the integrity of cell junctions. Mechanisms of sporoplasm release in myxozoans, however, remain unknown (see Chap. 13).

Malacosporean trophic stages exhibit conspicuous cell-cell junctions (Fig. 9.1f). All known species have an external (mural) epithelial layer in which cells are connected by apical complexes comprising outer apical adherens junctions and inner septate junctions (although these have mostly been referred to as tight and gap junctions, respectively; Canning et al. 1996, 2000, 2007, 2008; Okamura et al. 2002). Similar junctional complexes develop in the inner epithelium (in *Buddenbrockia* species), here with the adherens junctions facing the inner cavity and the septate junctions being more distal (Okamura et al. 2002; Canning et al. 2008). In both epithelia, the adherens junctions develop (or at least become visible) before the septate junctions. In myxoworms (worm-like malacosporean trophic stages) of *Buddenbrockia,* muscle develops between the two epithelial layers. There are no cell-cell junctions between muscle cells nor between muscle cells and cells of the inner or outer epithelia (Canning et al. 2008).

The outer wall of actinosporean pansporocysts is created by 2–8 flattened envelope cells that are interconnected by cell-cell junctions (Fig. 9.1g). These junctions have been described in several species (e.g. *Ellipsomyxa mugilis*, Rangel et al. 2012; Aurantiactinomyxon-type myxosporean, Morris 2012; *Triactinomyxon legeri*, Lom and Dykova 1992; *Aurantiactionmyxon*, *Myxobolus cultus*, Lom et al. 1997; *Sphaeractinomyxon ersei*, Hallett et al. 1998; *Tetraspora discoidea*, Hallett and Lester 1999; *Myxobolus cerebralis*, El-Matbouli and Hoffmann 1998). In all cases ultrastructure reveals clear characteristics of apical adherens junctions (as defined above), although these junctions have been variously named (e.g. as adherens junction, desmosome-like junction, cell junction, gap junction, electron dense junction, junction reminiscent of tight junction). Septate junctions either do not occur or are indiscernible (due to the narrow region of contact between the highly flattened envelope cells). In any case, some form of sealing (occluding) junction must be present because the inner space of the pansporocyst that accommodates the developing spores almost certainly has a chemical composition different to that of the outside medium (host intercellular or coelomic fluid). There is also good evidence for transcytotic uptake of nutrients (Fig. 9.1g, Hallett et al. 1998; Hallett and Lester 1999, and see Sect. 9.5.1 on Resorptive and secretory tissues) through the envelope cells, a process requiring epithelial characteristics (e.g. polarity and control of permeability).

Gap junctions have so far not been demonstrated in myxozoans by ultrastructural or experimental techniques. Their presence, however, is likely (see Sect. 9.5.2 on Nervous tissues). Other types of communicative junctions have been described. In early sporogonic stages of *Thelohanellus nikolskii*, two cells remain connected via a narrow channel that contains bundles of microtubules, forming an open cytoplasmic bridge (Desser et al. 1983). These cells then differentiate into a two-cell sporoblast with

pericyte and enclosed generative cell. A cytoplasmic bridge is also described between α and β cells in the pansporocyst of an *Aurantiactinomyxon*-type species (Lom et al. 1997). It is not clear how long these connections persist in myxozoans. One possibility is that they represent remnants of the midbody of cytokinesis and thus reflect a highly transient stage of cell division. Alternatively, cytokinesis may be arrested for a period of time. Midbodies are clearly present in cell divisions during malacosporean development (sporogony) in bryozoan hosts (see e.g. Fig. 2F in Okamura et al. 2002). Similar structures, although without clear microtubule involvement, have been shown between secondary cells of *Sphaerospora testicularis* (Sitjà-Bobadilla and Alvarez-Pellitero 1993b) and between secondary and tertiary cells in plasmodia of the myxosporean, *Ortholinea fluviatilis*, infecting fish (Figs. 43 and 44 in Lom and Dyková 1996). These structures have been interpreted as remnants of cytokinesis.

The function of these myxozoan cytoplasmic bridges is unclear, but similar structures in other metazoans facilitate transport of gene products, cytoplasmic components and organelles between cells enabling daughter cells to acquire diverging morphologies (e.g. in oogenesis; Fawcett 1961; de Cuevas et al. 1997; Pepling and Spradling 1998; Alexandrova et al. 2005). Such connections also synchronise cell cycles during embryonic development and spermatogenesis (Fawcett 1961) and are involved in nematoblast formation in *Hydra* (Fawcett et al. 1959; Fawcett 1961).

9.3.2 Junctions Between Myxozoans and Hosts

Unusual types of cell-cell junctions occur where parasite cells make contact with neighbouring host cells. This is mostly evident in coelozoic forms. It is not always clear whether these junctions directly connect parasite and host membranes or whether connection is via an intermediate ECM. In the latter case the junctions would have to be classified as cell-matrix junctions rather than cell-cell junctions. Below we describe some examples.

Zschokkella pleomorpha plasmodia form microvilli-like cytoplasmic extensions that interdigitate with microvilli of the host renal epithelium. In some areas membranes appose very closely and bar-like junctions reminiscent of septate junctions are seen (Lom and Dyková 1996). Plasmodia of several species attach to the gall bladder epithelium by large cytoplasmic protrusions that connect mostly, but not exclusively, to the junctions between epithelial cells (e.g. in *Ortholinea fluviatilis*, Lom and Dykovà 1996; *Myxidium trachinorum*, Canning et al. 1999; *Ellipsomyxa gobioides*, Azevedo et al. 2013; *Zschokkella mugilis*, Sitjà-Bobadilla and Alvarez-Pellitero 1993a). The type of junction facilitating these connections has not been investigated in detail. Similar junctions also occur between *Sphaerospora epinepheli* plasmodia and kidney tubule epithelium (Supamattaya et al. 1993). In *Triangulamyxa psittaca* septate junctions have been proposed to attach pseudopodia to the surface of the urinary bladder epithelium (Rocha et al. 2011). These regions of attachment are, however, much too large to be achieved by septate junctions and more likely represent fields of small spot-like adherens junctions, similar to those seen by Paperna et al. (1987) in *Myxidium giardi*. In *Ortholinea fluviatilis* additional desmosome-like junctions occur between the tips of the host microvilli and the plasmodial membrane (Lom and Dyková 1996). Neither membrane protrusions nor microvilli occur where *Myxidium gadi* plasmodia attach to fish gall bladder epithelial cells, but the parallel alignment of host and parasite membranes is suggestive of proper cell-cell junctions (Feist 1995). In *Myxidium giardii* rhizoid-like protrusions of the plasmodial membrane connect to the urinary bladder epithelial cells by spot-like (desmosomal) attachment points at regular intervals (Paperna et al. 1987). Plasmodial extensions also anchor *Henneguya laterocapsulata* to the malphigian cells of the host skin (Obiekezie and Schmal 1993). Junctions with parallel membranes and regularly

spaced bands reminiscent of septate junctions have been found between plasmodia of *Enteromyxum scophthalmi* and host intestinal epithelium (Redondo et al. 2003).

The formation of cell-cell junctions involves complex intercellular signalling and crosstalk and usually happens between cells belonging to the same tissue (Juliano 2002; Cereijido et al. 2004). Thus, it is unclear how junctions can be formed between cells of different species. It may be highly adaptive for the parasite to exploit the host cells' capabilities to form junctions in order, for instance, to subvert host responses by chemical subterfuge or simply to gain purchase and eliminate expulsion from coelozoic spaces. In turn, the host may alter the structure of its cell-junctions in order to prevent formation of junctions between host and parasite cells. Such dynamics could have accelerated the evolution of cell-cell junctions in both hosts and parasites, contributing perhaps to the unusual structure of myxozoan cell-cell junctions.

9.4 Extracellular Matrix and Cell-Matrix Junctions

The ECM is composed of polysaccharides and proteins. The former (especially glycosaminoglucans) form a gel-like substance which is stabilised by a dense network of interlinked proteins (especially collagen). While mesenchymal cells secrete ECM equally via their entire surface, epithelial cells are polarised, with a clear distinction between their apical and basal surfaces. This polarity facilitates the differential secretion of various types of ECM. Apically secreted ECM components often form protective layers of the epidermis, such as a glycocalyx or cuticle. The basal lamina is another specialised form of ECM which is secreted by the basal surface of an epithelium and appears ultrastructurally as a dense thin lining just beneath the cell membrane. The basal lamina is stratified, with an upper, electron dense zone (the *lamina densa*) proximal to the cell membrane, followed by a more electron lucid zone (the *lamina lucida*), which in turn is followed by a layer of normal ECM (the *lamina fibroreticularis*) resembling that around mesenchymal cells. Basal lamina occurs in association with epithelial cells and is one of the defining features of an epithelium. Formation of the basal lamina is specified by a unique set of genes, supporting homology of this feature across Eumetazoa (Hynes 2012).

Cells anchor to the ECM by two classes of junctional complexes (Table 9.1; Fig. 9.1a): hemidesmosomes and focal adhesions (Magie and Martindale 2008). These involve a transmembrane protein (integrin) that, on the plasma side of the membrane, connects to the cytoskeleton (to intermediate filaments in the case of hemidesmosomes and to actin in the case of focal adhesions). On the external side of the cell, integrin connects to ECM components. Ultrastructurally, both hemidesmosomes and focal adhesions appear as electron dense plaques on the plasma side of the cell membrane. Cell-matrix junctions can occur all around mesenchymal cells, whereas in epithelial cells, they are restricted to the basal side.

A distinct basal lamina containing fibrous collagen has been demonstrated in the invertebrate trophic stages of malacosporeans (Fig. 9.1h, i). In *Buddenbrockia plumatellae* myxoworms an extensive collagenous layer is found basal to the epidermis (Okamura et al. 2002; McGurk et al. 2006; Canning et al. 2008). In *T. bryosalmonae* (Canning et al. 2000), *Buddenbrockia allmani* (Canning et al. 2007) and the sac-forming *B. plumatellae* (Canning et al. 1996) this layer is less pronounced, but still visible. A clear lamina densa is not discernible in any malacosporean, although immediately below the epidermal cells the fibres are more concentrated and run in multiple directions. Muscle and connecting cells in myxoworms are in contact with the ECM layer and, although specific cell-matrix junctions have not been seen in detail, the muscle and connecting cells seem to be mechanically coupled to the basal lamina: the ECM layer is much thicker and has pronounced radial fibres near the connecting cells (Fig. 9.3d, e; Okamura et al. 2002). Mechanical connections between muscle cells and connecting cells also seem to involve cell-matrix junctions. The cells themselves are separated by a large gap,

which is filled with fibrillar material (Okamura et al. 2002; Canning et al. 2008), and cell-cell junctions are not evident.

The valve and capsulogenic cells that form the external wall of myxozoan spores demonstrate epithelial characteristics in the form of cell junctions, however a characteristic basal lamina has so far not been shown by ultrastructural investigation and is probably absent. Canning et al. (2007) report a flocculent matrix in the internal compartment of early spores of *B. allmani* that resembles an ECM-like component, but the nature and significance of this feature requires further investigation.

An apical glycocalyx- or cuticle-like ECM is present on the outer membrane of valve cells in several myxosporeans. It appears as a slightly granular continuous layer (Feist 1995; Fig. 23; Hallett et al. 1998), a dense granular continuous coat (Desser et al. 1983), or a dark fibrous layer sometimes restricted to the ridges of valve cells (Azevedo et al. 1989, 2011, 2012). Casal et al. (2003) found the space between the pericyte and developing spores to be filled with a loose flocculent material and Desportes-Livage et al. (1990) observed dense transmembrane particles on the surface of valve cells. Such extracellular layers may cause hardening of the spore valves. If distributed heterogeneously they may create the ridge-like structures characterising spores of some myxosporeans (Fiala and Bartosova 2010). In other cases the hardening agent is intracellular, located directly beneath the outer membrane of the valve cell (Lom and Dykova 1996; Lom et al. 1997; Morrison et al. 1996). In several cases both intra- and extracellular layers characterise the outer valve cell membrane (e.g. Azevedo et al. 2011, 2012; Rocha et al. 2013). Extensive secretions from valve cells have been observed in species of *Polysporoplasma* (Sitjà-Bobadilla and Alvarez-Pellitero 1995).

The agents responsible for hardening the spores are unknown. Using lectin histochemistry and light microscopy, Munoz et al. (1999) demonstrated chitin and α-galactose associated with the valves. They could not, however, determine whether these compounds reside within or on the surface of valve cells because light microscopy cannot resolve at this level. An electron dense layer around pansporocysts or sporoplasms is often reported (e.g. Siddall et al. 1995; Lom and Dykova 1992) and the surfaces of some coelozoic plasmodia bear microvilli-like processes connected by fine, fibrous glycocalyx-like strands (e.g. *Chloromyxum menthicirri*, Casal et al. 2009; and *Sphaeromyxa noblei*, Lom 2004). A thick layer of collagen surrounds plasmodia of *Myxobolus pendula* (Martyn et al. 2002), but it is not clear whether this is secreted by the parasite or whether it originates from modified surrounding host tissue. In most other plasmodia and pseudoplasmodia the cell membranes appear just slightly thicker and slightly more electron dense than those of other cells (e.g. secondary cells, host cells). Higher resolution images would be desirable to determine if this is due to an external secreted coat.

9.5 Myxozoan Tissues

9.5.1 Resorptive and Secretory Tissues

One characteristic eumetazoan feature obviously lacking in Myxozoa is a digestive tract. An intestinal tract, embryologically derived from the archenteron, with cells capable of extracellular digestion is regarded as the key innovation of the common ancestor of cnidarians, ctenophores and bilaterians. The intestinal tract is a self-contained compartment communicating to the exterior via mouth or mouth and anal opening. Gland cells interspersed in between normal endodermal epithelial cells secrete digestive enzymes into the lumen of the intestinal tract and nutrients released by extracellular digestion are taken up by the epithelial cells.

Uptake of nutrients from the host environment by myxozoans is thought to be accomplished by simple endocytosis (pino- or phagocytosis) at the external surface of the trophozoite. This surface is an epithelium in myxozoan stages that develop in invertebrates (i.e. malacosporean sacs and worms (Fig. 9.2) and myxosporean pansporocysts (Fig. 9.1g)), and is the cell membrane in myxozoan stages developing in vertebrates

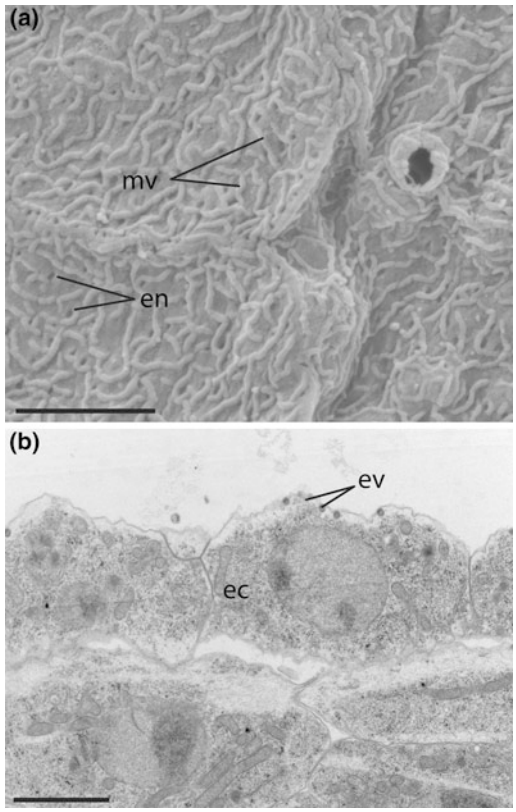


Fig. 9.2 Nutrient uptake in myxozoans. **a** SEM image of *Buddenbrockia* external surface showing microvilli and putative endocytotic pits. The large pore is a discharged polar capsule, which in some *Buddenbrockia* worm populations occur in the epidermis. **b** TEM of endocytosis in *Buddenbrockia* (photo: Alan Curry). *ec* epidermal cell, *ev* endocytotic vesicles, *mv* microvilli. *Scale bars* 2 µm

(plasmodia, pseudoplasmodia and uninuclear pericytes). Endocytotic vesicles have been demonstrated ultrastructurally in many species (e.g. *Theloanellus nikolskii*, Desser et al. 1983; *Myxidium mackei*, Helke and Poynton 2005).

Surface extensions potentially involved in uptake include microvilli, pseudopodia, and small invaginations of the plasma membrane (often termed pinocytotic channels). Microvillous surface extensions have been documented in several species (e.g. *Myxobolus insignis*, Azevedo et al. 2013; *Triactinomyxon drepanopsettae*, Morrison et al. 1996). Pansporocyst envelope cells in *Sphaeractinomyxon ersei* exhibit numerous membrane-bound vesicles and cell surface extensions to both the outside and inside of the pansporocyst. This is suggestive of transcytosis of nutrients, especially as the extensions become smaller once sporogony nears completion (Hallett et al. 1998). Pansporocyst cells of *M. cerebralis* exhibit only a few microvilli and pseudopodia (El-Matbouli and Hoffmann 1998). Vesicles interpreted as phagosomes have also been observed in *Triactinomyxon legeri* early (binucleate) stages as well as in pansporocysts (Lom and Dyková 1992). In many large plasmodia, the segregation of the cytoplasm into an outer granular layer (ectoplasma) and an inner endoplasma rich in organelles suggests an organisation that may reflect remnant resorptive and secretory functions of polarised epithelial cells.

There is evidence for secretion of material into the intercellular space surrounding myxozoan trophozoites, but it is not clear whether this promotes extracellular digestion. Sporoplasmosomes (membrane-bound vesicles) in trophic stages in fish are aligned directly beneath the cell membrane and show polarity towards it (e.g. in *T. bryosalmonae*; Feist 1997; Morris and Freeman 2010). In cases where nutrients are readily available (e.g. coelozoic forms in body cavity, bloodstream, lymph, etc.), secreted substances likely provide non-digestive functions, such as camouflage against the host immune system or excretion of waste products. In *M. cerebralis* there is evidence for the lysis of cartilage and phagocytosis of chondrocytes by the plasmodia (El-Matbouli et al. 1995; Feist and Longshaw 2006). An exceptional case is represented by *M. pendula*. Stages of this species developing in fish consist of an inner plasmodial generative region surrounded by a palisade layer of cells that secrete an extensive collagenous matrix (forming a cyst) and presumably undertake nutrient uptake (Martyn et al. 2002).

The interpretation that in myxozoans the intestinal tract is completely reduced and resorptive functions are taken over by the epidermis is obvious. In the parasitic cnidarian, *Polypodium hydriforme*, the epithelium of the intestinal tract (i.e. the endoderm) is external in parasitic developmental stages with a reversion to the normal (endoderm inside) condition when the animal leaves the host (Raikova 1980, 1994).

Such a development has not been shown in myxozoans, but the nature of the outer epidermis of malacosporeans has yet to be resolved. Epidermal uptake of dissolved nutrients is, however, undertaken in many free-living aquatic invertebrates, including cnidarians (see Chap. 3), but how much this contributes to general nutrition is unknown. In certain endoparasitic taxa, especially in platyhelminths but also in other phyla (Rhizocephala, Acanthocephala, Nematomorpha), epidermal uptake of nutrients is significant and may even exceed nutrient uptake via the intestinal tract (Halton 1997; Wilson 2012). This has led to reduction of the intestinal tract in several cases (e.g. cestodes).

Parasitic platyhelminths (Neodermata) have a syncytial outer layer (tegument), which significantly contributes to nutrition, but is also the main parasite-host interface. There are several hypotheses for the evolution of this syncytial condition (Dalton et al. 2004). For instance, it has been proposed that the absence of cell boundaries may reduce vulnerability to host defences (e.g. by digestive enzymes, phagocytes, or other components of the immune system). In addition, nutrients and other substances may be distributed more easily (especially laterally), while organelles and nuclei can be secured in regions less readily accessible to host defences. These scenarios may equally apply to myxozoans. The fact that syncytial trophozoites (plasmodia) mostly occur in vertebrates, which may present more challenging immune responses than invertebrates, provides support for the host-defence scenario. In the only known case in which a cellular outer layer exists (*M. pendula)*, the trophozoite is surrounded by a massive collagenous ECM (Martyn et al. 2002), potentially providing camouflage from host defences.

9.5.2 Nervous Tissues

Nervous tissues are characteristic of all metazoans except sponges. These range from the diffuse nerve nets that ramify throughout the body of cnidarians and ctenophores to the centralised nervous systems characteristic of bilaterians. Nervous coordination facilitates physical reactions that are perceived as animal-like and its putative absence is therefore hard to accept when there is evidence of concerted movement. On the face of it, malacosporean worms present such a paradox. Myxoworms exhibit helical swimming and stationary coiling in *B. plumatellae* (Canning et al. 2002; Gruhl and Okamura, pers. obs.), yet all structural investigations have so far failed to demonstrate nervous elements in *B. plumatellae* (or in any other myxozoan). The syncytial forms (plasmodia) of myxosporeans exclude multicellular nervous systems in these highly derived myxozoans. Recent research now provides evidence for neurotransmission in *Buddenbrockia* myxoworms, with a transcriptomic survey demonstrating expression of elements of neurotransmission pathways (Gruhl and Okamura, unpub. data). This suggests that, although nervous signal transduction occurs, nerve cells have reduced their characteristic features and are not recognised in structural investigations. Such effects have been demonstrated in nervous systems of other miniaturised animals (Niven and Farris 2012). *B. plumatellae* worms exhibit no notable taxes in response to light (Gruhl and Okamura, unpub. data), and sensory elements have never been encountered by ultrastructure or light microscopy. An absence of sensory elements is consistent with the absence of ciliary structures in all myxozoans. It is possible that the nervous system of *B. plumatellae* consists of only a few pacemaker neurons that excite the muscle cells. Excitation could then be passed on from muscle cell to muscle cell or through the rows of connecting cells that run longitudinally along the body axis in concert with the four muscle blocks (Okamura et al. 2002; Canning et al. 2008). Conduction via excitable epithelia stimulates the contraction of swimming muscles in many hydromedusans and siphonophores (Mackie 2004) and involves synaptic contacts or gap junctions between cells. Neither synapses nor gap junctions have, however, been found in myxoworms.

The activation of sporoplasms could involve communication that resembles nervous transmission. Upon contact with the host the polar

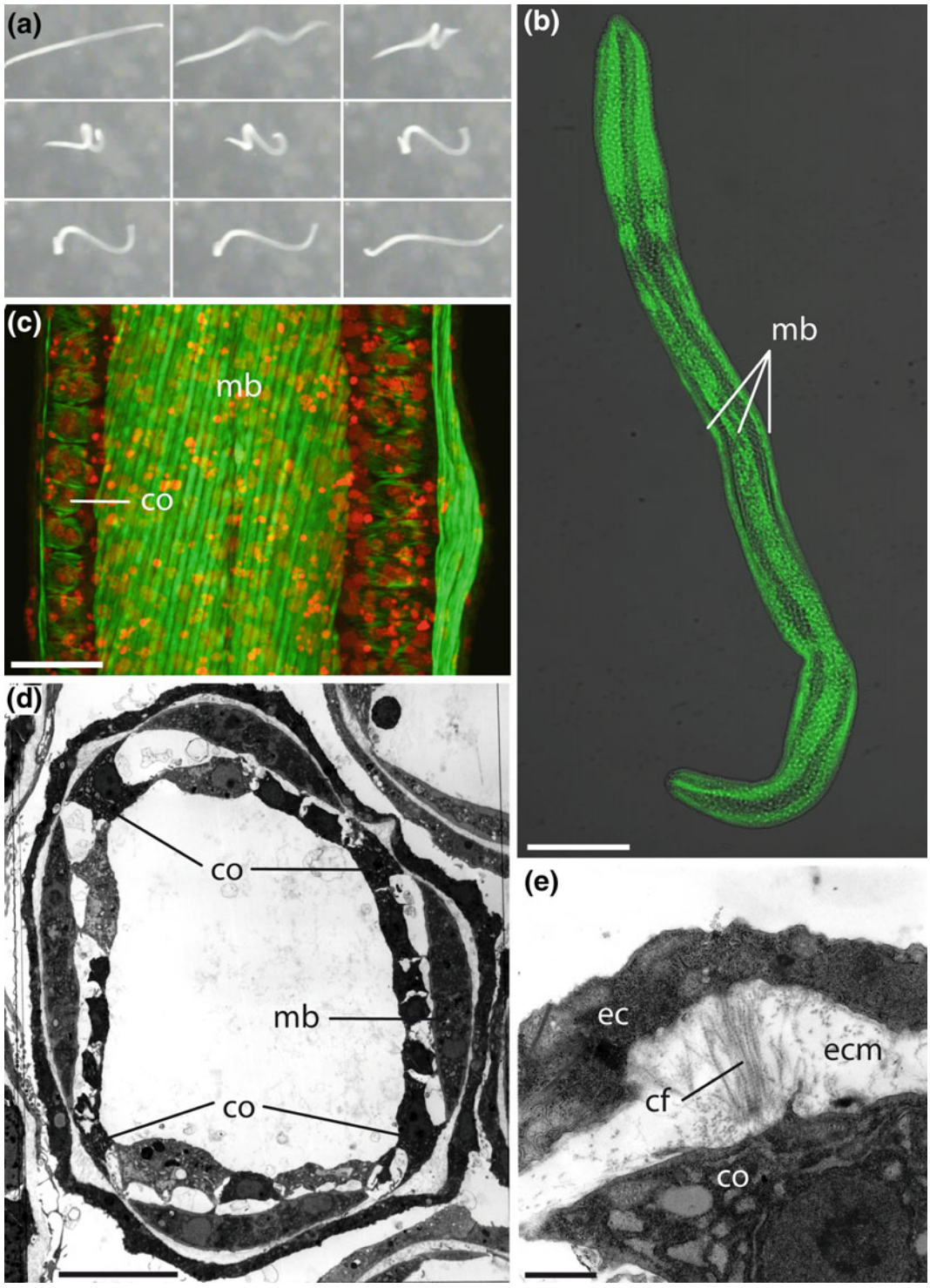
capsule discharges and the expelled polar filament anchors the spore to the host. At the same time (or shortly thereafter), the sporoplasms enclosed within spores initiate amoeboid movements to reach and penetrate the host tissue. Such movement could involve a signal transduction from the capsulogenic cells (acting as chemo- or mechanoreceptors) to the sporoplasms. Further study is required to elucidate how sporoplasms are activated (see Chap. 13 for more information).

9.5.3 Musculature

B. plumatellae and other undescribed worm-like malacosporeans (Canning et al. 2002; Hartikainen et al. 2014, see also Chap. 4) differ from all other myxozoan species known so far in having a highly motile trophozoite with distinctive musculature. Developing and mature myxoworms undergo rhythmic spiralling contractions within the body cavities of their hosts (phylactolaemate bryozoans). Myxoworms released from hosts exhibit helical swimming and stationary coiling (Fig. 9.3a; Schroeder 1912; Canning et al. 2002).

Cnidarian muscle systems are formed by epitheliomuscular cells from the endodermal or ectodermal layer. Basal processes in these cells contain myofilaments. The musculature of *B. plumatella*, however, does not consist of epitheliomuscular cells. Instead, the four longitudinal muscle blocks (Fig. 9.3b–d) are formed by independent cells that are separated from both the outer and inner epithelia by ECM (Fig. 9.3d; Okamura et al. 2002; Gruhl and Okamura 2012). Each muscle block consists of two rows of elongate muscle cells arranged at an angle of 12° with respect to the longitudinal axis of the worm (Fig. 9.3c). These four muscle blocks establish a muscle system characterised by chirality and tetraradial symmetry (Gruhl and Okamura 2012). The strong preference for a right-handed chiral pattern suggests genetic determination and selection. Cells of neighbouring muscle blocks are linked via a row of connecting cells (Fig. 9.3c–e) that exhibit an enhanced actin cytoskeleton (see above, Okamura et al. 2002; Gruhl and Okamura 2012). Connecting cells are likely to provide mechanical functions (Gruhl and Okamura 2012), but it is not known whether they contract or act purely as anchoring elements. Muscle development begins early in ontogeny with precursor cells recognisable in stages that begin elongation to the final worm-like form. In these stages, four cells, arranged in 90° quadrants, lie in an intermediate position between the developing outer (epidermal) and inner (sporogonic) epithelia. The presence of muscles is the main histological difference between myxoworms and other malacosporeans. Muscles are intimately linked with the differences in body axis polarities and overall body shapes between myxoworms and sac-forming malacosporeans.

The function of the musculature in myxoworms is elusive. Although, as discussed previously (see Chaps. 2 and 3), musculature most likely represents a primitive feature that has been lost repeatedly within Myxozoa (Hartikainen et al. 2014; and Chap. 4), it is certainly not a mere rudiment in myxoworms. The energetic costs of both formation and extensive use of musculature suggest a significant adaptive value. Worm movements inside the host body cavity may increase the flow of coelomic fluid, thus increasing the flux of nutrients and oxygen and hence their uptake by diffusion. However, in freshwater bryozoans the coelomic fluid is in constant circulation anyway, so this effect is potentially limited. Another possible function could be to increase the dimensions of the host's body cavity to create more space. This scenario, however, is unsupported by the sac-like, immotile parasite *T. bryosalmonae* having a greater effect on bryozoan host morphology (causing zooid gigantism; Hartikainen et al. 2013) than is evident for *B. plumatellae* (Hartikainen pers. comm.). Locomotion within the host could also help the parasite to spread within the collective colonial body cavity to reach unparasitised regions of the bryozoan colony. Helical swimming after release from the host potentially increases dispersal. Swimming may also increase transmission by mimicking prey, if the fish host feeds on small worm-like organisms (e.g. tubificids, chironomids).


(a)
(b)
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◀**Fig. 9.3** Musculature in *Buddenbrockia plumatellae*. **a** Light microscopy frames of swimming locomotion. **b** Confocal image of muscle block arrangement (whole mount, mature worm). **c** Detail of muscle cells (mature worm). **d** TEM cross section showing muscle block, inner epithelium and connecting cells. **e** TEM detail of connecting cell anchored to epidermis via extracellular matrix. Photos: **d** reproduced from Okamura et al. (2002) with permission from Cambridge University Press, **e** Alan Curry. *cf* collagen fibres, *co* connecting cell, *ec* epidermal cell, *ecm* extracellular matrix, *mb* muscle block. *Scale bars* **b** 200 μm, **c** 20 μm, **d** 10, **e** 1 μm

9.5.4 The Nature of Myxozoan Tissues

Only malacosporean trophic stages in invertebrate hosts display all criteria of possessing a bona fide epithelium. Nevertheless, many myxozoan cells and tissues show some characteristics that are clearly epithelial. These include septate and adherens junctions, ECM, and general cell polarity that enables directed transport uptake and secretion in multicellular forms. From an evolutionary perspective these features may be explained by reduction and modification in the course of general body simplification (see Chaps. 2 and 3) or as specific adaptations to the parasites' environment. The epithelial tissue characteristics retained in trophic stages of the Malacosporea include 1–2 epithelial layers, complex cell junctions, and a pronounced basal ECM. Myxosporean pansporocysts demonstrate cell-cell junctions and general cell polarity along with functions associated with epithelia (directed uptake, secretion, transcytosis). Similarly, spore walls (valve + capsulogenic cells) in both malacosporeans and myxosporeans present epithelial traits (polarity, cell-cell junctions, ECM) and functions (secretion, sealing). Thus, it is imprecise to suggest that myxosporeans lack tissues. Rather, they possess highly modified tissues that maintain traits and functions associated with a tissue-level of organisation. Even the syncytial plasmodial stages of myxosporeans sustain functions of epithelial layers (such as sealing, bordering, uptake, secretion) that are most likely directly derived from a former epithelial state.

Mesenchymal cells typically arise by the process of epithelial-mesenchymal transition. This may involve an epithelium completely disintegrating or epithelial cells leaving the epithelial layer, with neighbouring epithelial cells closing the resulting gap (Shook and Keller 2003; Magie and Martindale 2008). Notably, Canning et al. (1996) inferred the latter process for the removal of dying cells from the epidermal wall of a sac-forming malacosporean. In all malacosporeans examined to date the internal cells derive from an epithelium, a process that may be homologous to epithelial-mesenchyme transition. In *Buddenbrockia* myxoworms the muscle cells remain in a mesenchymal state.

9.6 Development

9.6.1 Gametogenesis

Sexual reproduction via fusion of haploid egg and sperm cells is a fundamental metazoan feature, although the mechanisms, timing, and location of germ cell specification differ across phyla. One particularly important distinction is between preformation and epigenesis—whether the germ cell lineage is segregated by maternally inherited determinants (preformation) or late in development by inductive signalling (epigenesis). In contrast to many laboratory model organisms, cnidarians and many other basal animal phyla, exhibit epigenesis (Extavour and Akam 2003). Generally, gametogenesis in cnidarians occurs in the mesoglea near the gastrodermis although in some hydrozoans this occurs near the epidermis. In anthozoans and scyphozoans primordial germ cells originate from the endoderm and germ cells aggregate in the mesenteries. In hydrozoans, primordial germ cells derive from somatic interstitial stem cells (I-cells), which are of ectodermal origin. Germ cell development occurs in medusae or in polyps when the medusa stage is reduced or absent. Distinguishing cnidarian germ cells from somatic cells by cytological features is difficult, especially during early stages of development. The use of molecular markers (for instance to detect *vasa* expression patterns; e.g. Extavour

et al. 2005; Shikina et al. 2012) to identify germ cells and to locate gametogenesis can thus be highly informative.

Parallels with gametogenesis in cnidarians are difficult to draw for myxozoans because it is highly modified and their gametes lack characteristic oocyte and spermatocyte features. Indeed, the only real clue to date relates to observations of meiosis. In malacosporeans, meiosis occurs in luminal cells that, in *Buddenbrockia* species, derive from the inner epithelial layer and proliferate within the internal cavity (Canning et al. 1996, 2000, 2002, 2007; Okamura 2002). In *T. bryosalmonae* sacs an inner epithelium is not present (Morris and Adams 2007), thus the origin of free luminal stages undergoing meiosis is elusive. Spore development in malacosporeans involves the aggregation of luminal cells to form multicellular spores (Canning and Okamura 2004). Whether these spores are formed entirely of haploid cells or contain both diploid and haploid cells is unknown. Canning et al. (2007) proposed that diploid cells may differentiate into valve and capsulogenic cells of spores and that the two sporoplasms are haploid (see Chaps. 3 and 8). Sporoplasms may therefore represent gametes. When diploidy is achieved is unknown and is discussed extensively in Chap. 3. Potential parallels in gametogenesis of myxozoans with other cnidarians include derivation of germ cell precursors from epithelial tissues and aggregation of germs cells during spore development.

We can infer few cnidarian parallels in gametogenesis of myxosporeans because of their extremely modified body plans. As in malacosporeans, meiosis in myxosporeans occurs during spore development in invertebrate hosts (El-Matbouli and Hoffman 1998; see Chaps. 3 and 8 for further discussion). Sporogenesis is initiated when two binucleate cells undergo plasmogamy (fusion) to form a stage with four nuclei. Further steps then lead to the production of two types of cells (termed α and β). According to the standard view both α and β cells undergo meiosis (El-Matbouli and Hoffman 1998), but a recent study proposes that meiosis only occurs in β cells (Morris 2012). The two scenarios differ in interpreting when fusion occurs to achieve diploidy (see Chap. 3 for discussion). This development is unique. Precursors to germ cells are not clearly derived from tissues. The only apparent potential parallel with cnidarian development may be the early aggregation of binucleate cells in the endoderm.

An intriguing question is whether myxozoan germ cells are derived from endodermal or ectodermal tissue. The latter would support an affinity with hydrozoans, in which gametes are known to originate from ectoderm. Resolving this question depends on the interpretation of germ layers in myxozoans (see below and Chap. 3). Topologically the germ cells of malacosporean worms are of endodermal origin and hence a feature not shared with hydrozoans. However, the development of endoderm as an external surface in the parasitic cnidarian, *P. hydriforme*, suggests the possibility that myxozoans could deploy similarly inverted epithelial surfaces.

9.6.2 Embryogenesis

How might the reduced diversity and aberrant characteristics of myxozoan tissues coincide with and be explained by differences in developmental pathways of myxozoans and free-living cnidarians? Myxozoan morphology is characterised by three unique features: (a) the extreme simplification and reduction of major characters present in the closest free-living relatives (cnidarians); (b) miniaturisation, and; (c) novel forms (spores). All of these features are clearly adaptations that evolved during the acquisition of an endoparasitic life style—the first two to exploit hosts and the last to achieve transmission. Major body-plan modifications are usually the result of changes in the developmental program of an organism. Both a decrease in body size and loss of characters can in principle be achieved by changes in the rates and timing of developmental events (heterochrony) (see also Chap. 3).

In all eumetazoans embryogenesis ensues when a zygote undergoes cleavage to form a blastula consisting of cells connected by junctions that line a hollow internal cavity. The next step is gastrulation, which entails the segregation

of ectoderm and endoderm and internalisation of the latter tissue layer. Many modes of gastrulation have been observed in cnidarians, including invagination, immigration, epiboly and delamination (Tardent 1978). It is increasingly clear that gastrulation is a complicated process that is difficult to define and study (see discussion in Hejnol and Martindale 2008). In bilaterians, gastrulation usually coincides with, or is followed immediately by, the formation of the third germ layer, the mesoderm.

During early stages of malacosporean development, groups of cells inferred to derive commonly from mitosis organise to form a layer of epidermal cells connected by cell junctions that encloses an irregular, loosely arranged mass of cells (Canning et al. 2007, 2008; Gruhl and Okamura 2012). This step likely corresponds to gastrulation, but it is currently impossible to infer the mode of gastrulation due to the low number of such stages investigated. Later stages of development differ between malacosporean species. In *T. bryosalmonae* the inner tissue remains compact, reminiscent of a solid gastrula (stereogastrula), a form that characterises many cnidarians. This inner tissue undertakes spore formation.

Buddenbrockia species form stages with two epithelial cell layers that are separated by a basal lamina. The inner epithelium surrounds a central cavity (Canning et al. 2007, 2008), which lacks communication to the exterior (there is no blastopore). Muscle cell precursors in myxoworms are embedded in the ECM between the inner and outer epithelium and are therefore technically to be regarded as mesodermal cells. Cnidarians are traditionally considered diploblastic organisms, with musculature formed by epitheliomuscle cells that reside in the endo- or ectoderm. This view, however, has recently been questioned (e.g., Martindale et al. 2004). The mesoglea in many cnidarians contains cells that originate from these epithelia (e.g. the ectodermally derived I-cells in hydrozoans). In addition, in some anthozoan polyps and in hydrozoan medusa buds muscles occur as independent tissues in the mesoglea and lack connections to endoderm or ectoderm. Such muscle is thus topologically mesodermal (Seipel and Schmid 2006), although appearing late in development and not during gastrulation as is case with bilaterian mesoderm. Topologically mesodermal musculature appearing during early development has been demonstrated in *Buddenbrockia* worms (Gruhl and Okamura 2012). The homology of these mesodermal-like tissues in cnidarians to bilaterian mesoderm remains to be demonstrated.

Myxosporean development is initiated by cell doublets (see Chap. 8 for review of early development). These stages may be associations of individual sporoplasms or they may be formed by mitosis and could thus represent early cleavage stages (2-cell embryos). Further development involves one cell (the secondary cell) being incorporated in another (the pericyte), probably by engulfment. A possible interpretation is that this is an extreme form of gastrulation with the gastrula stage consisting essentially of one ectodermal and one endodermal (or endomesodermal) cell.

Further developmental processes that play important roles in metazoan development, such as cell migration and apoptosis, are insufficiently documented at present in myxozoans.

9.7 Conclusions: Myxozoan Development in Perspective

The extreme morphological simplification of myxozoans illustrated by plasmodial forms that are capable of uptake and secretion and of supporting internal developmental stages demonstrates the power of selection for driving change in an inherently flexible cnidarian body plan. The adaptations that have enabled myxosporeans to exploit their hosts have obscured many cnidarian traits associated with development. Nevertheless vestiges of these traits remain evident as processes that were once carried out by tissues in their free-living ancestors. This view is supported by the presence of proper tissues in the more primitive malacosporeans which also illustrate a capacity for tissue loss (e.g. both endoderm and musculature are absent in many sac-forming taxa). The reappearance of myxoworms within the Malacosporea (Hartikainen et al. 2014, and see Chap. 4) on multiple occasions suggests that

the genetic basis for worm development has been retained. The developmental pathways that lead to sacs and myxoworms may be triggered by simple differences in gene regulatory networks—a scenario particularly supported by the fact that sacs and worms can be closely-related sister taxa. The reappearance of a cellular outer layer in the myxosporean, *M. pendula*, may similarly be postulated to reflect the expression of genes that are normally suppressed during myxosporean development. This, however, would imply that the relevant genes for tissue development have been retained over substantially longer periods of time in myxosporeans (perhaps as a result of pleiotropy) and are currently not expressed in all other myxosporeans described to date.

The early development of myxozoans is poorly understood but we are now in a position to evaluate this from a cnidarian viewpoint. Outstanding issues that remain to be resolved include the identification and formation of blastula and gastrula stages and the orientation of the ectoderm and endoderm (gastrodermis) of myxozoans.

We now have molecular tools (gene expression studies, genomics and transcriptomics) that will enable us to unravel further aspects of the evolution and development of the Myxozoa and can look forward to new insights on the evolutionary trajectories associated with the road to endoparasitism.

9.8 Key Questions for Future Study

- Are there recognisable germ layers in myxozoans?
- Does morphological variation within the Myxozoa result from heterochrony?
- Do myxoworms possess a highly reduced nervous system?
- Do physiological processes differ between malacosporean trophozoites (with a tissue level of development) and myxosporean trophozoites (characterised by syncytia)?
- Can germ cell markers be used to gain insights into gametogenesis and embryogenesis in myxozoans?
- What is the diversity of cell junctions and do they relate to phylogeny, hosts or tissue environment?
- What is the function of musculature in myxoworms and why has it been lost in other malacosporeans?

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Myxozoan Life Cycles: Practical Approaches and Insights

10

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Abstract

This chapter summarises the collective knowledge of myxozoan life cycles gained through experimental studies and observations. We review myxozoan life cycles, factors that influence parasite development and the conditions required for laboratory maintenance of invertebrate hosts. Unusual modes of transmission, including fish-to-fish and vertical transmission in invertebrate hosts are highlighted. We close by summarizing attempts to propagate myxozoans in vitro.

Keywords

Environmental factors · Laboratory culturing · Susceptibility · Invertebrate host · Fish-to-fish transmission · Vertical transmission · In vitro cultivation

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10.1 Well-Characterised Myxozoan Life Cycles

The first myxozoan life cycle—that of *Myxobolus cerebralis*, which causes salmonid whirling disease—was elucidated by Wolf and Markiw (1984). Prior to this revolutionary discovery, experimental transmission of myxozoans was usually unsuccessful. Both Uspenskaya (1957), Halliday (1976) observed that *M. cerebralis* spores must first be "aged" in sediment for up to 4 months before the parasite becomes infective. Hoffman and Putz (1969) reported the experimental transmission of whirling disease; however, their interpretation of results gained from fish-to-fish transmission by cohabitation has to be treated with caution, as direct transmission of *M. cerebralis* has never been documented. Most likely, unknown to the authors, the experiments were successful because the "spore-free" pond mud used in fish tanks contained oligochaete hosts. Molnár (1979) conducted fish infection experiments with *Myxobolus pavlovskii* from the gills of silver carp (*Hypophthalmichthys molitrix*). Parasite transmission was successful in one case only: with the use of muddy water. This finding reinforced the association of parasite infectivity with a muddy substrate, but the infectious agent remained unidentified.

Wolf and Markiw (1984) demonstrated unequivocally that the life cycle of *M. cerebralis* involves two spore forms, which develop in alternating vertebrate and invertebrate hosts (Fig. 10.1). This finding was a milestone in myxozoan research and significantly accelerated studies on myxozoan transmission and development. Since 1984, dozens of life cycles have been studied (Table 10.1), however most of the studies either replicate only partial life cycles (typically fish to worm transmission) or identify transmission of myxozoans based on DNA sequence matches. Holistic transmission studies can be difficult for several reasons: problems maintaining specific hosts under laboratory conditions, complexities of the transmission process and, for most species, a lack of knowledge of half the life cycle—the identity of the invertebrate host and morphology of the actinospore stage.

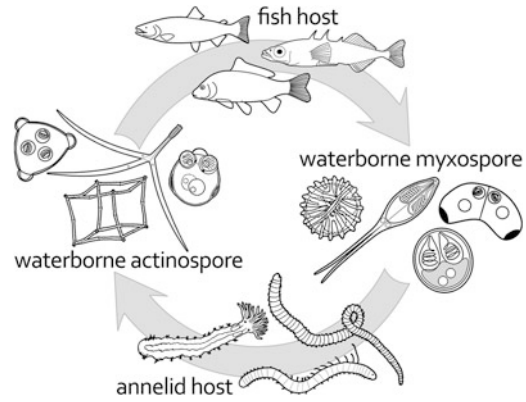

Fig. 10.1 Generic two-host myxozoan life cycle showing alternate vertebrate and invertebrate hosts, and the two morphologically dissimilar waterborne spore stages (modified from Atkinson 2011 by S. Atkinson)

Even though life cycles are inferred for some 50 myxozoan species (of >2,200 described taxa), laboratory transmission studies demonstrating transmission and development in both invertebrate and vertebrate hosts have been completed for five species only, four from Myxosporea, *M. cerebralis*, *Myxobolus pseudodispar*, *Myxobolus parviformis*, and *Ceratonova shasta* (syn. *Ceratomyxa shasta*) and one, *Tetracapsuloides bryosalmonae* from Malacosporea (Table 10.1).

10.1.1 *Myxobolus cerebralis*

The life cycle of *M. cerebralis* involves salmonid fishes and oligochaete worms. Infected *Oncorhynchus* spp. develop whirling disease while *Salmo* spp. (*S. salar* and *S. trutta*) develop limited clinical signs and fewer myxospores are produced (Hedrick et al. 1998; MacConnell and Vincent 2002; Steinbach Elwell et al. 2009). Intraspecific differences in host susceptibility have been shown for rainbow trout (*Oncorhynchus mykiss*) (Hedrick et al. 2003; Fetherman et al. 2011) and for the oligochaete *T. tubifex* (Beauchamp et al. 2001).

When waterborne *M. cerebralis* TAMs contact a susceptible fish, the sporoplasm penetrates then multiplies mitotically within the host epithelial cells (see Chap. 13 on host invasion). Sporoplasms migrate intercellularly in the fish epidermis and

Table 10.1 Summary of known myxozoan life cycles

Species	Actinospore	Experiment	DNA match	Biotope	Vertebrate host	Invertebrate host	Reference
Myxosporeans							
Ceratomyxa auerbachi	Tetractinomyxon	No	Yes	Marine	*Clupea harengus*	*Chone infundibuliformis*	Køie et al. (2008)
Ceratonova shasta	Tetractinomyxon	Complete f-w-f	100 %	Freshwater	*Oncorhynchus* spp.	*Manayunkia speciosa*	Bartholomew et al. (1997)
Chloromyxum auratum	Antonactinomyxon	Partial f-w	Yes	Freshwater	*Carassius auratus*	Mixed oligochaetes	Atkinson et al. (2007)
Chloromyxum schurovi	Neoactinomyxum	No	Yes	Freshwater	*Salmo salar, S. trutta*	*Eiseniella tetraedra*	Holzer et al. (2006)
Chloromyxum truttae	Aurantiactinomyxon	No	Yes	Freshwater	*Salmo salar*	*Stylodrilus heringianus*	Holzer et al. (2004)
Ellipsomyxa gobii	Tetractinomyxon	Partial w-f	Yes	Marine	*Pomatoschistus microps*	*Nereis* spp.	Køie et al. (2004)
Gadimyxa atlantica	Tetractinomyxon	Partial w-f	Yes	Marine	*Gadus morhua*	*Spirorbis* spp.	Køie et al. (2007)
Henneguya exilis	Aurantiactinomyxon	No	Yes	Freshwater	*Ictalurus punctatus*	*Dero digitata*	Lin et al. (1999)
Henneguya ictaluri	Aurantiactinomyxon	Partial w-f	100 %	Freshwater	*Ictalurus punctatus*	*Dero digitata*	Styer et al. (1991)
Henneguya nuesslini	Triactinomyxon	Partial w-f	100 %	Freshwater	*Salmo trutta, Salvelinus fontinalis*	*Tubifex tubifex*	Kallert et al. (2005a)
Hoferellus carassii (Germany)	Aurantiactinomyxon	Complete f-w-f	nd	Freshwater	*Carassius auratus*	Mixed oligochaetes	El-Matbouli et al. (1992b), Troullier et al. (1996)
Hoferellus carassii (Japan)	Neoactinomyxum	Partial f-w	nd	Freshwater	*Carassius auratus*	*Branchiura sowerbyi*	Yokoyama et al. (1993)
Hoferellus cyprini	Aurantiactinomyxon	Partial f-w	nd	Freshwater	*Cyprinus carpio*	*Nais* sp.	Grossheider and Körting (1992)
Myxidium giardi	Aurantiactinomyxon	Partial f-w	nd	Freshwater	*Anguilla anguilla*	*Tubifex tubifex*	Benajiba and Marquès (1993)
Myxidium truttae	Raabeia	No	Yes	Freshwater	*Salmo trutta*	*Tubifex tubifex*	Holzer et al. (2004)
Myxobilatus gasterostei	Triactinomyxon	No	99.8 %	Freshwater	*Gasterosteus aculeatus*	*Nais communis, Nais pseudobtusa*	Atkinson and Bartholomew (2009)
Myxobolus arcticus (Canada)	Triactinomyxon	Partial w-f	nd	Freshwater	*Oncorhynchus nerka*	*Stylodrilus heringianus*	Kent et al. (1993)
Myxobolus arcticus (Japan)	Triactinomyxon	Partial w-f	99.9 %	Freshwater	*Oncorhynchus masu, O. nerka*	*Lumbriculus variegatus*	Urawa (1994), Urawa et al. (2011)
Myxobolus bramae	Triactinomyxon	Partial f-w	nd	Freshwater	*Abramis brama*	*Tubifex tubifex*	Eszterbauer et al. (2000)
Myxobolus carassii	Triactinomyxon	Partial f-w		Freshwater	*Leuciscus idus*	*Tubifex tubifex*	El-Matbouli and Hoffmann (1993)
Myxobolus cerebralis	Triactinomyxon	Complete f-w-f	100 %	Freshwater	*Oncorhynchus mykiss*	*Tubifex tubifex*	Wolf and Markiw (1984), El-Matbouli et al. (1999b) etc.
Myxobolus cotti	Triactinomyxon	Partial f-w	nd	Freshwater	*Cottus gobio*	Mixed Oligochaetes	El-Matbouli and Hoffmann (1989)

(continued)

Table 10.1 (continued)

Species	Actinospore	Experiment	DNA match	Biotope	Vertebrate host	Invertebrate host	Reference
Myxobolus cultus	Raabeia	Partial w-f	99.4 %	Freshwater	*Carassius auratus*	*Branchiura sowerbyi*	Yokoyama et al. (1995), Eszterbauer et al. (2006)
Myxobolus dispar	Raabeia	Partial f-w	nd	Freshwater	*Cyprinus carpio*	*Tubifex tubifex*	Molnár et al. (1999), Holzer et al. (2004)
Myxobolus diversicapsularis	Triactinomyxon	No [a](Rácz et al. (2004)-yes)	99.6 %	Freshwater	*Rutilus rutilus*	*Tubifex tubifex*	(*M. intimus*-Rácz et al. (2004) Molnár et al. (2010)
Myxobolus drjagini	Triactinomyxon	Partial f-w	nd	Freshwater	*Hypophthalmichthys molitrix*	*Tubifex tubifex*	El-Mansy and Molnár (1997a)
Myxobolus erythrophthalmi	Triactinomyxon	No	99.9 %	Freshwater	*Scardinius erythrophthalmus*	*Isochaetides michaelseni*	Kelemen et al. (2009)
Myxobolus fundamentalis	Triactinomyxon	No	100 %	Freshwater	*Rutilus rutilus*	*Isochaetides michaelseni*	Borkhanuddin et al. (2013)
Myxobolus hungaricus	Triactinomyxon	Partial f-w	nd	Freshwater	*Abramis brama*	*Tubifex tubifex*	El-Mansy and Molnár (1997b)
Myxobolus lentisuturalis	Raabeia	No	99.8 %	Freshwater	*Carassius gibelio, C. auratus*	*Branchiura sowerbyi*	Caffara et al. (2009)
Myxobolus macrocapsularis	Triactinomyxon	Partial f-w	nd	Freshwater	*Abramis brama, Blicca bjoerkna*	*Tubifex tubifex*	Székely et al. (2002)
Myxobolus parviformis	Triactinomyxon	Complete f-w-f	99.9–100 %	Freshwater	*Abramis brama*	*Limnodrilus hoffmeisteri, Tubifex tubifex*	Kallert et al. (2005b)
Myxobolus pavlovskii	Echinactinomyxon	Partial w-f	100 %	Freshwater	*Hypophthalmichthys molitrix*	*Limnodrilus udekemianus*	Marton and Eszterbauer (2011)
Myxobolus portucalensis	Triactinomyxon	Partial f-w	nd	Freshwater	*Anguilla anguilla*	*Tubifex tubifex*	El-Mansy et al. (1998)
Myxobolus pseudodispar (Hungary)	Triactinomyxon	Complete f-w-f	100 %	Freshwater	*Rutilus rutilus*	*Tubifex tubifex, Limnodrilus hoffmeisteri, Psammoryctides* spp.	Székely et al. (1999, 2001), Marton and Eszterbauer (2012)
Myxobolus pseudodispar (Germany)	Triactinomyxon	Complete f-w-f	100 %	Freshwater	*Rutilus rutilus*	Mixed oligochaetes	Kallert et al. (2007)
Myxobolus rotundus	Triactinomyxon	Complete f-w-f	100 %	Freshwater	*Abramis brama*	*Tubifex tubifex*	Székely et al. (2009)
Myxobolus shaharomae	Triactinomyxon	No	100 %	Freshwater	*Alburnus alburnus*	*Isochaetides michaelseni*	Kelemen et al. (2009)

(continued)

Table 10.1 (continued)

Species	Actinospore	Experiment	DNA match	Biotope	Vertebrate host	Invertebrate host	Reference
Myxobolus wootteni	Triactinomyxon	No	99.9 %	Freshwater	*Rutilus rutilus*	*Tubifex tubifex*	Eszterbauer et al. 2006, Molnár et al. 2010
Parvicapsula minibicornis	Tetractinomyxon	Partial f-w	100 %	Freshwater	*Oncorhynchus* spp.	*Manayunkia speciosa*	Bartholomew et al. (2006)
Sigmomyxa sphaerica (syn. *Myxidium sphaericum)*	Tetractinomyxon	No	99.8 %	Marine	*Belone belone*	*Nereis pelagica*	Karlsbakk and Køie (2012)
Thelohanellus hovorkai	Aurantiactinomyxon	Partial f-w	99.8 %	Freshwater	*Cyprinus carpio*	*Branchiura sowerbyi*	Yokoyama (1997), Székely et al. (1998), Anderson et al. (2000), Eszterbauer et al. (2006)
Thelohanellus kitauei	Aurantiactinomyxon	No	99.4 %	Freshwater	*Cyprinus carpio*	*Branchiura sowerbyi*	Borkhanuddin et al. (2013)
Thelohanellus nikolskii	Aurantiactinomyxon	Partial f-w	nd	Freshwater	*Cyprinus carpio*	*Branchiura sowerbyi*	Székely et al. (1998)
Thelohanellus nikolskii	Aurantiactinomyxon	No	100 %	Freshwater	*Cyprinus carpio*	*Nais* spp.	Borkhanuddin et al. (2013)
Zschokkella mugilis	Unnamed type	No	100 %	Marine	*Mugilidae* spp.	*Nereis diversicolor*	Rangel et al. (2009)
Zschokkella nova	Siedleckiella	Partial f-w	nd	Freshwater	*Carassius carassius*	*Tubifex tubifex*	Uspenskaya (1995)
Malacosporeans							
Tetracapsuloides bryosalmonae	Malacospore	[b]Complete f-b-f	99.5 %	Freshwater	*Salmo trutta*, *Oncorhynchus mykiss*	*Fredericella sultana, Plumatella repens*	Feist et al. (2001), Morris and Adams (2006c)
Buddenbrockia plumatellae	Malacospore	Partial b-f	99.5 %	Freshwater	*Phoxinus phoxinus*	*Plumatella repens*	Grabner and El-Matbouli (2010)

For **myxosporeans** partial *f-w* transmission of myxospore (from fish) to worm (intraoligochaete development); partial *w-f* transmission of actinospore (from worm) to fish (intrapiscine development); complete *f-w-f* transmission of both myxospores and actinospores. For **malacosporeans** partial *f-b* transmission from fish malacospores (from fish) to bryozoan; partial *b-f* transmission of malacospore (from bryozoan) to fish; complete *f-b-f* transmission of spores from both fish and bryozoans. DNA match yes: undefined percentage value; 100 %: identical DNA fragment (SSU rDNA); *nd* no data. DNA match was detected on spore stages (i.e. actinospores and myxospores)

[a] The triactinomyxon detected by Rácz et al. (2004) in transmission experiments was misidentified as *Myxobolus intimus*, and DNA sequence analyses by Molnár et al. (2010) revealed that the obtained actinospores belonged to species *M. diversicapsularis*

[b] Transmission was brown trout to bryozoan to rainbow trout

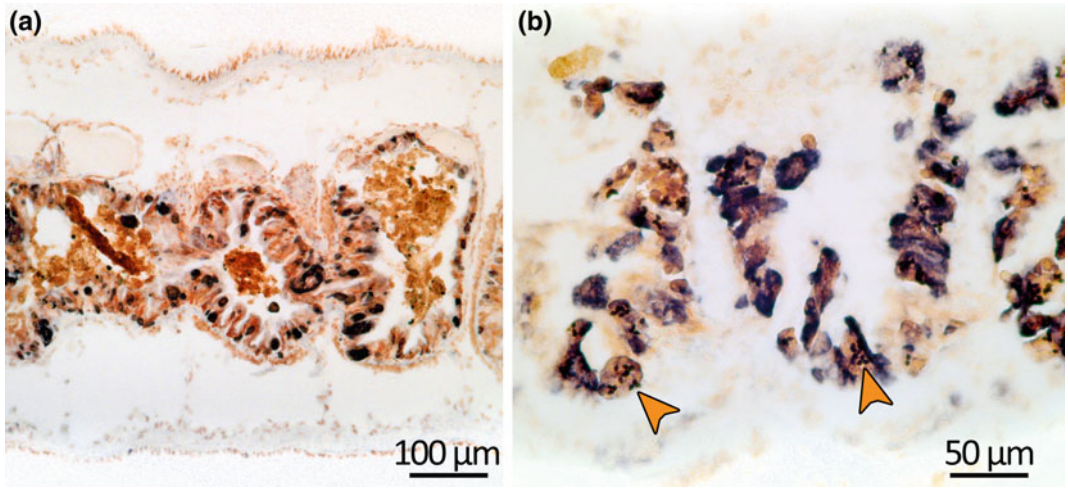

Fig. 10.2 *Myxobolus pseudodispar* developmental stages **a** 1 month, **b** 3 months post-exposure in the intestinal epithelium of its oligochaete host, stained *dark* by in situ hybridisation. Actinospore polar capsule triplets indicated by *arrowheads* (E Eszterbauer)

gill epithelium, then, uniquely among myxosporeans, migrate through the trout peripheral and central nervous system, with continued proliferation of cell-doublets, which started in the epithermis (El-Matbouli et al. 1999a). Sporogony occurs in the cartilage of skull and vertebrae about 90 days after infection (El-Matbouli et al. 1992a). Laboratory studies suggest that myxospores are released when infected fish die and autolyse or when they are consumed and excreted by predators (El-Matbouli et al. 1992a). Myxospores are then ingested by *T. tubifex*, in which they develop intercellularly in the intestinal epithelium. Spore development takes ~3 months and comprises proliferation, gametogony and sporogony (El-Matbouli and Hoffmann 1998; Morris and Freeman 2010). Mature, infective TAMs are released into water with faeces. It has not been proven experimentally that the parasite can be transmitted by direct feeding of fish with infected oligochaetes.

10.1.2 *Myxobolus pseudodispar*

Myxobolus pseudodispar is a common myxosporean parasite of cyprinid fishes in Europe. It develops mature spores intracellularly in skeletal muscle of roach (*Rutilus rutilus*), white bream (*Blicca bjoerkna*), common bream (*Abramis brama*), rudd (*Scardinius erythrophthalmus*), and bleak (*Alburnus alburnus*) (Molnár et al. 2002). The invertebrate host range of *M. pseudodispar* is similarly broad and includes *T. tubifex* Lineages I, II and III, *L. hoffmeisteri, Psammoryctides barbatus* and *P. moravicus* (Székely et al. 1999; Eszterbauer 2002; Marton and Eszterbauer 2012). The parasite enters the oligochaete through the gut epithelium and migrates along the basal lamina (Marton and Eszterbauer 2012) (Fig. 10.2). *T. tubifex* Lineage V can become infected, but the parasite does not mature, hence these worms are dead-end hosts and may serve as biological filters (Marton and Eszterbauer 2012).

Székely et al. (2001) report infection prevalence of 55–70 % in roach and showed the parasite could not be transmitted by feeding fish infected oligochaetes. Rácz (2004) found that TAM production started ~54 days post-exposure (dpe) at 19 °C, but only 35 dpe at 23 °C, and that temperature did not influence the total number of released TAMs. However, at higher temperatures, worms release TAMs for shorter periods and at higher intensity. Development is considerably slower at low temperatures (≤10 °C) (Rácz 2004, Eszterbauer personal observation).

10.1.3 *Myxobolus parviformis*

The parasite develops in small plasmodia in the gill lamellae of common bream *A. brama*, often with several other common *Myxobolus* species (Molnár and Székely 1999; Kallert et al. 2005b). These natural multiple infections can be problematic for life cycle studies, especially if the oligochaete species being used is not permissive for the *Myxobolus* species of interest, and instead one of the contaminating species is "selected for" (i.e. spores of the contaminating species are produced in the host). Kallert et al. (2005b) developed a protocol that combined experimental and molecular techniques to identify mixed infections, isolate myxospores/plasmodia of the target species and establish contamination-free laboratory life cycles. The first step is to add the potentially mixed myxospore sample to an established culture of known mixed oligochaete species (preferably laboratory-reared *Tubifex* spp. and *Limnodrilus* spp.). When actinospores are produced, individual infected worms are isolated and their actinospores classified by spore morphology and DNA sequencing. Once identified, these actinospores are used to infect naïve bream, which develop monospecific myxozoan infections from which plasmodia and myxospores can be characterised unambiguously. If the myxospores are confirmed as conspecific with the original actinospores, they can be harvested and used to infect additional oligochaetes. Periodic verification of spore morphologies and DNA sequences ensures the laboratory cycle is truly monospecific. This procedure effectively controls for contamination by non-target myxozoans, which can confound or even invalidate the results of transmission experiments using wild-sourced hosts.

In contamination-free monospecific cultures of *M. parviformis*, actinospore production by oligochaetes began 114 dpe, and lasted >3 months at 18 °C. Spore production could be prolonged in selected host specimens by keeping the oligochaetes between 4 and 6 °C for >300 d. In the fish host, mature myxospores were observed in numerous small cysts on gill lamellae 67 dpe (Kallert et al. 2005b).

10.1.4 *Ceratonova shasta* (Syn. *Ceratomyxa shasta*)

Ceratonova shasta is an intestinal parasite of salmon and trout. Its life cycle involves one of the few known species of freshwater polychaete worms. Originally identified as *Manayunkia speciosa*, molecular data indicate that this West coast polychaete is distinct from the type *M. speciosa* from the eastern U.S. (Bartholomew et al. 1997; Atkinson unpublished data).

To infect polychaetes, myxospores from the intestine of infected rainbow trout are added to culture tanks (see Sect. 10.2.1.1). The polychaetes ingest myxospores during feeding. In the gut, myxospores release their infective sporoplasms, which then penetrate the gut epithelium and migrate through nerves to the epidermis (Meaders and Hendrickson 2009). The parasite undergoes several stages of development and proliferation, including schizogony, gametogony and sporogony, with actinospore production 35–49 dpe at ~17 °C (Meaders and Hendrickson 2009). Parasite release appears to occur through the secretory pores in the epidermis (Bartholomew et al. 1997). Infection can be monitored by subsampling and examining polychaetes directly (Bjork and Bartholomew 2009a) or indirectly through PCR assay of polychaetes or filtered water samples (Hallett and Bartholomew 2006). To infect salmonid fish, water that contains actinospores from the polychaete system is placed into a tank with fish. Exposure time/dose is adjusted for the number of actinospores/L based on qPCR analysis of water samples, with as few as 1–5 actinospores constituting a lethal dose for susceptible fish strains (Bjork and Bartholomew 2009b). Actinospores attach to the fish gills and the infective sporoplasm penetrates between epithelial cells to reach the capillaries where the parasite multiplies (Bjork and Bartholomew 2010). Vegetative stages use the blood system to

reach the intestine where sporogony occurs 14–21 dpe at ~18 °C (Bjork and Bartholomew 2010).

10.1.5 *Tetracapsuloides bryosalmonae*

Laboratory experiments on the *T. bryosalmonae* life cycle currently rely on naïve host stocks of freshwater bryozoans (Bryozoa: Phylactolaemata) and brown or brook trout. *Fredericella sultana* is the most amenable bryozoan host for laboratory culture. Susceptible fish are exposed to either previously-infected laboratory grown bryozoans (Abd-Elfattah et al. 2014) or field-collected material (e.g. Longshaw et al. 2002). The infectivity of *T. bryosalmonae* spores released from bryozoans can be high (e.g. one spore per fish, McGurk et al. 2006) and short exposures (90 min) have been successful (Longshaw et al. 2002). Cohabitation with live (e.g. 8 h/day for 2 weeks) or dissected overtly infected (spore-producing) bryozoans for ~1–2 h has been used (Longshaw et al. 2002; Grabner and El-Matbouli 2008; Abd-Elfattah et al. 2014). Morris and Adams (2006c) exposed fish to water from a tank with infected bryozoans for 2 consecutive days, and observed spore development in fish 9 weeks post exposure (wpe; 18 °C). Grabner and El-Matbouli (2008) began exposing naïve bryozoans at 8 wpe of the fish (at 15 °C). Thus the rate of spore development and onset of PKD in fish depend on temperature and to a lesser extent on spore dose. Spore-filled sacs (overt infections) were observed to develop in some bryozoans 4 wpe to infected fish and within 2 months most bryozoans developed overt infections (Grabner and El-Matbouli 2008). Morris and Adams (2006b) observed overt infection development 42 dpe to infected fish. Once infected, the overt parasite stages in bryozoans are produced periodically, and observations suggest development times of a few days (Morris and Adams 2006b; Tops et al. 2006). Adjusting temperatures and food levels can be used to trigger the development of covert to overt infection in bryozoans and to help achieve maximum transmission success to fish (Tops et al. 2006; Hartikainen and Okamura 2012).

10.2 Laboratory Life Cycle Studies

Recognition of the complex nature of myxozoan life cycles has guided design of laboratory studies. Examination of myxozoan life cycles in vivo starts with identification of and access to susceptible naïve hosts. Laboratory conditions then need to be established that are both suitable for maintenance of these hosts and conducive to parasite transmission and proliferation. Below we describe some of the requirements and methods for undertaking laboratory studies.

10.2.1 Establishment of Stocks of Invertebrate Hosts

Ideally, infection-free stocks of suitable vertebrate and invertebrate hosts (Fig. 10.3) should be obtained, however often only wild stocks of unknown infection status are available. Establishment of infection-free stocks of invertebrate hosts derived from natural populations with some level of background infection can be time consuming: e.g. oligochaetes can remain infected for >12 months (El-Matbouli and Hoffmann 1998; Hallett et al. 2005) and bryozoans may harbour cryptic, avirulent malacosporean infections in colonies and in dormant resting stages (Hartikainen et al. 2013; Abd-Elfattah et al. 2014). Strategies for obtaining parasite-free invertebrate stocks include: rearing oligochaetes from cocoons, screening worm populations or portions of bryozoan colonies with parasite-specific PCR assays, rearing bryozoans from dormant propagules (statoblasts) produced by uninfected colonies, and producing clonal fragments of uninfected bryozoans by division of colonies (Kallert et al. 2005b; Grabner and El-Matbouli 2008; Hartikainen et al. 2013; Abd-Elfattah et al. 2014).

Invertebrate hosts of myxozoans are non-model organisms and the investment required to develop reliable culture systems can be substantial. While conditions for maintaining some invertebrate hosts in laboratory systems are well established (see Sects. 10.2.1.1 and 10.2.1.2), it can be challenging to determine optimal culture

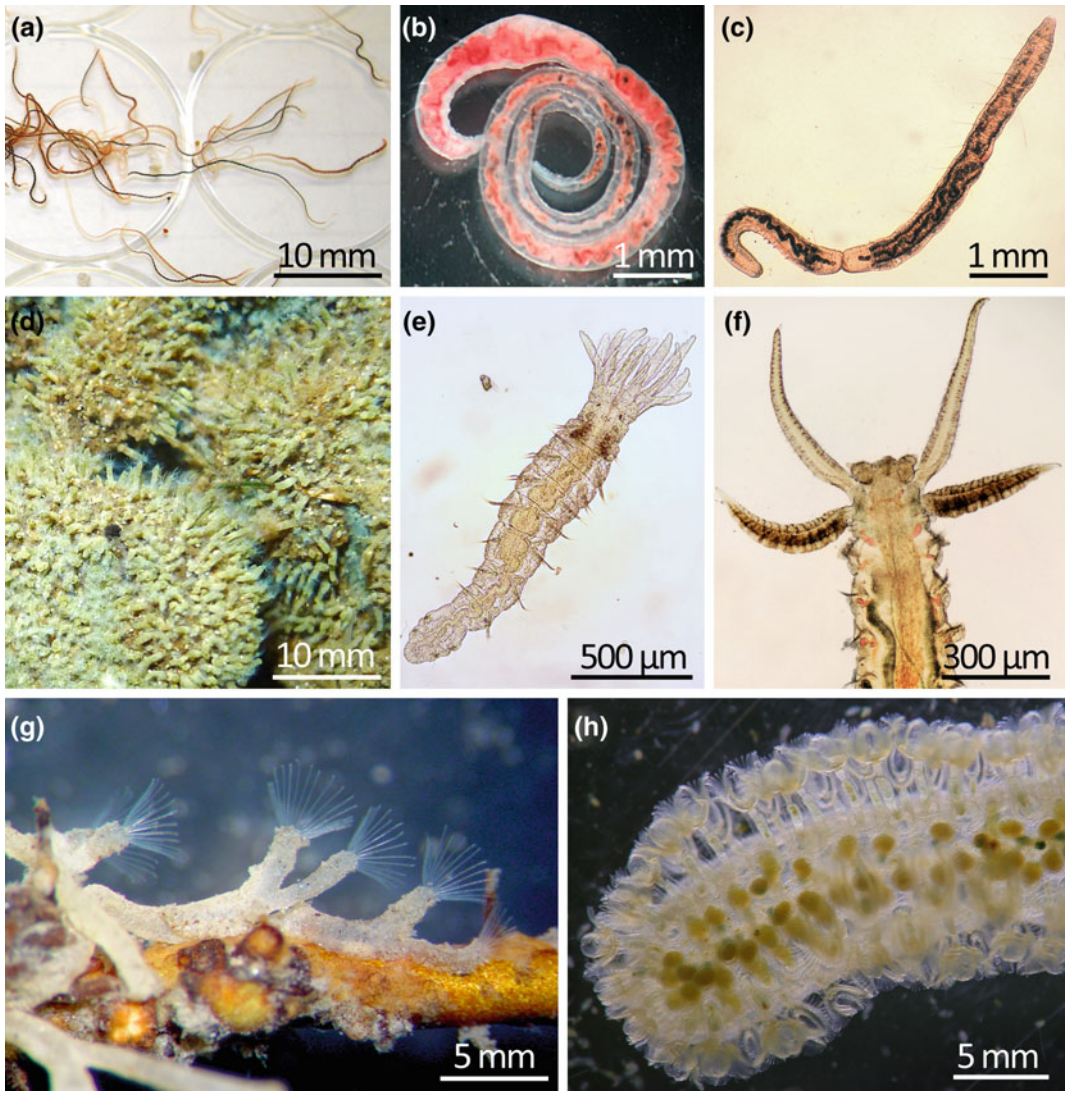

Fig. 10.3 Representatives of the three main groups of myxozoan invertebrate hosts: oligochaete worms **a**–**b** *Tubifex tubifex*, **c** *Nais* sp., polychaete worms, **d**–**e** *Manayunkia* sp., **f** *Streblospio* sp., freshwater bryozoans **g** *Fredericella* sp., **h** *Cristatella mucedo*. Images **a**–**g** by S. Atkinson, image **h** courtesy of B Okamura

conditions for some species, and to supply these with appropriate food. For example, challenges remain to effectively culture *Limnodrilus udekemianus*, the oligochaete host of *M. pavlovskii* (Marton and Eszterbauer 2011) and *Cristatella mucedo*, the bryozoan host of the sac-forming *Buddenbrockia plumatellae* (Okamura pers. comm.). An additional challenge is if the species or strain composition of an oligochaete culture changes over time, due to competition or fitness differences between worms or different effects of the parasite on the host. For instance, *M. cerebralis* reduces feeding activity and fecundity of *T. tubifex* and susceptible worm strains may be displaced by resistant strains (Shirakashi and El-Matbouli 2009). Sub-optimal conditions may result in failure to infect hosts, development after infection being aborted, or low prevalence and/or intensity of infection. In the following sections we review some specific challenges and solutions for maintaining invertebrate hosts in the laboratory.

10.2.1.1 Maintenance of Aquatic Annelids

The most common invertebrates used for freshwater myxosporean life cycle studies are annelid worm species belonging to family Naididae (formerly Tubificidae). *Tubifex* spp., *Limnodrilus* spp. and *Psammoryctides* spp. are able to reproduce under laboratory conditions, and parasite-free stocks can be reared from cocoons (Marton and Eszterbauer 2012). An equal mixture of autoclaved mud (that had been washed and stirred daily for ~10 days prior to use to remove toxic components) and autoclaved coarse sand was an optimal substrate for these worms. Additional organics, such as a mixture of minced frozen lettuce, *Spirulina* powder and frozen *Artemia* or bloodworms, can be added to the worm cultures once a week to stimulate reproduction (Kallert et al. 2005b; Marton and Eszterbauer 2012). Aeration reduces anaerobic microbial activity and build-up of hydrogen sulfide in the sediment (Eszterbauer personal observation). Pote et al. (1994) report a method for isolating and propagating infected *Dero digitata* in Petri-dishes. Mischke and Griffin (2011) developed a novel method for mass culture of *D. digitata*: paper towels were placed into 10.7 L dish pans into which 4 L of autoclaved pond water and 0.1 g of fish food were added. Cultures were maintained at room temperature (22–25 °C) and not aerated. After 5 weeks, thousands of worms could be harvested.

Maintenance of *B. sowerbyi* in culture is encumbered by slow development and low reproductive rates of these large tubificids (Carroll and Dorris 1972). Aston et al. (1982) reared eggs and juvenile worms over ~12 weeks in a substrate of one third activated sludge (10 % solids) and two thirds sand, in jars with aerated tap water. Highest reproduction and growth rates occurred between 21 and 29 °C, and the addition of 8 % cellulose improved worm body weight and egg production (Aston 1984). However, because of the relatively low production levels of these worms, most *B. sowerbyi* infection trials use worms collected from natural habitats (e.g. Molnár et al. 1999b; Liyanage et al. 2003).

A variety of worm culture conditions have been used, with variation in holding temperature, container design and substrate (Fig. 10.4). For example, for *M. parviformis*, naidid/tubificid worms were held at 18 °C in aerated 4 L tanks on

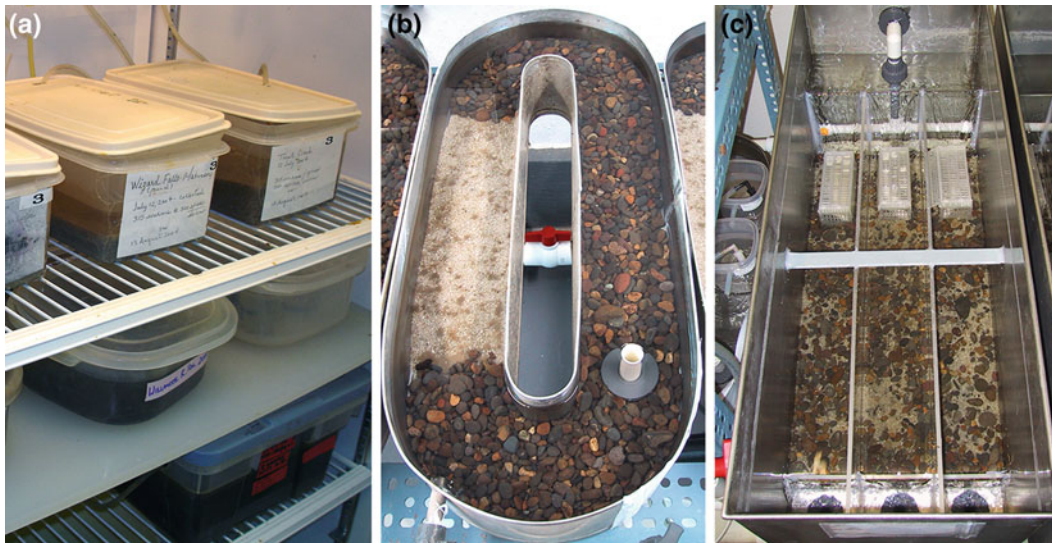

Fig. 10.4 Different oligochaete worm cultures: **a** 1–4 L aerated, static containers in a constant-temperature incubator, **b** 20 L recirculating 'donut' system with a variety of habitat substrates, **c** constant flow-through system with three replicate habitats and mesh boxes (*top*) for holding infected fish (S. Atkinson)

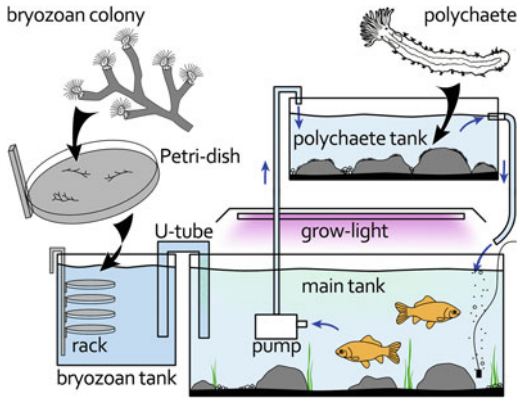

Fig. 10.5 Microcosm method for culturing bryozoans and freshwater polychaetes based on recirculating plankton-rich water from a main tank, which is illuminated and aerated to promote microbial growth. Bryozoan colonies grow on inverted plates, in a clean side tank with water exchanged with main tank through a combination of an airlift and siphon U-tube (Wood 1996). Polychaetes live on natural substrate in a header tank, which receives water pumped from main tank. Resident goldfish help to stabilise water chemistry and encourage productivity (S. Atkinson)

a substrate of coarse sand (Kallert et al. 2005a). For *Chloromyxum auratum*, *Limnodrilus hoffmeisteri* and *Lumbriculus variegatus* cultures were held at 14 °C in 4 L plastic containers with 1 L of autoclaved coarse sand and 2 L of aerated water (Atkinson et al. 2007). To study the intraoligochaete development of several freshwater *Myxobolus* spp., *T. tubifex* and *L. hoffmeisteri* were cultured at 18–22 °C with sterilized pond mud and aerated tap water in 5 L aquaria or plastic cups (El-Mansy and Molnár 1997b; Molnár et al. 1999a; Eszterbauer et al. 2000 etc.).

For *P. minibicornis* and *C. shasta* studies, freshwater polychaetes *Manayunkia* sp. were maintained at ambient lab temperatures (15–25 °C) with different tank configurations: 100 L aquaria with aeration (Bartholomew et al. 1997), recirculating 57 L tanks (Meaders and Hendrickson 2009) and flow-through 25 L tanks supplied with river water as a food source (Bjork and Bartholomew 2009b). The most stable long-term configuration comprised multi-tank recirculating 'microcosm' systems (Fig. 10.5, Alexander personal communication), similar to the microcosms used for growing bryozoans (Sect. 10.2.1.2). In both systems, a ~ 100 L illuminated, aerated aquarium containing several goldfish serves as a source of plankton, which is constantly pumped through smaller (10–20 L) tanks or trays in which the polychaetes are held on natural substrates (stones, sand or mud).

10.2.1.2 Maintenance of Bryozoans

Freshwater bryozoans can be grown in the laboratory using a "pond microcosm" or by providing a diet of cultured algae and protozoans. Pond microcosms (Fig. 10.5) are created by spiking illuminated, aerated aquaria with natural planktonic communities. Fish hosts considered non-susceptible to the target malacosporeans (e.g. *Carassius auratus* for *T. bryosalmonae* cultures) are placed in these aquaria to stimulate productivity (Wood 1971, 1996; Tops 2004) and the water is circulated through side tanks that house the bryozoans and protect them from predation by the fish in the main tank. Suitable surfaces for the establishment of bryozoan colonies are Petri-dishes hung upside-down to protect the colonies from settling particles. Bryozoans feed on various suspended particles that develop in the system, including algae, detritus and bacteria, and occasionally the systems may require spiking with further pond water to reintroduce food items. Addition of inorganic phosphorus and nitrogen to a bryozoan culture microcosm can result in increased photosynthetic and heterotrophic production of planktonic food for the hosts, with increases in intensities and prevalences of overt (spore-producing) *T. bryosalmonae* infections (Hartikainen and Okamura 2012). Microcosm systems have been used successfully to culture *Lophopus crystallinus* with the malacosporean parasite *Buddenbrockia allmani* (Hill and Okamura 2007), *Fredericella sultana* with *T. bryosalmonae* (Tops 2004; Hartikainen and Okamura 2012), and various plumatellid bryozoans with *B. plumatellae* (Grabner and El-Matbouli 2010, Hartikainen own observation). However, laboratory culture of *Cristatella mucedo* has not been successful (Hartikainen own observation). Microcosm culturing systems are well suited for ecological and evolutionary studies as they

generally promote strong growth rates and infection development (Tops et al. 2009; Hartikainen and Okamura 2012), and can be used to generate large numbers of replicates from the same clonally reproducing bryozoan genotype. Microcosms provide a mixed diet that supplants the need to culture an array of food items separately.

The second method involves specifically culturing food (algal and protist cultures) for the bryozoans (Morris et al. 2002; McGurk 2005; Grabner and El-Matbouli 2008; Abd-Elfattah et al. 2014). For example, Grabner and El-Matbouli (2008) used a mixture of algae (80 % of *Cryptomonas ovata* and 20 % of combination of *Cryptomonas* spp., *Chlamydomonas* spp., *Chlamydomonas reinhardii*, *Synecochoccus* spp. and *Synechococcus leopoliensis*) at a concentration of 6.5–7.5 × 10^6 cell mL^{-1}. Bryozoans have been maintained in buckets (Kumar et al. 2013b), aquaria filled with special culture medium (Morris et al. 2002) or custom-built recirculating culture systems (McGurk 2005). This method may be more tractable for transmission studies of novel parasites as it avoids the requirement for initial spiking with pond water and presence of fish. However, culturing algae for feeding is labour intensive and bryozoan growth rates achieved using this method remain unquantified. Hence, for study of known malacosporeans, microcosms are probably a better alternative.

Most bryozoan experimental work has focused on *F. sultana* since it is the most common host of the causative agent of salmonid proliferative kidney disease. Colonies of this species fragment naturally, resulting in viable branches drifting in the water column. Existing laboratory colonies can be propagated by artificial colony fragmentation: detaching branches with forceps and allowing them to attach to culture substrates via their growing tips. Alternatively, the proximal portion of the branch can be glued to a substrate. Given that malacosporean infections in bryozoans can be systemic, many portions of an infected colony can harbour parasite proliferative stages (Morris and Adams 2006b). This facilitates experimental studies as multiple genetically identical, infected hosts can be created. Bryozoan cultures can also be established by hatching uninfected, asexually produced resting stages (statoblasts). Great care must be taken in establishing stocks of putative naïve bryozoans as *T. bryosalmonae* can exist as covert infections (when only cryptic stages of parasites are present) in statoblasts (Hartikainen et al. 2013; Abd-Elfattah et al. 2014) and in detached branches from infected parent colonies (Morris and Adams 2006b). Molecular screening of colony portions and statoblasts from successive generations is recommended during establishment of naïve stock material (Hartikainen et al. 2013; Abd-Elfattah et al. 2014). Uninfected statoblasts can be collected and stored at 4 °C for several years. To stimulate synchronised hatching, statoblasts should be stored for at least 3 months.

10.2.2 Fish Hosts

The majority of myxozoans are fish host-specific (Lom and Dyková 2006), thus a particular host species or strain is required to establish the life cycle in a laboratory setting. For myxozoans that are able to infect multiple host species, not all hosts may be susceptible equally or develop equivalent disease signs. For example, *M. cerebralis* infects a range of salmonid hosts that vary in susceptibility (Halliday 1976; Hoffman 1990; Hedrick et al. 1998; MacConnell and Vincent 2002 etc.). Fish age and size may also affect parasite development. For instance, susceptibility of salmonid fry to *M. cerebralis* decreases with age and growth, as bone replaces cartilage (Halliday 1976; Markiw 1991, 1992; El-Matbouli et al. 1992a). In contrast, roach (*Rutilus rutilus*) of various sizes and ages are equally susceptible to *M. pseudodispar* (Eszterbauer unpub. data).

Local adaptation to fish hosts can confound life cycle studies. For instance, European strains of *T. bryosalmonae* can complete their life cycles in brown and brook trout but not in rainbow trout, which are native to North America (Grabner and El-Matbouli 2008; Kumar et al. 2013a). The observation of malacosporean-like spores in the

urine of rainbow trout infected in their native habitat only (Hedrick et al. 2004) suggests that rainbow trout in Europe are a dead end host to that parasite strain. Similarly, different genetic strains of *C. shasta* are adapted to different species of salmonid hosts (Atkinson and Bartholomew 2010).

Similar issues regarding host strain and susceptibility may apply to myxozoans and their invertebrate hosts. For example, *Tubifex tubifex* comprises a cryptic species complex, which consists of more than six genetic lineages (based on mitochondrial 16S rDNA) (Sturmbauer et al. 1999; Beauchamp et al. 2001; Marton and Eszterbauer 2012). Particular *T. tubifex* lineages are highly susceptible to *M. cerebralis* and *M. pseudodispar*, some are resistant or less susceptible, and others can become infected but the parasites do not reach maturity (Beauchamp et al. 2006; Baxa et al. 2008; Marton and Eszterbauer 2012).

Even though differences in susceptibility exist, there is little information available on the underlying mechanisms. There is limited experimental data available demonstrating infectivity of myxozoan isolates from one fish species and their ability to produce viable spores in a different fish species. We suggest publication of results from unsuccessful transmission experiments would provide a better overview on myxozoan host preference.

10.2.3 Experimental Transmission Regimes

Determination of appropriate experimental conditions requires careful consideration of multiple factors: e.g. spore dose, exposure regime (i.e. infection of hosts individually or in groups), water temperature, number of hosts, duration of the experiment and number of replicates. There is a relatively rich literature on *M. cerebralis* infection experiment design (El-Matbouli et al. 1999a; Stevens et al. 2001; Hedrick et al. 2003; Ryce et al. 2004; Baerwald et al. 2008; Eszterbauer et al. 2009; Kallert et al. 2009).

10.2.4 Infectious Dose

Depending on the aim of the study, doses to infect hosts can vary considerably. For *M. cerebralis*, experimental doses range from 10 to 1.4 million actinospores (triactinomyxons, TAMs) per fish. For infecting *T. tubifex* cultures, doses of 1,000–5,000 myxospores have been used (Bechara et al. 2002; Kelley et al. 2004; Baerwald et al. 2008; Eszterbauer et al. 2009; Severin et al. 2010 etc.). Although higher doses have been used to examine the parasite's portal of entry to fish using in situ hybridisation (Antonio et al. 1999), overdosing susceptible invertebrate hosts may cause their populations to crash. For instance, exposure to high concentrations of *C. shasta* actinospores resulted in high mortality of polychaetes (Atkinson unpublished observation).

Estimation of infectious dose in batch-exposure challenges is straightforward, through direct enumeration of actinospores in combination with testing their viability (e.g. with FDA/PI staining as per Yokoyama et al. 1997). When collection of spore material is complicated, co-habitation with infected hosts or exposure to water from aquaria housing infected hosts has been used successfully (McGurk et al. 2006; Grabner and El-Matbouli 2008; Hurst and Bartholomew 2012). Spore dose must then be estimated indirectly, e.g. via qPCR of water samples (Bettge et al. 2009a). An alternate approach is to dissect infected invertebrates to release spores in a limited volume of water, e.g. with *T. bryosalmonae* from bryozoans (Feist et al. 2001; Longshaw et al. 2002) and both *C. shasta* (Bjork and Bartholomew 2009b) and *Parvicapsula minibicornis* from polychaetes (Bartholomew et al. 2006).

10.2.5 Temperature

Several studies have shown that myxozoans have an optimum temperature range for development, e.g. 10–15 °C for *M. cerebralis*, with actinospore production decreasing outside this range. At >20 °C *M. cerebralis* TAM production declines, and the parasite may lose virulence

(El-Matbouli et al. 1999b; Blazer et al. 2003; Kerans et al. 2005). In the fish host, while higher parasite proliferation may occur at increased temperature, the fish immune system is also more active, possibly accounting for increased pathological effects (El-Matbouli and Hoffmann 2002; Bettge et al. 2009a,b). For example, at >15 °C trout infected with *T. bryosalmonae* show increased clinical disease signs and greater mortality (Clifton-Hadley et al. 1986; Hedrick et al. 1993). The same tendency was observed for *C. shasta* infection (Udey et al. 1975; Ray et al. 2012). In other host (e.g. cyprinids and oligochaetes) and parasite (e.g. *M. pseudodispar*) systems, parasite development may vary at suboptimal temperatures but usually is not aborted. For example, the intraoligochaete development of *M. pseudodispar* takes ~2 months at ~20 °C, 1–1.5 months at 28–30 °C, and over 3 months at <10 °C development (Marton and Eszterbauer 2012; Eszterbauer personal observation).

Temperature can affect the onset of spore production and the intensity of infection. For instance, higher temperatures result in increased infection intensity and an earlier onset of transmission stage production of *T. bryosalmonae* in bryozoan hosts (Tops et al. 2006).

10.2.6 Other Environmental Factors

Composition of the substrate and food availability may affect the health of invertebrate hosts and hence affect parasite production. For instance, Blazer et al. (2003) show that *T. tubifex* produce the greatest total number of *M. cerebralis* actinospores in mud, and the fewest in leaf litter.

Presence of non-target zooplankton and benthic suspension feeders may influence the number of infective stages in the experimental system. For instance, Rácz et al. (2006) observed a decrease in the number of floating actinospores in the presence of planktivorous copepods (*Cyclops* spp.). Hyperparasites of myxozoans (e.g. the microsporidian *Neoflabelliforma aurantiae*, which infects actinospore stages) can prevent viable spore production (Morris and Freeman 2010).

In flow-through systems, water velocity may influence parasite transmission. Bjork and Bartholomew (2009a) observed that higher flows correlated with high host polychaete density and low *C. shasta* infection prevalence. For *M. cerebralis*, low flows increased both parasite production and number of invertebrate hosts (Hallett and Bartholomew 2008). A temporary cessation/reduction in flow combined with aeration may therefore improve transmission success without adversely affecting invertebrate host health (Eszterbauer et al. 2009; Schmidt-Posthaus personal communication).

10.3 Alternative Modes of Transmission

Besides the common two-host life cycles, other means of transmission have been observed for myxozoans in laboratory studies. Here we review other modes of transmission by focusing on evidence for fish-to-fish transmission and for vertical transmission in invertebrate hosts. These processes are achieved without the production of spores.

10.3.1 Fish-to-Fish (Direct) Transmission

One of the first attempts to transmit Myxosporea experimentally between fish was by Hahn (1917), who inserted strips of *Myxobolus*-infected tissue into ‘pocket incisions’ in *Fundulus* sp. He (erroneously) concluded that “myxospores germinate when transplanted to another fish and produce schizonts in considerably less than one day”.

Deliberate, invasive transfer of stages between fish may be considered as a form of ‘transplantation’ of parasite cells rather than ‘direct transmission’. *Sphaerospora dykovae* (=*S. renicola*), the agent of renal sphaerosporosis in common carp, has extrasporogonic stages that also induce swimbladder inflammation (SBI) (Molnár 1984) as well as “UBO’s” (Unidentified Blood Organisms, or “Csaba bodies”) that circulate in the blood (Csaba 1976). Molnár and Kovács-Gayer (1986)

induced renal sphaerosporosis in carp by injection of SBI stages. Körting et al. (1989) transmitted *S. dykovae* between fish by intra-muscular injection of "Csaba" bodies from the blood of infected carp. Likewise, injection of blood developmental stages of *C. shasta* (Johnson 1980), PKX (Kent and Hedrick 1985) and *Kudoa thyrsites* (Moran et al. 1999) produced infections in fish. Oral intubation of gilt head sea bream *Sparus aurata* L. with *Enteromyxum leei*-infected gut tissue was also successful (Diamant 1997; Sitjà-Bobadilla et al. 2007), as were anal intubations (Estensoro et al. 2010). However, many other direct transmission trials were unsuccessful, e.g. attempts to transmit *Ceratomyxa puntazzi* by intra-coelomic injection of blood or extrasporogonic bile stages (Alama Bermejo et al. 2013), oral and anal intubations with *C. shasta* stages in salmonids (Johnson 1980), and intra-oesophageal intubations with *C. puntazzi* stages in *D. puntazzo* (Alama Bermejo et al. 2013).

The genus *Enteromyxum* is recognized currently as the only myxosporean taxon unequivocally capable of "natural" fish-to-fish transmission. *E. leei* is extremely pathogenic in high-density, intensive recirculated ponds and sea cage farms (Diamant et al. 2006b; Fleurance et al. 2008). Its rapid geographic spread is related to the wide range of susceptible hosts and extraordinary fish-to-fish transmission capabilities (Sitjà-Bobadilla and Palenzuela 2012). *E. leei, E. scophthalmi* and *E. fugu* have all been transmitted experimentally between fish, either by feeding on infected tissue ("orally"), by sharing space with infected fish ("cohabitation"), or by exposure to contaminated discharged water ("effluent"). Table 10.2 summarises fish-to-fish infection trials carried out with *Enteromyxum* spp.

Enteromyxum spp. are unique in the nature of their extrasporogonic, proliferative stages. They invade and disrupt the gut epithelium, causing detachment and excretion of infected tissue fragments (Diamant 1997, 1998; Yasuda et al. 2002; Redondo et al. 2004; Diamant et al. 2006a). Three *E. scophthalmi* developmental stages may be involved in the direct transmission process in turbot (Redondo et al. 2004). The key elements, the presporogonic development stages, occur mainly in the intestinal epithelium, but also in blood, hemopoietic tissue, muscle, heart, skin and gills, which suggests diverse infection routes. Invasion by *E. leei* involves a caspase-3 like enzyme and several other proteases possibly involved in intra-and extra-cellular digestion and destruction of immunocompetent molecules (Estensoro et al. 2009, 2013). Obliteration of host gut mucosa is fundamentally important for the liberation of infective *E. leei* stages into the water column where they can be picked up by new hosts. Transmission is temperature-governed, with the optimum of 18 °C in all three *Enteromyxum* species, although *E. leei* transmission can occur at 15 °C in *T. rubripes* (Yanagida et al. 2006) and *E. scophthalmi* at 12 °C in turbot (Redondo et al. 2002). At >20 °C *E. leei* outbreaks are common in sparid farms (Rigos et al. 1999; Diamant et al. 2006b; Fleurance et al. 2008) and Malabar grouper (*Epinephelus malabaricus*) cultures (China et al. 2013). Laboratory transmission of *E. leei* stages deriving from fish have been carried out with freshwater species, including zebrafish (*Danio rerio*) (Diamant et al. 2006a). Turbot (*Psetta maxima* L) was infected experimentally with *E. scophthalmi* (Redondo et al. 2002, 2004), as were tiger puffer (*Takifugu rubripes*) and red sea bream (*Pagrus major*) with *E. fugu* (Yasuda et al. 2002; Yanagida et al. 2004, 2008) (Table 10.2). The effluent infection method is used routinely in laboratory experimentation with *E. leei* (Sitjà-Bobadilla et al. 2007; Redondo et al. 2008; Estensoro et al. 2011, 2013). Although direct transmission occurs, this does not exclude the possibility of an invertebrate host (that host has not yet been identified for *E. leei*).

10.3.2 Vertical Transmission in Invertebrate Hosts

Vertical transmission is typically viewed as the transmission of infection from parents to sexual progeny (i.e. from a mother to an embryo). As described below, myxozoans achieve a form of vertical transmission whereby infection is passed to clonal propagules.

Table 10.2 Fish-to-fish infection trials with *Enteromyxum* spp.

Parasite species	Mode of infection	Donor fish	Recipient fish	Result	References
Enteromyxum leei	F	*Sparus aurata*	*Sparus aurata*	+	Diamant (1997), Diamant and Wajsbrot (1997), Sitja-Bobadilla et al. (2007)
	F	*Takifugu rubripes*	*Takifugu rubripes*	+	Yasuda et al. (2002)
	F	*Paralichthys olivaceus*	*Paralichthys olivaceus*	+	Yasuda et al. (2005)
	F	*Paralichthys olivaceus*	*Takifugu rubripes*	+	Yasuda et al. (2005)
	F	*Sparus aurata*	*Puntius tetrazona*	+	Diamant et al. (2006b)
	F	*Sparus aurata*	*Oreochromis mossambicus*	+	Diamant et al. (2006b)
	F	*Sparus aurata*	*Astronotus ocellatus*	+	Diamant et al. (2006b)
	F	*Sparus aurata*	*Danio rerio*	+	Diamant et al. (2006b)
	F	*Takifugu rubripes*	*Takifugu rubripes*	+	Yanagida et al. (2006)
	F	*Epinephelus malabaricus*	*Epinephelus malabaricus*	+	China et al. (2013)
	O	*Sparus aurata*	*Sparus aurata*	–	Estensoro et al. (2010)
	A	*Sparus aurata*	*Sparus aurata*	+	Estensoro et al. (2010)
	C	*Sparus aurata*	*Sparus aurata*	+	Diamant (1997), Diamant and Wajsbrot (1997), Sitja-Bobadilla et al. (2007)
	C	*Sparus aurata*	*Sciaenops ocellatum*	+	Diamant (1998)
	C	*Diplodus puntazzo*	*Diplodus puntazzo*	+	Golomazou et al. (2006), Muñoz et al. (2007)
	C	*Sparus aurata*	*Diplodus puntazzo*	+	Alvarez Pellitero et al. (2008)
	C	*Takifugu rubripes*	*Pagrus major*	+	Yanagida et al. (2008)
	C	*Epinephelus malabaricus*	*Epinephelus malabaricus*	+	China et al. (2013)
	E	*Sparus aurata*	*Sparus aurata*	+	Diamant (1997), Diamant and Wajsbrot (1997), Sitja-Bobadilla et al. (2007)
	E	*Sparus aurata*	*Sciaenops ocellatum*	+	Diamant (1998)
	E	*Takifugu rubripes*	*Takifugu rubripes*	+	Yasuda et al. (2002, 2005)
	E	*Takifugu rubripes*	*Pagrus major*	+	Yanagida et al. (2004, 2008)
E. scophthalmi	F	*Scophthalmus maximus*	*Scophthalmus maximus*	+	Redondo et al. (2002, 2004)
	C	*Scophthalmus maximus*	*Scophthalmus maximus*	+	Redondo et al. (2002, 2004)
	E	*Scophthalmus maximus*	*Scophthalmus maximus*	+	Redondo et al. (2002, 2004)
E. fugu	F	*Takifugu rubripes*	*Takifugu rubripes*	+	Yasuda et al. (2002, 2005)
	C	*Takifugu rubripes*	*Pagrus major*	–	Yanagida et al. (2008)
	E	*Takifugu rubripes*	*Takifugu rubripes*	+	Yasuda et al. (2002, 2005)
	E	*Takifugu rubripes*	*Pagrus major*	+	Yanagida et al. (2004, 2008)

F feeding, *O* oral intubation, *A* anal intubation, *C* cohabitation, *E* exposure to effluent. ± indicates successful/failed transmission, respectively

10.3.2.1 Myxosporea

Morris and Adams (2006a) demonstrated vertical transmission of myxosporeans in the freshwater oligochaete *Lumbriculus variegatus*, a species known to replicate through architomy (a form of fragmentation). Worms infected naturally with a triactinomyxon type actinospore were placed individually into dishes with sterilised mud. Architomy occurred in 2 worms resulting in an extra oligochaete per dish, which released actinospores. In another experiment, from a starting culture of 5 oligochaetes maintained in autoclaved mud, 13 were recovered after 2 months, all of which were infected with the myxosporean.

Vertical transmission is suspected also to occur with *Myxobilatus gasterostei*, a common parasite of three-spined sticklebacks (*Gasterosteus aculeatus*). The invertebrate host is the cosmopolitan, fissiparous naidid oligochaete *Nais communis* (Atkinson and Bartholomew 2009). Fission is primary mode of reproduction in naidines and division of worms every couple of days can rapidly effect high population densities (Bely and Wray 2004). Fission of an infected *N. communis* yielded daughter worms with parasite developmental stages on each side of the fission plane. These stages persisted in the daughter worms but spore development was not observed due to death of the hosts. Vertical transmission is likely to form an important propagation strategy for myxosporean spp. that infect annelids capable of fission (Morris and Adams 2006a).

10.3.2.2 Malacosporea

Clonal replication via colony fragmentation occurs widely in freshwater bryozoans and readily offers a route for vertical transmission. For instance, vertical transmission of *B. allmani* in *Lophopus crystallinus* colonies was observed to occur in 64/65 colony fission events (Hill and Okamura 2007). Morris and Adams (2006a) demonstrated that fragmentation (effected by agitation of colonies and by using a scalpel to sever individual colonies) of malacosporean-infected *F. sultana* colonies produced infected daughter colonies. Indeed fragmentation of *F. sultana* colonies has been used routinely to establish replicate colonies for experimental studies in microcosms and transmission studies in cultured food (i.e. algae) systems (e.g. Morris and Adams 2006c; Tops et al. 2006).

Another form of vertical transmission can occur through infection of bryozoan statoblasts (resistant asexual propagules that persist during unfavourable conditions) as demonstrated for *T. bryosalmonae* in *F. sultana* (Abd-Elfattah et al. 2014) and *B. allmani* in *L. crystallinus* (Hill and Okamura 2007). Infection of statoblasts occurs primarily during covert infections (Hill and Okamura 2007; Hartikainen et al. 2013; Abd-Elfattah et al. 2014). In practical terms, infection of statoblasts facilitates storage of both hosts and parasites, which can be later revived for experiments.

10.4 In Vitro Cultivation

The in vitro cultivation of myxozoan species would facilitate examination of parasite biochemistry, nutrient requirements, chemotherapeutant studies and development of pure parasite stocks, which could be used for downstream experiments free of contaminating host tissues (particularly for transcriptomics). In vitro methods align also with animal welfare goals by reducing in vivo studies.

Five publications report in vitro culture attempts using Myxozoa. Morris (2012), Redondo et al. (2003), and Tyutyaev (2008) recorded successful parasite replication, while Wolf and Markiw (1976), and Siau and Grassé (1977) observed sporulation but no additional replication. Sporulation in vitro is noted also in unpublished conference proceedings and experimental observations (e.g. Palenzuela et al. 1994; Yokoyama and Yukata 1999; Bartholomew pers. comm.). Below we outline procedures and results of the published studies.

Enteromyxum scopthalmi: Redondo et al. (2003) examined the suitability of a range of media formulations for co-culture of the parasite with turbot fin cell lines. Tested media formulations included: M199 (Medium 199), DME/F12 (Dulbecco's modified Eagles medium/Ham's

nutrient mixture F-12), HMEM (Eagle's minimum essential medium with Hank's salts), and L-15 (Leibovitz's L-15 medium). All were supplemented with 10 % heat inactivated foetal bovine serum (FBS), heat-inactivated turbot sera, conditioned media or a penicillin/streptomycin/amphotericin mixture. Osmolarities were adjusted to 350 mOsm kg^{-1} and a range of media pHs (6.5–8.5) were tested. None of the formulations was conducive for long term parasite growth, and parasites typically become senescent shortly after initiation of cultures. The best results were obtained with M199 supplemented with either turbot sera (5–10 %) or turbot sera with FBS (5 % each) using cell suspensions.

Myxobolus dispar: Tyutyaev (2008) trialled DMEM, L-glutamine and roach *Rutilus rutilus* muscle extract, under a CO_2-enriched atmosphere (the exact media formulation is not reported). A limited period of parasite replication was observed but this was not sustained.

Tetracapsuloides bryosalmonae: Morris (unpub. data) trialled L-15, L-15/ methylcellulose, RPMI-1640, Glasgow's media, TDL-15, DMEM, and Eagle's MEM, supplemented with foetal calf serum (FCS), dialysed FCS, horse serum, horse serum/FCS, rainbow trout serum (50:50), heat inactivated rainbow trout plasma or goat serum, all at 10 % concentration. Different sera concentrations and parasite preparations (homogenised tissue/ explants) were tested, with all media including an antibiotic mixture of penicillin or streptomycin. The best results were obtained with a serum reduced media (adapted from Cheng et al. 1993) of L-15, with 3 % FCS, 1 % heat inactivated rainbow trout sera, 2 mM L-glutamine, 0.3 % w/v/ glucose, 1 mg mL^{-1} bovine serum albumin (BSA), 0.1 mM non-essential amino acids, 10 μg mL^{-1} insulin, 10 μg mL^{-1} transferrin and 10 μg mL^{-1} selenium, which sustained parasite replication over 2 weeks. Sub-culturing attempts failed and substantial growth variation was observed among replicate cell wells.

These preliminary studies suggest there is much scope for the development of in vitro myxozoan cultures. The most successful results to date used fish sera/tissue, with <10 % BSA, which might indicate BSA is inhibitory at higher concentrations. Inclusion of BSA in fish cell cultures is known to maintain cells whose fatty acid profiles resemble those of cows rather than the fish from which they were obtained (Tocher et al. 1988). In addition, there may be other factors that myxozoans require which are not present in traditional fish cell line media.

10.5 Key Questions for Future Study

- Do species of *Enteromyxum* incorporate an invertebrate host in their life cycles?
- Can some myxozoan infections in fish be achieved by trophic transmission (e.g. when fish prey on infected worms)?
- How widespread is fish-to-fish transmission in myxozoans?
- How does vertical transmission in invertebrate hosts influence epidemiology of fish diseases?
- Can we improve on media for in vitro culture of myxozoans?

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Part III
Host-Parasite Interactions

Ecology and Evolution of Malacosporean-Bryozoan Interactions

11

Hanna Hartikainen and Beth Okamura

Abstract

The discovery that freshwater bryozoans are hosts of *Tetracapsuloides bryosalmonae*, the malacosporean causative agent of proliferative kidney disease (PKD) of salmonids prompted a flurry of research on the ecology of malacosporeans and their interactions with bryozoan hosts. This chapter provides an overview of malacosporean-bryozoan interactions by discussing effects of parasitism on host fitness and strategies of host exploitation through virulent overt and avirulent covert infections. Evidence for host-condition dependent development and substantial levels of vertical transmission in bryozoan propagules (statoblasts) and colony fragments suggests virulence strategies that are closely tied to clonal reproduction in bryozoan hosts. Although there is evidence for *T. bryosalmonae* strain variation and host specificity, there is much scope for examining patterns of infection in relation to host clonality and local adaptation. The development of overt infections in bryozoans in good condition suggests that environmental change (e.g. warmer temperatures, eutrophication) will entail changes in malacosporean-bryozoan interactions which may, in turn, relate to disease emergence in fish.

Keywords

Phylactolaemates · Colonial hosts · Covert infections · Virulence strategies · Parasitic castration · Vertical transmission · *Tetracapsuloides bryosalmonae* · PKD · *Buddenbrockia plumatellae*

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11.1 Introduction

The Malacosporea was described by Canning et al. (2002b) as a sister taxon to the Myxosporea and comprises species that utilise freshwater bryozoans (Bryozoa: Phylactolaemata) and fish as definitive and intermediate hosts, respectively.

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Interactions between the malacosporean *Tetracapsuloides bryosalmonae* and its most common bryozoan host, *Fredericella sultana*, have received a great deal of investigation because *T. bryosalmonae* causes the devastating Proliferative Kidney Disease (PKD) of salmonid fish. This chapter reviews how *T. bryosalmonae* and other malacosporeans interact with their freshwater bryozoan hosts based on evidence deriving from field and laboratory observations and from experimental studies. This leads us to consider unique strategies that malacosporeans appear to have evolved to exploit freshwater bryozoans as well as patterns of adaptation between parasites and hosts, host specificity and potential regulation of host populations (Wood and Okamura 2005). We conclude by examining broader ecological issues including the impacts of environmental change on host-parasite interactions and patterns of infection and disease.

11.2 Freshwater Bryozoans

Freshwater bryozoans are suspension-feeding, colonial animals commonly found in ponds, lakes and rivers where they attach to surfaces such as submerged roots, branches, rocks and macrophytes (Wood and Okamura 2005) (Fig. 11.1). Colonies grow by budding new colony modules (zooids) and can form dense stands during favourable conditions. Feeding currents generated by the ciliated tentacular crown (the lophophore) of each zooid (Fig. 11.1e) enable a mixed diet comprised of microalgae and bacteria (Raddum and Johnsen 1983; Kaminski 1984; Richelle et al. 1994). Bryozoans can contribute significantly to nutrient cycling (Sørensen et al. 1986) and create local hotspots of high food environments for associated microfauna (the 'aufwuchs') (Bushnell 1966; Bushnell and Rao 1979; Ricciardi and Reiswig 1994).

Phylactolaemates are viviparous hermaphrodites and propagate by both sexual and asexual reproduction (Wood 2001). Sexual reproduction in early summer results in short-lived, actively swimming larvae that quickly settle to form a new colony (see Fig. 11.2 for life cycle details). Larvae production appears to be rare in some species, e.g. *Fredericella sultana* (Wood 1973), while larvae are produced regularly in others, e.g. *Cristatella mucedo* (Uotila and Jokela 1995; Wöss 1996; Okamura 1997). The presence of non-maternal genotypes in brooded larvae of *Cristatella mucedo* (Jones et al. 1994; Freeland et al. 2003) provides evidence for outcrossing in phylactolaemates. Mechanisms of asexual propagation include fission of gelatinous colonies (Wood and Okamura 2005; Hill and Okamura 2007) (Fig. 11.1d) and fragmentation of tubular, branching colonies (Morris and Adams 2006c; Tops et al. 2006) (Fig. 11.2). In addition, all phylactolaemates produce numerous asexual, seed-like propagules called statoblasts, which are highly resistant, dormant stages that persist during unfavourable conditions (Figs. 11.1e and 11.2). The presence and viability of intact statoblasts in the faeces of waterfowl (Charalambidou et al. 2003; Figuerola et al. 2003) provide evidence that statoblasts may achieve widespread dispersal. Gene flow amongst bryozoan populations linked by waterfowl migratory routes (Freeland et al. 2000) and with the movements of birds tracked by ringing-data (Figuerola et al. 2005) helps to substantiate this inference. In temperate regions many species rely exclusively on statoblasts for survival during winter (e.g. *C. mucedo*) although some (e.g. *Lophopus crystallinus* and *F. sultana*) can also survive as colonies (Hill 2005; Wood and Okamura 2005).

Bryozoan populations are highly seasonal and densities fluctuate over both temporal and spatial scales consistent with a metapopulation biology (Karlson 1994; Wöss 1994; Vernon et al. 1996; Okamura and Freeland 2001). The propensity of many freshwater bryozoan populations to undergo extinction, dispersal and colonisation of distant water bodies will result in a network of interconnected host populations that malacosporeans may exploit.

11.3 Malacosporeans

A complete malacosporean life cycle is only known for one species, the causative agent of

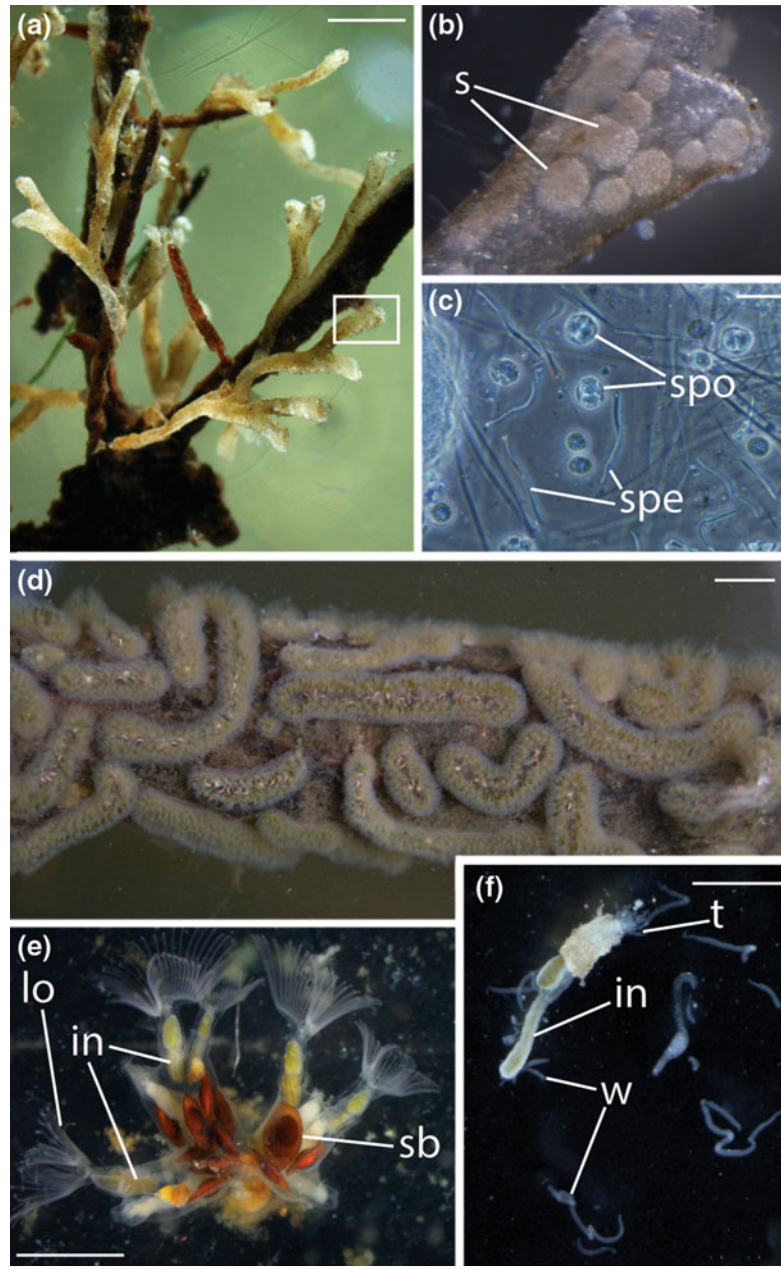

Fig. 11.1 Bryozoan hosts and malacosporean parasites. **a** A branching colony of *Fredericella sultana* attached to a tree root, the *white square* shows a branch tip. **b** Two *F. sultana* zooids with *Tetracapsuloides bryosalmonae* sacs. **c** The presence of both bryozoan sperm and spores of *T. bryosalmonae* in *F. sultana*. **d** *Cristatella mucedo* colonies divide by fission which can result in extensive colony cover of surfaces as shown here on a small branch. **e** *Lophopus crystallinus* colony undergoing statoblast production. **f** Dissected *F. sultana* zooid with *Buddenbrockia* sp. myxoworms attached to the intestine and free myxoworms that have spilled out from the coelomic cavity. *s* = sac, *spo* = spore, *spe* = sperm, *lo* = lophophore, *in* = intestine, *sb* = statoblast, *t* = tentacle, *w* = myxoworm. *Scale bars* refer to 1 mm apart from (**c**), where it is 50 μm

salmonid proliferative kidney disease (PKD), *Tetracapsuloides bryosalmonae*. In this species, fish-to-fish and bryozoan-to-bryozoan transmissions appear to be precluded (Ferguson and Ball 1979; D'Silva et al. 1984; Tops et al. 2004). Salmonids (brown trout, *Salmo trutta*) are confirmed fish hosts (Morris and Adams 2006b; Grabner and El-Matbouli 2008) but there is increasing evidence (see Chap. 4) that cyprinid and perciform fish are hosts of other malacosporeans (Grabner and El-Matbouli 2010; Bartošová-Sojková et al. 2014). No other invertebrate hosts have been identified despite attempts to screen a wide range of candidates including

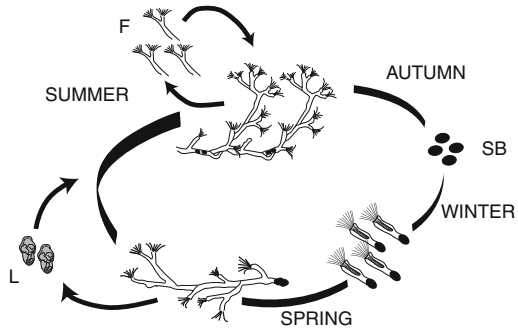

Fig. 11.2 Seasonal life-cycle of *F. sultana*, the most common host of *T. bryosalmonae*. *T. bryosalmonae* proliferates during spring and summer in bryozoan colonies as spore-producing and covert stages. During winter bryozoans persist as resting stages (statoblasts) although *F. sultana* can also persist as regressed colonies. Both may carry covert infections. Bryozoan larvae result from a brief sexual period in summer. Colonies grow rapidly during summer by asexual budding of zooids and disperse by fragmentation and re-attachment of branches. *f* = fragmentation; *l* = larvae; *sb* = statoblasts

freshwater planarians, cnidarians, chironomid larvae, molluscs, nymphs and nematodes, including ~30,000 individual oligochaetes (D. Morris, pers.comm.), as well as marine bryozoans (Okamura unpub. data).

Malacosporeans develop as sacs (e.g. *T. bryosalmonae*, *Buddenbrockia allmani*) (Fig. 11.1b) or worm-like (myxoworm) stages (*Buddenbrockia plumatellae*) (Fig. 11.1f) that become filled with infectious spores in the body cavity of freshwater bryozoans (see Chaps. 8 and 9 for further discussion of malacosporean development). Below we outline the different stages and processes associated with the invasion and development of malacosporeans in their bryozoan hosts. Most of our discussion refers to the relatively well studied *T. bryosalmonae/F. sultana* system but where possible we allude to other malacosporean/bryozoan systems.

11.3.1 Finding a Bryozoan Host

Spores that mature in kidney tubules of fish hosts ('fish malacospores') are released in low numbers with urine (maximum concentration estimate = 120 spores/ml urine; Hedrick et al. 2004). Spores will be greatly diluted upon release from fish and degrade relatively quickly (in <24 h) due to their soft, thin-walled valves (de Kinkelin et al. 2002). Transmission of spores to bryozoans is achieved by passive transport and encounter rates should increase with proximity to bryozoan hosts and with host density. These issues suggest that local host-parasite dynamics are likely to play an important role in transmission. Notably, bryozoans prefer to grow in sheltered locations that offer protection from overgrowth by algae, mainstream flows and sedimentation (Wood 2001). The extensive, three-dimensional root systems of riparian trees (e.g. alder, willow) are examples of such microhabitats and often support dense bryozoan growth (Wood 2001, Hartikainen, Okamura pers. obs). These same root systems also attract fish, especially juveniles, and are used as sheltering and feeding areas (Zika and Peter 2002). Indeed, the members of the aufwuchs community associated with dense bryozoan growth may represent important sources of food for juvenile fish. Spore encounter rates should therefore be greatly enhanced when urine is released in the same low-energy microenvironments as inhabited by bryozoans.

11.3.2 Infecting a Bryozoan Host

The route of infection to bryozoans has not been documented but may occur via capture and consumption of fish malacospores or when these spores directly impact the tentacles of the lophophore. This is likely to be facilitated by the lophophoral feeding currents which draw in particles of the size range of malacospores (~16 μm) (Hedrick et al. 2004; Riisgård et al. 2004). Sporoplasms are therefore anticipated to invade the host through the gut wall or through the tentacle sheath epithelium. Invasion through the general body wall is less likely as bryozoan colonies are protected by a stiff chitinous outer wall or a thick mucous matrix. Following successful invasion of the bryozoan host, the

parasite must establish an infection, multiply and eventually produce transmission stages.

11.3.3 Exploiting Bryozoan Hosts

Following infection, cryptic, presaccular, amoeboid stages may multiply within the bryozoan body wall (Canning et al. 2008) and as stages attached to the host peritoneum, retractor muscles and gut (Morris and Adams 2006a). These covert infections can be detected by e.g. PCR assays (Tops et al. 2009; Abd-Elfattah et al. 2013) and ultrastructure (Tops et al. 2009; Abd-Elfattah et al. 2013) but cannot be unambiguously detected by stereomicroscopy. Small presaccular stages have been observed circulating within the host body cavity (Morris and Adams 2006a) perhaps enabling systemic infection by spreading throughout the entire colony.

Field and laboratory data indicate that infections are highly persistent. For instance, covert infections are detected by PCR throughout the year within targeted wild populations (Tops 2004; Hill 2005) and overt infections (sac development—see below) have been observed in laboratory-grown bryozoans continuously for several months (Hill and Okamura 2007; Hartikainen and Okamura 2012). Early cryptic stages associated with covert infections are also able to gain entry to bryozoan statoblasts with the proportion of infected statoblasts ranging from 44 to 78 % (Hartikainen et al. 2013). Such infection is effective (Abd-Elfattah et al. 2013). Thus, spores produced within bryozoan colonies raised from infected statoblasts in turn are infectious to fish. Vertical transmission of both covert and overt infections is also common and is achieved during colony fission (Hill and Okamura 2007) and colony fragmentation (Morris and Adams 2006c). Hill and Okamura (2007) reported that overt *B. allmani* infections were transmitted to daughter colonies during 98.5 % of all observed *L. crystallinus* fission events (n = 65). In *F. sultana*, colony fragmentation and re-attachment of loose branches occurs naturally (Wood 1973) and can be routinely used to establish laboratory stocks (Tops et al. 2006; see also Chap. 10). Morris and Adams (2006b) divided a single overtly infected colony of *F. sultana* into four smaller colonies that continued to grow while retaining overt infection stages. In addition, Fontes et al. (unpub. data) collected drifting, detached branches of *F. sultana* downstream from dense populations on tree roots in three different river systems. PCR revealed that a substantial proportion of these branches were infected (maximum values ranged from 16 to 88 %). Thus, vertical transmission of both covert and overt stages during asexual colony propagation likely explains why infection prevalences can be locally very high and may result in patchy infections within populations. Transmission achieved via colony fragmentation and infection of statoblasts may also facilitate dispersal via drifting of infected bryozoans (Morris and Adams 2006c) or waterfowl vectors (Freeland et al. 2000).

Malacosporeans undergo subsequent development to spore-filled sacs or myxoworms causing overt infections (Canning et al. 2002a). Because overt infections can be observed with light microscopy, assessments of e.g. infection intensity during the course of infection are possible. Development of sacs and myxoworms can be very rapid and result in high parasite densities. For instance, Canning et al. (2002a) observed 22 *B. plumatellae* myxoworms to develop in 19 zooids of *Plumatella fungosa* within a period of 6 days. Sacs of *T. bryosalmonae* have been observed to develop within 3 days in colonies collected from the field and maintained in laboratory culture (Canning and Okamura 2004).

11.3.4 Developmental Cycling in Bryozoan Hosts

Evidence for periodicity in covert and overt infection development is wide-spread and such developmental cycling is likely to generally characterise malacosporeans. For instance, laboratory culturing of infected *L. crystallinus* colonies revealed a pattern of waxing and waning of

B. allmani sac production over 13 weeks. "Rest" periods of 1–2 weeks were observed between flushes of sac production (Hill and Okamura 2007). McGurk et al. (2006) observed continuous waves of myxoworm production for up to 3 months in *Plumatella repens* infected by *Buddenbrockia plumatellae* (McGurk et al. 2006). Similar patterns have been observed for *T. bryosalmonae* infections, with sacs being observed to appear and disappear rapidly (Tops 2004; Tops et al. 2009). The appearance and disappearance of overt infections of *T. bryosalmonae* in persistent field populations of *F. sultana* over annual periods (Tops 2004) provide further evidence that developmental cycling is a routine phenomenon.

11.3.5 Finding a Fish Host

Sac stages generally burst inside the bryozoan hosts and spores are released into the water column via the vestibular pore (McGurk et al. 2006). The water-borne spores attach to fish hosts via the extrusion of filaments from polar capsules (see Chaps. 2, 3 and 13 for further discussion of polar capsules and host attachment). The usual portal of entry to fish is via gills or skin and is achieved by the infectious amoeboid sporoplasms that creep out of spores (see Chap. 13).

Malacosporean myxoworms have been documented to actively exit the bryozoan host via the vestibular pore (Canning et al. 2002a; Morris et al. 2002) although spores released from myxoworms within colonies are also observed (Hartikainen pers. obs., Okamura pers. obs.). After departing their bryozoan hosts, myxoworms undergo periodic vigorous swimming that may facilitate spore dispersal away from the local microhabitat or attract predation from fish thus achieving transmission. An additional mode of transmission may be direct consumption of infected bryozoans by foraging fish (Dendy 1963; Applegate 1966), although it is not known if infections can be acquired in this way.

11.4 Effects on Bryozoan Hosts (Virulence)

Virulence is defined as a general capacity of parasites to inflict damage and reduce host fitness (Read 1994). Virulence of malacosporeans on bryozoan hosts is highly variable, often depending on developmental cycling as outlined below.

11.4.1 Host Growth and Mortality

Overt infections generally reduce colony growth rates. Tops et al. (2009) showed a 49 % reduction in growth of *F. sultana* colonies with overt *T. bryosalmonae* infections cultured at 20 °C when compared to colonies with no overt infections. Hartikainen and Okamura (2012) found that the reduced growth of overtly infected colonies was similar to that observed for colonies producing statoblasts, suggesting that sustaining overt infection and investing in statoblast production represent similar energetic costs for *F. sultana*. Both studies showed a lack of colony mortality associated with overt infection (apart from at the unrealistic, environmentally extreme condition of 20 °C; Tops et al. 2009) while Hartikainen and Okamura (2012) found that partial colony mortality (zooid mortality) was actually reduced relative to uninfected hosts during stressful conditions (low food treatment). No significant effects of covert infections on colony growth or mortality have been found (Hill and Okamura 2007; Tops et al. 2009). Laboratory studies on the sac-forming *B. allmani* in *L. crystallinus* have demonstrated that overt infections are associated with reduced zooid production and colony fission but, as for *T. bryosalmonae*, no significant impact on colony mortality was found.

Overt infections of *T. bryosalmonae* are associated with significant changes in host phenotype. Thus, zooids of *F. sultana* are wider and colonies consequently appear stunted (Hartikainen et al. 2013). Such morphological changes are most likely associated with differential resource allocation in overtly infected colonies.

The infected phenotype with wider zooids may ultimately lead to increased transmission since wider zooids may support larger sacs with more infectious spores. Covert infections exert no effect on host morphology (Hartikainen et al. 2013). Notably, infections of the myxosporean, *Myxobolus cerebralis*, are associated with increased growth and respiration in the annelid host, *Tubifex tubifex* (Elwell et al. 2009; Shirakashi and El-Matbouli 2009). Alexander et al. (see Chap. 12) speculate that such increased growth may benefit the parasite by providing increased resources for parasite proliferation which could, in turn, lead to increased transmission.

Overt infections of myxoworms are generally pathogenic for bryozoans although pathogenicity may be reduced by the responses of colonies. Thus, *B. plumatellae* infections were associated with death of *Hyalinella punctata* colonies in the field and in laboratory culture, possibly due to damage caused by the actively moving myxoworms within the host body cavity (Canning et al. 2002a). Canning et al. (2002a) also observed damage followed by death of zooids in infected portions of a colony of *Plumatella fungosa* after myxoworms had exited the colony in laboratory culture. However, the *P. fungosa* colony had responded to infection by resolving into uninfected and infected portions, possibly as a result of inward pinching of the endocyst to seal off infected regions. The uninfected portions of the colony remained alive and produced statoblasts during a further 33 days in culture. McGurk et al. (2006) showed relatively high pathogenicity caused by vermiform malacosporeans, although the infected *P. repens* colonies were sustained for several months by providing high levels of food in culture systems.

11.4.2 Host Reproduction

Cessation or drastic reduction in statoblast production is associated with overt malacosporean infections in all study systems investigated to date (Okamura 1996; Canning et al. 2002a; McGurk et al. 2006; Hill and Okamura 2007; Hartikainen and Okamura 2012). Overtly infected regions of colonies are effectively castrated although some statoblasts have been observed in hosts fed with high quality diets (Hartikainen and Okamura 2012). However, these statoblasts are generally malformed and exhibit markedly reduced hatching success (Hartikainen et al. 2013). In contrast, statoblast production is not affected in covertly infected colonies (Hill and Okamura 2007; Tops et al. 2009). It is not known how infections impact the production of larvae although sperm have been observed in *F. sultana* with overt infections of *T. bryosalmonae* (see Fig. 11.1c).

Current evidence from the *T. bryosalmonae/F. sultana* system suggests there is no fecundity compensation. Thus, no differences were observed in the onset of statoblast production in uninfected and covertly infected colonies (Hartikainen et al. 2013) when grown in the laboratory after hatching from statoblasts. Such results further underline the avirulent nature of covert infections.

Myxozoans may generally influence how their definitive hosts allocate their resources by influencing investment in reproduction. The above evidence suggests that malacosporeans alter investment in clonal reproduction (statoblasts). Chapter 12 reviews evidence that myxosporeans may influence host investment in sexual reproduction. In particular, reduced fecundity and testis resorption (host castration) have been noted in annelids sustaining myxosporean infections.

11.5 Developmental Triggers

Both high temperature and food levels have been shown to provoke the development of overt infections in *T. bryosalmonae*. Tops (2004) monitored a single bryozoan population monthly and showed an increased level of overt infections in spring/early summer and during late autumn, suggesting that overt infection development may be linked with increased food resources during the spring and autumn algal blooms and possibly

also with increasing temperatures in spring. To directly test the effects of temperature on infection development, Tops et al. (2006) measured the prevalence of overt and covert infections in colonies collected from a single site on three occasions (summer, autumn and winter) and subsequently maintained at three different temperature regimes (20, 14 and 10 °C) in laboratory culture. In all cases, overt infection prevalences increased with temperature. In a similar experiment, Hartikainen and Okamura (2012) exposed colonies to three different food levels generated by spiking microcosms with fertiliser to promote algal and bacterial growth. Both infection prevalence and intensity peaked at the highest food levels when the nutritional status of hosts was maximal. As all food treatments were conducted at 20 °C, very few infections remained covert. These experimental studies also demonstrated that the growth of the uninfected hosts increased with higher temperature (Tops et al. 2006) and food (Hartikainen and Okamura 2012) levels, thus indicating that overt infections are triggered by environmental conditions suitable for host growth. They also indicate that when environmental conditions do not support bryozoan growth (low temperature and food levels) *T. bryosalmonae* persists within bryozoan hosts as avirulent covert infections.

11.6 Virulence Strategies

The theory of virulence evolution proposes that trade-offs explain the evolution of different virulence strategies (Schmidt-Hempel 2011). For example, the transmission-virulence trade-off model predicts that an evolutionarily stable level of virulence would be achieved by balancing the costs of virulence (from e.g. host mortality) with the benefits of increasing host exploitation in order to maximise transmission (Frank 1996). To understand virulence evolution in malacosporeans it is thus necessary to consider how transmission during overt infections (relatively high virulence and resulting in horizontal transmission) and covert infections (very low virulence and resulting in vertical transmission) interact to maximise the life-time reproductive success of the parasite. We also consider how virulence may relate to host population regulation.

11.6.1 Transmission-Virulence Trade-Offs in Malacosporeans

Theory predicts that vertically transmitted parasites tend to evolve towards lower virulence than horizontally transmitted parasites (Lipsitch et al. 1995, 1996). This is because the reproductive interests of the host and parasite become aligned during vertical transmission and high virulence would risk killing the host before transmission to offspring takes place. The low virulence of covert infections that achieve vertical transmission via statoblasts and colony fragments supports this prediction. Overt infections are associated with higher virulence and may be associated with higher transmission success, at least initially, since spores are produced in large numbers during this phase. Infection by multiple competing parasite strains may alter host exploitation dynamics and give rise to parasite strains with higher virulence strategies. However, the presence of multiple parasites and competing infections within bryozoans remains currently untested.

When transmission success starts to decelerate at high virulence levels (as we suggest might be the case above), parasites should evolve towards intermediate virulence (reviewed in Schmidt-Hempel 2011). The developmental cycling between overt and covert infections may facilitate expression of such intermediate virulence in a resource-dependent manner. For covert and overt infection strategies to co-exist, overt infections should evolve to maximise horizontal transmission without killing the host. This is because overt infections that achieve horizontal transmission at the cost of killing/castrating the host would then preclude the subsequent transmission achieved by covert infections. The observed pulses of overt infections and the persistence of covert infections may therefore reflect

trade-offs that result in intermediate levels of virulence while maximising horizontal transmission. We note that the periodic development of overt infections is unlikely to be driven by the availability of fish since these hosts are present year-round. Fish susceptibility is also unlikely to influence parasite developmental cycles since fish immune responses are triggered by warmer temperature. Susceptibility should therefore be relatively low in spring when temperatures are rising and high in autumn when temperatures are falling, yet peaks in overt infection development are observed in the field at both these times (Tops 2004).

Host-condition dependent development may explain how such intermediate virulence is achieved, as development of overt infections in healthy hosts would allow tapping into resources destined for reproduction (causing temporary host castration), thus avoiding costs associated with survival when hosts are in poor condition. Evidence for such processes includes low colony mortality and castration observed in malacosporean-bryozoan systems and the link between parasite development and host condition (Hartikainen and Okamura 2012). The cue for overt infection development may be the greater energy sources available in healthy hosts, thus coordinating the energetically-demanding production of transmission stages to a period when the host is most likely to be able to sustain such development. Such host condition-dependent development could be explained by straightforward resource limitation acting on the parasite via the host health. Alternatively, it could reflect a parasite strategy to reduce energetic burden on hosts in poor condition to improve survival within the host (Hartikainen and Okamura 2012).

Pilot studies also suggest that parasite development may be triggered when host mortality is imminent (Fontes et al. unpub. data). Such terminal effort might contribute to the surge in overt infection development observed during autumn, when many colonies are regressing to statoblasts. For those parasites that were unable to infect statoblasts, killing the host may then maximise transmission. This scenario is consistent with occasional overt infections in field collected *F. sultana* colonies in winter months (Tops 2004) when low productivity may have created stressful conditions, with year-round infections in a recirculating fish culturing facility (de Kinkelin et al. 2002) and with infection of fish in winter months (Gay et al. 2001).

11.6.2 Virulence and Host Population Regulation

For host population regulation to occur readily, parasite effects should be exerted in a density dependent manner. Thus, virulence impacts should increase with increasing host population size (Anderson and May 1978; May and Anderson 1983) and should be randomly distributed across the host population. This could be achieved by rapid evolution of parasites to exploit and become more virulent in large populations. It could also be achieved by lower investment in immune responses by hosts in large populations, perhaps due to resource competition (for review, see Schmidt-Hempel 2011). No evidence for either process exists in bryozoans and malacosporeans. However, if malacosporeans spread efficiently with the expansion of their highly clonal host population (e.g. being transmitted between colony modules during colony growth and via vertical transmission in statoblasts and colony fragments), chronic infection may characterise colonies at high density. A synchronous transition to overt infection expression could lead to high mortality and might function in a density-dependent manner to regulate host population size. However, the host condition-dependent expression of virulent and avirulent malacosporean stages would suggest that when bryozoan populations would be most vulnerable to population regulation, the parasites would tend to revert to non-virulent infection strategies. Host condition-dependent development may therefore not preclude population regulation, but it may modulate the amplitude and frequency of fluctuations in host population density.

11.7 Local Adaptation

Little is currently known about what processes drive local adaptation of malacosporeans and their hosts and how such locally adapted populations of hosts and parasites translate into disease emergence in fish populations. Evidence for local adaptation would include exploitation of common host clones, strain variation and variation in parasite viability in hosts with which no long-term co-evolutionary history exists.

11.7.1 Host Clonality and Infection Prevalence

As bryozoan hosts have a propensity for high levels of clonal reproduction, repeated occurrences of the same genotypes are both expected and documented (e.g. Vernon et al. 1996; Hatton-Ellis et al. 1998; Freeland 2000) within populations. However, not all bryozoan populations are highly clonal. For example, Hartikainen and Jokela (2012) found that clonal diversities, characterised by the number of unique microsatellite multilocus genotypes (MLGs), were highly variable amongst *F. sultana* populations, with some populations being monoclonal (only one genotype present), while in others multiple genotypes were present. In some cases no repeated MLGs were found (Hartikainen and Jokela 2012). Given such variation in clonal diversity within host populations, it is interesting to consider how parasites may interact with or influence spatial and/or temporal fluctuations in the representation of host clonal genotypes.

Disproportionate infection of common host clones is a predicted outcome of host-parasite coevolutionary trajectories as parasites become locally adapted to the most abundant resource (Lively and Dybdahl 2000; Jokela et al. 2003). Thus, antagonistic host-parasite coevolution may explain fluctuations in host (and parasite) genotype frequencies, as the parasites track their hosts in a negative frequency dependent manner. Although time lags are anticipated, disproportionate infection of common clones would nevertheless provide evidence for local adaptation (Dybdahl and Lively 1995).

At present there is little evidence for such local adaptation in malacosporeans and bryozoans. Disproportionate infection of common clones (characterised by random amplified polymorphic DNA markers or RAPDs) of *Cristatella mucedo* by the sac-forming *Buddenbrockia plumatellae* was not detected, nor was parasitism linked with the decline of the most common clone over time (Vernon et al. 1996). However, this study was conducted before covert infections were recognised and they were not assayed. More recently, MLG analyses of *F. sultana* colonies collected from a focal tree root system revealed that a single genotype dominated the entire bryozoan population over a 10 year period (Hartikainen et al. in prep). Infection prevalences of this genotype by *T. bryosalmonae* were detected by PCR and were >90 % over the 10 years. A small number of colonies of two unique genotypes were detected in one year but vanished subsequently. These rare clones were not infected and thus this minor increase in population genetic diversity was linked with a decrease in infection prevalence, a pattern somewhat consistent with local adaptation. However, no turnover of genotype frequencies was detected despite the relatively long study period. Such patterns could indeed characterise bryozoan/malacosporean interactions, as malacosporeans may not be sufficiently virulent to give rare clones a fitness advantage that would result in genotype frequency fluctuations. Notably, modelling has suggested that even a small amount of vertical transmission, especially when the parasite is only weakly virulent, may slow or preclude cyclical dynamics (Greenspoon and M'Gonigle 2013). The relatively low virulence and vertical transmission of malacosporeans in bryozoans and the constant host genotype frequencies in the long term temporal study of a highly infected *F. sultana* population (Hartikainen et al. in prep.) provide support for model predictions.

The studies described above suggest that host populations with low genetic diversities may

promote local adaptation of parasites and function as major sources of infections for local trout populations. Variation in the susceptibility of rare and common clones to malacosporean infections remains unknown. However, if such variation exists it would provide evidence for antagonistic host-parasite co-evolution and would demonstrate a genetic basis for resistance and susceptibility as has been shown for the myxosporean, *Myxobolus cerebralis*, in tubificid worm hosts (Beauchamp et al. 2002).

11.7.2 Strain Variation and Host Specificity

Evolutionary processes such as mutation, random genetic drift, gene flow and natural selection can drive population divergence resulting in strain differentiation. Local adaptation of strains in *T. bryosalmonae* and other malacosporeans has not been directly investigated although there is some evidence for strain variation (ITS-1 region) and some circumstantial evidence for local adaptation relating to specificity to the fish host.

Evidence for strain variation is revealed in a phylogeographic study of *T. bryosalmonae* from North America and Europe based on analysis of 119 isolates characterised by Internal Transcribed Spacer 1 (ITS-1) sequences (Henderson and Okamura 2004). The study revealed relatively greater genetic diversity of North American isolates and suggested that *T. bryosalmonae* may have been introduced from North America to Europe prior to aquaculture activities, perhaps as infected statoblasts transported by birds. Although the gross patterns of strain variation revealed by this broad-scale ITS-1 phylogeny are robust, the ITS-1 locus has proven to be difficult to interpret especially at smaller spatial scales due to intragenomic variation and low phylogenetic signal (Hartikainen and Okamura, unpubl. data). Development of microsatellite and SNP markers will undoubtedly improve resolution for characterising any strain variation exhibited by *T. bryosalmonae* and other malacosporeans thereby revealing finer-scale geographic patterns.

Circumstantial evidence for local adaptation of *T. bryosalmonae* derives from the aquaculture industry with farming practices illustrating that severe disease may arise when exotic fish are infected by locally adapted parasites. In particular, rainbow trout are native to California and are widely used to stock fish farms throughout Europe and the United Kingdom. These farmed fish are highly susceptible to infection by local strains of *T. bryosalmonae* that develop in bryozoan populations in source waters, where natural fish hosts are brown trout (Morris and Adams 2006b; Grabner and El-Matbouli 2008). However, for the parasite, these rainbow trout will be a dead-end as mature spores do not develop. Controlled cross-infection studies using European and North American *T. bryosalmonae*, *F. sultana* and trout strains might enable demonstration of local adaptation of strains and assessment of strain-specific infectivity. Such studies might also reveal genetic signatures of resistance and tolerance in trout.

11.8 Host-Parasite Interactions, Environmental Change and Disease

Host-parasite interactions can be influenced by environmental variation. For instance, temperature or the availability of food for bryozoan hosts can determine infection outcomes and rates (see also Chap. 15). Below we explore how environmental change is posing new challenges and opportunities to malacosporeans and their bryozoan hosts by focusing on the *T. bryosalmonae*/*F. sultana* system. Such changes have important implications for the coevolutionary dynamics of hosts and parasites as well as consequences for the spread and severity of PKD.

11.8.1 Climate Change

Bryozoan populations are likely to respond positively to increasing temperatures as growth rates increase (Tops et al. 2009) in the first instance.

Nevertheless, temperature increases can be expected eventually to become detrimental so predicting the impacts of climate change in the long term is difficult. Even in the intermediate term, impacts of climate change on infection prevalence may be unpredictable. For example, moderate temperature increases are likely to promote the growth and density of bryozoan populations and may enable many populations to overwinter as colonies. Production of infectious spores in such overwintering colonies may increase the infective period and dose to trout, although the impact on bryozoan hosts is less clear (Tops et al. 2006). Climate change may thus act to increase the incidence of PKD—as suggested by recent northerly outbreaks in association with warmer waters (Sterud et al. 2007; Kristmundsson et al. 2010). On the other hand, young trout exposed to *T. bryosalmonae* during cold winter months may develop natural immunity whereby the immune reaction to *T. bryosalmonae* is precluded when temperatures increase the following year (Clifton-Hadley et al. 1984; Foot and Hedrick 1987; de Kinkelin and Loriot 2001). However, the development of natural immunity is not well understood currently and infection outcomes are likely to depend on a balance of factors that are modified by the local environment and the genetic background of the hosts and parasites.

11.8.2 Eutrophication

Freshwater bryozoans are broadly distributed and tolerate widely varying environmental conditions (Wood 2001; Wood and Okamura 2005). They generally flourish in meso- to oligotrophic waters (Bushnell 1974; Sládecek 1980) but many plumatellids seem to prefer very eutrophic conditions (Dendy 1963; Jónasson 1963; Job 1976; Mukai et al. 1979). *F. sultana* inhabits both high elevation oligotrophic localities as well as lowland eutrophic lakes (Raddum and Johnsen 1983). There is considerable evidence that bryozoan biomass production rates are higher in eutrophic waters, possibly due to increased food availability. For instance, *Plumatella fungosa* grew faster and developed greater mass in eutrophic lakes (Jónasson 1963), and bryozoan growth was greater in ponds receiving agricultural runoff than in unimpacted ponds (Dendy 1963). *F. sultana* colonies grew faster in highly productive microcosms spiked with high nutrients relative to those receiving low nutrients (Hartikainen and Okamura 2012), and bryozoan statoblast concentrations were found to be orders of magnitude higher in rivers characterised by high nutrient levels than in rivers with medium or low nutrient levels (Hartikainen et al. 2009). Finally, bryozoan biomass in eutrophic rivers and lakes can be very high (e.g. 103–1,313 g/m^2 in the River Meuse (Henry et al. 1989) and 1,600 g/m^2 in Danish lakes (Jónasson 1963).

As outlined earlier, *T. bryosalmonae* undergoes prolific growth when bryozoan hosts are supported by high food levels. It is therefore unsurprising that the incidence and severity of PKD have been linked with eutrophication (El-Matbouli and Hoffmann 2002; Wahli et al. 2002; Borsuk et al. 2006) and it might be expected that a reduction in eutrophication could ameliorate disease. Indeed, the diversion of an effluent outlet was followed by a decrease and finally disappearance of PKD from a rainbow trout farm downstream (El-Matbouli and Hoffmann 2002). However, whether this reflected improvement in fish health with a change in water quality or decreases in bryozoan host abundances is unknown (El-Matbouli and Hoffmann 2002).

11.8.3 Environmental Change and Disease

There is now substantial evidence that PKD is an emerging disease which is increasing in geographic range, incidence and severity (Okamura et al. 2011). Much of this may be explained by the influence of environmental change on the development of and interactions between *T. bryosalmonae* and its bryozoan host, *F. sultana*.

T. bryosalmonae appears to be highly adapted to exploit the clonal nature of bryozoans and this enables long term persistence within host populations. This persistence results in a disease reservoir which underlies the annual outbreaks of PKD on fish farms as well as the development of clinical disease in regions where PKD has not previously been recognised (Sterud et al. 2007; Kristmundsson et al. 2010). As the environment continues to change we predict that cases of clinical PKD will continue to be reported in novel regions. Variability in waterfowl movements in response to environmental change may further contribute to future patterns of disease outbreaks as a result of co-dispersal of parasites with dormant statoblasts.

11.9 Conclusions

The discovery that freshwater bryozoans are hosts of the causative agent of PKD (*T. bryosalmonae*) prompted a flurry of research on the ecology of malacosporeans and their interactions with bryozoan hosts. This body of research suggests that *T. bryosalmonae* has evolved strategies to persistently exploit bryozoan hosts by undergoing host-condition dependent development (cycling between virulent overt and avirulent covert infections) and substantial levels of vertical transmission during clonal propagation of bryozoans (via statoblasts and colony fragmentation). Vertical transmission is also evident in other malacosporean-bryozoan systems but wider study is required to characterise malacosporean-bryozoan interactions in general. What is evident, however, is that the development of sporogonic stages (sacs and myxoworms) is extremely rapid (within days) and presumably results from cryptic stages associated with the body wall. The development of overt infections when conditions are conducive for bryozoan growth (e.g. warmer temperatures and eutrophic waters) suggests that environmental change will influence malacosporean infection dynamics in bryozoans with implications for disease emergence in fish.

11.10 Key Questions for Future Research

- Can bryozoans escape infection by fragmenting (e.g. by sealing off portions of colonies, autotomising branches, undergoing fission)?
- How important are birds as dispersal vectors for malacosporeans?
- Can resistant bryozoan genotypes be identified and used to displace highly susceptible populations?
- Do bryozoans and *T. bryosalmonae* exhibit local adaptation and strain differentiation that relate to virulence and susceptibility?
- Do multiple infections occur and how do they influence infection dynamics in bryozoan hosts?

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Annelid-Myxosporean Interactions

12

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Abstract

This chapter provides an overview of the diversity of annelids parasitized by myxosporeans in both marine and freshwater systems and reviews the interactions between myxosporeans and their annelid hosts during the so-called actinospore phase of the myxosporean life cycle. Both host and environmental factors can influence infection. Our chapter examines these influences from the initial infection of annelids through to the release of spores infectious to fish. Topics covered include how myxosoporean infections may relate to the trophic ecology of annelid hosts and what factors may influence dispersal and increase encounter rates of infectious spores with hosts. We also review the little that is known about annelid-myxosporean interactions, focusing on patterns of host specificity, host susceptibility and host immune defenses. Finally, we examine development within and effects on annelid hosts. To illustrate these interactions we draw primarily on examples from the relatively well-studied freshwater salmon parasites *Myxobolus cerebralis* and *Ceratonova shasta,* and interactions with their respective annelid hosts.

Keywords

Actinospore phase · Definitive host · Host-parasite interactions · Oligochaetes · Polychaetes · *Manayunkia speciosa* · *Tubifex tubifex*

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12.1 Introduction

Myxozoans parasitize annelids and freshwater bryozoans as invertebrate hosts. Annelids are exclusively parasitized by myxozoans belonging to the Myxosporea, while bryozoans are parasitized by the Malacosporea. Some 2,200 species of myxosporeans have been described (Lom and Dyková 2006; Yokoyama et al. 2012), of which some 180 species or types[1] have been described from annelids.

The myxosporean life cycle involves the alternative formation of two distinct spores that are produced during the myxospore and actinospore phases of development (Lom and Dyková 2006). The myxospore phase includes the infection of lower vertebrates (namely fishes) by actinospores and culminates in the formation of myxospores (myxospores also form in other vertebrates but the complete life cycle of these species is unknown; see Chap. 7). The actinospore phase begins with the infection of annelids by myxospores and culminates in the formation of actinospores. Direct transmission between annelid hosts has not been observed but transmission from mother to daughter during paratomy can occur as demonstrated by Triactinomyxon type in *Lumbriculus variegatus* (Morris and Adams 2006) and *Myxobilatus gasterostei* in *Nais communis* (Atkinson and Bartholomew 2009). Annelids are considered definitive hosts because meiosis occurs prior to the production of actinospores. However, this has only been observed for a subset of species (i.e. *M. cerebralis* (El-Matbouli and Hoffmann 1998), *Ceratonova* (syn *Ceratomyxa*) *shasta* (Meaders and Hendrickson 2009), *Myxobolus pseudodispar* (Alvarez-Pellitero et al. 2002) and Unicapsulactinomyxon type (Rangel et al. 2011)).

[1] Actinospores were originally described as species within the Class Actinosporea, but the discovery that actinospores and myxospores are alternate stages in a common life cycle led to suppression of the Actinosporea and assignment of both actinospore- and myxospore-bearing taxa to the Class Myxosporea. Taxa described based on actinospores alone (i.e. myxospore-less members) were then considered as species inquirendae and are now referred to as types within collective groups (formerly genera) (Kent et al. 1994).

We begin by reviewing the diversity and ecology of annelid hosts parasitized by myxosporeans. We then summarize interactions between myxosporeans and annelids throughout the actinospore phase—from myxospore encounter to actinospore release. Chapter 11 provides a complementary review on malacosporean-bryozoan host interactions and Chap. 13 reviews the recognition and invasion of vertebrate hosts by myxozoans.

12.2 Annelid Hosts

Myxosporeans parasitize both freshwater and marine annelids. Polychaetes (~168 species; Glasby and Timm 2008) are less common than oligochaetes (~1,100 species; Martin et al. 2008) in freshwater environments, whereas oligochaetes (~200 species; Giere and Pfannkuche 1982; Giere 2006) are less common than polychaetes (~12,000 species; Dauvin et al. 1994) in marine environments. Freshwater oligochaete hosts include tubificids (hereafter referred to as 'tubificoid naidids' due to reclassification that assigns the Tubificidae to the Naididae; Erséus et al. 2008), naidids and lumbriculids (Marquès 1984; Brinkhurst 1991, 1996; Yokoyama and Urawa 1997). Freshwater polychaete hosts are represented by only a single sabellid species to date (Bartholomew et al. 1997, 2006). Marine annelid hosts include tubificoid naidid oligochaetes (Caullery and Mesnil 1905; Ikeda 1912; Marquès 1984; Roubal et al. 1997; Hallett et al. 1997a, 1998, 1999, 2001; Hallett and Lester 1999), serpulid, onuphid and spionid polychaetes (Køie 2000, 2002, 2003; Køie et al. 2004, 2007, 2008; Rangel et al. 2009, 2011, 2012; Karlsbakk and Køie 2012), and sipunculids (a group of unsegmented annelids) (Ikeda 1912).

The first freshwater myxozoan life cycle was elucidated in 1983 (Markiw and Wolf 1983) and the first marine life cycle was resolved 21 years later (Køie et al. 2004). The majority of resolved life cycles involve freshwater oligochaetes, and two species of tubificoid naidids (*Tubifex tubifex* and *Branchiura sowerbyi*) are involved in over half (16 and eight, respectively) of these life cycles

(Table 12.1). The life cycles of five marine myxosporeans have now been described, all of which involve polychaete hosts (Karlsbakk and Køie 2012). Annelids associated with resolved marine life cycles include nereidid, serpulid, and sabellid polychaetes. Table 12.1 provides details and references regarding annelid hosts for all myxosporeans whose life cycles have been resolved.

The diversity of annelid hosts involved in resolved myxosporean life cycles (Table 12.1) is narrow in comparison to the diversity of annelid hosts from which actinosporean stages have been described. Notably, actinospores have been reported from a variety of marine and estuarine oligochaetes (Hallett et al. 2001) and myxosporeans are known from fishes in these environments (e.g. Hallett et al. 1997b; Hallett and Diamant 2001). Thus myxosporeans infecting at least some marine oligochaetes are expected to be linked eventually with vertebrate hosts. Likewise, actinospore stages (e.g. Rosser et al. 2014) described from freshwater annelids may eventually be linked with vertebrate hosts.

12.3 Annelid Trophic Ecology and Myxospore Morphology

Myxospores exhibit a range of morphologies (see morphotypes in Lom and Dyková 2006). Some myxospores appear to be adapted for settling whereas others display features that may facilitate floating. For instance, negatively buoyant myxospores that are spherical (with low surface areas, e.g. most myxobolids) may be adapted for deposition with small particles (Lemmon and Kerans 2001; Kerans and Zale 2002). This may increase the likelihood of consumption by deposit feeding annelids. The longer processes of some myxospores (e.g. henneguyoids) could prolong suspension, facilitating consumption by filter feeders. The evolution of myxospore morphotypes has not yet been considered in the context of interactions with their annelid hosts. However, as consumption of myxospores may be the main or only invasion route (see later discussion), then annelid host trophic ecology may exert selection on myxospore morphology.

Most oligochaetes are collector gatherers that consume sediment particles, digesting the associated bacteria and organic matter (Brinkhurst et al. 1972). Thus, myxospores trapped in or deposited on sediments may be encountered and ingested. The freshwater polychaete, *M. speciosa*, is likely to be a facultative suspension and deposit feeder as this appears to be the case for other *Manayunkia* species (Fauchald and Jumars 1979; Stocking and Bartholomew 2007). It may therefore encounter myxospores in both suspended and in deposited form. The varied trophic ecology of marine polychaetes may allow these hosts to encounter both suspended and deposited myxospores. For example, *Nereis* species are omnivorous and undertake carnivory, deposit- and filter-feeding opportunistically. *Spirorbis* species and the sabellid, *Chone infundibuliformis,* are filter feeders and may be infected exclusively by suspended myxospores. However, the extent that facultative deposit- and filter-feeding (e.g. Taghon et al. 1980) may apply to various marine annelids remains to be determined.

12.4 Annelid-Myxosporean Interactions

A great deal of our knowledge of annelid-myxosporean interactions is based on myxosporeans associated with economically important fish parasites. Whether these annelid-myxosporean interactions are broadly representative warrants consideration, and we include information for other annelid-myxosporean systems when it is available. Table 12.2 summarises information about the development of myxosporeans in various annelids.

The remainder of this chapter focuses on development in annelid hosts and host-parasite interactions during the actinospore phase. Our discussion is largely focused on three myxosporean parasites of salmonids, *M. cerebralis*, *C. shasta* and *Parvicapsula minibicornis*, as these have received a great deal of attention because of the high value placed on salmonid conservation. All three parasites are problematic in North America where they have ecological and

Table 12.1 Diversity of annelid hosts involved in resolved myxosporean life cycles

Habitat	Host Identity	Myxosporean (actinospore morphotype) (References)
Freshwater	**Oligochaeta Naididae** *Tubifex tubifex*	*Henneguya nuesslini* (triactinomyxon) (Kallert et al. 2005a); *Myxidium giardi* (aurantiactinomyxon) (Benajiba and Marquès 1993); *M. truttae* (raabeia)(Özer and Wootten 2000; Özer et al. 2002a,b; Holzer et al. 2004); *Myxobolus bramae* (triactinomyxon) (Eszterbauer et al. 2000); *M. carassii* (triactinomyxon) (El-Matbouli and Hoffman 1993); *M. cerebralis* (triactinomyxon) (Markiw and Wolf 1983; Wolf and Markiw 1984; El-Matbouli et al. 1999b); *M. dispar* (raabeia) (Molnár et al. 1999a); *M. diversicapsularis* (triactinomyxon) (Hallett et al. 2005; Molnár et al. 2010); *M. drjagini* (triactinomyxon) (El-Mansy and Molnár 1997b); *M. hungaricus* (triactinomyxon) (El-Mansy and Molnár 1997a); *M. macrocapsularis* (triactinomyxon) (Székely et al. 2002); *M. parviformis* (triactinomyxon) (annelid identification unclear: authors also report infection in *Limnodrilus hoffmeisteri*) (Kallert et al. 2005b); *M. portucalensis* (triactinomyxon) (El-Mansy et al. 1998); *M. rotundus* (triactinomyxon) (Hallett et al. 2005; Székely et al. 2009); *M. wootteni* (triactinomyxon) (Eszterbauer et al. 2006; Molnár et al. 2010); *Zschokkella nova* (siedleckiella) (Uspenskaya 1995)
	Branchiura sowerbyi	*Hoferellus carassii* (neoactinomyxum) (Yokoyama et al. 1993); *Myxobolus cultus* (raabeia) (Yokoyama et al. 1995; Eszterbauer et al. 2006); *M. lentisuturalis* (raabeia) (Janiszewska 1955; Caffara et al. 2009); *Sphaerospora renicola* (neoactinomyxum) (Grossheider and Körting 1993; Molnár et al. 1999b); *S.* sp. (guyenotia) (Eszterbauer and Székely 2004; Eszterbauer et al. 2006); *Thelohanellus hovorkai* (aurantiactinomyxon) (Yokoyama et al. 1993; Yokoyama 1997; Székely et al. 1998; Anderson et al. 2000); *T. nikolskii* (aurantiactinomyxon) (Székely et al. 1998); *Zschokkella* sp. (echinactinomyxon) (Yokoyama et al. 1991)
	Limnodrilus hoffmeisteri	*Myxobolus intimus* (aurantiactinomyxon) (Rácz et al. 2004), *M. parviformis* (triactinomyxon) (annelid identification questionable; authors also report infection in *T. tubifex*) (Kallert et al. 2005b)
	Isochaetides michaelseni	*Myxobolus erythrophthalmi* (triactinomyxon), *M. shaharomae* (triactinomyxon) (Székely et al. 2014)
	Dero digitata	*Henneguya exilis* (aurantiactinomyxon) (Lin et al. 1999); *H. ictaluri* (aurantiactinomyxon) (Styer et al. 1991)
	Nais communis	*Myxobilatus gasterostei* (triactinomyxon) (Atkinson and Bartholomew 2009)
	Nais sp.	*Hoferellus cyprini* (aurantiactinomyxon) (Grossheider and Körting 1992)
	Lumbriculidae *Eiseniella tetraedra*	*Chloromyxum schurovi* (neoactinomyxum) (Ormieres and Frezil 1969; Holzer et al. 2006)
	Stylodrilus heringianus	*Chloromyxum truttae* (aurantiactinomyxon) (Marquès 1984; Holzer et al. 2004); *Myxobolus arcticus* (triactinomyxon) (Kent et al. 1993)
	Lumbriculus variegatus	*Myxobolus arcticus* (triactinomyxon) (Urawa and Awakura 1994; Yokoyama and Urawa 1997)
	Polychaeta Sabellidae *Manayunkia speciosa*	*Ceratonova shasta* (tetractinomyxon) (Bartholomew et al. 1997); *Parvicapsula minibicornis* (tetractinomyxon) (Bartholomew et al. 1997, 2006)

(continued)

Table 12.1 (continued)

Habitat	Host Identity	Myxosporean (actinospore morphotype) (References)
Marine	**Oligochaeta**	Hosts known from Naididae and Lumbriculidae but none known for resolved life cycles
	Polychaeta Nereididae *Nereis diversicolor*	*Zschokkella mugilis/Ellipsomyxa mugilis* (unnamed type) (Rangel et al. 2009, 2012)
	Nereis pelagica	*Sigmomyxa sphaerica* (tetractinomyxon) (Karlsbakk and Køie 2012)
	Nereis spp.	*Ellipsomyxa gobii* (tetractinomyxon) (Køie et al. 2004)
	Sabellidae *Chone infundibuliformis*	*Ceratomyxa auerbachi* (tetractinomyxon) (Køie et al. 2008)
	Serpulidae *Spirorbis* spp.	*Gadimyxa atlantica* (tetractinomyxon) (Køie 2002; Køie et al. 2007)

Information provided under Host Identity relates to annelid class, family and the species that is identified as the definitive host. Information provided under Myxosporean relates to actinospore morphotype

Table 12.2 Myxosporean development in annelid hosts including target infection site (where infection develops), site of mature actinospores, site of actinospore release, and degree days (DD) required for actinospore development

Myxosporean/ Annelid host	Infection site	Mature actinospores	Actinospore release	DD	Reference
Myxobolus cerebralis/Tubifex tubifex	Intestinal epithelium	Lumen	Anus	1,170–1,518	El-Matbouli and Hoffmann (1998), Kerans et al. (2005)
M. bramae/T. tubifex	Intestinal epithelium	Lumen	Anus	1,832.5	Eszterbauer et al. (2000)
M. hungaricus/T. tubifex	n.d.	n.d.	n.d.	2,040	El-Mansy and Molnár (1997a)
M. drjagini/T. tubifex	n.d.	n.d.	n.d.	1,820	El-Mansy and Molnár (1997b)
M. intimus/ Limnodrilus hoffmeisteri	Intestinal epithelium	n.d	n.d	997.5	Rácz et al. (2004)
M. macrocapsularis/ T. tubifex	n.d.	n.d.	n.d.	1,386–2,079*	Székely et al. (2002)
M. pseudodispar/ Unknown oligochaete	n.d.	n.d.	n.d.	1,634	Székely et al. (1999), (2001)
M. cotti/Unknown oligochaete	n.d.	n.d.	n.d.	2,062.5	El-Matbouli and Hoffmann (1989)
Thelohanellus hovorkai/ Branchiura sowerbyi	n.d.	n.d.	n.d.	800–1,395	Liyanage et al. (2003)
Parvicapsula minibicornis/ Manayunkia speciosa	Coelom	Coelomic cavity	Gonadal pores	n.d.	Bartholomew et al. (2006)
Ceratonova shasta/M. speciosa	Gut epithelium and epidermis	Epidermis	Epidermis-secretory pores and gonoducts	829.5	Bartholomew et al. (1997); Meaders and Hendrickson (2009)

Degree days were calculated by multiplying the average experimental temperature by total days to actinospore detection (n.d. = no data)

*Authors reported 'room temperature' and we assumed 21 °C

economic impacts. Although they share many characteristics, these parasites also demonstrate important differences that could influence interactions with their annelid hosts. *M. cerebralis* is endemic to Europe, but has achieved a much broader distribution through anthropogenic activities, and most of the empirical work has been conducted on North American isolates of *T. tubifex*. By contrast, *C. shasta* and *P. minibicornis* have only been reported from their native ranges in North America, and the few studies of their hosts have been conducted on isolates from basins in which the parasites are endemic.

We develop our review in the context of constraints in transmission (Fig. 12.1). Transmission is affected by a variety of host and environmental factors that operate variously from myxospore encounter to actinospore release. These factors act during the actinospore phase to influence (1) myxospore dispersal and host encounter, (2) annelid host-parasite interactions including invasion and proliferation, and (3) actinospore release (Fig. 12.1).

12.4.1 Myxospore Dispersal and Annelid Host Encounter

The actinospore phase begins when myxospores are encountered and ingested by a permissive annelid host—one that supports the production of transmission stages. Myxospores must therefore survive long enough to be ingested by their target host. Myxospore survival (viability) has not been widely characterized, but evidence suggests that myxospores can tolerate a range of stressors. For example, the myxospores of *M. cerebralis* remain viable following exposure to temperatures ranging from −20 to +60 °C (Hoffman and Markiw 1977) and digestion by piscivores (Hoffman and Putz 1969; El-Matbouli and Hoffmann 1991; Koel et al. 2010). Recent

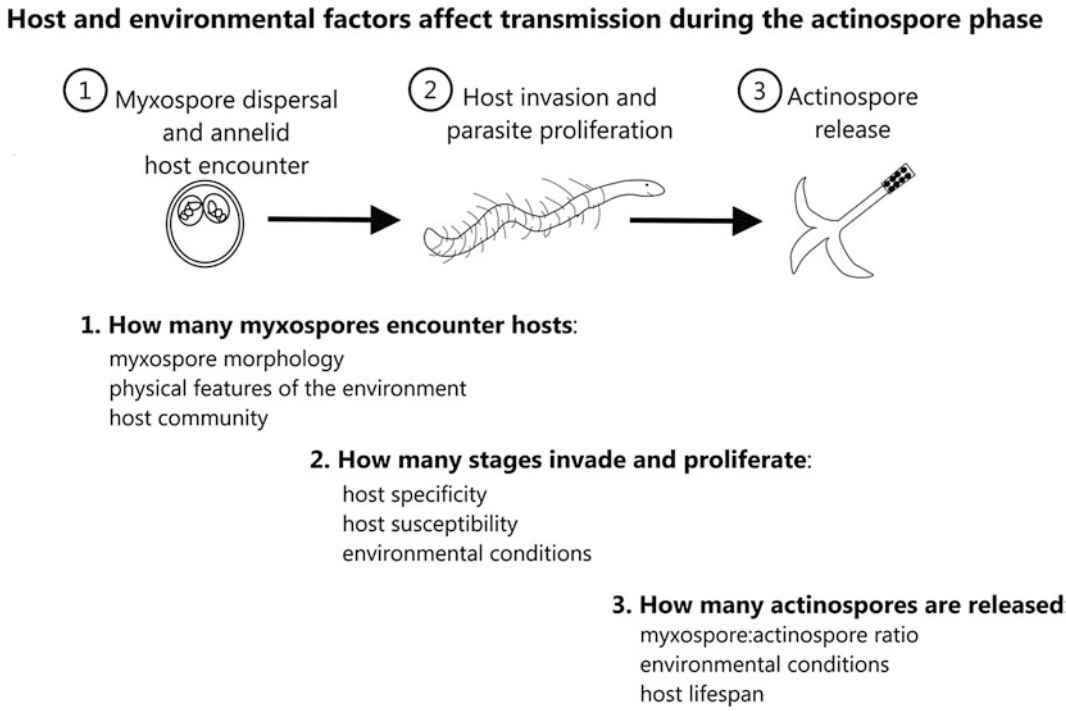

Fig. 12.1 Factors affecting parasite transmission during the actinospore phase of the myxosporean life cycle. Abiotic and biotic factors affect (*1*) myxospore dispersal and host encounter, (*2*) annelid host-parasite interactions including invasion and proliferation, and (*3*) actinospore release

investigations, however, have indicated that *M. cerebralis* myxospores lose their infectivity after ultraviolet irradiation or complete desiccation (Hedrick et al. 2008) (in contradiction to previous reports; Hedrick et al. 2000). Myxospore viability likely varies considerably among species. The myxospores of *C. shasta,* for example, appear less tolerant of thermal stressors than those of *M. cerebralis*. This is suggested by reduced viability of *C. shasta* myxospores following freezing (S. Hallett, unpublished data) and also by exposure to temperatures between 7 and 15 °C which reduced viability by 50–80 % after 3 months and by >90 % when exposed to temperatures between 18 and 23 °C for 1 month (Chiaramonte 2013).

Myxospores must not only survive long enough to be ingested by their target hosts, but because of the patchy distributions that often characterize annelid hosts, they may require dispersal from the point of myxospore release to target host habitats. The distribution and dispersal of myxospore stages are not well understood because it is difficult to detect myxospores in the environment (e.g. Gates 2007). Myxospore dispersal is most likely passive, influenced by a combination of environmental conditions and myxospore morphology. Through their effects on spore deposition and re-suspension, environmental factors (including depth, flow, and the nature of the substratum) may influence spore distribution at the local scale (Kerans and Zale 2002; Elwell et al. 2009b; Jordan 2012). The magnitude and duration of peak runoff (MacConnell and Vincent 2002) and predator foraging activities that move infected fish and myxospores (Koel et al. 2010) may also play a role in spore dispersal.

Once in the host environment, rates of myxospore encounter may be influenced by characteristics of myxospores, features of the host environment, and structure of the host community. The spherical myxospores of *M. cerebralis* are negatively buoyant and adhere to small particles, which facilitates settling out with fine sediments (Lemmon and Kerans 2001) where they will then be available for ingestion by deposit-feeding *T. tubifex*. The comparatively elongate myxospores of *C. shasta* may be retained in suspension for some time and may therefore be ingested during filter feeding by *M. speciosa* (see Sect. 12.3 for additional discussion).

Annelids actively select particles while foraging (Matisoff et al. 1999; Rodriguez et al. 2001). Myxospores that are similar to their host's food source are therefore more likely to be ingested than those that are dissimilar. For example, myxospores may be more likely to be consumed if they fall within the range of particle sizes selected by hosts or are coated with surficial protein or bacteria. It is possible that myxosporeans influence host particle selection by manipulating rates of encounter with and ingestion of myxospores by annelids.

Myxosporeans utilize a variety of portals for entry into fish, and we would expect the same for annelid host invasion. Myxospore ingestion, however, is the only invasion route described so far for annelids. As additional systems are studied, we may discover that myxosporeans are also able to penetrate annelid host epidermis or gills, as in fish.

Environmental conditions in the host's habitat may also influence rates of myxospore encounter, for instance through their effects on host distribution. For example, environments characterized by nutrient enrichment and increased siltation tend to support high densities of *T. tubifex* (Brinkhurst and Jamieson 1971; Lazim and Learner 1987; Juget and Lafont 1994; Krueger et al. 2006). Similarly, environments impacted by deep recirculating eddies retain particulate organic material and tend to support high densities of *M. speciosa* (Stocking and Bartholomew 2007; Jordan 2012). These areas are also where dead fish tend to be deposited and where myxospores are most likely to settle out (Kerans and Zale 2002).

Variation in the biotic environment may also influence myxospore encounter. For instance, the rate of encounter may be reduced by a high proportion of non-permissive annelids that occupy a similar ecological niche as permissive hosts. Non-permissive hosts may become infected but actinospores do not develop. Attempts to

test the effects of annelid community composition on host encounter under controlled laboratory conditions have given conflicting results. In several studies, mixed cultures of permissive and non-permissive *T. tubifex* produced fewer actinospores (Beauchamp et al. 2006; Baxa et al. 2008) and exhibited lower infection prevalence (Zielinski et al. 2011) than monocultures of permissive *T. tubifex* when exposed to the same doses of *M. cerebralis* myxospores. In the mixed cultures, non-permissive *T. tubifex* may have reduced encounter of viable myxospores by permissive *T. tubifex* through inactivation (R. Lamb, L. Stevens, D. Rizzo, N. Fytilis and B. Kerans, unpublished data). In contrast to the aforementioned studies, Elwell et al. (2006) observed no difference in infection prevalence or actinospore production when comparing mixed cultures of non-permissive and permissive *T. tubifex* with monocultures of permissive *T. tubifex*. Differences in methodologies from myxospore dose, to host strain and density, to choice of parasite detection assay could explain the discrepancies in the results of the above studies. Despite these caveats, there is considerable evidence that non-permissive hosts may reduce the number of myxospores available to permissive hosts by consuming and deactivating myxospores. A body of evidence now suggests that consumption of free-living parasite stages (e.g. eggs, miracidia, cercaria; reviewed in Johnson et al. 2010) can significantly contribute to food webs. It is likely that myxozoan spores make similar contributions and that these may influence myxospore dose (see Sect. 12.4.2.5).

12.4.2 Host Invasion and Development Within Hosts

Invasion of annelid hosts by myxosporeans is poorly understood compared with well-studied pathogens such as bacteria and viruses. In this section we consider issues relating to host invasion and development. In particular, we examine specificity of myxosporeans for annelid hosts and review annelid host susceptibility, immune defenses and the invasion of and development within annelids. Finally, we describe the relationship between the number of myxospores presented to annelid hosts (the myxospore dose) and the numbers of actinospores that are subsequently released, and how this varies for different myxosporeans.

12.4.2.1 Specificity

Myxosporeans exhibit varying degrees of specificity for annelid hosts just as they do for fish hosts. Some appear to be generalists, capable of infecting and proliferating in multiple annelid host species (e.g. *Sphaeractinomyxon ersei*, see Hallett et al. 2001), whereas other species appear to be more specific. The only known oligochaete host of *M. cerebralis* is *T. tubifex*. Actinospores were not produced in other oligochaetes (including *Limnodrilus hoffmeisteri, Ilyodrilus templetoni, Quistadrilus multisetosus, Tubifex ignotus*, *Lumbriculus variegatus,* and *Aeolosoma* spp.) following experimental exposures to *M. cerebralis* myxospores (Wolf et al. 1986; El-Matbouli and Hoffmann 1998; Beauchamp et al. 2002; Kerans et al. 2004). Several marine myxosporeans infect multiple annelid host species (e.g. *E. gobii*, Table 12.1). In contrast, most freshwater myxosporeans (nearly 90 % of those with known hosts) appear to be highly host specific, capable of infecting only a single oligochaete species (Table 12.1) (Xiao and Desser 1998; Hallett et al. 2001). Further studies are required to determine if these contrasting patterns of annelid host specificity are generally characteristic of marine and freshwater myxosporeans.

12.4.2.2 Host Susceptibility

Annelid hosts exhibit variable degrees of susceptibility to myxosporeans. For instance, susceptibility to *M. cerebralis* varies amongst six genetically distinct lineages of *T. tubifex* (referred to as Lineages I–VI) (Sturmbauer et al. 1999; Beauchamp et al. 2001; Arsan et al. 2007; Crottini et al. 2008). Lineages V and VI do not produce actinospores following experimental

exposure to myxospores (Beauchamp et al. 2006; Elwell et al. 2006; Zielinski et al. 2011). Lineage I exhibits more variability—actinospores were produced by at least one strain of Lineage I (Kerans et al. 2004) but not by another strain when exposed to myxospores under controlled laboratory conditions (e.g. Arsan et al. 2007).

Data suggest that *T. tubifex* lineages that do not produce actinospores can be infected but that transmission stages may not be produced. Infection is implied because even though actinospores are not produced in these hosts, exposed hosts exhibit reduced survivorship, growth and/or reproduction (Elwell et al. 2006). Thus, *T. tubifex* lineages that do not produce actinospores are most likely non-permissive rather than resistant. Histological studies, however, should be conducted to confirm this conclusion.

Strains of *T. tubifex* belonging to Lineage III are generally considered permissive hosts because most produce actinospores when experimentally infected with *M. cerebralis* (Stevens et al. 2001; Kerans et al. 2004; Rasmussen et al. 2008; Hallett et al. 2009). However, actinospore production (when myxospore dose is held constant) varies considerably among strains (see Markiw 1992; Baxa et al. 2008; Zielinski et al. 2011). At least one strain of Lineage III did not produce any actinospores when experimentally infected (Baxa et al. 2008). Whether a similar spectrum of susceptibility is common within species of other annelid hosts is not yet known.

The likely potential for strain variation in parasites adds further complexity to annelid-myxosporean interactions. For instance, *C. shasta* genotypes are linked with different susceptibilities to infection amongst fish species and strains (Atkinson and Bartholomew 2010; Hallett et al. 2012). The question of whether these *C. shasta* genotypes are linked to differential susceptibilities to infection amongst *M. speciosa* strains has yet to be addressed.

12.4.2.3 Host Immune Defenses

The lack of actinospore development in non-permissive *T. tubifex* suggests the possible involvement of immune responses. For instance, ingested *M. cerebralis* myxospores were observed to hatch and invade the intestinal mucosa of Lineage V worms, but failed to develop and disappeared (El-Matbouli et al. 1999a). Parasite development progresses further in non-permissive Lineage III worms, but is arrested and the stages persist in the intestine (Baxa et al. 2008). Annelid immune defenses are, however, poorly understood and have not been investigated at all in the context of myxosporeans.

Invertebrates in general lack an adaptive immune system so immune responses are based entirely on the innate immune system. Innate defense mechanisms in invertebrates include the use of RNA interference (RNAi), pattern recognition receptors (PRRs), anti-microbial peptides (AMPs), phagocytic cells, production of toxic oxygen and nitrogen metabolites, and melanization pathways (Kopp and Medzhitov 2003). It would be expected that annelid immune responses to myxosporean infections could be mediated by these defense mechanisms but none have been reported in infected annelids. It would be interesting to test whether the immune response of *T. tubifex* to *M. cerebralis* involves serine protease dependent pathways that facilitate or impede *M. cerebralis* proliferation and development, similar to those detected in fish hosts (Baerwald et al. 2008). If the immune response to *M. cerebralis* is distinct for different genetic lineages of *T. tubifex*, then investigating the role of specific signaling pathways involving molecules that mediate the innate immune response would be informative.

12.4.2.4 Intra-Annelid Development

Invasion begins once myxospores have been ingested. Polar filaments extrude and attach to the gut epithelium, effectively anchoring the myxospore. Myxospore shell valves then open along the suture line and the sporoplasm penetrates between the host's epithelial cells. This process takes approximately 5 days for *M. cerebralis* in *T. tubifex* at 13 °C (El-Matbouli and Hoffmann 1998) but only 2–24 h for *C. shasta* in

M. speciosa at 15.5 °C (Meaders and Hendrickson 2009) (Fig. 12.2). The parasite migrates to the target tissue (intestinal epithelium or body wall). After a proliferative phase (schizogony), gametogony occurs followed by the formation of actinospores (sporogony) (Fig. 12.2).

Myxosporean parasites invade a variety of tissues in their annelid hosts. The intestinal epithelium is a common site of infection in freshwater oligochaete hosts and coelomic infections are commonly reported in marine oligochaete and polychaete hosts (Table 12.2). Whereas *C. shasta* proliferates in the body wall, a sympatric parasite (*P. minibicornis*) proliferates in the coelom of *M. speciosa*. Niche partitioning may explain these differences in target tissues, reducing competition within the annelid in the case of mixed infections (although these may be rare; see Yokoyama et al. 1991; Hallett et al. 2001). A similar partitioning may underlie tissue specificity in fish hosts (see Chap. 16). Mature actinospores have also been observed in blood (undescribed aurantiactinomyxon, S. Atkinson, Oregon State University, personal communication) and gonadal tissues (Køie 2002). Regardless of the infection site, localized infections are common when infection is early or light, whereas parasite proliferation is far more dispersed in heavy infections. For example, El-Mansy et al. (1998) observed *M. portucalensis* spores in centrally located intestinal segments in moderate infections and in most segments in severe infections.

Rates of proliferation and actinospore release may be influenced by the locations of infections. For example, there is a ten-fold difference in the numbers of *P. minibicornis* versus *C. shasta* actinospores that develop in *M. speciosa* (Bartholomew et al. 2006). This may relate to differences in the capacity of coloemic compartments and epithelial layers (e.g. the body wall) to support parasite development. The location of infection (tissue tropism) may provide clues regarding the route of actinospore release

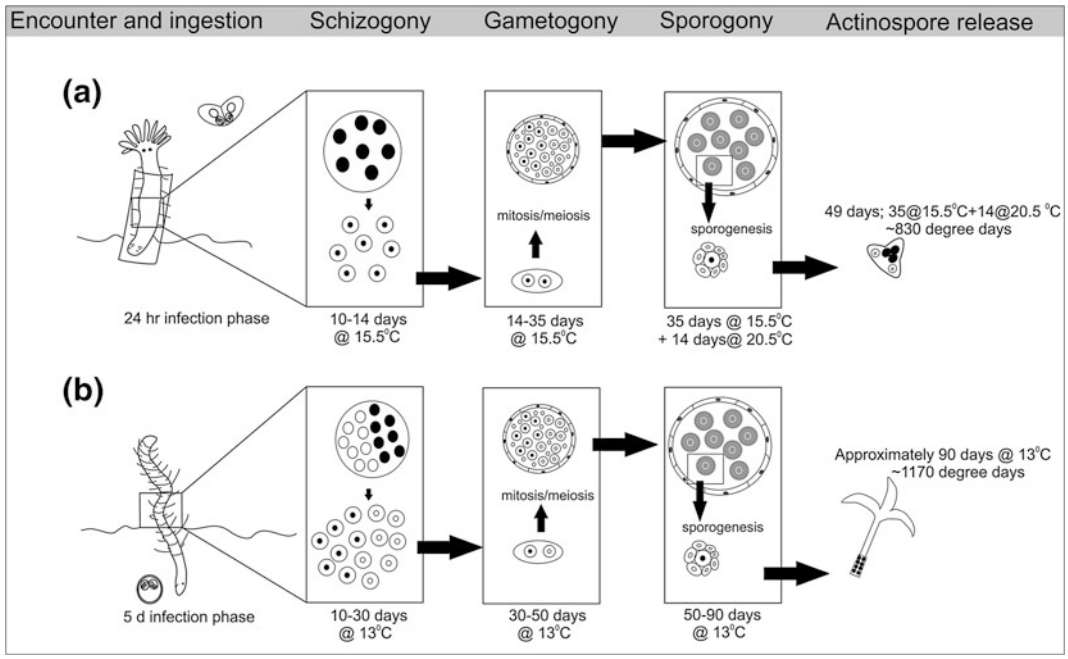

Fig. 12.2 Development of **a** *Ceratonova shasta* in the body wall tissue of *Manayunkia speciosa* and **b** *Myxobolus cerebralis* in the intestinal epithelial tissue of *Tubifex tubifex*. After correcting for temperature differences between development, the progression from myxospore to actinospore is faster for *C. shasta* (~830 degree days; 35 days @ 15.5 °C plus 14 days at 20.5 °C) than for *M. cerebralis* (~1,300 degree days; 90 days @ 13 °C)

(Table 12.2). Myxosporeans that target host intestinal tissues appear to be released from the anus (egestion). By contrast, those that target the coelom or reproductive organs appear to be released from proximally located tissues. How the spores traverse epithelial barriers and whether there is a general correlation between the target tissue and the route of actinospore release require further study. Asynchronous development in annelids characterizes all myxosporeans and enables long term release of parasites over the host's life span.

Temperature can affect the rate of parasite development and actinospore release. Elevated temperatures are correlated with earlier release of *M. cerebralis* actinospores from *T. tubifex* and higher total actinospore production (El-Matbouli et al. 1999b; Blazer et al. 2003; Kerans et al. 2005). Differences in experimental conditions, however, frequently limit the comparisons of development times. Characterising actinospore development and release as a function of degree-days (as has been done for *M. cerebralis*; Kerans et al. 2005) facilitates comparisons, and illustrates the wide variability in degree-days required to complete the actinospore phase. There is evidence that degree days from myxospore to actinospore also vary in other myxosporeans (Table 12.2). Factors other than temperature (e.g. host size, nutritional state or stage) may also influence parasite development but have not been investigated.

12.4.2.5 Myxospore Dose

The relationship between myxospore dose and infection has not been widely studied. In most infection experiments, harvested myxospores are added unquantified to annelid cultures. A few detailed laboratory studies suggest that a threshold dose is required for infections to develop. For instance, infection of polychaete hosts was achieved with exposures of 10 *C. shasta* myxospores per worm but not with exposure to a single myxospore per worm (Hallett, unpublished data). Similarly, Elwell et al. (2009a) reported that exposure to >1 <10 spores per worm was sufficient to infect *T. tubifex* but achieved less than 10 % prevalence of infection. Infection prevalence reached 100 % when exposures were based on 50 or more myxospores per worm. It is important to note that Elwell et al. measured prevalence of infection as the proportion of individuals releasing actinospores. Many studies on myxosporeans report infection prevalence based on the detection of parasite DNA by PCR (e.g. Krueger et al. 2006; Stocking and Bartholomew 2007; Alexander et al. 2011). To more effectively characterize host-parasite dynamics investigations should confirm actinospore development (for instance by their release) since some infections may never produce mature spores.

The number of actinospores generated over the course of an infection can vary substantially —for instance from 100s to 100,000s (Gilbert and Granath 2001; Hallett et al. 2001; Yokoyama et al. 1991; Liyanage et al. 2003). The ratio of the number of myxospores estimated to enter a host (based on exposure dose) versus the number of actinospores that are estimated to exit a host (based on release) also varies. Markiw (1986) noted a ratio of 1:47 for *M. cerebralis* in highly susceptible *T. tubifex*. In *Branchiura sowerbyi*, a dose of 20,000 *Thelohanellus hovorkai* myxospores per host produced an estimated 504 actinospores per host, suggesting that only 0.02 actinospores were formed per myxospore (Liyanage et al. 2003; whether all 20,000 myxospores were actually ingested was not measured and seems unlikely). A dose threshold (e.g. for *M. cerebralis* in *T. tubifex*; Elwell et al. 2009a) may contribute to differences in the ratios of myxospores to actinospores. The relationship between myxospore dose and actinospore production would be clarified by determining the difference between dose (number of myxospores in the water or sediments), the number of myxospores ingested (more difficult to quantify), and the number of actinospores that are released per infected host. We note in passing that an exposure dose of 10–100 *M. cerebralis* actinospores can initiate infection in a susceptible fish (Markiw 1992; Hedrick et al. 2003) but millions of myxospores can form in this host (e.g. Halliday 1973).

12.5 Infection Prevalence

Although prevalence of infection can reach 100 % in laboratory exposures (e.g. for *M. cerebralis* in *T. tubifex*; Hallett et al. 2009; Elwell et al. 2009a) it is considerably lower in natural populations (e.g. 2.5 % for *Neoactinomyxum eiseniellae* sp. inquir. infections (Holzer et al. 2004), 1–20 % for *M. cerebralis* infections (DuBey and Caldwell 2004; Krueger et al. 2006; Lodh et al. 2011; Alexander et al. 2011), 4.4 % for Echinactinomyxon sp. infections (Yokoyama et al. 1993), 12 % for total actinosporean infections in marine oligochaetes (Hallett et al. 2001), 1 % for total actinosporean infections in freshwater oligochaetes (Xiao and Desser 1998)). These naturally low infection levels likely reflect reduced encounter rates of myxospores, biotic complexity, and availability of permissive annelids in the aquatic environment.

12.6 Effects of Infection on Annelid Hosts

The effects of myxosporean infections are primarily sub-lethal and include reduced fecundity and tissue damage. Effects on fecundity are commonly reported in both naturally and experimentally infected annelid hosts. Under experimental conditions, *M. cerebralis* infections reduced production of progeny biomass in permissive *T. tubifex* (Elwell et al. 2009a; Alexander 2010). High doses of *M. cerebralis* also reduced progeny biomass in non-permissive *T. tubifex* (Elwell et al. 2009a). One explanation for reduced fecundity is that infection may alter the allocation of host resources away from reproductive investment. For example, in male *M. speciosa*, *P. minibicornis* infections were associated with resorption of gonads (Bartholomew et al. 2006). Likewise, male *N. diversicolor* infected with *E. mugilis* and *Diopatra neapolitana* infected with Unicapsulactinomyxon were characterized by fewer gametes in comparison with uninfected worms (Rangel et al. 2009, 2011). The diversion of host resources away from reproduction, resulting in lower fecundity, is supported by observations of increased growth and respiration in *T. tubifex* over the course of *M. cerebralis* infections (Elwell et al. 2009a; Shirakashi and El-Matbouli 2009). Increased host growth may benefit the parasite by providing increased tissue for proliferation. Malacosporeans appear to exert parallel effects, causing gigantism in zooids of bryozoan colonies that may provide greater space for parasite proliferation (Hartikainen et al. 2013) and causing temporary castration of these colonial hosts (Hartikainen and Okamura 2012).

Host tissue damage is also likely during parasite invasion or release but has not been widely reported. However, Alexander (2010) suggested that weight loss in infected *T. tubifex* in the first 5 days following exposure to *M. cerebralis* could be related to gut epithelial damage incurred during invasion. Actinospore release may also cause extensive host damage, depending upon mode of exit. Those released through the body wall likely cause damage that may expose the host to infection by other organisms (e.g. bacteria and Unicapsulactinomyxon; Rangel et al. 2011). Additional effects of infection on annelid hosts may vary according to host stage or sex but have not been widely investigated.

Issues that would benefit from future study include whether infection causes host castration or influences sex ratios and host reproductive strategies (e.g. allocation to fission versus sexual reproduction). Many of these effects have been documented for malacosporeans infecting freshwater bryozoans and appear to be related to the highly clonal life cycles of bryozoan hosts (see Chap. 11 for review). The effects of infection may also be influenced by co-infections or hyperparasitism, for instance with microsporidians (Morris and Freeman 2010). The latter may easily be overlooked.

12.7 Conclusions

Our review leads us to conclude that knowledge of interactions between annelids and myxosporeans during the actinospore phase is meager. Marine species in particular are highly

understudied while further investigations are required to determine whether we may generalize from insights gained by examining a few systems. We urge future researchers to incorporate degree days to characterize myxospore to actinospore development to enable comparative insights. Further issues in need of investigations include tissue tropism, characterizing host ecological niches, and clearly defining the relationship between myxospore dose and actinospore production. Appreciation of these and other issues will help us to better understand myxosporean-annelid interactions which, in turn, will provide insights on the evolution of myxosporeans.

12.8 Key Questions for Future Research

- Does myxospore morphology affect encounter by annelid hosts?
- Do properties of myxospores (e.g. surface proteins) affect ingestion by annelid hosts?
- Does actinospore morphology correlate with site of development in annelid hosts (tissue tropism)?
- Are there other routes of actinospore release besides pores and egestion?
- How representative is current knowledge of annelid-actinosporean interactions?

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Transmission of Myxozoans to Vertebrate Hosts

13

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Abstract

This chapter reviews how waterborne myxozoan stages achieve transmission to vertebrate hosts, a process that depends on attachment to a host and subsequent invasion. Such transmission has largely been examined for actinospores that are produced by myxosporeans infecting annelid hosts, but we also consider malacosporean stages released from freshwater bryozoan hosts where possible. We review the functional morphology of spores and how this relates to the invasion process and consider cues that initiate the discharge of polar capsule filaments for attachment and sporoplasm activation. We summarize initial invasion steps and discuss factors that may influence transmission ranging from spore viability and infectivity to host cues. We also describe what is known about portals of entry to fish hosts and how non-specific host recognition may enable some myxosporeans to infect a broad range of hosts. Such non-specificity could promote diversification if speciation follows the acquisition of new hosts. We conclude by recommending experimental procedures to be adopted when handling and harvesting actinospores for experimental studies.

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13.1 Introduction

With the growing economic importance of fish diseases caused by myxozoans, research over recent decades has resulted in a greatly improved understanding of the life cycles and transmission of this enigmatic endoparasitic group. Despite reports of direct transmission in very few species (e.g. *Enteromyxum* spp.), all fully resolved myxozoan life cycles involve alternation between invertebrate and vertebrate hosts. Transmission to vertebrate hosts, predominantly fish, is achieved by highly specialized waterborne spores expelled from the invertebrate host. These immotile stages are adapted for dispersal, host recognition and attachment, the latter achieved by means of extrusible filaments from the characteristic polar capsules. The floating spores are relatively short-lived and, being immotile, rely on water currents to carry them to the vicinity of hosts prior to attachment and subsequent release of infectious sporoplasms. The actinospore transmission stages are generally released in large numbers and face obstacles such as predation, erroneous invasion attempts, and mechanical damage. They may also have to withstand physiological or osmotic stress prior to achieving transmission. The tremendous diversity of myxozoan transmission stage morphologies, host ranges, and habitats reflects the evolutionary need for efficient and quick host recognition and invasion mechanisms that have to be functional in a hostile environment by only a few specialized cell types.

Knowledge on how transmission to vertebrate hosts is achieved is crucial for understanding the epidemiology of diseases caused by myxozoans. It is also fundamental for studying myxozoan life cycles, understanding host specificity and elucidating the molecular aspects of myxozoan development. This chapter provides an overview of how myxozoans manage to complete transmission to vertebrate hosts by examining attachment to hosts, how sporoplasms are activated for invasion, and what factors may influence transmission success. In particular, we review morphological and functional features of myxozoan spores, host recognition and specificity, invasion steps and the respective mechanisms. We conclude by reviewing experimental procedures and laboratory handling for investigations that involve actinospores.

13.2 Transmission Stages

13.2.1 Functional Morphology of Actinospores

For most myxozoans, the vertebrate-infecting stage is not yet known. Nevertheless, a number of actinospore 'types' have been described (Lom and Dyková 2006) and these are typically triradiate with either three or six caudal processes that arise from the spore's valve cells. The triactinomyxon type (Figs. 13.1 and 13.2) is the most common and possesses three valve cells (and associated caudal processes) and three polar capsules. Actinospore morphological features are highly variable, particularly the caudal processes, which are inflated osmotically when spores are released into water. These processes produce the characteristic final shape of the actinospore and lend buoyancy so the spore is able to drift passively in the water column.

Variation in the length, shape, diameter, and symmetry of the caudal processes has been proposed to be adaptive for particular habitat conditions (Fig. 13.2), although very different types may be present in the same habitat (Xiao and Desser 1998b). Actinospores in standing waters have caudal processes whose collective large surface areas enhance buoyancy enabling them to remain in the water column for long periods. In contrast, neoactinomyxon-type actinospores

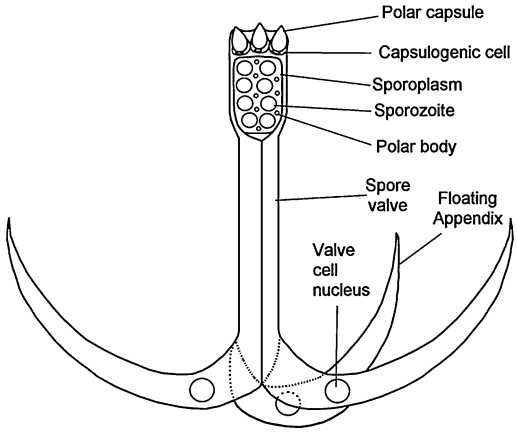

Fig. 13.1 Schematic structure of a triactinomyxon actinospore (Kallert)

have very small caudal processes which may be advantageous for infecting bottom dwelling fish species such as cyprinids. The caudal processes may also play a role in host attachment, although this requires experimental study. For instance, the very long appendices of certain aurantiactinomyxon-type spores may become entangled with gill filaments while very small caudal processes may decrease drag during attachment to fish living in fast flowing waters. Some actinospores form clusters (e.g. synactinomyxon-type spores), with spores attached by the tips of their caudal processes to form cuboidal or spherically-shaped congregations of actinospores, with the apical (polar-capsule bearing) regions orientated outwards. Presumably, this is to increase the likelihood of transmitting a number of parasites infecting an individual fish host. Intraspecific variation in actinospore size and shape has been reported from the same host (Hallett et al. 2004). Whether this variation is functionally significant remains unknown.

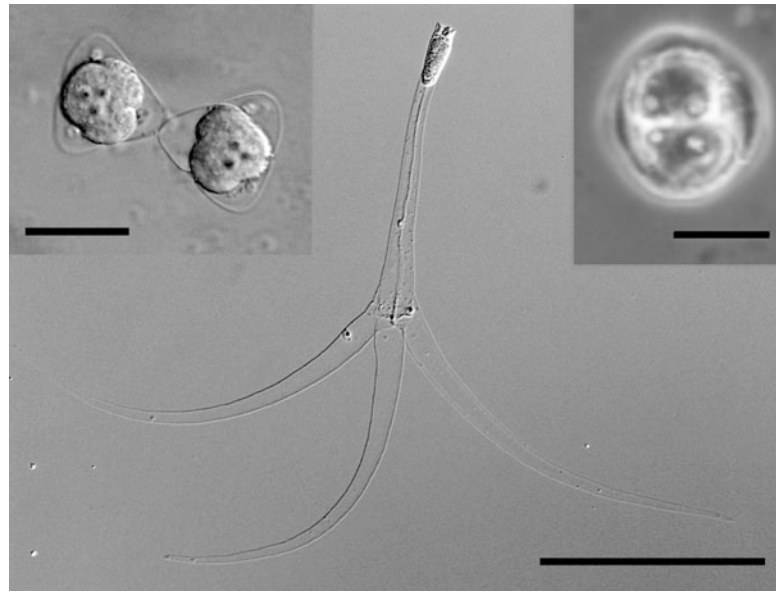

Fig. 13.2 Wet mount of a *Henneguya nuesslini* actinospore (triactinomyxon) (*central image*). See Fig. 12.1a for features. Scale bar = 100 μm *Left insert* Aurantiactinomyxon type spore from *Branchiura sowerbyi*, Scale bar = 20 μm *Right insert* malacosporean spore from *Fredericella sultana*. Scale bar = 10 μm (Kallert, Grabner, Székely)

Another important actinospore feature is the number of secondary sporozooite cells enclosed within the primary sporoplasm cell. The function of sporozooites is to ultimately infect host tissues. Sporozooite numbers vary from two to over 60 (e.g. eight in *Myxobolus pseudodispar* and 64 in *M. cerebralis*). Some species may form less than their maximum number of sporozooites (Lom and Dyková 1992), which may be linked to host nutritional status or species. The polar capsules are located apical to the sporoplasm-harbouring style, often at the end of valve shell protrusions. Their conical plug or stopper often protrudes through the gaps where the surrounding valve cells do not overlap (Fig. 13.3). In the more compact spore types, such as the flower-shaped aurantiactinomyxon, the polar capsules cluster centrally on one side, sometimes beneath a hump-like protrusion of the surrounding valve cells. The number of polar capsules and valve cells is usually the same and is an important species character. The capsulogenic cells are tightly joined to the valve cells through septal complexes or gap/tight junctions (see Chap. 8 for further discussion of cell junctions in Myxozoa) that are maintained throughout sporogenesis.

An often overlooked structural feature of many actinospores is the endospore unit, which consists of a membrane-sheath that encloses the sporoplasm primary cell with the capsulogenic cells attached apically. Its purpose is most likely to maintain the integrity of the sporoplasm during host attachment after the valve cells have been shed. Scanning electron microscopic examination shows that the endospore of *M. cerebralis* is a fibrous structure that remains after the sporoplasm cells have penetrated the fish surface (El-Matbouli et al. 1999b). The endospore structure has also been observed in *M. parviformis* (Kallert et al. 2005a) and *M. pseudodispar* (Kallert et al. 2007).

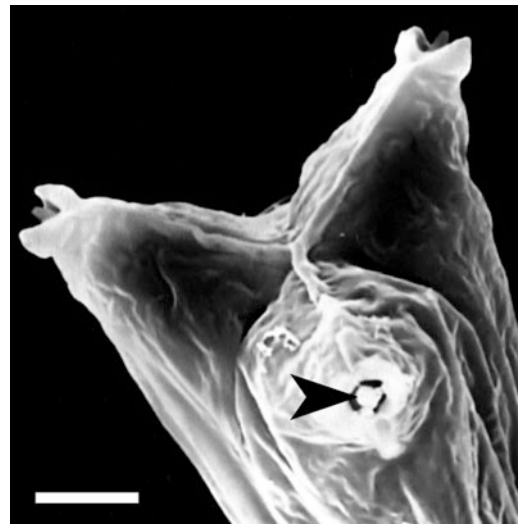

Fig. 13.3 Apical view of *Myxobolus cerebralis* actinospore in scanning electron microscopy. *Note* the protruding capsule tip stoppers (*cones*, *arrowhead*) through lip-like valve shell structures. Scale bar = 2 µm (Kallert)

13.2.2 Malacosporean Transmission Stages

Vertebrate-infecting malacosporean spores (see Fig. 13.2) develop inside spherical sac-like or motile worm-shaped stages, which are located in the body cavity of freshwater bryozoan hosts (Canning and Okamura 2004). The mechanism of spore-release from bryozoans is not yet clear but is likely to be via the vestibular pore from which bryozoan dormant stages (statoblasts) are released (Canning et al. 2002). Compared to most actinospores, the equivalent malacosporean spores released from bryozoans are small (19–20 µm), spherical and lack appendages. They possess four spherical polar capsules, four capsulogenic cells, eight valve cells (McGurk et al. 2005) (although recent confocal microscopy studies suggest there are more; Gruhl and Okamura, unpub. data) and two sporoplasms, each containing a secondary cell (Canning et al. 1996, 2000, 2007; McGurk et al. 2005). Ultrastructurally, specific types of sporoplasmosomes were detected in various developmental stages of malacosporeans, separate distinct from those observed in myxosporeans (Canning et al. 2000, 2002, 2007; Morris and Adams 2006a, 2007). Laboratory studies have indicated similar activation with polar capsule discharge and emission reactions comparable to those of myxosporean actinospores (Grabner and El-Matbouli 2010b; Grabner and Kallert, unpub. data).

13.2.3 Actinospore Release

Vegetative and sporogonic stages of actinosporeans are typically located intercellularly in the intestinal epithelium or in the coelom of annelid hosts (see Chap. 11 for discussion of different tissue tropisms in annelid hosts). Many actinospores are released through the digestive system, thus spore release may depend on feeding activity and may be influenced by temperature (El-Matbouli et al. 1999a). Seasonality in actinospore release varies among myxosporean species. Xiao and Desser (1998a) observed no apparent temporal fluctuations in spore emergence, whereas Yokoyama et al. (1993) reported that several actinospores in a goldfish pond emerged from oligochaetes between spring and summer when susceptible goldfish fry were present. The release of *M. cultus* actinospores from the oligochaete, *Branchiura sowerbyi*, was observed to peak around midnight implying actinospores emerge when fish are inactive at the bottom of ponds (Yokoyama et al. 1993).

13.3 Transmission Stage Longevity and Infectivity

Unlike myxospore stages whose hardened valves confer prolonged resistance and infectivity for months to years, infectivity of actinospores lasts for a period of days (Yokoyama et al. 1993; Xiao and Desser 1998b). For instance, at temperatures around 15 °C, *M. cerebralis* actinospores must invade their host in less than 60 h after release (Markiw 1992). Differences in estimated life-spans of actinospores (often referred to as viability or longevity) are likely to reflect different assay techniques. Ageing experiments have confirmed that periods of actinospore durability and reactivity differ among myxozoan species (Kallert and El-Matbouli 2008) (Fig. 13.4). The lowest 'life-span' was found for *M. cerebralis* actinospores. The viability of *Henneguya nuesslini* and *M. pseudodispar* actinospores was significantly longer upon storage, particularly at lower temperatures. Storage at lower temperatures yielded higher viability in all species. Reports of relatively long survivability of actinospores (up to two weeks) are presumably based on visible cellular breakdown (Yokoyama et al. 1993; Xiao and Desser 2000). The vital staining technique using fluorescein diacetate and propidium iodide (FDA/PI) yielded estimates of viability between 3 days and 4 weeks (Markiw 1992; Yokoyama et al. 1997; Özer and Wootten 2002; Wagner et al. 2003). Most of these studies on actinospore viability show a clear temperature-dependence while none of them address whether polar capsules are functional. Infectivity of actinospores over different time periods determined by infection experiments with susceptible fish hosts (El-Matbouli et al. 1999b) provide better inferences of the true life-span of actinospores in natural environments.

Factors influencing infectivity besides durability include both a viable sporoplasm primary cell and functional polar capsule discharge (see below) for host attachment. Reactivity of actinospores may be influenced by several factors and certain isolates may not be reactive at all. For instance, defective polar capsules have been observed which are smaller than normal and show aberrant morphology (Kallert, unpub. data). It is unknown if spores with such non-functional polar capsules arise from development in unsuitable oligochaete hosts or deleterious mutations. It is possible that physical factors, such as osmotic or pH gradients, may influence spore development. Markiw (1992) observed dead sporoplasm cells but intact polar capsules within the same spores. In some cases actinospores with damaged processes, missing polar capsules or deformed or dull appearing sporoplasms remain fully infective (see Sect. 13.5.1).

Due to their soft valve cells, bryozoan-borne malacospores are very short lived and lose infectivity in less than 24 h (de Kinkelin et al. 2002). Short life-span, small size and fragility of malacosporean actinospores make laboratory infection experiments difficult, as spores cannot be obtained efficiently by filtration of water. So far, the only useful methods for fish infection were either cohabitation of fish with infected bryozoans colonies (e.g. Feist et al. 2001; McGurk et al. 2006; Morris and Adams 2006b,

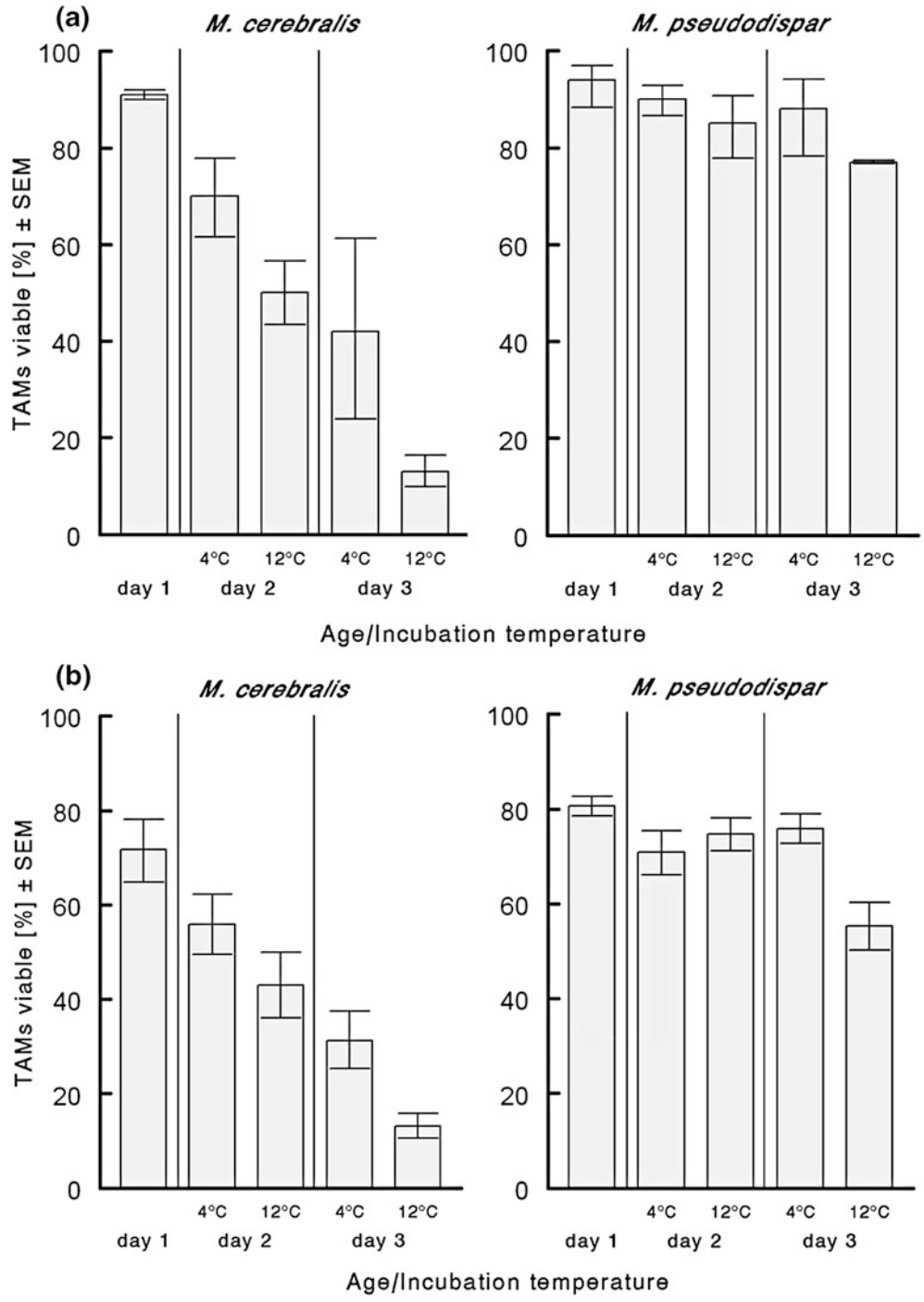

Fig. 13.4 Percent viable actinospores (TAMs) of two myxozoan species determined by **a** FDA/PI-double-staining (fluorescence microscopy) and **b** morphological characters (light microscopy) on the date of harvest (day 1) and after storage at 4 and 12 °C on two subsequent days (days 2 and 3). Total range of number of specimens per day/temperature **a** 30–42 (*Myxobolus cerebralis*), 65–86 (*Myxobolus pseudodispar*). All combinations per species tested in 3 replicates **b** 135–157 (*Myxobolus cerebralis*), 87–137 (*Myxobolus pseudodispar*), all combinations per species tested in 6 replicates. SEM = standard error of the mean (from Kallert and El-Matbouli 2008, reprinted with permission from Folia Parasitologica)

2008; Grabner and El-Matbouli 2008, 2010a), exposure to homogenates of infected bryozoans colonies (Longshaw et al. 2002) or dissection of mature spore sacs to obtain defined numbers of spores (McGurk et al. 2005; Grabner and El-Matbouli 2010b). Fish have also been infected successfully by intraperitoneal injection of disrupted parasite material (Feist et al. 2001).

13.4 Invasion Process

13.4.1 Invasion Course and Speed

Most of our knowledge of the vertebrate invasion process of Myxozoa is based on studies of myxosporeans. Host encounter is likely to be transient so polar capsule discharge must be suitably timed to ensure a high likelihood of successful attachment. The apical region of actinospores must be in close proximity to the host surface (i.e. within the length of the polar filament). The combined sequence of actinospore invasion events is schematically shown in Fig. 13.5. After anchoring the spore via the polar filament to the host surface, the apical valve shell sutures open possibly due to hydrodynamic forces (e.g. drag) acting on the extended caudal processes which are then discarded. The endospore unit remains on the host surface enabling sporoplasm penetration. Unfortunately, virtually nothing is known about the invasion of vertebrate hosts by malacosporean stages owing to difficulties in obtaining naturally released mature spores and their minute size.

The activation response speed and excitability of invasion reactions are known to differ among species although polar capsule discharge itself is uniformly rapid. Kallert et al. (2005b) have shown that a sequential stimulation of chemical and mechanical host cues elicits polar capsule discharge in *M. cerebralis*. Yokoyama et al. (2006) observed slow and fast reactivity of two myxozoan species infecting different fish hosts: *Thelohanellus hovorkai* sporoplasms were released over a 30 min exposure time to host mucous, whilst *M. arcticus* actinospores reacted instantly. Exposure of single isolated *M. parviformis* actinospores to both host (bream) mucous and mechanical stimulation, as conducted with

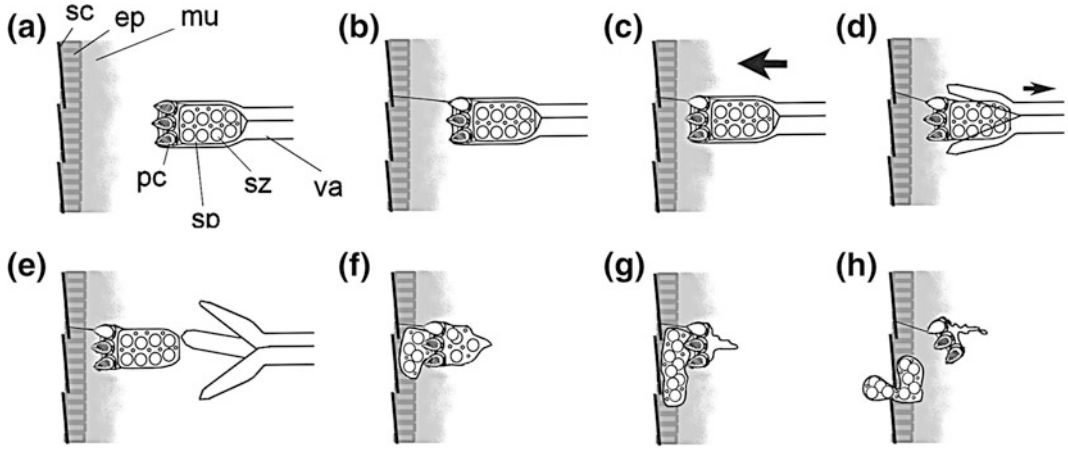

Fig. 13.5 Inferred schematic course of events during host invasion by triactinomyxon-type actinospores. **a** Actinospore apical region encountering fish host surface and chemosensory stimulation. **b** Polar filament discharge after mechanical stimulus upon contact. **c** Rapid adduction (*arrow*) of the actinospore apex towards the host surface by means of filament constriction. **d** Opening of the apical valves along sutures and development of parachute-like form (*arrow*). **e** Endospore remains attached while the valve shell is discarded. **f** Active emergence of the sporoplasm. **g** Fully emerged sporoplasm leaving the endospore sheath on host surface. **h** Penetration of the sporoplasm through the host integument and movement towards deeper layers. *sc* fish scales, *ep* fish epidermis, *mu* mucous layer, *pc* polar capsule, *sp* sporoplasm, *sz* sporozooites, *va* spore valves (modified from Kallert et al. 2007, reprinted with permission from Cambridge University Press)

M. cerebralis (Kallert et al. 2005b), elicited no reactions at all. The notably concealed polar capsule apertures of certain actinospores (e.g. those of *M. parviformis*) contrast with the more exposed polar capsules of other species (e.g. *M. pseudodispar*). The excitability threshold may relate to the relative degree of exposure of polar capsules. For instance, the polar capsules of the aurantiactinomyxon-type spore of *T. hovorkai* are counter-sunk below the apical valve cell margins. This morphological arrangement may result in the slow discharge onset of *T. hovorkai* actinospores in contrast to the fast discharge of actinospores in species such as *M. cerebralis* and *H. nuesslini* whose polar capsules have strikingly protruding tips. Since high flow rates may limit the time for actinospores to attach to their fish hosts (Hallett and Bartholomew 2008) the transmission of slow reacting species may be adversely affected by high water flow. The relative exposure timing for polar capsule discharge could be an adaptation to the speed of host recognition, based on different stimuli required in different habitats and hosts.

13.4.2 Attachment by Polar Capsule Discharge

The feature that most strikingly connects myxozoans to their putative cnidarian ancestors is the intracellular nematocyst-like polar capsule produced within a capsulogenic cell. Its function is to anchor the actinospore to the host integument to enable host invasion. Polar filament discharge is irreversible and the associated polar capsule cell quickly disintegrates. Undischarged polar capsules are filled with an electron-dense mass surrounding the internal coiled filament. This electron dense mass is absent after discharge, which can thus be inferred by electron lucent polar capsules. The conical stopper on the capsule tip often bears ridges on its surface and points through the apical valve openings. Video analysis of polar filament discharge in *M. cerebralis* actinospores has shown that complete extrusion requires less than 10 ms (Kallert et al. 2007). The polar filaments of *M. cerebralis* and *H. nuesslini* are sticky and rapidly elongate and then retract to about half their length in milliseconds after discharge (Kallert et al. 2007). The spore is thus moved by about 35 µm towards the host surface due to the adhesion of the filament. This intrinsic mechanism effects very close contact with the epithelial surface of the fish host.

As a mechanical stimulus, a thigmotropic (touch sensitive) mechanism is assumed. The mechanism of perception is unknown and so is the cellular mechanism that triggers mechanical sensitivity. Presumably, the protein cone covering the capsule tip has a crucial role in triggering the reaction. Uspenskaya (1982) assumed that Ca^{2+} ions were involved in the polar filament discharge mechanism and suggested that it is an active process due to the action of contractile proteins. Discharge rates of actinospores in Ca^{2+}-containing water were doubled compared to those in Ca^{2+}-deficient water of the same osmolality (Kallert et al. 2007). Extreme pH values favour spontaneous discharge (Smith 2001).

13.4.3 Sporoplasm Emergence and Host Penetration

Once the spore has attached to the host, the function of the sporoplasm is to disseminate the infective germ cells (sporozooites) into the host tissue (Fig. 13.6). This emission and penetration process is achieved by active movement via pseudopodia and amoeboid plasticity and is possibly facilitated by proteolytic activity (Adkison and Hedrick 2001). Sporoplasms of *M. cerebralis* can be found in superficial trout tissues only minutes after exposure (El-Matbouli et al. 1999b). Exposure of *Henneguya ictaluri* actinospores to the gills of channel catfish was observed to elicit sporoplasm protrusion and contraction, and pseudopodial amoeboid movement persisted for several hours until the sporoplasms were rounded up and detached from the valve cells of spores (Pote and Waterstrat 1993). Experimental observations have indicated that the apical spore opening does not occur passively

by an intrinsic mechanism prior to sporoplasm emission, since activated and discharged actinospores, after fish mucous stimulation, do not automatically open (Kallert and El-Matbouli 2008). Enhanced movement of the sporoplasms and active rearrangements of the polar capsules have been frequently observed by the authors during incubation of actinospores with fish mucous homogenate in the absence of mechanical stimuli. This suggests that the sporoplasm primary cells can recognize host cues even inside the undischarged actinospore. Thus, although polar filament discharge facilitates sporoplasm emission, it is not vital for sporoplasm activation. Sporoplasms generally appear to be osmotically sensitive and can only be kept viable in suitable Ringer solutions. The primary cell of the sporoplasm usually degrades after host penetration to release the secondary cells that either become blood stages (e.g. some *Sphaerospora* spp.) or invade other tissues that are appropriate for subsequent development.

13.4.4 Portals of Entry and Initial Invasion Steps

Portals of entry of actinospore sporoplasms have been demonstrated by fluorescent labelling (Yokoyama and Urawa 1997) and include gills, skin and the buccal cavity for *M. cerebralis* (Antonio et al. 1999) and gills for *T. hovorkai* and *Sphaerospora truttae* (Yokoyama and Urawa 1997; Holzer et al. 2003). In contrast, *H. ictaluri* infects via the intestine (Belem and Pote 2001). Consumption of infected oligochaetes may also serve as a route of fish infection by myxosporeans, although not by all species as exemplified by the transmission of *M. pseudodispar* (Székely et al. 2001). To date, the route and mechanism of malacosporean entry into fish hosts has only been studied for *Tetracapsuloides bryosalmonae,* although there is evidence that other malacosporeans also infect and develop spores in the kidney tubules of fish (Grabner and El-Matbouli 2010b; Bartošová-Sojková et al. 2014). There is no clear concensus on the route of malacosporean entry. Longshaw et al. (2002) detected stages of *T. bryosalmonae* by in situ hybridization (ISH) in skin epidermal mucous cells of rainbow trout one minute post exposure (p.e.) of fish to homogenized infected bryozoan colonies. *T. bryosalmonae* stages in gills were rarely encountered in their study. In contrast, attachment of *T. bryosalmonae* spores to the gill epithelium of rainbow trout was observed five minutes p.e. via ISH studies (Grabner and El-Matbouli 2010b). Initial penetrated stages

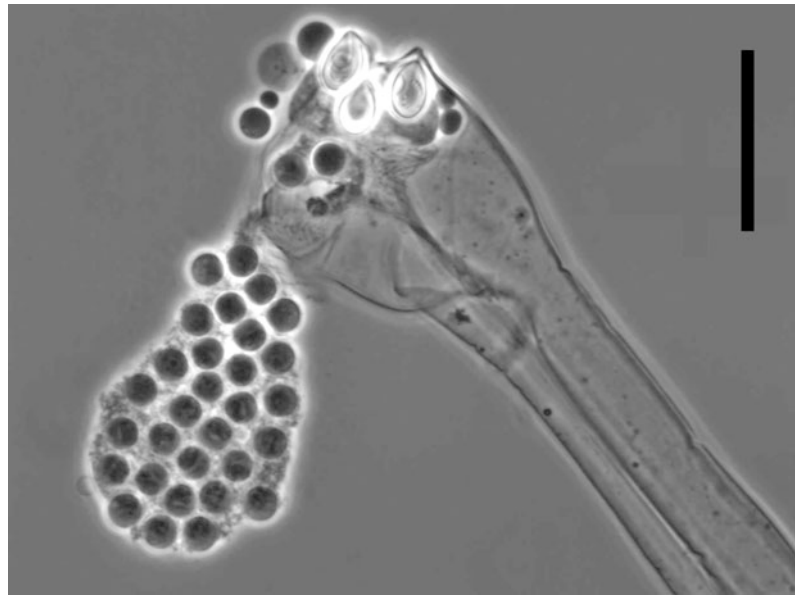

Fig. 13.6 Apically emerging sporoplasm containing secondary sporozooites from *Myxobolus parviformis* actinospore. Scale bar = 20 µm (Kallert)

were subsequently seen in the gills 30 min p.e., and no stages were detected attached to the outer surface or inner layers of the skin epithelium. Earlier ISH studies of both rainbow (Morris et al. 2000) and brown trout (Holzer et al. 2006) similarly concluded that the gills are the major route of entry of *T. bryosalmonae* into fish hosts.

Initial events during host invasion have been studied most intensively in the myxosporean *M. cerebralis*. After sporoplasm penetration, intracellular multiplication within the first 60 min p.e. has been observed prior to intercellular migration in the epidermis and gill epithelium. This is followed by the disintegration of the syncytial primary cell encapsulating the secondary sporozooites, facilitating invasion of the host epidermal or gill epithelial cells (El-Matbouli et al. 1999b). Secondary cell proliferation is promoted by rapid synchronous mitosis (Daniels et al. 1976; El-Matbouli et al. 1995) resulting in production of cell-doublets via endogenous division of secondary cells into an enveloping cell and an inner cell. After entry into the host cell cytoplasm, cell-doublets that survive traverse the host cell plasmalemma to enter the extracellular space. The presporogonic stages migrate deeper into subcutaneous layers concomitantly with continued proliferation of cell-doublets. In other myxozoans, such as *T. bryosalmonae* and *Sphaerospora* spp., presporogonic proliferation is observed in tissues other than those in which sporulation occurs and is known as extrasporogonic development.

Amoeboid cells derived from malacosporean spores enter the fish host's vascular system, where they accumulate and, after migration, undergo further development in target organs (Morris et al. 2000; Longshaw et al. 2002; Grabner and El-Matbouli 2010b). Unicellular extrasporogonic stages multiply in blood and proliferate in kidney interstitial tissue, where presporogonic stages can be found as cell doublets, consisting of a typical primary cell and a secondary cell. So-called S-T doublets migrate to the kidney tubule lumen, where the parasites transform into a pseudoplasmodium each developing into a single spore subsequently (Kent and Hedrick 1985, 1986; Morris and Adams 2008).

Extrasporogonic developmental stages in blood are commonly found among the members of the genus *Sphaerospora*. Blood stages found in common carp (*Cyprinus carpio*) were designated by Molnár (1980) as "Csaba-parasites" (C-protozoa) and by Lom et al. (1983) as "Unidentified Blood Organism" (UBO). These parasites were identified as the developmental stages of *S. dykovae* (syn *S. renicola*) (Csaba et al. 1984; Grupcheva et al. 1985) which was experimentally confirmed by Molnár (1984). Kovács-Gayer et al. (1982) and Körting (1982) described the occurrence of myxosporean developmental stages in the swimbladder of *Sphaerospora*-infected common carp. Such parasites, which seemed to represent intermediates of blood and renal stages were named "K protozoa or K stage" (in reference to the initials of Prof. Körting and Dr. Kovács-Gayer). Baska and Molnár (1988) characterized blood stages from six other cyprinid species, suggesting that other myxosporeans (most likely closely related *Sphaerospora* species) possess extrasporogonic blood stages. Extrasporogonic proliferation of *S. dykovae* begins with a small primary cell that contains a secondary cell. Remarkably, primary cells constantly rotate in the blood stream (Lom and Dyková 1992). The function of this movement has not been clarified, although it has been suggested this may be linked to host immune evasion (Hartigan et al. 2013).

13.4.5 Specific Versus Non-specific Host Recognition

Whether actinospores are able to react specifically to susceptible host species has been a matter of considerable debate. *T. hovorkai* has been reported to react specifically to certain cyprinids (Yokoyama et al. 1997). In a later study *T. hovorkai* actinospores discharged only in response to the mucous of the susceptible host, whereas *M. arcticus* actinospores reacted to both non-susceptible and susceptible hosts (Yokoyama et al. 2006). El-Matbouli et al. (1999b) concluded, based on the number of spores that

attached to host surfaces, that *M. cerebralis* actinospores distinguish carp from rainbow trout. The speed of sporoplasm release has been observed to vary in the presence of mucous from different fish species (Xiao and Desser 2000). Thus, besides differences in reactivity, specific reactions could never been shown conclusively.

Other studies provide further evidence for a lack of host specificity during initial invasion steps. Actinospores of several myxosporeans have been shown to react to mucous of a variety of fish species. Field-collected actinospore isolates of different morphotypes reacted to mucous of salmon, trout, stickleback and bream by polar filament discharge and sporoplasm emission in studies by McGeorge et al. (1997) and Özer and Wootten (2002). Similarly, raabeia-type actinospores of *M. cultus* reacted to mucous from common carp, loach, rainbow trout, catfish, and Japanese eel, as well as to mucin from bovine submaxillary gland (Yokoyama et al. 1995a). Actinospores of *H. nuesslini*, *M. parviformis*, *M. pseudodispar* and *M. cerebralis* readily respond to mucous of roach, rainbow trout, bream and common carp (Kallert et al. 2005b, 2007, 2011; Kallert, unpub. data). More detailed studies have found no significant differences in the numbers of actively emerging *M. cerebralis* sporoplasms on epithelial surfaces of susceptible and resistant strains of rainbow trout and of common carp upon experimental exposure (Kallert et al. 2009). Stages of *M. arcticus* have been observed penetrating non-susceptible salmonids, although no further reproduction and development was reported (Yokoyama et al. 2006). The reduction in myxospore load in rainbow trout infected with *M. cerebralis* actinospores that were preincubated with common carp specimens, suggests that decoy fish species may lower spore abundance. Furthermore, a similar number of actinospores were observed to attach and penetrate gill tissue of the less susceptible Hofer strain rainbow trout (Kallert et al. 2009).

The discovery of non-specific invasion behaviour of several actinospores from limnic European Myxosporea further indicates that host specificity in Myxosporea is presumably not a result of parasite choice (Kallert et al. 2011). In myxosporeans, the positive effect of specific recognition on transmission is presumably outbalanced by the benefits of a rapid response upon contact with any fish to increase the likelihood of a successful host invasion. The lack of specificity in discharge may be compensated for by the release of considerable numbers of transmission stages. Such non-specific host recognition may provide a means of acquiring of new host species, thus promoting diversification if speciation follows infection of novel hosts. Non-specificity of host recognition may therefore have contributed to the great radiation exhibited by the Myxosporea (see Chap. 3 for further discussion).

13.4.6 Host Cues

For successful transmission to fish, accidental polar capsule discharge upon contact with other surfaces must be avoided. In actinospores, this is achieved by perception of chemical discharge triggers prior to activation of mechanical sensitivity. As is evident from the above discussion, numerous studies have shown reactivity of various actinospore samples to fish mucous substrates (Uspenskaya 1995; Yokoyama et al. 1995a, b, 2006; McGeorge et al. 1997; Xiao and Desser 2000; Özer and Wootten 2002; Kallert et al. 2005b, 2007, 2009, Kallert and El-Matbouli 2008). However, the most abundant teleost mucous components, carbohydrates and amino compounds, are unlikely to be involved owing to their ubiquitous distribution in aquatic environments. Yokoyama et al. (1995a) found that a low molecular (<6 kDa) fraction from *Carassius auratus* mucous elicited sporoplasm release from *M. cultus* actinospores. Further analyses of mucous factors led to the discovery of the nucleosides inosine, 2′deoxyinosine, and guanosine as natural triggers of polar filament discharge and sporoplasm activation for *M. cerebralis*, *H. nuesslini,* and *M. pseudodispar* (Kallert et al. 2010, 2011). It is likely that these molecules could be involved in host sensing for other actinospores. The advantage of using nucleosides as discharge triggers may relate to the rather hydrophobic nature of nucleosides along with

their unavoidable release from fish hosts. Inosine is presumed to be an exometabolite intermediate that is excreted into the skin mucous of fish as a result of cellular energy (ATP) consumption and cell death. Thus, for at least some species inosine may indicate close proximity to a potential fish host. There may be other substances present in fish mucous that contribute to triggering discharge and it is possible that associated electrolytes and amino acids help to maintain solubilized inosine levels in mucous. Knowledge of host recognition cues could enable biological control of disease by provoking premature polar filament discharge and sporoplasm release in the absence of fish hosts or by co-stocking with non-susceptible fish.

13.4.7 Genes Activated During Host Invasion

Eszterbauer et al. (2009) have studied genes expressed in *M. cerebralis* actinospores that may be involved in host invasion. The transcriptomes of non-activated, naturally shed actinospores and in vitro activated actinospores of *M. cerebralis* were compared using suppressive subtractive hybridization (SSH). The activation assay was based on host recognition cues (chemical and mechanical stimuli) described by Kallert et al. (2005b) (see Sect. 13.5.2). The relative expression profile of selected transcripts was investigated in tissue of experimentally infected rainbow trout by quantitative real-time PCR. Increased expression was detected for four genes during host penetration. Expression levels that peaked in activated actinospores were significantly lower in non-activated actinospores. The four selected parasite genes were identified as actin-related protein 3 homologue (ARP3), ubiquitin-conjugating enzyme E2 of the UBC complex, a frequenin-like Ca^{2+}-binding protein, and an unknown protein containing pleckstrin-homology and Zn^{2+}-binding FYVE domains. Functional studies are now required to confirm whether these genes play a role in host invasion (Eszterbauer et al. 2009). Genes expressed during the initial stages of host recognition and invasion could in future be evaluated as potential parasite antigens to target for vaccine development to enable control of myxozoan diseases.

13.4.8 Early Parasite Stages in Susceptible and Non-susceptible Hosts

Even though fish mucous is a potent defensive barrier, sporoplasms are able to penetrate susceptible fish host tissue without being affected by host immune factors that are present in fish mucous such as lysozyme, antimicrobial peptides, or complement. Comparative studies have shown that mucous from both susceptible and non-susceptible fish is ineffective in breaking down actinospore sporoplasms of *M. cerebralis*, *H. nuesslini* and *M. pseudodispar* (Kallert et al. 2012). But sporoplasms of the same species exposed to blood sera showed significantly increased cellular breakdown in non-susceptible host serum relative to serum from susceptible host fish. The serum from non-susceptible hosts was considerably less effective over time in this study, with the exception of *M. cerebralis*. In this species, both the primary and secondary cells were affected in much shorter times than in the other two.

To date, little is known on the fate of early invaded stages in non-susceptible hosts before and after the sporoplasm primary cell disintegrates. Observations on the early development of *M. cerebralis* in susceptible and non-susceptible hosts provided insights on factors that may influence transmission. For instance, challenges of non-salmonid fish and the amphibian *Rana pipiens* with *M. cerebralis* have demonstrated specificity towards salmonids at a very early developmental stage (El-Matbouli et al. 1999b), with only few or no early developmental stages detected in non-susceptible fish tissues. It is possible that rapid host immune responses in the outermost cells or tissues are sufficient to prevent infection in non-susceptible hosts or that specific (cellular or antigen) cues are lacking for

development to proceed. Since specific pathogen free (naïve) blood sera were effective in damaging myxozoan transmission stages (sporoplasm and sporozooites) (Kallert et al. 2012), innate immunity factors, such as complement or complement induced factors, may play a central role in survival of early stages determining transmission success. It is also unknown whether the observed host specificity may be linked to the parasite's adaptation for specific immune evasion that only works in susceptible host species.

13.5 Practical Research Aspects

13.5.1 Collecting Functional Actinospores

Actinospores are delicate stages that require care when handling and harvesting for transmission studies. If proper steps are not taken, researchers may experience problems when experimentally infecting fish. Stimulated or blocked polar capsule discharge by inappropriate buffers must be avoided. Small molecules that pass through the valve membranes or interfere with osmotic balance can artificially cause discharge (Kallert, unpub. data). Sporoplasm emission can also be influenced. For instance, sporoplasm emergence from *M. cultus* actinospores was found to be inhibited in PBS solutions with a concentration greater than 5 mM (Yokoyama et al. 1995a). Visual indicators for non-viable or damaged actinospores include ruptured valve cells, a dull grey appearance of the sporoplasm in phase-contrast microscopy, sporoplasm elongation (to about 2–3 times its original size), dispersed sporoplasm germ cells that are very easy to discern, and dislocation of the sporoplasm cells from the apex towards the style.

A problem in harvesting actinospores for laboratory work is that actinospores of different ages accumulate in cultures. Variation in proportions of non-infective actinospores may cause problems in infection trials when, for example, a dose-effect is under investigation. Such isolates cannot be considered a homogenous sample since spores may differ in viability and infectivity. It is therefore important to conduct viability tests and morphological checks to determine optimal harvesting times and storage conditions that optimise longevity. Exposure to mucous or inosine solutions can be used to check reactivity (see below). In our experience, actinospores used in transmission studies should be no older than 48 h. A further consideration is that batches of actinospores may include those that either entirely lack or bear non-functional or dysplastic (malformed) capsules. This should be checked by phase contrast microscopy (30–50 specimens). Polar capsule function and sporoplasm emission should also be checked. The former can be assessed by addition of urea or ammonia solutions, which instantly causes discharge in actinospores. Further discharge and activation tests with mucous homogenates or inosine solutions are also advisable. This is best measured by mechanical stimulation after mixing with mucous or inosine followed by examination with phase contrast microscopy (Kallert et al. 2005b).

Once the actinospores are harvested they should be kept cool at all times. Concise enumeration of viable spores by filtering of infected oligochaete culture supernatants is imperative. Spores should be added to glass infection containers filled with chilled filtrate water prior to the addition of fish and mixed to ensure dispersion of parasites. If possible, bulk infections of fish should be avoided, since in such setups, homogeneous spore solutions and comparable doses per fish are almost impossible to achieve. Incubation times are dependent on fish species and may range from 3 h to overnight with aeration from 1 h post challenge. Fish often show jerking movements during incubation presumably caused by sporoplasm penetration. To avoid overinfection, preliminary dosage tests are advisable, especially when juvenile fish are used. For *M. cerebralis*, actinospore doses from 1,000 to 5,000 spores per rainbow trout between 3 and 6 months of age can be used. Smaller doses are advisable for younger fry. By experimental

infections, McGurk et al. (2006) evaluated the spore dose required to cause infection of rainbow trout by *T. bryosalmonae* and found that a single spore can be enough to cause proliferative kidney disease in rainbow trout.

13.5.2 In Vitro Activation

For future functional and genetic studies, an important prerequisite will be the in vitro activation of actinospores. The activation of sporoplasm primary cells, which remain quiescent in water, is elicited by mucous in small scale reaction volumes. Thus, parasite movement, active emission, and penetration attempts can be observed after approximately 3–5 min. Large numbers of spores can be activated simultaneously. Eszterbauer et al. (2009) developed an in vitro assay for the activation of *M. cerebralis* actinospores in multi-well culture plates by the addition of rainbow trout mucous homogenate and the application of mechanical stimulation by vortexing. The activation efficiency was examined in each well by counting activated and non-activated spores. An actinospore was considered activated if its sporoplasm was either released from the valve or actively moved inside the spore style. Sporoplasm release rates were significantly different between the activated and non-activated samples, the latter receiving neither mucous or vortexing treatments. The high rate of activation (over 90 % of spores reacting to both stimuli) showed that the method can produce high yields of activated actinospores.

Contamination by fish DNA from fish mucous may be problematic when using the above methods. For genetic studies in particular, the use of mucous substrates is not advisable owing to the risk of such contamination. Instead, pure chemical solutions, appropriately buffered to avoid osmotic imbalance of parasite cells, should be used. The low solubility of pure inosine and guanosine in water and their reduced efficiency over time, renders them partially unsuitable for use, but these problems might be resolved by substances such as inosine-arginine salt which incorporates both high activation properties and full water solubility (Kallert et al. 2014).

13.6 Key Questions for Future Studies

- Why do actinospores possess multiple polar capsules?
- Are there further chemical host cues for actinospores and malacospores?
- What is the location and nature of the chemo- and mechanoreceptors in actinospores?
- What is the mechanism generating the dynamic forces for polar capsule discharge?
- Are actinospores with one discharged polar filament still infective?
- How is orientation and tissue detection inside the vertebrate host achieved?
- What is the function of blood stages in many myxozoans?
- What factors are involved in vertebrate host specificity?

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Fish Immune Responses to Myxozoa

14

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Abstract

Myxozoans evoke important economic losses in aquaculture production, but there is almost a total lack of disease control methods as no vaccines or commercial treatments are currently available. Knowledge of the immune responses that lead to myxozoan elimination and subsequent disease resistance is vital for shaping the future development of disease control measures. Different fish immune factors triggered by myxozoan parasites are reviewed in this chapter. Detailed information on the phenotypic and underlying molecular aspects of innate and adaptive responses, at both cellular and humoral levels, is provided for some well-studied fish-myxozoan systems. The importance of the local immune response, mainly at mucosal sites, is also highlighted. Myxozoan tactics to disable or avoid immune responses, such as modulation of immune gene transcription and immune evasion, are also reviewed. The existence of innate and acquired resistance to some myxozoan species suggest promising possibilities for controlling myxozooses through immune-based strategies, such as genetic selection for host resistance, vaccination, immune therapies and administration of immunostimulants.

Keywords

Immune evasion · Host resistance · Innate response · Adaptive response · Transcriptional modulation · Histopathology

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14.1 Introduction

It is generally agreed that the teleost and mammalian immune systems share a similar repertoire of molecules and cells involved in innate and adaptive immune responses. In recent years, our knowledge of the immune system of fish (Zhang et al. 2010a; Fillatreau et al. 2013; Fischer et al. 2013) and immune responses against some parasites (e.g. flagellates (Woo and Ardelli 2014) and microsporidia (Rodríguez-Tovar et al. 2011)) has greatly increased. Nevertheless, information is only available for particular fish species and many questions regarding parasite infections remain. This chapter reviews the immune responses of fish, the secondary hosts in the complex life cycle of myxozoans (see Chaps. 2, 3, and 10), by focussing on innate and adaptive immune processes evoked by myxozoan infection. We also review how myxozoans may deal with immune responses, including modulation of immune gene transcription and immune evasion.

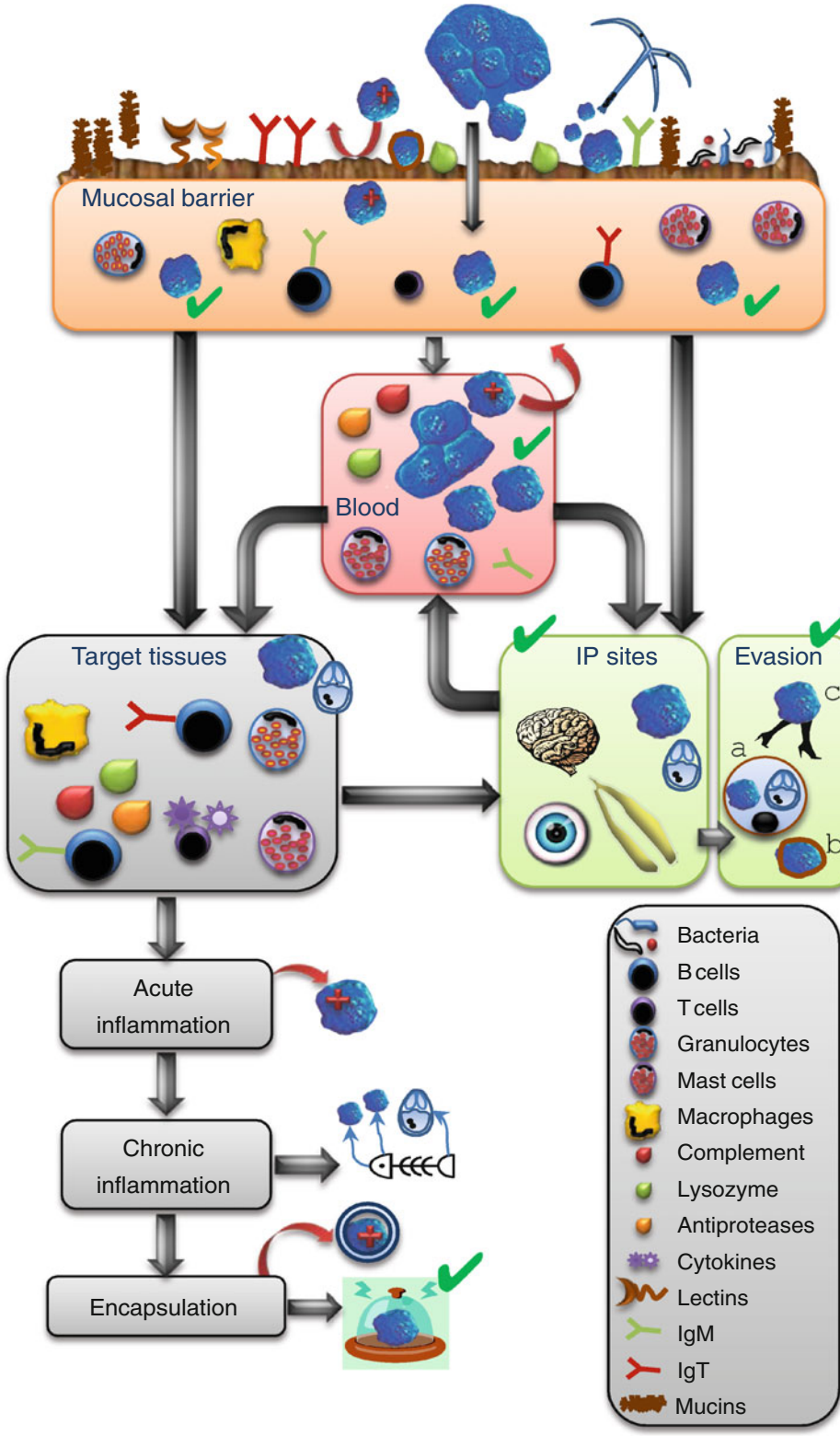
The different immune factors of fish and their interactions with parasite stages are schematically presented in Fig. 14.1. When infectious stages of myxozoans contact fish mucosal surfaces, they may be rejected by immune molecules present in fish mucous. If not eliminated, myxozoan stages will encounter a formidable array of cell-mediated and humoral immune responses that arise in the hosts' mucosal or epithelial tissues. These must, in some way, be subverted by myxozoan parasites in order to reach target tissues. After passing through mucosal or epithelial barriers, parasitic stages reach target tissues, including immunoprivileged sites (see Sect. 14.6.1), via the blood where they display different immune evasion strategies. Some myxozoans are eliminated in the blood owing to the expression of an array of cellular and humoral immune factors. Once in the target host tissues, myxozoan parasites evoke the activation of host immune mechanisms that are regulated by the interplay of both immunoactivating and immunosuppressive cytokine molecules. Acute or chronic disease pathology then develops, depending on the host-myxozoan model. Eventually myxozoan parasites may be encapsulated or finally cleared from the host. Some parasites, however, may survive within granulomatous tissue, leading to chronic disease pathology and even death of the host with the subsequent release of the parasite to the environment.

14.2 Innate Immune Response and Histopathology

The innate or non-specific immune response is the first line of defence of fish against parasites and is based on cellular and humoral factors. Many myxozoan species cause little or no host cellular response, especially coelozoic forms (Lom and Dyková 1992). In some cases, the absence of a cellular reaction is due to development in immunoprivileged sites of the organism (see Sect. 14.6). On the other hand, some myxozoans trigger an excessive immune response leading to an immunopathological condition. Below we review insights about innate immune responses and the associated histopathology based on several well-studied fish-myxozoan systems.

14.2.1 Formation of Granulomata and Melanomacrophage Centres

The most common histopathological reaction to myxozoan infections is the formation of granulomata, which encapsulate parasitic stages by connective and epithelioid tissue layers, with the aim of isolating the parasite and preventing its dispersal to surrounding tissues (Fig. 14.2f). This occurs in *Ceratomyxa shasta*-resistant hosts, in which parasites captured in granulomatous lesions are lysed (Hallett and Bartholomew 2012). The efficacy of encapsulation is often limited. For instance, in *Myxobolus pendula* gill infections, the capsule formed around the complex cysts does not sufficiently inhibit diffusion of oxygen and nutrients to the parasite (Koehler et al. 2004). In *Sphaerospora testicularis*-infected European sea bass (*Dicentrarchus labrax*), large granulomata reduce germinal tissue of the testis in the following spawning season


Mucosal barrier
Blood
Target tissues
IP sites
Evasion
a
b
c
Acute inflammation
Chronic inflammation
Encapsulation
Bacteria
B cells
T cells
Granulocytes
Mast cells
Macrophages
Complement
Lysozyme
Antiproteases
Cytokines
Lectins
IgM
IgT
Mucins

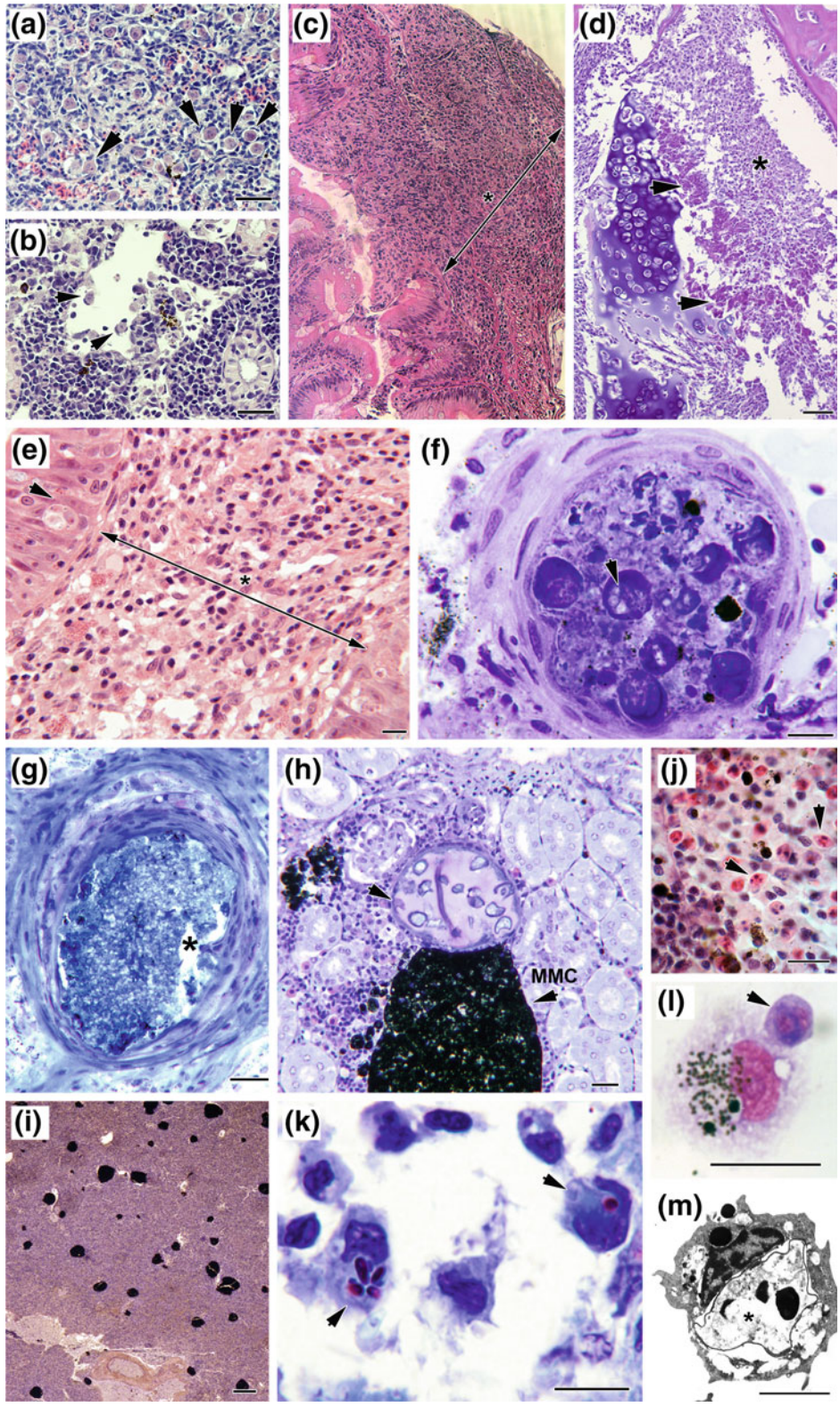
◀ **Fig. 14.1** Diagrammatic representation of parasite strategies and host immune factors activated in myxozoan infections. Infective stages of myxozoans (from actinospores or myxosporean proliferative stages) get in contact with the host through mucosal surfaces at the gills, skin and the gastrointestinal tract. At this first contact, the parasite can be rejected by the many molecules present in the mucous, which is made of a mesh of mucins in which commensal microbiota, lysozyme, lectins, and immunoglobulins can interact. If the parasite survives this first barrier, it encounters different cell types (macrophages, granulocytes including mast cells, B cells, T cells) and immunoglobulins at the subsequent mucosal layer (composed of epithelium and dermis/lamina-propria-submucosa). After passing the mucosal barrier, parasitic stages can proliferate and travel through the blood to the final target tissue or arrive at immunoproviledged sites such as eyes, gonads or brain, or, otherwise, display different immune evasion strategies such as intracellular disguise (*a*), antigen masking (*b*) or fast proliferation (*c*). Some myxozoans can be eliminated during their blood passage thanks to different cellular (granulocytes/mast cells) and humoral factors (lysozyme, complement, antiproteases, immunoglobulins), whereas others survive. Once in the target tissues, the host engages again different cellular factors and Th1- Th2, Th17 and Treg cytokines play an important role in activating or suppressing them and the molecules they produce. This interplay induces acute and chronic inflammation that, depending on the host-myxozoan model, may end up with encapsulation and final clearance of the parasite. However, some parasites may survive within the granuloma or the inflammation can lead to immunopathological consequences and even death of the host, with the subsequent release of the parasite to the environment

(Fig. 14.2g) (Sitjà-Bobadilla and Álvarez-Pellitero 1993).

The principal leucocyte types involved in granuloma formation are macrophages and lymphocytes. Other cell types can also be involved, including neutrophils, eosinophilic granule cells, multinucleated giant cells and fibroblasts. Granulomata are often accompanied by focal development of melanomacrophage centres (MMC) (Fig. 14.2h) (e.g. in lymphohaemopoietic organs of the head kidney and spleen). An increase in the number of MMCs was reported in the spleen of *Enteromyxum leei*-infected gilthead sea bream (*Sparus aurata*) (henceforth referred to as GSB) (Fleurance et al. 2008) and *Enteromyxum scophthalmi*-infected turbot (Sitjà-Bobadilla et al. 2006; Ronza et al. 2013). However, a significant increase in the percentage of splenic surface occupied by MMCs was observed only in GSB exposed to but not infected by *E. leei* (Fig. 14.2i) (Estensoro et al. 2014). These increases are associated with the inflammatory response onset and in some cases with the presence of engulfed parasites in MMCs. It is possible that rapid MMC proliferation in the spleen may help to combat the parasite. MMCs have been suggested to be the primitive analogues of the germinal centres of lymph nodes in mammals (Tort et al. 2003; Vigliano et al. 2006). Their role in the fish immune response involves antigen retention and processing and thus in the maintainience of humoral memory (Agius and Roberts 2003). Macrophages are not only involved with forming MMCs, but also engulf parasites directly (Fig. 14.2j–m).

14.2.2 Other Cellular Responses

In *Tetracapsuloides bryosalmonae*-infected rainbow trout (*Oncorhynchus mykiss*), the proliferation of leucocytes (mainly macrophages and lymphocytes) invokes a more diffuse granulomatous-like reaction (Fig. 14.2a, b), leading to renal tissue hyperplasia (Proliferative Kidney Disease, PKD) (Chilmonczyk et al. 2002; Schmidt-Posthaus et al. 2012). Similarly, in *C. shasta*-infected salmonids, a vigorous host response consisting of lymphocytes and eosinophilic granular leucocytes is mounted (Fig. 14.2c) (Bartholomew et al. 1989). The pronounced inflammatory response of channel catfish (*Ictalurus punctatus*) against *Henneguya ictaluri* in Proliferative Gill Disease contributes to the breakdown of the gill filament cartilage (Lovy et al. 2011). The chronic cartilaginous inflammation induced by *Myxobolus cerebralis* trophozoites in susceptible salmonids is responsible for cartilage degeneration (Fig. 14.2d) (MacConnell and Vicent 2002). A massive hyperplasia of the submucosa occurs in the intestine of *E. leei*-infected sparids due to the proliferation of heterogeneous leucocytes (Fig. 14.2e). Some authors have pointed out that


(a)
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MMC
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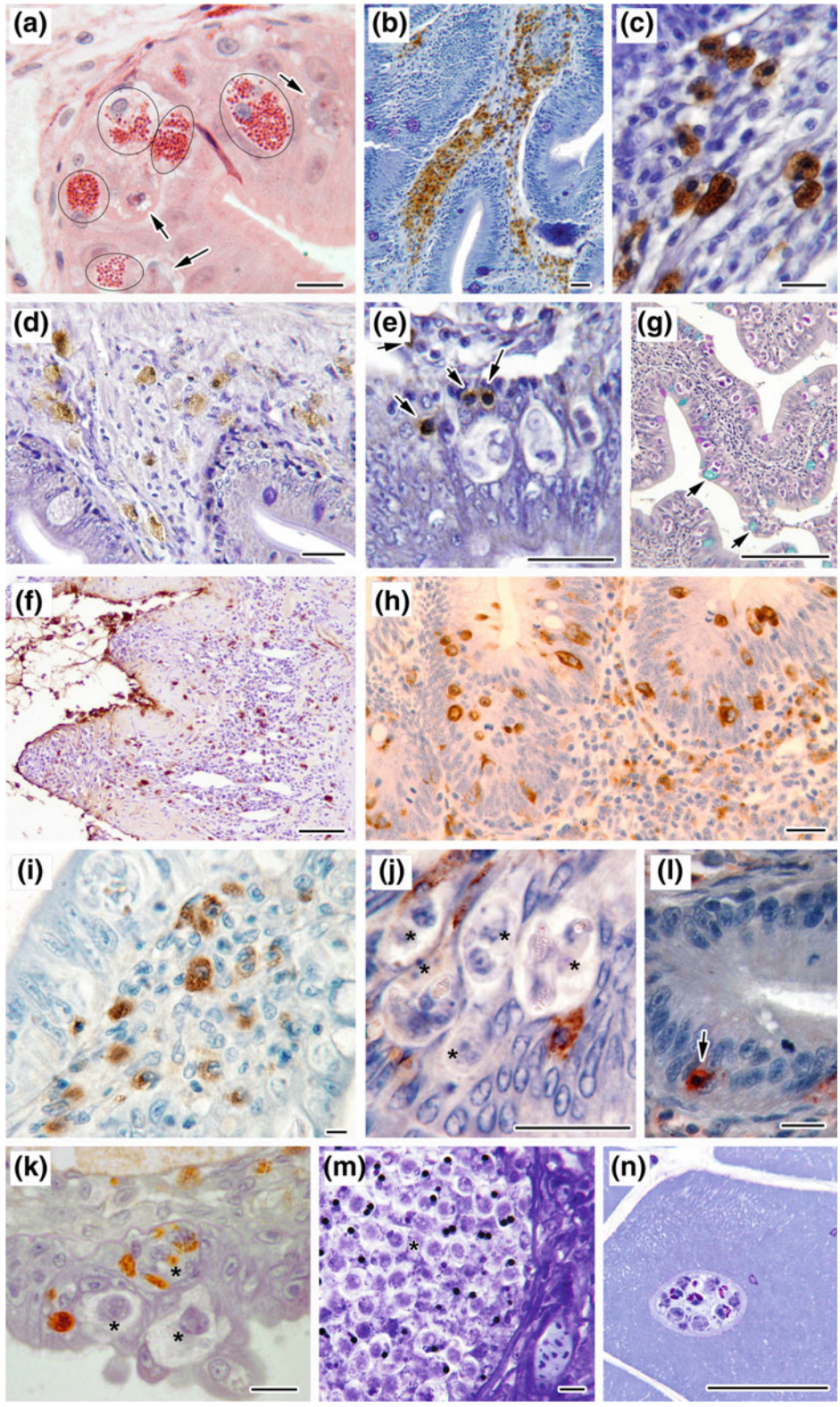
◀ **Fig. 14.2** Photomicrographs showing a variety of immune responses in fish tissues infected by different myxozoan parasites. **a**, **b** Rainbow trout kidney infected by *Tetracapsuloides bryosalmonae*, showing the typical granulomatous reaction; *arrows* in **a** point to parasite stages and *arrows* in **b** points to a thrombus in a vessel. **c** Rainbow trout intestine showing a massive leucocyte proliferation in the submucosa (*arrowed line with* *) due to *Ceratomyxa shasta*. **d** Granulomatous reaction in the rainbow trout cartilage infected by *Myxobolus cerebralis,* showing infiltration with mainly macrophages (*) and partly degenerated myxozoan parasites (*arrows*). **e** Hyperplasic submucosa (*arrowed line with* *) of gilthead sea bream infected by *Enteromyxum leei*; *arrow* points to the parasites located in the epithelium. **f** Fibrotic encapsulation of *Sphaerospora* (formerly *Polysporoplasma*) *sparis* in the glomerulus of gilthead sea bream, spores appear necrotic (*arrow*). **g** Fibrotic encapsulation of *Sphaerospora testicularis* in the tesis of European sea bass; the parasite is completely destroyed (*). **h** Accumulation of melanomacrophage centres (*MMC*) around a *S. sparis*-infected glomerulus in the trunk kidney of gilthead sea bream. Spores are still visible in the glomerulus (*arrow*). **i** Spleen of gilthead sea bream exposed to *E. leei* showing a high number of MMC (*dark spots*). **j** MMC interspersed in the kidney of a *Myxidum giardi*-infected eel (*arrows*: myxospores). **k** Macrophages engulfing *Kudoa thrysites* spores (*arrows*) free in the muscle of Atlantic salmon. **l** Engulfment of a *Ceratomyxa sparusaurati* trophozoite *(arrow*) by a gilthead sea bream head kidney macrophage after 24 h of in vitro incubation. **m** Transmission electron micrograph of a European sea bass macrophage with an engulfed and partially destroyed spore (*) of *Sphaerospora dicentrarchi*. Stainings: Hematoxylin and eosin (**a**–**e**, **i**, **j**), toluidine blue (**f**–**h**, **k**), May-Grunwäld Giemsa (**i**). Scale bars: 100 μm (**i**), 50 μm (**d**) 25 μm (**a**, **b**), 20 μm (**j**, **h**), 10 μm (**f**, **g**, **k**, **l**), 0.35 μm (**m**). Images courtesy of: Dr. J. Bartholomew (Oregon State University, USA) (**c**), Dr. K. Molnár (Hungarian Academy of Sciences) (**j**). Images original from the authors: A S-B (**e**–**i**, **k**–**m**), H S-P (**a**, **b**, **d**)

such severe inflammatory reactions are found in unusual hosts for such myxozoans (Kent and Hedrick 1985). Furthermore, reactions against a myxozoan, even within a given host, can vary considerably depending on the infected tissue. For example, *Parvicapsula minibicornis* induces inflammatory cells to infiltrate the gills of sockeye salmon (*Oncorhynchus nerka*), whereas no cellular host response is noticed in the mesonephros around or within infected glomeruli (Bradford et al. 2010).

The types of leucocytes involved in local responses against myxozoans vary depending on the myxozoan-fish model and site of infection. For example, when *T. bryosalmonae* first enters the fish gills a granulomatous reaction, dominated by infiltration with macrophages, is induced in the filaments (Schmidt-Posthaus et al. 2013). Once in the kidney, macrophages surround the parasites in the vessels and proliferating lymphocytes account for the lymphoid hyperplasia and gross kidney swelling response that is characteristic of PKD. Finally, granulomatous-like reactions are established in the renal interstitium during the advanced stages of this disease (Hedrick et al. 1993; Bettge et al. 2009).

In general, phagocytes and granulocytes are the most abundant immune cells in mucosal infections (skin, gills and intestine). Neutrophils and other granulocytes (mast cells and eosinophil-like cells) dominate in the gill inflammatory infiltrate found in Proliferative Gill Disease (Lovy et al. 2011). Polymorphonuclear leucocytes, fibroblasts and macrophages are abundant in *C. shasta*-infected intestines (Bartholomew et al. 1989). A succession of granulocytes and lymphocytes is described in the oedemata surrounding *M. pendula* branchial cysts in creek chub (*Semotilus atromaculatus*) (Martyn et al. 2002). Some granulocyte subsets increase during enteromyxosis in sharpsnout sea bream (*Diplodus puntazzo*) (Fig. 14.3a) (Álvarez-Pellitero et al. 2008), whereas acidophils decrease at both local and systemic levels in GSB (Estensoro et al. 2014). Acidophilic granulocytes act as professional phagocytes in the latter. Considered the functional equivalents of mammalian neutrophils, acidophilic granulocytes can be rapidly recruited from head kidney and are the most abundant circulating granulocytes (Sepulcre et al. 2002; Chaves-Pozo et al. 2005; Estensoro et al. 2014). Mast cells are massively recruited to the intestine in *E. leei* infections (Fig. 14.3d, e) (Katharios et al. 2011; Estensoro et al. 2014), and are often associated with chronic inflammation. When activated, mast cells degranulate releasing hormonal mediators and histamine analogues in the extracellular environment, where they act as pro-inflammatory moderators of the inflammation reaction. Importantly, however, only fish belonging to the order Perciformes show typical mast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◀ **Fig. 14.3** Photomicrographs of fish tissues infected by different myxozoan parasites. **a** Eosinophilic granular cells (*encircled*) in the intestinal epithelium of sharpsnout sea bream infected by *Enteromyxum leei*. *Arrows*: parasitic stages. **b** Intestine of *E. leei*-infected gilthead sea bream showing acidophilic granulocytes immunostained (*brown cells*) in the submucosa with the G7 antibody. **c** Detail of the G7-immunostained cells. **d** Intestine of *E. leei*-infected gilthead sea bream showing mast cells immunostained (*brown cells*) in the submucosae with an anti-histamine antibody. **e** Detail of mast cells (*arrows*) at the base of the epithelium. **f** Intestine of *E. scophthalmi*-infected turbot with abundant iNOs$^+$ cells in the submucosae (*brown cells*). **g** Intestine of *E. leei*-infected gilthead sea bream showing scarce goblets cells (in *blue*, *arrows*); parasite stages appear fuchsia-coloured. **h** Intestine of *Ceratomyxa shasta*-infected rainbow trout showing Ig$^+$ cells in the epithelium and the submucosae (*brown cells*). **i** Intestine of *E. leei*-infected gilthead sea bream showing IgM$^+$ cells (*brown cells*) in the submucosae and epithelium. **j** Detail of IgM+ cells in the epithelium, surrounding parasite stages (*). **k** Intestine of *E. scophthlami*-infected turbot showing IgM$^+$ cells (*brown cells*) in the epithelium, surrounding parasite stages (*). **l** IgT$^+$ intraepithelial lymphocyte (*arrow*) in the intestine of a naïve rainbow trout. **m** *Sphaerospora testicularis* myxospores (*) in the lumen of a seminiferous tubule of European sea bass. **n** Intracellular *Kudoa thyrsites* spores in the muscle of Atlantic salmon. Stainings (when not immunostained): Hematoxylin and eosin (**a**), alcian-blue PAS (**g**), toluidine blue (**m**, **n**). Scale bars: 100 μm (**g**), 50 μm (**f**, **n**), 20 μm (**b**, **d**, **e**, **h**, **j**), 10 μm (**a**, **c**, **k**, **l**, **m**), 5 μm (**i**). Illustrations courtesy of: Dr. J. Bartholomew (Oregon State University, USA) (**h**), Dr. M.I. Quiroga (University of Santiago de Compostela, Spain) (**f**, **k**), Dr. C. Tafalla (INIA, Spain) (**l**). Illustrations original from the authors: A S-B (**a**–**e**, **g**, **i**, **j**, **m**, **n**). n was photographed from material provided by Dr. W. Marshall (Centre for Aquatic Health, Canada)

cells containing histamine (Mulero et al. 2007). Large numbers of degranulated cells are also observed within the gill lesions produced by *H. ictaluri* (Lovy et al. 2011). Immune cell degranulation acts selectively against the parasite, but, when excessive, can have deleterious immunopathological consequences for the host, even causing death.

Phagocytes play important roles in the host's fight against parasites but their activity can be modulated by myxozoan infections. Some enteromyxoses induce an increase in the respiratory burst of circulating phagocytes (Sitjà-Bobadilla et al. 2006, 2008), but a decrease in the activity of head kidney phagocytes (Cuesta et al. 2006b). Furthermore, in vitro studies demonstrated that some myxozoans induce the production of reactive oxygen species (ROS) (Muñoz et al. 2000a). Reactive nitrogen species are also produced by activated phagocytes and are used as cytotoxic effector molecules against fish pathogens. The production of reactive nitrogen species is controlled by the inducible nitric oxide synthase (iNOS) enzyme. The number of putative iNOS immunoreactive cells in the intestine and lymphohaematopoietic organs of *E. scophthalmi*-infected turbot was significantly higher and increased as infection progressed (Fig. 14.3e). The high presence of iNOS$^+$ cells in the gut lamina propria in severe enteromyxosis might contribute to epithelial injury and desquamation (Losada et al. 2012). In *E. leei*-infected sharpsnout sea bream, serum nitric oxide values gradually increased after exposure to the parasite (Golomazou et al. 2006), whereas in GSB they were reduced or unchanged in fish fed a fish oil or vegetable diet, respectively (Estensoro et al. 2011). Species more susceptible to enteromyxosis may exhibit increased levels of nitric oxide (NO) and iNOS$^+$ cells.

Another leucocyte type involved in innate immunity in mammals is the natural killer (NK) cell. Two types of NK cell homologues have been described in fish: nonspecific cytotoxic cells (NCC) and NK-like cells. Cell-mediated cytotoxicity is known to increase in *E. leei*-exposed GSB (Cuesta et al. 2006b).

Rodlet cells (RC) generally increase in infected tissues or are observed in close vicinity to myxozoans, sometimes even discharging their rods (Leino 1996; Palenzuela et al. 1999; Muñoz et al. 2000b; Gorgoglione and Sommerville 2010). RCs are, however, very scarce in sharpsnout sea bream, even when infected by *E. leei* (Álvarez-Pellitero et al. 2008). RCs are thought to be connected with inflammatory cells and host defence against parasites (Reite 2005), but their function is still controversial and their specific role in myxozoan infections requires clarification.

14.2.3 Humoral Responses

Some humoral innate factors, such as peroxidases, lysozyme or complement, are variably involved in myxozoan infections. Upon *E. leei* exposure, serum peroxidase increases in sharpsnout sea bream (Muñoz et al. 2007), whereas serum and leucocyte peroxidases are depleted in GSB (Cuesta et al. 2006a, b; Sitjà-Bobadilla et al. 2008). Lysozyme activity is significantly depleted in GSB naturally infected with *Sphaerospora* (formerly *Polysporoplasma) sparis* (Karagouni et al. 2005) and in *E. leei*-exposed GSB (Sitjà-Bobadilla et al. 2008). In contrast, in other myxozoan infections or after immunisation, lysozyme levels are increased (Muñoz et al. 2000b; Sitjà-Bobadilla et al. 2006; Foott et al. 2004).

Fish serum complement is also variably involved in myxozoan infections. Plasma complement activity of infected salmon is not altered until advanced ceratomyxosis (Foott et al. 2004). In *Enteromyxum* spp. infections, the activity of the complement alternative pathway is initially increased (or unaltered) in response to parasite exposure, and generally decreases at later stages of infection (Cuesta et al. 2006a; Sitjà-Bobadilla et al. 2006). Some parasite-specific glycans or surface-associated carbohydrate moieties may activate the complement system through the lectin pathway (Kaltner et al. 2007; Redondo et al. 2008; Redondo and Álvarez-Pellitero 2009; Estensoro et al. 2013a). The lowered serum in vitro activity following heat inactivation and withdrawal of bivalent metal ions (Kallert et al. 2012) suggests that complement or complement induced factors are responsible for cellular disintegration of some myxozoans (*M. cerebralis*, *Henneguya nuesslini* and *Myxobolus pseudodispar*). The exhaustion of complement can have immunopathological consequences, due to the accumulation of immune complexes that are not lysed by complement. For instance, the immune deposits found at the basement membrane of the glomeruli of *Myxobolus*-infected barbels (*Barbus graellsii*) seem to be the cause of membranous glomerulonephritis (Peribáñez et al. 1993).

Several myxozoan parasites are known to produce proteases. For example, the early stages of *H. ictaluri* release proteases that degrade collagen and support trophozoite growth within the gill filament (Lovy et al. 2011). Serine proteases of *M. cerebralis* digest and cause lysis of rainbow trout cartilage (Kelley et al. 2004), and *Kudoa thyrsites* proteases are reponsible for histolysis characteristic of the invoked disease (Langdon 1991; Funk et al. 2008). Host antiproteases counteract the action of parasitic proteases. Examples include the increase in serum α-2 Macroglobulin (α-2 M), (one of the most versatile anti-proteases) in *E. scophthalmi*-parasitized turbot (Sitjà-Bobadilla et al. 2006) and of total serum antiproteases in *E. leei*-parasitized sharpsnout sea bream (Muñoz et al. 2007).

The mucosal surface is relevant for the outcome of the disease, not only because it is a complex organisation of epithelium, immune cells and resident microbiota (Rombout et al. 2011; Gómez et al. 2013), but also because the mucous layer may favour or impede pathogen entrance. Mucins are the main structural component of the mucous and are secreted by goblet cells, forming a mesh-like structure. Turbot (Bermúdez et al. 2009) and GSB (Fleurance et al. 2008; Estensoro et al. 2012a) with advanced enteromyxoses had decreased numbers of goblet cells. In heavily parasitized intestinal areas goblet cells were even absent in GSB. The mucous of infected GSB had higher glycosylation levels and terminal glycosylation of mucous proteins, depending on the intestinal segment affected (Estensoro et al. 2013b).

14.3 Adaptive Immune Response

The very first attempts to detect specific antibodies against myxozoans failed (Pauley 1974; Halliday 1976; Siau 1980; Bartholomew et al. 1989). This led to the suggestion that fish were unable to mount an adaptive or specific immune response against myxozoans. The presence of specific antibodies has, however, been unambiguously reported in fish infected by *M. cerebralis* (Hedrick et al. 1998), *Myxobolus artus* (Furuta et al. 1993), *T. bryosalmonae* (Saulnier and de Kinkelin 1996), *C. shasta* (Bartholomew 2001), *E. scophthalmi* (Sitjà-Bobadilla et al.

2004) and *E. leei* (Estensoro et al. 2010a). The speed of antibody production is relatively low in comparison with available data for fish immunized or vaccinated with other parasites. Specific antibodies were not present until 12 weeks after exposure of rainbow trout to *M. cerebralis* (Ryce 2003), whereas anti-*T. bryosalmonae* antibodies were detected as early as 6 weeks post exposure (p.e.) (Hedrick et al. 1991). Specific antibodies were detected as soon as 48 days p.e. in turbot exposed to *E. scophthalmi* if fish belonged to previously exposed stocks. If fish belonged to naïve stocks, they developed the disease and died without producing antibodies at 40–49 days p.e. (Sitjà-Bobadilla et al. 2006, 2007b). Further information on gene expression of immunoglobulins (Ig) is provided in Sect. 14.4.

Myxozoan infections evoke changes in numbers and distributions of B cells and plasma cells. For instance, there is a significant decrease in the number of B cells positive for IgM (the main serum immunoglobulin in fish) (IgM^+ B cells) in the head kidney and spleen of turbot (Bermúdez et al. 2006), but an increase in number in the head kidney of GSB in well-established infections (Estensoro et al. 2014). Both fish species demonstrate a very high recruitment of IgM^+ B cells and plasma cells at the intestine (Fig. 14.3i–k) (Bermúdez et al. 2006; Estensoro et al. 2014), particularly in the lamina propia-submucosa and epithelium, where they can be observed surrounding parasite stages. These observations challenge the paradigm that teleost intraepithelial lymphocytes (IEL) are exclusively T cells, and confirm recent observations in rainbow trout (Ballesteros et al. 2013) (Fig. 14.3l).

The speed of the recruitment of IgM^+ B cells depends on the route of infection in GSB, being higher in fish infected by anal intubation than by exposure to parasite-contaminated effluent (Estensoro et al. 2012b, 2014). Interestingly, in *C. shasta*-infected rainbow trout a large accumulation of IgT^+ cells (but not IgM^+ B cells) in the lamina propria was coincident with an increase in the total amount of IgT (specialised mucosal immunoglobulin) and specific IgT antibodies in the intestinal mucous, whereas IgM could not be found at this site (Zhang et al. 2010b). These findings show once more the importance of mucosal immunity and in particular the role of a specialized IgT in fish (Salinas et al. 2011; Gómez et al. 2013).

14.4 Changes in Immune Gene Expression in Response to Myxozoan Infection

An important prerequisite for understanding the nature of parasite-mediated modulation of immune processes is to know the immune mechanisms and molecules that result in chronic disease or resistance to infection. The majority of immune genes examined in studies on myxozoans encode innate immune molecules. Unlike bacterial infections, myxozoan-mediated changes in gene expression have often been reported to be weak, transient or down-regulated in infected fish. Data from fish genomes and transcriptomes have tremendously increased in our understanding of fish immune genes, providing new opportunities to explore, in detail, the impact of myxozoans on fish adaptive immunity (Laing and Hansen 2011, Secombes and Wang 2012). However, it is important to keep in mind that changes in gene transcription may not necessarily reflect changes in functional proteins. Moreover, the multiple isoforms of immune molecules in some fish genomes may differ functionally (Husain et al. 2012; Hong et al. 2013; Wang et al. 2014). Nevertheless, transcriptional profiling during myxozoan infection (RNA-seq) is a powerful tool providing insights into the immune mechanisms shaping chronic disease or protective immunity. Knowledge gained by such studies may identify means of rebalancing immune processes to affect parasite clearance (Álvarez-Pellitero 2008; Sitjà-Bobadilla 2008; Gorgoglione et al. 2013). Below we review evidence for transcriptional modulation of immune genes in several well-studied myxozoan infections.

14.4.1 Transcriptional Modulation During *T. bryosalmonae* Infections

Expression of tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and cyclooxygenase (COX)-2 isoforms in rainbow trout is refractory towards *T. bryosalmonae* infection, potentially reflecting the unresponsiveness of immune cells expressing these molecules due to a skewing of pro-inflammatory mechanisms towards an anti-inflammatory phenotype (Gorgoglione et al. 2013). Evidence for this is provided by the over-expression of putative anti-inflammatory molecules, including IL-6, IL-10a/b, IL-11, nIL-1F, (the IL-1β antagonist), and suppressors of cytokine signalling (SOCS) 1/3 (Costa et al. 2011; Wang et al. 2011; Gorgoglione et al. 2013). Importantly, the severe PKD pathology in rainbow trout (in Europe), provoked by extrasporogonic stages, does not occur to the same extent in brown trout (*Salmo trutta*) (Morris and Adams 2008). Fish IL-10 has been shown to suppress pro-inflammatory cytokines, phagocytosis and respiratory burst activity, all of which are refractory or suppressed by PKD (Chilmonczyk et al. 2002; Grayfer et al. 2011). The antimicrobial proteins, cathelicidin isoforms 1 and 2 and hepcidin are highly up-regulated during PKD. The former has been ascribed cytokine modulating activities in fish and so may contribute to the apparent anti-inflammatory phenotype in PKD (Bridle et al. 2011; Gorgoglione et al. 2013). A dominant anti-inflammatory phenotype could be attributed to the proliferation of lymphocytes characteristic of PKD. Although fish macrophage marker genes are refractory to or suppressed by PKD, trout macrophages remain abundant in granulomatous lesions (Hedrick et al. 1993; Gorgoglione et al. 2013). Furthermore, the up-regulation of molecules indicative of macrophage activity (e.g. IL-6, IL-12, and IL-34) suggests macrophages to be important in PKD pathogenesis (Gorgoglione et al. 2013; Wang et al. 2013, 2014).

PKD studies suggest a link between disease pathology and dysregulated over-expression of T helper (T_H) cell-like genes (Gorgoglione et al. 2013). For instance, aberrant T cell activity has been linked to pathogen-mediated chronic pathology and over-expression of antibodies in mammals (Taylor et al. 2012). Unregulated T_{H17} and T_{reg} responses are linked to granulomatous lesion formation and reduced vaccine responsiveness both of which are observed during PKD, thus, implicating T_{H17} and T_{reg}-like activities in PKD pathogenesis (Chilmonczyk et al. 2002; Wen et al. 2011; Taylor et al. 2012). T_{H1}-like activities in PKD are exemplified by up-regulation of the master T_{H1} transcription factor, T-bet, and T_{H1} cytokines (Wang et al. 2010). T_{H2} and T_{reg}-like activities in PKD were evidenced by up-regulation of the master transcription factors, GATA3 and FOXP3, and their associated cytokines, respectively (Gorgoglione et al. 2013). Rainbow trout homologues of mammalian T_{H17} cytokines were up-regulated during PKD despite the refractoriness of the master T_{H17} transcription factor, ROR-γ (Gorgoglione et al. 2013). Functional studies in rainbow trout have demonstrated pro-inflammatory activities of IL-17A/F2a and confirmed IL-21 as a key regulator of B and T cell-like activities upregulating IL-10, IL-22, interferon (IFN)-γ, CD4, T-bet and GATA3, whilst maintaining CD8 and IgM transcripts in head kidney leucocytes (Wang et al. 2011; Monte et al. 2013).

14.4.2 Transcriptional Modulation During *E. leei* Infections

Pro-inflammatory cytokines were down-regulated and refractory in infected and non-infected *E. leei*-challenged GSB, respectively (Sitjà-Bobadilla et al. 2008). Expression of α-2 M was up-regulated in the intestine of *E. leei*-infected GSB (Sitjà-Bobadilla et al. 2008). α-2 M silences pathogen proteases and inhibits pro-inflammatory cytokines in higher vertebrates (James 1990; Armstrong and Quigley 1999). Up-regulation of this molecule could account for the concurrent down-regulation of pro-inflammatory cytokines during enteromyxosis. cDNA microarray expression analysis of *E. leei*-challenged resistant and susceptible GSB signified a global down-regulation of innate immune and acute phase

response genes in infected GSB relative to non-infected fish (Davey et al. 2011). Interplay between host and parasite proteases, protease inhibitors, apoptotic mechanisms, cell proliferation, and antioxidant defense genes was evident in both *E. leei* challenged groups. Down-regulation of innate immune or acute phase genes was associated with the complement system and normal homeostatic mechanisms in infected and non-infected fish, respectively. Mannose binding lectin (MBL)2, a known pathogen resistance marker in other fish species and an activator of the lectin-mediated complement pathway (Holmskov et al. 2003), was down-regulated in *E. leei* infected GSB (Davey et al. 2011). Conversely, MBL2 expression along with other genes, such as LPS-binding protein and MHC class II, could signify a global antiparasitic response.

Endocrine factors have important immune modulatory activities in fish (Yada 2007). Growth hormone receptor (GHR)-1 and insulin-like growth factor (IGF)-1 were down-regulated in the kidney of *E. leei*-infected fish and up-regulated in non-infected fish (Sitjà-Bobadilla et al. 2008). Other immune-related genes examined in the intestine of *E. leei*-infected GSB include mortalin (a heat-shock protein) and glutathione peroxidase (an anti-oxidant enzyme), the former being up-regulated and the latter down-regulated (Sitjà-Bobadilla et al. 2008). Modulation of endocrine and other immune-related genes was also observed in a recent transcriptomic study in order to assess changes associated with a vegetable oil replacement (66VO) diet compared to a fish oil diet in *E. leei* infected GSB (Calduch-Giner et al. 2012). Disease outcome was more pronounced in fish fed 66VO (see other effects in Sect. 14.5), consistent with the number and magnitude of differentially expressed genes. Four expression clusters reflected disease progression. Clusters 1 and 2 contained strongly and moderately up-regulated genes, respectively, with the first including a marker of alternative macrophage activation (arginase-1) and the second, genes encoding Ig chains, IL-6, and IFN-related genes. Strongly and moderately down-regulated genes were included in cluster 3 (with complement components and lectins among other immune genes) and 4 (grow factors, e.g. GHR1 and IGF-binding protein 4), respectively. The pro-inflammatory molecule, metallothionein was strongly down-regulated by infection, especially in 66VO fish, suggesting a potential role in parasite resistance.

Recent studies have linked mucin expression to *E. leei* susceptibility in GSB, with the molecule I-Muc being down-regulated in parasitized fish (Pérez-Sánchez et al. 2013). Such changes in mucins could be responsible for changes in bacterial adherence to the host intestinal epithelium (Estensoro et al. 2013b). Mucins are directly antiparasitic and involved in immune regulation during parasite infection with transcriptional up- and down-regulation being linked to parasite expulsion and chronic infection, respectively (Hasnain et al. 2013).

14.4.3 Transcriptional Modulation During *M. cerebralis* Infections

M. cerebralis infection of rainbow trout is apparently characterized by a transient up-regulation of pro-inflammatory genes, including IL-1β 1/2 and COX-2 (Severin and El-Matbouli 2007). Thus, in whirling disease, pro-inflammatory mechanisms may remain intact at the early stages of infection. In support of this, the anti-inflammatory molecule, transforming growth factor (TGF)-β, was initially down-regulated in susceptible fish and up-regulated at later stages. Intriguingly, a cDNA microarray study revealed the prominence of the interferon system in both TL and GR infected fish (Baerwald et al. 2008). The putative antiviral molecules ubiquitin-like protein 1, interferon regulatory factor (IRF)1, IRF7, and GCHV-induced gene 2 (Gig2) were up-regulated in both resistant and susceptible fish. Metallothionein isoform B was specifically up-regulated in parasite exposed resistant fish (Baerwald et al. 2008), whereas a trout metallothionein homologue was up-regulated during *T. bryosalmonae* infection (Burki et al. 2013). Thus, the potential roles of metallothioneins in myxozoan infection require further clarification.

Recent *M. cerebralis* studies have focused on known upstream immune regulators of metallothioneins, including IL-1β, the signal transducer and activator of transcription (STAT)3 and innate immune effector molecules (including IFN-γ, IRF1, and iNOS) (Baerwald 2013). Trout metallothionein isoforms A and B were up-regulated in parasite-challenged resistant fish, further implicating metallothionein isoforms in parasite resistance mechanisms. IL-1β exhibited a biphasic up-regulated expression profile in challenged susceptible and resistant fish, STAT3 exhibited a biphasic profile in resistant fish implicating STAT3 as an additional biomarker of *M. cerebralis* resistance (Baerwald 2013). IFN-γ and IRF1 were up-regulated in both susceptible and resistant trout 24 h post challenge, albeit to a greater extent in susceptible fish, whereas iNOS was only up-regulated in challenged susceptible fish. Trout homologues of the macrophage marker gene (e.g. natural resistance–associated macrophage protein (NRAMP α/β)) are implicated in pathogen killing by macrophages, phagocytosis, and regulation of pro-inflammatory genes (Lang et al. 1997). Both isoforms of NRAMP were down-regulated in susceptible relative to resistant fish, implicating these genes in *M. cerebralis* resistance. iNOS was confirmed to be up-regulated in *M. cerebralis* challenged susceptible fish, whilst arginase-2, was up-regulated in both susceptible and resistant challenged fish (Rucker and El-Matbouli 2007).

14.4.4 Comparing and Contrasting Transcriptional Modulation

The prominent involvement of macrophages suggests that innate immune responses play a key role in whirling disease susceptibility and resistance. In contrast, PKD pathology is linked to lymphoid tissue proliferation, specifically in relation to IgM-negative lymphocytes (Chilmonczyk et al. 2002). Both IgM and IgT transcripts were up-regulated during PKD, with IgT strongly correlating with both parasite burden and disease progression (Gorgoglione et al. 2013). IgT transcripts are also highly up-regulated (correlating with accumulation of IgT^+ B cells) in the intestines of trout surviving infection with *C. shasta* (Zhang et al. 2010b). The same laboratory has also described the elevation of the anti-inflammatory cytokine, IL-10 in IgM^+ and IgT^+ B cells that could signify the presence of regulatory B cells in fish (Takizawa et al. 2013). In *E. leei*-infected GSB intestine, IgM up-regulation correlated closely with the accumulation of IgM^+ B cells, particularly in fish fed the 66VO diet (Estensoro et al. 2012b). As indicated in Sect. 14.3, an important role of IgT in mucosal immunity has been proposed (Zhang et al. 2010b), although its role in non-mucosal immunity needs to be clarified. Interestingly, only IgM and IgT transcripts corresponding to the secretory forms were up-regulated during PKD (Gorgoglione et al. 2013). Whether the proliferating lymphocytes are IgT^+ B cells or T cells is currently unknown. In *C. shasta* infected intestine, IgM^+ and IgT^+ B cells express CCR7, a chemokine receptor binding the mammalian homeostatic chemokines CCL19 and CCL21 (Ordás et al. 2012). Interestingly, upregulation of a trout CCL19-like chemokine (CK10) during PKD was closely correlated to disease progression, thus indicating a link between over-expressed homeostatic activity and lymphocyte proliferative responses (Gorgoglione et al. 2013).

The transcriptional profiling of myxozoan infections will continue to prove highly valuable in assessing the underlying immune mechanisms shaping susceptibility and resistance in the context of disease pathogenesis and parasite clearance, respectively. However, there is a growing need to develop new functional tools, in order to fully elucidate fish-myxozoan interactions and fish diseases in general.

14.5 Host and Environmental Factors Affecting Fish Immune Response

Environmental factors affecting the immune response of fish are well known (Bowden 2008), and myxozoan infections are typically influenced by environmental conditions (for more details see

also Chap. 15). The effects of these environmental factors can involve changes in disease severity and disease.

14.5.1 Water Temperature

Water temperature can modulate the immune system efficiency due to the poikilothermic nature of fish. Temperatures below or above the thermal limit can influence both innate and adaptive immune responses (Ainsworth et al. 1991; Le Morvan et al. 1998; Martins et al. 2011). It is still unclear if these changes are directly driven by temperature or if they are due to changes in oxygen supply. Furthermore, it is still unclear whether temperature-dependent differences in myxozoan infections, such as those due to *T. bryosalmonae* (Bettge et al. 2009; Schmidt-Posthaus et al. 2013), *M. cerebralis* (Vincent 1998), *C. shasta* (Ray et al. 2012) and *Enteromyxum* spp. (Redondo et al. 2002; Yanagida et al. 2006; Estensoro et al. 2010b), are driven exclusively by changes in the immune response or also by effects on myxozoan proliferation.

14.5.2 Altitude

Increasing altitude involves changes in water temperature and ultraviolet B irradiation (UVB). UVB radiation is known to have various effects on the immune system of fish. Some authors have demonstrated reduced resistance to bacteria and parasites in irradiated fish (Markkula et al. 2007). To date no experimental data are available on the effects of UVB in myxozoan infections. Indirect evidence comes exclusively from epidemiological data. For instance, brown trout infected with *T. bryosalmonae* were almost exclusively below an altitude of 800 m above sea level (Wahli et al. 2008) (see also Chap. 15).

14.5.3 Stress

Stress, induced by different external or internal factors, is known to modulate the immune system of fish. This may occur through the interaction of corticosteroid receptors and pro-inflammatory cytokines (Stolte et al. 2008; Verbug-Van Kemenade et al. 2011). The direct effect of steroids on myxozoan infections is poorly known, but the little evidence available indicates that steroids increase fish susceptibility. High mortality of sharpsnout sea bream heavily infected with *Ceratomyxa diplodae* was sustained under a long term treatment with reproductive steroids (Katharios et al. 2007). Rainbow trout treated with cortisol implants had 20 times the density of *T. bryosalmonae* interstitial stages compared with non-treated fish, but showed less interstitial inflammation (Kent and Hedrick 1987). Exposure of *T. bryosalmonae*-infected rainbow trout to low or high levels of dietary estradiol had no influence on disease prevalence, parasite load or cumulative fish mortality (Burki et al. 2013).

14.5.4 Pollution

Pollution can affect myxozoan infections. For example, PKD prevalence increased at polluted sites in comparison to sites with lower levels of organic compounds (El-Matbouli and Hoffmann 2002). An increase in the prevalence of different myxozoans in relation to eutrophication (Marcogliese and Cone 2001), sewage effluents (Bucher and et al. 1992) or polluted waters (Cone et al. 1997) has also been reported. However, it is unclear whether these effects are due to increased invertebrate host populations, increased host stress, impaired immune response, respiratory impairment (e.g. gills irritation) or less oxygen.

14.5.5 Nutrition

Nutrition acts directly on the fish immune system (Oliva-Teles 2012), and the complex relationship between nutrition, immune status and parasitic infections is well recognized (Fekete and Kellems 2007). The only well documented case for the effect of host nutrition on myxozoan infections is for *E. leei*. Replacement of fish oils by

vegetable oils in a plant protein based diet (66VO) increased disease progression in GSB (Estensoro et al. 2011) (see also Chap. 15). The main difference between the two diets tested was the fatty acid profile (the 66VO diet had lower levels of n-3 long-chain polyunsaturated fatty acids). Dietary fatty acids are capable of modulating the immune system in mammals (De Pablo and De Cienfuegos 2000) and n-3 long-chain polyunsaturated fatty acids have well recognized anti-inflammatory properties (Calder 2007). Nevertheless, other factors (such as an effect of nutrition on mucosal immunity or the integrity of the intestinal tract) may have contributed to disease progression.

14.5.6 Host Factors

Aspects of hosts clearly affect the disease outcome in different myxozoan infections, the most studied being host age and host size. There is a minimum size or age for the development of a specific immune response. Young fish are generally more susceptible to diseases and vaccination has no effect in very early stages (Ellis 1988). However, the situation may be more complex, additional interacting factors may intervene (see also Chap. 15). For example, brown trout (>1 year) infected with *T. bryosalmonae* showed lower infection prevalence and less severe pathology compared to young-of-the-year brown trout. The inflammatory responses of both age classes were, however, comparable (Schmidt-Posthaus et al. 2013).

14.6 Immune Evasion Strategies

Parasites have evolved a range of mechanisms to evade the immune response and host immune systems have, in turn, evolved means of counteracting them. Such coevolutionary dynamics are characteristic of host-parasite interactions. Myxozoans, like many other parasites (Sitjà-Bobadilla 2008), can evade host responses by masking their presence or developing strategies to combat them.

14.6.1 Exploiting Immunologically Privileged Sites

One form of immune evasion is to occupy immunologically privileged sites (IPS) within the host body, where the immune system has either highly specific or reduced surveillance capability. The most common IPS in fish are: the central nervous system (CNS), the eyes, and the testes. The immune privilege of the CNS is indispensable in view of damage limitation to a sensitive organ with poor regenerative capacity. A number of *Myxobolus* species show tropism for the brain and spinal cord (see Chap. 15) and others multiply exclusively in nervous tissue before reaching the target tissue for sporogony. The penetrating triactinomyxon-sporoplasms of *M. cerebralis* reach the cartilage via peripheral nerves and the CNS (El-Matbouli et al. 1995). It is likely that this route is chosen to avoid immune responses whilst allowing proliferation in large numbers before reaching other tissues with greater immune surveillance. Neurotropic myxobolids, such as *M. neurophilus* in *Perca flavescens* (Khoo et al. 2010) or *M. hendricksoni* in fathead minnows (Mitchell et al. 1985), induce minimal or no inflammation, even when large portions of the brain (optic lobes, cerebellum, ventricles and meninges) are replaced by the parasite.

Eyes constitute another IPS because portions of the eyes are isolated during embryonic development and thus are recognized as non-self (Koevary 2000). Several myxospores can be found in the eyes (either exclusively or in addition to the brain) and most of them belong to the genus *Myxobolus* (see Chap. 15). Extrasporogonic stages of *Sphaerospora elegans* have been found in the *rete mirabile* of the eye (Lom and Dyková 1992).

The immuneprivileged nature of testis is not only due to the blood-testis barrier, but also to differential immunological factors (Fijak and Meinhardt 2006). The main function of this IPS is to avoid any autoimmune reaction against gametes, which have undergone recombination during meiosis and are thus allogenic for the host. Damage to gametes would pose a threat to the survival of offspring. *S. testicularis* efficiently

exploits the IPS condition of the seminiferous tubules of European sea bass, proliferating within their lumen without invoking any cellular reaction (Fig. 14.3m). Only when the testicular cysts are disrupted does the infection spread to the interstitial tissue and the immune cellular effectors appear (Sitjà-Bobadilla and Álvarez-Pellitero 1993). The route of entry of this myxosporean into the seminiferous tubules and the immune evasion strategy that enables it to reach the target site are unknown.

14.6.2 Intracellular and Antigen Disguises

Another evasion strategy used by myxozoans is intracellular disguise. This enables them to hide from the immune system within a host cell. Myxozoans that develop inside oocytes not only avoid the immune response, but also find a highly suitable habitat rich in nutrients. Plasmodia and spores of *Kudoa ovivora* proliferate within the internal oocyte layer and vitelline granules. The latter are gradually reduced until oocytes are fully occupied by parasitic stages and exceed the dimensions of healthy oocytes (Swearer and Robertson 1999). The cnidarian, *Polypodium hydriforme*, demonstrates a similar strategy, being an intracellular parasite of eggs of acipenseriform fish (Raikova 1994). Other *Kudoa* species are intracellular in myocytes (Fig. 14.3n), and do not elicit any cellular response as long as the host cell remains intact (Morado and Sparks 1986).

Antigen-based strategies include antigen mimicry or masking, antigen sequence diversity and polymorphism, and sharing and shedding of parasite antigens. Such strategies have been extensively documented for several mammalian parasites. Limited evidence also suggests that myxozoans exhibit such strategies. An example may be the modified antigen expression, especially involving the glycoprotein Mcgp33, that has been reported in different developmental stages of *M. cerebralis* (Knaus and El-Matbouli 2005a).

14.6.3 Dealing with Host Immune Responses

Some myxozoans induce immunosuppression to avoid host reactions. For instance, *M. cerebralis*-infected rainbow trout challenged with the bacterium *Yersinia ruckeri* exhibit lower survival, a more rapid onset of mortality, or both, compared to non-parasitized fish (Densmore et al. 2004). Immunosuppression can be a consequence of parasite-induced anorexia. Reduced food intake in infected hosts, which can end in cachexia, has been described for several myxozoans (Wang et al. 2005; Palenzuela 2006). Immunosuppression can also be caused by reducing the proliferative capacity of lymphocytes or the phagocytic ability of macrophages, by inducing apoptosis of host leucocytes, or by exhausting innate humoral factors. The phagocytic and/or the respiratory burst activities were decreased in GSB infected by *S.* (ex *Polysporoplasma*) *sparis* (Karagouni et al. 2005). Other myxozoan infections selectively depress phagocytic activity (Chilmonczyk et al. 2002), lymphocyte proliferative capacity (Densmore et al. 2004), or the number of some types of leucocytes in lympho-haematopoietic organs (Cuesta et al. 2006b; Álvarez-Pellitero et al. 2008) or in blood (Sitjà-Bobadilla et al. 2006).

Innate immune humoral factors can be significantly decreased in parasitized fish as indicated in Sect. 14.2. Transcriptional studies may provide us with important clues to the immune mechanisms exploited by myxozoan parasites to evade host immunity. The refractoriness of myxozoan resistance marker genes (such as STAT3 and metallothionein in *M. cerebralis* and *E. leei* susceptible fish) may suggest that myxozoan parasites exploit immune pathways involving these molecules to evade host immunity (Baerwald et al. 2008; Calduch-Giner et al. 2012; Baerwald 2013). Similarly, the modulation of genes encoding innate, pro-inflammatory and immunosuppressive molecules (SOCS-1 and -3) may also illustrate immune evasion strategies of myxozoans (Wang et al. 2011; Gorgoglione et al. 2013). Host SOCS

molecules may have been targeted by pathogens ranging from viruses to eukaryotic parasites owing to the cytokine modulating activities of these molecules (Delgado-Ortega et al. 2013). Similarly, the suppression of MBL-2 during enteromyxosis may be indicative of parasite immune evasion, as has been demonstrated for fungal pathogens (Koneti et al. 2008; Davey et al. 2011).

14.7 Immune-Based Strategies for the Control of Myxozoans

As inferred from the above sections, we are far from a comprehensive understanding of fish immune responses elicited against myxozoans. However, the information gathered to date enables us to identify promising opportunities for controlling these organisms.

14.7.1 Genetic Selection for Host Resistance

One possibility for disease control is to exploit the innate or natural resistance against Myxozoa observed in some fish species and strains, by means of genetic selection. The presence of natural resistance is implied by both interspecific and intraspecific differences in susceptibility in several systems (Table 14.1). In only a few cases have particular immune factors involved in such differences been suggested. For example, brown trout, Atlantic (*Salmo salar*) and coho (*Oncorhynchus kisutch*) salmon showed a considerably higher number of eosinophilic granule cells in *M. cerebralis*-affected areas compared to rainbow trout (Hedrick et al. 1999). In sharpsnout sea bream no lysozyme was detected in either *E. leei*-infected or healthy animals (Golomazou et al. 2006; Álvarez-Pellitero et al. 2008). The absence of lysozyme could therefore contribute to the high pathogenicity of *E. leei* in sharpsnout sea bream (Álvarez-Pellitero et al. 2008).

It is important to appreciate the potential complexity of resistance and its heritable basis. For example, rainbow trout strains resistant to *C. shasta* are susceptible to *M. cerebralis*, suggesting that different mechanisms might be specifically involved in the resistance to each myxozoan (Hedrick et al. 2001). Quantitative genetic studies and genome-wide mapping (Baerwald et al. 2011; Fetherman et al. 2012) provide evidence that resistance to *M. cerebralis* is heritable; and is based on a single quantitative trait locus (QTL) which explains a large percentage of the phenotypic variance contributing to whirling disease resistance in rainbow trout (Dionne et al. 2009) found that different MHC alleles were associated with different levels of susceptibility and resistance to *M. cerebralis*. In contrast, resistance to *C. shasta* is linked to multiple loci in rainbow trout (Nichols et al. 2003), however, protection can be overwhelmed by high and continuous exposure to *C. shasta* (Bjork and Bartholomew 2010).

14.7.2 Vaccination

A second strategy for controlling myxozoan disease is the development of vaccines to promote acquired resistance in fish upon re-exposure. For *M. cerebralis,* acquired immunity was found only among previously exposed fish that developed active infections (Hedrick et al. 1998). Similarly, trout that had recovered from clinical PKD were found to be resistant to reinfection if an active infection was previously induced (Foott and Hedrick 1987). Sharpsnout sea bream that recovered from *E. leei* infection were refractive to the disease in a second exposure (Golomazou et al. 2006). *E. leei*-infected red sea bream (*Pagrus major*) surviving mortalities in Japanese farms do not seem to have recurrent infections (Yanagida et al. 2008). The association between this acquired immunity and the production of specific antibodies has been demonstrated for turbot surviving *E. scophthalmi* epizootic outbreaks (Sitjà-Bobadilla et al. 2007b). It is speculated that these protective responses reflect a repertoire of non-specific immune factors that limit ceratomyxosis (Bartholomew 1998) and contribute to *M. cerebralis* resistance (Ryce 2003). In turbot enteromyxosis, antibody responses are slow in onset (50–100 days p.e.) compared to cellular and

Table 14.1 Host fish species and strains for which innate resistance against different myxozoan parasites has been described

Innate resistance	Myxozoan species	Host species	Reference
Inter-specific differences	*Ceratomyxa shasta*	Salmonids	Bartholomew 1998
	Enteromyxum leei	Sharpsnout sea bream, European sea bass, gilthead sea bream & many others	Sitjà-Bobadilla and Palenzuela 2012
	Enteromyxum scopthalmi	Turbot, sole	Sitjà-Bobadilla and Palenzuela 2012
	Henneguya ictaluri	Cat fishes	Griffin et al. 2010
	Kudoa amamiensis	Amberjacks	Sugiyama et al. 1999
	Myxobolus cerebralis	Salmonids	Hedrick et al. 1998; Vincent 2002; Blazer et al. 2004
	Thelohanellus nikolskii	Cyprinids	Molnár 2002
	Tetracapsuloides bryosalmonae	Salmonids	Arkush and Hedrick 1990; Grabner and El-Matbouli 2009
Intra-specific differences	*Ceratomyxa shasta*	Salmonids	Bartholomew 1998; Bjork et al. 2014
	Enteromyxum leei	Gilthead sea bream	Jublanc et al. 2006; Sitjà-Bobadilla et al. 2007a
	Enteromyxum scopthalmi	Turbot	Quiroga et al. 2006; Sitjà-Bobadilla et al 2006
	Myxobolus cerebralis	Rainbow trout	Hedrick et al. 2003; Wagner et al. 2006
	Myxobolus wulli	Allogynogenetic gibel carp	Zhang et al. 2010a
	Tetracapsuloides bryosalmonae	Atlantic salmon	Quigley and McArdle 1998
		Rainbow trout	Grabner and El-Matbouli 2009
		Cutthroat trout	Wagner et al. 2002
		Steelhead	Densmore et al. 2001

humoral effectors (20 days p.e.) (Sitjà-Bobadilla et al. 2006, 2007b). If innate immune responses to myxozoans can be activated more rapidly and to a greater extent this may accelerate the production of specific antibodies.

There is an empirical basis to search for suitable parasite antigens capable of triggering a protective immune response for several myxozooses. This is a complex task since myxozoans have several parasitic stages possibly with different antigenic profiles owing to adaptation to different hosts and environments in their life cycle. As an example, monoclonal antibodies raised against mature *K. thyrsites* spores do not react with stages found in exposed fish, suggesting they are antigenically distinct stages (Young and Jones 2005). Antigenic profiles have been obtained using classical approaches, such as enzyme-linked immunosorbent assay (ELISA), dot-blot, western-blot, lectin-blot, indirect fluorescent antibody technique (IFAT) and immunohistochemistry for a number of myxozoans, including *S. dicentrarchi* (Muñoz et al. 2000c), *E. leei* (Estensoro et al. 2013a), *M. cerebralis* (Kaltner et al. 2007; Knaus and El-Matbouli 2005b), *K. thyrsites* (Chase et al. 2001), *H. salminicola* (Clouthier et al. 1997), *M. rotundus* (Lu et al. 2002; Zhang et al. 2006) and *T. bryosalmonae* (Saulnier et al. 1996; Saulnier and de Kinkelin 1996).

More recently, other molecular approaches have become available such as Expression

Library Immunization (ELI). One such study, integrating cDNA-based ELI (cDELI) with next generation sequencing approaches, is currently underway in the search for protective *T. bryosalmonae* antigens (Secombes and Holland, personal communication). ELI or cDELI consists of the systemic screening of any given genome or transcriptome to identify potential vaccine candidates. The essential concept is to employ the host immune system to select the best vaccine candidates against a particular disease. The entire genome or transcriptome of a pathogen is cloned into genetic vectors optimized for eukaryotic expression under the control of a powerful cytomegalovirus (CMV) promoter to create a library that would express all the open reading frames (ORFs) of a pathogen. Purified plasmid DNA from this library is inoculated into animals, usually in subgenomic pools of clones, to induce immune responses against the cloned antigens. The immunized animals are then challenged with the pathogenic organism to see which clones induced protective immunity and therefore which pool of antigens should be further deconvoluted to individual protective antigens (Talaat and Stemke-Hale 2005).

Another antigen screening option is the Serial Analysis of Gene Expression (SAGE) of the pathogen (Ye et al. 2002). This is also promising for myxozoans, since the transcriptome and genome of several species is already engaged. This will offer a comprehensive catalogue of genes encoding all the potential proteins of the pathogen, with the potential to rationally select vaccine candidates rather than empirically test them one at a time. Furthermore, the strategy of antigen prediction is independent of the need to culture the pathogen in vitro, a bottleneck for myxozoan research. A complete genome sequence can be screened using bioinformatic algorithms to select ORFs encoding putative surface-exposed or secreted proteins, to select proteins with homology to known virulence factors or protective antigens from other pathogens, and to filter for proteins with no similarity to proteins of the host. After candidate antigens are identified in silico, they are produced as recombinant proteins or DNA vaccines and their immunogenicity is assayed to measure their potential as vaccine candidates.

14.7.3 Immune Therapies

An alternative strategy to vaccination for disease control is the development of specific immune therapies, designed to rebalance immune responses. This approach aims to alleviate the chronicity and intensity of myxozoan-mediated immunopathology in fish hosts. This has been demonstrated recently in mammals through the development of small peptide antagonists of SOCS proteins, by SOCS gene knockdown (Delgado-Ortega et al. 2013). Antibody-based neutralization of over-expressed IL-17 (one of the causative factors of chronic immunopathology and granuloma formation in helminth infections) has been shown to enhance parasite-specific antibodies and to reduce disease pathology and parasite prevalence (Wen et al. 2011). Mammalian MBL has some benefits towards relieving the intensity of chronic infections through the use of recombinant protein (Casanova and Abel 2004). Trout SOCS and IL-17 homologues up-regulated during PKD and *E. leei*-mediated suppression of GSB MBL-2 could be similarly controlled to facilitate immune protection and parasite clearance. Looking further ahead, tailoring fish vaccines by incorporating molecular adjuvants may enable the construction of highly efficacious vaccines priming the immune response and providing long-lasting immunological memory. This approach has been highly successful in mammalian vaccines utilizing a variety of cytokines, including IL-12, IL-18, and granulocyte monocyte colony stimulating factor (GMCSF) (Toka et al. 2004; Kathuria et al. 2012). Such advances, however, will need to go hand-in-hand with the continued functional characterization of fish immune molecules and pathways and, ultimately, the immune cell populations shaping disease pathogenesis and pathogen resistance.

14.7.4 Immunostimulants

Different commercial aquafeeds are nowadays formulated to include immunostimulant compounds, such as vitamins, nucleotides, minerals, glucans, prebiotics, probiotics, and herbal extracts. They are thought to enhance the fish basal immune system, the mucosal barriers, and the overall potential to fight against pathogens (Dalmo and Bøgwald 2008; Merrifield et al. 2010; Kiron 2012). However, their usefulness in myxozoan infections has not been fully determined, and it depends on the type of immune factor enhanced and its involvement in the immune response against each specific myxozoan. A diet including a glucan from yeast and the antiparasitic drug fumagilin decreased the number of *O. mykiss* infected with *C. shasta*, but did not improve the overall survival rate (Whipple et al. 2002). Similarly, bioactive, natural extract-supplemented feeds, although not preventing infection, decreased the intensity of infection and associated intestinal lesions of *E. scophthalmi* infected turbot and significantly increased survival rate (Palenzuela et al. 2009).

14.8 Conclusions

In the current chapter we have shown the complexity of the different immune factors evoked by the challenge of aquacultured fish species with myxozoan parasites, and the parasite strategies that have evolved to evade host immune responses. The importance of the local immune response and the interplay of host and environmental factors have been highlighted. Differences in the knowledge of the many fish-myxozoan models are outstanding, with most of the information coming from few species, and nothing is known about how invertebrates fight myxozoan infections. The existence of innate and acquired resistance to some myxozoan species opens promising possibilities to prevent myxozooses and therefore to alleviate the important economic losses provoked by them worldwide. We have come a long way in characterising the fish immune response, but still many aspects await to be deciphered. Only integrated and concerted research efforts will enable the identification of means to control diseases caused by myxozoans and to avoid their global dispersal in farmed fish stocks.

14.9 Key Questions for Future Studies

- What are the key immunoregulatory molecules associated with disease susceptibility and intensity?
- What methods can be explored to culture myxozoans in vitro?
- Are myxozoan strategies to deal with host immune response similar in fish and invertebrates?
- What are the protective mechanisms involved in disease resistance, and hence the pathways that need to be triggered by vaccination?
- What are the main myxozoan virulence-associated targets for the development of drugs and vaccines?

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Host and Environmental Influences on Development of Disease

15

Heike Schmidt-Posthaus and Thomas Wahli

Abstract

While many myxozoan parasites produce asymptomatic infections in fish hosts, several species cause diseases whose patterns of prevalence and pathogenicity are highly dependent on host and environmental factors. This chapter reviews how these factors influence pathogenicity and disease prevalence. Influential host factors include age, size and nutritional state. There is also strong evidence for host strains that vary in resistance to infection and that there is a genetic basis for resistance. A lack of co-evolutionary processes appears to generally underly the devastating impacts of diseases caused by myxozoans when introduced fish are exposed to novel parasites (e.g. PKD in rainbow trout in Europe) or when native fish are exposed to an introduced parasite (e.g. whirling disease in North America). Most available information on abiotic factors relates to water temperature, which has been shown to play a crucial role in several host parasite systems (e.g. whirling disease, PKD) and is therefore of concern in view of global warming, fish health and food sustainability. Eutrophication may also influence disease development. Abiotic factors may also drive fish disease via their impact on parasite development in invertebrate hosts.

Keywords

Pathology · Host age and size · Host identity · Disease resistance · Temperature · Eutrophication

15.1 Introduction

Many myxozoan parasites appear to have little effect on their fish hosts, producing asymptomatic, subclinical infections. However, several species cause recognizable diseases and elevated

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mortalities and it is increasingly clear that the outcome of such infections can be highly dependent on environmental and host specific factors. Because these diseases are often associated with economically significant hosts they have been the focus of considerable study by pathologists.

Pathologists typically examine disease in populations by assessing patterns of disease prevalence, and the pathology and pathogenicity associated with parasites and pathogens. Pathology is often used synonymously with disease and is defined as structural changes to the cells in an organism. Pathology can be induced by infectious or non-infectious causes. The capability of an infectious agent to induce pathology or disease in a host is referred to as pathogenicity. The pathogenicity of an agent depends on a wide variety of factors related to the host and the environment, and these factors form the basis of this review. Here we adopt a broad definition of environment and consider how both biotic (specific attributes of the host) and abiotic factors (such as temperature) are aspects of the environment experienced by the host and are therefore influencing pathogenicity and patterns of infection of myxozoans in fish.

15.2 Host Factors Influencing Pathogenicity and Infection

Hosts may resist infection by a pathogen via: (a) innate resistance, which is not necessarily pathogen specific, or (b) acquired resistance, which is based on immune processes specific to an infecting pathogen. Pathogenicity will therefore reflect the parasite's ability to evade both non-specific resistance mechanisms of the host and specific acquired immune responses. Pathogenicity will also be related to the parasite's ability to prevent induction and expression of host immunity. The latter can occur when the pathogen causes immunosuppression (see Chap. 14 for further cover of these topics). Below we review how various host factors (which are summarised in Table 15.1) may influence pathogenicity.

15.2.1 Host Age, Size and State

The development and severity of *Myxobolus cerebralis* associated pathology is dependent on the age of the salmonid host when first exposed to the triactinomyxon stage (Baldwin et al. 2000; Hedrick et al. 1999a, b; Ryce et al. 1998). Trophozoite stages of *M. cerebralis* invade and destroy cartilage, mainly of the head and spinal region (Fig. 15.1), and initiate a granulomatous reaction. The mechanism by which the parasite invades the fish epidermis, migrates through host tissues and promotes disruption of chondrocytes is still mainly unresolved. However, subtilisin-like serine proteases produced by the parasite spores seem to facilitate these processes and are therefore key factors for pathogenesis (Kelley et al. 2004; Dörfler and El-Matbouli 2007). Host age related differences in pathogenicity are at least partly dependent on the degree of ossification, with pathogenicity decreasing with increasing host age because the parasite's ability to invade the cartilage becomes limited. However, resistance to infection also depends on the size of the fish at a certain age (Ryce et al. 2004, 2005), which may relate to stage of development of the nervous system (Ryce et al. 2005) (Table 15.1). While fish age-dependent pathogenicity caused by *M. cerebralis* is relatively well understood, the influence of age in other systems is far less clear.

Host age-dependent differences in disease prevalence have been recorded for *Sphaerospora testicularis* infections in sea bass, *Dicentrarchus labrax* L. (Sitjà-Bobadilla and Alvarez-Pellitero 1993) and were attributed to host maturation. A progressive increase in infection levels of *S. testicularis* was observed in cultured males during the spawning season. Younger perch (*Perca fluviatilis*) were found to be more heavily infected by *Henneguya creplini* on the gills (Haaparanta et al. 1994), but the reason for this age dependency in infection remains unknown. Quiroga et al. (2006) found a weight dependent susceptibility of turbot (*Scophthalmus maximus*) for *Enteromyxum scophthalmi*, whereby prevalence in fish of 201–300 g was higher than in fish <200 and >300 g in weight.

Table 15.1 Fish host factors influencing pathogenicity of various myxozoan infections

Factor	Myxozoan species	Host species	Association of factor and effect in host species	Reference
Host factors				
Age	*Myxobolus cerebralis*	*Oncorhynchus mykiss*	+	Baldwin et al. (2000), Hedrick et al. (1999a, b), Ryce et al. (1998, 2004, 2005)
		Salmo trutta	+	Baldwin et al. (2000), Hedrick et al. (1999a, b)
	Tetracapsuloides bryosalmonae	*Salmo trutta*	+	Schmidt-Posthaus et al. (2013)
	Sphaerospora testicularis	*Dicentrarchus labrax*	+	Sitja-Bobadilla and Alvarez-Pellitero (1993)
	Henneguya creplini	*Perca fluviatilis*	±	Haarparanta et al. (1994)
Size	*Myxobolus cerebralis*	*Oncorhynchus mykiss*	+	Ryce et al. (2004, 2005)
Weight	*Enteromyxum scophthalmi*	*Scophthalmus maximus*	+	Quiroga et al. (2006)
Gonadal maturation	*Sphaerospora testicularis*	*Dicentrarchus labrax*	+	Sitja-Bobadilla and Alvarez-Pellitero (1993)
Host strain	*Ceratomyxa shasta*	*Oncorhynchus mykiss*	+	Atkinson and Bartholomew (2010), Buchanan (1983), Currens et al. (1997), Bartholomew et al. (2001)
		Oncorhynchus kisutch	+	Hemmingsen et al. (1986)
	Myxobolus cerebralis	*Oncorhynchus clarkii*	+	Wagner et al. (2002)
		Oncorhynchus mykiss	+	Arkush et al. (2002), Baerwald et al. (2008), El-Matbouli et al. (2002), Hedrick et al. (2003), Schisler et al. (2006), Fetherman et al. (2012)
	Tetracapsuloides bryosalmonae	*Oncorhynchus mykiss*	±	Grabner and El-Matbouli (2009)
	Enteromyxum leei	*Sparus aurata*	+	Sitja-Bobadilla et al. (2007)
	Henneguya ictaluri	*Ictalurus punctatus*	±	Bosworth et al. (2003)

Compilation of references according to host factor, myxozoan, host species and a possible association between factor and effect in host species

In brown trout (*Salmo trutta*) infected with *T. bryosalmonae*, fish older than 1 year have been characterised to have lower infection prevalence and less pathology compared to young-of-the-year brown trout (Schmidt-Posthaus et al. 2013). Whether this difference in disease development relates to fish age or reflects acquired immunity (see Chap. 14) has still to be elucidated. However, young-of-the-year rainbow trout (*Oncorhynchus mykiss*) exposed to river water containing *T. bryosalmonae* in the autumn showed reduced disease in the following year (Foott and Hedrick 1987; Morris et al. 2005) suggesting partial immunity may be involved at least in this system (see also Chap. 14).

An effect of nutrition of fish hosts on disease caused by *E. leei* provides evidence that fish state can influence pathogenicity. Thus, Estensoro et al. (2011) showed that partial replacement of fish oil by vegetable oils (VO) altered the fatty

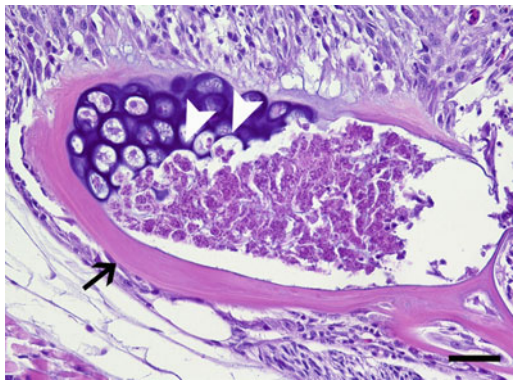

Fig. 15.1 *Myxobolus cerebralis* in the head region of a rainbow trout (*Onchorhynchus mykiss*); parasites invade and destroy the cartilage (*white arrowheads*) while the bone tissue is unaffected (*black arrow*), HE stain, bar = 25 μm

acid composition. This had a modulating effect on disease signs and outcome in gilthead sea bream, both being more severe in VO fed fish. An effect of the dietary composition on the immune system could also contribute to poor condition of the vegetable oil fed fish (see Chap. 14).

15.2.2 Host Identity

Differences in pathogenicity of a parasite in different susceptible fish host species have been recorded for several myxozoan diseases. For instance, rainbow trout are highly susceptible to whirling disease, whereas lake trout (*Salvelinus namaycush*), brown trout, Atlantic (*Salmo salar*) and coho salmon (*Oncorhynchus kisutch*) are considerably more resistant (Blazer et al. 2004; Hedrick et al. 1999a, b; O'Grodnick 1979). Rainbow trout were found to have high numbers of sporoplasm cells of *M. cerebralis* in the epidermis, while coho salmon had significantly fewer sporoplasm cells that were able to invade the epidermis after attachment by triactinomyxon actinospores (Adkison et al. 2001). Thus, host resistance or parasite ability to invade appears to vary amongst host species, but the reason for these differences is unresolved.

Host identity may also influence spore production. For instance, in Europe, spores of *T. bryosalmonae* are produced in kidney tubules and excreted in the urine of brown and brook trout (*Salvelinus fontinalis*). Despite spores being identifiable in the tubules of introduced rainbow trout, excretion of spores infective to European bryozoans has never been shown (Morris and Adams 2006; Grabner and El-Matbouli 2008; Schmidt-Posthaus and Wahli, pers. obs.).

As stated above many myxozoans cause little or no pathogenicity to their hosts (Kent et al. 2001; Lom and Dyková 1992). This likely reflects the outcome of long term co-evolutionary processes involving counter-adaptations on the part of both parasites (to host immune responses) and of hosts (to parasite virulence), resulting in finely-tuned virulence and immune responses. Evidence for such dynamics is available in the form of genotype-by-environment interactions for certain myxozoan/fish systems. For instance, changing environmental conditions can cause less adapted host strains or hosts that are introduced to novel environments succumb to disease more likely. The absence of co-evolutionary dynamics may explain some of the patterns described above, such as lack of development of infective *T. bryosalmonae* spores in the kidney of rainbow trout introduced to Europe (Morris and Adams 2006; Grabner and El-Matbouli 2008) as well as the high mortality of rainbow trout to whirling disease relative to e.g. brown trout, Atlantic salmon and lake trout (Blazer et al. 2004). This greater susceptibility of rainbow trout in North America to whirling disease is likely explained by the introduction of *M. cerebralis* with brown trout into the United States (Hoffman 1990). This introduction resulted in catastrophic declines in native rainbow and cutthroat trout populations (Hedrick et al. 1999b; Nehring and Walker 1996; Nehring et al. 1998; Vincent 1996) and the disease continues to be a major health issue for these species in the USA (Gilbert and Granath 2003; Krueger et al. 2006). Atkinson and Bartholomew (2010) similarly found greater mortality of highly susceptible non-native rainbow trout relative to native Chinook salmon. Such species-dependent differences in susceptibility are increasingly being linked with the exposure of hosts to exotic parasites.

There is increasing evidence that genetic background or "strain" can influence susceptibility to disease within fish species (Table 15.1). *C. shasta* occurs in salmonids in freshwater environments of the Pacific Northwest region of North America. However, *C. shasta* remains limited to certain river systems and this limited distribution is reflected in patterns of resistance among salmon and trout, with relative resistance to infection and disease in fish populations where the parasite is endemic. Steelhead trout (*Oncorhynchus mykiss*) originating from different geographic regions exhibit considerable differences in mortality rates after exposure to river water containing *C. shasta* spores (Buchanan et al. 1983). While strains originating from coastal areas, where *C. shasta* does not occur, showed high susceptibility resulting in an almost total loss of fish, three strains from the Columbia River basin, where *C. shasta* is known to be abundant, showed no mortality. Further, Atkinson and Bartholomew (2010) found that different genotypes (based on ITS-1) of *C. shasta* along the course of the Klamath River were related to differences in survival of infected fish and that parasite genotypes displayed differences in host specificity. Their findings suggest that selection for resistance in the host population and adaptation of the parasite to the host play important and complementary roles in determining the pathogenicity of particular host-parasite combinations.

A number of studies provide support that differing susceptibility to *C. shasta* has a genetic basis (Ibarra et al. 1992; Bartholomew 1998; Bartholomew et al. 2001; Nichols et al. 2003). For example, differences in resistance to *C. shasta* in relation to the origin of coho salmon were demonstrated by Hemmingsen et al. (1986). *C. shasta*-infected coho salmon from a Columbia River stock showed higher survival than fish from two coastal stocks, while survival of crossbreeds was intermediate. In another study, Currens et al. (1997) compared the degree of genetic introgression and susceptibility to *C. shasta* between native rainbow trout strains and a rainbow trout population that had been stocked for several decades. Considerable differences in allele frequencies were observed between native and stocked fish populations. Exposure to *C. shasta* resulted in differences in survival, which was enhanced in native fish and reduced in stocked fish. Populations with an allele composition based on a mixture of alleles from native and stocked fish showed an intermediate survival. The authors therefore concluded there was a relationship between introgression and susceptibility to infection. Bartholomew et al. (2001) presented data indicating that resistance of rainbow trout to infection by *C. shasta* is a dominant trait. Progeny with at least one parent from a resistant stock showed similar survival to exposure to *C. shasta* as non-mixed progeny from the resistant stock (both >99 %), whilst the survival of progeny from the susceptible stock was less than 20 % (Bartholomew et al. 2001).

Strain dependent resistance to *M. cerebralis* in different species of salmonids have repeatedly been reported, e.g. for cutthroat trout (*Onchorhynchus clarki*) (Wagner et al. 2002). Differences have also been found for rainbow trout, whereby a strain bred in Germany ("Hofer-strain" or "German strain") proved to be much more resistant than North American rainbow trout strains (El-Matbouli et al. 2002; Hedrick et al. 2003). A selective attachment of spores to salmonid species and strains was not demonstrated (Kallert et al. 2009). However, Schisler et al. (2006) obtained evidence that the differences in resistance between the "Hofer" and North American rainbow trout strains had a genetic basis. Crossbreeding of resistant and susceptible rainbow trout strains showed that the resistance is heritable (Schisler et al. 2006; Fetherman et al. 2012), and breeding experiments revealed that inbred progeny were more susceptible to *M. cerebralis* than outbred fish (Arkush et al. 2002). In addition to the differences in salmonid strain resistance, there are considerable differences in susceptibility to the parasite in the invertebrate host, *Tubifex tubifex*. In particular, genetic differences in *T. tubifex* populations were found to be related to different susceptibilities to *M. cerebralis* infection and these differences translated into different degrees of infection in the resident trout populations (Beauchamp et al. 2002).

Infection experiments have demonstrated that resistance to one pathogen is not related to resistance to another. Whirling disease-resistant German rainbow trout and North American rainbow trout strains were susceptible to *T. bryosalmonae*, *Yersinia ruckeri* and viral haemorrhagic septicaemia virus (El-Matbouli et al. 2009). Similarly, *C. shasta* resistant salmonids were susceptible to *M. cerebralis* infections (Hedrick et al. 2001). Baerwald et al. (2011) demonstrated that a single quantitative trait locus (QTL) is responsible for whirling disease resistance in rainbow trout. A further QTL region involved in resistance to *C. shasta* has been found on the same chromosome (Nichols et al. 2003). However, as demonstrated by Hedrick et al. (2001), resistance to one parasite does not mean resistance to another and it is unlikely that the same QTL region is responsible for resistance to both parasites.

Except for the examples mentioned above, there is only very limited information available on strain dependent resistance in other myxozoans. Preliminary investigations in rainbow trout strains indicated that differences in susceptibility to *T. bryosalmonae* might exist (Grabner and El-Matbouli 2009). Sitjà-Bobadilla et al. (2007) infected different strains of gilthead sea bream (*Sparus aurata*) with *Enteromyxum leei* and found evidence for resistant strains, but the basis of resistance remains unknown. Exposure of channel catfish (*Ictalurus punctatus*) full sib families to *Henneguya ictaluri* revealed differences in susceptibility that may have a genetic basis (Bosworth et al. 2003).

15.3 Abiotic Factors Influencing Infection and Pathogenicity

15.3.1 Temperature

Temperature has been widely demonstrated to influence infection development and pathogenicity of parasites and pathogens (e.g. Medoff et al. 1986; Mitchell et al. 2005). Evidence that temperature can influence both infection and pathogenicity of myxozoans is available for *M. cerebralis* (Baldwin et al. 2000), *T. bryosalmonae* (Bettge et al. 2009; Schmidt-Posthaus et al. 2012, 2013), *C. shasta* (Ray et al. 2012; Udey et al. 1975), and *Enteromyxum* spp. (Yanagida et al. 2006) as described below (and see Table 15.2).

Severity of *M. cerebralis* infection has been correlated with mean water temperature or temperature variations in rainbow trout, brown trout and Yellowstone cutthroat trout (Baldwin et al. 2000; Murcia et al. 2006; Vincent 1998). In contrast, the mortality of rainbow trout infected with *C. shasta* was independent of water temperature (Udey et al. 1975). However, water temperature influenced the period of time before death (Udey et al. 1975; Ray et al. 2012), indicating that water temperatures have pervasive effects on the pathogenicity of the parasite. *Enteromyxym* spp. have also been shown to cause a temperature dependent increase in disease rate in tiger puffer, *Takifugu rubripes* (Yanagida et al. 2006). While *E. fugu* was consistently detected with a high prevalence of infection (60–100 %) at temperature ranges between 10 and 25 °C, no sporulation occurred at 10 and 15 °C water temperature. In contrast, development of *E. leei* and onset of disease were suppressed by low water temperatures (<15 °C) with promotion of development and increase of disease rate at a temperature increase to 20 °C (Yanagida et al. 2006).

T. bryosalmonae can cause mortality at temperatures of 15 °C or above (Bettge et al. 2009; Clifton-Hadley et al. 1987; Ferguson 1981; Hedrick et al. 1993) (Fig. 15.2). The driver/s for increased mortality at higher water temperatures are not completely resolved. The histological lesions (Fig. 15.3) provoked by infection were shown not to be temperature dependent nor were there temperature-related differences in severity (Schmidt-Posthaus et al. 2012) (Figs. 15.2 and 15.3). Mortality at higher water temperature is probably a combination of higher oxygen demands due to increased metabolism and increased demands on osmoregulation at higher temperatures together with reduced kidney function due to infection. A direct effect of the higher temperature (18 °C) on fish survival was not evident (Bettge et al. 2009). However, histological

Table 15.2 Environmental factors influencing pathogenicity of various myxozoan infections

Factor	Myxozoan species	Host species	Effect observed in	Reference
Environmental factors				
Temperature	*Ceratomyxa shasta*	*Oncorhynchus kisutch*	Mortality, time before death	Ray et al. (2011), Udey et al. (1975)
		Oncorhynchus mykiss	Mortality, time before death	Udey et al. (1975)
		Oncorhynchus tshawytscha	Mortality, time before death	Ray et al. (2010)
	Myxobolus cerebralis	*Oncorhynchus mykiss*	Mortality, severity of histological lesions	Baldwin et al. (2000), Schisler et al. (2000), Vincent (1998)
		Oncorhynchus clarkii	Mortality, severity of histological lesions	Murcia et al. (2006, 2011)
	Tetracapsuloides bryosalmonae	*Oncorhynchus mykiss*	Mortality	Bettge et al. (2009), Clifton-Hadley et al. (1987), Ferguson (1981), Hedrick et al. (1993), Schmidt-Posthaus et al. (2012)
		Salmo trutta	Disease occurence	Wahli et al. (2008)
Eutrophication	*Tetracapsuloides bryosalmonae*	*Oncorhynchus mykiss*	Disease prevalence	Matbouli and Hoffmann (2002)
		Salmo trutta fario	Disease prevalence	Matbouli and Hoffmann (2002)
	Zschokkella nova	*Cottus gobio*	Disease prevalence	Bucher et al. (1992)
	Myxobolus procerus	*Percopsis omiscomaycus*	Infection rate	Cone et al. (1997)
Nutrition	*Enteromyxum leei*	*Sparus aurata*	Disease signs and outcome	Estensoro et al. (2011)

Compilation of references according to environmental factor, myxozoan, host species and the effect which was recorded in the fish host

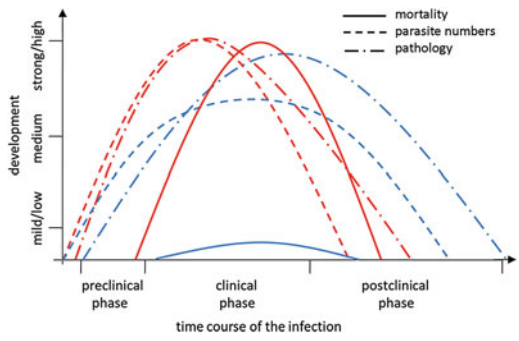

Fig. 15.2 *Tetracapsuloides byosalmonae* in rainbow trout (*Oncorhynchus mykiss*); disease associated mortality (*continuous line*), parasite numbers (*dotted line*) and associated pathological lesions (*dashed line*) at low (12 °C) (*blue lines*) and high (18 °C) (*red lines*) water temperature

lesions developed more quickly at higher water temperature and there was evidence for enhanced kinetics of parasite proliferation as an accelerated increase of parasite numbers (Bettge et al. 2009; Schmidt-Posthaus et al. 2012) (Fig. 15.2). Parasite numbers were observed to be higher in fish kept at higher water temperature when assayed as parasite counts in histological and immunohistological slides (Bettge et al. 2009).

A possible contributor to temperature-related disease development in fish is the effect of temperature on the development of myxozoans in their invertebrate hosts. In *M. cerebralis* infections, production, release, and survival of the infective stage from *Tubifex tubifex* have been correlated with water temperature (Markiw 1992;

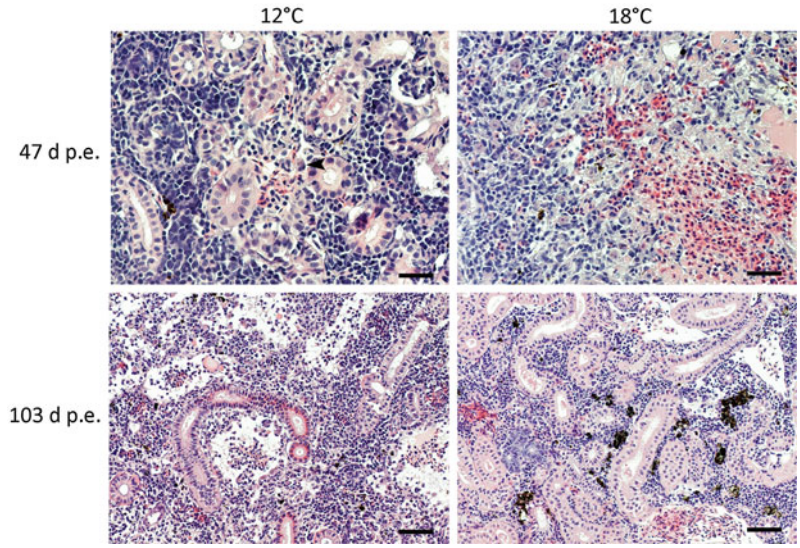

Fig. 15.3 Histological lesions in rainbow trout (*Oncorhynchus mykiss*) exposed to *T. bryosalmonae* 47 days post exposure (p.e.) and 103 days p.e., respectively, animals were kept at 12 or 18 °C; 47 days p.e. trout kept at 12 °C showed only slight changes in the renal interstitium and scattered parasites (*arrowhead*) in the vascular lumen, surrounded by inflammatory cells, trout kept at 18°C showed widespread necrosis and hemorrhage in the interstitium and multiple parasites; 103 days p.e. trout kept at 12 °C showed interstitial necrosis, hemorrhage and edema, whereas trout kept at 18 °C at this point point showed a completely restored renal morphology. HE stain, 47 days p.e.: bar = 25 μm, 103 days p.e.: bar = 50 μm

El-Matbouli et al. 1999). The optimal temperature for spore development and maturation in the invertebrate host was revealed to be 15 °C, with inhibition of spore maturation at lower temperatures and spore degeneration at higher temperatures (El-Matbouli et al. 1999). Temperature dependent proliferation of *T. bryosalmonae* spores in the invertebrate host *Fredericella sultana* has similarly been shown (Tops et al. 2006), with increasing temperatures between 10 and 20 °C accelerating and prolonging the production of infective spores. For *C. shasta*, a correlation has been shown between water temperature and higher mortality and faster mean days to death (Bjork and Bartholomew 2009; Hallett and Bartholomew 2011; Ray and Bartholomew 2013). Such studies suggest that in general the greater availability of infectious spores shed by the annelid host as driven by temperature may contribute to temperature-dependent disease development in fish. This in turn may result in increased fish mortality (Ray et al. 2012).

The above studies provide collective evidence that temperature often exerts a strong impact on disease development. This constitutes a worry for conservation of endangered fish stock and for sustainable food production in view of global warming.

15.3.2 Other Environmental Factors

Results from different investigations suggest that eutrophication may variously influence myxozoan infections in fish (Table 15.2). El-Matbouli and Hoffmann (2002) studied the effect of sewage treatment plant effluents on the prevalence of PKD in farmed and wild trout. They found that disease prevalence decreased dramatically in both wild and farmed fish populations after the effluents were diverted. Marcogliese and Cone (2001) reported an increase in prevalence of infected fish and in infracommunity richness of myxozoans in relation to increasing eutrophication in the

St. Lawrence River. A relation between sewage effluents and prevalence of *Zschokkella nova* infection in bullhead (*Cottus gobio*) was similarly reported by Bucher et al. (1992) while Cone et al. (1997) suggested that an increased infection rate of trout-perch (*Percopsis omiscomaycus*) by *M. procerus* in Duluth harbor when compared to other areas of the Great Lakes was related to nutrient enrichment. The authors of both studies hypothesized that the effect could reflect increased invertebrate host populations or debilitated health of fish hosts due to sewage effluents or nutrient enrichment. Evidence that nutrient enrichment indeed can enhance invertebrate host populations (Hartikainen et al. 2009; Hartikainen and Okamura 2012) with a consequent effect on the development of infectious spores (Hartikainen and Okamura 2012) is provided by studies of *T. bryosalmonae* in its bryozoan host, *F. sultana*. The same was shown for increased *M. cerebralis* infections in salmonids in the Lostine River, Oregon, due to enhanced density of *T. tubifex* colonies in areas with high organic matter (Sandell et al. 2001). In contrast, eutrophication had no influence on infection prevalence in *Henneguya creplini* infections in the gills of perch in Finland (Haaparanta et al. 1994).

The most comprehensive surveys of a myxozoan associated disease in wild fish populations have been conducted in Switzerland, where *T. bryosalmone* infected brown trout were generally found below an altitude of 800 m above sea level (Wahli et al. 2008). In a few cases, where rivers were drained by shallow lakes, infected fish were found above this altitude. The altitude effect on PKD occurrence was therefore attributed to water temperature (Wahli et al. 2008).

Other factors that have been correlated with disease development include specific conductivity of water (Murcia et al. 2011; Sandell et al. 2001) or gas supersaturation (Schisler 1999). Different salmonid species infected with *M. cerebralis* showed most severe lesions in the cranial and jaw cartilages in stream sites with a combination of high temperature and low specific conductivity (Murcia e al. 2011). Mortality due to *M. cerebralis* infection in rainbow trout was significantly increased when animals were exposed to additional stressors, like elevated temperature, bacterial infections and/or gas supersaturation (Schisler 1999).

15.3.3 Multiple Factors and Root Causes

As developed above, there is good evidence that both temperature and eutrophication may influence infection prevalence, pathogenicity and host mortality. In addition, there is evidence that both may influence invertebrate host populations, the proliferation of myxozoans in these invertebrate hosts and the responses of fish hosts. Thus a multiplicity of factors is likely to contribute to fish disease. A largely unresolved issue relates to the root causes of various environmental effects. Thus, it is unclear to what extent environmentally-driven effects on disease development relate to variation in parasite development versus variation in host resistance and susceptibility.

15.4 Key Questions for Future Research

- How do multiple sources of environmental variation influence disease development?
- What is the genetic basis of resistance?
- Are temperature and eutrophication effects on disease development largely attributable to host stress?
- Does infection by multiple spores result in greater pathogenicity?
- How is pathogenicity influenced by co-infection with other parasites and pathogens?

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Specificity of Infection Sites in Vertebrate Hosts

16

Kálmán Molnár and Edit Eszterbauer

Abstract

Most myxozoans infecting fish are host-, tissue- and organ-specific parasites. The host specificity of most myxosporean species is rather narrow, with the parasites able to develop in only one host species or in closely related fishes. There are species, however, with relatively wide host ranges, infecting genetically distant host species. Tissue selection is a key taxonomic feature for histozoic myxozoans, as they develop mature spores in only one tissue type. Histozoic species may form plasmodia in epithelial, muscular, cartilaginous and connective tissues, inside nerve cells and in blood vessels, etc. Development of plasmodia often occurs in a well-defined part of an organ. For example in the gills, different species develop in or among lamellae, in different tissues of gill filaments or inside the cartilaginous gill arch. Other species may develop plasmodia in different organs composed of the same tissue type. Using examples, this chapter reviews infection specificity, characterizes how infections are manifested in various tissues and organs, and discusses how infections relate to both parasite and host phylogeny.

Keywords

Host specificity · Site preference · Tissue tropism · Vertebrate host · Histozoic · Coelozoic · Host phylogeny

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16.1 Introduction

The Malacosporea contains a small number of species with only one receiving extensive histological investigation in vertebrate hosts. In contrast, members of the Myxosporea are known to cause diseases in many fish and thousands of species have been described (Lom and Dyková 2006). Consequently, knowledge on the development of Myxosporea in fish hosts is more extensive, and data reveal a diversity of patterns of exploitation of hosts, organs and tissues. The taxonomy of this group has been based mainly on myxospore morphometrics, and since the number of characters is limited, this has led to the putative identification of the same species from large geographic areas, very different hosts (euryxenic species) and as parasites of various organs and tissues within hosts. Recent studies, however, show that myxosporeans of fish are mostly host-specific parasites, typically infecting only one host species (oioxenic species) or a limited number of closely related species (stenoxenic species) (Hedrick et al. 2001; Urawa et al. 2011).

Organ and tissue specificity of myxozoans have been rather neglected. Although some species are described as developing in specific tissues, in general, the locations of other parasites have been reported broadly, for instance, as in the gills, fins or gut, with no details on the affected tissue or the preferred site within the infected organ. Molnár (1994, 2002a, b) pointed out that *Myxobolus* spp. are highly host-specific parasites that develop plasmodia in a certain type of tissue in a specific part of a given organ.

This chapter presents examples of specificity of myxozoans in relation to hosts and location within hosts and then goes on to review the development of infections in different sites within fish. We demonstrate with examples that tissue specificity is the most important tropism-related factor that determines the location of the plasmodial development of myxosporeans. We conclude by considering how patterns of specificity may relate to phylogeny.

16.2 Host Specificity

The host specificity of most myxosporean species is rather narrow, with the parasites able to develop in only one host species or in closely related fishes. There are species, however, with relatively wide host ranges, infecting genetically distant host species. The host specificity of myxosporeans infecting fishes in the former Soviet Union was examined by Shul'man (1966). He listed 43 species infecting a single host species, 20 species specific to fishes from one genus, and 68 species that were able to infect members of one fish family. These species formed the majority of species (89 %) that were host specific to some extent. The minority of species (17 species) were able to infect fish hosts belonging to unrelated fish families.

Host specificity has historically been inferred on the basis of recorded natural infections in fish (Shul'man 1966). However, since the two-host life cycles of some myxozoans are now known, host specificity for both the fish and invertebrate host may now be examined experimentally. Hedrick et al. (2001) demonstrated that *Myxobolus cerebralis* actinospores were capable of infecting different salmonids (*Oncorhynchus mykiss*, *O. clarki*, *O. tshawytscha* and *Salvelinus confluentus*), but the parasite could not colonize *Thymallus arcticus,* only *T. thymallus*. Considering that the natural host of *M. cerebralis* in Europe is brown trout (*Salmo trutta*), this parasite species is considered stenoxenic as it is able to infect at least four salmonid genera.

The difficulties in distinguishing between myxosporeans with very similar spore morphologies led to the use of molecular biological methods for determining the relation among isolates collected from different host species. For example, using both experimental infections and molecular methods, Urawa et al. (2011) found that myxospores of *Myxobolus arcticus* developing in masu salmon (*Oncorhynchus masu*) and their actinospores from *Lumbriculus variegatus* in Japan, and myxospores of *M. arcticus* of the sockeye salmon (*Oncorhynchus nerka*) and their

actinospores released from *Stylodrilus heringianus* in Canada, had identical SSU rDNA sequences. These spores were also very similar in morphology. Nevertheless, the actinospore isolated from *L. variegatus* from Japan did not infect the type host sockeye salmon under experimental conditions. It was concluded that the American and Japanese *M. arcticus* were unique but conspecific strains.

Besides *M. cerebralis* and *M. arcticus*, another example of stenoxeny is *Myxobolus alvarezae*. Cech et al. (2012) reported that *M. alvarezae* is able to infect only members of the genus *Leuciscus* (ide *L. idus* and asp *L. aspius*), whereas two morphologically similar, but genetically distinct species (*Myxobolus dujardini* and *M. sitjae*) were found in chub, a leuciscine fish but member of another tribe of the subfamily Leuciscinae, and white bream representing another subfamily (Abraminae), respectively, are considered oioxenic.

Available data suggests strict host specificity (oioxeny) for *Myxobolus drjagini* developing cysts on the head of silver carp (*Hypophthalmichthys molitrix*). The majority of myxosporean spp. infecting the common carp (*Cyprinus carpio*) (e.g. *M. dispar, M. basilamellaris, M. cyprinicola, Thelohanellus nikolskii, T. hovorkai, T. kitauei, Sphaerospora dykovae, S. molnari*) seem to be oioxenic, infecting only this fish species (Dyková and Lom 1988a).

The above examples that mainly represent histozoic freshwater platysporines suggest that myxosporeans in general show high host specificity. However, marine species, including members of the histozoic genus *Kudoa,* show a range of host specificity (Yokoyama et al. 2012). Some species appears to be oioxenic, while others such as *Kudoa thyrsites* are euryxenic, infecting fishes from different orders (Moran et al. 1999; Whipps and Kent 2006). *Enteromyxum leei* is another example of euryxeny. The parasite infects a number of genetically distant marine fishes, which is facilitated by the ability of presporogonic developmental stages to transmit directly between fish.

Host specificity in malacosporeans is well known only for one species, *Tetracapsuloides bryosalmonae*. This stenoxenic myxozoan is able to infect several salmonid fish species. However, clear differences in susceptibility were detected among rainbow trout (*Oncorhynchus mykiss*) and brown trout (Kumar et al. 2013). Experimental studies by Grabner and El-Matbouli (2010) indicated that *Buddenbrockia plumatellae* could be transmitted only to minnow (*Phoxinus phoxinus*), while another *Buddenbrockia* sp. was infective to common carp but not to minnow.

16.3 Tissue and Organ Specificity

Tissue and organ specificity can be considered as the location in which the parasite develops into mature spores, in contrast to the locations that are a part of the migration route of the parasites' developmental stages. The development of many well-studied myxosporean species is strictly connected to a specific tissue of the host. Thus, as described below, there are species that develop only in the epithelial, muscular, cartilaginous and connective tissues, inside nerve cells and in the lumen of blood vessels. Usually, myxosporeans develop in a certain tissue of a single organ. These organ-specific species start and finish their development in a well-defined organ, or in most cases, in a specific part of the organ. In other cases, the organ specificity is less emphasized and plasmodia develop in the same tissue type in different organs (e.g. *Myxobolus gayerae, Myxobolus pfeifferi*) (Molnár et al. 2014). Another example is *Thelohanellus nikolskii,* the plasmodia of which develop in the cartilaginous fin rays of the common carp fry, while in older fish this parasite forms plasmodia in the cartilage of the scales (Moshu and Molnár 1997). The latter case represents strict tissue specificity and a lack of organ specificity.

Histozoic myxosporeans demonstrate pronounced tissue specificity. Several species develop in epithelial tissues, forming small plasmodia and developing intercellularly in the epithelium between two neighboring gill lamellae (e.g. *Myxobolus pavlovskii*), while others form large plasmodia in the multilayered epithelium of a single gill filament (e.g. *M. dujardini*). Infection of the skin is mostly

restricted to the epithelium of the scales and the fins and it is relatively rare in scale-less skin. On the other hand, some highly pathogenic myxosporeans (e.g. *Ceratonova shasta, Enteromyxum* spp.) infect the intestinal epithelium, developing intercellularly as small, individual pansporoblasts. Infections in kidney are located mostly in the tubules. In some of these cases, small plasmodia (e.g. those of *Hoferellus* spp.) divide in the tubular epithelium, but form their spores coelozoically in the tubular lumen. Myxosporean infection in the epithelium of liver is rarely documented, although the major part of the liver is composed of epithelial cells. On the other hand, early stages of *Chloromyxum, Myxidium* and *Zschokkella* spp. are frequently found in the epithelium of bile ducts and gall bladder.

Histozoic and coelozoic species are distinguished depending on the site selection within an organ (Lom and Dyková 2006; Feist and Longshaw 2006). Histozoic species form vegetative stages and spores in a certain tissue of a specific organ, whereas for coelozoic species a part of the process of sporogony takes place in the lumen of urinary and biliary ducts, in the urinary bladder or in the gall pouch. The extrasporogonic stages of most *Sphaerospora* spp. circulate in the blood, but sporogonic stages and mature spores develop in urinary channels. A typical histozoic species is *M. cerebralis,* which develops spores in the cartilage after migration through the skin epithelium and the peripheral nerves (El-Matbouli et al. 1995). For *C. shasta*, sporoplasm cells migrate via the blood stream from the portal of entry to the organ where sporogony takes place (Bjork and Bartholomew 2010).

Little is known about site selection of malacosporeans. Presporogonic stages of malacosporeans seem to develop intercellularly, likely propagating in capillaries (Fig. 16.1a). Their sporogony, however, takes place in renal tubules (Fig. 16.1b) as in many coelozoic myxosporeans. Myxosporean site selection and histology of different myxosporean species has been reviewed by Dyková and Lom (2007).

16.3.1 Infections in Gills

The majority of known myxosporean species infect gills (Molnár 2002a), probably because this location serves as an easy option for spore release (Eszterbauer et al. 2013). Myxosporean plasmodia might develop in or among gill lamellae, in gill filaments and inside the cartilaginous gill arch.

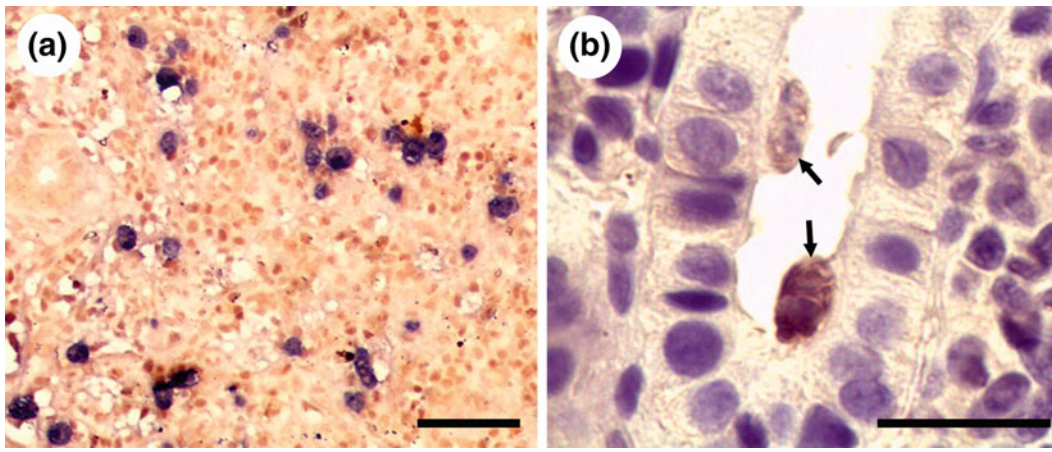
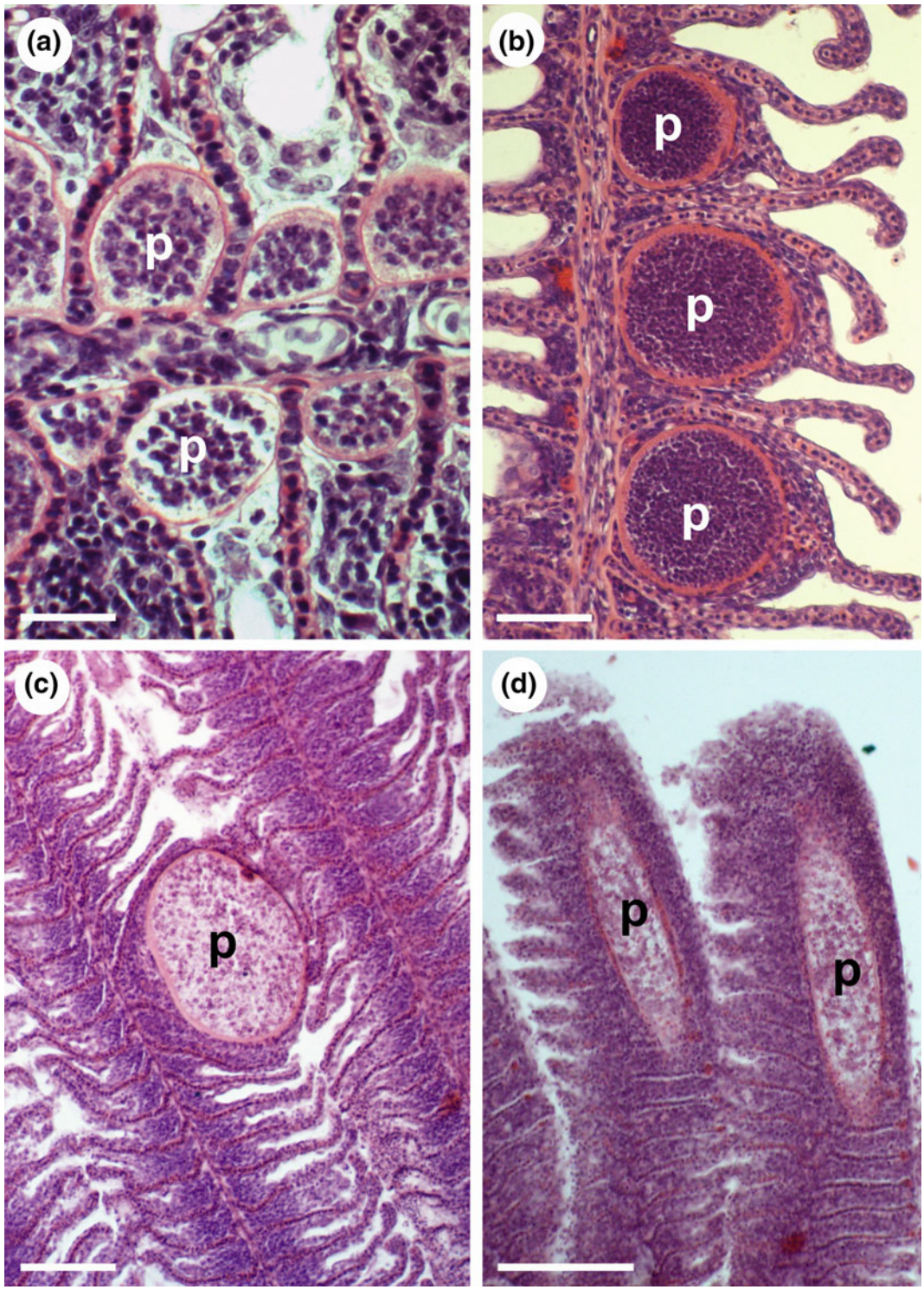


Fig. 16.1 **a** Extrasporogonic stages of *Tetracapsuloides bryosalmonae* (*dark-blue* colouration) in the renal interstitium of *Oncorhynchus mykiss*. ISH section, BCIP/NBT with Bismarck *brown* Y counterstain. Bar = 40 µm. **b** Spores of *Tetracapsuloides bryosalmonae* (*arrows*) in the lumen of the urinary channel of *Salmo trutta*. IHC section, DAPI with hematoxylin counterstain. Courtesy of Dr. Daniel Grabner and Prof. Mansour El-Matbouli. Bar = 20 µm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◀**Fig. 16.2** **a** Interlamellarly locating small plasmodia (*p*) of *Myxobolus impressus* in the arteries of the gill filament of *Abramis brama*. H and E. Bar = 20 µm. **b** Intralamellarly developing young plasmodia (*p*) of *Henneguya creplini* in the gill filament of *Sander lucioperca*. H and E. Bar = 20 µm. **c** An intralamellarly developing large *Myxobolus intimus* plasmodium (*p*) in a gill filament displacing the neighboring lamellae. H and E. Bar = 20 µm. **d** Large plasmodia (*p*) of *Myxobolus sommervillae* in the arteriae of the gill filament of *Rutilus rutilus*. H and E. Bar = 100 µm

Two specific forms of lamellar type infection can be distinguished. In the interlamellar type of infection, as represented by *Myxobolus pavlovskii* from silver carp *Hypophthalmichthys molitrix* the plasmodia develop in the stratified epithelium between gill lamellae (Fig. 16.2a). In the majority of cases, the plasmodia fill only the interlamellar space (small-cyst type), but occasionally plasmodia displace adjacent gill lamellae and form relatively larger cysts. In the intralamellar type of infection, plasmodia are formed in the sinusoids of the gill lamellae. Small or large plasmodia, depending on myxosporean species, may occur within single lamellae. The most common type is relatively small plasmodia dilating the lamella symmetrically. This type is characteristic of some *Henneguya* species, such as *H. psorospermica* from pike *Esox lucius* and *H. creplini* from pike-perch *Sander lucioperca* (Fig. 16.2b). Plasmodia of other species grow so large that they may deform the adjacent gill lamellae (Fig. 16.2c). A special form of intralamellar location was observed for *Myxobolus persicus* from *Barbus grypus*. Its plasmodia start developing in the basal membrane between the endo- and epithelial-layers of gill lamellae Masoumian et al. (1994).

Some myxosporeans develop large plasmodia inside the gill filaments. A vascular type of infection, where plasmodia establish inside the arteries of gill filaments, is the most common of these. *Myxobolus bramae* from *Abramis brama*, *M. muelleri* from *Squalius cephalus* and *M. dispar* from *Cyprinus carpio* exhibit this type of development. Small plasmodia colonize the gill, accumulate in the arteries of gill filaments, and fuse together forming a large plasmodial conglomeration (Fig. 16.2d). Other species like *Myxobolus dujardini* from *Squalius cephalus* and *M. alvarezae* from *Aspius aspius* form large plasmodia in the stratified epithelium of the filament, both in areas lacking lamellae and in the interlamellar epithelium (epithelial type) (Fig. 16.3a). Large plasmodia complexes with several lateral branches are formed by the fusion of small plasmodia. Less frequently, plasmodia (e.g. *M. rutili*) may develop in the connective tissue around the filament arteries. In rare cases (e.g. for *Myxobolus feisti*), plasmodia start developing in the connective tissue of blood vessels, which are in association with the cartilaginous gill rays. Cartilaginous tissue may overgrow the connective tissue, thereby surrounding and enclosing the plasmodia (Fig. 16.3b).

In basifilamental development, plasmodia develop in the subepithelial tissue between gill filaments on the surface of the gill arch. Parts of the plasmodia often penetrate into the cavity of the gill arch (Fig. 16.3c). In some species infecting cyprinid fishes (*Myxobolus fundamentalis, M. pfeifferi, M. gayerae*), plasmodial development takes place inside the cartilaginous gill arch (Molnár et al. 2010, 2012). Less frequently, the formation of plasmodia occurs in the cartilaginous matrix of the gill arch (e.g. *Myxobolus intrachondrealis* in common carp, or *M. albi* in marine goby *Pomatoschistus microps*). Contrary to the cyst-forming myxosporeans, some *Sphaerospora* spp. (e.g. *S. molnari* and *S. carassii*) cause multiple infections in the gill epithelium. Subsequent to their extrasporogonic development in the blood, these species begin sporogony intercellularly and form spores both in the interlamellar epithelium and in the epithelium of filaments (Fig. 16.3d). For *Parvicapsula pseudobranchicola*, a pathogenic species of Atlantic salmon *Salmo salar*, similar multiple infections occur in the pseudobranchia, while in the air-breathing African catfish solitary large plasmodia of *Henneguya suprabranchiae* are formed in the suprabranchial organ.

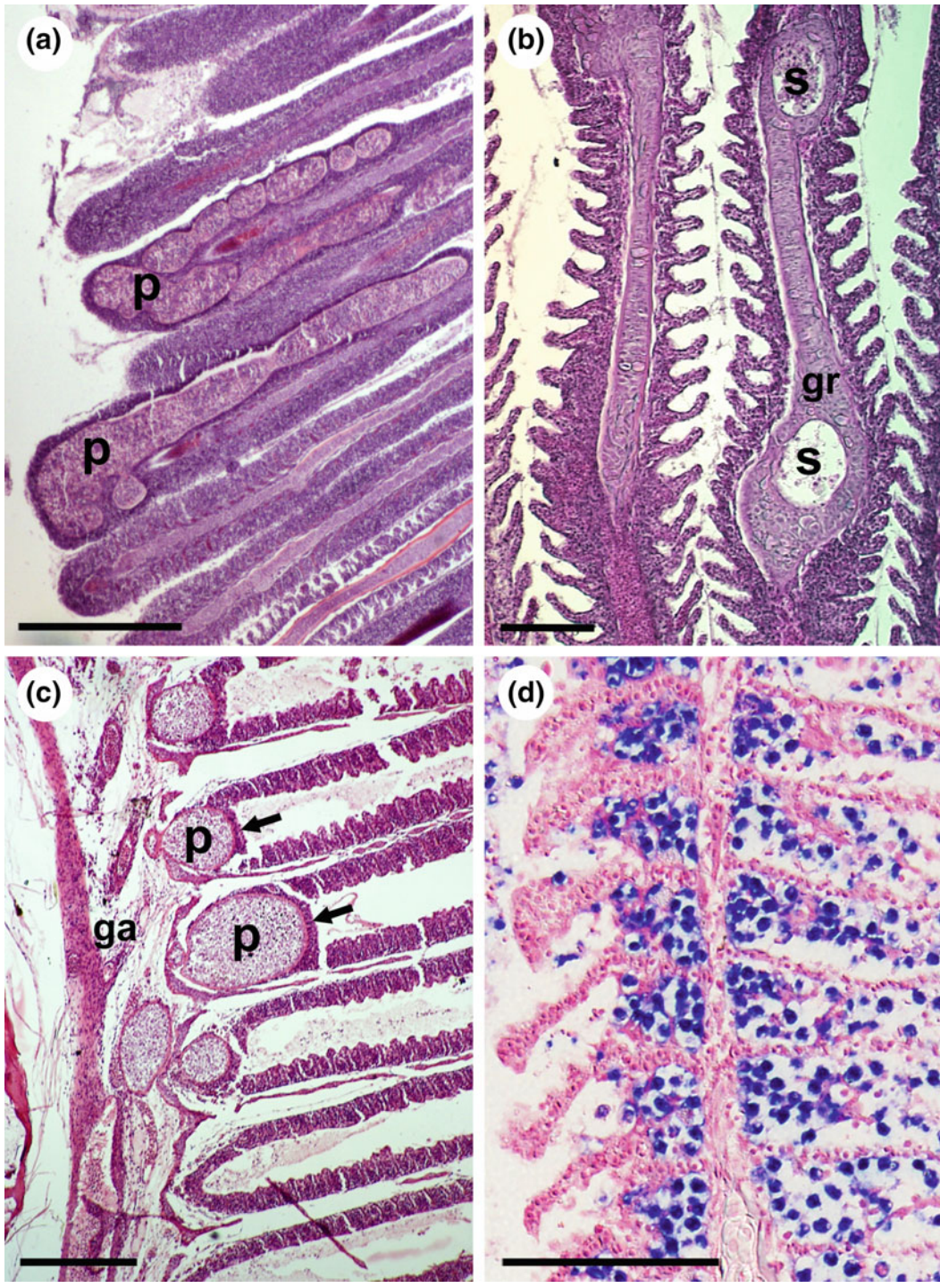

Fig. 16.3 **a** Large plasmodia (*p*) of *Myxobolus dujardini* developing in the epithelium of the gill filaments in *Squalius cephalus*. H and E. Bar = 500 µm. **b** Spores (*s*) of a *Myxobolus* sp. in a cartilaginous gill ray (*gr*) of *Lepomis gibbosus*. H and E. Bar = 100 µm. **c** Plasmodia (*p*) of *Myxobolus basilamellaris* locating basifilamentally under the basal epithelium (*arrows*) and in the cartilaginous gill arch (*ga*) of *Cyprinus carpio*. H and E. Bar = 100 µm. **d** Spores of *Sphaerospora molnari* (*dark-blue* colouration) in the interlamellar epithelium of the gill filament of *Cyprinus carpio*. ISH section, Vector blue with nuclear fast *red* counterstain. By the courtesy of Dr. Astrid Holzer. Bar = 50 µm

16.3.2 Infections in Skin

Myxosporean infection in skin is rare but easily observable. In heavy infections of *S. molnari,* which normally infects the gills of common carp, pansporoblasts and spores may develop also in the multilayered epithelium of the skin. *Myxobolus dermatobius* in Japanese eel *Anguilla japonica* and *M. kotlani* in European eel *Anguilla anguilla* produce a series of small cysts in the integumental connective tissue. Some other species, however, might form well observable, large plasmodia in the skin. For example, conspicuous bulgings from the skin are produced by *Myxobolus turpisrotundus* in Prussian carp *Carassius gibelio*. These represent large plasmodia surrounded by strong capsules formed by a collagenous fibroblast membrane (Zhang et al. 2010b).

Skin infections commonly occur in the scales. Small plasmodia of *Myxobolus squamae* and *M. squamaphilus* in barbel (*Barbus barbus*) and in common bream (*Abramis brama*), respectively, develop on the inner side of scales (Fig. 16.4a). They are in close contact with the cartilaginous layer, where they form a great number of small plasmodia with a small number of spores. On the other hand, *Thelohanellus nikolskii* in common carp and *Myxobolus episquamalis* in mullet *Mugil cephalus* form large plasmodia on the outer surface of the apical part of the scales (Egusa et al. 1990).

16.3.3 Infections in Fins

Infections in fins occur in three typical locations (Molnár 2002b). In the first case, plasmodia are located in the skin doublets between fin rays, and they develop in the loose connective tissue layer. Such site selection was observed for *Myxobolus portucalensis* infection of eel, *M. alburni* infection of bleak *Alburnus alburnus*, *M. caudatus* infection of barbel and *Myxobolus wootteni* infection of roach *Rutilus rutilus* (Fig. 16.4b). Some species develop inside the lumen of fin rays, with plasmodia occurring between the two hemisegments constituting the bony fin-rays. *Henneguya cutanea* in common bream and *Myxobolus diversus* in goldfish (*Carassius auratus*) are examples of such infections. The ectoplasm of the plasmodia of both species is bordered by a collagenous capsule surrounded by a flattened cell layer of dense connective tissue. The third location of infection is on the surface of fins, as represented by *Thelohanellus nikolskii*, which forms plasmodia of substantial size (1–5 mm in diameter) on fin surfaces of common carp. Plasmodia are surrounded by a relatively thick collageneous capsule formed by periosteal cells.

16.3.4 Infections in the Eye

The best known representative of eye infection is *Thelohanellus oculileucisci* in roach that infects the vitreous humour (Lom et al. 1987). However, infection with small plasmodia is common in the cornea and sclera in cyprinid, percid and centrarchid fishes, for example *Myxobolus heterolepis* in *Notropis heterolepis, M. corneus* in *Lepomis macrorchirus*, *M. magnus* in *Gymnocephalus cernua* and *M. scleroperca* in *Perca flavescens* (Muzzal 1995), while *M. cordeiroi* in *Zungaro yahu* infects the connective tissue of corneal stroma (Adriano et al. 2009).

16.3.5 Infections in Kidney

Myxospores and developmental stages of several myxozoan species are frequently found in the kidney. In most cases, the occurrence and development of these parasites can be attributed to a specific part or tissue of this organ. Five major sites of infection were distinguished (Molnár 2007).

Most coelozoic myxozoans developing spores in the kidney have a presporogonic stage in the blood or in the renal glomeruli before sporogony in the kidney tubules or in the ureter. Members of the genus *Sphaerospora* are common inhabitants of the renal tubules of freshwater fishes. Of the about 50 described *Sphaerospora* spp., *S. dykovae* is the best known (Dyková and Lom 1982).

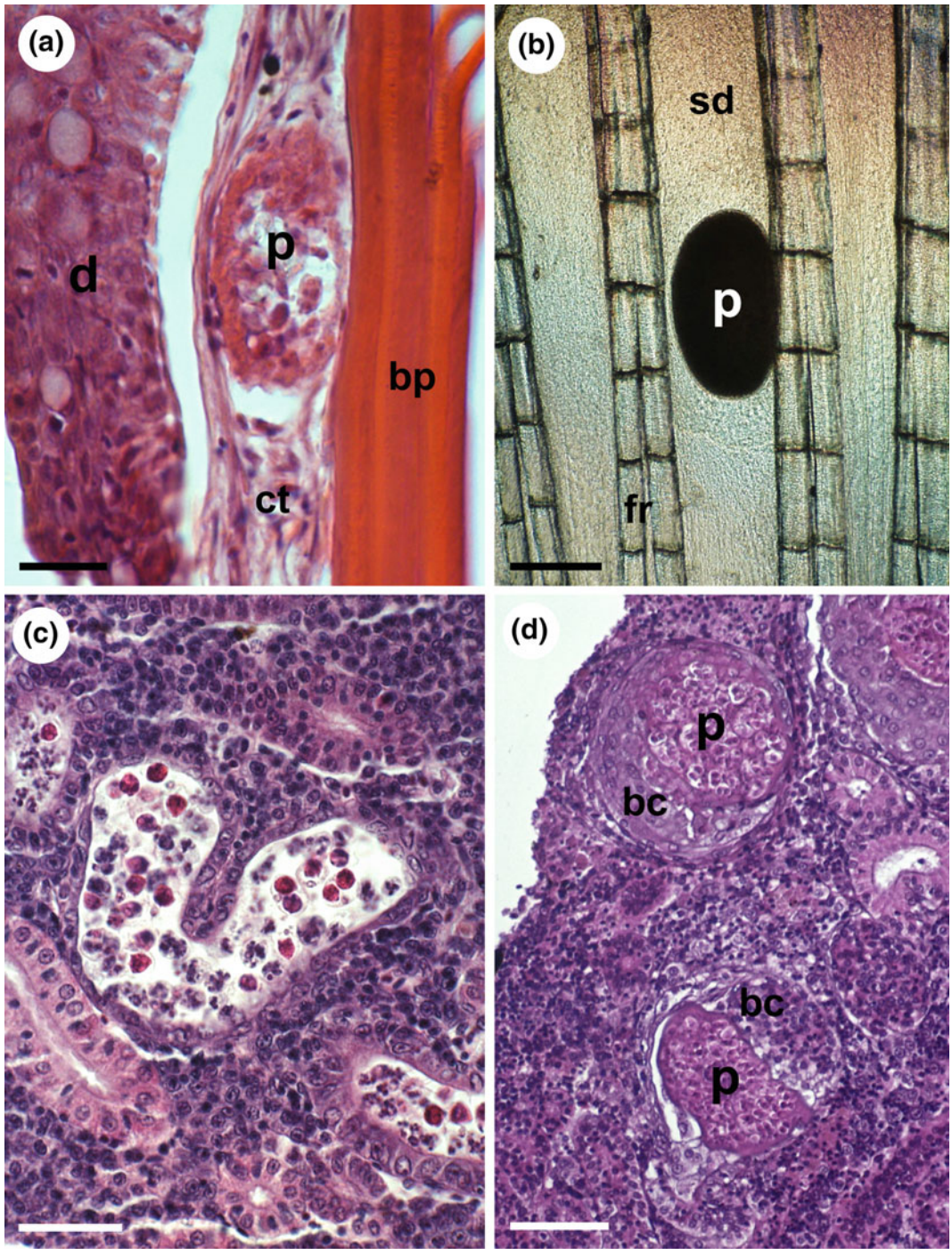

Fig. 16.4 **a** Small plasmodium (*p*) of *Myxobolus squamae* in the subdermal connective tissue (*ct*) between the dermis (*d*) and the bony plate of the scale (*bp*) of *Barbus barbus*. H and E. Bar = 20 µm. **b** *Myxobolus wootteni* plasmodium (*p*) in the skin duplet (*sd*) between two fin rays (*fr*) in *Rutilus rutilus* Bar = 1 mm. **c** Spores of a *Sphaerospora* sp. in the lumen of the urinary channel in the kidney of *Scardinius erythrophthalmus*. H and E. Bar = 20 µm. **d** *Myxidium rhodei* plasmodia (*p*) developing in the renal glomerula inside the Bowman capsules (*bc*) of *Rutilus rutilus*. H and E. Bar = 20 µm

This species has an extra-sporogonic blood stage, but its sporogony takes place in the lumen of the urinary channels (Fig. 16.4c). Blood stages enter the kidney tubules via the glomeruli of Bowman's capsules. Developing pseudoplasmodia and mature spores are usually found simultaneously in the renal tubules. For the Malacosporea, tissue specificity of *Tetracapsuloides bryosalmonae* is well known. Its presporogonic stages proliferate mostly in the renal interstitium but they are found all over the fish body (systemic infection). However, mature spores are found only in the renal tubules (Kent and Hedrick 1986).

In kidney infections of *Myxidium* and *Chloromyxum* vegetative stages often occur inside the Bowman capsule, with early developmental stages found in the renal corpuscles. Plasmodia containing primary, secondary and tertiary cells enter the tubules and form spores during the coelozoic stage. *Parvicapsula* spp. of marine fishes, including the highly pathogenic *P. minibicornis,* have a similar developmental cycle. In some *Myxidium* spp. development and sporulation takes place entirely inside the Bowman capsule, as exemplified by *M. rhodei* in roach (Fig. 16.4d).

Some members of the genera *Myxobilatus* and *Hoferellus* have an intraepithelial stage in the tubular epithelium and a coelozoic stage in the urinary channels. Vegetative stages of these myxosporeans develop in the epithelial cells of the renal tubules (Fig. 16.5a), then enter the lumen of the tubules and continue their development coelozoically. Mature spores are usually found in the urinary bladder. In the course of the development of *Hoferellus gilsoni* in European eel (Lom et al. 1986), pseudoplasmodia forming 4–8 spores attach firmly to the urothelium of the urinary bladder (Fig. 16.5b). The development of *Sphaerospora tincae* in tench *Tinca tinca* is characterized by an intercellular stage. Its pseudoplasmodia may be found throughout the renal interstitium among haematopoetic cells, and sporogony takes place in the head kidney.

Large groups of myxospores are commonly seen in the interstitial tissue of the kidney. Two types of these spore-conglomerates can be distinguished. In the first type, the spores originate from large plasmodia that develop among the haematopoietic cells. In the second, aggregations of spores captured by macrophages are found within melano-macrophage centers. These spores apparently translocate to the kidney from other organs, such as *Myxobolus pseudodispar* from the muscle of roach. These two types of "cyst-like" formations can easily be distinguished during the early stage of plasmodial development. In most cases, plasmodia of type 1 species (e.g. *Myxobolus erythrophthalmi* from rudd *Scardinus erythrophthalmus*) start their development in renal blood vessels (Fig. 16.5c) (Molnár et al. 2009). These young *Myxobolus* plasmodia contain multinucleated pansporoblasts and immature spores. More mature plasmodia are filled with hundreds of spores, and are bordered by a single cell layer of connective tissue. However, in progressed cases, a connective tissue capsule, composed of several layers can be formed around them, and it can be difficult to distinguish plasmodia and spore aggregations in melano-macrophage centers. The latter may be recognized by the presence of macrophage like cells, often containing melanin (Fig. 16.5d).

16.3.6 Infection in Intestine

A few myxosporeans form large plasmodia in the intestine (Fig. 16.6a). Of these, plasmodia of *Thelohanellus kitauei* are particularly known to cause swellings that block the lumen of the intestine in common carp (Egusa and Nakajima 1981). Most myxosporeans that develop in intestine, like *Enteromyxum leei* and *E. fugu* in marine fishes, form small pansporoblasts and spores intercellularly in the intestinal epithelium, although *E. fugu* develops also on the epithelium attached to the intestinal mucosa (Alvarez-Pellitero et al. 2008; Tun et al. 2002). Data presented by Palenzuela et al. (2002) and Redondo et al. (2003) suggest early developmental stages of *Enteromyxum scophthalmi* might also occur intracellularly. Early trophozoites of *C. shasta* were found in the connective tissue layers between the outer muscularis and epithelial

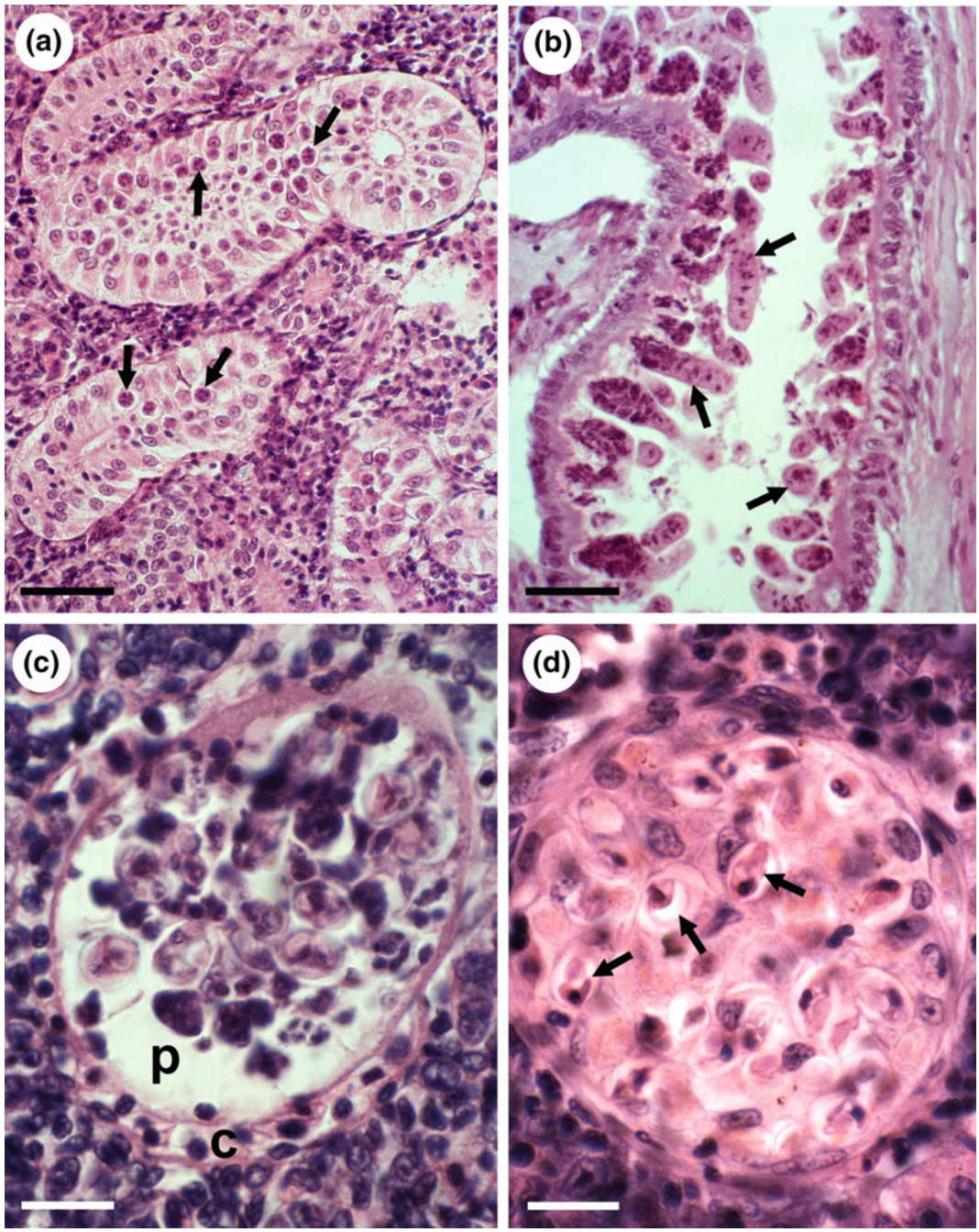

Fig. 16.5 **a** Developing trophozoites (*arrows*) of *Myxobilatus legeri* inside epithelial cells of the urinary channels in *Scardinius erythrophthalmus*. H and E. Bar = 20 µm. **b** Small plasmodia (*arrows*) of *Hoferellus gilsoni* attached to the wall of the urinary bladder of *Anguilla anguilla*. H and E. Bar = 50 µm. **c** Spores and pansporoblasts in a plasmodium (*p*) of *Myxobolus erythrophthalmi* developing in a capillary (*c*) in the kidney of *Scardinius erythrophthalmus*. H and E. Bar = 10 µm. **d** *Myxobolus* spores (*arrows*) among macrophage cells in a melano-macrophage centre (*mc*) of the kidney of *Scardinius erythrophthalmus*. H and E. Bar = 10 µm

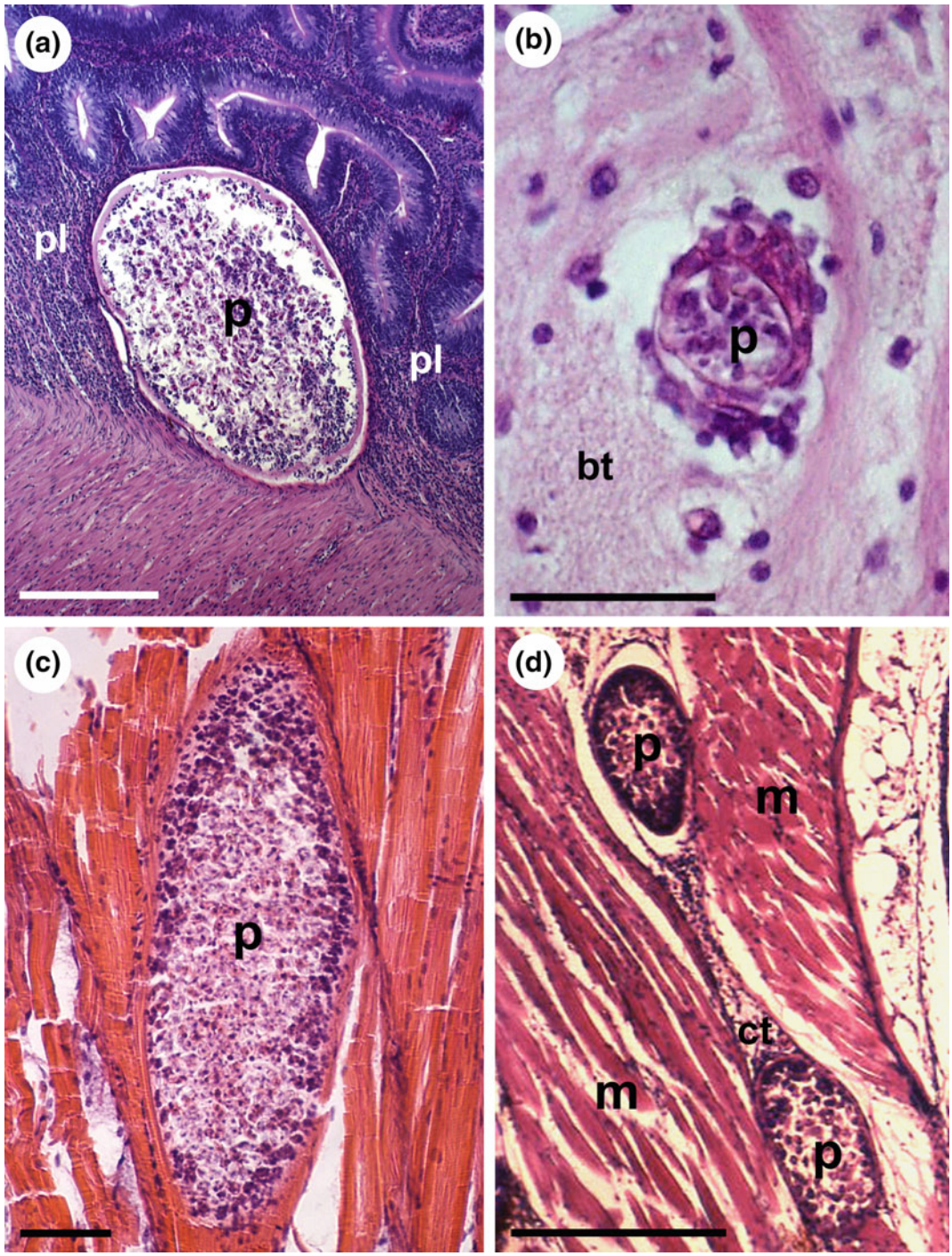

Fig. 16.6 **a** A large *Myxobolus gayerae* plasmodium (*p*) in the propria layer (*pl*) of the intestine of *Squalius cephalus*. H and E. Bar = 200 µm. **b** Cross-section of a small *Myxobolus encephalicus* plasmodium (*p*) inside a capillary in the brain tissue (*bt*) of *Cyprinus carpio*. H and E. Bar = 20 µm. **c** *Myxobolus pseudodispar* plasmodium (*p*) in a muscle cell of *Scardinius erythrophthalmus*. H and E. Bar = 100 µm. **d** *Thelohanellus hovorkai* plasmodia (*p*) in the intermuscular connective tissue (*ct*) among muscle cells (*m*) of *Cyprinus carpio*. H and E. Bar = 50 µm

layers of the intestinal caeca, then later on the surface of the epithelial cells (Bartholomew et al. 2004).

16.3.7 Infections in Liver and Gall Bladder

Infection of the liver with large plasmodia of cyst-forming myxosporeans is relatively rare. In many cases, liver infections occur together with infection of other organs. For example, *M. shaharomae* in rudd infects the blood vessels of the kidney, spleen and intestine as well as the liver. However, for *Myxobolus dombrovskayae* in *Sarcocheilichthys sinensis, M. grandis* in *Notropis buccatus*, and *Henneguya kayarensis* in the marine fish *Galeoides decadactylus*, infections are restricted to the liver parenchyma.

Infections of the biliary ducts and the gall bladder are more common. Infection with *Myxidium, Zschokkella, Chloromyxum* and *Ceratomyxa* spp. are frequently observed in the gall bladder, with early development in the walls of the bile ducts and the liver parenchyma (Dyková and Lom 1988b, 2007). An infection with large polysporic plasmodia and spores of *Myxidium tuanfengensis* were reported in the liver parenchyma of *Leptobotia teniops* (Gong et al. 2003). This location and developmental type is also characteristic of the mammal myxosporean *Soricimyxum fegati* infections in shrews (Prunescu et al. 2007) and of the avian myxosporean *Myxidium anatidum* infections in ducks (Bartholomew et al. 2008).

16.3.8 Infections in the Nervous System

Several *Myxobolus* spp, such as *M. arcticus, M. kisutchi, M. neurobius, M. neurotropus, M. fryeri* and *M. murakamii,* have been described from the central and peripheral nerve tissues of salmonid fishes (Urawa et al. 2009). Plasmodia of *M. encephalicus* from common carp and *M. balantiocheili* from tricolour sharkminnow *Balantiocheilos melanopterus* develop in the vascular system of the brain and meninges (Fig. 16.6b) (Dyková et al. 1986; Levsen et al. 2004), while *M. fryeri* forms plasmodia inside the nerve cells (Ferguson et al. 2008). Plasmodia of some *Kudoa* spp., (e.g. *K. neurophila* and *K. yasunagai*) develop plasmodia in nerve cells (Grossel et al. 2003, Zhang et al. 2010a). Intracellular developmental stages of an unidentified species were also found in pericytes of the brain capillaries of the mole *Talpa europaea* (Friedrich et al. 2000). Although *M. cerebralis* develops spores in the cartilage of skull, it has been shown that its presporogonic stages reach the cartilage via peripheral nerves and the CNS (El-Matbouli et al. 1995).

16.3.9 Infections in Cartilage

Among chondrophilic species, *M. cerebralis* is the most studied. Its small plasmodia develop in the cartilage of the skull and vertebrae, compress the brain and medulla spinalis and affect swimming behaviour. *M. buckei,* a parasite of the spinal column, develops in the cartilage and embryonic notochord in the intervertebral spaces. Infection of skeletal bones by myxosporeans is rarely observed, however, their development in bony elements of scales and fins is more common. Periosteal cells and cells of the dense connective tissue often produce collagenous material in which myxosporeans may develop. After calcification of the collagen, large plasmodia developing in this tissue (e.g. for *Thelohanellus nikolskii* and *Myxobolus tauricus*) are often surrounded by a thick capsule made of connective tissue.

16.3.10 Infections in Musculature

Infection of muscle tissue is common both in marine and freshwater fishes. Three major forms of myxosporean infection in musculature can be distinguished. Plasmodia located intracellularly within muscle cells are the most common (Fig. 16.6c). Development commences as spindle-shaped plasmodia, which are centrally located in the cell, surrounded by the rest of the

cytoplasm. Infected cells then become completely filled with spores, and the cytoplasm is no longer observable. Of the marine species, the best known is *Kudoa thyrsites* in salmonid fishes and *Kudoa paniformis* in North Pacific hake *Merluccius productus*. Both species are known from their liquefying effect on the fish muscle (Whipps and Kent 2006). In European freshwater fishes, *Myxobolus cyprini*, a parasite of common carp is well-known (Molnár and Kovács-Gayer 1985). Heavy infections of *M. musculi* occur in barbel. Roach and other cyprinids may become severely infected by *M. pseudodispar* (Molnár et al. 2002). Similar heavy infections occur in Malaysian cyprinid fishes (*Osteochilus* spp.) due to *M. terengganuensis* and *M. tasikkenyirensis* (Székely et al. 2009). In American cyprinids, *M. ridouti* and *M. ridgwayi* occur in muscles of *Pimephalus notatus* and in *Semotilus atromaculatus*, respectively. Intramuscular *Myxobolus* infections have also been recorded from non-cyprinids; for example, *M. insidiosus* in Pacific salmon and *M. intramusculi* in trout-perch *Percopsis omiscomaycus* (Easy et al. 2005).

In addition to intramuscular development, plasmodia also may develop in the intermuscular connective tissue of the musculature. Plasmodia of *Myxobolus sandrae* from the pike-perch are located within the wide myosepta (Molnár and Székely 2014). Plasmodia developing in the intermuscular connective tissue also occur in *Thelohanellus hovorkai* infections in common carp (Fig. 16.6d) and *M. pfeifferi* infections in Iberian barbel *Luciobarbus bocagei* (Molnár et al. 2012). In North America, *Myxobolus procerus* represents this type of infection by parasitising intermuscular tissues of the trout-perch (Cone et al. 1997). *M. dogieli* in the heart of common bream represents a special case of infection in intermuscular connective tissue. Its plasmodia only develop in connective tissue of the bulbus arteriosus and ventricle, and never enter muscle cells (Molnár et al. 2008). Bones in the intermuscular septa and spinal column are in close association with skeletal muscles and may become infected by myxosporean species with collagenophilic histotropism. For example, *Myxobolus tauricus* infects the fin rays, but its cysts are frequently found in muscle bones of Iberian and common barbel. Plasmodia of this species are located in the dense, collagenous connective tissue covering the bones in the muscle (Molnár et al. 2012; Molnár and Székely 2014).

16.3.11 Infections in the Swimbladder

Some myxosporeans, like *Myxobolus cycloides* forms large flat plasmodia in the connective tissue of the swimbladder wall (Holzer and Schachner 2002). Swimbladder inflammation caused by extrasporogonic stages of *S. dykovae* in common carp often occurs when the so-called K-stages of the parasite block the capillaries of rete mirabile.

16.3.12 Infections in Multiple Sites

Some myxosporean infections are not restricted to particular locations. For instance, infections in the abdominal cavity may affect several organs. *Kudoa ciliatae* is found throughout the serosal surface of the intestinal tract of *Sillago ciliata*, affecting pyloric caeca, intestinal mesentery and liver as well (Hallett et al. 1997). *Myxobolus colossomatis* in *Colossoma macropomum* and *M. cordeiroi* in *Zungaro yahu* were found in most organs in the connective tissue. Similarly loose organ specificity characterizes other species with a strict histotropism to connective tissue. Of them, *M. cuneus* from *Piaractus mesopotamicus* and *Thelohanellus hovorkai* from common carp are often found in different organs. Several species infect the serosa of the abdominal cavity, of them *Myxobolus pangasii* forms plasmodia only on the serosa of the spleen in sutchi catfish *Pangasianodon hypophthalmus* (Molnár et al. 2006).

Several myxosporeans are able to develop in the connective tissue. They form plasmodia in connective tissue inside the gill arch and gill filaments (*Myxobolus fundamentalis*, *M. rutili*) or in the intermuscular connective tissue (*M. sandrae, Thelohanellus hovorkai*) (Molnár and Kovács-

Gayer 1986). Some species with a tropism to connective tissue might infect different organs, e.g. *M. gayerae* forms plasmodia both in the gill arch and the wall of the intestine. Blood has mesenchymal origin and it is considered as a specialized form of connective tissue. Host blood represents an important location in the development of Myxozoa. Most myxosporeans start their development in small capillaries or blood vessels. A special form of this type of infection is the intralamellar infection where small plasmodia are formed in gill filament lamellae (e.g. *Myxobolus muellericus, Henneguya creplini*). For other myxosporean spp., large-sized plasmodia are formed in arteries or veins of different organs, mainly in gills (e.g. *Myxobolus muelleri*).

16.4 Sex-Specific Infection

A number of myxozoans exhibit sex specificity, infecting fish ovaries rather than testes (Sitjà-Bobadilla 2009). Sex-specific gonad infection may be caused by the specific need of the parasite, as oocytes and spermatozoa differ in composition and volume. Trophozoites of *Sphaerospora testicularis* in European sea bass *Dicentrarchus labrax* develop within the seminal fluid and feed on spermatozoa. The timing of parasite infection seems to be in synchronization with spawning, as immature sea bass with developing testes have never been found to be infected (Sitjà-Bobadilla and Alvarez-Pellitero 1993).

Myxozoans infecting gonads can alter natural processes of sex reversal in hermaphroditic fish species. For instance, a higher proportion of male sea bass infected by *S. testicularis* (Sitjà-Bobadilla 2009) possess ovo-testis (ovarian tissue in testes) possibly as a result of hormonal dysfunction invoked by the destruction of Sertoli cells with steroidogenic functions (Nóbrega et al. 2009). Females of *Thalassoma bifasciatum* with *Kudoa ovivora* infections in the ovary change sex earlier and at a smaller size, thus gaining a reproductive advantage. Such sex change represents 'host death' from the parasite's point of view, as the host tissue required for sporogony disappears (Schärer and Vizoso 2003).

16.5 Infection Specificity and Phylogeny

Characterization of myxozoan species using DNA sequence data allows connections to be drawn between host, organ and tissue specificity and the genetic relationships between species (Andree et al. 1999). As traditional species identification was based mainly on the myxospore morphology, the first phylogenetic findings based on SSU rDNA revealed the necessity of using further characters when designating species (e.g. Andree et al. 1999). One of these characters, host specificity, complemented the interpretation of phylogenetic findings by dividing species into groups with strict and broad host ranges. Organ, and particularly tissue specificity, brought new insights into the myxozoan phylogeny when these relationships could be examined in relation to site preference.

Early SSU rDNA based phylogenetic studies of *Myxobolus* spp. in salmonid and cyprinid fishes found that tissue tropism correlated better with the groupings than spore morphology or host species (Andree et al. 1999; Eszterbauer 2004). It was suggested that that new species may evolve through contact with 'new' fish hosts, and that the parasite's site preference (i.e. organ and/or tissue tropism) generally remained unchanged (Eszterbauer 2004).

Holzer et al. (2004) came to the conclusion that although relatedness according to localization was dominant in the phylogeny of freshwater myxosporeans, tissue tropism is probably not the only factor driving myxozoan evolution. Nevertheless, they concluded that myxosporeans inhabiting the urinary system clearly cluster together in SSU rDNA phylogenies, independent of host species or spore morphology. Whipps et al. (2004) found the site of infection to be an important criterion in molecular phylogenetic relationships of some *Kudoa* species. Heiniger et al. (2013) showed a clear separation of somatic, muscle-dwelling species from intestinal species, based on SSU rDNA data supporting the idea that tissue tropism is a distinguishing character between morphologically similar species.

As additional SSU rDNA sequences became available, Fiala and Bartosová (2010) analyzed the evolutionary impact of site preference, particularly organ specificity. Their findings revealed that two main splits from coelozoic to histozoic site preference occurred in myxozoan evolution, once for multivalvulids and enteromyxids, then later for *Myxobolus* and *Henneguya* spp. The analysis of organ specificity suggested that the first myxozoan inhabited the excretory system, then gall bladder. Intestine, muscle and gill infections appeared later in evolution (see Chap. 4).

Some myxozoans are able to infect a broad range of hosts that are not closely related, while others show specificity to hosts that are phylogenetically closely related. Molnár et al. (2014) showed that for *Myxobolus* spp., both host relatedness and spore morphology correlate with genetic relationship among species, thus they were relevant features in the taxonomy of genus *Myxobolus,* similar to tissue tropism. Alama-Bermejo et al. (2011) studied *Ceratomyxa puntazzi* and three other ceratomyxids from the closely related fish species *Diplodus annularis* and *Sparus aurata*, which share the same habitat, and molecular data suggested that the genus *Ceratomyxa* is host-specific in Mediterranean sparids. Gunter et al. (2009) studied *Ceratomyxa* spp. from closely related host species (i.e. confamilial) as well as from different host orders in Australia, to provide a clue to host specificity. Of the 32 examined *Ceratomyxa* species from Queensland, only *Ceratomyxa talboti* has been found in more than one host species, showing that marine *Ceratomyxa* spp. are generally restricted to a single host species (Gunter et al. 2009). Burger and Adlard (2011) identified 18 different fish species representing six different fish families that were susceptible to *Kudoa thalassomi*, the parasite species thereby having a remarkably broad host range, similar to *Kudoa thyrsites*. Studying *Myxobolus* spp. known for their strict host specificity, Molnár et al. (2011) demonstrated that some members of the genus may infect several closely related cyprinids. For example, *Myxobolus macrocapsularis*, a parasite of the gill filaments of *Blicca bjoerkna*, is able to infect *Abramis brama* as well. In the same way, *Myxobolus bliccae,* which develops plasmodia in the arteries of gill filament in *B. bjoerkna*, can also occur in *Vimba vimba* (Molnár et al. 2011). On the basis of our present knowledge, it seems that most myxozoans tend to infect one host species or closely related hosts, however experimental (i.e. cross-infection) data or more thorough surveys involving other putative host species might alter this trend.

16.6 Suggestions for Future Studies

In the description of novel myxozoan species, more attention should be paid to host, tissue and organ specificity. A basic requirement is the exact identification of the host and the host tissue in which the mature spores develop. In addition, the early stage of intrapiscine development should be studied in detail to widen our knowledge on the changes of tissue preference over development. To better understand host specificity, cross-infection experiments involving closely related fish species should be performed. The host range of malacosporean species in particular requires further investigation.

16.7 Key Questions for Future Study

- Are all early myxozoan stages intracellular?
- Does tissue specificity reflect competitive interactions and niche differentiation amongst myxozoans?
- What factors determine broad versus narrow host specificity?

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Part IV
Disease Ecology

Comparative Epidemiology of Myxozoan Diseases

17

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Abstract

Epidemiological studies are crucial to understand infectious diseases in both captive and free-ranging fish. Such studies on myxozoan fish parasites are rare or incomplete, owing to the complexity of myxozoan life cycles, confounding environmental factors and difficulties of data collection. Here we discuss how epidemiological data can be gathered for myxozoans and then compare the epidemiology of seven economically and/or ecologically important myxozoan species: *Myxobolus cerebralis*; *Ceratonova shasta*; *Tetracapsuloides bryosalmonae*; *Henneguya ictaluri*; *Enteromyxum leei*; *Kudoa thyrsites*; and *Parvicapsula pseudobranchicola*. These species were selected due to their representation in the literature, breadth of habitats and range in life histories. Specifically, we synthesise epidemiological information in relation to: life cycles; habitat types; distribution; fish host disease characteristics; actinospore and malacospore prevalence in water, infectivity and longevity; parasite transmission modes; infection prevalence and mortality in invertebrate and fish hosts; seasonality and environmental and biotic factors. We also consider available treatment and control strategies and how progress may be made in understanding myxozoan epidemiology by incorporating new research approaches.

Keywords

Free-ranging fish · Captive fish · Invertebrate hosts · Data collection · Environmental effects · Modelling

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17.1 Introduction

The Myxozoa is a group of mostly aquatic parasitic animals with complex life cycles alternating between an invertebrate definitive host and a vertebrate intermediate host. Some 2,200 species have now been described in two myxozoan classes, the Malacosporea and the Myxosporea (Lom and Dyková 2006). Transmission is achieved by spores that develop within the invertebrate and vertebrate hosts. Intermediate hosts of myxozoans are mostly teleost fishes (class Actinopterygii), captive (i.e. fishes bred in captivity for the aquaculture, aquarium and pet-shop trades) as well as free-ranging (i.e. fishes in natural environments). Some myxosporeans exploit reptiles (Barnard and Upton 1994), amphibians (Kudo and Sprague 1940), mammals (Prunescu et al. 2007) and birds (Bartholomew et al. 2008). Life cycles are inferred for some 50 myxozoan species and completed for only 5 (see Chap. 10).

Several myxozoans cause economically important diseases that damage organs, reduce growth and cause death in free-ranging and captive fish populations. For instance, *Tetracapsuloides bryosalmonae* causes proliferative kidney disease (PKD) which has been estimated to result in losses of £2.5 million/year to the UK trout farming industry (Morris and Adams 2008). Epizootics (animal disease outbreaks, Subasinghe et al. 2001), such as those of PKD, can frequently be the most profit-limiting factor not only in aquaculture (Bruno and Ellis 1996), but also in the ornamental fish trade and sport fishing (see Peeler and Taylor 2011). Understanding the drivers of epizootics is the remit of epidemiology, which is defined as the study of the occurrence of infectious diseases, their origins and pattern of spread through populations. The aims of epidemiology are to investigate the cause, ecology and control of disease (Georgiadis et al. 2001). Studying epidemiology in practice involves obtaining data on prevalence and severity of infection and determining and characterising factors influencing these estimates. Epidemiological studies on Myxozoa should include a combination of data from: fish hosts; invertebrate hosts; and the environment. The data needs for fish hosts include mortality, infection prevalence and severity in both captive and free-ranging fish populations. Invertebrate hosts should be evaluated for their presence and abundance as well as the prevalence and severity of infection. Water samples may also be collected to obtain data on the presence and abundance of myxozoan spores in the environment. Epidemiological studies on myxozoan fish parasites are rare or incomplete, owing to the complexity of myxozoan life cycles, confounding environmental factors and difficulties of data collection.

Here we discuss how epidemiological data can be gathered for myxozoans and then compare the epidemiology of seven economically and/or ecologically important myxozoan species: *Myxobolus cerebralis*, *Ceratonova shasta*, *T. bryosalmonae*, *Henneguya ictaluri*, *Enteromyxum leei*, *Kudoa thyrsites*, and *Parvicapsula pseudobranchicola*. These species were selected due to their representation in the literature, breadth of habitats and range in life histories. Specifically, we synthesise epidemiological information in relation to: life cycles; habitat types; distribution; fish host disease characteristics; actinospore and malacospore prevalence in water, infectivity and longevity; parasite transmission modes; infection prevalence and mortality in invertebrate and fish hosts; seasonality and environmental and biotic factors. We also consider available treatment and control strategies and how progress may be made in understanding myxozoan epidemiology by incorporating new research approaches.

17.2 Gathering Epidemiological Data

The most obvious and common method to study the prevalence and severity of infection in free-ranging or captive fish populations is to sample fish directly. Fish samples are collected by electrofishing, screw traps, gill nets, seining, trawling, weirs and hook and line fishing. Depending upon available and approved protocols (e.g. American Fisheries Society-Fish Health Section Blue Book 2012), ecological status and the economic value of individual fish (usually low), fish may be sampled lethally or non-lethally. Fish

infected with intestinal parasites such as *C. shasta* can be sampled non-lethally using either an intestinal lavage (Coley et al. 1983) or an intestinal swab (Fox et al. 2000) for adults and juveniles, respectively. However, lethal sampling is the most common procedure for routine diagnostic tests of fish (Oidtmann et al. 2013). Tissue may be taken for wet mount observation of spores (species identification), histology (for information on site and severity of infection within the host, e.g. Neudecker et al. 2012; True et al. 2013) or highly sensitive and specific molecular analyses that increase infection detection (e.g. PCR for pathogen detection and identification, quantitative PCR for pathogen quantification, Bartholomew et al. 2004). The higher sensitivity of molecular methods enables detection of light and/or early infections but researchers should bear in mind that detection of a pathogen does not necessarily indicate disease. Statistical analyses of parasite prevalence estimates should incorporate the effects of imperfect parasite detection (Miller et al. 2012). The larger the sample size the more accurate the estimate of prevalence and if prevalences are low this can entail substantial numbers of fish. For instance, 150 fish are required to detect a 2 % prevalence assuming a perfect test at 95 % confidence level (and no population clustering, Oidtmann et al. 2013). Captive fish generally are easy to sample and obtain large sample sizes. Studies of free-ranging fish, however, often rely on limited and opportunistic sampling due to moral, aesthetic, political, social and financial considerations (Elliott 1994; Crisp 2000). Accurate sampling of diseased fish, however, may be difficult. For instance, in populations of free-ranging fish diseased individuals may be quickly removed by predators, although direct demonstrations of this hypothesis are rare (Miller et al. 2014). Furthermore, accurately assessing mortality rates in fish populations requires controlling for the proportion of deaths over time caused by other factors. Finally, inferring where and when infection of free-ranging fish occurred and the duration of exposure to a parasite may be obscured by fish movements (e.g. migration). In captive fish, accurate sampling of diseased individuals may be difficult as they are amongst thousands of other fish (Oidtmann et al. 2013). Sampling the appropriate age/size class before, during and following a suspected outbreak may help to minimise this sampling problem in captive fish. Aquaculture practices also allow the random sampling of fish during grading, vaccinations, stocking or transfers at set times or intervals. Such random sampling of free-ranging populations is difficult (Oidtmann et al. 2013).

As a proxy for sampling free-ranging fish, sentinel fish exposures can be performed where groups of uninfected fish are held in cages at a relevant site for several days to weeks (e.g. Stocking et al. 2006; Wise et al. 2008; Granath and Vincent 2010). The fish may then be processed in the field or transported to a laboratory where they can be held on pathogen-free water and monitored for the onset of disease signs prior to sampling. Despite potentially providing an under- or over-estimation of parasitism (e.g. due to having a fixed location or being affected by other pathogens), sentinel fish exposures can provide information on the incidence of infection and parasite load at a specific location because the duration of exposure is known. Sentinel exposures are not compromised by horizontal transmission from other fish (since this is absent in most myxozoans) but interpreting mortalities may be complicated by the presence of other non-target pathogens. In captive populations, the resident fish are effectively a sentinel population exposed to water sources and other factors affecting transmission.

Searching for invertebrate hosts in the field can provide information on point sources of fish infection. Invertebrate hosts are not typically highly valued economically or ecologically, and sampling may require fewer permits. Sampling efforts can be hampered by patchy distributions or low abundances of hosts and of associated myxozoan infections (Stocking et al. 2007; Zielinski et al. 2011). A large sample size may therefore be required to address confidence intervals (see Granath and Vincent 2010). Invertebrate hosts are frequently targeted for management applications.

However, the fact that these hosts are unknown for many myxozoans is a limiting factor for many epidemiological studies.

An alternative to sampling hosts is direct sampling of waterborne parasite stages. Water samples can be collected manually or using a programmable automated sampler (Hallett et al. 2012). Myxozoans with large spores (e.g. actinospores of *M. cerebralis*) can be concentrated on a glass bead filter and quantified microscopically (Lukins et al. 2003). This approach can be used to process large volumes of water but lacks specificity because actinospores of several species are morphologically similar. A more specific method incorporates quantitative PCR. Protocols for the filtration, DNA extraction and molecular analysis of water samples to determine parasite density have been developed for at least the following five myxozoans: *C. shasta* (Hallett and Bartholomew 2006), *P. minibicornis* (Hallett and Bartholomew 2009) and *T. bryosalmonae* (Fontes et al., unpub. data) in rivers; *H. ictaluri* in aquaculture ponds (Griffin et al. 2009); and *Ceratomyxa puntazzi* in seawater (Alama-Bermejo et al. 2013). Water sampling can be more cost effective and less labour intensive than direct sampling of hosts and avoids lethal host sampling. It also enables more frequent, extensive and faster data collection and generation and thus provides higher resolution of temporal and spatial distributions of actual transmission stages and semi-real time data ($\sim$1 week from collection). Such data may be important to collect since infection pressures may alter during the course of an epizootic, causing changes in parasite distributions (Taylor et al. 2011). However, molecular analysis of water samples, while specific, sensitive and quantitative, does not distinguish between infectious stages (data will reflect concentrations of both myxospores and actinospores), nor does it indicate parasite viability.

By combining several approaches, multiple data can be collected in tandem and relationships, such as dose and mortality in specific fish strains, can be determined (e.g. Hallett et al. 2012). Whatever the selected approach, consistency in methodology and the provision of explicit details across samples is essential for insightful long-term comparisons. Furthermore, collaborations among epidemiologists, ecologists, veterinarians, fish health managers and aquaculturists are important to study epizootics in aquatic environments (Georgiadis et al. 2001). Data can be analysed in multiple ways to identify the routes of parasite transmission, to examine disease impacts by theoretical modelling and risk assessment (see Chap. 20) and to undertake biostatistical and economic analysis (Thrusfield 2013). Captive fish populations are ideal for developing and testing epidemiological models, particularly in freshwater, because they can provide large homogeneous groups of individuals and various factors can be easily manipulated. Control measures can also be more readily implemented in aquaculture than in natural settings. Observational studies are, however, difficult to fund in cases where the host is not of commercial interest since they can be costly and labour intensive (Peeler and Taylor 2011).

17.3 Comparative Epidemiology

Here we compare and contrast the epidemiology of seven myxozoans associated with economically important diseases: *M. cerebralis*; *C. shasta*; *T. bryosalmonae*; *H. ictaluri*; *E. leei*; *K. thyrsites*; and *P. pseudobranchicola*. Specifically, we synthesise available epidemiological information in relation to various aspects of their life cycles, ecology, environment and effects on fish hosts.

17.3.1 Life Cycles, Habitat and Distribution

The hosts associated with the complex life cycles of the seven focal myxozoan species are provided in Table 17.1. For some, the entire life cycle is resolved, while in others only the fish host is known (e.g. *E. leei*). All seven parasites occur in the temperate regions of the northern hemisphere (USA, Europe and Asia; Table 17.1). Four species (*M. cerebralis*, *C. shasta*, *T. bryosalmonae* and *H. ictaluri*) occur in fresh waters

and appear to be host-specific at the family level infecting mostly salmonid fishes as intermediate hosts (except *H. ictaluri* which infects catfish). The marine myxosporeans, *E. leei* and *K. thyrsites* have a broad fish host range (Whipps and Kent 2006). *P. pseudobranchicola*, also a marine species, has been found only in anadromous salmonids. Oligochaetes or polychaetes are used as definitive hosts apart from the malacosporean, *T. bryosalmonae*, which uses freshwater bryozoans.

17.3.2 Fish Host Disease

Mature spores released by the definitive host contact the fish skin or gills whereupon amoeboid sporoplasms penetrate the epithelium and begin to multiply (see Chap. 13 for review of host invasion processes). Early parasite stages migrate to the final site of infection where further proliferation occurs and spores develop. Spores may be released back into the water via defecation or with urine to re-infect the definitive hosts. Alternatively, spores in tissues may only be released upon death of fish hosts (Table 17.2). Myxospores from both *M. cerebralis* and *C. shasta* are mostly released from dead fish (spawned adults for *C. shasta*) although there have been reports of release from live fish (e.g. juveniles in *C. shasta*). *T. bryosalmonae* is released from live fish but only from certain species of salmonids (see Table 17.2); non-transmitting fish act as dead-end hosts. Similarly, not all *M. cerebralis*-infected fish develop clinical disease (i.e. resistant fish; MacConnell and Vincent 2002); these hosts may act as cryptic carriers.

The diseases considered here may affect various organs and tissues (see Table 17.2), in some cases directly causing death (e.g. *C. shasta*, Udey et al. 1975; and *M. cerebralis*, Granath and Vincent 2010). Sub-clinical-PKD stimulates the fish's nonspecific defense mechanisms (Foott and Hedrick 1990), which in combination with secondary infections and stressful conditions can lead to high mortalities (Seagrave et al. 1981; Schmidt et al. 1999). Sub-lethal effects of *T. bryosalmonae* infections include reduced biomass and fish health, assessed by hepatic 7-ethoxy-resorufin O-deethylase (EROD) activity, organ histopathology and gross biometric indices (Zimmerli et al. 2007). *M. cerebralis* infections cause a tail chasing movement (whirling) in fish 2–3 months after infection (Schaperclaus 1931). Infections cause lethargy in fish for *C. shasta* (Bartholomew et al. 1989) and *P. pseudobranchicola* (Karlsbakk et al. 2002) and this effect is likely to occur for the other focal species.

Naïve fish of any life stage are susceptible to myxozoan infections. However, juvenile fish are most at risk from disease caused by *M. cerebralis*, *T. bryosalmonae* and *H. ictaluri* whereas adult fish are most at risk of contracting soft flesh disease which is caused by *K. thyrsites* (see Table 17.2). Parasite development and disease progression is temperature dependent. For example, the mean time from infection with *C. shasta* to death for rainbow trout *Oncorhynchus mykiss* is 19 days at 20.5 °C but increases to 55 days at 12 °C (Udey et al. 1975). There is evidence that fish are able to recover from myxozoan disease (for references see Table 17.2). However, infections may persist for months or even years after disease recovery (e.g. *T. bryosalmonae*; Abd-Elfattah et al. 2014). Knowledge of myxozoan diseases (i.e. how the parasite develops within fish hosts and what clinical symptoms arise) can make the assessment of disease prevalence in fish populations more specific. If a particular life-stage is more susceptible to a disease, then resources can be aimed at focusing on those life stages when funding is scarce for epidemiological studies.

17.3.3 Actinospore and Malacospore Prevalence in Water, Infectivity and Longevity

The number of spores present in water samples has been estimated by real-time PCR for both *C. shasta* (Hallett and Bartholomew 2006; Hallett et al. 2012) and *H. ictaluri* (Griffin et al. 2009). These studies demonstrate a temporal and spatial variation in actinospore concentration for both

Table 17.1 Characteristics of the life cycle, habitat and distribution of seven myxozoan species

Parasite	Myxozoan class	Habitat	Distribution	Definitive host		Intermediate host	
				Class	Species	Family	Species
Myxobolus cerebralis	Myxosporea	FW	USA, Europe, Asia, Africa, Oceania (Barnard and Upton 1994; Hallett and Bartholomew 2012)	Oligochaeta	*Tubifex tubifex* (Markiw and Wolf 1983)	Salmonidae	Most salmonids (El-Matbouli et al. 1999a; Kallert et al. 2009)
Ceratonova shasta	Myxosporea	FW	Pacific Northwest USA (Wales and Wolf 1955)	Polychaeta	*Manayunkia speciosa* (Bartholomew et al. 1997)	Salmonidae	Most salmonids (Bartholomew 2012)
Tetracapsuloides bryosalmonae	Malacosporea	FW	Europe, western North America (Hedrick et al. 1993)	Phylactolaemata	E.g. *Fredericella sultana* (Longshaw et al. 1999)	Salmonidae and Esocidae	Most salmonids, northern pike (Ferguson and Needham 1978; Seagrave et al. 1981; Hedrick et al. 1984; Ellis et al. 1985; Bucke et al. 1991)
Henneguya ictaluri	Myxosporea	FW	USA (Pote et al. 2000)	Oligochaeta	*Dero digitata* (Burtle et al. 1991; Styer et al. 1991)	Siluriformes	Channel catfish (Pote et al. 2000)
Enteromyxum leei	Myxosporea	SW	Mediterranean, Japan (Yasuda et al. 2005; Rigos and Katharios 2010)	na	na	Sparidae, Tetraodontidae, Paralichthyidae, Mugilidae, amongst others	E.g. gilthead seabream, tiger puffer (Diamant et al. 1994; Yanagida et al. 2004)
Kudoa thyrsites	Myxosporea	SW	Pacific and Atlantic Oceans (Langdon et al. 1992)	na	na	Salmonidae, Gempylidae, Merlucciidae, Pleuronectidae and Aulorhynchidae	E.g. Atlantic salmon, Pacific hake, and tube-snout (Kabata and Whitaker 1981; Harrell and Scott 1985; Shaw et al. 1997)
Parvicapsula pseudobranchicola	Myxosporea	SW	Norway (Sterud et al. 2003)	na	na	Salmonidae	Rainbow trout, Atlantic salmon, sea trout, Arctic charr (Karlsbakk et al. 2002; Nylund et al. 2005; Jørgensen et al. 2011)

All parasites presented affect captive and free-ranging fish, except *Henneguya ictaluri* which has only been reported from captive fish.
FW freshwater, *SW* seawater
na data not available

Table 17.2 Characteristics of the diseases in fish caused by seven myxozoan parasites

Parasite	Disease	Main affected tissues	Susceptible life stages	Spore release	Recovery
Myxobolus cerebralis	Whirling disease	Central nervous system, cartilage (El-Matbouli et al. 1995, 1999a)	Juveniles (before the cartilage ossifies and central nervous system is fully developed, Ryce et al. 2005)	From live fish (Taylor and Haber 1974; Nehring et al. 2002) but mostly from dead fish (Hedrick et al. 1998; Hallett and Bartholomew 2008)	Yes (Miller and Vincent 2008; Granath and Vincent 2010)
Ceratonova shasta	Enteronecrosis	Intestine (Bjork and Bartholomew 2010)	Juveniles (True et al. 2013) Adults (Foott et al. 2009), particularly those pre-spawning (Zinn et al. 1977; Ratliff 1981; Ching and Munday 1984a, b; Mann et al. 2011)	From juvenile fish but mostly from spawned dead adults (Fogerty et al. 2012)	Yes (Bjork et al. 2014; Ibarra et al. 1994)
Tetracapsuloides bryosalmonae	Proliferative kidney disease	Kidney (Hedrick et al. 1993)	Juveniles (Feist and Longshaw 2006)	Urine of brown trout (Morris and Adams 2006b) and brook trout but not from rainbow trout or grayling (Grabner and El-Matbouli 2008).	Yes (Foott and Hedrick 1987)
Henneguya ictaluri	Proliferative gill disease	Gills (Bowser and Conroy 1985; MacMillan et al. 1989)	Juveniles (Wise et al. 2004)	na	Yes (Wise et al. 2008)
Enteromyxum leei	Enteromyxosis	Gut epithelium (Diamant 1992; Diamant et al. 1994; Diamant 1997)	na	na	na
Kudoa thyrsites	Soft flesh disease	Musculature (Willis 1949)	Adults (Shaw et al. 1997; St-Hilaire et al. 1998)	na	Yes (Whitaker and Kabata 1987; Moran et al. 1999)
Parvicapsula pseudobranchicola	Parvicapsulosis	Pseudobranchs (Karlsbakk et al. 2002; Sterud et al. 2003)	Smolts transferred to salt water (Sterud et al. 2003)	na	na

na data not available

species. Just a few spores released from invertebrates are sufficient to infect fish (e.g. one *T. bryosalmonae* malacospore, Longshaw et al. 2002; McGurk et al. 2006; 2–20 *M. cerebralis* actinospores, Hallett and Bartholomew 2008; one *C. shasta* actinospore, Bjork and Bartholomew 2009), which facilitates transmission to fish hosts. Accuracy of epidemiological models could be improved by incorporating the rates of spore release from fish and invertebrate hosts.

The longevity of both spore stages is temperature dependent. Spores released from invertebrates may be viable for just a few days, such as those of *T. bryosalmonae* (12–24 h at 16–17 °C; De Kinkelin et al. 2002), or persist for a week or longer, such as those of *M. cerebralis* (6–15 days at 7–15 °C; Markiw 1992, El-Matbouli et al. 1999b) and *C. shasta* (6–18 days at 4–20 °C; Bjork 2010). Similarly, myxospores can have relatively long or curtailed life-spans depending on temperature. For instance, at 18 °C, 15 % of *C. shasta* spores were viable after 1 month and 10 % after 2 months. At 21 °C, 5 % of *C. shasta* spores were viable after 1 month and 0 % after 2 months. However, at 6 or 14 °C, 50 % are viable after 3 months (Chiaramonte 2013).

17.3.4 Parasite Transmission

The aquatic environment provides a variety of possible transmission routes for myxozoan spores, creating a potentially complex contact network. Control of contact with spores will therefore be difficult, particularly in the vast marine environment. Hence, the more we understand about how myxozoans are transmitted between and within hosts the easier it will be to identify potential mechanisms of disease control through epidemiological modelling as the models developed will be closer to reality (see Sect. 17.3.7).

Natural horizontal transmission is a rapid means of transmitting infection from fish-to-fish that is rare in the Myxozoa and of the seven focal myxozoan species, has only been shown for *E. leei*. In captive fish populations this is achieved during cohabitation (31.6 % success), by ingestion of stages present in the effluent from a tank with diseased fish (33.3 % success) and by the ingestion of infected gut tissue (13 % success) (Diamant 1997). Such transmission must occur quickly since the developmental stages of *E. leei* are short-lived. Free-ranging fish could be reservoirs for the infection (Golomazou et al. 2006). Myxozoans with the capacity for horizontal transmission between fish will pose the most serious threats for aquaculture and conservation (Feist and Longshaw 2006) as infectious spores from definitive hosts may either be unnecessary for disease development or the life cycle may have evolved to a simple one-host system.

Vertical transmission in definitive hosts appears to be a common feature of malacosporeans (see Chap. 11 for further review). *T. bryosalmonae* has been shown to pass to dormant asexual propagules (statoblasts) that undergo dispersal (Abd-Elfattah et al. 2013) and also to daughter colonies produced by fragmentation and fission (e.g. Morris and Adams 2006a; Hill and Okamura 2007). Vertical transmission of myxosporeans occurs within two annelid species: *Lumbriculus variegatus* (unidentified triactinomyxon, Morris and Adams 2006a); and *Nais communis* (*Myxobilatus gasterostei*, Atkinson et al. 2009). There is no evidence for vertical transmission in fish. Vertical transmission may enable indefinite persistence in highly clonal local populations of bryozoans thereby contributing to annual outbreaks of PKD (Okamura et al. 2011) and enabling parasite co-dispersal across great distances with bryozoan hosts (Abd-Elfattah et al. 2013).

17.3.5 Infection Prevalence and Mortality in Fish and Invertebrate Hosts

Studies recording infection prevalence and mortality of fish hosts have been conducted for most of the seven focal myxozoan species. Prevalence estimates are widely available for captive fish and less abundant for free-ranging fish. For instance, no prevalence data is available for

E. leei in free-ranging fish populations whereas there are many reports in captive fish (e.g. Diamant et al. 2006; Pellitero et al. 2008). Mortality estimates however, are rarely reported and, as expected, are mostly available for captive fish as they are easier to obtain. Mortality estimates for free-ranging fish are only available for *C. shasta* (40–50 %, True et al. 2013) and *T. bryosalmonae* (70–80 %, Sterud et al. 2007). Prevalence of infection in captive fish generally does not vary as much as those in free-ranging fish populations and often reaches much higher levels. For example, prevalences of *T. bryosalmonae* range from 60 to 100 % in captive fish (e.g. Seagrave et al. 1981; Clifton-Hadley et al. 1987; Skovgaard and Buchmann 2012) and from 0 to 100 % in free-ranging fish (e.g. Feist et al. 2002; Wahli et al. 2007). Also, *C. shasta* prevalence estimates can reach levels above 90 % in captive fish (Tipping 1988; Bartholomew et al. 2004) whereas in free-ranging fish these levels range from 0 to 100 % (Slezak 2009; Foott et al. 2010; True et al. 2013). Prevalence estimates are typically much higher than mortality estimates, which tend to vary from relatively low to high values in most of our focal myxozoan taxa. For example, in captive fish, mortality estimates of *E. leei* range from 0.001 to 7.7 % (e.g. Diamant 1992; Cuesta et al. 2006) whereas prevalence estimates range from 30 to 100 % (e.g. Athanassopoulou et al. 1999; Rigos et al. 1999; Diamant et al. 2006; Golomazou et al. 2006; Muñoz et al. 2007; Alvarez-Pellitero et al. 2008). Also, mortality of rainbow trout attributed to infection by *T. bryosalmonae* may be very low in its native region (0.04–6 %, Foott and Hedrick 1987) but very high when naïve fish are exposed to exotic strains of the same species (10–75 %, Ferguson and Needham 1978; Ferguson and Ball 1979; Ferguson 1981).

Prevalence and mortality estimates can be difficult to compare because of variation in the methodologies used. Some studies collecting prevalence data focus on juvenile fish (e.g. Mo et al. 2011), others on adults (e.g. Foott et al. 2010) and some on both (e.g. Langdon et al. 1992; Shaw et al. 1997; St-Hilaire et al. 1998). Also, there are instances where prevalences are estimates for whole catchments (e.g. Peeler et al. 2008) and others individual rivers (e.g. Zimmerli et al. 2007), each of which will vary in size. Mortality rates may vary in timescale and are often confused with percentage mortality although prevalence per se is not linked with time. For example, the percentage mortality of *K. thyrsites* in captive fish is analysed per day in Harrell and Scott (1985) whereas that of *H. ictaluri* is analysed over a two-year period in Bowser and Conroy (1985). Comparisons between mortality estimates can also be compromised if these include large confidence intervals due to small sample sizes. In addition, myxozoan co-infections are not uncommon and further confound data analyses. Finally, the geographical region as well as specific aquaculture facilities may have significant effects on prevalence and mortality as a result of different environmental conditions (see Sect. 17.3.6).

Despite the often severe disease signs associated with myxozoan infections, fish often recover (Table 17.2). Therefore for certain myxozoans, such as *C. shasta*, percent mortality, rather than prevalence of infection, may be a more informative measure of population level effects (Hallett et al. 2012). For others however, prevalence of infection is the only measure that is possible to obtain, as the parasite does not cause mortality of fish hosts (e.g. *K. thyrsites*, Dawson-Coates et al. 2003). A further issue to be aware of is that many mortality estimates are likely to be made specifically because of severe disease outbreaks (e.g. Sterud et al. 2007). Thus background mortality levels may be poorly understood. Despite these various caveats, there is no doubt that some myxozoan species can cause substantial mortality in fish populations (see Chap. 21).

Mortality effects on free-ranging fish populations have been demonstrated for *M. cerebralis*, *C. shasta* and *T. bryosalmonae* and include reductions in survival, recruitment and catch declines (see Chap. 21). Infections of rainbow trout by *M. cerebralis* can reduce survival and recruitment of the age = 0 class and alter population structure (Granath and Vincent 2010). *M. cerebralis* infections may also change the overall species composition as susceptible trout are replaced by more

resistant species (Baldwin et al. 1998; Granath et al. 2007; Miller and Vincent 2008). Infections of *C. shasta* are similarly associated with reduced survival when fish migrate through areas of high parasite density (Fujiwara et al. 2011), thus reducing recruitment and the number of returning adult Chinook salmon *Oncorhynchus tshawytscha* (Stone et al. 2008; Fujiwara et al. 2011).

Prevalence of the focal taxa in definitive hosts is not well studied, in part because most of these hosts are unknown. Infection levels are generally low for myxosporeans (e.g. *M. cerebralis* <10 %, Rognlie and Knapp 1998; Zendt and Bergersen 2000; Beauchamp et al. 2002; DuBey and Caldwell 2004; Granath and Vincent 2010; and *C. shasta* <1–8 %, Stocking and Bartholomew 2007; Alexander et al. 2014; see also Chap. 12). Prevalence estimates are often higher for *T. bryosalmonae* in bryozoan host populations (e.g. up to 15 % based on observation of mature spore-producing stages; Tops et al. 2006) and sometimes reach 100 % based on PCR assays (Fontes et al., unpub. data). The lack of detection of covert infections in bryozoan populations (see Chap. 11) will underestimate infection prevalence estimates when these are based on dissection and visual observations of mature spore-producing stages. In contrast to the vertebrate host, mortality estimates for invertebrate hosts of myxozoans are sparse, derived only from laboratory experiments with *M. cerebralis* and *T. tubifex,* and tend to report survival rather than mortality. *M. cerebralis* can affect overall survival of *T. tubifex* populations exposed to *M. cerebralis* with survival inversely proportional to parasite production level (Hallett et al. 2009; see Chap. 12 for sub-lethal effects of myxosporeans on annelids).

17.3.6 Seasonality and the Effect of Environmental and Biological Factors

Seasonality of myxozoan parasites is generally one of the first epidemiological factors to be analysed, hence information is available for most of our species of interest. Myxozoan-caused fish diseases in temperate regions tend to occur from spring to autumn, when temperatures are higher (~15–20 °C) (see Table 17.3). Spore release from invertebrate hosts tends to occur mostly in spring and summer, which may be an adaptation to synchronise with hatching and growing seasons of larval fish (Yokoyama et al. 2012), whereas periods of fish spore release are more variable (see Table 17.3). For example, *M. cerebralis* myxospores may be released from salmonids throughout the year, following development of clinical whirling disease or death post spawning. Although infection is largely seasonal and fry are most sensitive, infection of salmonids can occur at any life stage since they possess cartilage (the target tissue) in their skeleton throughout their lives (Markiw 1991).

Temperature exerts the clearest and most consistent effects on the focal myxozoan species. Optimal temperatures for the development of clinical disease tend to be around 15–20 °C in temperate regions (see Table 17.3). The temperature ranges for invertebrate and fish spore release have been assessed for a limited number of species and hosts (Table 17.3). Infections tend to decrease below optimal temperatures although sometimes fish still become or remain infected (e.g. *C. shasta*, Ching 1984; *T. bryosalmonae*, Gay et al. 2001). At higher temperatures invertebrate hosts are generally more abundant (e.g. Hartikainen et al. 2009). This greater abundance of invertebrate hosts along with the direct effects of temperature in accelerating the development of myxozoans (e.g. El-Matbouli et al. 1999b; Tops et al. 2006) are likely to contribute to higher infection prevalences, greater severity of infection and increased mortality of fish (see various examples in Table 17.4). Thermal accumulation rates (degree-days) may be a more accurate indicator (Chiaramonte 2013) as high *M. cerebralis* infection risk occurs over a broader temperature range (Neudecker et al. 2012).

Other environmental and biological factors also affect myxozoan infections (Fig. 17.1). Table 17.4 summarises the results of many studies that provide evidence for environmental and biological factors that impact on disease development. Common factors include

Table 17.3 Timing and optimal temperatures that promote disease development and spore release and the effects of temperature on various aspects of the biology of the focal myxozoan species

Parasite	Timing and optimal temperature			Temperature effects
	Fish disease	Invertebrate host spore release	Fish host spore release	
Myxobolus cerebralis	Spring creeks: Late fall to early spring at 4.5–13 °C (Neudecker et al. 2012) Rivers: Late spring to early fall (Thompson and Nehring 2000; Sandell et al. 2001; Downing et al. 2002; Murcia et al. 2006; Neudecker et al. 2012) at 1.7–12.5 °C (Neudecker et al. 2012)	Timing coincides with emergence of juvenile fish (MacConnell and Vincent 2002; Neudecker et al. 2012) and occurs at 10–15 °C (Halliday 1976; El-Matbouli et al. 1999b)	All year (Markiw 1991) at 15–17 °C (Halliday 1976)	• Higher temperatures reduce actinospore viability (Markiw 1992; El-Matbouli et al. 1999b), accelerate infection development (although infections can be purged at >15 °C, El-Matbouli et al. 1999b) and increase infection prevalence and intensity in free-ranging fish (Baldwin et al. 2000; Hiner and Moffitt 2001; De la Hoz Franco and Budy 2004; Krueger et al. 2006) • High temperatures and low specific conductivity jointly increase pathology (Murcia et al. 2011) • Temperature increases morbidity and mortality (Schisler et al. 2000)
Ceratonova shasta	April–November peaking in the summer at 18 °C (Ray et al. 2012)	Early spring, when salmonids migrate, (Hallett et al. 2012) peaks at 18 °C decreasing at 23 °C (Ray and Bartholomew 2013)	Juveniles: Late spring/early summer Adults: Autumn/winter	• Infections can occur at 4 °C (Ching 1984) but disease develops at >10 °C (True et al. 2013) • Higher temperatures increase replication within fish (Bartholomew 1998) and accelerate disease progression in fish (Udey et al. 1975; Hallett et al. 2012; Ray et al. 2012)
Tetracapsuloides bryosalmonae	April–October (Clifton-Hadley et al. 1985; Foott and Hedrick 1987) peaking in May (Ferguson and Ball 1979) at 12–20 °C (Clifton-Hadley et al. 1985; Foott and Hedrick 1987)	Spring and autumn (Tops et al. 2006)	na	• PKD inhibited at <12 °C (Ferguson 1981) but the parasite is still transmitted to fish (Gay et al. 2001) • Disease progresses more quickly at >15 °C and is associated with increased *T. bryosalmonae* numbers in the kidney and host mortality (Bettge et al. 2009a, b) • High temperatures promote the growth of *Fredericella sultana* (Hartikainen et al. 2009; Tops et al. 2009; Hartikainen and Okamura 2011) and provoke, accelerate and prolong overt infections (Tops et al. 2006)

(continued)

Table 17.3 (continued)

Parasite	Timing and optimal temperature			Temperature effects
	Fish disease	Invertebrate host spore release	Fish host spore release	
Henneguya ictaluri	15–20 °C	Spring (more severe) and autumn (Wise et al. 2004)	Mid-summer and mid-winter (Griffin et al. 2009)	• At > 19 °C disease signs develop (MacMillan et al. 1989), • At >24 °C disease severity increases, at <15 °C disease disappears (Wise et al. 2004)
Enteromyxum leei	22–25 °C (Athanassopoulou et al. 1999; Rigos et al. 1999)	na	na	• Parasite development and diseases suppressed at <15 °C (Yanagida et al. 2006)
Kudoa thyrsites	Summer and autumn (Moran et al. 1999)	na	na	na
Parvicapsula pseudobranchicola	Spring at 10–16 °C (Sterud et al. 2003; Nylund et al. 2005)	Summer (Sterud et al. 2003; Nylund et al. 2005)	na	• At low temperatures (10-12 °C) post-smolts may be infected but spores do not develop until the following year • At higher temperatures (12–16 °C) spore formation can be completed during the summer and autumn in captive salmon introduced in the sea in the spring • Temperature is potentially important for parasite and definitive host longevity and reproduction

na data not available

Table 17.4 Biotic and abiotic (excluding temperature) factors that have been linked with disease development

Parasite	Factor	Effect
Myxobolus cerebralis	Invertebrate host genotype	Variation in susceptibility and actinospore production in different lineages of *Tubifex tubifex* (Sturmbauer et al. 1999; Beauchamp et al. 2002; DuBey et al. 2005; Elwell et al. 2006; Arsan et al. 2007; Baxa et al. 2008; Hallett et al. 2009)
	Actinospore dose	Lesions only develop in fish exposed to sufficient number of actinospores (Hedrick et al. 1999)
		Duration of exposure affects development and severity of disease (Bartholomew et al. 2003)
	Host species/strain/stock	Differential development and severity of disease according to fish host species, strains and stocks (see Hallett and Bartholomew 2012)
	Sediment type	Silt and clay harbour more infected worms than other substrates (Krueger et al. 2006) and worms residing in silt or mud (at the same flow) produce more actinospores than those in sand (Arndt et al. 2002; Blazer et al. 2003). However, Neudecker et al. (2012) found no significant association between fine sediment abundance and infection severity
	Water flow	High flows may scour and remove preferred oligochaete habitat, dilute infectious stages and decrease transmission of actinospores to fish (Hallett and Bartholomew 2008)
		Lower flows favour higher retention of spores and transmission of myxospores to oligochaete hosts (Kerans and Zale 2002) resulting in higher prevalence of infection in both oligochaete and fish hosts and higher infection severity in fish (Hallett and Bartholomew 2008)
Ceratonova shasta	Parasite genotype	Different genotypes exert different pathogenicities on salmonid hosts (Atkinson and Bartholomew 2010a, b; Hurst and Bartholomew 2012)
	Invertebrate host genotype	Polychaete host genotype has no effect on susceptibility, regardless of parasite genotype (Hallett and Bartholomew 2012; S. Atkinson, pers comm.)
	Actinospore dose	Both elevated temperature and/or dose can overcome resistance (Foott et al. 2007)
		Actinospore dose exerts variable responses (e.g. 40 % mortality is reached with 10 spores/L for Chinook salmon but half that for coho salmon, Hallett et al. 2012)
		Time to death is independent of spore dose in Chinook salmon but is negatively correlated with spore dose in coho salmon (Hallett et al. 2012)
	Fish strain	Infection severity varies with fish strains (Bartholomew 1998)
	Host size and age	Fish size and age are unrelated to infection (Bjork and Bartholomew 2009)
	Non-native salmonids' stocking	Stocked rainbow trout can become infected and produce large numbers of parasites amplifying existing parasites (Hurst and Bartholomew 2012)
	Water flow	Flow is the most important abiotic factor in transmission after temperature (Ray et al. 2012; Ray and Bartholomew 2013)
		Flow affects dispersal and concentration of waterborne stages (actinospores, myxospores) and thus exposure dose (Fujiwara et al. 2011). Stream discharge is correlated with survival rate of hatchery-released juvenile fish (Fujiwara et al. 2011)
		Actinospore transmission is greatly reduced above a velocity threshold of ~0.2–0.3 m/s (Ray 2013) above which infection prevalence is reduced (Bjork and Bartholomew 2009)

(continued)

Table 17.4 (continued)

Parasite	Factor	Effect
Tetracapsuloides bryosalmonae	Fish species	Some species may not transmit infection to bryozoans possibly because they are inappropriate hosts for spores to develop (Grabner and El-Matbouli 2008)
	Fish size, age and diet	Juvenile (Feist and Longshaw 2006), small (Grabner and El-Matbouli 2009) and well-fed fish (Seagrave et al. 1981) are more affected by the parasite
	Clonality of freshwater bryozoans	Clonal reproduction may enable a single genotype to spread in local habitats (Okamura and Hatton-Ellis 1995)
	Eutrophication	Eutrophication is linked with bryozoan abundance (Hartikainen et al. 2009) and enhanced food for bryozoans resulting from nutrient enrichment promotes higher infection intensities and increases bryozoan host growth (Hartikainen and Okamura 2011)
		Water pollution has been linked with PKD prevalence and increases in disease severity (El-Matbouli and Hoffmann 2002; Zimmerli et al. 2007)
	Salinity	Mortality in Atlantic salmon transferred to seawater is reduced post-infection (O'Hara 1985)
Enteromyxum leei	Fish species	Sharpsnout seabream is highly susceptible to *E. leei* (Alvarez-Pellitero et al. 2008)
	Fish diet	High fat contents in fish diets (i.e. 17–22 %) are associated with a higher prevalence and intensity of infection as well as faster parasite establishment (Athanassopoulou et al. 1999; Rigos et al. 1999; Estensoro et al. 2011)
	Salinity	Salinity below 8 ‰ effectively controls infection (Yokoyama and Shirakashi 2007)
Kudoa thyrsites	Salinity	Higher disease prevalences are linked with higher salinities in Atlantic salmon (Harrell and Scott 1985)
Parvicapsula pseudobranchicola	na	na

na data not available

invertebrate host genotype, actinospore dose (*M. cerebralis* and *C. shasta*) and the species, strains, ages and sizes of fish hosts (*M. cerebralis*, *C. shasta*, *T. bryosalmonae* and *E. leei*). Fish host diet (quantity and quality) has been reported as important for the development of both *T. bryosalmonae* and *E. leei*. High water flow may minimize infection prevalence and severity of diseases caused by *M. cerebralis* and *C. shasta* by reducing transmission. The effects of these environmental factors can be explored as potential management tools (see Sect. 17.3.7).

Factors that promote parasite dispersal, incidence and persistence in host populations are fundamentally related to life histories of hosts and parasites. Persistence over broad spatial scales may be promoted by low fish host specificity and wide distributions of both fish and invertebrate hosts in *E. leei, K. thyrsites.*

17.3.7 Using Epidemiology to Inform Control

Currently there are no treatments or vaccines available for myxozoan infections (Yokoyama et al. 2012). Control strategies for captive fish are mostly preventive and involve altering the fish's living conditions. These include: (1) adopting good management techniques such as reducing stock densities and improving water quality to reduce fish stress, which supresses the immune system (e.g. *T. bryosalmonae*, Environment Agency 2011); (2) using pathogen-free water,

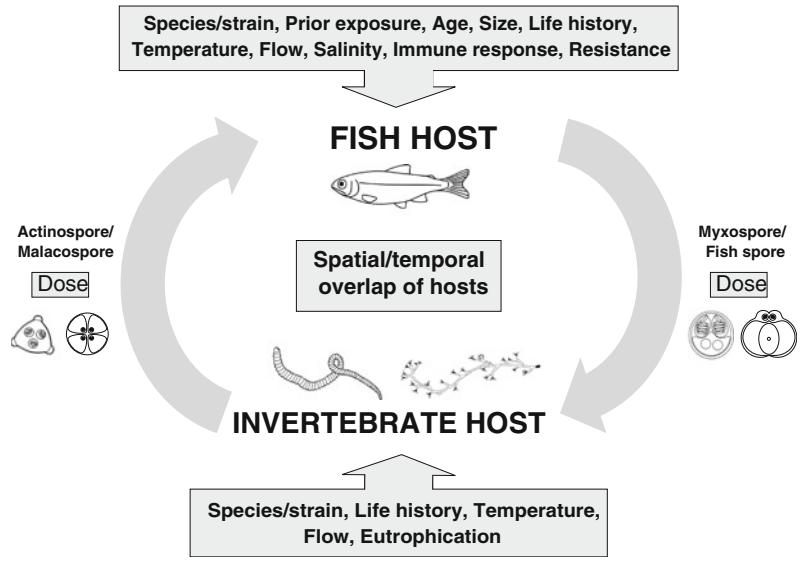

Fig. 17.1 General summary of the main biotic and abiotic factors (*in boxes*) known to affect myxozoan development and disease severity in hosts

which can be achieved by spring water, sand filtration, chlorination low salinity (e.g. *E. leei*, Yokoyama and Shirakashi 2007), exposure to UV (e.g. *M. cerebralis*, Hedrick et al. 2012) or ozone (e.g. *C. shasta*, Tipping 1988). Converting ponds and raceways to concrete and regularly cleaning and disinfecting to kill both spores and invertebrate hosts are useful for managing whirling disease caused by *M. cerebralis*. Another set of strategies focuses on the nature of the fish stocks. Such strategies include: (1) exposing fish in the autumn to promote acquired immunity towards PKD in the following year (Ferguson and Ball 1979); (2) avoiding fish exposure to peak parasite densities to avoid losses (e.g. *C. shasta*, Hendrickson et al. 1989; *H. ictaluri*, Wise et al. 2004; *M. cerebralis*, Kallert et al. 2009); (3) stocking resistant fish strains (e.g. *C. shasta*, Buchanan et al. 1983; Hendrickson et al. 1989; *M. cerebralis*, Schisler et al. 2006; Kallert et al. 2009); and (4) removing sexually mature fish from the stocks before they are harvested to reduce infection prevalence as these are more likely to become infected (e.g. *K. thyrsites*, St-Hilaire et al. 1998).

The invertebrate hosts represent other potential targets for disease control (Bartholomew et al. 2005; Alexander et al. 2014). However, removing or reducing invertebrate host populations from natural systems is likely to be impossible, ineffective and impractical given the wide yet patchy distribution of some species and particularly considering the connectivity of river systems, which will promote re-colonisation by the invertebrate hosts. Another factor that renders control of invertebrate populations, and thus the parasite, difficult is dispersal by animal vectors. For example, infected bryozoan statoblasts may be dispersed by waterfowl (Okamura et al. 2011) and *M. cerebralis* myxospores by avian piscivores (Koel et al. 2010).

Epidemiological models can be developed and parameterised using data on host-parasite interactions under varying environmental conditions to investigate how infections progress and how disease is affected by environmental factors. The development of such models involves describing how a parasite transmits and infects different hosts through mathematical expressions that define how the parasite moves between compartments (e.g. different host stages, the environment). Such modelling attempts to achieve a balance between reality and simplicity and has been conducted for *M. cerebralis* (Turner et al. 2014) and *C. shasta* (Ray 2013; Ray et al. 2014; see Chap. 19). Epidemiological modelling has identified targeting infectious spores and oligochaete hosts as the most effective strategies

for management and control of whirling disease (Turner et al. 2014). By conducting model sensitivity analysis Ray (2013) showed that the transmission of myxospores from adult salmon to polychaete hosts is one of the most influential parameters in determining the persistence of the *C. shasta* population. Thus, manipulation of winter transmission may be the most effective management action to reduce enteronecrosis. One method that could reduce rates of contact between myxospores and polychaetes is increasing discharge during the winter to remove spores from the system, reduce successful foraging of spores and dislodge worms (and thus reduce host density).

Three modelling approaches incorporate water sample data (parasite density) for *C. shasta*. A survival analysis model incorporates water data to predict overall and rates of *C. shasta*-induced mortality in free-ranging populations of juvenile Chinook and coho salmon *O. kisutch* to identify management strategies (Ray et al. 2014). Models to predict timing and level of actinospore production based on temperature and flow (N. Som USFWS, personal communication) and prevalence of infection in out-migrating juvenile salmon based on total actinospore density and exposure duration (R. Perry, USGS, personal communication) are being developed. These three models will be incorporated into a salmon production model to predict the population level effects of *C. shasta* and to improve long-term management of fish populations, for instance by providing better escapement goals and harvest quotas (R.A. Ray, personal communication).

Risk assessments may be used in parallel with epidemiological models to identify the most likely risk and establishment factors for infections. Risk assessments may also inform which control strategies will be more effective (see Chap. 20). For instance, a risk assessment model developed by Ayre et al. (2014) identified available habitat (as defined by gradient and elevation) for the invertebrate host as the factor with the greatest effect on the likelihood of infection of fish by *M. cerebralis*. Although prevalence of stream barriers also affected the risk outcome, these barriers may be effective in reducing risk of exposure to fish populations. Based on such factors obtained through risk assessments, risk maps may be created. Risk mapping is a tool that correlates epidemics to the terrain and the likelihood of occurrence. Geographic Information Systems (GIS) can be used to generate risk maps, highlighting areas according to the probability of contracting epidemics (low-to-high risk scores), which can aid in the monitoring and control of myxozoan diseases. Risk maps have not yet been undertaken for myxozoan parasites. However, they have been created for other fish parasites including koi herpesvirus (KHV) and spring viremia of carp (SVC) which allowed efficient use of health management resources directed towards higher risk animals and geographic areas for early disease detection (Thrush and Peeler 2013).

17.4 Conclusions and Recommendations for Future Investigation

The seven myxozoan species examined here exhibited some similarities in patterns of development and host exploitation and how these may be linked with environmental and biotic factors. Nevertheless, many gaps in understanding myxozoan epidemiology remain and there is great scope for further investigation, particularly as diseases are of growing concern for food security and in view of the effects of environmental change on aquaculture. Epidemiological studies of aquatic diseases generally lack datasets required to parameterise theoretical models that could predict such future effects. For instance, large-scale nationwide datasets of myxozoan diseases (e.g. mortalities and prevalences in aquaculture) would enable epidemiologists to build statistical and mathematical models.

Epidemiological tools developed for examining disease in terrestrial systems may provide new insights when applied to the emergence and control of aquatic diseases (Peeler and Taylor 2011). Network analysis examines the patterns of exchange of resources among individuals (Haythornthwaite 1996), or in the case of parasitology, the patterns of transmission of parasites among

hosts. Networks provide a flexible framework that enables analysis at the individual, dyadic and network levels and network analysis has been used extensively to understand the ecology of parasite transmission in human host populations and the emergence of epizootics (Godfrey 2013). Network analysis could be applied to myxozoan systems, provided appropriate types of data are collected (e.g. Thrush and Peeler 2006) and myxozoan life cycles and transmission routes are understood. Finally, molecular epidemiological approaches could also be used in more complex ways, for example by genotyping hosts and parasites (Cacciò and Ryan 2008) or by identifying genes underlying functional traits.

Other areas for future epidemiological research and disease control relate to the exploitation of resistant host strains and the significance of disease sinks and carriers. For instance, the ingestion of myxospores by non-susceptible oligochaetes (Baxa et al. 2008) may reduce transmission to susceptible hosts (other worms). In addition, not all myxozoan-infected fish develop spores (MacConnell and Vincent 2002). Thus non-transmitting fish may act as disease sinks. This effect could be substantial if the large stocks sustained in aquaculture are involved. For instance, captive rainbow trout in Europe and the UK may act as sinks for *T. bryosalmonae* since European *T. bryosalmonae* spores do not develop in these exotic fish, which are native to the west coast of North America. On the other hand, 'resistant' fish may still act as carriers for whirling disease. Such fish do not become clinically diseased, but, depending on their level of resistance, viable myxospores may still form (MacConnell and Vincent 2002).

Although epidemiological data are available for all of the seven myxozoans reviewed here, it is clear that *M. cerebralis*, *C. shasta* and *T. bryosalmonae* have been much more extensively studied. Notably, the life cycles of these three species are resolved, thus epidemiological data for both invertebrate and vertebrate hosts have been obtained. The three species are also all associated with freshwater environments whose nature may facilitate sampling and data collection. Finally, all three myxozoans cause diseases of salmonids. Because salmonids are highly valued for commercial and sport fisheries, funding for investigating these diseases may be more readily obtained. Our comparative approach also underscores that there are more studies of myxozoan infections in captive than in free-ranging fish. This no doubt partly reflects the necessity of reducing economic losses to aquaculture arising from myxozoan diseases. Despite the difficulties associated with accurately assessing disease effects in free-ranging fish populations, further epidemiological studies are required to identify more informed strategies for disease control in these populations. Such studies may be crucial for the protection of some of the most familiar and emblematic residents of fresh waters whose iconic nature confers high societal value and signifies substantial importance to human well-being.

17.5 Key Questions for Future Study

- Are there major differences in the epidemiology of diseases in freshwater versus marine environments and of captive versus free-ranging fish hosts? If so, what are the reasons for these differences and can they be exploited for their counterparts?
- How do disease sinks and disease carriers impact disease epidemiology?
- How widespread is vertical transmission of myxozoans among invertebrates?
- How does vertical transmission influence predictions of epidemiological models?
- What are the invertebrate hosts of marine myxozoans, *E. leei*, *K. thyrsites* and *P. pseudobranchicola*?
- What types of epidemiological models are most appropriate for myxozoans?

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Myxozoans on the Move: Dispersal Modes, Exotic Species and Emerging Diseases

18

Sascha L. Hallett, Ashlie Hartigan, and Stephen D. Atkinson

Abstract

Increased global connectivity and shorter transportation times translate to a higher probability and frequency that hosts and their parasites will be introduced to foreign locations and will arrive viable. However, myxozoans are heteroxenous with two obligatory hosts and two free-living life stages, so even if a parasite is introduced, a myriad of factors must align for establishment to occur. Associated with successful introductions are ecological and economic impacts. Unless there is an established monitoring program, it is difficult to detect the emergence of a myxozoan before clinical signs become overt. In this chapter we examine features of myxozoan parasites that promote or impede dissemination and establishment. We present examples of modes of myxozoan introduction and dispersal, both natural and human-mediated. We review known and novel species responsible for emerging diseases, and recently identified species that have the potential to cause disease. We conclude by outlining strategies to understand and limit myxozoan introductions.

Keywords

Dissemination · Introduction · Exotic parasite · Natural dispersal · Anthropogenic dispersal · Emergent disease

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18.1 Introduction

Aquatic parasites may be dispersed either discretely as waterborne infectious stages (for example eggs, spores, cercariae of trematodes and nauplii of sea lice) or with movement of infected hosts. For myxozoans, natural dissemination at local geographic scales, such as within a river basin, occurs both via infectious waterborne spore stages (actinospores, myxospores or malacospores) and by trophic and vegetative stages within hosts. At broader spatial scales, these parasites are most likely transported only within the safety of their hosts, with migratory vertebrates (e.g. fish, birds) facilitating the longest non-anthropogenic portage. Human movement of myxozoans, either as spores or within infected hosts, may occur at all spatial scales. Increased global connectivity and shorter transportation times translate to a higher probability and frequency that hosts and their parasites will be introduced to distant locations and will be viable when they arrive.

Myxozoans have indirect life cycles with multiple host species and environmental stages, so when they are introduced to a novel location several requirements must be satisfied for establishment to occur. Despite these prerequisites, myxozoan introductions *have* occurred. It is likely that ever changing environmental conditions and the global expansion of aquaculture are accelerating introductions of both known myxozoans and of novel, emergent, pathogenic species, causing subsequent ecological and economic impacts. Unless there is an established monitoring program, however, it is difficult to detect the emergence of a myxozoan before clinical signs become overt.

In this chapter, we summarise those features of myxozoan biology that facilitate or impede their dispersal and establishment in new environments. We discuss different modes—both natural and human mediated—by which myxozoans may be dispersed, and discuss examples. We largely focus on infections in the most well-known host group—fishes. Infections in invertebrate and other vertebrate hosts are also considered. We conclude with a summary of emerging diseases caused by myxozoans and strategies for mediating their effects on free-ranging and farmed fish populations.

18.2 Myxozoan Features that Influence Dissemination and Establishment

Myxozoans have complex life cycles that involve development in invertebrate and vertebrate hosts, to form waterborne, infectious spores. Two main myxozoan groups are recognised—myxosporeans (which produce distinct myxospores and actinospores) and malacosporeans (which produce similar malacospores). Myxozoan traits that influence their potential for dissemination and establishment at different stages in their life cycles are listed in Table 18.1. Below we expand on the significance of these traits.

Myxozoan spores have different morphologies and longevities. Myxospores, which typically develop in a vertebrate, are the most resilient, being able to withstand extremes in temperature, desiccation, extended dormancy in sediments and passage through the digestive tract of piscivorous predators (e.g. *Myxobolus cerebralis* and *Ceratonova shasta*, see Hallett and Bartholomew 2012). Malacospores, which develop in bryozoans and fishes, and actinospores, which develop in annelids, tend to be more fragile and short-lived (see Yokoyama et al. 2012; Hallett and Bartholomew 2012). Asynchronous maturation of actinospores (e.g. see Hallett et al. 1998) and non-lethal exit of mature spores enables long-term release, possibly for the duration of the host's lifespan (e.g. see Gilbert and Granath 2001). Mature spores may persist within hosts prior to release. For example, myxospores of *M. cerebralis* and *C. shast*a over-winter within fish carcasses although freezing may reduce viability (El-Matbouli and Hoffmann 1991; Hedrick et al. 2008; Hallett unpublished data). Cryptic stages of malacosporeans can overwinter in bryozoan statoblasts (Abd-Elfattah et al. 2013). Whether actinospore

Table 18.1 Characters of myxozoans that are likely to influence their dissemination and establishment

Promote	Impede
Transmission via waterborne stages (actinospores, myxospores, malacospores)	Indirect life cycle—both invertebrate and vertebrate host must be present
Resilient myxospore stage (in myxosporeans)	Temperature-dependent development in hosts
Motile worm stage (in some malacosporeans)	Temperature- and salinity-dependent longevity of waterborne stages
Transmission via waterborne developmental stages (*Enteromyxum* species only)	Survive only in moist or fully aquatic environment
Vertical transmission in invertebrate hosts (e.g. *Tetracapsuloides bryosalmonae* in clonal bryozoans and *Myxobilatus gasterostei* in fissiparous naids)	Fragile, short-lived actinospore (myxosporeans) and malacospore (malacosporeans)
Amplification in both invertebrate and vertebrate hosts	Lack of viability of trophic stages outside hosts
Persistence (long-term sustained infections) in both invertebrate and vertebrate hosts	
Protection within hosts	
Rarely—transmission among vertebrate hosts (invertebrate host not needed)(e.g. *E. leei* fish-to-fish)	
Rarely—broad host range (e.g. *E. leei*, *Kudoa thyrsites*)	

stages of myxosporeans can achieve a similar persistence within invertebrate hosts is unknown. The oligochaete, *Tubifex tubifex*, can encyst to avoid desiccation or starvation (Anlauf 1990) but it is unclear whether a myxozoan infection can remain viable within the encysted worm. Myxosporean spores are transported passively by water currents. Actinospores may possess inflatable caudal processes that aid buoyancy. Although some malacosporeans (e.g. *Buddenbrockia)* have a motile worm stage, the effect of this mobility is likely to be limited since worms rapidly tire and become moribund (Okamura, personal communication).

Elucidated life cycles indicate that myxosporeans must sequentially infect alternate hosts. Therefore, both a susceptible invertebrate *and* vertebrate host must generally be present in a novel location and have sufficient spatial and temporal overlap with the infectious stages for myxozoan establishment. The likelihood of a suitable host being present is influenced by both host specificity and host ranges. For instance, *M. cerebralis* infects only Salmonidae whereas *Kudoa thyrsites* infects fish species across 18 families (Lom and Dyková 2006). On the other hand, *M. cerebralis* infects only one invertebrate species, *T. tubifex*, but this organism is widespread.

In contrast to myxosporeans, if malacosporeans arrive in an exotic location (e.g. when infected bryozoan statoblasts are dispersed; Abd-Alfattah et al. 2013), they may not require a vertebrate host, at least in the short term, to become established. This is because malacosporeans can persist in bryozoan populations for many years via clonal reproduction of hosts and extensive vertical transmission (see Okamura et al. 2011 and references therein). Indeed, there is evidence for infection in bryozoan populations in sites where putative vertebrate hosts are presently absent (Canning and Okamura 2004).

18.3 Modes of Myxozoan Introduction, Dispersal and Amplification

The principal natural and human-mediated mechanisms of myxozoan dispersal are summarised in Table 18.2. Natural dispersal occurs at all spatial and temporal scales, though arguably this has a lower potential for longer range and shorter time scale expansion than human-mediated pathways. Human-mediated spread of organisms covers the full spectrum of geographic scales. Typically this may occur over shorter time scales

and greater geographic distances than natural dispersal and may circumvent natural barriers (e.g. mountain ranges and oceans).

There are three possible outcomes for the introduction of myxozoan-infected hosts into a novel habitat, regardless of whether the event is natural or human-mediated (Fig. 18.1). The scenarios probably apply to both vertebrate and invertebrate hosts, but examples are known only for fishes and amphibians.

A. **Infection of native hosts with exotic parasites**: In this scenario, competent vertebrate and invertebrate hosts are present in the new location and these naïve populations become infected with the exotic parasite. The classic example is the salmonid myxosporean, *M. cerebralis*, which spread from Europe into North America (see also Sects. 18.3.5 and 18.3.9). A second example is *Thelohanellus nikolskii*, which was co-introduced to Europe from Asia with Amur carp (*Ctenopharyngodon idella*). Hungarian carp stocks became heavily infected and the parasite is now responsible for one of the most common carp diseases in Hungary and several neighbouring countries (Molnár 2002). The invertebrate host for both myxozoans, *T. tubifex*, is ubiquitous and was already present at the foreign locations (Kathman and Brinkhurst 1998; Székely et al. 1998).

B. **Infection of exotic hosts with native parasites**: In this second scenario, the introduced host may be freed of its own myxozoan parasites post-introduction (either due to absence of infection in translocated hosts or absence of a suitable alternate host in the new environment) but becomes infected with novel species (Torchin and Mitchell 2004). In some cases the introduced host amplifies these parasites and permits them to "spillback" into the native host population (Torchin et al. 2003; Kelly et al. 2009). For example, the cane toad (*Rhinella marina*) was introduced from Puerto Rico via Hawai'i (Easteal 1981) to northern Australia in 1935 as a biological control for agricultural pests. The toad acquired two Australian species of myxozoans, *Cystodiscus axonis* and *C. australis*, known from native amphibians and provided a spillback and dissemination mechanism for those myxozoan species to the native frog population (Hartigan et al. 2011). An alternate outcome is that the native parasite infects the exotic host but does not develop transmissible spores, thus the exotic host acts as a sink for native parasites and may provide relief for indigenous hosts (Tompkins and Poulin 2006; Poulin et al. 2010). Although this process has not been reported for myxozoans, laboratory experiments support the concept: *M. cerebralis* actinospores can attach to carp—an unsuitable host—thereby decreasing available infectious stages and reducing the subsequent myxospore load in compatible trout (Kallert et al. 2009). See also Sect. 18.3.5 for examples of fishes introduced to new locations for aquaculture, which then acquired novel endemic myxozoans.

C. **Exotic hosts lose parasites in new range**: A third scenario is that the introduced host becomes freed from its myxozoan parasites if either the parasite species is not indigenous in the new location or if an alternative competent host is not present to continue the life cycle of the exotic parasite (parasite release hypothesis; Dunn 2009; Ross et al. 2010). For example, salmonids introduced into Australia are considered freed from *M. cerebralis*. Importation and breeding of salmonids has occurred in Australia since the 1800s with live imports until the 1960s (MacCrimmon and Marshall 1968; Cadwallader 1996). There are self-sustaining populations of trout in six of the eight states/territories where they would be in contact with *M. cerebralis*-invertebrate hosts, *T. tubifex* (see Pinder and Brinkhurst 2000), yet no whirling disease has been reported (DAFF 2009). In a comparison of myxozoans from sympatric exotic poeciliids and phylogenetically unrelated but

Table 18.2 Natural and anthropogenic modes of introduction, dispersal and amplification of myxozoan parasites

Natural	Human-mediated
Migration or straying of vertebrate host (particularly fishes)	Aquaculture and mariculture
Ingestion of infected vertebrate hosts	Commercial fishing: dumping of bycatch and processing waste
Migration of bird host (paratenic)	Aquatic pet trade
Dispersal of invertebrate host	Commercial transfers, stocking into natural waters
Translocation with habitat fragments	Recreational fishing
Host range shifts with environmental change	Alteration of waterways
Parasite spillover from introduced to native hosts	Shipping and trade: contaminated ships, ballast water, contaminated goods

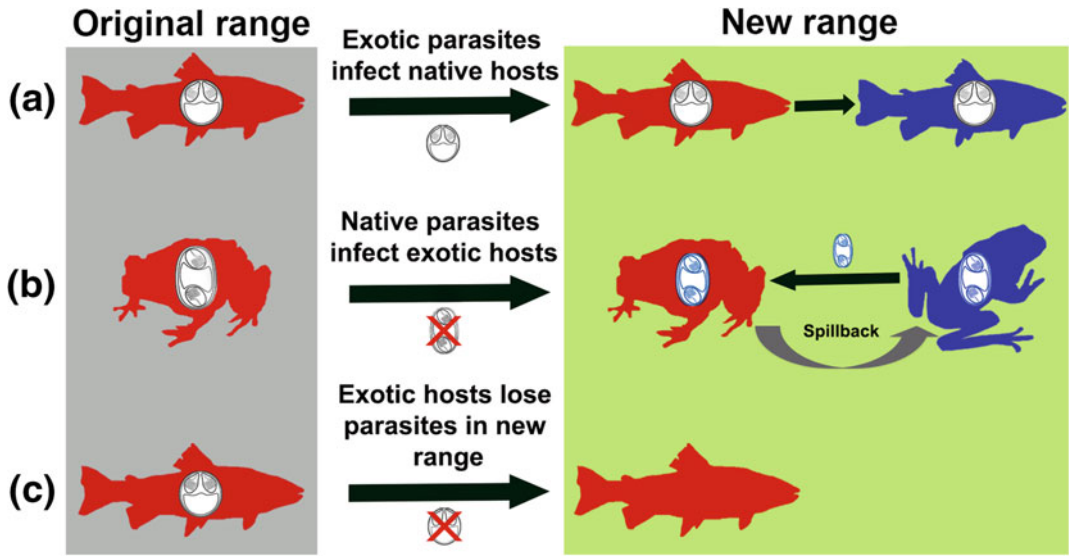

Fig. 18.1 Three scenarios for the introduction of myxozoans (indicated as infectious spores within and released from hosts) with their host into a novel habitat, regardless of whether the event is natural or human-mediated. While the outcomes are applicable to both the vertebrate and invertebrate host, examples are known only for fishes and amphibians. **a** Infection of native hosts with exotic parasites: compatible vertebrate and invertebrate hosts are present in the new location and become infected with the exotic parasite (e.g. spread of *Myxobolus cerebralis* from Europe into North America; co-introduction of *Thelohanellus nikolskii* to Europe from the Far East with Amur carp). **b** Infection of exotic hosts with native parasites: exotic hosts can act as sinks for native parasites and provide some relief for native hosts, or exotic hosts can amplify native parasites and permit them to "spillback" into the native host population (e.g. the cane toad introduced into Australia acquired and disseminated two native amphibian myxosporeans). **c** Exotic hosts lose parasites in new range: the introduced host is freed of its myxozoan parasites if either the parasite is not already present in the new location or if a compatible invertebrate host is not present (parasite release hypothesis) (e.g. introduced salmonids in Australia were "released" from their native *M. cerebralis* infections). For further details, see Sect. 18.3

ecologically similar native Australian species, two to five myxozoan species across four genera (*Myxidium*, *Zschokkella*, *Myxobolus* and *Henneguya*) were found in the native fishes but none was found in the exotic fishes (Dove 2000). The infection status of the poeciliids at the time of introduction is, however, unknown.

18.3.1 Migration or Straying of Fish Host

Diadromous fishes, such as salmonids and eels, can migrate long distances and spread myxozoans from one river or basin to another. Both *C. shasta* and *M. cerebralis* have been detected in stray anadromous salmonids. The introduction of *M. cerebralis* into a Columbia River tributary (USA) from an enzootic tributary has been attributed to straying host fish (Engleking 2002; Zielinski et al. 2010). Over evolutionary time, anadromous fishes may transfer myxozoan species between freshwater and marine habitats (see Chap. 3).

18.3.2 Piscivorous Predators and Waterfowl

Myxospores can remain intact and viable after passage through the digestive system of fishes, birds and mammals (e.g. *M. cerebralis*: Taylor and Lott 1978; El-Matbouli and Hoffmann 1991; El-Matbouli et al. 2005; Koel et al. 2010). Bald eagles (*Haliaeetus leucocephalus*) can excrete myxospores for 2–3 days after consuming a *M. cerebralis*-infected fish (Arsan and Bartholomew 2008) and these birds can travel >200 km/day during migration (Kerlinger 1995). Migratory waterfowl are a possible vector for *Tetracapsuloides bryosalmonae* if the parasite is protected within its bryozoan host statoblasts. The statoblasts may pass through the digestive tract of waterfowl or become attached to their feathers (Bilton et al. 2001; Charalambidou et al. 2003; Figuerola et al. 2004). Infected statoblasts translocated via waterfowl may have introduced *T. bryosalmonae* to southern Europe from North America, prior to more recent human introductions (Henderson and Okamura 2004).

18.3.3 Dispersal of Invertebrate Host

Most invertebrate hosts for myxozoans are aquatic annelids (oligochaetes, polychaetes or sipunculid worms) or freshwater bryozoans, which can be dispersed with water currents. Downstream movement in rivers may occur following a high flow event in which polychaetes are scoured from boulders or bryozoan colonies are fragmented. Bryozoan hosts may be dispersed to new river basins via statoblasts on birds (see Sect. 18.3.2).

18.3.4 Translocation of Habitat Fragments and Hosts

Assemblages of hosts and parasites may be transported along with habitat components (e.g. rafting of woody debris, vegetation, and other natural or human-made items) that are translocated as a result of floods (within river basins) or tsunamis (across oceans). For example, the tsunami associated with the 2011 Japanese earthquake generated marine debris, which was then transported via currents and wind across the Pacific Ocean. Most notably, two segments of floating dock beached 15–21 months after the earthquake on the Pacific Northwest coast of North America. Both were teeming with hitchhikers, including >25 species of non-benthic Japanese polychaetes (Miller 2013; Carlton 2013; Miller et al. 2014), which are potential hosts for myxozoans.

18.3.5 Aquaculture and Mariculture

Fish farming has been responsible both for the creation of artificially large and dense populations of native fishes, and for translocation and subsequent propagation of non-native fishes. These pursuits have variously provided novel hosts, increased local host densities and inadvertently introduced parasites along with the cultured host. Consequently, several myxozoan species have emerged as threats to pisciculture and new cases continue to arise (see Sect. 18.4). High infection levels can result in economic losses because of mortalities or suboptimal fish condition (reduced growth, damaged product). For example, in western Canadian mariculture,

endemic *K. thyrsites* became prevalent in introduced Atlantic salmon. Infections can result in post-mortem myoliquifaction and render fillets unmarketable (Moran et al. 1999). In the Red Sea, native *K. iwatai* produced heavy infections in gilt head sea bream (*Sparus aurata*) when this fish species was introduced from the Mediterranean for cage culture (Diamant et al. 2005). In Japan, heavy outbreaks of native *K. amamiensis* occurred in exotic yellowtail (*Seriola quinqueradiata*) and amberjack (*S. dumerilii*) (Sugiyama et al. 1999). In German hatcheries, whirling disease was unheard of until endemic *M. cerebralis* and imported North American rainbow trout crossed paths. Subsequently, the parasite was accidentally introduced to the eastern USA and decimated several trout hatcheries that then had to close (Bartholomew and Reno 2002). Indigenous *C. shasta* was unknown until Californian hatchery trout (USA) developed enteronecrosis (Noble 1950). Similarly, *Henneguya ictaluri* was undescribed until after proliferative gill disease impacted the American channel catfish industry (Pote et al. 2000). More recent appearances are described in Sect. 18.4.

Establishment of farmed populations outside of their native ranges can facilitate exotic myxozoan spread via escaped infected fish. In the Canary Islands, sea bass (*Dicentrarchus labrax*) infected with *Sphaerospora testicularis* are known to escape from fish farms, and there is concern for parasitic castration of neighbouring native fish species (Toledo-Guedes et al. 2012). The exotic myxozoan likely arrived with infected juveniles from mainland hatcheries as this parasite infects both reared and wild sea bass in the Mediterranean (Sitjà-Bobadilla and Alvarez-Pellitero 1990). In some cases aquaculture may have no impact on local wild fish populations. For instance, infectious spores of native *T. bryosalmonae* are not produced in exotic rainbow trout farmed in the UK (Bucke et al. 1991; Morris et al. 1997) and, consequently, fish farms will not amplify disease in local wild fish populations.

18.3.6 Commercial Fishing

An estimated 7.3 million tonnes of fish and shrimp were dumped overboard from commercial boats yearly between 1992 and 2001 (Kelleher 2005). These fishes are often juvenile, low value or restricted species, which die and are thrown overboard without treatment so as to not exceed the catch limit (Zeller and Pauly 2005). Since moribund and dead fishes are easy prey, dumping them away from the catch site could spread myxozoans.

The processing of wild-caught and farmed infected fishes may spread myxozoans through effluent and discarded fish solids, depending on the site and size of discharge (Arsan and Bartholomew 2008). However, high temperatures during the processing of by-products to fish meal may destroy spores of many species.

18.3.7 Aquatic Pet Trade

The aquatic pet animal trade moves millions of fishes, reptiles and amphibians each year and is thus very likely a primary mode for international transport of myxozoan parasites. There are, however, few published surveys of myxozoans in these animals. Evans and Lester (2001) identified *Chloromyxum* sp. in four of six shipments of sucking catfish (*Gyrinocheilus aymonieri*) imported from Singapore to Australia, and Garner et al. (2008) found 11 cases of *Sinuolinea* infections in weedy sea dragons (*Phyllopteryx taeniolatus*) imported from Australia to the United States. Marine and fresh water fishes imported into the USA, including kuhli loach (*Pangio kuhlii*), purple tang (*Zebrasoma xanthururm*) and debauwi catfish (*Pareutropius debauwi*), were found infected with *Myxobolus*, *Zschokkella*, *Sphaerospora* and *Henneguya* (Atkinson, unpublished data). It is curious that this number is not higher, but likely reflects a lack of screening effort or the exclusion of

myxozoans from parasitological surveys. Latent infections with myxozoans are probably far more common than realised. A New Zealand import risk analysis for ornamental fishes recognised the risk of introduction of *Enteromyxum leei* via this route and recommended targeted surveillance (Biosecurity 2005).

Common goldfish (*Carassius auratus*) are cultured and transported widely as an ornamental species, as a 'feeder' fish for other fishes and reptiles, and as live bait for recreational fishing. In the USA, this species is a widespread invasive and continues to be introduced into the wild through escape from hatcheries and ponds, release from aquaria, and disposal of bait fish (Nico et al. 2014). A survey of goldfish from a national USA pet store chain, revealed that 50 % of fish were infected with up to three species of six myxozoans, representing *Sphaerospora*, *Myxobolus*, *Hoferellus*, and *Zschokkella* (see Hallett et al. 2006b). Species of the first three genera were also observed in fish sampled directly from an Arkansas goldfish farm (Karandashova 2008): *Sphaerospora* sp., *Myxobolus diversus*, *Myxobolus cultus*, and *Hoferellus carassii*. Another species, *Chloromyxum auratum*, was prevalent in goldfish illegally introduced into an Oregon reservoir, and was thought to have been co-introduced with its vertebrate host since none of the local native fishes was infected. An appropriate invertebrate host must already have been present to sustain infections in the goldfish population (Hallett et al. 2006b). Another carp myxozoan, *Myxobolus koi*, was introduced with its host *Cyprinus carpio* from Asia to the UK and the USA, and has caused mortalities of koi in ornamental ponds (Crawshaw and Sweeting 1986; Camus and Griffin 2010).

Non-fish vertebrates, particularly frogs and turtles, harbouring myxozoan parasites have also been transported globally. One white-lipped tree frog *Litoria infrafrenata* purchased online was sent to the Czech Republic from Indonesia with *Cystodiscus* spores in the gallbladder and *Myxobolus* spores in the gonads (I. Fiala, personal communication). Both parasites have been linked to disease and could be a conservation issue for rare species (Sijtà-Bobadilla 2009; Hartigan et al. 2013). Invasive red-eared slider turtles (*Trachemys scripta*) have been identified in the western USA with *Myxidium* species (Atkinson, unpublished data).

A less-considered but nevertheless existing transit route for myxozoans is via commercial trade of oligochaetes of mixed species as live food for aquarium fishes. Widespread human-mediated worm transport occurs within Europe (from Romania and Hungary to Germany; Hallett et al. 2006a) and also between Europe and the USA (Lowers and Bartholomew 2003). Spores were observed exiting oligochaetes for several weeks post-purchase. European petshop oligochaetes including *Tubifex tubifex*, *Limnodrilus hoffmeisteri* and *L. udekemianus* shed 12 actinospore types from four collective groups (Hallett et al. 2003, 2004, 2005, 2006a). European tubificoid oligochaetes sold in North America shed seven actinospore types from one collective group (Lowers and Bartholomew 2003).

18.3.8 Recreational Fishing

Angling can lead to the inadvertent transfer of myxozoans and their hosts within or among rivers or basins. Spores may be introduced in bait fish (see Sect. 18.3.7), undrained water, and adhering to fishing gear or equipment. Both the invertebrate host and spores may be transported in muddy equipment. Public education and proper cleaning and disinfection of fishing-related items (clothes, waders, equipment, boats, trailers) could reduce further transfers (Steinbach Elwell et al. 2009).This approach has been used extensively to combat *M. cerebralis* in the USA.

18.3.9 Commercial Transfers and Stocking

M. cerebralis serves as a classic case of global dissemination of a myxozoan parasite through commercial transfers and stocking. Although the parasite is endemic in wild German trout, whirling disease emerged in that country as a malady of cultured non-native trout. The parasite

spread to other countries within Europe and beyond, including South Africa, New Zealand and the USA, heavily impacting both wild and cultured salmonid populations (Bartholomew and Reno 2002). Unintentional introductions of *M. cerebralis* are largely attributable to the human transfer of subclinically-infected fishes. Subsequent widespread establishment of the parasite was facilitated by the broad natural distribution of its invertebrate host, *T. tubifex*.

18.3.10 Alteration of Waterways

The Suez Canal connected the Red Sea with the Mediterranean in 1869 and has since allowed the westerly immigration of more than 80 species of Lessepsian fishes (Otero et al. 2013). Despite the influx of exotic hosts and that myxozoans are known from the Red Sea (e.g. Ali et al. 2006; Diamant et al. 2004), mostly non-myxozoan parasites are documented as being co-introduced (e.g. Diamant et al. 1999, 2014). However, *Ceratomyxa* sp., *Ortholinea* sp. and *Zschokkella icterica* of definite Red Sea origin have been recovered from invasive rabbitfish *Siganus* spp. from the Mediterranean coast of Israel (Diamant 2010).

18.3.11 Shipping and Trade

Shipborne transportation of commercial goods has dispersed thousands of aquatic organisms via the fouled hulls of cargo ships or released ballast water (Torchin et al. 2002; Gozlan et al. 2010). Although no myxozoan-specific surveys have been conducted, accidental introductions may include both infected fish and invertebrates. For example, yellowfin goby (*Acanthogobius flavimanus*) was probably introduced in the 1960s via ballast water from Japan to the Suisun Marsh, San Francisco Estuary, USA (Dill and Cordone 1997). In 2005, 45 yellowfin goby were determined by PCR to be infected with a *Henneguya* species that was closely related (but not identical) to a known *Henneguya* from gobies in Japan (Baxa et al. 2013). Without baseline data about the *Henneguya* fauna in the estuary prior to the introduction and the absence of this species in the native range of the yellowfin goby in Japan, we do not know from which direction the parasite came.

Agriculture and horticulture account for thousands of unintentional translocations around the world with no control for invertebrates in the soil of plants, or frogs and reptiles within timber or produce. In Minnesota, USA, 10 kinds of aquatic invertebrates were identified in 40 interstate horticulture shipments, and two of the orders also contained live fish (Maki and Galatowitsch 2004). The survival of such potential hosts during transport is promoted by refrigeration, faster shipping times and open trade agreements. The only demonstrated example of a myxozoan being dispersed through accidental transport of infected hosts was where myxozoan-infected amphibians were transported extensively within and beyond Australian borders in banana boxes (Hartigan et al. 2012).

18.4 Emerging Myxozoan Diseases

We define emerging diseases as those that have not been detected previously in a population. In Sects. 18.4.1–18.4.3, we review examples and patterns of emerging myxozoan diseases in fishes, including both introduced and native myxozoan species that infect wild and captive host populations. Many new myxozoan species are described each year, and many probably remain to be discovered (e.g. see Chaps. 1, 4 and 7). Although all have the potential to cause disease, most cause inconspicuous infections. In Sect. 18.4.4, we highlight novel species discovered through piscicultural pursuits that may become problematic as well as recently described species from non-poikilothermic hosts with the potential to emerge as disease agents.

18.4.1 Catalysts of Disease Emergence

The emergence of a disease typically follows a change in community structure (e.g. host species or density, parasite species or density), or habitat (e.g. physical architecture, temperature, environmental degradation). An overarching phenomenon that will influence the outcome of introductions and emergence of diseases is climate change (for review see Harvell et al. 2002; Okamura and Feist 2011). There is often a time lag following an alteration before an emerging disease or its affects become apparent. For example, it may take years for *M. cerebralis* to propagate to levels high enough to cause overt disease (Bartholomew and Reno 2002). When a myxozoan disease emerges in a host population, identification of the causative agent can be problematic due to insufficient or nonexistent species records (see Chap. 4). In addition, poor understanding of which myxozoans are present naturally in a system may obscure the nature and timing of disease emergence. The presence of severe myxozoan infections may go unnoticed in wild fish populations because diseased fish disappear quickly unless there is a mass impact. Emerging diseases are therefore more obvious in captive populations, which are regularly monitored. The gradual nature of parasite establishment, lack of basic data and constraints in disease detection can all hinder our understanding and management of disease problems.

18.4.2 Patterns and Dynamics of Emerging Diseases

Emergent myxozoans have been reported from both free-ranging and cultured vertebrate populations, and some species have impacted both (e.g. *M. cerebralis, C. shasta* and *T. bryosalmonae*). In some cases emergent myxozoan diseases associated with aquaculture may impact local (free-ranging) fish of the same species (or family). These may involve endemic or introduced myxozoans. It is clear that problematic species are being revealed worldwide, from tropical (e.g. Red Sea) to cold temperate conditions (e.g. Iceland, Norway). The examples described below demonstrate some of the complex patterns and dynamics of emerging diseases caused by myxozoans. These, and other examples of emergent myxozoan diseases are summarized in Table 18.3.

Enteronecrosis of salmonids, caused by the endemic myxozoan *C. shasta*, emerged initially as a hatchery disease in non-sympatric hosts in the Pacific Northwest of the USA in the 1950s (Noble 1950). Epizootics in captivity were reduced through changes in management practices. Decades later, in the 2000s, enteronecrosis began to noticeably impact free-ranging salmonids in some river basins with regulated flows. Although *C. shasta* and its salmonid hosts have likely co-existed for considerable time, anthropogenic changes to the river systems have altered host-parasite dynamics. The construction of dams has tempered natural high flow events associated with winter precipitation and spring snow melt, and consequential scouring and movement of sediment rarely occurs. The resultant stable habitat favours the invertebrate host of *C. shasta* (Alexander et al. 2014).

Emaciation disease, caused by *E. leei*, emerged in sea bream (*Sparus aurata*) in the early 1990s. In the Mediterranean, infections were sparse in wild populations but widespread in mariculture (Diamant et al. 2006; Palenzuela 2006). In the Red Sea, *E. leei* infections in cage-cultured sea bream spilled-over to native wild fish species, but these infections disappeared soon after local culture ceased and the farms were dismantled (Diamant, unpublished data). Elsewhere, *E. leei* continues to emerge as a threat in cultured marine fishes, such as for tiger puffer (*Takifugu rubripes*) in Japan (Yasuda et al. 2002), but spillover has not yet been detected. Unlike most myxozoans which require two host species to continue their life cycle, *E. leei* can be transmitted horizontally, from fish to fish (Diamant 1997). Furthermore, it has low host specificity. These attributes may be particularly permissive for disease emergence as was exemplified by an epizootic that occurred during an aquarium exhibition, in which enteritis spread to

Table 18.3 Examples of myxozoan species associated with emergent diseases

Myxozoan	Host	Location	Effect on host	References
Parvicapsula pseudobranchicola	Atlantic salmon (*Salmo salar)*	Norway	Pale, swollen, destroyed pseudobranchs; mortalities	Karlsbakk et al. (2002), Jørgensen et al. (2011)
Kudoa islandica	spotted wolffish (*Anarhichas minor)*	Iceland	Post mortem somatic myoliquifaction; financial losses	Kristmundsson and Freeman (2014)
Tetracapsuloides bryosalmonae	Salmonids—wild Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta fario),* arctic char (*Salvelinus alpinus*)	Norway, Switzerland, Iceland	Proliferative kidney disease; mortalities in Norway and Iceland, population decline in Switzerland	Sterud et al. (2007), Schmidt-Posthaus et al. (2013), Kristmundsson et al. (2010)
Myxobolus acanthogobii	Japanese mackerel *(Scomber japonicus)*	Japan	Brain, spinal curvature; marketability	Yokoyama et al. (2005a)
Henneguya lateolabracis	Chinese sea bass (*Lateolabrax* sp.)	Japan	Enlarged bulbus arteriosus, anaemic gills; chronic mortalities	Yokoyama et al. (2003)
H. pagri	Red sea bream (*Pagrus major)*	Japan	Anaemic gills, an enlarged bulbus arteriosus and internal haemorrhaging	Yokoyama et al. (2005b)
Enteromyxum leei	sea bream(*Sparus aurata*), tiger puffer (*Takifugu rubripes*)	Mediterranean, Red Sea, Japan	Emaciation disease; mortalities	Diamant et al. (2006), Palenzuela (2006), Yasuda et al. (2002)
Ceratonova shasta	Salmonids—Chinook (*Oncorhynchus tshawytscha*) and coho (*O. kisutch*)	USA	Enteronecrosis; mortalities	Noble (1950), Fujiwara et al. (2011)

All except *Henneguya lateolabracis* appear to be endemic

25 fish species (Padrós et al. 2001). Consequently, *E. leei* was identified as a high risk for sharpsnout seabream (*Diplodus puntazzo*) culture (Sánchez-García et al. 2014).

In Japan, two species of *Henneguya* have emerged as mariculture pathogens: *H. pagri* in Red Sea bream (*Pagrus major)* and *H. lateolabracis* in Chinese sea bass (*Lateolabrax* sp.). The former may have been already present in Japan but was not described until the disease outbreaks occurred. The latter was also undescribed until disease emergence but was introduced with juvenile hosts from China to Japan and was then unintentionally propagated along with its host (Yokoyama et al. 2003, 2005b). Effects on wild fish populations are unknown.

Although no myxozoan is known to undergo development within a human host, *Kudoa* infections in fish somatic muscle have recently been associated with allergic reactions and food poisoning (See also Chap. 7). *K. septempunctata* has been identified as the culprit when infected raw farm-reared olive flounder (*Paralichthys olivaceus)* imported from South Korea to Japan are consumed (Kawai et al. 2012). Allergic reactions have also been reported when Chilean

hake (*Merluccius gayi gayi*) infected with a *Kudoa* sp. has been consumed in Spain (Martínez de Velasco et al. 2008). Some of the more visible macroscopic myxozoan plasmodia or pseudocysts in fish fillets (e.g. *H. salminicola*, *K. thyrsites*) are completely harmless to humans.

18.4.3 Declining Emerging Diseases

Several previously emergent myxozoans no longer impact certain fish populations. For diseases in aquaculture this can relate to changes in hatchery practices. For example, ozonation of incoming water is used to inactivate infectious spores for *C. shasta*. Conversion of sediment-based raceways to concrete is used to reduce invertebrate host habitat for *M. cerebralis*. Fish farmers expose fish fry to *T. bryosalmonae* infections when temperatures drop in autumn. This effectively immunizes fish to disease development in the following year when temperatures increase but involves substantial time and effort and reduces production (Okamura et al. 2011). Wild fish populations may develop disease resistance and recover naturally. This has occurred in certain North American salmonid populations with *M. cerebralis* (Granath and Vincent 2010).

18.4.4 Novel Myxozoan Species as Causes of Emerging Diseases

The diversity of known and potentially undetected myxozoan species represents a rich pool of pathogens that can cause emerging diseases, particularly under intensive farming conditions or as a consequence of environmental change. Parasitological screening of candidate fish species and regular health surveillance of cultured fish species is therefore becoming routine as aquaculture is developing rapidly in many countries. Novel myxozoans are often discovered in the process. Table 18.4 provides examples of myxozoan species that affect host health or potential marketability and are therefore a cause for concern.

Table 18.4 Examples of myxozoan species discovered during parasitological assessments of candidate piscicultural species or health surveillance of cultured fishes

Myxozoan	Host	Location	Effect on host	References
Unicapsula pflugfelderi	striped seabream (*Lithognathus mormyrus)*	Mediterranean	Numerous plasmodia in skeletal muscle repulsive, may reduce host marketability	Alama-Bermejo et al. (2009)
Henneguya caudalongula	cultivated curimbatá (*Prochilodus lineatus)*	Brazil	Heavy infections likely compromise gill function	Adriano et al. (2005)
Ortholinea aurata	gilthead seabream (*Sparus aurata)*	Portugal	Urinary bladder (high infection prevalence but no pathology)	Rangel et al. (2014)
Kudoa neurophila	juvenile striped trumpeter (*Latris lineata*)	Australia	Infects central nervous system and causes severe meningoencephalomyelitis; mortalities	Grossel et al. (2003)
Myxobolus tambroides	Malaysian mahseer (*Tor tambroides)*	Malaysia	Gill deformation	Székely et al. (2012)
M. lentisuturalis	goldfish (*Carassius auratus auratus)*	Italy	Muscle lesions, macroscopic humps	Caffarra et al. (2009)

Most myxozoans represented appear to be native but previously unknown. The author's observations suggest that these myxozoan species may impact fish culture (see Effect on host). *Unicapsula pflugfelderi* is a new host record and *Myxobolus lentisuturalis* is a new host and geographical location record; the remainder is new species records

Myxozoans in novel vertebrate host groups have been described over the past decade (see Chap. 7) and some of these may become problematic. Examples include liver infections with *Myxidium* spp. that contribute to poor health of North American waterfowl (Bartholomew et al. 2008) and *Soricimyxum* spp. that cause inflammation and lesions in livers of European shrews (Prunescu et al. 2007; Dyková et al. 2007, 2011; Székely et al. 2011). Because of the sporadic nature of these observations and small sample groups, the population level impacts of these myxozoans on their homeothermic hosts are unknown.

18.5 Strategies to Understand and Limit Introductions

Diagnostic, monitoring and modeling approaches improve our understanding of myxozoans as agents of emerging diseases and inform management choices to limit myxozoan introductions. Below we describe how these approaches can be used to minimise introductions and curb disease emergence. Refer also to Chap. 21, which focuses specifically on mitigating myxozoan disease impacts on wild fish populations.

18.5.1 Baseline Monitoring

Detection of true invasives is impossible without knowing what myxozoan species are endemic. Collection of baseline data and having complete species descriptions provide answers to basic questions when a novel disease agent is indicated, such as: What is the parasite? Is it, or a close relative, a problem elsewhere? Was the parasite already present but not problematic? What is the natural host range of this parasite? Does a new record reflect true introduction or increased monitoring efforts? Examination of historical records (e.g. fisheries data) and specimens (e.g. in museum collections, zoological gardens, commercial aquaria) may confirm whether an infection is novel or has been present for some time (Hartigan et al. 2010). Emerging technologies, including deep sequencing of environmental DNA, can be exploited for detecting existing and novel species (e.g. Hartikainen et al. 2014).

18.5.2 Phylogeographic Studies

Comparison of variation in the genetic structure within and among parasite populations may reveal the mechanism and timeframe of dispersal. Low level intraspecific variation among DNA sequences of an informative gene reflects a high level of relatedness amongst geographically distant populations and hence evidence for recent transfer associated with anthropogenic actions. For example, European and North American isolates of *M. cerebralis*, which has been spread via trout culture, vary by <1 % in their ssrRNA and 1.7 % in their ITS-1 genes suggesting a recent transfer from Germany to the USA (Whipps et al. 2004; Arsan et al. 2007; Chap. 4). In contrast, distinct (1.9–11.9 % divergence) ITS-1 populations of *T. bryosalmonae* on both continents do not implicate fisheries activities for the distribution of this species (Henderson and Okamura 2004). Similarly, the presence of genetically distinct geographic populations of the marine myxozoan *K. thyrsites* suggests that the global distribution of the non-host specific parasite is natural and unaffected by anthropogenic activities (Whipps and Kent 2006). (See Chap. 4 regarding *C. shasta* and *Parvicapsula minibicornis* in the Pacific Northwest of North America).

18.5.3 Diagnostic Tools

Sensitive and specific tools, such as PCR, are used to detect and identify early or light infections in hosts, prior to development of clinical disease signs or mature spores. Surveillance of target problematic species using molecular analysis (qPCR) of water samples offers a non-lethal, semi-realtime, quantitative approach (e.g. in river water: Hallett and Bartholomew 2006; Hallett

et al. 2012 for *C. shasta*; Hallett and Bartholomew 2009 for *P. minibicornis*; in seawater: Alama-Bermejo et al. 2013 for *Ceratomyxa puntazzi*). This approach is recognised as an important monitoring tool (Sitjà-Bobadilla and Palenzuela 2012). Surveillance of spore levels informs proactive management. For example, qPCR is used to monitor the weekly abundance of waterborne *C. shasta* in the Klamath River, USA. In spring 2014 when parasite levels and river water temperature surpassed a disease-relevant threshold for endangered coho salmon (*O. kisutch*; see Hallett et al. 2012), water was released from a reservoir (a pulse flow) in an attempt to reduce disease effects (S. Hallett, unpublished data). PCR proved to be an effective aquaculture health management tool for *K. neurophila*, which causes severe meningoencephalomyelitis in juvenile striped trumpeter (*Latris lineata*). The assay can be used to screen fish for infection and also confirmed entry of the infectious stage via the hatchery intake water (Grossel et al. 2005). Subsequent ultraviolet irradiation treatment of incoming seawater to the hatchery prevents infection in juveniles (Cobcroft and Battaglene 2013) and may reduce potential economic impacts.

18.5.4 Risk Assessments

Development of predictive models, either quantitative or qualitative, can identify parasites that have the potential to impact aquacultural enterprises. For example, a qualitative risk analysis to determine the likelihood and consequence of parasite transfer from local wild yellowtail kingfish (*Seriola lalandi*) to farmed kingfish in South Australian sea-cage aquaculture identified *Kudoa* sp. and *Unicapsula seriolae* among metazoan species that present the highest potential for negative consequences (Hutson et al. 2007). Models can also determine the most likely route of introduction of problematic myxozoans (see Arsan and Bartholomew 2008, 2009). A better understanding of how the nature of the release affects success and the parasite threshold required for establishment would inform assessments. For instance, introductions can result when owners release low numbers of aquarium fish or when fisheries managers stock large numbers of fish for anglers. Laboratory experiments suggest that one fish infected with *M. cerebralis* is sufficient for transmission to downstream *T. tubifex*, which can complete the life cycle (Hallett and Bartholomew 2008). Future risk assessments need to account for increased pressure on food production and climate change. Chapter 20 provides further discussion of risk assessments for the evaluation of myxozoan disease impacts.

18.5.5 Management Strategies

Aquaculture facilities managers may alter farm conditions in response to myxozoans (endemic or exotic) that are potential or actual threats. This may involve, for example, delaying release of hatchery fish into infective waters, constructing concrete-bottom raceways, treating incoming water, or surveying source stocks for parasites prior to transport (Hallett and Bartholomew 2012). This latter approach is limited by available knowledge and detection assays. A cautionary tale is that of European glass eels (*Anguilla anguilla*) that were imported from Atlantic coast rivers to Hungary for farming. The eels were examined proactively for *Myxidium giardi* but latent infections were not detected. The infections subsequently became overt and initially were presumed to originate at the eel farm (Székely et al. 1988). This occurrence underscores that understanding the progression and presentation of infection of various myxozoans is essential for comprehensive screening.

18.5.6 Education and Legislation

Biosecurity measures and regulations at state and national levels may restrict transport of potentially infected hosts. Adequate veterinary controls and proper quarantines have been indispensable

to avoid the widespread contamination of Mediterranean areas by *Ceratomyxa*, *Enteromyxum*, *Myxobolus* and *Polysporoplasma* species (Sánchez-García et al. 2014). Education strategies for transport by members of the general public have included point-of-contact information (placards, brochures) at pet shops, boat ramps and fish cleaning stations to inform owners and recreational fishers. There is a need, however, for the development and implementation of quarantine measures in emerging industries such as amphibian and reptile farms for food production in Asia. Some commercial pathways exist outside of effective legislation. For instance, suppliers and purchasers in illegal wildlife trade and online sales are likely to ignore dangers of introductions. Furthermore, purchasing fish, amphibians and reptiles does not always require a license and therefore there is no associated education about disease or quarantine procedures.

Quarantine of incoming animals for myxozoan parasites is not practical in many circumstances. Vertebrates may not exhibit external disease signs, lethal sampling may only be possible for subsampling large populations of fishes (e.g. precluding sampling individual aquarium fishes) and non-lethal sampling (e.g. anal swabs, gill snips, fin punch biopsies, filtration of holding water) has not been developed for all species (see Hallett and Bartholomew 2012 for *M. cerebralis* and *C. shasta*). Furthermore, myxozoans may continue to develop and persist for weeks or months within introduced hosts. Some myxozoans exit their invertebrate host while it is alive, in which case the accompanying water can be screened for waterborne stages, however, the technique will not detect immature infections.

18.6 Conclusion

The spread of myxozoan infections to new parts of the world is an ongoing, dynamic process taking place in the setting of a rapidly changing global environment. Myxozoans are disseminated via a range of natural and human-mediated routes and exhibit a variety of traits linked to transmission, propagation, persistence and host utilization. These characteristics enable them to exploit new hosts and habitats and to cause severe emerging diseases in host species of economic and conservation importance. Emerging diseases may also be caused by endemic myxozoans when changing abiotic or biotic conditions drive disease outbreaks. Fish farming, stocking and global trade are making a considerable contribution to myxozoan dissemination and amplification. Our limited knowledge of the diversity and biological complexity of this group impedes our ability to monitor or block the movement of these parasites. Effective identification of emerging myxozoans is only possible after collection of baseline biodiversity and taxonomic data, which are essential for the unambiguous identification of pathogens and the diseases they cause.

18.7 Key Questions for Future Studies

- How can we best acquire baseline datasets on myxozoans to provide a context for understanding myxozoan diseases in local environments?
- What are the life cycles of myxozoans that may be threats or may currently cause emerging diseases?
- What are thresholds for the establishment of myxozoans?
- How does the nature of myxozoan release affect establishment success?
- Can technologies be developed to quickly and differentially assess the presence and abundance of actinospores and myxospores in the environment?
- Should we consider adopting quarantine procedures for animals potentially carrying myxozoan infections and what should these be?

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Modeling the Effects of Climate Change on Disease Severity: A Case Study of *Ceratonova* (syn *Ceratomyxa*) *shasta* in the Klamath River

19

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Abstract

Shifts in future temperature and precipitation patterns will have profound effects on host-parasite interactions and the dynamics of disease in freshwater systems. The aims of this chapter are to present an overview of myxozoan disease dynamics in the context of climate change, and to illustrate how these might be predicted over the next several decades by developing a case study of disease dynamics of *Ceratonova* (syn *Ceratomyxa*) *shasta* in the Klamath River, California USA. Our case study introduces a model ensemble for predicting changes in disease dynamics under different climate scenarios (warm/dry, moderate/median, and cool/wet) from 2020 to 2060. The ensemble uses Global Circulation Models (GCMs) and basin scaled models for the Klamath River to generate predictions for future water temperature and river discharge. The environmental data are used as inputs for a predictive model and a degree day model to simulate effects of climate change on polychaete host populations and on *C. shasta* spore viability, respectively. Outputs from these models were then used to parameterize an epidemiological model to predict changes in disease dynamics under each climate scenario. The epidemiological model outputs were measured against baselines established using real data for low (2006), high (2008) and intermediate (2011) disease risk years. In general, the epidemiological model predicts that except for infrequent high discharge years, *C. shasta* dynamics will be

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similar to the high disease risk baseline (2008). This suggests that the recovery and management of Klamath River salmon will continue to be impacted by *C. shasta*.

Keywords
Epidemiological models · Disease risk · Disease dynamics · Phenology · Host-parasite interactions · *Manayunkia speciosa*

19.1 Overview of Climate Change Effects

Global Circulation Models (GCMs) simulate the circulation of the atmosphere and ocean and are the principal tools used to predict future climate change. Outputs from GCMs depend on the model type (Table 19.1). Most GCMs provide predictions for atmospheric temperatures and precipitation. Atmospheric temperature shifts are the most striking predictions from these models. Globally, air temperatures are predicted to increase by ~2–4 °C over the next century (Solomon et al. 2007), with the magnitude of increase varying spatially as a function of distance from the equator. Increases of ~7 °C are predicted for arctic regions whereas only slight (1–2 °C) increases are predicted for equatorial regions (Solomon et al. 2007). The GCM predictions for atmospheric temperatures also vary seasonally. For example, data for the northern hemisphere predict that winter air temperatures will warm at approximately twice the rate of summers (Solomon et al. 2007). Increasing air temperatures are the most consistent predictions from the group of commonly used GCMs.

The GCMs also predict significant changes in precipitation patterns, but with greater uncertainty than for air temperature. Most GCMs predict shifts in the timing and form of precipitation, rather than net changes in the amount. Thus, winters will be wetter and summers drier (Mote 2003; Frei et al. 2006). These shifts will be linked with changes in precipitation form: as temperatures increase precipitation will increasingly fall as rain instead of snow, especially at lower elevations (<2,000 m) (Regonda et al. 2005). The effects of shifts from snowmelt to rainfall will include earlier onset of spring runoff, higher magnitude discharge over a shorter period of time, and reduced water availability in summer months (Regonda et al. 2005). Because precipitation determines the hydrological regimes of freshwater ecosystems, any change in the type and timing of precipitation is likely to have direct and indirect effects on the characteristics of freshwater ecosystems (Poff 1992).

Aquatic environments will also be susceptible to eutrophication caused by increasing temperatures, decreased precipitation, and rising nutrient inputs (Moss et al. 2011). These effects will vary depending on the characteristics of the water body (Gerten and Adrian 2001). For some aquatic environments this will affect changes in trophic structure due to increased sedimentation of organic matter resulting in less oxygen in bottom waters and sediments (Moss et al. 2011).

Shifts in temperature and precipitation will also alter the phenology (or timing) of biological events, with important consequences for disease dynamics. A recent meta-analysis determined that increased temperature was associated with the earlier occurrence of emergence, migration, or reproduction in 62 % of 677 terrestrial and aquatic species (Parmesan and Yohe 2003). The relationship between precipitation and phenological change/shifts is not as clear as for temperature (e.g. Peñuelas et al. 2004). For example, shifts that result in higher magnitude maximum (peak) flows (in winter/spring) and lower base flows (in summer) may alter the stability of host habitats, possibly reducing the overlap between hosts and parasites. However, the effects that shifts in precipitation are predicted to have on phenology often contrast with those predicted for temperature and both co-vary. Consequently, the types and directions of phenological responses to shifts in precipitation can be difficult to predict *a priori*.

19.2 Effects of Climate Change on Myxozoan-Host Interactions

Organismal responses to climate change effects are frequently non-linear and opposing (Altizer et al. 2013). For example, both parasite replication and parasite mortality rates are non-linear and increase with water temperature up to a threshold (Patz et al. 2003). When opposing factors such as replication and mortality both increase with temperature, the net effect can be difficult to predict. Predicting the effects of climate change on myxozoan disease is additionally complicated by the complexity of myxozoan life cycles, as the effects of each parameter must be considered in the context of both the vertebrate and invertebrate hosts and for each free-living spore stage. In this section, we review some of the potential effects of climate change on interactions between aquatic myxozoans and their hosts.

19.2.1 Water Temperature Effects

Water temperature influences each phase of the myxozoan life cycle, from interactions within hosts to spore viability (Chaps. 14 and 16). Consequently, temperature is likely to be a primary driver of myxozoan disease dynamics. The effects of increased water temperature on interactions between fish hosts and myxozoan parasites commonly manifest as more rapid disease progression and increased mortality. These effects are well documented for the myxosporeans *Myxobolus cerebralis* (Halliday 1973; Baldwin et al. 2000; Bettge et al. 2009), *Ceratonova shasta* (syn. *Ceratomyxa shasta*, Udey et al. 1975; Ray et al. 2012) and *Henneguya ictaluri* (Griffin et al. 2008), and for the malacosporean *Tetracapsuloides bryosalmonae* (Bettge et al. 2009). The effects likely extend to other pathogenic myxozoans. In addition to the increased rate of parasite replication at higher temperatures, temperature effects on fish host immune function also play a role in determining the rate of disease progression (see Chap. 13).

Water temperature also affects invertebrate hosts and interactions with their myxozoan parasites (see Chaps. 10 and 11). In general, individual and population growth rates of invertebrate hosts increase with temperature (Hogg et al. 1995; Hogg and Williams 1996) up to host thresholds. The relationship between temperature and parasite proliferation in invertebrate hosts has not been widely characterized but likely also increases until the upper thermal tolerance of the parasite or hosts is exceeded. For example, development and release of *M. cerebralis* actinospores in the invertebrate host (*Tubifex tubifex*) increase with temperature up to ~20 °C (El-Matbouli et al. 1999; Blazer et al. 2003; Kerans et al. 2005), but at temperatures ≥25 °C the host appears to clear or purge the parasite (El-Matbouli et al. 1999), suggesting *M. cerebralis* may have an upper thermal tolerance between 20 and 25 °C. A similar relationship between water temperature and spore production was noted for *T. bryosalmonae*, but an upper thermal limit was not identified (Tops et al. 2006).

The longevity of myxozoan stages in the environment is inversely correlated with water temperature (Yokoyama et al. 1995; El-Matbouli et al. 1999; Foott et al. 2007; Kallert and El-Matbouli 2008, and see Chap. 12). However, actinospores may be more negatively affected by temperature than myxospores, which are comparatively stable due to the hardened valves that surround the sporoplasm (Hedrick et al. 2008). For example, *M. cerebralis* actinospores remain viable for ~15 days at 15 °C and only 1 day at 23 °C, whereas myxospores remain viable for >60 days at <10 °C and 7 days at 22 °C (El-Matbouli et al. 1999; Hedrick et al. 2008; Kallert and El-Matbouli 2008). Similar relationships were observed for *C. shasta*: actinospores were viable for ~7 days at 4 °C and for ~4 days at 20 °C and myxospores persisted for >150 days at 4 °C and for 50 days at 20 °C (Foott et al. 2007; Bjork 2010; Chiaramonte 2013).

Increasing water temperatures may affect disease dynamics indirectly through effects on host distribution and on host and parasite

phenology. Shifts in the distributions of host species in response to changing environmental conditions may allow myxozoans to disperse further upstream or into previously uninhabited locations. Shifts in host distributions over larger spatial scales could result in major changes in parasite distributions. For example, Okamura et al. (2011) predicted increased ranges and/or range shifts to more northern latitudes or higher elevations for *T. bryosalmonae* in response to warmer temperatures. Shifts in host distributions may in turn affect the timing or duration of host-parasite interactions. Warmer temperatures will alter life cycle phenology, potentially resulting in longer periods of invertebrate host and parasite reproduction as well as increased numbers of parasite life cycles completed within a year (see Marcogliese 2001).

19.2.2 Precipitation and Discharge Effects

The effects of climate driven changes in precipitation (considered as discharge) on phases of the myxozoan life cycle may be just as important as those of temperature. The role of precipitation in these interactions is, however, less clear in the context of future climate scenarios. The predicted shifts in precipitation from snowpack runoff to rain will affect freshwater habitat stability through differences in the timing and magnitude of discharge. Decreased magnitude discharges may increase habitat (e.g. fine sediment) available for invertebrate hosts (Marcogliese 2001). The concomitant lower water levels may also cause vertebrate hosts to aggregate in greater densities. An increased overlap between high densities of hosts (vertebrate and invertebrate) and parasites can lead to greater infection prevalence and disease severity (Izyumova 1987; Holmes 1996).

Several studies have examined the effects of water velocity, which is influenced by discharge, on interactions between myxozoans and their hosts. When transmission and infection dynamics of *M. cerebralis* were examined at low and high velocity in a laboratory experiment, Hallett and Bartholomew (2008) observed that prevalence of infection in *Tubifex tubifex* (invertebrate host) and actinospore densities in water were higher in the low velocity treatment. Prevalence and severity of infection in the fish hosts were also higher in the low velocity treatment. Ray and Bartholomew (2013) similarly observed an inverse relationship between velocity and *C. shasta* transmission to fish hosts in a laboratory challenge, as did Bjork and Bartholomew (2009) for *C. shasta* transmission to its invertebrate host, *Manayunkia speciosa*. Although the mechanism (s) have not been identified, spore attachment to fish hosts (e.g. Ray and Bartholomew 2013) and invertebrate host density and foraging success (and hence ingestion of infectious spores; see Chap. 12) (Bjork and Bartholomew 2009; Jordan 2012) are likely reduced at higher velocities.

19.2.3 Nutrient Effects

Climate related changes in water quality (nutrient effects) may also affect disease dynamics through their effects on hosts. The amount of organic material in a stream is positively associated with the occurrence of several invertebrate hosts. For example, abundance of *T. tubifex* is correlated with high organic material (Allen and Bergersen 2002; Kaeser et al. 2006). Similarly, greater abundances of bryozoans occur in rivers with higher nutrient levels (Hartikainen et al. 2009) and increased growth and higher intensity of infection with *T. bryosalmonae* in bryozoans at higher nutrient/food levels (Hartikainen and Okamura 2012). Eutrophication also appears to influence outbreaks of proliferative kidney disease (PKD) caused by *T. bryosalmonae* infection. Following diversion of effluent from sewage treatment, the prevalence of PKD was reduced in hatchery and wild fish sampled downstream (El-Matbouli and Hoffmann 2002). Thus,

increased nutrient input or reduced water quality may exacerbate disease risk through effects on invertebrate hosts.

19.3 Modeling Approaches for Predicting the Influence of Climate Change on Disease in Aquatic Systems

Bioclimatic models (also known as envelope models, ecological niche models or species distribution models) are primarily applied to zoonoses (e.g. Olwoch et al. 2003; Ogden et al. 2006; Zhou et al. 2008). Such models can be statistical, predicting range shifts from correlations between climate variables and disease responses, or mechanistic, predicting change in spatial patterns of disease from inferred effects on physiological factors (Jeschke and Strayer 2008). Despite their limitations these models can be useful for predicting trends and relationships between climatic and response variables and to guide management actions. Meta-analysis has been used to examine broad patterns by synthesizing results from numerous small-scale correlative studies or experiments. This approach can provide evidence for responses to climate change by quantitatively summarizing data from multiple studies (Root et al. 2003). Statistical approaches have been used to examine correlations between disease incidence or severity and climate patterns that vary over space or time (e.g. Chaves and Pascual 2006). For aquatic parasites, the most informative modelling approaches will likely include a combination of correlative analyses that describe variation under current conditions, and predictive models that forecast disease dynamics under future conditions. The development of predictive models for most aquatic parasite infections and diseases is, however, constrained by a lack of baseline data and by the complexity of host-parasite interactions.

In this case study, we present an approach consisting of a combination of meta-analysis, and statistical and mathematical models to predict the dynamics of *C. shasta* in the Klamath River basin under several future climate scenarios.

19.4 Case Study: *Ceratonova shasta* and the Future Klamath River Basin

Ceratonova shasta is endemic to river systems throughout the Pacific Northwest region of North America. The parasite causes enteronecrosis (ceratomyxosis) in salmon and trout. Disease impacts on fish populations vary widely, from largely unnoticeable to highly significant effects (Ching and Munday 1984; Hallett and Bartholomew 2011). The Klamath River, California, is one of the more heavily affected rivers, and despite an intensive hatchery enrichment program (from Iron Gate Hatchery), salmon populations have continued to decline, in part due to high *C. shasta*-related mortality (Stocking et al. 2006; Fujiwara et al. 2011; True et al. 2011). Therefore, managing the parasite is a high priority in this river.

In the following sections we describe a model ensemble for predicting the dynamics of *C. shasta* under different future climatic scenarios (Fig. 19.1). The ensemble includes: (1) GCMs to predict future air temperatures and precipitation patterns (Table 19.1) and a fine-scale climate change model to predict future stream temperatures and discharge in the Klamath River (Perry et al. 2011), (2) polychaete models to predict changes in invertebrate host populations under different discharge scenarios (Wright et al. 2014; Alexander et al. unpub. data), (3) a degree-day model to predict *C. shasta* spore viability and number of annual generations under different temperature scenarios (Chiaramonte 2013), and (4) an epidemiological model to predict how *C. shasta* dynamics may respond to future climate scenarios (Ray 2013; Ray et al. unpub. data). The first model (Perry et al. 2011) used downscaled GCM data to provide water temperature and discharge data for the Klamath River and the latter three models are derived from empirical data. Below, we describe each model, the inputs and outputs for each model, and how these outputs are used in the epidemiological model to predict changes in *C. shasta* dynamics under the different future climate scenarios.

Table 19.1 Global Circulation Models (GCMs) for warm/dry and cool/wet climate scenarios and the predicted climatic factors for the Klamath River by decade from 2020 to 2060. Current conditions from 2006, 2008, and 2011 are shown as a baseline to compare the future river conditions including number of days <4 °C, max spring temperatures, and maximum discharges by Greimann et al. (2011)

Model name	Modeled year (disease status/ discharge)	Maximum discharge (CMS)	Maximum discharge (CFS)	Maximum spring temp (°C)	Maximum summer temp (°C)	#Days <4 °C
Baselines (current conditions)	2006 (low disease)	339.6	12,000	17.8	22	31
	2008 (high disease)	100.23	3,544	21.7	24.7	34
	2011 (moderate disease)	161.31	5,700	19.6	21.8	8
MIUB (warm/dry)	2046 (min)	32.97	1,164	23.4	25.6	0
	2039 (median)	52.59	1,857	20.7	24.5	0
	2015 (max)	474.02	16,738	22.4	24.4	0
GFDL (avg/avg)	2046 (min)	34.98	1,235	19.9	25.7	0
	2040 (median)	90.62	3,200	20.2	24.6	0
	2015 (max)	943.96	33,332	20.5	23.5	5
MRI (cool/wet)	2046 (min)	34.41	1,215	21.9	24	0
	2061 (median)	78.47	2,771	24.6	26.8	41
	2048 (max)	634.93	22,420	21	24.8	39

MIUB Meteorological Institute of the University of Bonn
GFDL Geophysical Fluid Dynamics Laboratory
MRI Meteorologic Research Institute
CMS discharge $m^3\ s^{-1}$
CFS discharge $ft^3\ \ s^{-1}$

19.4.1 Environmental Data Models

We selected three GCMs to provide temperature and precipitation predictions: (1) MIUB—warm/dry, (2) GFDL—average temperature and precipitation, and (3) MRI—cool/wet (Table 19.1, Fig. 19.1). The GCMs were selected to represent a wide range of conditions for both temperature and precipitation (based on the quantile rankings for predicted temperature and precipitation). We note that other factors will be affected by climate change (e.g., acidification, UV-radiation), but limit our analyses to temperature and precipitation predictions provided by the different models.

Future (2012–2061) Klamath Basin water temperature and discharge values were estimated from a calibrated one-dimensional water model (RBM10, Perry et al. 2011). This RBM10 model was developed using data from GCMs ($\sim$275 km^2) downscaled to provide more refined watershed-specific estimates (270 m^2) as described by Flint and Flint (2008).

Data Inputs: (1) Temperature and precipitation from GCMs, (2) basin scaled air temperature and discharge from an environmental model for the Klamath Basin (Flint and Flint 2008).

Data Outputs: Water temperature and maximum discharge at study location (Fig. 19.2) for each of three climate scenarios (min, median and max) for each GCM (Table 19.1, Perry et al. 2011).

19.4.2 Polychaete Predictive Model

To predict the effects of climate change on polychaete hosts, we used two modelling approaches: (1) two-dimensional hydraulic models (2DHMs) and (2) a logistic regression model (Fig. 19.1) (Alexander et al. unpub. data). The purpose of the hydraulic model was to predict depth, velocity, and substrate data. These variables are the inputs needed for the logistic regression model, which predicts the probability

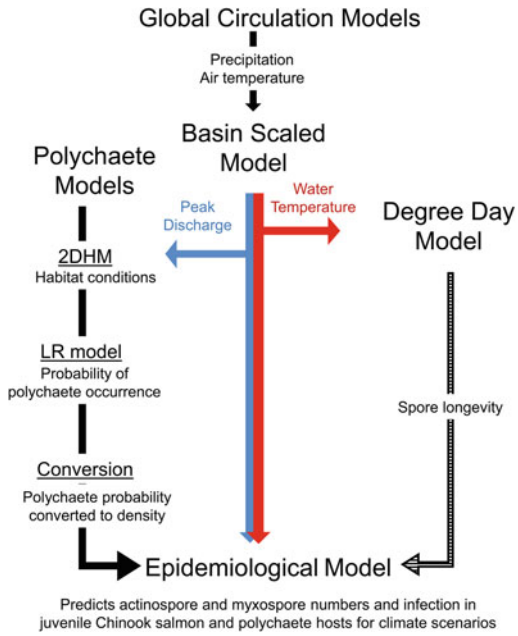

Fig. 19.1 Conceptual diagram of model ensemble and sources of data and data flow among the different models. Model outputs are represented by *smaller font*. *2DHM* 2 dimensional hydraulic model, *LR* logistic regression. The *dashed arrow* from the degree-day model indicates that this model was not used in this analysis as there was not a significant difference in degree-day accumulations between the current and future temperature scenarios in the Klamath River basin

of polychaetes being present at specific locations. Together, these models predict the probability that polychaetes will occupy specific locations based on maximum winter discharge predicted for each of the modeled future climate scenarios. 2DHMs have been developed for three sections of the Klamath River (Wright et al. 2014) in which prevalence and severity of *C. shasta* infection is high in juvenile salmon (Hallett and Bartholomew 2006; Hallett et al. 2012). The logistic regression model developed by Alexander et al. (unpub. data) uses data outputs from the 2DHMs to predict the probability of polychaete presence at specific x, y locations.

Data Inputs for 2DHM: Maximum discharge predicted from the basin scaled model (Table 19.1).

Data Outputs from 2DHM: Depth, velocity, and shear stress at 0.5 m grid cells at each site. These data are coupled with a substrate map created in 2011 (Wright et al. 2014).

Data Inputs for logistic regression model: Depth, velocity, and substrate at maximum discharge for each of the modeled future climate scenarios.

Data Outputs from logistic regression model: The probability of polychaetes at 122,747 x, y data points approximately 0.5 m grid cells apart. The probabilities were weighted by grid cell size and then averaged to obtain an overall probability of occurrence for the entire study reach. To obtain polychaete densities for input into the epidemiological model, we adjusted a baseline density (measured in 2010, Jordan 2012) by the difference in the modeled probability of polychaetes for each climate scenario. This required two steps. First, the probability of polychaete presence was modeled for the baseline (2010) and compared to the modeled probability of polychaetes for each climate scenario. Second, the baseline density was adjusted by the difference between each modeled scenario and the baseline. Thus, a 20 % decrease in the probability of polychaete presence was translated into a 20 % decrease in the baseline density. The baseline density was estimated using data from Beaver Creek (Jordan 2012), a site for which long term data for parasite density and prevalence of infection in sentinel fish are available (Hallett et al. 2012) and for which Wright et al. (2014) developed a 2DHM.

19.4.3 Degree Day Model

Chiaramonte (2013) developed a degree day model for the *C. shasta* life cycle that uses the accumulation of thermal units to estimate the time to completion of a single generation and the longevity of each spore stage (Fig. 19.1). Results from a combination of field and laboratory studies identified a temperature threshold of ~4 °C, below which development of *C. shasta* is inhibited. This is significant because if winter temperatures increase, as predicted, there is a potential for year round release of *C. shasta* actinospores. In the salmon host, myxospore

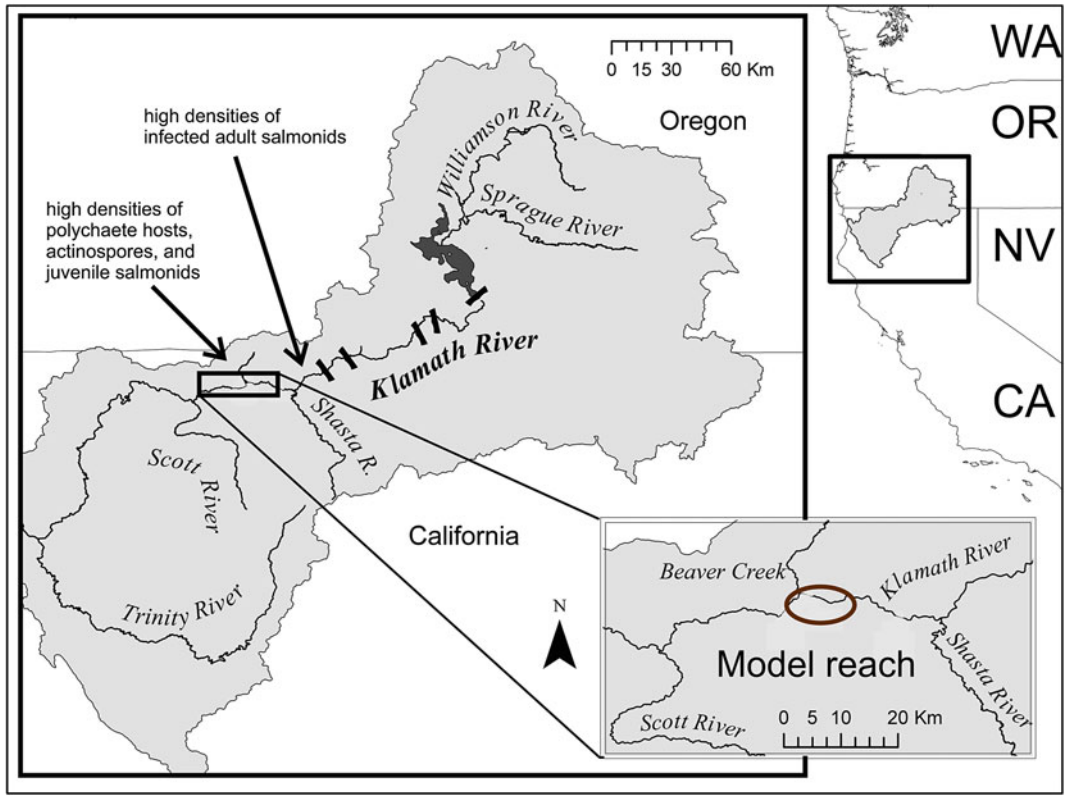

Fig. 19.2 Location of case study reach on the Klamath River, California. *Black perpendicular lines* indicate location of dams, the lowermost of which is a barrier to anadromous salmonids. High densities of infected adult salmonids are found in the reach immediately downstream from the lowermost dam, and high densities of polychaetes, actinospores, and juvenile salmonids are observed in the reach downstream from the confluence with the shasta River

development occurs in ~282 degree days. About 651 degree days are required for development and release of actinospores from the polychaete host. Thus, one complete generation of *C. shasta* requires ~934 degree days at >4 °C, assuming instantaneous transmission to the next host. This is about the number of degree days required from spawning to emergence of fry.

Data Inputs: Water temperatures predicted from the downscaled GCMs were used for each water year type (2006, 2008, 2011) and future climate scenario (MIUB, GDFL, MRI) (Table 19.1). Data included the number of days from November 1st to March 31st where water temperatures were <4 °C and the mean water temperature in spring, summer, and fall.

Data Outputs: Longevity of myxospores and actinospore stages.

19.4.4 Epidemiological Model

Ray (2013) developed an epidemiological model for the *C. shasta* life cycle that incorporates the rates of spore production from, and transmission to, their respective hosts, and mortality rates of host and parasite stages. This model is comprised of a series of differential equations that mathematically represent phases of the *C. shasta* life cycle. We have further developed the model to include a periodicity function to more accurately capture the timing of life cycle phases (Ray et al. unpub. data, Box 19.1).

Box 19.1 Epidemiological model periodic equations and descriptions. κ (kappa) is a periodic "switch" function that activates different parts of the model corresponding to the season in which they occur. κ is "switched on" in the spring with a value of 1 and "switched off" (value of 0) in the other seasons. ω denotes a maturation rate term for juvenile salmon that results in a loss of juveniles but a gain in adult salmon (here we simplify and assume instantaneous maturation).

Equation (19.1) Actinospores are produced (λ_a) from infected polychaetes (P^*) in either the spring or fall and are lost due to natural mortality (ν_a; related to water temperature) or transmitted (β) to juvenile (S_J) or adult (S_A) salmon hosts. Equation (19.2) Myxospores are produced (λ_m) from infected juvenile (S_J^*) and adult (S_A^*) salmon hosts and are lost due to natural mortality (ν_m) or are transmitted (β) to polychaete (P) hosts. Equation (19.3) Polychaetes are assumed to have year round production (λ_P) and natural mortality rates (μ_P). Polychaetes become infected when the myxospore stage is transmitted (β) in either the winter, from infected adult salmon, or summer, from infected juvenile salmon. Equation (19.4) Infected polychaetes are produced in the winter and summer from the transmission (β) of myxospore stage and are lost due to natural mortality (μ_P). Equation (19.5) Adult salmon return in the fall (uninfected, λ_A) and we assumed no *C. shasta* related pre-spawn mortality. Adult salmon become infected when the actinospore stage is transmitted (β) from infected polychaetes in the fall. In the winter all adult salmon succumb to natural mortality (μ_{SA}). Equation (19.7) In most systems, Juvenile salmon are produced in the spring from returning adults the previous fall (f_{SA}) but due to the significant and consistent hatchery production in the Klamath River system we assume this value to be constant. Juvenile salmon are lost due to transmission of the actinospore stage (β), natural mortality (μ), and maturation to the adult stage (ω). Equation (19.8) Juvenile salmon become infected when the actinospore stage is transmitted (β) from infected polychaetes in the spring and are removed from the system due to mortality (μ_{SJ}^*, both natural and parasite induced).

$$da/dt = \kappa_{Sp}(t)(\lambda_a P^* - \nu_a a - \beta a S_J) + \kappa_F(t)\,(\lambda_a P^* - \nu_a a - \beta a S_A) - \kappa_{Su}(t)(\nu_a a) - \kappa_w(t)(\nu_a a) \tag{19.1}$$

$$dm/dt = \kappa_W(t)(\lambda_m S_A{}^* - \nu_m m - \beta m P) + \kappa_{Su}(t)\,(\lambda_m S_J{}^* - \nu_m m - \beta m P) - \kappa_{Sp}(\nu_m m) - \kappa_F(\nu_m m) \tag{19.2}$$

$$dP/dt = \kappa_W(t)(\lambda_P P - \mu_P P - \beta m P) + \kappa_{Su}(t)\,(\lambda_P P - \mu_P P - \beta m P) - \kappa_{Sp}(\mu_P P) - \kappa_F(\mu_P P) \tag{19.3}$$

$$dP^*/dt = \kappa_W(t)(\beta m P - \mu_P P^*) + \kappa_{Su}(t)\,(\beta m P - \mu_P P^*) - \kappa_{Sp}(t)(t)(\mu_P P^*) - \kappa_F(t)(\mu_P P^*) \tag{19.4}$$

$$dS_A/dt = \kappa_F(t)(\lambda_A - \beta a S_A) - \kappa_W(t)\mu_{SA} S_A + \kappa_{Sp}(t)\omega_{S_J} \tag{19.5}$$

$$dS_A{}^*/dt = \kappa_F(t)\beta a S_A - \kappa_W(t)\mu_{SA}{}^* S^*{}_A \tag{19.6}$$

$$dS_J/dt = \kappa_{Sp}(t) f_{SA} - \kappa_{Sp}(t)(\beta a S_J + \mu_{SJ} S_J + \omega_{SJ} S_J) \tag{19.7}$$

$$dS_J{}^*/dt = \kappa_{Sp}(t)(\beta a S_J - \mu S_J{}^*) - \kappa_{Su}(t)(\mu_{S_J}^* S_J^*) \tag{19.8}$$

Data Inputs: Predicted (min, mean, max) spring, summer, and fall water temperatures from the GCMs and the basin scaled models were used as inputs into the epidemiological model to parameterize parasite induced mortality and myxospore production in the salmon host (Fig. 19.1, Table 19.2). Predicted maximum winter discharges from GCMs were input into the epidemiological model to parameterize myxospore transmission rates to the polychaete host. Changes in occurrence and availability of polychaete habitat were used as a proxy metric for changes in polychaete population densities for the different climate scenarios. The differing spore longevity rates from the degree-day model were used to parameterize spore survival.

In addition to data provided from models in this ensemble, data from a long-term longitudinal survey were incorporated. Data from sentinel ('caged') fish and water samples collected since 2006 were used to parameterize parasite distribution and density, and the severity of infection in fish at the study site (Ray et al. 2010; Hallett et al. 2012). Data from out-migrating juvenile salmon were used to parameterize prevalence of infection in juveniles (True et al. 2013).

Data Output: Changes in the population size (or population growth) of actinospores, myxospores, infected polychaetes and infected juvenile Chinook salmon over a 12 month time frame for each of the different climate scenarios and water years.

19.4.5 Predicted Climate Scenarios and Their Effects on the *C. shasta* Life Cycle

The environmental data models predict important differences in temperature and significant differences in discharge in the Klamath River under all future climate scenarios (Flint and Flint 2008; Perry et al. 2011) (Table 19.1). Spring water temperature is predicted to increase by ~1–3 °C between 2012 and 2060, depending on the climate scenario (Perry et al. 2011). In the Klamath River, where salmonids already exist near their upper thermal tolerance limits (<20 °C) this seemingly small temperature increase may be particularly significant. Overall, net precipitation is predicted to remain the same as current conditions, but summer precipitation is predicted to decrease by 1–3 mm, with the balance

Table 19.2 Epidemiological model assumptions and starting values for density of polychaetes (Poly) and infected polychaetes (Inf Poly), parasite transmission, and mortality rates for actinospores in spring (A_{sp}) and fall (A_f), myxospores in winter (M_W), and infected juvenile salmon (*Inf JS*)

Scenario	Density		Transmission	Mortality rates			
	Poly	Inf Poly	Rate	A_{sp}	A_f	M_W	Inf JS
2008	100,000	20,000	2.00E−06	0.20	0.20	0.067	0.05
2011	76,000	15,200	1.30E−06	0.1	0.1	0.01	0.04
2006	36,000	7,200	5.70E−07	0.067	0.067	0.067	0.033
MIUB min	100,000	20,000	6.00E−06	0.2	0.2	0.01	0.05
MIUB med	97,000	19,400	4.00E−06	0.2	0.2		0.04
MIUB max	22,000	4,400	4.20E−07	0.2	0.2		0.04
GDFL min	100,000	20,000	6.00E−06	0.125	0.1		0.04
GDFL med	90,000	18,000	2.20E−06				
GDFL max	1,000	200	2.13E−07	0.125		0.067	0.04
MRI min	100,000	20,000	6.67E−06	0.125		0.01	0.05
MRI med	92,000	18,400	2.50E−06	0.25	0.25	0.067	0.05
MRI max	1,000	200	3.17E−07	0.125	0.25	0.067	0.05

Starting values for actinospores (1,000), myxospores (2,500), adult salmon (0.01), infected adult salmon (0), juvenile salmon (1), infected juvenile salmon (0), myxospore mortality (0.02), polychaete mortality (0.01), adult salmon mortality (*winter* 1; all die), and juvenile salmon mortality (0.002) were held constant across modeled scenarios

(+1–14 mm) occurring in winter (Barr et al. 2010). Maximum winter discharge is predicted to vary significantly among and within the different climate scenarios and the majority of the differences we detected among modeled response variables (actinospores, myxospores, polychaete populations) appear to be driven by discharge.

The epidemiological model predicts substantial variation in numbers of actinospores, myxospores, infected polychaetes and infected juvenile salmon among the modeled climate scenarios (Fig. 19.3a–d). The outputs are interpreted relative to model outputs for three baselines for the years 2008 (high mortality in salmonids), 2011 (intermediate mortality), and 2006 (low mortality), when inputs were based on real data collected in the Klamath River. In general, many of the future values for actinospores, myxospores, infected polychaetes, and infected juvenile salmon were predicted to be similar to the 2008 baseline values. This provides compelling evidence that *C. shasta*-induced mortality will remain high in the Klamath River. Predicted effects of climate scenarios on life cycle phases and model limitations are discussed below.

19.4.5.1 Actinospores

Under current climate conditions, actinospore release begins in early spring (3rd time step—roughly March or April, Fig. 19.3a) as water temperatures increase above 10 °C. Densities peak in late spring and a second peak, of lower magnitude, generally occurs in the fall. The model predicts that actinospore numbers during juvenile salmon migration will be similar to 2008 (high disease risk) for all scenarios except the

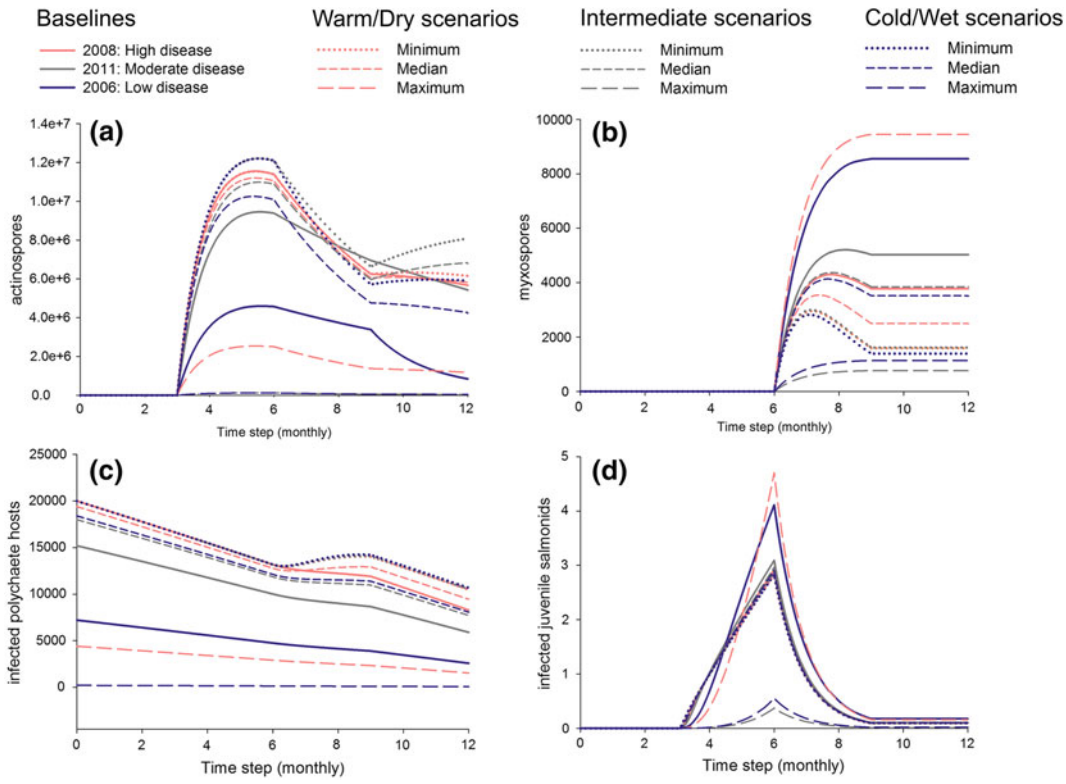

Fig. 19.3 Epidemiological model predictions for numbers of **a** actinospore **b** myxospore, **c** infected polychaete, **d** infected juvenile salmon populations under nine future climate scenarios, including *minimum*, *median* and *maximum* for MIUB (warm/dry), GDFL (intermediate) and MRI (cool/wet). Baselines (2006, 2008, 2011) are shown as *solid lines*. Time steps are based on monthly periods (from Jan to Dec)

maximum scenarios for all climate models, where actinospore numbers decrease below 2006 (low disease risk) levels. A limitation of the current model is that it does not capture interannual variability in start and peak dates of actinospore release because the periodicity function is based on a monthly time step. Our empirical data demonstrate earlier and longer periods of actinospore detection in warmer years (2008 and 2009) than in cooler years (2010 and 2011, Chiaramonte 2013). Because these effects may counteract or offset the increased spore mortality at higher temperatures, we recognize this may be an important limitation of the model.

19.4.5.2 Myxospores

Under current conditions, myxospore production occurs in late spring (outmigrating juvenile salmon) and in late fall to early winter (upstream migrant adult salmon). The epidemiological model predicts myxospore production in late spring through winter (time steps 6–12; Fig. 19.3b). The model also predicts that myxospore levels will be lower or equal to 2008 baseline level. We would expect 2008 myxospore levels to have been high relative to 2006 and 2011 baselines but the model predicts the opposite. One explanation for this is that under high disease risk conditions juvenile fish may die faster than myxospores develop. A limitation of the current model is that it doesn't reflect that the majority of myxospores are likely derived from the infected spawning adults as their carcasses decompose (late fall to winter). As with the actinospore predictions, the model fails to capture interannual variability in myxospore production, likely as a result of the relatively constant juvenile and adult salmon populations (see starting values for adult returns, Table 19.2).

19.4.5.3 Polychaete Hosts

Under current conditions, polychaete populations expand in late spring, peak in summer and decline in fall (Jordan 2012). Overwintering mature (adult) polychaetes that consume myxospores in late fall or early winter are assumed to be the source of actinospores released in spring. The epidemiological model predicts that under future climate scenarios, numbers of infected polychaetes will be similar to the 2008 baseline (high), except under all maximum scenarios, when densities of infected polychaetes are predicted to be lower than the 2006 (low disease risk) baseline (Alexander et al. 2014). This illustrates the importance of maximum discharge. The model predicts declining numbers of infected polychaetes from time steps 0 to 5, which we attribute to (1) a lack of myxospore input, and (2) natural polychaete mortality in winter to spring (time steps 0–5). During time steps 6–9, the model predicts an increase in numbers of infected polychaetes under all minimum climate scenarios and the warm/dry median scenario, which we attribute to (1) myxospore input from infected juvenile salmon, and (2) polychaete population growth during spring-summer.

For the purpose of examining the effects on juvenile salmon, we focus on model predictions between time steps 5 and 7 (when juvenile salmon are present). During these time steps, water temperatures are >10 °C, and the model predicts high numbers of infected polychaetes (except under maximum discharge scenarios, Fig. 19.3c), which corresponds to the high numbers of actinospores (Fig. 19.3a). As actinospore numbers begin to decline (model time step 6), the increase in numbers of infected polychaetes is likely due to new infections arising from myxospore inputs derived from infected juvenile salmon (see below).

19.4.5.4 Juvenile Salmonid Hosts

Under current conditions the numbers of juvenile salmon released from Iron Gate hatchery are relatively constant and we observe substantial interannual variation in infection rates and numbers of infected juveniles (Ray et al. unpub. data). The epidemiological model predicts that under most climate scenarios, numbers of infected juvenile salmon will be similar to 2008, a year in which high mortality was observed (Fig. 19.3d). However, under cool/wet and intermediate climate scenarios at maximum

discharge, which are predicted to occur at rare and irregular intervals, the numbers of infected juvenile fish will be low relative to present-day conditions (e.g. reduced by one third), indicating the effects of *C. shasta* on juvenile salmon may be less dramatic in those year types. Interestingly, the epidemiological model predicts that numbers of infected juvenile salmon will be highest under the warm/dry maximum discharge and 2006 baseline scenarios. This result may be explained by slower parasite induced mortality at lower temperatures, resulting in greater numbers of juveniles that take longer to be removed from the (modeled) population. This explanation is supported by the high infection prevalence among the outmigrant fish sampled during 2006 (True et al. 2011). Alternatively, this may represent another model limitation that could be resolved by separately modeling juvenile mortality rates and prevalence of infection.

19.5 Conclusions

The most striking prediction from the epidemiological model is that only two scenarios predict that the numbers of infected juvenile salmon will be lower than most present-day conditions. For the majority of the scenarios, the numbers of infected salmon will be similar to the 2008 baseline, a year in which high mortality was observed. One important limitation of our epidemiological model is that *C. shasta* life cycle parameters (e.g. actinospore production, polychaete birth/death, transmission rates, etc.) cannot yet be modeled on a daily time step. Future iterations of this model should begin to address these limitations as data become available.

In our case study high winter discharge had the greatest effect on disease dynamics. Smaller polychaete host populations predicted under high discharge scenarios likely produce fewer actinospores, which in turn should result in lower numbers of infected juvenile salmon, while the number of infected juvenile fish was predicted to decrease under cooler temperature regimes. Surprisingly, we did not see a dramatic effect of increasing temperature, which is likely a result of the Klamath system already being near the upper thermal limit for both the parasite and salmon host. This is evidenced by the summer maximum temperatures ranging between 22 and 27 °C, regardless of climate scenario. Another effect of temperature that was not detected in our model is the increase in numbers of infected juvenile salmon compared to the 2008 baseline (a warm/dry year with high disease-related mortality) (True et al. 2011). One explanation for this result is that the thermal tolerance of juvenile salmon is not incorporated in our model. If it had been, the abundance of infected individuals would decrease in the summer months once temperatures exceed 23–24 °C (e.g. Udey et al. 1975; Richter and Kolmes 2005).

Climate change will alter host-parasite interactions, however the magnitude and direction of overall effects are difficult to predict and may be context specific. Our case study exemplifies this complexity in examining the potential effects of temperature and precipitation-driven climate change on the *C. shasta* life cycle in the Klamath River. The study demonstrates the types of data required to develop and parameterize models that can be used to predict *C. shasta* dynamics. We hope that our case study exemplifies how ensemble modelling may be applied for predicting disease dynamics in other pathogenic myxozoans.

19.6 Key Questions for Future Research

- Will increasing temperatures cause changes in parasite virulence that affect model predictions?
- Will changes in precipitation be more influential than temperature changes at host or parasite range limits?
- Will the effects of other anthropogenic factors (e.g. dams, pollution) mask the predicted effects of climate change on disease?
- Can conceptual models be developed for myxozoans for which there are limited data on the effects of climate-related parameters on life cycle stages?

- Is this model ensemble approach applicable to other river systems and to other myxozoans?
- How will myxozoans infecting terrestrial and avian hosts be affected by climate change?

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Risk Assessments and Approaches for Evaluating Myxozoan Disease Impacts

20

Jerri L. Bartholomew and Billie Kerans

Abstract

Risk assessments are increasingly being used as tools to examine the possibility and consequences of transboundary introductions of pathogens and parasites of important aquaculture species. Developing these tools for myxozoan parasites is complicated by their two-host life cycles and our lack of basic data for many parameters. The distributions of many myxozoans are undocumented, techniques used for detection of myxozoan may vary in sensitivity and specificity, and often, the invertebrate host and its ecological requirements are poorly understood. Risk assessments for myxozoans have been performed for a variety of reasons and incorporate different geographic scopes and management questions. In this chapter, we review the general components of a risk assessment, discuss specific data requirements for myxozoan risk assessments, and provide examples of myxozoan risk assessments in relation to importation of food fish, emerging aquaculture species and wild fisheries, and pathogen transmission from aquaculture to wild fish.

Keywords

Release assessment · Exposure assessment · Consequence assessment · Import risk assessment · Risk analysis

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20.1 Risk Analysis and Assessment Methods in Aquatic Animal Health

A risk assessment is typically part of the larger process of risk analysis, which is a structured way to determine: what can go wrong (hazard identification), the chances that the specific hazard occurs and the consequences of that occurrence (risk assessment), and what can be done to reduce the chances or the consequences of the hazard (risk management) (see Fig. 20.1). Risk assessments provide information about the nature, magnitude, and likelihood of undesirable events and are used in decision making for e.g. disease management. Although we include some description of the process as background for understanding the myxozoan risk assessments in this chapter, we refer the reader to other papers for how to develop a risk assessment and for overall reviews of risk analysis methods (NRC 1983; Covello and Merkhofer 1993; MacDiarmid 1997; Vose 2000; Murray 2002; Peeler et al. 2006, 2007; Peeler and Taylor 2011; Oidtmann et al. 2013).

In aquatic animal health, risk assessments have been used primarily in the form of import risk analysis (IRA) to examine risks to animal and human health that are associated with international trade in animals or animal products (Peeler et al. 2007). Guidelines for IRA described in the Aquatic Animal Health Code [Office International des Epizooties (OIE) 2003] and are based on the Covello-Merkhofer model (Covello and Merkhofer 1993). A similar risk assessment developed by the European Food Safety Authority (EFSA 2012) for animal welfare has also been applied to aquatic animal disease (Sánchez-García et al. 2014). In these risk assessment models, carefully defining the scenario (hazard and scope) is an important part of the process and is done prior to developing the risk assessment. Hazards are commonly defined as pathogens known to cause mortality in a certain host species or region. In an IRA this is often developed as a list of known potential pathogens associated with the commodity in the exporting (source) region. In risk assessments for emerging aquaculture species and wild fisheries the definition of a hazard can be expanded to include previously undescribed pathogens identified when new host species are brought into aquaculture, and pathogens benign in one host species or stock that cause adverse effects when introduced into a naive population. Determining whether a particular hazard requires further consideration depends on whether the pathogen is exotic to the importing region, whether it is listed and thus requires consideration, and whether it is likely to result in significant disease. If a potential pathogen does not meet these criteria then a risk assessment may be concluded at this

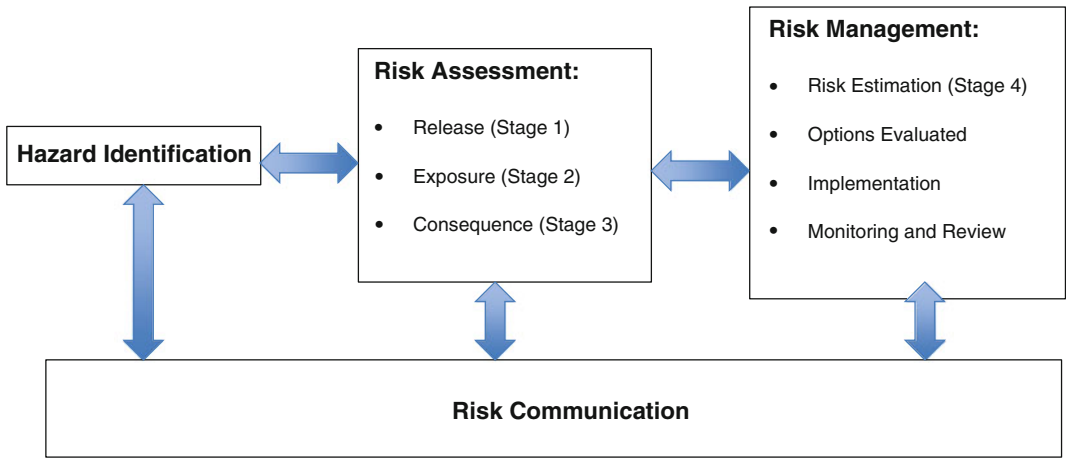

Fig. 20.1 Structure of the risk analysis process (modeled after Covello and Merkhofer 1993; Peeler et al. 2007)

point. The scope of a risk assessment can be narrow or broad; for instance, an assessment can be restricted to a single water body or fish farm, or can encompass an ocean or entire fishery. Defining the scope will depend on the management questions.

The OIE and EFSA models are similarly structured, dividing the assessment into four stages (Fig. 20.1). Stages 1–3 are associated with the risk assessment phase of the risk analysis process. Stage 1, the release assessment, examines pathways for introduction of the hazard. Stage 2, the exposure assessment, examines the pathways required for the hazard to occur after introduction. Stage 3, the consequence assessment, identifies the adverse effects of the hazard on the population at risk. Finally, Stage 4, the risk estimation, is associated with the risk management phase of the risk analysis process and integrates the previous three stages into a risk estimate for a specific case by building a scenario tree that sequentially examines the risk factors and their potential outcomes. Risk assessments for aquatic pathogens have generally focused on the first three stages, and these will be discussed further below.

Risk assessments can be either qualitative, where risks are expressed in general terms like "high" or "negligible", or quantitative. Those for aquatic pathogens have been, in large part, qualitative, as reliable data on host population size, infection prevalence and other important characteristics of wild populations are often lacking. A qualitative assessment is often sufficient to support a decision process when the scope and terms used to describe risk are well defined. Qualitative risks become semi-quantitative when the assessments are assigned a numerical value (e.g. high risk = >90 % probability). Quantitative assessments are less common for a variety of reasons that include lack of data, a high degree of uncertainty and difficulties in communicating these outcomes to stakeholders (Peeler et al. 2006). Approaches for quantitative assessments incorporate uncertainty and variability in data and include Monte Carlo simulations (e.g. Bruneau 2001; Ayre et al. 2014) and Bayesian belief networks (e.g. Ayre et al. 2014).

20.2 Data Required for Conducting Myxozoan Risk Assessments

By its very nature, a risk assessment integrates data from multiple sources and requires knowledge of the biology of the infectious agent, including its transmission and dispersal routes, characteristics of the receiving environment, and the demographic and ecological traits of the hosts involved. Even within a particular species or ecosystem, some information will be lacking and may require reliance on expert opinion. The assessment begins with the assumption that the hazard is not already present within the defined geographic scope, which itself is not always certain. For instance, data on parasite presence and distribution in wild populations or larger geographic regions are often lacking or insufficient. Thus, carrying out a risk assessment requires compiling the best information available and identifying data gaps. These data gaps may significantly increase the uncertainty of the risk assessment; however, as these questions are resolved by research and monitoring, the quality of the risk assessment can be expected to improve. The conclusions may then be either supported or modified. In the following sections we consider the type of data that may be required for Stages 1–3 of a risk assessment for myxozoans.

20.2.1 Release Assessment

Myxozoans may be introduced by stocking or movements of fish through aquaculture practices, in fresh or frozen fish product, by natural movements of fish, by dispersal of parasite stages in water, through recreational, restoration or commercial activities, or by release of spores from piscivorous fish or mammals (Fig. 20.2; see also Chap. 18). When evaluating the likelihood of introduction by these routes, the presence and distribution of the parasite(s) in the source area should ideally be known. Movement of live fish is generally considered the most likely route of introduction (Hedrick 1998); thus when considering risks posed either by aquaculture/stocking

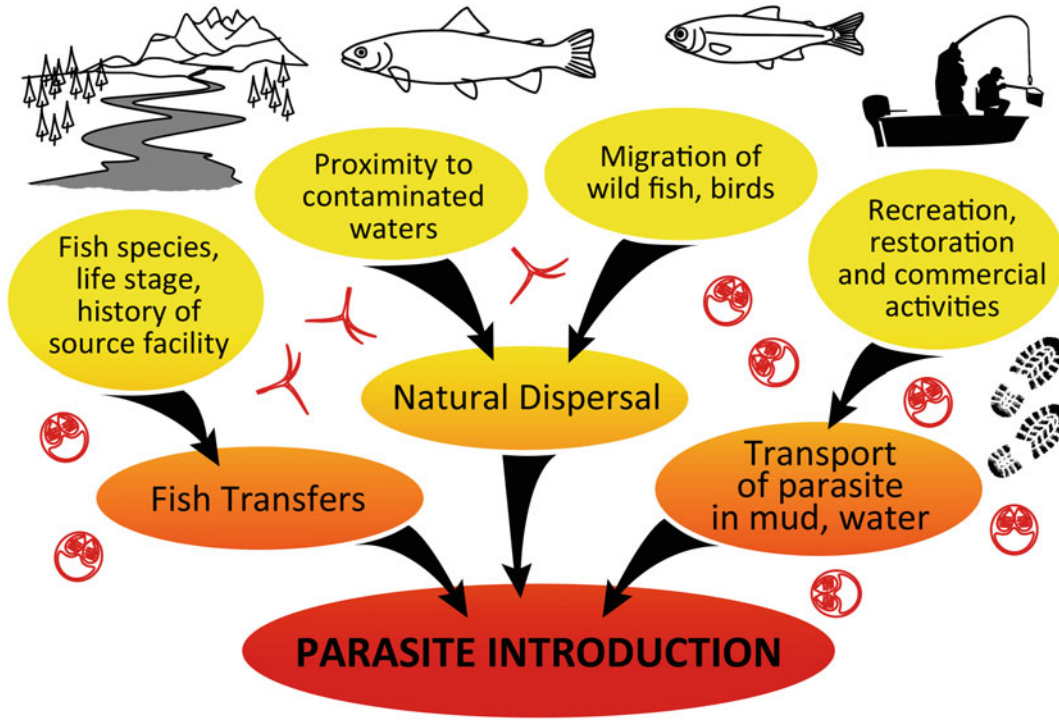

Fig. 20.2 Release assessment involves characterizing potential pathways for introduction of myxozoan parasites (e.g. as spores or via infected hosts) (diagram courtesy Stephen Atkinson)

or naturally migrating fish, knowing the infection prevalence in the source host population is important. The frequency of severe infections, which is generally related to the susceptibility of the source host population(s), is also important as it affects the numbers of parasite introduced. For example, susceptibility to *Myxobolus cerebralis* varies widely among the salmonid species, as does its proliferation within hosts [e.g., lake trout (*Salvelinus namaycush*) are refractory to infection, rainbow trout (*Oncorhynchus mykiss*) are highly susceptible, and variation in disease susceptibility and parasite proliferation exists among subspecies of cutthroat trout (*O. clarki*) (MacConnell and Vincent 2002; Vincent 2002)]. Additional considerations apply in evaluating the risk of importing fish as a commodity and include the tissue in which the parasite localizes, the method of preservation and the intended use. For instance, importation of fish fillets is less likely to be a high risk for introduction of *M. cerebralis* compared with whole fish.

Myxozoans may also disperse naturally with their invertebrate hosts. For example, *Tetracapsuloides bryosalmonae* may be dispersed in the detached fragments (Morris and Adams 2006) and dormant stages (statoblasts) (Abd-Elfattah et al. 2014) of bryozoans (See Chap. 11). The likelihood of natural dispersal of both invertebrate hosts and of waterborne parasite stages is difficult to predict and requires data on proximity to source areas, which may not be well documented, as well as on survival of each of the two spore stages. Invertebrate hosts are less likely to be intentionally moved than fish hosts, but introductions can occur as a result of recreation or commercial activities. For example, actinospores have been detected in oligochaetes imported as food for ornamental fish (Lowers and Bartholomew 2003). Invertebrate host susceptibility also may differ. For example, there is considerable variation in propagation of *M. cerebralis* within genetic lineages of *Tubifex tubifex* (Rasmussen et al. 2008). Parasite dissemination may also be facilitated by migratory fishes

and piscivorous birds (Koel et al. 2010), although many factors would need to align for long-distance introduction to occur by these routes (Arsan and Bartholomew 2008). Thus, particularly for natural dispersal, distance from source areas is likely to be inversely related to the likelihood of introduction (Schisler and Bergersen 2002).

The frequency of an event occurring, whether it be stocking, commodity importation, recreation or natural dispersal, also affects the likelihood of introduction. Even infrequent events may promote introductions when the assessment is expanded over sufficiently long timescales. Further examples of data used for conducting a release assessment are summarized in Table 20.1 (see Pathways considered). The outcome of a release assessment can be expressed as either probabilities or degrees of likelihood of introduction occurring, and may include a scenario tree that identifies and ranks introduction pathways.

20.2.2 Exposure Assessment

A variety of factors affect the ability of a parasite to become established and proliferate once it has been introduced (Fig. 20.3). Foremost of these is the presence of both susceptible fish and invertebrate hosts, which in most cases must overlap both spatially and temporally for the parasite life cycle to establish. For this reason, myxozoans with complex life cycles may not be as likely to establish as directly transmitted species. Information is generally available on fish species present, but data on presence, abundance and distribution of invertebrate hosts is less common. In addition, susceptibility to parasite infection is often variable within both vertebrate and invertebrate host species. In the invertebrate host, identifying susceptible strains is further complicated by morphologically similar species (Lodh et al. 2011, 2013), cryptic host lineages (Beauchamp et al. 2001) and covert infections

Table 20.1 Examples of data required for conducting a release assessment

Introduction route	Data needs
Fish transfers	Infection prevalence in introducing population
	Capacity of host to proliferate parasite
	Frequency of introduction
	Rate/mode of parasite release
Invertebrate host dispersal	Likelihood of natural dispersal
	Capacity of host to proliferate parasite
Dispersal of parasite stages	Proximity from source area
	Longevity of myxospore
	Longevity of actinospore
	Migration corridors for piscivorous birds
Recreational/restoration/commercial activities	Proximity to source areas
	Frequency of activity
	Longevity of myxospore
	Longevity of actinospore
Commodity	Product type (e.g. frozen, fresh)
	Tissue of parasite localization
	Intended use (human consumption, fish food component)
	Size and frequency of importation
	Longevity of myxospore under storage conditions

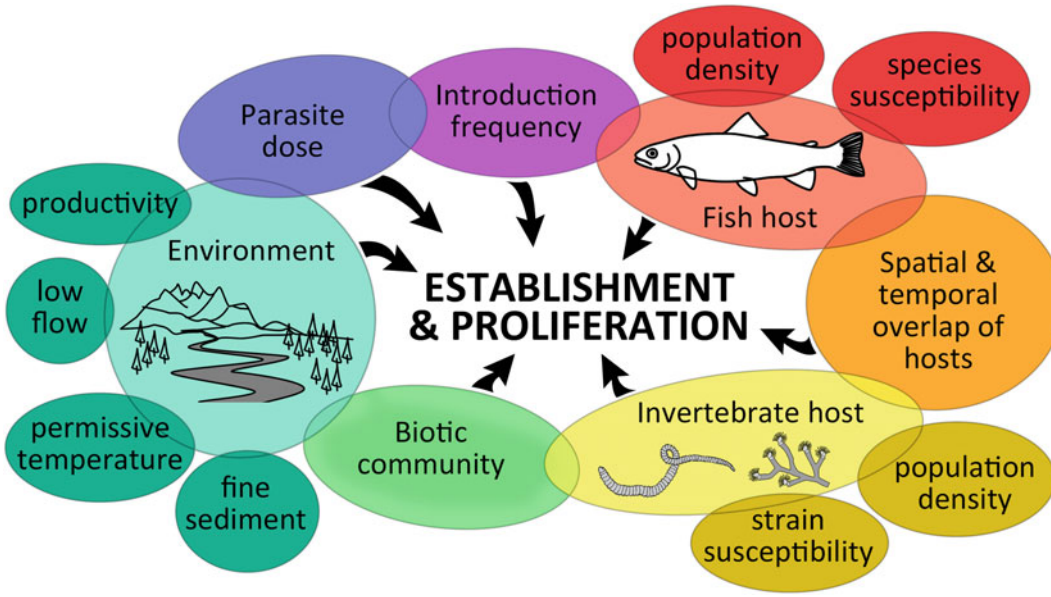

Fig. 20.3 Factors that affect the ability of myxozoans to establish and proliferate in new environments and that are relevant to exposure risk assessment (diagram courtesy Stephen Atkinson)

(Henderson and Okamura 2004; Okamura et al. 2011).

Modes of parasite transmission and release affect likelihood of establishment and persistence. Species that are able to transmit directly between fish, like *Enteromyxum leei* (syn *Myxidium leei*), represent a high disease risk [as has occurred in a public aquaria that introduced infected individuals (Padrós et al. 2001)]. Other species, like *Parvicapsula minibicornis*, are shed in the urine and thus may be widely dispersed as fish move (Kent et al. 1997). In contrast, *M. cerebralis* is not released until death of the host, and its success in establishing in new localities is likely to rely on the broad distribution of its invertebrate host (Hallett and Bartholomew 2011). Vertical transmission of myxozoans in bryozoan hosts via statoblasts and colony fragmentation (Okamura et al. 2011; Abd-Alfattah et al. 2014) and in oligochaete hosts during asexual propagation (Morris and Adams 2006) may also allow myxozoans to persist in the absence of a suitable fish host.

Establishment can also be influenced by environmental factors such as temperature, sediment size and water velocity, as has been shown for *M. cerebralis* (e.g., Kerans et al. 2005; Krueger et al. 2006; Hallett and Bartholomew 2008; McGinnis and Kerans 2013) and *T. bryosalmonae* (Tops et al. 2006), either through interactions of the environment with the invertebrate and fish hosts or with the transmission stages of the parasite. Productivity of a system may promote establishment and proliferation of myxozoans through effects on invertebrate hosts. For example, bryozoan growth and abundance (Hartikainen et al. 2009; Hartikainen and Okamura 2012) as well as spore production (Hartikainen and Okamura 2012) increase with nutrient levels. The biotic community itself may influence establishment. For *M. cerebralis*, where both highly and less susceptible *T. tubifex* lineages and non-susceptible oligochaetes are sympatric, the less susceptible lineages and non-host worms may be able to remove myxospores from the environment, reducing production of actinospores by the susceptible lineage (Lamb et al. unpub. data) and consequently reducing infection in the fish.

Because the likelihood of establishment is affected by all of the above factors, it may take years or decades before a myxozoan life cycle becomes established. This has been documented

for *M. cerebralis* in the Deschutes River, Oregon, USA, where myxospores were detected in straying adult salmon for decades before the infectious stage of the parasite was finally confirmed in sentinel fish (Zielinski et al. 2010). This illustrates how the dynamics of the interactions between introduced parasites and their hosts are marked by real or apparent time delays. In the case of the straying salmon above, we would not know whether *M. cerebrali*s was introduced and went undetected for years until it was able to proliferate to a detectable level, or whether it just took a long time for establishment to occur.

20.2.3 Consequence Assessment

Assessing the likelihood of adverse effects is the step with the highest level of uncertainty, and depends not only on the biology of the host at risk and the environment, but also on management aims. Consequence assessments often involve economic evaluation of the potential outcomes weighed against the costs of control measures. Thus, they are likely to be highest for myxozoans that decrease the market value of economically important species of fish and for those for which detection would result in regulatory restrictions.

20.3 Factors Affecting the Uncertainty of a Risk Assessment

Each step of a risk assessment has a degree of uncertainty. Some of this uncertainty is inherent to the system (stochastic) and can be quantified. Uncertainty will also arise from a lack of data (epistemic) and can be decreased by improving data quality. Reliable detection of the pathogen is a primary source of uncertainty, especially since visual diagnosis of myxozoans is often confounded by similar or cryptic species. For example, in surveys for *M. cerebralis* in Idaho, USA, a morphologically similar species of myxozoan was detected in the brain and spinal cord of rainbow trout (Hogge et al. 2004, 2008). Although this new species could be distinguished from *M. cerebralis* using molecular techniques, it could be easily confused with *M. cerebralis* during routine monitoring. The proliferation of molecular methods has greatly increased detection accuracy and has thus improved data quality; however, validated assays are available for only a few myxozoans (American Fisheries Society-Fish Health Section Blue Book 2012).

Another source of uncertainty in a release assessment is the accuracy of data on infection prevalence in the source fish or invertebrate host populations, which dictates the rate of parasite introduction. Estimation of infection prevalence in fish is affected by the sensitivity of the assay, the proportion of fish sampled and the performance of the diagnostic test (Thorburn 1996; Bruneau 2001; OIE 2003; Williams and Moffitt 2010; Oidtmann et al. 2013). For instance, an assay that is 95 % efficient will still miss infections in 1 of 20 fish, even when the animals are examined at a 100 % sampling effort. This false negative rate is multiplied further by the uncertainty introduced by the confidence limits imposed by the sample size. Thus, highly infected animals should be relatively easy to detect in a population, but if infection prevalence is low or infection levels are below the detection threshold of the assay there may be a substantial risk of unintentional introduction of the parasite. This has certainly been the case for *M. cerebralis*, which was widely disseminated prior to the development of robust detection methods (Bartholomew and Reno 2002).

Estimates of infection prevalence in source host populations will also vary with the sampling approach. Sampling of captive populations is more easily accomplished and efforts can be targeted to species or age classes in which detection of mature spores is most likely (Ryce et al. 2004). For wild populations there is likely to be less control over the sampled population and surveillance strategies will need to be carefully considered (Oidtmann et al. 2013). For example, sampling at defined locations or only during certain months can result in design effects, especially when site-level factors affect infection

prevalence (Peeler et al. 2008). In addition, heavily infected wild fish may be underrepresented as a result of disease-related mortality or predation. Sampling methods may also introduce bias. For example, electrofishing may bias towards larger fish and seining toward smaller or slower fish that are unable to avoid the net.

Data obtained from sampling wild populations can be supplemented with other sorts of data to address specific questions. For example, sentinel cage studies have been widely used to determine the distribution and seasonal occurrence of certain myxozoans (e.g. Gay et al. 2001; Sandell et al. 2001; Stocking et al. 2006; Alama-Bermejo et al. 2013). Other methods for obtaining information on parasite distribution and prevalence include water sampling in combination with molecular methods (Hallett and Bartholomew 2009; Griffin et al. 2009; Hallett et al. 2012) and literature survey, or retrospective surveillance (Burkhardt-Holm and Scheurer 2007). One unique study utilized museum specimens to study the emergence of *Myxidium immersum* after invasion of the cane toad in Australia (Hartigan et al. 2010).

20.4 Myxozoan Risk Assessments

Risk assessments for myxozoans have been developed for a variety of reasons, thus they incorporate different geographic scopes and management questions. Examples of these are presented below, with additional studies summarized in Table 20.2, which appears at the end of this section.

20.4.1 Commodity Import Risk Assessments

The first import risk assessments (IRA) to examine the likelihood of introductions and potential impacts of myxozoans were produced by regulatory authorities in New Zealand (MacDiarmid 1994; Stone et al. 1997) and Australia (Kahn et al. 1999) for trade of products for human consumption. These assessments evaluated a range of wild, ocean-caught Pacific salmon products, from frozen fillets to chilled, headless, eviscerated fish. Each assessment considered a variety of myxozoan pathogens from the source region. The assessments generally concluded that, given the widespread distribution of myxozoans and potential for subclinical infections, the likelihood of infected salmonids being harvested and processed for human consumption is high. However, because myxozoan life cycles are indirect and include a specific invertebrate host, it was considered unlikely that viable myxospores would be introduced into an area with an appropriate invertebrate host.

Considerations for individual myxozoan species were also in general agreement regarding a low likelihood of introduction. Although introduction of *T. bryosalmonae* would constitute some level of risk, the parasite is not considered a problem of salmonids from the source region (Pacific coast of North America), is not found in the muscle and is not a disease of adult, market size fish (the rationale being that adult fish should have acquired immunity when exposed to the parasite as juveniles). Similarly, *Parvicapsula* sp. is not known to produce its spore stage in salmonids and thus flesh containing the parasite would not be a vehicle of transmission. For *Ceratonova shasta* (syn. *Ceratomyxa shasta,* Atkinson et al. 2014), the concept of "marker diseases" (diseases that have a restricted geographic range and are reliably diagnosed) was introduced. Based on that concept, the risk of *C. shasta* was low because of the apparent lack of spread of this parasite despite the large volume of exports of salmonids from this region. Processing would not remove *Kudoa* sp., and *H. salminicola*, as they are associated with muscle tissue, but filleting would increase the likelihood of heavily infected product being detected and disposed of, and *Kudoa* is already widely present in the marine environment. Similar to *C. shasta, H. salmonicola* has a limited distribution and has not been found in other countries that import Pacific salmon. Sources of uncertainty were insufficient information on the length of time the

parasites would remain viable in the tissue, the presence of appropriate invertebrate hosts to complete the life cycles and additional pathways for introduction of *Kudoa* sp. through marine hosts.

An exception to the general concurrence of the assessments was that for *M. cerebralis* in Australia. The Australian IRA concluded that there was a low probability of import/release as a consequence of importing eviscerated rainbow trout. However, because any risk was considered unacceptable this warranted risk management in the form of restricting import of juvenile rainbow trout. This resulted in requirements for documented health surveillance and certification of the source population, and removal of the head and gills (Kahn et al. 1999). Because whirling disease had already been reported in New Zealand (Hewitt and Little 1972), it was not further considered in the New Zealand IRA.

20.4.2 Risks for Emerging Diseases of Aquaculture Species

Risk assessments for emerging diseases of aquaculture species identify myxozoans that are: (1) the most likely to establish in an aquaculture scenario, (2) already present with potentially negative consequences and (3) potentially difficult to manage. As part of an assessment of the southern bluefin tuna (*Thunnus maccoyii*) industry in Tasmania, a risk estimation matrix was developed for each potential pathogen using information provided by the industry and a review of the literature (Nowak et al. 2003). The only myxozoan hazard identified was an incompletely described species of *Kudoa* found in the muscle tissue. Although the *Kudoa* sp. was considered to have a moderate probability of entry and spread, the consequences of establishment were deemed to be very low as the prevalence of lesions is only about 1 % and infection does not result in postmortem myoliquefaction or mortality. Thus the overall risk rating was negligible, with a recommendation that further research be conducted on the epidemiology and control of this parasite.

An assessment of disease risk to sustainability of yellowtail (*Seriola lalandi*) aquaculture in Australia considered four myxozoan species (*Ceratomyxa seriolae, C. buri, Kudoa* sp., *Unicapsula seriolae*) (Hutson et al. 2007). The consequences of the two *Ceratomyxa* species establishing and proliferating were regarded as negligible as the species infect the gall bladder with no apparent pathological changes or mortality associated with infections. Infections of *U. seriolae* and *Kudoa* sp. are associated with muscle degeneration and post-mortem myoliquefaction and because of the potential negative impact on marketability were considered to have a low consequence of establishment in aquaculture. Management of these parasites is problematic because of the lack of effective chemotherapeutants, while the inability to avoid exposure is exacerbated by the lack of knowledge of their life cycles. However, as these species were not considered endemic in the immediate area of the proposed aquaculture the likelihood of establishment was considered negligible.

A risk assessment for parasites of sharpsnout seabream (*Diplodus puntazzo*) was conducted to determine if this particular fish species provided a good option for diversification of aquaculture in Spain (Sánchez-García et al. 2014). The assessment included parasites present in farmed gilthead seabream (*Sparus aurata*), as the probability of cross infection between the species is high. Two myxozoan species were identified among the three parasites responsible for high mortalities. *Enteromyxum leei* was considered to pose a high risk for culture of sharpsnout bream and *Ceratomyxa sparusaurati* was associated with a moderate risk level, capable of reducing market value and causing pathology, but not causing mass mortality.

20.4.3 Extension of Risk Assessments to Wild Fisheries

The most common application of risk assessments for myxozoans has been to examine risks of introduction or spread into an area where a pathogen is not believed to exist in wild

populations. Both quantitative and qualitative risk assessments for *M. cerebralis* have been conducted for this purpose. For example, to inform a ban on the importation of live salmonids into Alberta, Canada to minimize the risk of *M. cerebralis* introduction, the probability of importing live farmed salmon from facilities incorrectly classified as non-infected (i.e. a false-negative classification) was estimated using Monte Carlo simulations (Bruneau 2001). The study highlighted the importance of diagnostic accuracy, sample size, and the use of robust testing protocols developed in response to the whirling disease outbreak in the Rocky Mountain region of the USA.

In response to the declines of rainbow trout populations in the Rocky Mountain region in the 1990s as a result of whirling disease, a coalition of scientists developed a conceptual risk assessment framework that could be adapted to a variety of scenarios to identify pathways of spread of *M. cerebralis* and high risk habitats (Bartholomew et al. 2005). This framework identified many of the risk factors detailed above (Sect. 20.3; Figs. 20.2 and 20.3). The associated release assessment identified the transfer of subclinically infected fish as the most commonly occurring route of introduction. Natural dispersal of infected fish in large interconnected systems was also considered to be a likely dissemination path, and there was at least a small possibility that birds could disseminate spores through their feces. Other possible pathways included dispersal of the oligochaete host and spore stages through recreational, restoration and commercial activities; however, less was known about how much these activities contributed to *M. cerebralis* introductions. The exposure assessment identified introduction size (dose) and frequency as the most significant risk factors associated with establishment and spread of *M. cerebralis*. Evidence for this was a positive relationship between the level of stocking of infected salmonids and the prevalence of infection and myxospore concentration in resident salmonids in some systems (Nehring 2006). *M. cerebralis* was most likely to establish and proliferate in systems with both large populations of highly susceptible salmonids and large populations of *T. tubifex* that were dominated by highly susceptible worm strains. Environmental characteristics (water velocity, sediments, temperatures) were important as well. Examples of high risk habitats include areas of low flows that contain fine sediments, and spring creeks that have relatively uniform year-round temperatures (Neudecker et al. 2012). Consequences of introductions were identified to be most severe when the most susceptible size or age (e.g., less than 9 weeks old) and species (e.g., rainbow trout) are coincident with high numbers of waterborne *M. cerebralis* actinospores (Bartholomew et al. 2005).

Building upon this framework, Arsan and Bartholomew (2008) used a qualitative approach to examine the risk of introducing *M. cerebralis* into the state of Alaska, USA. This study integrated the assessment with targeted sampling and oligochaete susceptibility challenges to test assumptions. Detection of *M. cerebralis* DNA at a hatchery considered high risk (Arsan et al. 2007) altered the assessment, which then identified stocking of these fish as the highest risk introduction route. Establishment risk was highly variable between rivers because of juvenile salmon species composition, water temperatures, presence of *T. tubifex* populations, and the variability of the *T. tubifex* lineages in their ability to propagate the parasite. Areas where introduction was considered most likely were high-use sport fisheries, but even within those systems the probability of establishment and proliferation was low.

In the Columbia River basin Oregon, USA, where *M. cerebralis* has been detected since the 1980s (Lorz et al. 1989), assessment of the risk of further dissemination into lower basin tributaries explored three elements of dispersal: stocking infected fish, natural dispersal by migratory birds and straying anadromous salmon, and recreational activities (Arsan and Bartholomew 2009; Engelking 2002; Zielinski 2008). Interestingly, in the Willamette River (lower Columbia River tributary) stray adult salmon were considered a low risk based on low straying rates and non-detection of infection in those fish (Arsan and Bartholomew 2009). In contrast, salmon stray with greater frequency into the Deschutes River

(mid-Columbia River tributary) and approximately 20 % of these fish were infected with *M. cerebralis* (Engelking 2002; Zielinski et al. 2010). Dispersal by migratory birds and recreational activities were considered low risks because of the large distances to endemic areas and the number of events that are needed to align for successful introduction (Arsan and Bartholomew 2009).

Quantitative approaches have also been used to explore risks from *M. cerebralis* across a broad ecological landscape. For example, an assessment of high elevation waters in Colorado, USA demonstrated that within-drainage distance to the nearest *M. cerebralis*-positive water and abundance of *T. tubifex* habitat were more important predictors of establishment than accidental stocking events (Schisler and Bergersen 2002). In another study, the probability of whirling disease infection for two strains of cutthroat trout (*Oncorhynchus clarki*) across their native range was estimated using a Bayesian Belief Network model (Ayre et al. 2014). Available habitat for *T. tubifex* (defined by gradient and elevation) had the greatest influence on infection likelihood, but stream barriers reduced risk and offered a means for controlling disease spread. This model was adapted to provide individual, parameterized models for different management areas, and relative risk distributions for whirling disease infection for each fish strain across their ranges were developed using Monte Carlo simulations (Ayre et al. 2014).

20.4.4 Risk of Pathogen Exchange from Aquaculture to Wild Fish

With the exception of the widespread dissemination of *M. cerebralis*, examples of myxozoans establishing in wild populations as a result of aquaculture are difficult to confirm. Most risk assessments have focused on the risks to aquaculture species via transfer from wild to farmed fish. Risks of cultured to wild fisheries have only been recently considered, although reviews on ecological risks of aquaculture have pointed to the potential for myxozoan transmission (e.g. Arechavala-Lopez et al. 2013). A European Union project (Raynard et al. 2007) reviewed disease interactions and pathogen exchange between farmed and wild finfish in Europe and found evidence for transmission of *E. leei* in the Mediterranean. The ability of *E. leei* and other members of that genus to infect a broad host range and transmit directly between fish (Diamant 1997; Redondo et al. 2004) poses a high risk for transmission, with cultured populations amplifying the parasite and wild fish serving as reservoirs of infection. Seabream escapees infected with *E. leei* were collected as far as 5 km from sea cages, although there was no evidence for transfer of the infection to wild fish (Diamant et al. 2007).

20.4.5 Effectiveness of Management Decisions Arising from Risk Assessments

There is some evidence that management resulting from risk assessments has been effective. For example, the lack of detection of the myxozoans considered in the Australian IRAs (Hutson et al. 2007; Kahn et al. 1999; Nowak et al. 2003) may be due to the stringent precautions taken because of the high risk attributed to *M. cerebralis* introduction. However, the IRA did not predict the emergence of a new myxozoan threat: *K. neurophila* in striped trumpeter (*Latris lineata*) (Grossel et al. 2003). The concept of a marker disease, referring to a pathogen that has a geographically restricted range and thus a reduced risk, does have some merit, at least for *C. shasta*. For this myxozoan, the restricted geographic range of the invertebrate host has likely limited its dissemination outside of the Pacific Northwest. Thus, for the aquacultured fish species for which risk assessments were conducted, there has been limited evidence for emergence of a myxozoan that threatens the sustainability of their culture. However, these industries are relatively young and it remains to be seen whether this remains the case.

Table 20.2 Summary of risk assessments on myxozoans

Type of risk assessment	Pathogens considered	Scope	Risk elements included	Pathways considered
Import risk assessments				
MacDiarmid (1994)	*Ceratonova* (*Ceratomyxa*) *shasta, Henneguya salminicola, Kudoa thrysites*, *Parvicapsula* sp., *Tetracapsuloides bryosalmonae*	New Zealand	Release Exposure Consequence Management	Importation of wild ocean-caught Pacific salmon from the USA and Canada for human consumption
Stone et al. (1997)	*C. shasta, Chloromyxum* sp., *H. salminicola, Kudoa* sp., *Myxidium truttae, Parvicapsula* sp., *T. bryosalmonae*	New Zealand	Release Exposure Consequence Management	Importation of headed, gilled and eviscerated salmonids for human consumption
Kahn et al. (1999)	*C. shasta, Chloromyxum* sp., *H. salminicola, M. truttae, Parvicapsula* sp., *T. bryosalmonae, Myxobolus cerebralis, Henneguya* spp., *Kudoa* sp., *Sphaerospora* sp.	Australia	Release Exposure Consequence Management	Importation of Pacific salmon, trout and marine non-salmonid fish
Emerging aquaculture species				
Nowak et al. (2003)	*Kudoa* sp.	Tasmania	Release Exposure Consequence	Transmission of endemic pathogens to aquacultured bluefin tuna
Hutson et al. (2007)	*Ceratomyxa seriolae, Ceratomyxa buri, Kudoa* sp., *Unicapsula seriolae*	Australia	Release Exposure Consequence	Transmission of endemic pathogens to aquacultured yellowtail
Sánchez-García et al. (2014)	*Ceratomyxa* sp., *Ceratomyxa diplodae*, *Ceratomyxa puntazzi*, *Ceratomyxa sparusaurati*, *Enteromyxum leei*, *Myxobolus* sp., *Polysporoplasma sparis*	Western Mediterranean	Release Exposure Consequence	Transmission of endemic pathogens to aquacultured sharpsnout seabream
Wild fisheries				
Bruneau (2001)	*M. cerebralis*	Alberta, Canada	Release	Importation of live, farmed salmonids from the USA or other Canadian provinces
Engelking (2002)	*M. cerebralis*	Deschutes River, Oregon USA	Release	Passage of adult salmon above a migration barrier
Schisler and Bergersen (2002)	*M. cerebralis*	High elevation lakes, Colorado, USA	Exposure	Stocking infected fish; distance to known positive waters; abundance of suitable habitat
Bartholomew et al. (2005)	*M. cerebralis*	1. General framework 2. Deschutes River, Oregon, USA	Release Exposure Consequence Risk estimation	Examples to provide a framework for conducting risk assessments

(continued)

Table 20.2 (continued)

Type of risk assessment	Pathogens considered	Scope	Risk elements included	Pathways considered
Arsan and Bartholomew (2008)	*M. cerebralis*	Alaska, USA	Release Exposure Consequence	Human movement of salmonids; commercial activities; recreational activities; natural dispersal
Arsan and Bartholomew (2009)	*M. cerebralis*	Willamette River, Oregon, USA	Release Exposure Consequence	Stocking infected fish; natural dispersal by migratory birds and anadromous salmon; recreational activities
Zielinski (2008)	*M. cerebralis*	Deschutes River, Oregon, USA	Release Exposure Consequence	Human movement of salmonids; commercial activities; recreational activities; natural dispersal
Stinson and Bartholomew (2012)	*C. shasta*	Deschutes River, Oregon, USA	Release Exposure Consequence	Human movement of salmonids; natural dispersal
Ayre et al. (2014)	*M. cerebralis*	Southwestern USA	Exposure Spread Establishment	Quantitative approach to estimate likelihood of infection and spread throughout the current range of two native cutthroat trout
Exchange between wild and farmed fish				
Raynard et al. (2007)	*Parvicapsula pseudobranchiola*, *E. leei*, *P. sparis*, *Myxobolus* spp., *Enteromyxum scophthalmi*, *Ceratomyxa* spp., *Sphaerospora* spp., *Kudoa* spp., *Henneguya* spp., *Leptotheca sparidarum* n. spp.	European Union	Release Exposure Consequence	Transmission from aquaculture facilities, human movement of fish
Toledo-Guedes et al. (2012)	*Sphaerospora testicularis*	Canary Islands	Release	Escaped cultured fish

Outcomes of risk assessments for wild populations are often difficult to assess. In the Deschutes River, OR, USA several risk assessments (Engelking 2002; Zielinski 2008) identified stray adult salmon from endemic portions of the Columbia River watershed as the most likely source of *M. cerebralis* introduction, and major spawning tributaries as the most likely locations for parasite establishment. Since that time, parasite establishment has been documented in a spawning tributary, as predicted (Zielinski et al. 2010). Detection of the parasite above migration barriers in the Deschutes system has not occurred, possibly because of the management restrictions put into practice to limit those introductions. As predicted, the parasite has not been detected in the Willamette River basin, with the exception of a private hatchery in the lower basin where the introduction source is unknown (Bartholomew et al. 2007). For Alaska (Arsan and Bartholomew 2008), the risk assessment was effective in identifying the location where the parasite would most likely be detected (Arsan et al. 2007), but whether the lack of further detection is a result of changes in hatchery practice or in detection methods is unclear. Subsequent closure of the hatchery eliminated the stocking risk leaving recreation and angler activity as the highest risk pathways for introduction from outside the state.

20.5 Conclusions

Risk assessment is a practical tool that can be used in a variety of applications, from assessing disease risk associated with commerce to managing natural resources. Because methodologies have largely been adapted from human and terrestrial applications, they could be improved for myxozoan applications by better information on factors affecting transmission in the aquatic environment and a better understanding of how myxozoans persist in their vertebrate and invertebrate hosts. Although risk assessments for many myxozoans are constrained by a lack of data on geographic distribution, infection prevalence in the fish host, and by incomplete knowledge of life cycles, identification of these data gaps is an important component of a risk assessment and can be used to guide future research. Use of risk assessments and risk-based surveillance methods is likely to increase with increasing dependence on aquaculture for food security (Oidtmann et al. 2013) and as we attempt to manage natural resources in the face of changing climate conditions. The latter application presents particular challenges, as it will involve incorporating several levels of predictive models, beginning with various climate scenario models and using these to predict effects on key environmental parameters (e.g. flow, nutrients, sedimentation) that affect myxozoan disease (see Chap. 19).

20.6 Key Questions for Future Research

- How can we better approximate transmission risks for species with complex and with direct life cycles?
- Are some species at a low risk for dissemination because of characteristics of their invertebrate hosts (e.g. restricted geographic range, inability to reproduce clonally, narrow environmental tolerance)?
- How can surveillance programs be improved to provide more meaningful data on infection prevalence and severity in wild populations?
- How will changing environmental conditions (e.g. water temperature, flow regimes, sedimentation, eutrophication) that result from global warming and anthropogenic modifications affect current risk assessments?
- Can estimates be improved by incorporating mathematical disease modeling approaches within a risk analysis framework?

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Mitigating Myxozoan Disease Impacts on Wild Fish Populations

21

Simon R.M. Jones, Jerri L. Bartholomew, and Jin Yong Zhang

Abstract

We review evidence for population-level impacts of infections for four economically important myxosporean parasites: *Myxobolus cerebralis*, the cause of salmonid whirling disease (WD); *Tetracapsuloides bryosalmonae*, the cause of proliferative kidney disease (PKD) of salmonids, *Ceratonova* (syn. *Ceratomyxa*) *shasta*, the cause of enteronecrosis of salmonids and *Myxobolus honghuensis*, the cause of pharyngeal myxosporidiosis in gibel carp. WD is associated with a decline of rainbow and cutthroat trout populations in the intermountain region of the western USA and PKD with the decline of brown trout populations in Switzerland. Severe enteronecrosis in up to 62 % of outmigrating juvenile Chinook salmon combined with high mortality in sentinel fishes supports a potential adverse population-level impact of *C. shasta* infection. Similarly, declines in Crucian carp abundance in China have been associated with severe *M. honghuensis* infections. Accurate interpretation of impacts on wild populations is challenged by a general absence of long-term datasets

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providing information on population structure and abundance, disease prevalence and severity and on associated anthropogenic and natural factors that contribute to disease severity or host susceptibility. Efforts to mitigate adverse effects of these diseases in wild populations include the application of more sensitive methods to detect the parasites in fish, invertebrate hosts and water, use of risk assessments in fisheries management, use of temperature and silt or sediment control strategies within riparian habitats, and alternative stocking practices that take advantage of age-specific differences in host susceptibility or temperature regimes that are not conducive to disease development.

Keywords
Disease mitigation · Parasite ecology · Population · Surveillance

21.1 Introduction

Infections with myxozoans occur frequently in aquatic vertebrates, particularly fishes, and many of these infections result in pathology. Despite evidence of these adverse effects in individual fish, most studies assess relatively few animals over a short time span within a restricted geographic area and therefore have limited capacity to comment on population level impacts. Some myxozoan infections are exceptional in that impacts on populations are better documented, especially for economically or culturally valuable species in freshwater and marine ecosystems. Examples of such infections include *Myxobolus cerebralis*, the cause of salmonid whirling disease (WD), *Tetracapsuloides bryosalmonae*, the cause of proliferative kidney disease (PKD) in salmonids, *Ceratonova* (syn *Ceratomyxa*) *shasta*, the cause of enteronecrosis (ceratomyxosis) in Pacific salmon and *Myxobolus honghuensis*, the cause of pharyngeal myxosporidiosis in gibel carp (*Carassius auratus gibelio)*. This chapter focuses on these examples, providing evidence of population level impacts and strategies for the mitigation of these impacts (Fig. 21.1). Chapter 17 provides a complementary overview of the comparative epidemiology of myxozoan diseases, including several discussed here.

21.2 *Myxobolus cerebralis*: Whirling Disease

Salmonid whirling disease is caused by infection with *Myxobolus cerebralis* (see Chaps. 10, 15). Briefly, clinical signs include erratic swimming behavior or whirling, a blackened caudal extremity and death. Distortion of the affected cartilage and damage to the central nervous system gives rise to clinical signs of the disease. There is evidence that the ancestral range of *M. cerebralis* was Eurasia, where brown trout (*Salmo trutta*) and possibly other salmonids were typically asymptomatic hosts. However, anthropogenic movements of infected fish resulted in the present widespread distribution of the parasite in 26 countries and in 22 states in the United States of America (USA) (Granath and Vincent 2010). Although the parasite has a wide host range among salmoniform fishes, including mountain whitefish (*Prosopium williamsoni*) and European grayling (*Thymallus thymallus*), susceptibility to disease varies considerably among species and among strains of fish within susceptible species (MacConnell and Vincent 2002). The severity of WD also depends on fish age and size, water temperature and infectious dose (See Steinbach Elwell et al. 2009). The latter is determined by the abundance and genetic

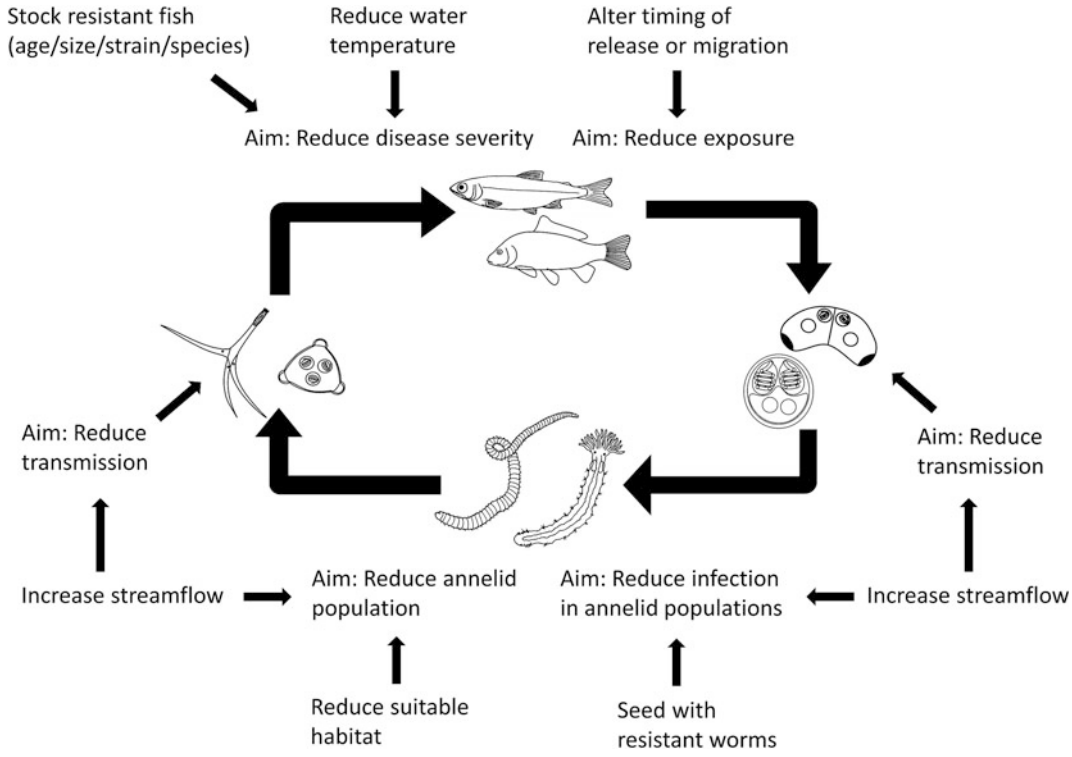

Fig. 21.1 Generalized strategies for reducing impacts of myxozoans on wild fish populations

lineages of the oligochaete *Tubifex tubifex* (which acts as the determinate host in the life cycle of *M. cerebralis*; see Chap. 12 for review of annelid-myxozoan interactions) (Beauchamp et al. 2002). Thus, benthic habitats suitable for *T. tubifex* and water temperature are predictors of infectious dose.

21.2.1 Evidence for Population Impacts

The history and dissemination of *M. cerebralis* and the impact of WD within the USA has been well documented by Bartholomew and Reno (2002). The disease has been examined best in controlled laboratory and field exposures and during infections in commercial brook trout (*Salvelinus fontinalis*) and rainbow trout (*Oncorhynchus mykiss*) production facilities. Thus, by the mid-1980s WD was well established in trout hatcheries and farms, with management practices minimizing impacts. By the mid-1990s however, WD was recognised to underly dramatic declines in several trout populations in Montana, Colorado and Utah (Nehring and Walker 1996; Hedrick et al. 1998). Research on the interacting roles of natural and human risk factors for WD suggests that the measurable population level effects are largely because indigenous rainbow and cutthroat trout *O. clarki* are highly susceptible to infection by this exotic parasite and to the development of disease (Granath et al. 2007). *M. cerebralis* was introduced into the Rock Creek drainage in Montana in the early to mid-1990s and the density of catchable (>15 cm) rainbow trout began to decline between 1993 and 1996 to lowest levels in 2004 (Granath et al. 2007). A concomitant failure of recruitment of infected juveniles into older age classes may explain the subsequent reduction in the abundance of larger rainbow trout. Despite this, the total salmonid abundance in affected streams was relatively stable because of a concurrent increase in the abundance of

introduced brown trout, possibly reflecting their higher natural resistance to the disease. Similar failures of juvenile rainbow trout recruitment documented in the Upper Colorado River basin have been linked to *M. cerebralis* infections (Thompson et al. 2002; Schisler et al. 2000).

The rapid expansion of *M. cerebralis* to an essentially global distribution speaks to the ease of establishment in freshwater ecosystems. This reflects the widespread distribution of susceptible fish and oligochaete hosts and of unregulated anthropogenic and natural processes that facilitate dissemination (for further discussion see Chap. 18). In contrast, once established, *M. cerebralis* is unlikely to be eradicated. For instance, in trout production facilities eradication requires stringent measures including depopulation and cessation of water flow (Bartholomew et al. 2007). The ubiquitous distribution of *T. tubifex* in freshwater ecosystems suggests that effective strategies for mitigating the disease in wild populations will require management of anthropogenic risk factors.

21.2.2 Strategies for Mitigating Impacts

Methods developed for the detection of *M. cerebralis* in fish vary in their utility because of differences in sensitivity and specificity (see Table 21.1, adapted from Steinbach Elwell et al. 2009, which summarises such detection methods). Confirmation of infection by regulatory authorities requires microscopic detection of myxospores following enzymatic digestion of the cranium and axial skeleton from fish that have had a minimum exposure time of 6 months or 1,800 degree-days. Furthermore, it is important that a sufficiently large sample size is obtained from the target population to ensure statistical power. For example, 60 samples are required from a population with an assumed prevalence of 5 % to obtain a 95 % probability of detecting at least one infection. Myxospores may be subsequently identified to species by using a highly specific polymerase chain reaction (American Fisheries Society 2012). A variety of molecular or serological detection assays have also been developed for fish, oligochaete hosts, water or sediment.

Fisheries managers and other decision makers conduct business despite environmental and biological uncertainties. Risk assessment for WD provides a management tool that attempts to compensate for this uncertainty by applying a qualitative or quantitative approach that systematically integrates and interprets factors known to contribute to the introduction and establishment of *M. cerebralis* and to disease. Risk assessment thus contributes a scientific basis for decision making. Several risk assessments have focused on the dispersal of *M. cerebralis* into drainage basins where previously it had not been detected (Bruneau 2001; Engelking 2002; Schisler and Bergersen 2002; Bartholomew et al. 2005; Arsan and Bartholomew 2008, 2009). The topic of risk assessment is discussed in detail in Chap. 20.

Mitigation of WD via habitat manipulation primarily targets the invertebrate host and waterborne infectious spores (myxospores and triactinomyxons (TAMs)) of *M. cerebralis*. It is well known that benthic oligochaete populations in fish culture facilities are minimized or eliminated through the use of concrete raceways instead of earthen ponds (Hoffman and Hoffman 1972). Further strategies include drying-out of raceways combined with chemical disinfection (Wagner 2002). However, the efficacy of chemical treatment may be reduced on the desiccation-resistant stages of *T. tubifex*. To be effective, alterations to benthic invertebrate communities must be combined with the treatment of incoming water, using filtration to exclude TAMs or myxospores or to kill them by means of chemical or physical methods (Wagner 2002). Ideally, combined mitigation strategies will compensate for gaps in effectiveness associated with single methods.

In contrast to carefully managed and controlled aquaculture environments, the options for mitigating WD in wild populations are limited. Habitat manipulation and control of water quality and fish movements are often neither economical nor practical, given the remote or mountainous terrain in which disease impacts are greatest. However, Allendorf et al. (2001) suggest that stream habitat alteration and degradation due to municipal, agricultural or mining practices are linked with the presence of *M. cerebralis*.

Table 21.1 A summary of tools for detection of *Myxobolus cerebralis*

Sample	Method	Sample type	Detection target	Age of host	Specificity[a]	Sensitivity	Application[b]
Fish							
	Plankton centrifugation	Skull and spine	Myxospore	>6 months	++	++	Diagnostics
	Pepsin-Trypsin digest (PTD)	Skull and spine	Myxospore	>6 months	++	++	Diagnostics inspection–screening[c]
	Histology	Head, gill, nares or operculum	Myxospore, developmental stages	>6 months	+++	++	Diagnostics inspection–confirmation[d]
	Polymerase chain reaction (PCR)	Half head or cranial punch	DNA	>4 months	+++	+++	Diagnostics inspection–confirmation[d, e]
	PCR	Non-lethal (e.g. fin or gill clip)	DNA	>4 months	+++	++	Research
	Quantitative PCR (QPCR)	Half head or cranial punch	DNA	Any age	+++	+++	Diagnostics research
	In Situ-hybridization (ISH)	Head, gill, nares or operculum	DNA	Any age	+++	++	Diagnostics research
	Myxo-Loop-Mediated isothermal amplification (LAMP)	Cranial punch	DNA	>6 months	+++	+++	Research
	Enzyme-linked Immunosorbent assay (ELISA)	Serum	Antibody	Any age	Unknown	Unknown	Research
	Immunofluoresence	Tissue section	Myxospore, developmental stages	Any age	Unknown	Unknown	Research
Tubifex	PCR	Whole or half worm	DNA	Any age	+++	+++	Research
	Release of TAMS	Whole worm (water sampled)	Triactinomyxon	>65 days (post exposure)	++	++	Research
	Histology	Whole worm	Triactinomyxon, developmental stages	Any age	++	++	Research
	In situ-hybridization	Whole worm	DNA	Any age	+++	++	Research

(continued)

Table 21.1 (continued)

Sample	Method	Sample type	Detection target	Age of host	Specificity[a]	Sensitivity	Application[b]
Water							
	Filtration	Variable volume	Triactinomyxon	N/A	+	+	Research
	Tamometer	120 liters	Triactinomyxon	N/A	+	++	Research
	Sentinel fish	50 fish per cage	Triactinomyxon[f]	<9 weeks	+++	++	Research
Sediment							
	Centrifugation	0.1–1 gram	Myxospore	N/A	+	+	Research

These methods have applications for research, diagnostics and fish health inspections. Specificity = ability of the method to differentiate among other parasites; Sensitivity = method can detect *M. cerebralis* at low concentrations

[a] Criteria for specificity

+ spores can be differentiated morphologically but sample source doesn't eliminate similar species

++ spores can be differentiated morphologically and sample source eliminates most similar species

+++ assay specific for *M. cerebralis*

[b] Diagnostic methods are those described in the AFS-FHS Blue Book (2007) Sect. 1: Diagnostic Procedures for Finfish and Shellfish Pathogens; those specified for inspection purposes are for regulatory purposes and are detailed in Sect. 2: USFWS/AFS-FHS Standard Procedures for Aquatic Animal Health Inspections. Research methods are either not considered validated or are standard methods used on non-traditional samples (e.g. PCR on worms)

[c] Fish health inspection protocols require screening by examination for spores in cranial cartilage processed by PTD

[d] Fish health inspection protocols require confirmation by identification of parasite stages in histological sections or by amplification of parasite DNA by PCR

[e] The PCR assay approved for fish health inspections specifically uses the primers by Andree et al. (1998)

[f] Sentinel fish are used to detect presence of the parasite (triactinomyxons stage) in water; however, one of the assays used to detect infection in fish must be used to confirm infection status

Managing sediment deposition or reducing temperatures in streams may therefore mitigate against disease development, although this is most likely to be effective in relatively short (<100 m) river reaches. For example, fish have been segregated from ideal *T. tubifex* habitat through the use of in-stream berms (Thompson and Nehring 2003, 2004; Waddle and Workman 2004; Waddle and Terrell 2006). The effects of small-scale habitat manipulations on tubificid abundance and genetic diversity on disease development were variable. These approaches were judged to lack sufficient promise to warrant further efforts at the study sites in Colorado (Thompson 2011). The association of tubificid oligochaetes with silt clay and fine sand sediments has allowed development of scale-dependent, predictive habitat models to identify and quantify the capacity of habitats to support *M. cerebralis* (Anlauf and Moffitt 2008, 2010). This approach has confirmed that percent of slow water habitat is a good predictor of tubificid benthic habitat and that decreases in conifer cover and increased agricultural activities are associated with elevated oligochaete densities (Anlauf and Moffitt 2010).

The suitability of a stream to support *M. cerebralis* may also be influenced through passive restoration practices such as excluding livestock grazing, logging or road development immediately adjacent to streams (Steinbach Elwell et al. 2009). Such activities create buffer zones that improve riparian habitat by minimizing silt deposition and allowing natural shade to regulate water temperature. The limited data presented by Hansen et al. (2006) suggested a slight increase in the prevalence of *M. cerebralis* in Bonneville cutthroat trout following the introduction of cattle to a site in Utah.

Efforts to seed streams with *M. cerebralis* resistant *T. tubifex* genotypes are based on the possibility that resistant worms will consume viable myxospores resulting in a possible reduction in the infection pressure by TAMs. However, the ephemeral occurrence of resistant Type V genotypes at a small number of locations within a watershed in Montana (Thompson et al. 2008) does not suggest this to be a viable mitigation strategy.

In areas where wild fish populations are stocked, stocking of larger fish may reduce the prevalence and severity of WD because of the size-related reduction in susceptibility (Wagner 2002). However, many wild populations at risk of WD are not stocked and spawn naturally. There is some evidence that several years of exposure to highly lethal *M. cerebralis* infections has imposed selection for elevated resistance within some wild populations. Thus, after several years of decline following the first detection of *M. cerebralis* in rainbow trout in Harrison Lake, Montana, trout abundance subsequently increased (Miller and Vincent 2008). Concurrently, rainbow trout from Harrison Lake were shown to be more resistant to disease development than other stocks (Vincent 2002; Wagner et al. 2006). Later, controlled exposure studies demonstrated reduced histological severity of *M. cerebralis* lesions in juvenile rainbow trout that were progeny of younger Harrison Lake rainbow trout parental crosses compared with the severity in progeny from older parental crosses, supporting the inheritance of resistance in the former (Miller and Vincent 2008). Similarly, there is evidence that the recent declining intensity of *M. cerebralis* infection in rainbow trout from some sites in the Rock Creek drainage (Montana) may also be attributed to increased resistance to disease development (Granath and Vincent 2010). It is therefore possible that direct management to mitigate WD in wild trout populations may become unnecessary if the effects of the disease are off-set by the gradual selection for disease resistant phenotypes. Further research and surveillance are required to assess the resistance status of the numerous affected populations and to examine the potential for selection of increased disease resistance among various susceptible species and stocks. The identification of stocks with elevated levels of natural disease resistance may also form the basis of carefully designed breeding trials for the production of native trout stocks with increased disease resistance.

Future efforts should focus on preventing introductions into previously naïve populations and should include, but not be limited to:

- Routine monitoring of infection status in cultured stock
- Treating effluent from all trout production facilities
- Prohibiting transplanting infected fish, regardless of infection status at destination
- Prohibiting the use of trout as baitfish
- Providing information to recreational fishers regarding disinfection of clothing, gear and boats
- Prohibiting the collection and selling of wild tubificid oligochaetes

21.3 *Tetracapsuloides bryosalmonae*: Proliferative Kidney Disease

Proliferative kidney disease (PKD) is a disease of salmonids caused by infection with *Tetracapsuloides bryosalmonae* (see Chaps. 10, 15). Briefly, fish become infected with spores released from the definitive hosts, colonial freshwater bryozoans belonging to the genera *Fredericella*, *Pectinatella*, *Plumatella* or *Cristatella* (Okamura et al. 2001) (see Chap. 11 for review of bryozoan-myxozoan interactions). The close relationship of *T. bryosalmonae* to other taxa isolated from freshwater bryozoan hosts suggests that PKD may be a disease caused by several species (Hartikainen et al. 2014). The parasite first replicates in the blood of the fish host and is disseminated via the circulatory system to several organs and tissues. Clinical signs of PKD include anaemia, splenomegaly and renomegaly, the latter caused by acute to chronic inflammation induced in the renal interstitium by parasitic stages. Severe inflammation in the kidney is associated with mortality, which ranges from 5 to 100 % among infected fish (Hedrick et al. 1993). Temperature is an important determinant of PKD, both in bryozoan (see Chap. 11) and fish hosts. Consequently, PKD is recognized as a seasonal disorder of fish, and although the disease may be observed at cooler temperatures it is typically associated with water temperatures greater than 15 °C, and has been linked to climate change (Hari et al. 2006; Okamura et al. 2011). Transmission and infection can occur at 9 °C (Gay et al. 2001), however, the severity and rate of progression of pathological lesions increase with temperature (Bettge et al. 2009; Schmidt-Posthaus et al. 2012). The absence of a measurable relationship in the latter study between parasite intensity and fish mortality indicates that mortality can be attributed directly to the temperature-driven inflammation. Similarly, overt infections in bryozoans occur more frequently at 20 °C and tend to be associated with larger numbers of parasites, compared with infections at 10 or 14 °C (Tops et al. 2006). Severe PKD and mortality are most frequently observed in naïve, young-of-the-year fish (MacConnell and Peterson 1992; Hedrick et al. 1993).

21.3.1 Evidence for Population Impacts

Population-level impacts of PKD have been documented in Norway and Switzerland. The example from Norway (Sterud et al. 2007) is notable because of the northerly latitude and may be indicative of the recent warming trend in coastal waters of northern Norway (Sundby and Nakken 2008). Between 2002 and 2006 (with the exception of 2005), elevated mortality among Atlantic salmon (*Salmo salar*) fry, up to 120 fish day^{-1}, was noted in the River Åelva on the Norwegian central coast. River water temperatures were on average 2.1° warmer in the summer of 2006 (17.5 °C) compared with 2005 (15.4 °C), with 24 and 5 days, respectively, in which the temperature exceeded 18 °C (Sterud et al. 2007). Fry (age 0+) densities in 2005 and 2006, determined by electrofishing, were 102 ± 62 and 13 ± 12 fish m^{-2}, respectively. Grossly, kidneys in six of eight 0+ moribund fry were pale and enlarged and microscopic examination revealed diffuse edema and interstitial granulomatous inflammation with an infiltrate of macrophages and lymphocyte-like cells. Polymerase chain reaction amplified a small subunit ribosomal RNA gene sequence with 99.7 % identity to that of *T. bryosalmonae*. Thus, Atlantic salmon fry in

the River Åelva experienced recurrent outbreaks of PKD resulting in a measureable decrease in fry density when years with and without mortality were compared. There was evidence that parr abundance (age 1+, 2+, 3+) in 2006 was elevated compared with 2005.

The decline in brown trout catch in Swiss lowland rivers for over 20 years appears to be related to the interacting effects of infection with *T. bryosalmonae*, increased temperature and reduced habitat (including water) quality (Borsuk et al. 2006; Burkhardt-Holm 2008; Zimmerli et al. 2007; see also Chap. 15). *T. bryosalmonae* infections occur widely in brown trout populations in streams primarily at elevations below 800 m (Wahli et al. 2002, 2007, 2008). By late summer at three sites along the Lyssbach River, all young-of-year brown trout were infected and more than 95 % showed renal pathology consistent with PKD. In contrast, while the prevalence of infection in age 1+ trout was 92 % or higher, renal pathology was evident in no more than 40 % of the fish, providing additional support for the impact of PKD in young or naïve salmonids (McConnell and Peterson 1992; Schmidt-Posthaus et al. 2013). Although decreased catches of brown trout have been correlated with the presence of PKD in Switzerland (Fischnetz 2004). Wahli et al. (2007) found no statistically significant evidence for a relationship between declining trout stocks in Switzerland and the presence of PKD. Thus, the role of PKD in exacerbating high mortality typically experienced among juvenile salmonids is not clear and it will be important to determine at the population level, the extent to which PKD mortality is additive or compensatory. If additive, overall survival to 1+ fish is reduced in the presence of the disease. If compensatory, survival to 1+ fish is not measurably different than in the absence of PKD, implying that PKD kills fish otherwise destined to die from other factors. While more research is required to determine the relative importance of infection with *T. bryosalmonae* in contributing to juvenile salmonid mortality, the roles of temperature and habitat quality in addition to the acquisition of protective immunity following recovery from severe infection (Hedrick et al. 1993), suggest options for mitigation strategies.

21.3.2 Strategies for Mitigating Impacts

Given their widespread distribution and diversity in freshwater ecosystems, efforts to manage bryozoan populations are likely to be impractical and highly likely to have adverse ecological consequences. Notably, outbreaks of PKD in northern regions where the disease was previously unrecorded imply a broad distribution of infected bryozoans and of infected fish that do not normally develop disease symptoms (Okamura et al. 2011). Such novel disease outbreaks are therefore expected to increase and the distribution of PKD will continue to extend northward as increasing surface water temperatures promote disease development (e.g. Sterud et al. 2007).

Okamura et al. (2011) explored possible future scenarios of *T. bryosalmonae*—salmonid interactions by considering the cumulative influences on salmonid populations of habitat loss, over-fishing and climate change. Mitigating the influence of PKD is likely to be largely limited to stocking or habitat manipulation in the case of wild populations or to husbandry practices in farmed populations. Building on an abundance of experimental evidence (e.g. Clifton-Hadley et al. 1986; de Kinkelin and Loriot 2001), trout farmers have adopted husbandry strategies to mitigate the risk of PKD. For example in Italy and the U.K., the risk is reduced when trout are exposed to infectious waters in the autumn when temperatures are decreasing (Longshaw et al. 2002; Okamura et al. 2011). Such exposure precludes the disease developing in the following summer when temperatures increase. As trout must be reared in *T. bryosalmonae*-free water (e.g. from wells or springs) prior to autumn exposure, this strategy may not always be feasible and is likely to incur additional production costs. It has long been recognized that fish which recover from PKD are resistant to clinical disease following a subsequent exposure (Ferguson 1981; Foott and Hedrick 1987) and that both innate and adaptive immune mechanisms are elicited during infection (Sitjà-Bobadilla 2008) (see also Chap. 14 which describes fish immune systems and provides specific discussion of immunological responses

to *T. bryosalmonae*). Presumably, it is such immunity against clinical disease elicited in the autumn-stocked trout that protects the fish as temperatures warm the following spring. For wild populations, a similar autumn-stocking strategy may be envisioned in which juveniles are held on well water until stream temperatures have declined below 15 °C. Alternatively, manipulation of water flow from dams may influence water temperature (Ward and Bonar 2003) and may, in some circumstances, reduce PKD impacts.

21.4 *Ceratonova* (Syn *Ceratomyxa*) *shasta:* Enteronecrosis (Ceratomyxosis)

In contrast to many myxozoans, the geographic range of *Ceratonova* (syn *Ceratomyxa*) *shasta* is limited with infections only reported in salmonids from the Pacific Northwest region of North America. The life cycle of *C. shast*a is completed in the freshwater polychaete, *Manayunkia* sp (Bartholomew et al. 1997; see Chaps. 10 and 12); thus this parasite infects Pacific salmon only during their freshwater residency. Being anadromous, infection of fish typically occurs during their seaward migration as juveniles and their return to spawning grounds as adults. Individuals of species that remain in freshwater, such as rainbow trout and kokanee salmon (*O. nerka*), are likely susceptible to infection throughout their life. Infection occurs when the parasite enters the gills and begins replication in the adjacent blood vessel, releasing parasite stages into the bloodstream. The parasite migrates to the intestine, where it continues to proliferate, invoking a severe inflammatory response and causing tissue necrosis. In susceptible fish the parasite disseminates to other tissues and ultimately causes host death. Clinical disease signs vary with host species, but may include distension of the abdomen with ascites and haemorrhaging of the vent (Bjork and Bartholomew 2010).

21.4.1 Evidence for Population Impacts

A failed enhancement program was the first indication that *C. shasta* could impact fish outside hatcheries. Thus, in the Willamette River, Oregon, USA, more than one million juvenile coastal steelhead (*O. mykiss*) were stocked over a 10-year period (1966–1975) and few, if any, adults returned (Buchanan and Sanders 1983; Bartholomew 1998). The following year, juvenile steelhead were sourced from within the basin and adult returns from this release increased to 7.5 %. Subsequent studies supported the premise that salmon strains that co-occur with the parasite were resistant to disease compared with strains from non-endemic waters (Sanders et al. 1972; Buchanan and Sanders 1983; Hoffmaster et al. 1988; Bartholomew 1998). These studies directed future re-introduction and stocking programs which have, for the most part, reduced mortality from *C. shasta*.

Impacts of *C. shasta* on natural salmon populations are more difficult to estimate and are largely inferred from capture studies conducted in the Columbia (Oregon/Washington, USA including the Willamette and Deschutes Rivers, Oregon), Fraser (British Columbia, Canada) and Klamath (Oregon/California, USA) river basins. In each study, migrating juvenile salmon were captured during their out migration (typically May-Sept) and mortality was either determined directly by holding the fish until they died from infection (Columbia and Fraser River basin studies) or estimated by quantifying parasites in intestinal tissue using molecular methods and histology (Klamath basin study). In the first of these studies, wild, age-0 Chinook salmon (*O. tshawytscha*) were captured weekly in the Deschutes River in 1978–1979. Mortality varied between months and years. Peak mortalities of 56 and 90 % in July of each year indicated that the parasite was a significant mortality factor for the wild fishery (Ratliff 1981). Juvenile Chinook salmon were collected before entering the

Columbia River estuary in 1983–1984, and of these, 10.6 % died with signs of enteronecrosis (Bartholomew et al. 1992). The catch included both age-0 and yearling Chinook, and mortality did not differ significantly between the year classes (9 % of age-0 fish; 11 % of yearling fish) or study years, but again was highest in the July capture group. Smaller numbers of coho salmon (*O. kisutch*) and steelhead trout were also taken in that study, and mortality for these species was 5 and 12 %, respectively. The Willamette River study (reviewed in Bartholomew 1998) focused on steelhead smolts collected prior to entering the Columbia River, spanned four years (1992–1995) and covered a range of environmental conditions. Mortality fluctuated between years from 31 % (flood, moderate temperature) to 88 % (drought, high temperatures), and fish of wild and hatchery origin and of different ages (age-0 to yearlings) were similarly affected. This dramatic difference in mortalities linked with river conditions supported recommendations to set minimum flows for that river. These studies also demonstrate that fish age (age-0 versus yearling) and origin (wild versus hatchery) do not affect the outcome of infection.

In the Fraser River to the north, 3.3 % of the juvenile Chinook salmon collected during 1985–1987 died from *C. shasta* infection (Margolis et al. 1992). Here, there was a significant difference in infection prevalence associated with age (age-0 fish had a higher infection prevalence than yearling fish), which was attributed to the longer migration period of younger fish and thus their arrival in the lower river during the period when infectious dose was highest. In contrast to the strong correlation between disease resistance and fish strain origin observed in other rivers (reviewed by Bartholomew 1998), disease challenges of Fraser River fish strains invariably resulted in moderate to high mortality (Ching and Munday 1984). This decreased disease resistance supports the suggestion that timing of migration enables Fraser River fish to avoid exposure to high parasite numbers. Thus *C. shasta* may not be as important a selection for these fish. This is likely true for fish populations in other northern rivers, where water temperatures limit parasite development (and run timing is more discreet). These two naturally evolved strategies, avoidance of parasite exposure through migrational timing and development of disease resistance, may illustrate that in different systems host-parasite interactions may reflect different drivers.

The most comprehensive studies of *C. shasta* effects on juvenile Chinook salmon focus on the Klamath River population and include molecular assays to determine infection prevalence and severity in out migrating hatchery-origin fish (True et al. 2010, 2013), measures of parasite density in the water (Hallett and Bartholomew 2006; Hallett et al. 2012) and infection in sentinel fish (Stocking et al. 2006; Ray et al. 2014). These complementary approaches were conducted over a period of 10 years. Synthesis of these data shows wide annual variations in mortality, with severe infections in 17–62 % of outmigrant fish and 0–100 % mortality in sentinel fish. Parasite densities in water provided some predictive capability, with mortalities >40 % mortality in sentinel fish correlating with densities of 10 or more parasites/l of river water (Hallett et al. 2012). Multivariate and factor analyses support the hypothesis that ceratomyxosis affects juvenile Chinook salmon populations in the reach of the river where infection intensity is highest (Fujiwara et al. 2011). Water temperature and discharge (water velocity) were found to affect disease transmission and severity (Bjork and Bartholomew 2009; Ray and Bartholomew 2013), in keeping with observations of mortality associated with these factors in the Willamette River.

The outcome of infection in adult salmon is even more difficult to predict as these fish typically are multiply infected and immune suppressed. There is evidence that *C. shasta* infections contribute to prespawn mortality (Sanders et al. 1970; Chapman 1986), most likely in fish that either have long migration routes or migrate when temperatures are high.

21.4.2 Strategies for Mitigating Impacts

Strategies for reducing ceratomyxosis initially focused on the salmon host. Intraspecies variations in disease susceptibility occur as a result of differences in inherent immunity, parasite strain virulence (Bartholomew 1998; Atkinson and Bartholomew 2010; Hurst and Bartholomew 2012) and life histories that minimize exposure to the parasite (Margolis et al. 1992). These differences provide opportunities for reducing disease effects by stocking resistant strains of fish in endemic areas, or by avoiding periods of high parasite exposure either by altering hatchery release times or enhancing natural fish runs with earlier or later migration times.

Water temperature has the most direct effect on the parasite, dictating the timing of actinospore release and rates of parasite replication in both hosts (Ray and Bartholomew 2012), and years characterized by low water temperature are associated with decreased disease risk. However, because the ability to reduce water temperature is limited to highly regulated systems allowing cool water releases from dams, preservation of cool water refugia may become even more important under future climate conditions (Chiaramonte 2013). Temperature and flow (velocity) are inextricably linked, and the effects of flow alone are difficult to measure. However, because this is one variable that can be manipulated in a managed river, it is important to characterize its effects. Two laboratory studies that attempted to do this demonstrated reduced parasite transmission to both hosts and reduced disease severity in fish at higher flow velocities (Bjork and Bartholomew 2009; Ray and Bartholomew 2013). Based on these data, an epidemiological model of *C. shasta* (Ray 2013) predicts that decreasing the transmission rate of myxospores released from adult salmon to winter polychaete populations could be the most effective means of disrupting the parasite's life cycle. In a managed river this rate could be affected by intentional discharges of dam water after adult salmon have spawned and released myxospores. Thus, both the magnitude and timing of discharge events are critical.

Although an intuitive solution, the epidemiological model predicted that simply reducing myxospores is not feasible because a small proportion of adult salmon are responsible for contributing large numbers of spores, and there is no method to detect these "high contributors" (Ray 2013). Similarly, reducing population densities of the polychaete host did not significantly affect the model until population densities approached zero. However, reducing invertebrate host densities could be effective if additional parameters such as myxospore transmission rate are affected or if highly infected polychaete populations are targeted. Thus the most effective control strategies would involve multiple parameters; for example, altered flows could decrease polychaete densities and influence spore transmission rates. The effectiveness of altered flows is being tested in the Klamath River by releasing a pulse of reservoir water when parasite densities reach levels that cause disease in salmon (Hallett et al. 2012; Bartholomew et al. unpublished data).

21.5 *Myxobolus honghuensis*: Pharyngeal Myxosporidiosis

Pharyngeal myxosporidiosis is a recently described disease of carp caused by infection with *Myxobolus honghuensis* (Xi et al. 2011; Liu et al. 2012; Zhao et al. 2013). Clinical disease signs include anorexia, lethargy, sluggish swimming, exophthalmos, severe pharyngitis and death. The heavily infected pharynx becomes swollen, nodular and severely damaged. These nodular lesions rupture and liquefy immediately following death of the fish (Xi et al. 2011). Infections have been associated with mass mortality in pond-cultured gibel carp, especially in Northern Jiangsu province, China, over the past 20 years.

21.5.1 Evidence for Population Impacts

Crucian carp (*Carassius auratus auratus*) have been cultured for a long time and historically, were a commercially important species in China.

However, since the early 1980s, all female gibel carp, bred by allogynogenesis, have been extensively introduced into aquaculture throughout China to replace crucian carp. The myxozoan fauna of the gibel carp is similar to that of crucian carp (Chen and Ma 1998; Zhang et al. 2005). During the past 20 years, numbers of wild crucian carp have declined in most Chinese watersheds, especially in areas where the gibel carp are cultured. Although the reason for this decline is not known, it was speculated that pathogens may have spilled out from populations of cultured fish causing negative effects on wild populations. Since 2009, consecutive monthly sampling and examination of wild crucian carp and their myxozoan fauna has been conducted in Yancheng, Jiangsu province and Honghu, Hubei province, where gibel carp are intensively cultured and pharyngeal myxosporidiosis is a severe endemic disease. Results showed that: (1) the number of wild crucian carp is far less than historical values; (2) the average prevalence of *M. honghuensis* in wild crucian carp from the two regions is 72 %, although the infection intensity is not very high and (3) some dead crucian carp were intensively infected by *M. honghuensis*. Based on these observations, subsequent research examined the relationship between *M. honghuensis* infections in populations of the wild and cultured carp species. This investigation has been underway for 3 years; however, interpreting population-level effects is limited by insufficient historical data. It is possible that the wild crucian carp serve as a reservoir for the infection in cultured species. Elevated prevalences of infection in cultured species, may in turn serve to amplify the rate of transmission back into the wild population.

21.5.2 Strategies for Mitigating Impacts

The most practical way to limit the prevalence and severity of *M. honghuensis* in wild carp is to control pharyngeal myxosporidiosis in cultured gibel carp as this would reduce the number of myxospores being released back into the freshwater environment. To this end, more sensitive methods are being developed (e.g. LAMP and QPCR) to monitor the abundance of the infective agents in effluent of ponds where the disease occurs and in water near the ponds (J.Y. Zhang, unpublished data). Combinations of chemical, ecological and husbandry measures have been used in attempts to control *M. honghuensis* in pond-cultured gibel carp. A recent study showing that a benthic strain of *Aeromonas veronni* secretes chitinase, which can degrade myxospores of *Thelohanellus kitauei* (Liu et al. 2011), suggests a possible alternative control method for pharyngeal myxosporidiosis. Rotation of cultured fish species and allowing ponds to remain fallow have also been suggested in the regions where *M. honghuensis* infections are too severe to control.

21.6 Conclusions

Diseases caused by the myxozoan parasites *Myxobolus cerebralis*, *Tetracapsuloides bryosalmonae*, *Ceratonova shasta* and *Myxobolus honghuensis* are associated with measurable changes in some affected wild fish populations. These impacts are influenced by environmental factors which affect both invertebrate and fish hosts. The extent to which these factors may be manipulated to mitigate the impacts will be contextual and dependent on mutual benefits to stakeholders sharing the relevant fisheries habitats. A general absence of systematic surveillance limits our ability to fully assess both the magnitude of adverse effects and any benefits of mitigation efforts.

21.7 Key Questions for Future Study

- How can we collect, coordinate and integrate long-term fisheries-relevant datasets needed to develop epidemiological models and risk assessments?
- Can studies be designed to determine whether mortality is compensatory or additive? In other words, does the disease kill fish that were already destined to die or does it impose an increased level of mortality?

- Can strategies be developed to limit the transmission of pathogens from aquaculture to wild populations?
- What are the roles of environmental variables in driving aspects of the parasite life cycle as they relate to development in the invertebrate host, infectivity to the fish host and fish host susceptibility?

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Glossary

Actinospore Spore stage of myxosporeans that develops in definitive hosts (annelids) and is infective for the vertebrate. Many types are characterized by triradiate symmetry and composed of three polar capsules, three caudal projections and one sporoplasm with many infectious germ cells

Actinosporean See myxosporean

Bryozoa Attached colonial invertebrates in marine and freshwater habitats that use tentacular crowns for suspension feeding and constitute a relatively large phylum with at least 6,000 species. Mostly found in marine habitats

Capsulogenic cell See polar capsule

Cnidaria A phylum containing >10,000 species of aquatic animals, characterised by having stinging cells (cnidocytes). Best known cnidarian groups include jellyfish, corals and anemones, the vast majority of which are free living organisms. Myxozoans have recently been confirmed as parasitic cnidarians

Coelozoic Character of myxozoans inhabiting cavities of body organs such as the gall bladder or urinary tract. They may attach to walls or float freely in these cavities and invade epithelial cells in early stages

Complement A heat-labile cascade system of many serum glycoproteins that interact to provide many of the effector functions of humoral immunity and inflammation, including vasodilation and increase of vascular permeability, facilitation of phagocyte activity, and lysis of certain foreign cells. It can be activated via either the classical, alternative or lectin pathways. In fish, complement factors are also found in skin mucous as well as serum where they are known to be important effectors of the innate immune defence

Covert (silent) infection Stage of malacosporean infection in which the parasite is cryptic, occuring as single cells (or early developmental stages) associated with the body wall. Covert infections are associated with low energy costs to the host and require PCR for detection as they cannot be assessed by stereomicroscopy (see overt infection)

Cryptomitosis (also called closed mitosis) Mode of nuclear division, especially in parasitic protists, during which the nuclear membrane is not disassembled (as opposed to the normal open mitosis)

Definitive host (also: final host, primary host) Host in which sexual reproduction occurs. In myxozoans this is signified by meiosis during actinospore (Class Myxosporea) or malacospore (Class Malacosporea) formation

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Degree days The total amount of heat required, between the lower and upper thresholds, for an organism to develop from one point to another in its life cycle

Diheteroxenous life cycle Complex or indirect parasite life cycle involving two hosts

Endogeny Situation where one cell (secondary cell) is completely embedded in the cytoplasm of another (primary) cell

Enzootic A disease of animal populations, analagous to endemic diseases in human populations

Endospore Membrane surrounding the sporoplasm in some myxozoan spores

Epizootic A disease that occurs in an animal population at a rate greater than what is expected based on recent experience, analogous to epidemic in human populations

Extrasporogony A proliferative phase in the developmental cycle of myxozoans. It occurs in tissues or organs different from the final site of sporogony and does not lead to the formation of spores. Sometimes, even once sporogonic stages have developed, extrasporogonic stages persist, allowing for additional parasite proliferation

Extrusomes Membrane-bound structures in some eukaryotes which, under certain conditions, discharge their contents outside the cell. For example, nematocysts and polar capsules

Fish malacospore Spore produced by a malacosporean in its intermediate host

Gametogony Development of the pansporocyst stage during the actinospore phase of development. Initiated when a binucleate cell stage undergoes mitotic division to yield a tetranucleate cell

Generative nuclei Generative nuclei occur in myxosporean plasmodia and are destined for cells involved in spore formation (as opposed to somatic nuclei; see entry)

Gonochoric Having just one of at least two distinct sexes in any one individual organism

Heteroxenous life cycle Complex or indirect parasite life cycle involving more than one host

Histozoic Character of myxozoans infecting solid tissues of hosts such as musculature, kidney, spleen, liver, intestine, cartilage or gill

Horizontal transmission Transmission of a parasite that achieves infection of new hosts, excluding transmission to offspring (see vertical transmission)

Infection intensity The number of parasites in an infected host

Infection prevalence The proportion of infected hosts among all the hosts examined

Intermediate host (syn.: secondary host) Host in which myxospores (Class Myxosporea) or fish malacospores (Class Malacospore) develop and proliferate asexually

Isogamy Mode of sexual reproduction involving fusion of very similar gametes. Appears to characterise Myxozoa. Anisogamy or oogamy (fusion of very dissimilar egg and sperm cell) is the rule in metazoans

Malacospore Infectious stage of malacosporeans produced in definitive host (freshwater bryozoans), typically spherical and containining 4-8+ soft shell valves, 2-4 polar capsules, and 1-2 sporoplasms. See also fish malacospore

Malacosporean Member of Class Malacosporea

Merogony Asexual replication process involving nuclear division followed by cytoplasmic division. Used to describe myxosporean proliferation in annelid hosts

Myxospore Infectious stage of myxosporean to annelid hosts. Myxospores develop in fish hosts and are composed of 2–15 shell valves enclosing 1–15 polar capsules and one to several sporoplasms

Myxosporean Member of Class Myxosporea (old terminology includes actinosporean, actinosporidian, TAM)

Myxoworm Worm-like trophozoite of malacosporeans in bryozoan hosts

Myxozoan Member of the Phylum Myxozoa (old terminology includes myxosporidian, myxosporidan)

Nematocysts Intracellular organelles characteristic of cnidarians composed of a capsule containing an eversible thread used for anchoring, defense or prey capture

Overt (observable) infection Stage of a malacosporean infection in which spore-producing sacs or myxoworms are found in the body cavity of bryozoans. Overt infection stages can be readily observed by stereomicroscopy (as stages within or released from bryozoan hosts)

Pansporocyst Multicellular spore-forming stages of myxosporeans developing in annelid hosts

Pansporoblasts Myxosporean stage consisting of a group of cells formed by a pericyte cell and the progeny of a sporogonic cell (the sporoblast)

Paratenic host Hosts that serve to transfer infective stages from one host to another but in which parasites do not develop (as opposed to dead-end hosts)

Paratomy A form of asexual reproduction where the animal splits in a plane perpendicular to the antero-posterior axis (in budding the body axes need not be aligned)

Pericyte A cell derived from generative cells, which surround sporogonic cells (e.g. during the process of endogeny) and presumably assist in nutrition

Plasmodium Developmental stage of myxosporeans composed of a cell with many vegetative nuclei. Within plasmodia endogenously produced generative cells form spores (in mono-, di-, tetra- or polysporic fashion)

Plasmotomy The division of a multinucleate cell into further multinucleate or into uninucleate cells

Polar capsule Intracellular organelle formed inside capsulogenic cells of myxozoan spores containing an extrusible polar filament. Homologous to cnidarian nematocysts

Polar filament Tube-like structure coiled within the polar capsule which extrudes to anchor the spore to the host

Post-mortem myoliquefaction Enzymatic degradation of musculature of marine fish caused by *Kudoa* myxozoans after fish harvest. Often referred to as ‘soft flesh’, ‘milky fish’ or ‘milky condition’

Presporogonic Stages prior to spore development

Primary cell (mother cell) Initial cell of the myxosporean cycle inside which secondary, tertiary, quaternary cells or spores develop

Pseudoplasmodium Plasmodium (see entry) with only one vegetative nucleus. Mono- or disporic

Schizogony Multiple sequential divisions of a mother cell giving rise to multiple daughter cells

Shell valves Outer cells of myxozoan spores that adhere to each other along a suture line to enclose and protect polar capsules and sporoplasms. Shell valves of myxospores are hardened, those of malacospores and most actinospores are soft and do not cover the exit point of the polar filament

Somatic nuclei Somatic nuclei are present in myxosporean plasmodia (see also generative nuclei)

Spore Transmission stage of myxozoans (see actinospore, fish malacospore, malacospore, myxospore). Comprised of valve cells, capsulogenic cells and sporoplasms

Sporoblast Cluster of sporogonic cells (inside a plasmodium, pseudoplasmodium, or pansporocyst) that gives rise to a single spore. In some myxozoan species the sporoblast is enclosed by a pericyte

Sporogonic cells Cells producing spores by division and differentiation

Sporogony Process of spore formation in myxozoans. Involves cell divisions, differentiation, motility, and formation of cell junctions

Sporoplasm Amoeboid cell, sometimes harbouring secondary cell (sporozoite, sporoplasm germ cell) that exits the spore (following polar capsule attachment and the opening of shell valves) to penetrate and infect the next host

Sporoplasmosomes Characteristic membrane-bound, electron-dense structures present in some myxozoans. In Malacosporea sporoplasmosomes have been documented in pre-saccular stages and in sporoplasms in bryozoan hosts and in proliferating single cell stages in fish. In myxosporeans, sporoplasmosomes occur in sporoplasms and lack the central lucent area present in those of malacosporeans

Sporozoite See sporoplasm

Statoblasts Dormant, asexually-produced propagules of freshwater bryozoans that survive adverse conditions, composed of disc-like buds surrounded by a hard shell

Suture See shell valves

Synapomorphy A trait that is shared by two or more taxa and inferred to have been present in their most recent common ancestor, whose own ancestor, in turn, is inferred not to possess the trait

TAM Triactinomyxon actinospore stage of *Myxobolus cerebralis*. Used as a generic term for actinospore by some authors

Trophozoite Vegetative (trophic) stages, in the form of active, worm-like organisms (myxoworms) or inert, closed sacs in malacosporeans, pansporocysts and amoeboid plasmodia in myxosporeans, and amoeboid pseudoplasmodia in both malaco- and myxosporeans

Tubificoid naidid *Tubifex tubifex* and other oligochaetes formerly classified as tubificids/Tubificidae (Erseus et al. 2008)

Valve See shell valves

Vertical transmission Transmission of a parasite from an individual to its progeny

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