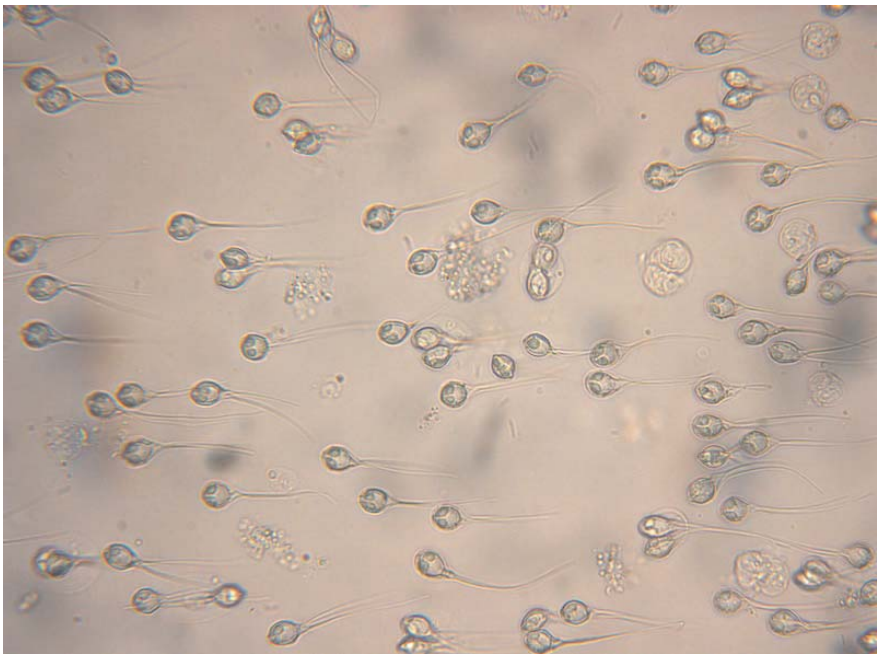
JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE
332

Hanna Ahonen

Spore-Forming Parasites Infecting Muscles of Freshwater Fishes

- Ecology and Epidemiology



JYVÄSKYLÄN YLIOPISTO

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UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2017

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Cover picture: Myxospores of *Henneguya zschokkei* by Hanna Ahonen

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To the memory of my beloved mother ♥

ABSTRACT

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Spore-forming parasites infecting muscles of freshwater fishes – ecology and epidemiology
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Yhteenveto: Kalan lihasta infektoivat itiöloiset – ekologia ja esiintyminen

Fish parasites may potentially harm fisheries and aquaculture. Infected fish are unusable, even though they would not pose a direct risk to human health. The aim of this thesis was to investigate the ecology and epidemiology of three previously unknown or poorly known spore-forming parasites infecting muscles of economically important fish species. The first novel microsporidian species, here described as *Microsporidium luciopercae*, was found from pike-perch (*Sander lucioperca*) and European perch (*Perca fluviatilis*) and formed opaque-looking patches in the muscles. The second novel microsporidian species, *Myosporidium spraguei*, was found from pike-perch and burbot (*Lota lota*) and occurred within xenomas in the musculature. The third muscle-dwelling parasite, *Henneguya zschokkei* (Cnidaria), formed white plasmodia in the muscle of whitefish (*Coregonus lavaretus*). Among the six study lakes, the prevalence of *M. luciopercae* in wild pike-perch and perch varied from 0 to 1 %, while *M. spraguei* was more common (prevalence of 5–26 % in pike-perch and 65 % in burbot). *M. spraguei* was strongly aggregated and most abundant in the middle-sized (37–45 cm) pike-perch and in larger burbot (> 35 cm), but the infection was not related to host sex, sampling season or host condition. In the case of *H. zschokkei*, in two inland fish farms, one year-old whitefish were virtually uninfected, but the prevalence of infection was higher in 2 and 3 year old fish (up to 36 % of 3 year old fish were infected). New infections appeared seasonally in July-August. Neither the size of plasmodia nor the proportion of different plasmodium types changed from 2 to 3 year old fish, suggesting slow development of *H. zschokkei* in whitefish. However, the proportion of infected fish with melanised plasmodia increased with fish age, suggesting an age-dependent host response against this strongly aggregated parasite.

Keywords: Aquaculture; *Henneguya zschokkei*; host-parasite relationship; fishery, *Microsporidium luciopercae*; *Myosporidium spraguei*; parasitism.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals (I–IV).

I Jones S., Ahonen H., Granlund L., Arsiola T. & Taskinen J. 2017. Two novel microsporidia in skeletal muscle of pike-perch *Sander lucioperca* and burbot *Lota lota* in Finland. *Journal of Parasitology* 103: 95–102.

II Ahonen H.S., Granlund L., Arsiola T. & Taskinen J. Infection of fish hosts by the microsporidian parasites *Microsporidium luciopercae* and *Myosporidium spraguei*. Manuscript.

III Ahonen H.S., Koskinen H., Valtonen E.T. & Taskinen J. 2017. *Henneguya zschokkei* (Myxozoa) infection in cultured whitefish: Age-dependence, seasonality and distribution within host. *Aquaculture* 470: 164–168.

IV Ahonen H.S., Osman Z.F., Valtonen E.T. & Taskinen J. *Henneguya zschokkei* (Myxozoa) infection in cultured whitefish (*Coregonus lavaretus*): Plasmodium types and sizes, sex-dependent infection and melanization. Submitted manuscript.

The table shows the contributions of the authors in the original papers. Initials stand for the following authors: Hanna Ahonen, Jouni Taskinen, E. Tellervo Valtonen, Simon Jones, Tiina Arsiola, Lars Granlund, Zul Faizuddin Osman and Heikki Koskinen.

	I	II	III	IV
Original idea	HA, ETV, JT	HA, JT	HA, ETV, HK, JT	HA, ETV, JT
Experimental design	SJ, HA, JT	HA, JT	HA, HK, ETV, JT	HA, ETV, JT
Data collection	HA	HA, LG	HA	HA, ZFO
Data analysis	SJ	HA, TA, LG, JT	HA, JT	HA, JT
Writing	SJ, HA, TA, LG, JT	HA, TA, LG, JT	HA, ETV, JT	HA, JT

1 INTRODUCTION

Parasites are inducing epidemics in both wild and cultured fish (e.g. Diamant 1992, Hyvärinen *et al.* 2009, Lafferty *et al*. 2015). Infections can be economically harmful causing losses to production, for example by affecting the quality or the condition of fish or directly killing the fish. There is a range of examples showing how parasites have caused destruction in fish industry – e.g. *Gyrodactylus salaris* destroyed several wild salmon populations in Norway (Johnsen and Jensen 1986) and very harmful Myxozoa parasite, *Myxobolus cerebralis,* is causing neurological damage and increasing mortality in wild and cultured salmonid populations in the United States (e.g. Steinbach Elwell *et al*. 2009). In Finland, fish bacteria diseases, especially *Flavobacterium columnare* and *Flavobacterium psychrophilum,* are harmful pathogens in fish farms increasing mortality (Anon. n.d.a.). In addition, some protozoa infections (e.g. *Ichthyobodo necator*, *Ichthyophthirius multifiliis* and *Capriniana piscium*) are problematic, especially with fingerlings (Valtonen and Koskivaara 1994). Because of significant losses, studies on parasite ecology and epidemiology are essential in developing control and preventive methods against the infections. An adequate fish health management and biosecurity are also essential for sustainability of the fisheries industry (Anon. 2016).

1.1 The Microsporidia

Species of the phylum Microsporidia are intracellular, spore-forming parasites infecting various invertebrates and all classes of vertebrates worldwide. Host taxa include e.g. protists, bryozoans, nematodes, oligochaetes, honeybees, fishes and human. Some species are known to be host-specific while others are able to infect many host species (Vávra and Larsson 2014). More than 1 500 species are known and new species are continuously discovered (Sprague *et al*. 1992, Lom and Nilsen 2003, Stentiford *et al*. 2013, Vávra and Lukeš 2013, Becnel *et al*. 2014). Probably, the first reported microsporidia–infection was *Glugea anomala* infection in stickleback fish already in the 1800s (Gluge 1838, cited by Franzen 2008). Classification of microsporidians has been difficult because of the

complexity of their life cycles and morphological structure, which is atypical for eukaryotes – e.g. Golgi system, mitochondria and peroxisomes are lacking (Canning and Lom 1986, Weiss 2001, Corradi and Keeling 2009). During the last 150 years, Microsporidia has been suggested to be e.g. yeast-like fungi, different kinds of protozoa and the Archezoa (Corradi and Keeling 2009). Today, based on both molecular and morphological methods, these parasites are considered as "the earliest diverging clade of fungi" (Capella-Gutiérrez *et al*. 2012).

Microsporidians have several distinct developmental stages in their complex life cycle, which include the proliferative phase (merogony), sporogonic phase (sporogony) and mature infective spores (Canning and Lom 1986, Bigliardi 2001, Capella-Gutiérrez *et al*. 2012, Vávra and Lukeš 2013). Fish microsporidia can be embedded directly in the cytoplasm, or rarely in the nucleus of the host cell, and this could destroy the cell or cause enormous hypertrophy of the cell to form xenomas (Lom and Dyková 2005, Stentiford *et al*. 2013, Palenzuela *et al*. 2014). In the host, spores may also infect surrounding cells and this may increase the intensity of infection (Solter and Maddox 1998). Mature spores may be released from the host via the skin, faeces, urine, or at the death of the host (Keeling and Fast 2002). Microsporidia spores are highly resistant to environmental changes (Li *et al*. 2003), but only mature spores may survive outside the host (Corradi and Keeling 2009). Transmission mechanisms are poorly known. Although horizontal transmission is believed to be the main mechanism, vertical transmission is also possible in some species. Some microsporidia species may use both transmission mechanisms (Canning and Lom 1986, Dunn and Smith 2001, Dunn *et al*. 2001, Phelps and Goodwin 2008) e.g. *Pseudoloma neurophilia* infection in zebrafish (*Danio rerio*) (Kent and Bishop-Stewart 2003). Horizontal transmission mechanism can be more virulent than vertical, because vertical transmission requires that the host reaches sexual maturity whereas horizontal transmission does not and can cause the death of the younger fish without jeopardizing the transmission (Dunn and Smith 2001). Some species are known to use intermediate hosts for transmission (usually some insect species) (e.g. Becnel 1992).

Microsporidians are known to cause severe diseases and reductions in fish populations (Lom and Dyková 1992, Lom 2002). Only few treatments are available to prevent the infections and Fumagillin is the most widely used drug for treating fish microsporidiosis (Kent *et al*. 2014). Microsporidia infection has been found to cause muscle destruction (e.g. Figueras *et al*. 1992, Shaw and Kent 1999, Costa *et al*. 2016), impaired swimming ability (Sprengel and Lüchtenberg 1991, Figueras *et al*. 1992), reduced fecundity (Summerfelt 1964, Wiklund *et al*. 1996) and growth (Matthews and Matthews 1980, Figueras *et al*. 1992, Palenzuela *et al*. 2014). However, typical Microsporidia infection is chronic in immunocompetent hosts but may develop lethal disease if host's immune condition is compromised (Texier *et al.* 2010). Some microsporidia infections have been associated with immune suppression in the host which may increase the severity of disease (Palenzuela *et al*. 2014) or affect fish susceptibility to infection by other pathogens (Wongtavatchai *et al*. 1995).

Almost half of all known microsporidians infect aquatic hosts (Stentiford *et al*. 2013) and approximately 156 species from 14–21 genera are listed to infect fish by now (Lom and Nilsen 2003, Stentiford *et al*. 2013, Vávra and Lukeš 2013, Phelps *et al*. 2015). In Finland, six fish-infecting microsporidia species were known prior to the present study (Table 1) (Valtonen *et al*. 2012).

TABLE 1 Microsporidia species observed in fishes of Finland (Valtonen *et al*. 2012) before the present study.

Microsporidia	Fish host	Organ
Glugea hertwigi	European smelt (*Osmerus eperlanus*)	Intestinal epithelium. Also in other organs if heavy infection
Glugea fennica	Burbot (*L. lota*)	Subcutaneous tissues
Glugea anomala	Three-spined stickleback (*Gasterosteus aculeatus*) and ninespine stickleback (*Pungitius pungitius*)	Subcutaneous tissues
Loma acerinae	Ruffe (*Gymnocephalus cernuus*)	Intestinal epithelium. Also in other organs if heavy infection
Ovipleistophora mirandellae	Ruffe and roach (*Rutilus rutilus*)	Ovaries
Pleistophora sp.	Burbot	Muscle

1.2 The Myxozoa

The Myxozoa belong to the phylum Cnidaria (Chang *et al*. 2015). These endoparasites have typically two-host life cycles involving invertebrate and vertebrate hosts. Two clades are recognized: the Myxosporea with 2 180 species and the Malacosporea with 4 species. The definite hosts for malacosporeans are freshwater bryzoans and the only known intermediate hosts are fishes. Definitive hosts for myxosporeans usually are annelids, and rarely sipunculid worms, while the intermediate hosts are mostly fishes but also amphibians, birds, reptiles or even small mammals (Lom and Dyková 1992, 2006).

Most of the Myxozoa infections are more or less harmless to fish host, but species causing severe diseases have also been reported (Lom and Dyková 1992, Kent *et al*. 2001, Yokoyama *et al*. 2012). There is no efficient treatment available for myxozoan infections in fish (Funk *et al*. 2008, Yokoyama *et al*. 2012) and this is one of the reasons why economic losses in fisheries and aquaculture can be very high. For example *Myxobolus cerebralis* causes whirling diseases and *Tetracapsuloides bryosalmonae* the proliferative kidney diseases (PKD) in salmonids (e.g. Kent *et al.* 2001, Lom and Dyková 2006) – both being economically most harmful fish diseases.

In Finland, only one Malacosporea species has been observed. PKD has been found few times since 2006, mostly in Åland but also once in north Finland, near Lake Inarijärvi, probably carried from abroad with stocked brown

trout (*Salmo trutta*) (Anon. n.d.b) Myxosporea species are more common in Finland, 14 species have been observed (Table 2) and two of them are infecting the muscle of fish i.e. H*enneguya zschokkei* (e.g. Luther 1909, Valtonen *et al*. 1988) and *Myxobolus pseudodispar* (Hakalahti-Sirén and Valtonen 2012).

TABLE 2 Myxosporea species observed in fishes of Finland (Hakalahti-Sirén and Valtonen 2012).

Myxosporea	Fish host	Organ
Henneguya zschokkei	Whitefish (*C. lavaretus*) and vendace (*C. albula*)	Muscle
Henneguya oviperda	Pike (*Esox lucius*)	Ovaries
Henneguya creplini	Perch (*P. fluviatilis*)	Gills
Henneguya psorospermica	Pike and perch	Gills
Myxidium rhodei	Roach (*R. rutilus*) and bream (*Abramis brama*)	Kidney
Myxidium lieberkuehni	Pike	Renal corpuscle, urinary bladder
Chloromyxum truttae	Brown trout (*S. trutta*) and whitefish	Gall bladder
Myxobolus bramae	Roach	Gills
Myxobolus elegans	Roach	Gills
Myxobolus macrocapsularis	Roach	Gall bladder
Myxobolus muelleri	Roach	Kidney
Myxobolus pseudodispar	Roach	Muscle
Myxobolus rutili	Roach	Gills
Zschokkella nova	Cyprinidae sp.	Gall bladder

The knowledge of these parasites increased significantly in the 1980s when two-host life cycle was described for the first time from *Myxobolus cerebralis* (Markiw and Wolf 1983, Wolf and Markiw 1984). The classification has been based largely on the spore morphology (Lom and Noble 1984), but recently, phylogenetic classification by SSU rDNA analyses has increased the knowledge of the relation between species.

1.2.1 The genus *Henneguya*; *Henneguya zschokkei*

Henneguya (Thèlohan 1892), is a widespread genus including at least 204 species infecting both marine and freshwater fishes. Complete life cycles are known only from four species, *H. exilis* (Lin *et al*. 1999), *H. ictaluri* (Pote *et al*. 2000), *H. nuesslini* (Kallert *et al*. 2005) and *H. mississippiensis* (Rosser *et al*. 2015) (Fig. 1). *Henneguya* species infect many organs in host, such as gills, heart, ureters, dermis, muscles and kidneys (Pote *et al*. 2000, Eiras 2002). Although many *Henneguya* species are harmless to their host, some species are also known to cause diseases in both cultured and wild fish (Lom and Dyková 1992, Molnàr 2002, Lom and Dyková 2006, Eiras and Adriano 2012). For example, *H. ictaluri* is an agent of the proliferative gill diseases (PGD) of *Ictalurus punctatus* (Pote *et al*.

2000) and *H. lateolabracis* is causing cardiac henneguyosis with chronic mortalities in *Lateolabrax* sp. in Japan (Yokoyama *et al*. 2003).

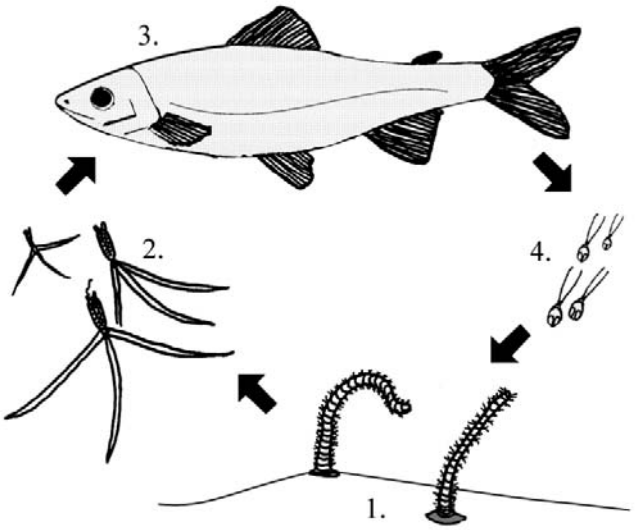

FIGURE 1 Life cycle of the four known *Henneguya* species involving two hosts. The definitive host is an oligochaete (1.), from which the freely floating actinospores are released into the water (2.). Actinospores infect the intermediate host, fish (3.), in which the second spore stage, myxospores developes. Mature myxospores (4.) may be released from fish e.g. via faeces, urine, eggs or at the death of the fish, and sink to the bottom to start the life cycle again.

In Finland, *H. zschokkei* is the only *Henneguya* species observed from wild whitefish (Table 2). *H. zschokkei* forms white plasmodia, which are full of myxospores (Fig. 2), in the skeletal muscles of fish. Plasmodia can be up to 2 cm in length. The first published case of this parasite in Finland was in the early 1900s (Luther 1909) and since then the occurrence has been documented in many Finnish lakes (Levander *et al*. 1901, Luther 1909, Levander 1914, Valtonen *et al*. 1988, Leinonen and Mutenia 2009) and from the Baltic Sea (Fagerholm and Valtonen 1980). *H. zschokkei* has also been observed from vendace (*C. albula*) (Hyvärinen *et al*. 2009) and in recent years it has appeared to infect cultured whitefish.

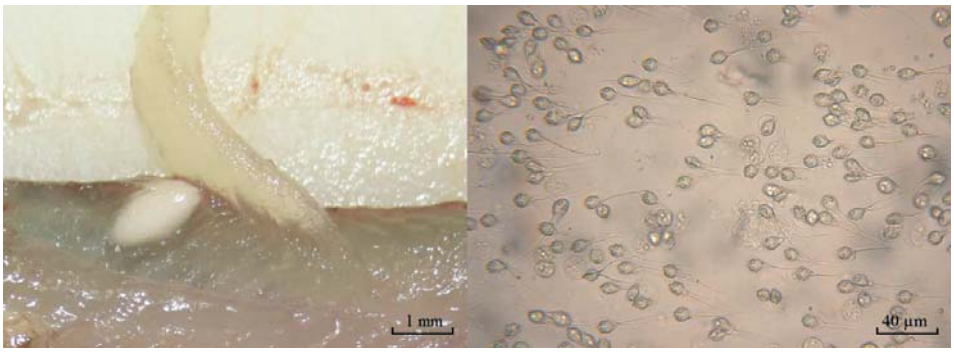

FIGURE 2 Plasmodium (oval, whitish cyst on the left; length ~ 1.5 mm) and myxospores (~ 45 µm) (right) of *Henneguya zschokkei*.

Infection by *H. zschokkei* has also been connected to other salmonid species although the genetic similarities between these infections have not been studied (Lom and Dyková 1992, Kent *et al*. 2001, Eiras 2002). *H. zschokkei* has been suggested to be synonym with *H. salminicola* which is known to infect salmonids e.g. in North America and Asian coastal waters (Boyce *et al*. 1985). Both species form large intermuscular plasmodia. In the gene bank they have sequence data which differ slightly from each other, but because of homology of sequences it is difficult to separate the species (Kallert *et al*. 2005). Several studies have shown that myxosporeans are host-specific parasites. Usually they can infect one host species or a limited number of closely related species (Eiras and Adriano 2012, Yokoyama *et al*. 2012). In Finland, all observations of *H. zschokkei* are from whitefish and vendace (e.g. Valtonen *et al*. 1988, Hyvärinen *et al*. 2009, Leinonen and Mutenia 2009). However, more studies are needed to identify the taxonomic relationship between *H. zschokkei* and *H. salminicola*.

1.3 Fish species of the study, and their economic significance

Fishery and aquaculture has an important role in the local economy in Finland. A total of 35 million kg of fish were caught by professional and recreational fishermen in 2014 (Anon. n.d.c). The most important species in inland professional fishery are vendace (*C. albula*), pike-perch (*S. lucioperca*), perch (*P. fluviatilis*) and whitefish (*C. lavaretus*). Fish farming is also an important industry in Finland; 14.9 million kg of fish was cultured in fresh and brackish waters in 2015 (Anon. n.d.c). The most important species is rainbow trout (*Oncorhynchus mykiss*) with 95 % of all production. The second most important species is whitefish with a production of 0.8 million kg. Aquaculture is producing a lot of fish also for stocking purposes. A total of 31 million fry were farmed in order to be stocked in year 2015. Whitefish and pike-perch are the most common stocking species in Finland (Anon. 2016b).

Pike-perch (*S. lucioperca*) is a predatory fish living in lakes and coastal waters of Europe (Wootton 1990). In Finland, it is one of the most valuable and popular species in commercial and recreational fishing. In 2015, the total catch was 4.1 million kilos (Anon. n.d.c). Pike-perch is also widely used in fish stockings; 6.6 million fingerlings were produced in 2015 (Anon. n.d.c). In recent years, the culture of pike-perch for food purposes has also increased (Jokelainen *et al*. 2009).

European perch (*P. fluviatilis*) is a predator fish species living in throughout Eurasia and North-America (Muus and Dahlstrøm 2005). Total catch was 9.4 million kg in 2015 (Anon. n.d.c).

Burbot (*L. Lota*) is the only member of Lotidae family which lives in freshwater. This predatory fish is widely distributed throughout the northern hemisphere (Maitland and Lyle 1991). In Finland, the main fishing season is winter when burbot is spawning. Total catch of burbot in Finland was half a million kilos in 2015 (Anon. n.d.c).

Whitefish (*C. lavaretus*) is a widely distributed fish species in northern Europe (Svärdson 1979). In Finland, two native *Coregonus* species occur, *C. lavaretus* and *C. albula* (vendace), and an alien species, *C. peled,* from Siberian has been stocked since 1965 (Salonen and Mutenia 1992). In 2015, the total whitefish catch was 1.7 million kilos. It is the second most important cultivated fish species in Finnish fish farms (Anon. 2016b). The amount of whitefish cultivated was 0.8 million kg of food fish and 17 million fingerlings for stocking purposes during 2015 in Finland (Anon. n.d.c).

1.4 Aims of the thesis

An unidentified Microsporidia, which formed opaque-looking patches in the muscles of pike-perch and perch, started to emerge in the catch of professional fishermen in some Finnish lakes since early 2000's. I started my PhD project in 2010 to study the microsporidia in order to get research-based information that could help to manage this new parasite infection. Investigations revealed not just one, but two microsporidia species new to science. The aim of the first article (I) was to describe these microsporidia species. The purpose of the II was to find out whether the same parasite infected both pike-perch and perch, determine how widely these previously unknown microsporidians are occurring in Finnish lakes and fish populations, what are the factors affecting their occurrence and do they harm the fish host.

In conjunction with the microsporidia, the aim was to investigate the occurrence of another type of musculature-infecting parasite, the Myxozoa, *H. zschokkei* in fish farm environment (III and IV). Previously, published information of this parasite is available only from lakes, while infection patterns at fish farms are unknown. *H. zschokkei* is a problematic parasite in fish farms because infected fish cannot be recognized until slaughter. Previously it was not known, when the cultured whitefish was infected by *H. zschokkei* and at what

age the infection becomes visible. Thus, the aim was also to investigate when *H. zschokkei* infection appears in cultured whitefish and how the infection develops. In addition, the aim was to study the factors affecting the abundance of *H. zschokkei* infection (III and IV).

Overall, this theses focuses on muscle-infecting, spore-forming parasites occurring in economically important fish species, with no (microsporidians) or limited (*H. zschokkei*) published information available. The knowledge of infection dynamics, occurrence and host-parasite relationships is important for developing preventative and control methods against these parasites in the future.

2 MATERIALS AND METHODS

2.1 The Microsporidia (I and II)

2.1.1 Fish material and examination

Samples of pike-perch were collected from lakes Haukivesi (560 km^2), Höytiäinen (283 km^2), Koirusvesi (0.4 km^2), Pielinen (894 km^2) and Päijänne (1 083 km^2) by gillnets (Fig. 3). A total of 495 pike-perch were studied in 2010–2014. Samples of perch (n = 284) were caught from three lakes: Haukivesi, Päijänne and Konnevesi (187 km^2). Furthermore 58 burbot were caught by gillnets from Lake Haukivesi in April 2011. Fish were transported to the laboratory alive in lake water or dead on ice. For article I, the fish were examined and microsporidia samples dissected within 24 h after fishing. Samples were preserved in 2.5 % glutaraldehyde in 0.1 M Sörensen´s phosphate buffer for electron microscopy, in neutral buffered 10 % formalin for light microscopy and in 95 % ethanol for DNA extraction. Fisherman sent samples from infected perch from Lake Pielinen in 2011 (see analysing of these samples below). In II pike-perch were filleted and cut into thinner slices (5–10 mm), pressed between two glass plates and examined using a dissection microscope with 6.3–25 x magnification. A compound microscope with 400 x magnification was used to verify unclear infections. *M. luciopercae* infection was determined as infected/uninfected, but numbers of xenomas of *M. spraguei* were counted from muscle slices of pike-perch and determined from three random 1 x 1 cm area from skinned burbot. In each case, the fish size was also measured.

FIGURE 3 Locations of study lakes (I and II) (picture by Vesa Saarikoski).

2.1.2 Histology

Neutral buffered 10 % formalin-fixed samples from infection of pike-perch were dissected, dehydrated in an alcohol gradient, cleared in xylene and embedded with paraffin. Each sample was cut into 3 µm slices and set onto a glass slide and stained using haemotoxylin and eosin or Gram stains. Samples were examined with a compound microscope.

2.1.3 Electron microscopy

Glutaraldehyde-fixed tissue (from infection of pike-perch) was washed with 0.1 M Sörensen´s phosphate buffer, fixed in osmium tetroxide (1 %) for 1 h, washed and dehydrated through a across series of ethanol in acetone (100 %). After incubation in 50:50 mixture of acetone:epoxy resin overnight, followed by two changes in the epoxy resin prior to polymerization at 60 °C overnight, tissue samples were cut to semi-thin (1–2 µm) and ultrathin (70–90 nm) sections. Semi-thin sections were stained with Toluidine blue and examined with a light microscope. Ultrathin sections were attached to the uncoated copper grids,

stained with uranyl acetate and washed three times with double distilled water, stained with lead citrate for three minutes before washing again and examining with an electron microscope. Morphology and dimensions were evaluated using ultrathin preparations viewed in electron micrographs.

2.1.4 DNA extraction, amplification and sequencing

DNA was extracted from the muscle samples preserved in ethanol (pike-perch and burbot) into AE buffer and DNA content was quantified with a Nanodrop-1000 spectrophotometer. Ten different oligonucleotide primers (I; Table 1) were used to amplify and to sequence (SSU) rDNA (Jones *et al*. 2012). PCR products were cleaned with commercial kit (ExoSap-IT) and sequence reactions were performed with BDT V3.1. Thereafter, commercial kits (Qiagen Dye-Ex 2.0) were used to purify reaction products, and 16-capillary 3130xl genetic analyser was used to obtain sequences.

Samples from muscle tissue of infected perch were preserved in ethanol and the DNA was extracted with a commercial kit (E.Z.N.A Tissue DNA Kit). PCR amplification of microsporidian rDNA was performed with two primers, 18 F and 580 R. The right sized PCR products were cut from a gel with ethidium bromide and purified with a commercial kit. PCR product was inserted into a plasmid and grown in a bacterial suspension. The sequencing was done at A.I. Virtanen institute, University of Eastern Finland, with MegaBASE 750 and assembled with Geneious Pro software.

2.1.5 Alignment and phylogenetic analyses

Sequencher 4.9 was used to edit and assemble sequences, and contigs were subjected to Blast analysis to identity with the most related archived sequences. Phylogenetic relationships were inferred using the general-time-reversible model with gamma distribution set to 3 (without gaps or missing data).

2.1.6 Statistical analyses on *M. spraguei* (II)

Differences in the prevalence of *M. spraguei* between lakes and fish sexes were tested using Pearson χ^2-test. One-way ANOVA was used to analyse differences in intensity between lakes, sexes, study years and season. Independent Samples *t*-test was used to compare the Fulton's condition index of infected and uninfected pike-perch.

Difference in the mean intensity of infection (only infected individuals included) between the middle-sized and large pike-perch was analysed using *t*-test with Log_{10}-transformed xenoma number as the response variable. Pike-perch individuals were categorized as small (< 370 mm), middle-sized (370–450 mm) and large (> 450 mm) according to their total length. The lower boundary value, 370 mm, was obtained from the previous legal minimum length of pike-perch in Finnish legislation. The upper boundary value, 450 mm, was set to the mid-point between 370 mm and the maximum pike-perch length in the Lake

Päijänne material (since the highest sample size was available from that lake). All statistical analyses were performed using IBM SPSS statistics Version 22.0.

2.2 The Myxozoa (III and IV)

2.2.1 Fish material and examination

H. zschokkei infection was studied in cultured whitefish (*C. lavaretus*) from two fish farms in southern (III) and northern Finland (IV). All fish were transported dead on ice or frozen to the laboratory. The first fish farm, (owner: Natural Resources Institute Finland) cultured fish in indoor-fiber glass tanks. A total of 1 599 fish (age-group 1+: n = 1054; age-group 2+: n = 381 and age-group 3+: n = 164) were examined during summer months in 2008 and 2009 (III). Half of 1+ old fish were stored after first summer at the Konnevesi Research Station (University of Jyväskylä) and examined the next year. The fish farm and the research station are located in the same watershed of the River Kymijoki and the distance between them is 50 km. The second fish farm (private company) cultured fish in floating cages in the stream. A total of 499 fish (1+: n = 151; 2+: n = 142 and 3+: n = 206) were examined between years 2009–2011 (IV).

All the fish were measured, filleted and cut to thin (5 mm) slices. *H. zschokkei* plasmodia were counted using light table (3+; III) or a dissection microscope with 6.3–25 x magnification (1+; III and 1–3+; IV). Age-, size- (III and IV), sex- (IV) dependent aspects of *H. zschokkei* infection were analysed from the data. Locations of plasmodia were recorded from infected fillets (III). Size, melanisation rate and firmness of plasmodia were measured (IV).

2.2.2 Statistical analyses with respect to *Henneguya zschokkei*

Differences in the prevalence of *H. zschokkei* between age groups, sexes, melanisation and plasmodia types were tested using Pearson χ^2-test (III and IV). The intensity between age groups, sexes and the mean proportion of type 2 plasmodia were analysed with One-way ANOVA and nonparametric Wilcoxon signed ranks test (III and IV). Locations of plasmodia in fish were tested also with nonparametric Wilcoxon signed ranks test (III). Independent Samples *t*-test was used to compare condition index (Fulton's condition factor) of infected and uninfected whitefish (III). To account for multiple tests, the Bonferroni correction was applied (III). Statistical analyses were performed with IBM SPSS statistics Version 22.0.

3 RESULTS AND DISCUSSION

3.1 Microsporidians in muscle of pike-perch and burbot

3.1.1 *Microsporidium luciopercae* in pike-perch and perch

Opaque-looking patch-forming microsporidia from pike-perch was described and named as *Microsporidium luciopercae* (I) (Fig. 4). The infection was identified in perch also (II). Spores were monokaryotic and ovoid: 4.6 µm long and 2.8 µm wide. Spore exospore was thinner than endospore, polar filament was isofilar and approximately 25 coils were found in single rank. Mature spores were in direct contact with sarcoplasma with no xenoma formation. *M. luciopercae* infection caused whitish patches in the muscle of fish, are actually aggregates of countless numbers of spores. Maximum likelihood analysis (numbers KX351969 and KU302782 in GenBank) (I; Fig. 4) clustered *M. luciopercae* with the *Microsporidium cerebralis* and *Pseudoloma neurophilia*. *M. cerebralis* is infecting the spinal cord and hind brain of cultured Atlantic salmon (*Salmo salar*), and increased lightly the mortality of fish and affected the balance of the fish causing abnormal spiral swimming behavior (Brocklebank *et al*. 1995). *P. neurophilia* is infecting the nervous system of zebrafish (*Danio rerio*) (Cali *et al*. 2011). It has also been found infecting the muscles and ovaries of fish (Kent and Bishop-Stewart 2003), as well as increase the mortality and morbidity of zebrafish (De Kinkelin 1980). In this thesis, the infection was studied only from the muscle tissue. In the future, it would be worth determining whether it occurs also in other organs. It may have an impact e.g. on parasite transmission; if ovaries are infected spores maybe release via eggs (see Dunn *et al*. 2001).

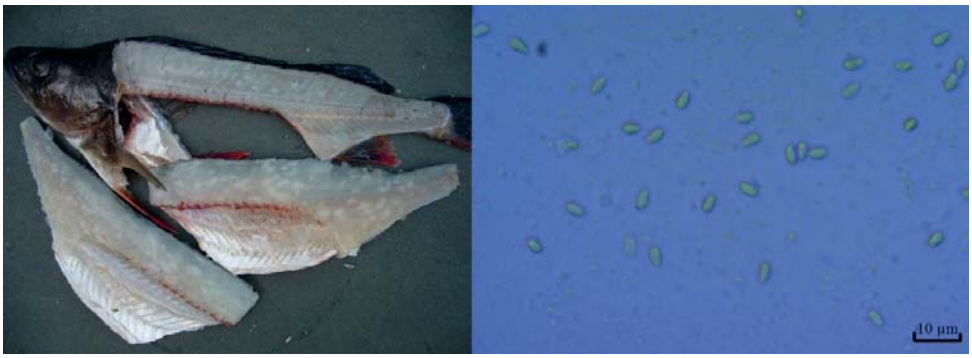

FIGURE 4 *Microsporidium luciopercae* spore-patches (left) in muscles of perch (photo by Hannu Hupli). *M. luciopercae* spores (right).

The occurrence of *M. luciopercae* was examined in pike-perch from five lakes and in perch from three lakes. It was found only in few fish individuals from Lake Haukivesi, Lake Pielinen and Lake Konnevesi (Table 3) (II). *M. luciopercae* infected fish are unmarketable for aesthetic reason. However, the whitish patches become invisible during the post-mortem changes in fish muscles. Nevertheless, possible changes in the muscle structure do not disappear; the infection causes degeneration and necrosis to the myocytes (I). Infection by *M. luciopercae* induced damage that resembles the liquefaction of muscles of marine fishes caused by e.g. *Microsporidium seriolae* (Sano *et al*. 1998). Similarly, *Heterosporis sutherlandae* is causing muscle necrosis in walleye (Phelps *et al*. 2015), and *Pleistophora* species are known to cause damage in the muscle of many fish species (e.g. Lom and Dyková 1992).

TABLE 3 Number of pike-perch and perch studied from five lakes with mean total length (mm) and mass (g) of fish and prevalence (%) (with 95 % confidence interval) of *Microsporidium luciopercae* infection.

Species and lake	n	Length	Mass	Prev. % (95% C.I.)
Pike-perch				
Haukivesi	158	407	725	0.6 (0.1–3.5)
Höytiäinen	46	465	949	0.0 (0.0–7.7)
Koirusvesi	30	404	453	0.0 (0.0–11.4)
Pielinen	71	394	556	1.4 (0.8–9.7)
Päijänne	190	402	692	0.0 (0.0–2.0)
Perch				
Haukivesi	222	127	35	0.0 (0.0–1.7)
Konnevesi	5	191	-	20.0 (0.0–62.5)
Päijänne	58	127	40	0.0 (0.0–6.2)

Some microsporidia species are known to increase mortality or decrease the condition of host. For example, heavy infection of *M. seriolae* may kill the host whereas a lightly infected fish can survive (Sano et al. 1998). Also closely related *P. neurophilia* increased mortality and morbidity of the host (De Kinkelin 1980).

Sprengel and Lüchtenberg (1991) found that infection of *Pleistophora ladogensis* decreased the maximum swimming speed of fish host when compared with non-infected fish. This decrease was even higher when fish was co-infected by both *P. ladogensis* and *Pseudoterranova decipiens* (Nematoda). Reduction in swimming speed may increase vulnerability to predation. These observations give a reason to suspect that *M. luciopercae* has some negative effects on fish. The present study did not find any effect on fish condition and there was no difference in mass and length between infected and uninfected fish. However, if infection remains rare, *M. luciopercae* is not threatening fishery.

3.1.2 *Myosporidium spraguei* in pike-perch and burbot

The second microsporidia species found during the sampling of *M. luciopercae* was described as *Myosporidium spraguei*. The spores were monokaryotic and ovoid: 3.8 µm long and 2.4 µm wide (Fig. 5). Exospore and endospore were equal in thickness, polar filament was isofilar and approximately 12 coils were found in single rank. The spores were aggregated within sporophorous vesicles which occurred inside the xenomas. Size of xenomas varied from 250–275 µm long and 50–175 µm wide. *M. spraguei* was observed from two hosts, pike-perch and burbot (Fig. 6). Morphological analyses were based on the infection in pike-perch only (I). Description of *M. spraguei* infection in burbot was based on the molecular analyses.

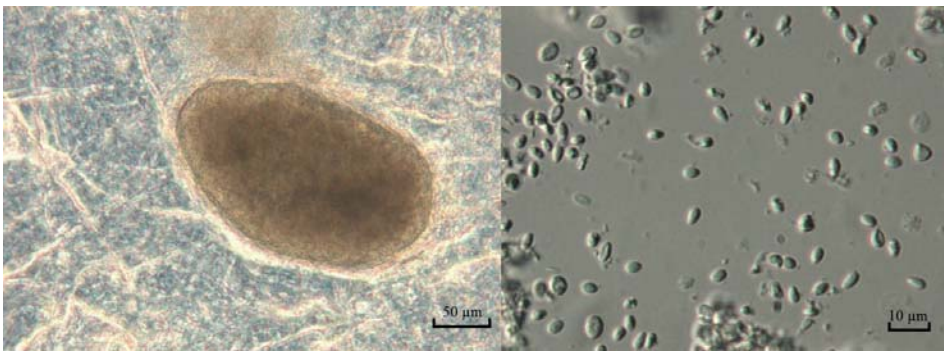

FIGURE 5 *Myosporidium spraguei* xenoma (left, length 265 µm) from muscle of pike-perch. *M. spraguei* spores (right).

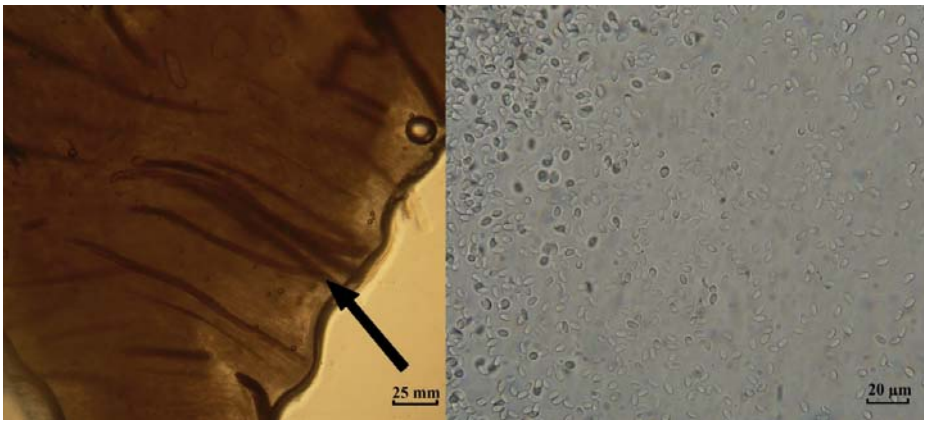

FIGURE 6 Long xenomas of *Myosporidium spraguei* from burbot (left, length up to 1–2 cm). *M. spraguei* spores from burbot (right).

M. luciopercae and *M. spraguei* showed major differences in DNA sequences (sequences shared ~78–79 % identity), and also morphological features differed; both have monokaryotic nucleus and isofilar polar filaments, the number of the coils inside the spores differed between the species. Maximum likelihood analysis (numbers KX351970, KX351971 and KU302781 in GenBank) (I; Fig. 4) clustered *M. spraguei* with two muscle-infecting species: *Microsporidium prosopium*, which is a parasite of the mountain whitefish (*Prosopium williamsoni*) (Kent *et al*. 1999) and *Myosporidium merluccius* which are infecting hake (*Merluccius* sp.) (Baqueiro *et al*. 2005): Impacts of infections of *M. prosopium* and *M. merluccius* on fish hosts were not studied.

M. spraguei was very common in Lakes Koirusvesi, Pielinen and Päijänne where more than 20 % of pike-perch were infected (II). In Lake Haukivesi, the infection was rare, with only 5 % of fish infected. *M. spraguei* was not found from Lake Höytiäinen (Table 4). The occurrence of infection was not associated with season, sex of fish or study year (II). Costa *et al*. (2016) studied *P. ladogensis* in smelt and found a clear seasonal fluctuation in the prevalence with the highest prevalence during summer months. Prevalence of infection also increased with age of the fish. In the present study, *M. spraguei* was strongly aggregated. In 90 % of the infected pike-perch, the infection intensity ranged from 1 to 24, with heavily infected fish having 60–2000 xenomas. This was also seen among burbot, where 14 % of infected fish were heavily infected. Aggregation is a typical phenomenon of parasite-host relationships (Shaw and Dobson 1995).

TABLE 4 Number of pike-perch studied with mean total length (mm) and mass (g) of fish, prevalence of *Myosporidium spraguei* infection (and 95 % confidence interval) and mean intensity (± s.e.).

Lake	n	Length	Mass	Prev. % (95% C.I.)		Mean int. ± s.e.
Haukivesi	158	407	725	5.1	(2.6–9.7)	85.0 ± 47.4
Höytiäinen	46	465	949	0.0	(0.0–7.7)	-
Koirusvesi	30	404	453	23.3	(11.8–40.9)	102.6 ± 75.7
Pielinen	71	394	556	23.9	(15.5–35.0)	167.7 ± 118.8
Päijänne	190	402	692	25.9	(19.6–31.9)	14.4 ± 6.6

Decreasing prevalence with host age has been considered as an indication of parasite-induced host mortality or acquired immunity (e.g. Perrin and Powers 1980, Anderson and Gordon 1982, Pekcan-Hekim *et al*. 2005). The mean intensity of *M. spraguei* infection in pike-perch from Lake Päijänne was statistically marginally lower in the large-sized fish when compared to middle-sized individuals. This indicates that there is a trend for a peak infection intensity among the middle-sized fish (Fig. 7), suggesting either parasite-induced host mortality or acquired immunity in older fish. Although no negative affect to fish condition was found, studies on other muscle-infecting microsporidia species suggest that infection may affect the swimming ability and even mortality of fish (Perrin and Powers 1980, Anderson and Gordon 1982, Sprengel and Lüchtenberg 1991, Pekcan-Hekim *et al*. 2005). In addition, Costa *et al*. (2016) observed that *P. ladogensis* infection in skeletal muscle of smelt caused necrosis and destruction of muscle cells.

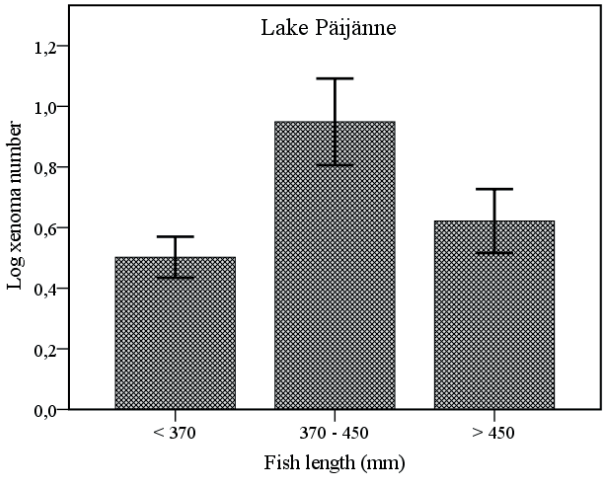

FIGURE 7 Log_{10} xenoma number (± s.e.) of *Myosporidium spraguei* in small (< 370 mm total lenght, n = 16), middle-sized (370–450 mm, n = 19) and large (> 450 mm, n = 13) pike-perch from Lake Päijänne in combined materials from years 2010–2014.

In Lake Haukivesi, *M. spraguei* prevalence was high in burbot (65 %) but in pike-perch it was only 5 %. According to results by Voronin (1978, as cited in Costa *et al*. 2016) revealed that the prevalence of *P. ladogensis* in burbot and European smelt (*O. eperlanus*) varied between study lakes and between host species from the same lake, being higher in burbot than in smelt. It was suggested that the typical host for *P. ladogensis* is burbot and smelt is the secondary host (Voronin 1978; as cited in Costa *et al*. 2016). From *M. spraguei*, two small subunit ribosomal RNA gene (SSU) sequence genotypes were observed. Type A was found in pike-perch and burbot from Lake Haukivesi, and type B was represented in pike-perch from Lake Päijänne. Similarity between type A and type B was 98.3 % (I). It is probable that these types are races or subspecies of the same species. Probably also the mixing of these two types is limited or negligible because of the distance between the catchment areas. However, further studies are necessary to resolve the relationship of the *M. spraguei* representing different SSU ribosomal RNA sequence types. To prevent the spread of *M. spraguei*, it is important that dead and unhealthy fish should not be returned into the lake.

3.2 *Henneguya zschokkei* in the muscle of cultured whitefish

Diseases related problems form one of the biggest causes of economic losses in aquaculture. Intensive farming is known to enhance parasite transmission due to high host density. In addition, stressful environment in fish farms, other pathogens and low genetic variation affect fish immune defense (Meyer 1991, Murray and Peeler 2005). In natural lakes, the prevalence of *H. zschokkei* varies depending on *Coregonus* species and water body. Valtonen *et al*. (1988) found *H. zschokkei* infection prevalence of 11.1, 16.4 and 6.8 % in *C. lavaretus*, *C. widegreni* and *C. acronius*, respectively. Leinonen and Mutenia (2009) studied *H. zschokkei* infection in *C. lavaretus* and it was below 30 % both in lakes Porttipahta and Lokka, while *C. peled* was not infected.

In the present study infection by *H. zschokkei* was rarely observed in 1+ fish, and in general, in both fish farms studied the first plasmodia appeared at the earliest in third summer in 2+ fish in July-August (Table 5). Seasonality was clearly seen as new infections became visible in July–August. Studies on Myxozoa diseases have shown that the water temperature determines seasonality of disease outbreaks at farms (Okamura *et al*. 2015). Experimental studies of Myxozoa have shown that parasites usually need 2–6 months to reach 'maturity' in fish host (Kent *et al*. 1993, Yokoyama *et al*. 1993). Myxospores within plasmodia of *H. nuesslini* were notable not until 102 days post-exposure when fish were kept at 18 °C (Kallert *et al*. 2005). Temperature is affecting the life cycle, development (e.g. El-Matbouli *et al*. 1999, Tops *et al*. 2006) and pathogenicity of the parasite (e.g. Ray *et al*. 2012, Schmidt-Posthaus *et al*. 2012). Haaparanta *et al*. (1994) observed that *H. creplini* plasmodia followed clear

seasonality and mature spores started to appear in early spring in gills of *P. fluviatilis*.

The prevalence of *H. zschokkei* did not increase significantly between 2+ and 3+ year old fish from either of the fish farms (III and IV). Two types of plasmodia were observed, both in 2+ and 3+ year old fish (IV). Type 1 seemed to be an earlier stage of plasmodium as the plasmodium wall was still undeveloped and more immature myxospores were observed than from type 2. Type 2 was also harder than the type 1 plasmodium. The mean (± s.e.) length of plasmodia was 1.6 ± 0.01 mm. The size of plasmodia did not increase between 2 and 3 years old fish, either between plasmodia types and rates (IV), suggesting a slow development rate of *H. zschokkei* in whitefish. According to Boyce *et al*. (1985), the prevalence and intensity of *H. salminicola* infection were higher in species of the genus Salmon that spend the longest time in freshwater before the sea migration. Thus, *H. salminicola* is infecting salmonids in their spawning and hatching sites (Boyce *et al*. 1985). Many Myxozoa species are known to release actinospores from invertebrate hosts in spring and summer, possibly synchronized with hatching and growing of larval fish (Yokoyama *et al*. 2012). Wild European whitefish (*C. lavaretus* L.) is also a migratory fish species. In nature, whitefish is typically hatching and spawning in freshwater (e.g. rivers) and migrating to feeding area (sea or lake) where it will stay for several years before coming back to lay eggs at the age of 4–6 years. Whitefish spawns in the fall and fry hatching in the early spring during ice break-up (Leskelä *et al*. 2002). Slow development of *H. zschokkei* might be an adaptation to the whitefish life cycle where fish are spending many years at the feeding area, before returning to the breeding site. It is known that species that form spores in the cartilage, brain, visceral organs and muscle release spores into the water after the death of the host (Gómez *et al*. 2014). It is also likely that *H. zschokkei* myxospores are not release until the infected whitefish dies—for example after spawning.

Location of plasmodia in fillet was studied and most of the plasmodia were observed from the middle part of the fish where the mass of the muscle was the highest (III). Oliva *et al*. (1992) found from another muscle-infecting myxozoan, *Kudoa sciaenae,* that the greatest number of plasmodia was behind the head or the anterior part of the fish where the dry weight of the fillet was the highest. Although the intensity of infection was highest in the largest muscles, *H. zschokkei* infection had no effect on the condition factor of fish. Also another muscle-infecting myxozoan, *Kudoa thyrsites*, had no effect on survival of the fish, though it caused "soft flesh" condition after death (e.g. Funk *et al*. 2008). Although I did not find any evidence that *H. zschokkei* infection harms the fish, it is plausible to assume that big plasmodia in high numbers within the musculature are harmful.

TABLE 5 Number of fish studied (n), prevalence (with 95 % confidence interval) and intensity ± s.e. of *Henneguya zschokkei* infection in 1+, 2+ and 3+ year old cultured whitefish from the Konnevesi Research Station (RS) and from two fish farms (FF; III and IV).

	1+			2+			3+		
	n	Prev. % (95 % C.I.)	Int.	n	Prev. % (95 % C.I.)	Int.	n	Prev. % (95 % C.I.)	Int.
RS (III)	573	0.4 (0.1–1.2)	1 ± 0	-	-	-	-	-	-
FF (III)	481	0.0 (0.0–0.8)	-	381	13.1 (10.0–16.9)	11.0 ± 2.5	164	17.1 (12.1–23.6)	8.1 ± 1.6
FF (IV)	151	0.0 (0.0–2.5)	-	142	29.6 (22.7–37.5)	4.6 ± 0.7	206	36.4 (30.1–43.2)	6.1 ± 1.1

Many Myxozoa diseases cause very little or no cellular response in host (Lom and Dyková 1992). Immune responses of hosts against myxozoan parasites vary depending on the target tissue of the plasmodia and myxozoan species, as well as on the host individual (Gómez *et al*. 2014). The best known immune response by the host is encapsulating and isolating the plasmodium from surrounding tissues (Sitjá-Bobadilla *et al*. 2015). However, efficiency of the encapsulation in eliminating the plasmodium is limited (Koehler *et al*. 2004). Melanisation is also associated with parasite infections (e.g. Chapman and Hunter 1954, Agius and Roberts 2003). The melanisation rate of plasmodia seems to increase with the age of fish (IV). It can be a reaction of fish immune defense (reviewed by Agius and Roberts 2003) or accumulation of parasites breakdown products (Stehr and Whitaker 1986). Indeed, some fish species (e.g. Molnàr 2002) and strains (e.g. Bartholomew 1998, Hedrick *et al*. 2003) show resistance against myxozoan diseases. Better understanding of the genetic and immunological mechanisms of fish against myxozoan is important when developing methods for controlling these parasites (Gómez *et al*. 2014). In addition, resistance of whitefish against *H. zschokkei* could perhaps also be increased via selective breeding program of whitefish (Kause *et al*. 2011, see also Gómez *et al*. 2014) in the future.

Control of *H. zschokkei* in fish farming would be important because Finland is widely stocking whitefish, to avoid the spread of *H. zschokkei* to lakes via stocking programs. On the other hand, effectiveness of fingerling stocking is questionable if *H. zschokkei* infection pressure is very high (see Leinonen and Mutenia 2009). Control of myxozoan infection within fish is difficult because there is no effective treatment available (see Yokoyama *et al*. 2012). Usually, traditional fish farms take incoming unfiltered water from the lake above. If *H. zschokkei* occurs in wild whitefish population in the lake above the fish farm, there is a risk that the parasite may also spread to cultured whitefish or vice versa if cultured fish are infected. Both fish farms in the present study receive unfiltered water from a stream/lake where *H. zschokkei* occurs. Preventative methods when trying to reduce the spread and occurrence of Myxozoa in fish farms include using parasite-free or filtered water supply, treating the incoming water with UV-radiation or ozone (e.g. Tipping 1988, Hedrick *et al*. 2000, 2012), keeping fish in good condition and decreasing the stress of fish (see Sitjá-Bobadilla *et al*. 2015), use recirculating aquaculture systems (RAS) at least when fingerlings are cultured for stocking purposes and removing dead and infected fish, so they do not spread infection.

4 CONCLUSION

The purpose of this study was to increase the knowledge of poorly known spore-forming parasites which are infecting economically important fish species in lakes and fish farms.

Two novel Microsporidia species were found and described – one from the skeletal muscle of pike-perch and perch, *M. luciopercae,* and another from the muscles of pike-perch and burbot, *M. spraguei*. Genetically *M. luciopercae* was identical in two fish hosts, pike-perch and perch, while *M. spraguei* has two races or subspecies with different DNA-sequences in the two fish hosts, pike-perch and burbot.

M. luciopercae was rare whereas *M. spraguei* was common in the study lakes. The highest *M. spraguei* infection abundances occurred in the middle-sized pike-perch, which may indicate parasite-induced host mortality of older fish. In addition, the infection of *M. spraguei* in burbot was easily visible by the naked eye. However, also *M. luciopercae* causes degeneration and necrosis to the myocyte, leading to damage of the fillet structure. Therefore, both parasites are a potential threat to inland fisheries and aquaculture, and their occurrence and possible spread should be monitored.

The Myxozoa parasite *H. zschokkei* was common in both of the studied fish farms. Probably the determining factors contributing to the parasitism include the incoming water (occurrence of *H. zschokkei* in source water body) and transmission possibilities of the parasite within the farm (earth ponds or tanks hosting oligochaetes). Development of *H. zschokkei* plasmodia and spores seems to be a very slow process, requiring 2 years to become visible in the fillet.

Knowledge of these parasites which are infecting economically important fish species – although not dangerous for humans – is essential for the development of methods to mitigate their negative effects in the future. In addition to fishing, these species either have spread (*H. zschokkei*) or can potentially spread (*M. luciopercae* and *M. spraguei*) to fish culture systems. Usually muscle-dwelling parasites are transmitted upon death and decay of the host. Thus, an important action to prevent the spread of these three parasites is

removing dead and infected fish from water / fish tank, and using water from parasite-free source, if possible.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Kalan lihasta infektoivat itiöloiset – ekologia ja esiintyminen

Niin järvissä kuin kalanviljelylaitoksillakin esiintyy kalaloisia, jotka voivat aiheuttaa taloudellista haittaa heikentämällä kalan terveyttä tai hankaloittamalla kalan kaupallista käyttöä. Väitöskirjassani tutkin kolmea itiöitä muodostavaa kalaloista, *Microsporidium luciopercae*, *Myosporidium spraguei* ja *Henneguya zschokkei*, jotka eivät ole vaarallisia ihmisille, mutta ovat kalataloudellisesti ongelmallisia infektoidessaan kalan lihasta. Yhteistä näille loisilla on se, että ne loisivat kalataloudellisesti tärkeissä kalalajeissamme vaikeuttaen kalojen myyntiä. Aikaisempi tietämys näistä loisista on ollut joko hyvin rajallista (*H. zschokkei*) tai sitä ei ole ollut ollenkaan (*M. luciopercae* ja *M. spraguei*).

2000-luvun alussa kalastajat huolestuivat kuhan ja ahvenen lihakseen ilmestyneistä vaaleista laikuista muutamissa järvissä. Väitöskirjatyössäni keräsin kuhanäytteitä viidestä järvestä (Haukivesi, Höytiäinen, Koirusvesi, Pielinen ja Päijänne), ahvennäytteitä kolmesta järvestä (Haukivesi, Konnevesi ja Päijänne) ja madenäytteitä Haukivedeltä. Heti tutkimuksen alkuvaiheessa selvisi, että yhden tuntemattoman Microsporidi-lajin lisäksi kuhan lihaksessa esiintyy toinenkin laji, joka muodostaa vain mikroskoopin avulla havaittavia pieniä ksenomia eli kystejä. Kuhan lihaksesta löytyi siis yhden lajin sijaan kaksi tieteelle uutta Microsporidia-loista, joiden lajikuvaukset ovat osa väitöskirjaani. Vaaleita läikkiä kuhan ja ahvenen lihakseen muodostava laji on *M. luciopercae* -läikät ovat loisen itiötihentymiä. Toinen laji, *M. spraguei*, muodostaa ksenoman, jonka sisässä itiöt ovat ja sitä esiintyi sekä kuhassa, että mateessa.

M. luciopercae oli hyvin harvinainen tutkimusjärvissä. Sitä löydettiin vain yksittäistapauksissa Haukivedeltä, Konnevedeltä, Pieliseltä ja Päijänteeltä. Loinen aiheuttaa lihassoluissa kuoliota ja lihaksen rakenteen muuttumista, mistä syistä *M. luciopercae* -loisittu kuha on myyntikelvoton. *M. spraguei* muodostaa kuhassa ~250 µm:n kokoisia ksenomia, joita ei voi havaita paljain silmin. Mateessa *M. spraguei* -loisen ksenomat ovat hyvin erottuvia, jopa 1–2 cm pitkiä kystejä, mistä syystä loisittu made on usein myyntikelvoton. *M. spraguei* -infektio oli yleinen tutkimissani järvissä; Päijänteellä, Pielisellä ja Koirusvedellä yli 20 % kuhista oli loisittu. Haukiveden madepopulaatiossa *M. spraguei* -loisen esiintyvyys oli yli 65 %. Tutkimuksessani ei tullut ilmi, että näillä Microsporidia-loisilla olisi vaikutusta kalan kuntoon, mutta aiemmissa tutkimuksissa lihaksessa loisivat mikrosporidit on liitetty mm. kalan lihasvoiman heikkenemiseen. Tämä voi vaikuttaa mm. verkkokuolleisuuteen ja lisätä mahdollisuutta joutua petokalan saaliiksi.

Kolmas tutkimuskohteeni oli siian rakkoloisio (*H. zschokkei*) ja sen esiintyminen kahdella sisämaan kalanviljelylaitoksella 1-, 2- ja 3-vuotiaissa siioissa. Aikaisemmin ei ollut tietoa, minkä ikäisessä kalassa loisen itiöitä sisältävät plasmodium-kystit ovat havaittavissa ja kuinka infektio kehittyy. Tutkimuksessa selvisi, että loinen näkyy mikroskoopin avulla käytännössä

aikaisintaan 2–vuotiaissa kaloissa, heinä–elokuusta alkaen. Tutkimuksessani kävi myös ilmi, että *H. zschokkei* oli hyvin yleinen ko. kalanviljelylaitoksilla; 17–36 % 3-vuotiaista siioista oli infektoituneita. Vuoden kestävän seurannan aikana rakkoloision kystien koko ei kasvanut merkitsevästi siirryttäessä 2-vuotiaista 3-vuotiaisiin kaloihin. Lihaksesta löytyi kahdentyyppisiä kystejä, jotka edustivat todennäköisesti aikaisempaa ja myöhempää kehitysvaihetta. Näiden kystityyppien koossa tai esiintymissuhteessa ei kuitenkaan ollut eroa 2- ja 3-vuotiaiden siikojen välillä. Kystien keskikoko oli alle 2 mm, kun taas luonnosta pyydetystä, vanhemmasta siiasta, ei ole mitenkään harvinaista löytää 1–2 cm:n kokoluokkaa olevia kystejä. Tuloksista voidaan päätellä, että *H. zschokkei* -loisen kehittyminen ja sen plasmodium-kystien kasvu siiassa on hidasta. Tutkin myös immuunipuolustukseen liitettyä ominaisuutta, melanisaatiota. Osalla plasmodium-kysteistä oli pinnallaan tummia pisteitä, jotka tulkittiin kalaisännän immuunipuolustukseen liittyväksi melanisaatioksi. Tutkimuksessa selvisi, että 3-vuotiaiden kalojen plasmodium-kystien pinnalla oli suhteessa enemmän melanisaatiota kuin 2-vuotiailla kaloilla. Tämä voi olla merkki siitä, että siialle syntyy immuunivastetta *H. zschokkei* -loista kohtaan. Melanisaation roolia ja merkitystä kalan immuunivasteessa siian rakkoloisiota vastaan olisi syytä selvittää tulevaisuudessa.

Tutkimieni itiöloisten itiötihentymät (laikut, ksenomat ja plasmodium-kystit) sisältävät lukemattoman määrän loisten itiöitä. Jotta infektioiden leviämistä voitaisiin vähentää tai estää, infektoitunut kala tulisi hävittää niin, etteivät itiöt pääse leviämään takaisin veteen infektoimaan uusia isäntiä. Esimerkiksi pyydykseen kuollut kala voi olla infektoitunut, ja järveen palauttamisen sijaan olisi parempi kompostoida se infektion leviämisen ehkäisemiseksi. Kalastuslaki (58 §) kuitenkin edellyttää mm., että pyyntimittojen vastainen kala, esim. alamittainen kuha, pitää laskea takaisin veteen. Kalanviljelyssä ennaltaehkäisy on yksi tärkein toimenpide loistartuntojen ehkäisemisessä. Tuloveden lähteen valitsemisessa tulisikin huomioida se, etteivät loiset pääse laitokselle veden mukana.

Väitöskirjatyöni lisäsi merkittävästi tietämystä luonnossa ja kalanviljelyssä tavattavista *M. luciopercae* -, *M. spraguei* - ja *H. zschokkei* -loisista. Tuloksiani voidaan käyttää hyväksi, kun kehitetään keinoja loisten torjumiseksi tai niiden leviämisen ehkäisemiseksi.

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ORIGINAL PAPERS

I

TWO NOVEL MICROSPORIDIA IN SKELETAL MUSCLE OF PIKE-PERCH *SANDER LUCIOPERCA* AND BURBOT *LOTA LOTA* IN FINLAND

by

Simon Jones, Hanna Ahonen, Lars Granlund, Tiina Arsiola & Jouni Taskinen
2017

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II

INFECTION OF FISH HOSTS BY THE MICROSPORIDIAN PARASITES *MICROSPORIDIUM LUCIOPERCAE* AND *MYOSPORIDIUM SPRAGUEI*

by

Hanna Ahonen, Lars Granlund, Tiina Arsiola & Jouni Taskinen

Manuscript

III

HENNEGUYA ZSCHOKKEI (MYXOZOA) INFECTION IN CULTURED WHITEFISH: AGE-DEPENDENCE, SEASONALITY AND DISTRIBUTION WITHIN HOST

by

Hanna Ahonen, Heikki Koskinen, E. Tellervo Valtonen & Jouni Taskinen 2017

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Henneguya zschokkei (Myxozoa) infection in cultured whitefish: age-dependence, seasonality and distribution within host

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Abstract

Whitefish (*Coregonus lavaretus*) is an important species for European fisheries and aquaculture. Recently, the myxosporean *Henneguya zschokkei* has been observed to infect the muscles of whitefish in fish farms. Plasmodia of *H. zschokkei* prevent marketing of the fillet and cause economic losses. The factors associated with occurrence of the parasite in fish farms have not been investigated previously. We studied age-dependence, seasonality, and within-host distribution of *H. zschokkei* plasmodia in farmed whitefish by examining a total of 1,599 fish. Distribution of plasmodia within the fish was not uniform. When the fillet was divided into parts, the number of plasmodia was positively correlated with the mass of the part, suggesting that larger muscles of whitefish may be particularly susceptible to, or preferred by *H. zschokkei*. Plasmodia were rarely found in 1 y old whitefish (prevalence 0.2 %). The youngest age group substantially infected was 2 y old fish with the prevalence of 13.1 %, which did not differ statistically from 3 y old fish with 17.1 % infected. Mean intensity of infection in these three age groups was 1.0, 11.0 and 8.1 plasmodia per fish, respectively. The infections started to appear in August both in 1 and 2 y old fish, which suggests a seasonal hatching of parasite actinospores (the infective stage released from the definitive host). Condition of fish was not related to the number of plasmodia.

Statement of relevance: This is the first study of *H. zschokkei* in whitefish (*C. lavaretus*) in fish farm. There is a need for increased knowledge of *H. zschokkei* infection in cultured whitefish, as this species is being largely used in aquaculture and for stocking purposes.

1. Introduction

Parasitic diseases represent a severe threat to fisheries and aquaculture production around the world, causing significant economic losses through reduced growth, morbidity and mortality of fish (e.g., Williams and Jones, 1994; Woo, 1995; Lom and Dyková, 2006; Lafferty et al., 2015). Whitefish (*Coregonus lavaretus*) is an economically important fish species with a wide range of occurrence in the northern hemisphere. For example, whitefish is one of the most popular fish species in professional, sport and commercial fishery in Finland, with a total catch of 1.8 x 10^6 kg in 2014 (Natural Resources Institute Finland), and a total fish farm production of 1.2 x 10^6 kg per year for food (Savolainen, 2014). *Henneguya zschokkei* (Myxosporea) infects coregonids, including whitefish, in Europe and Russia (Lom and Dyková, 1992). *H. zschokkei* infection was recognized in Finland already in the early 1900s (Luther, 1909). Thereafter, it has been documented in a number of Finnish lakes (e.g., Valtonen et al., 1988; Hyvärinen et al., 2000; Leinonen and Mutenia, 2009).

In recent years, this myxosporean has also been found in fish farms with plasmodia up to 20-30 mm in size found in the muscles of whitefish. The presence of such plasmodia makes fish unattractive and thereby hamper whitefish fishery and aquaculture.

Some authors have suggested that *H. zschokkei* and *Henneguya salminicola* are synonyms for the same species (e.g., Boyce et al., 1985; Lom and Dyková, 1992). *H. salminicola* is commonly known to infect salmonids in the North America and Asian coastal waters (Boyce et al., 1985). Although parasitized fish can be found from the sea, Boyce et al. (1985) suggest that *Henneguya* infections take place in the freshwater habitats.

Class Myxosporea belongs to the Phylum Cnidaria (Chang et al., 2015) and harbor endoparasites with a life cycle involving an invertebrate and a vertebrate host. Typically, the definitive host of Myxosporea is an oligochaete and the intermediate host is a fish. The oligochaete host of *H. zschokkei* is not known. Majority of myxosporean infections are harmless to fish, although serious diseases are known (e.g., Lom and Dyková, 1992; Kent et al., 2001). Myxosporeans clearly have substantial economic impacts on fisheries and aquaculture (e.g., Diamant, 1992) particularly because there are no effective treatments or vaccines available (e.g., Gómez et al., 2014).

Here, we address several basic questions that are essential in preventing or mitigating the harm of *H. zschokkei* on whitefish aquaculture and fisheries. (i) What is the distribution of infection within an individual whitefish? It is known that *H. zschokkei* plasmodia occupy muscles of host fish (Eiras, 2002), but the spatial distribution pattern of plasmodia within the musculature has not been studied. When processing the fish fillet it will be useful to know where the plasmodia occur within the muscles, in order to, perhaps, develop measures cutting out the plasmodia from the muscles. (ii) Are there certain seasons when infection is more intense, perhaps due to the life cycle of the parasite, or seasonal behaviors of the intermediate host? (iii) Does the parasite infect certain age classes of whitefish? In addition, it would be important to know the development rate of the parasite, i.e., at what age the plasmodia become visible in fish. Finally, (iv) it is also unclear whether the infection affects the condition of the fish. For these reasons, the aims of the present study were to investigate the development rate, age-dependency of infection, seasonality of occurrence and the spatial distribution of plasmodia within the muscles, as well as differences in condition factor between *H. zschokkei* -infected and uninfected whitefish in a (freshwater) fish farm.

2. Material and methods

2.1. Whitefish material

Whitefish specimens were obtained from the Tervo fish farm (Natural Resources Institute Finland). Three age groups were investigated: 1 y old (year class 2008), 2 y old (year class 2007) and 3 y old (year class 2005) fish (Table 1). Age group 1 y old fish also included whitefish which were moved for purpose of another experiment, at the age of 0 y old fish (after first summer) from the fish farm to Konnevesi Research Station (University of Jyväskylä) and studied at the age of 1 y old fish. Whitefish used in the present study were grown in fiber glass tanks kept indoor, and were fed with dry pellets. Water supply to the fish farm was from Lake Nilakka (surface area 168 km^2, average depth 4.9 m) and to the research station from Lake Konnevesi (surface area 200 km^2, average depth 12.5 m). *H. zschokkei* has been found in wild whitefish population in both lakes. The fish farm and research station are located in the watershed of the River Kymijoki, flowing to the Gulf of Finland in the Baltic Sea. The distance between the fish farm and the research station is 50 km.

2.2. Examination of fish

In 2008, 3 y old whitefish (n = 164) were examined fresh in June, July and August from the fish farm (Table 2). In 2009, approximately 50 2 y old fish whitefish were collected every second week from May to September from the fish farm (n = 381), and stored in -20 °C for a later examination. Similarly, in 2009, approximately 50 1 y old fish whitefish from the fish farm (n = 481) and the research station (n = 573) were collected every second week from May to October and stored in -20 °C to be examined later (Table 3).

The mass and length of each fish was measured. In 2008, 3 y old fish were filleted into 5 mm slices and *H. zschokkei* plasmodia were observed visually on a glass table using transmitted light. In 2009 (1 and 2 y old fish) *H. zschokkei* plasmodia were examined by pressing muscle tissue samples between two glass plates (8.0 x 20.0 cm in size) and using a dissection microscope with 6.3-25 x magnification (Olympus SZX9). Occurrence of *H. zschokkei* myxospores within plasmodia was microscopically verified using 400 x magnification (Motic B series).

In the case of 2 y old fish from the fish farm in 2009, position of each plasmodium was recorded. Each fillet was divided dorso-ventrally and anterior-posteriorly into six parts, totaling 12 parts per one fillet (Fig. 1). Total number of *H. zschokkei* plasmodia per part was quantified by combining numbers of plasmodia from both sides of the fish. To be able to estimate the mass of muscles in each of the 12 parts, a separate sample of whitefish (n=11) with same age and size as in 2009 was collected, muscles divided in 12 parts as in 2009, and weighed to the nearest mg.

2.3. Statistics

Pearson Chi-Square Test was used to analyse differences in prevalence of infection between age groups, prevalence being defined as the proportion of infected fish among all fish in the sample. Differences between locations within fillet (both sides of fish combined) in the mean number of plasmodia were estimated with nonparametric Wilcoxon Signed Ranks test. We used One-way ANOVA to analyse the intensity of infection of *H. zschokkei* plasmodia in whitefish among age groups, mean intensity being defined as the mean number of plasmodia per infected fish. Condition index of fish was calculated using the formula $K = 100 \times (\text{weight}/\text{length}^3)$ and compared between infected and uninfected fish with independent samples t-test. All statistical analyses were performed with SPSS Version 22.0. To account for multiple tests, the Bonferroni correction was applied to p-values so that 0.05 was divided by the number of tests to achieve critical p-value α.

3. Results

In the fish farm, none of the 1 y old fish were infected, and in the research station the prevalence of infection among 1 y old fish was 0.4 % (Table 1). Difference between the fish farm and research station among the 1 y old fish in the prevalence of infection was not significant ($\chi^2 = 0.344$, df = 1, $P = 0.588$). Prevalence of infection in 2 and 3 y old fish was 13.1 and 17.1 %, respectively (Table 1), being statistically significantly higher than among the 1 y old fish (all studied 1 y old fish combined) ($\chi^2 = 134.0$, df = 1, $P < 0.001$ and $\chi^2 = 168.4$, df = 1, $P < 0.001$, respectively). However, prevalence of *H. zschokkei* infection did not differ significantly between 2 and 3 y old whitefish ($\chi^2 = 1.459$, df = 1, $P = 0.227$). The two fish from age group 1 y old fish were infected only with a single *H. zschokkei* plasmodium whereas in 2 and 3 y old fish individuals the maximum number of plasmodia per fish was 101 and 32 respectively. However, heavily infected fish were rare; most of the infected fish had only 1-20 plasmodia, showing an aggregated distribution. Mean (± s.e.) intensity (and median) of *H. zschokkei* infection in the age group 1, 2 and 3 y old fish was 1.0 ± 0, 11.0 ± 2.5 (median 3.0) and 8.1 ± 1.6 (median 5.5) (Table 1), respectively. The difference was not significant between 1, 2 and 3 y old fish (One-way ANOVA; df = 2, F = 0.711, P = 0.494).

The infection was notable for the first time in August from 1 and 2 y old fish. The first infected individual was found in the 1 y old whitefish in August and the second one in October from the

research station. In 2 y old fish, the prevalence of infection was zero from the beginning of June to 13th of July. When the first *H. zschokkei* observation was made in early August almost one third of the fish were infected (Table 3). There was a statistically significant increase in the prevalence of infection among 2 y old fish from 13th of July to 3rd of August (Table 3, Pearson $\chi^2 = 19.392$, df = 1, $P = 0.001$). From early August on, the parasite was present in 22.0-36.7 % of fish with mean intensities varying from 7.9 to 18.9 plasmodia per fish to the end of the study period in September (Table 3). There was no statistically significant difference between sampling dates from 3rd of August to 7th of September in the prevalence of infection (Pearson $\chi^2 = 2.814$, df = 3, $P = 0.421$) or in the intensity of infection (One-way ANOVA, df = 3, F = 1.100, P = 0.359) among the 2 y old fish.

Among the 3 y old whitefish, plasmodia of *H. zschokkei* were already evident in the first samples collected in June, and were present throughout the study period till August. The monthly prevalence of infection varied from 6.9 to 26.5 % (Table 2). Prevalence of infection in July was significantly lower than in June or August ($\chi^2 = 6.315$, df = 1, $P = 0.012$ and $\chi^2 = 8.833$, df = 1, $P = 0.003$, respectively). The mean monthly intensities of infection, varying from 6.4 to 14.4 (Table 2), did not differ from each other among the 3 y old whitefish (One-way ANOVA; df = 2, F = 1.914, P = 0.169).

Mean (± s.e.) intensity of infection was higher on the dorsal side of the whitefish, 6.2 ± 1.3, compared to the ventral side, 4.8 ± 1.3 (n = 50) (Wilcoxon Signed Ranks Test, Z = -2.444, $P = 0.015$). Mass-related intensity, i.e. the mean (± s.e.) intensity of infection per mass (g) of muscle part (plasmodium number/mean weight of the part) did not differ between dorsal (0.10 ± 0.02) and ventral (0.10 ± 0.03) side (Wilcoxon Signed Ranks Test, Z = -0.391, P = 0.696). Highest numbers of plasmodia were observed in the middle section of the fish (areas D2-D5 and V2-V5; Fig. 2). Statistically significant differences between parts within the fish fillet were mainly characterized with the most anterior and most posterior sites having lower number of plasmodia than the middle parts (Table 4 and Table 5). There was a statistically significant positive correlation between the mass of the muscle and number of plasmodia in the muscles (from dorsal: Pearson Correlation = 0.856, $P = 0.030$, N = 6 and from ventral Pearson Correlation = 0.951, $P = 0.004$, N = 6) (Fig. 3).

Finally, there were no differences in the condition factor between infected and uninfected fish, as analysed for the 2 y old fish from August to September (Independent Samples t-test, t = 0.004, df = 178, P = 0.997) and from 3 y old fish from June to August (Independent Samples t-test, t = 0.000, df = 161, P = 1.000).

4. Discussion

Studies on Myxozoa diseases have shown a connection between water temperature (seasonality) and outbreak of the diseases. The diseases usually tend to occur when the water temperature is high, ~ 15-20 ˚C. This is perhaps not surprising because temperature is known to affect both the parasite occurrence in fish and the release of spores from invertebrate hosts (e.g., El-Matbouli et al., 1999; Tops et al., 2006). In our study, water temperatures started to increase in April and the highest temperatures were observed in July and August. We found a clear seasonal pattern in the infection dynamics. In the 1 and 2 y old fish, new infections appeared in August. This indicates a distinct seasonal rhythm in the release of *H. zschokkei* actinospores from the definitive hosts, oligochaetes. These results are in accordance with previous studies on temperature dependence of Myxozoa species in fish hosts that have suggested a seasonal release of actinospores in *Myxobolus cerebralis* (El-Matbouli et al., 1999), *Ceratonova shasta* (Hallett et al., 2012), *Tetracapsuloides bryosalmonae* (Tops et al., 2006) and *Henneguya ictaluri* (Wise et al., 2004). Alternatively, if the actinospores release and infection of fish are not temporally limited on seasonal basis, the current result suggests that at least the development of plasmodia in fish follows a clear seasonal pattern – with a burst of microscopically visible, new infections in August. Haaparanta et al. (1994) showed a clear

seasonality in the life cycle and infestation of host fish by *Henneguya creplini* in Finland. New infections appeared on the gills of *Perca fluviatilis* in July, and mature plasmodia with fully developed myxospores were found mainly in April-May. After that the old plasmodia disappeared from the gills so that in June the fish mostly had no *H. creplini* infections. This suggests that the life cycles of *H. zschokkei* and *H. creplini*, with respect to seasonal infection of fish, may follow a similar pattern. The difference with these parasites, however, is that the plasmodia of *H. creplini* in gills rupture, release myxospores and disappear every summer while *H. zschokkei* stay in the host musculature probably until the host dies.

Henneguya species can increase mortality of fish and hamper fish farming, but most of them are quite harmless (Lom and Dyková, 2006). In our study we did not find any evidence that the parasite has negative effect on the condition of the host as measured by a coefficient index based on the size of the fish. Moreover, we did not observe any fish mortality during our study. Nevertheless, it should be noted that if the plasmodia do not stop growing, it is possible that the harmful effects of *H. zschokkei* is amplified in older fish, and requires further study.

What can we deduce from these results about the exposure of fish to *H. zschokkei* actinospores and the development of plasmodia? Although the present results showed that the 1 y old whitefish could be infected by *H. zschokkei* (extremely rarely; 2 cases out of 1054 fish, 0.2 %), in practice the youngest age group infected was 2 y old fish. Judging from the seasonally synchronized appearance of infections in fish in August, the exposure of fish to *H. zschokkei* actinospores is probably also seasonal. However, the precise time of this event remains unknown.

Similarly, we acknowledge that the age of fish at the time of exposure to the parasite is unknown. Moran et al. (1998) found that myxospores of *Kudoa* sp. are apparent in the host six months after infection. Szèkely et al. (2001) infected parasite-free roach fingerlings with actinospores of *Myxobolus pseudodispar* and young plasmodia were observed 80 days after infection. However, in our study all fish were kept in equal conditions, in indoor tanks up to the age of 2 y old fish and, thus, up to the outbreak of the infection. Therefore, it is reasonable to assume that all of the present fish have been exposed to *H. zschokkei* actinospores throughout their lives. If the exposure has started already at the age of 0 y old fish, but the plasmodia are found (predominantly) not until 2 y old fish of age, it is likely that the development of *H. zschokkei* inside the fish may be very slow. If the parasite development within fish tissue would be quick (weeks) we should have probably found a high prevalence of infection in 1 y old whitefish.

The parasite plasmodia in whitefish muscles were aggregated. Most of the infected fish had only 1-20 plasmodia. Distribution of the number of plasmodia resembled the negative binomial distribution which is common in most parasite populations in their host (Shaw and Dobson, 1995). It might suggest differences in susceptibility among individuals if one assumes equal exposure in the tanks. Most of the plasmodia located in the middle of fillet where the mass of the muscle were highest. Correlation was positive between muscle mass and amount of the plasmodia in the site. Studies on the prevalence of *Kudoa sciaenae* had shown that the prevalence and intensity was highest behind the head and in the anterior part of fish, which was also the highest mean dry weight of the muscle (Oliva et al., 1992). However, plasmodia of *H. zschokkei* were not accumulated in any single part of the fillet, so cutting the fish in a particular way to separate plasmodia may not be possible. On the other hand it is possible to remove plasmodia from fresh fillet, if infection intensity is low.

Controlling Myxozoa diseases is difficult because there are no treatments or vaccines available (Yokoyama et al., 2012). Thus, preventive measures to avoid infections are very important. These include e.g., reduction of stress (Sitjá-Bobadilla et al., 2015), improving water quality by filtering incoming water or using clean water supplies from parasite-free water bodies (e.g. *Enteromyxum leei*, Yokoyama and Shirakashi, 2007), exposure to ozone (e.g., *Ceratomyxa shasta,* Tipping, 1988) or UV *(*e.g., *M. cerebralis*; Hedrick et al., 2000*;* Hedrick et al., 2012). Our study provides the first insight into the seasonality and development of the myxosporean parasite *H. zschokkei* infecting

commercially important whitefish stocks. To mitigate the harmful effects of the parasite on whitefish aquaculture and fisheries, more research on the life cycle, including identification of the definitive oligochaete host of *H. zschokkei*, is needed.

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Tables

Table 1. Sampling year, numbers of whitefish studied (n), fish age, mean length (mm) and mass (g) of fish, with prevalence and mean ± s.e. and median intensities of infection of *H. zschokkei* infection in the fish farm (FF) and research station (RS). Different superscript letter denotes statistically significant difference between groups.

	Sampling year	n	Age	Length/Mass	Prev. (%)	Intensity	Median
FF	2008	164	3	259 / 155	17.1[b]	8.1 ± 1.6	5.5
FF	2009	381	2	244 / 153	13.1[b]	11.0 ± 2.5	3
FF	2009	481	1	159 / 036	0.0[a]	-	-
RS	2009	573	1	180 / 047	0.4[a]	1.0 ± 0	1

Table 2. Three years old whitefish (year class 2005) from the fish farm studied in 2008. Months of collection (2-4 samples per month combined), numbers of fish studied (n), mean length (mm) and mass (g) of fish, with prevalence and mean ± s.e. and median intensities of infection and range of plasmodium numbers per fish.

Month	n	Length/Mass	Prevalence	Intensity	Median	Range
June	43	272 / 185	23.3 %	6.4 ± 2.2	3.5	0-24
July	72	249 / 128	6.9 %	14.4 ± 5.3	16.0	0-32
August	49	262 / 169	26.5 %	7.0 ± 1.9	5.0	0-28

Table 3. Two years old whitefish (year class 2007) from the fish farm studied in 2009. Dates of collection, numbers of fish studied (n), mean length (mm) and mass (g) of fish, with prevalence, mean ± s.e. and median intensities of infection and range of plasmodia numbers per a fish at farm.

Date	n	Length/Mass	Prevalence	Intensity	Median	Range
02.06.	50	227 / 110	0	0	0	0
11.06.	50	243 / 126	0	0	0	0
29.06.	50	228 / 129	0	0	0	0
13.07.	51	238 / 146	0	0	0	0
03.08.	49	243 / 137	30.6 %	7.9 ± 2.4	3.5	1-39
13.08.	50	252 / 166	22.0 %	8.7 ± 3.1	4	1-34
24.08.	50	258 / 190	24.0 %	18.9 ± 8.5	5	1-101
07.09.	30	284 / 262	36.7 %	9.3 ± 3.9	3	1-41

Table 4. Paired comparisons (Wilcoxon Signed Ranks Test) of mean numbers of plasmodia of *H. zschokkei* in the dorsal part of fillet of 2 y old whitefish from the fish farm. Six sites from anterior to posterior end are marked from D1 to D6 (Fig. 1). To account for multiple comparisons, critical *P*-value was set to 0.0033 (0.05 / 15). Statistically significant differences are given in bold.

	D1	D2	D3	D4	D5	D6
D1	*	**< 0.001**	**< 0.001**	**< 0.001**	**0.001**	0.436
D2	**< 0.001**	*	0.505	0.194	0.257	**< 0.001**
D3	**< 0.001**	0.505	*	0.560	0.088	**< 0.001**
D4	**< 0.001**	0.194	0.560	*	0.017	**< 0.001**
D5	**0.001**	0.257	0.088	0.017	*	**< 0.001**
D6	0.436	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	*

Table 5. Paired comparisons (Wilcoxon Signed Ranks Test) of mean numbers of plasmodia of *H. zschokkei* in the ventral part of fillet of 2 y old whitefish from the fish farm. Six sites from anterior to posterior end are marked from D1 to D6 (Fig. 1). To account for multiple comparisons, critical *P*-value was set to 0.0033 (0.05 / 15). Statistically significant differences are given in bold.

	V1	V2	V3	V4	V5	V6
V1	*	**0.001**	**< 0.001**	**< 0.001**	0.050	0.096
V2	**0.001**	*	0.038	0.014	0.159	**0.001**
V3	**< 0.001**	0.038	*	0.060	0.009	**< 0.001**
V4	**< 0.001**	0.014	0.060	*	**< 0.001**	**< 0.001**
V5	0.050	0.159	0.009	**< 0.001**	*	**0.003**
V6	0.096	**0.001**	**< 0.001**	**< 0.001**	**0.003**	*

Figures

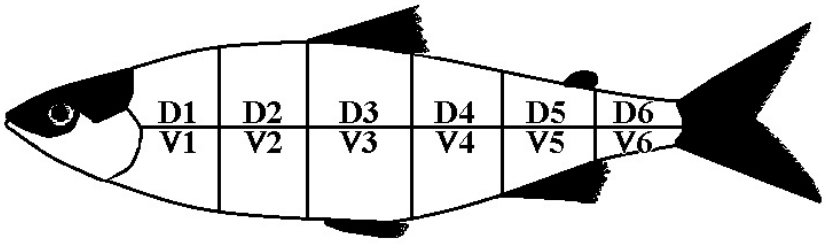

Fig. 1. "Fish map" used to record locations of *H. zschokkei* plasmodia within the 2 y old fish. Dorsal (D) and ventral (V) side of the fish was divided into six separate sites.

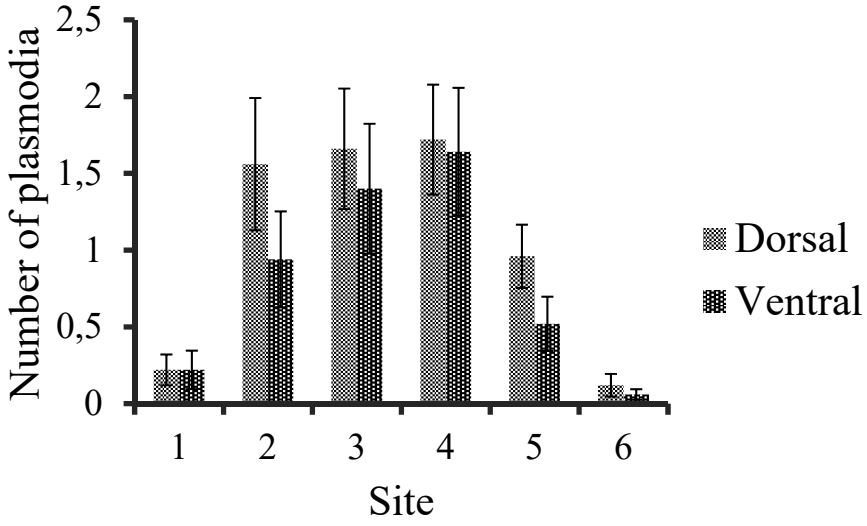

Fig. 2. Distribution of *H. zschokkei* plasmodia within fish, i.e., mean (± s.e.) numbers of plasmodia in different sites of fillet (for positions of the sites, see Fig. 1). Based on 50 2 y old infected whitefish from fish farm studied in 2009.

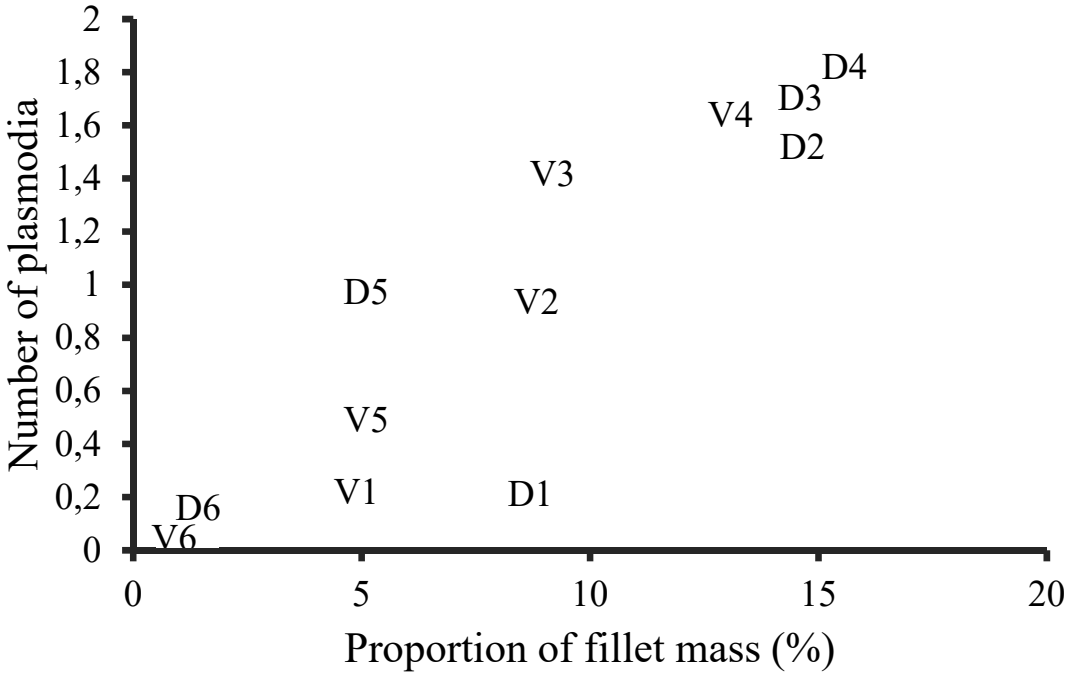

Figure 3. Site specific numbers of *H. zschokkei* plasmodia plotted against site specific muscle masses within fillet (for positions of the sites, see Fig. 1).

IV

HENNEGUYA ZSCHOKKEI (MYXOZOA) INFECTION IN CULTURED WHITEFISH *(COREGONUS LAVARETUS):* PLASMODIUM TYPES AND SIZES, SEX-DEPENDENT INFECTION AND MELANIZATION

by

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