Master's thesis

Development of *Henneguya zschokkei* (Myxozoa: Myxosporea) actinospores in oligochaetes and plasmodia in whitefish *Coregonous lavaretus*

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ABSTRACT

The aim of this study was to describe the life cycle of *H. zschokkei* by observing the development of actinospores in oligochaete and the morphological development of *H. zschokkei* cysts in whitefish. Laboratory observation of seasonal occurrence of actinospores sampled from oligochaetes from RTKL Tervo Fish Farm revealed 6 types of actinospores from collective groups of Triactinomyxon, Raabeia and Aurantiactinomyxon in 2010, and 3 types of actinospores in 2011. Both years showed similar patterns with the release of actinospores peaking in both summer (June and July) and winter (January and February) times. The infection experiment was not successful in infecting *Coregonus lavaretus* with myxozoan parasite *Henneguya zschokkei*. In addition, 336 individuals of whitefish *C. lavaretus* from fish farm were examined to study the factors influencing the prevalence and intensity of infection of *H. zschokkei*. Three types of cysts were observed in this study namely cyst type-1 (mature plasmodium cyst), cyst type-2 (intermediate plasmodium cyst) and cyst type-3 (immature cyst). A pattern was observed with high prevalence of type 3 cyst (small cyst without myxospore) in young *C. lavaretus* and high prevalence of type-1 cyst in older *C. lavaretus*. Melanisation of cysts were also observed especially in older *C. lavaretus*. The prevalence of *H. zschokkei* was influenced by host age (42.0 % in 2 year old; 0.7 % in 1 year old) and host size but not by host sex. Based on these findings, the prevalence of *H. zschokkei* infection and the development of cyst were associated with host age.

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TIIVISTELMÄ

Henneguya zschokkei on pääjaksoon Myxozoa (luokka Myxosporea) kuuluva loinen, jonka pääisäntä on harvasukamato (Oligochaeta) ja väli-isäntä siika (*Coregonus lavaretus*). Loinen, jota kutsutaan siian rakkoloisioksi (’piimäsiika’) aiheuttaa kalataloudellista haittaa, koska sen kystit esiintyvät siian lihaksessa alentaen siikafileen kaupallista arvoa. Työn tarkoituksena oli tutkia rakkoloision elämänkiertoa harvasukamatoisännän osalta, jota ei vielä tunneta. Lisäksi tavoitteena oli tutkia loisen plasmodium-kystien kehittymistä siiassa. Harvasukamatoisäntää etsittäessä otettiin pohjanäytteitä Riista- ja kalatalouden tutkimuslaitoksen Tervon yksikön poistouomasta 2010 ja 2011 ja seurattiin Myxozoa actinospoorien parveilua harvasukamadoista laboratoriossa kesäkuulta seuraavan vuoden helmikuulle. Molempina vuosina actinospooreja parveili kesä-heinäkuussa sekä toisen kerran tammi-helmikuussa. Siian rakkoloision actinospooreja ei kuitenkaan itiöiden joukosta löytynyt, vaikka laitoksella oli aikaisempina vuosina esiintynyt loista siioissa. Infektiokokeessa yritettiin tartuttaa siiasta saatuja myxospooreja harvasukamatoihin. Madot kerättiin Jyväskylän alueen lammikoista, joissa siikaa ei esiinny, keväällä 2011 ja altistettiin myxospoorisuspensiolle, joka tehtiin a) tuoreista plasmodium-kysetistä saaduista spooreista, b) vanhentuneista plasmodiumeista saaduista spooreista (80 vrk säilytys 5ºC vedessä ennen infektiokoetta) sekä c) kontrollisuspensiolle (pelkkä vesi). Koe tehtiin laboratoriossa 8ºC lämpötilassa sekä luonnon lämpötilaa noudattaen siten, että actinospoorien erittymistä seurattiin kesäkuulta seuraavaan helmikuuhun saakka. Infektiokoe ei onnistunut; *H. zschokkei* actinospooreja ei saatu. Kemijärvellä sijaitsevan à Pohjois-Suomessa sijaitsevasta kalanviljely-yksiköstä otettiin 1+ ja 2+ -ikäisiä viljelysiikoja tutkittavaksi heinäkuussa 2011, loka-marraskuussa 2011, helmikuussa 2012 ja huhtikuussa 2012, yhteensä 336 kalaa. 1 + -ikäisissä kaloissa (n = 151) *H. zschokkei* – loisinnna prevalenssi oli 0.7 % ja 2+ -ikäisissä (n = 185) 42.0 %. 2+ -ikäisissä siioissa matureiden, kypsien plasmodium-kystien osuus ja sekä kypsien plasmodioitten määrä, loisinnan kokonaisprevalenssi, plasmodium-kystien keskikoko ja melanisoitujen plasmodioitten osuus kasvoivat vuodenaikaisesti kesästä seuraavaan huhtikuuhun, mutta pienten, immaturien kystien osuus ja lukumäärä alenivat. Tulos kuvaa siian rakkoloision plasmodium-vaiheen ajallista kehittymistä kalaisännässään.

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1. INTRODUCTION

Myxozoa are endogenic parasites to a wide range of animal groups such as fish, bryozoan, mammals and insects. Phylum Myxozoa consists of many parasite species in aquatic ecosystems and they are known for causing disease in some commercially important fish (Kent and Fournie 2007). *Henneguya zschokkei* is commonly found in salmonids and coregonids (Woo *et al.* 2002). In Finland, parasitic behavior of a myxozoan species *H. zschokkei* causes threat to whitefish (*Coregonus lavaretus*) culture. Myxozoan life cycle consists of 2 phases, a myxospore stage and an actinospore stage. *H. zschokkei* itself forms myxospores in whitefish and actinospores in final hosts, the oligochaete worms. They form plasmodium cysts in muscle tissue of the fish and decrease the aesthetic value of the fish. Although not proven pathogenic to whitefish, *H. zschokkei* ruptured cysts will cause ulcers on fish, perfect for secondary pathogens. Whitefish can survive the parasite but infected fish is unsuitable for market and causes economic loss (Woo *et al.* 2002).

Knowledge about the myxozoan biology in the fish host is still considerably limited, and although various studies have been carried out explaining their life-cycles, the life cycle of *H. zschokkei* is not known. Currently there is no treatment available against *H. zschokkei* infection. Manual selection of healthy fish is used to discriminate the infected ones.

This research is important in order to understand the life cycle of *H. zschokkei*. The aim of this study was to describe the life cycle of *H. zschokkei* by looking at the development of actinospores in oligochaete and the morphological development of *H. zschokkei* plasmodium cysts in whitefish.

The study was done by observing the effect of two different temperatures on the shedding of actinospores from oligochaete. Additionally, seasonal occurrence of the actinospores from a fish farm was observed in order to understand the effect of temperature to the release of actinospores. The morphological development of *H. zschokkei* plasmodium cysts in whitefish was also observed. Factors like host age, sex, seasonality were investigated to see if they have an influence on the plasmodia and myxospore development inside the fish host.

2. BACKGROUND

2.1. Myxozoa

Myxozoans are parasites in aquatic invertebrate and vertebrate hosts. They were once regarded as protozoa because of having simple body form especially in Myxosporea but the discovery of a primitive myxozoan *Buddenbrockia* in bryozoans upgrades them to metazoa (Lom and Dyková 2006). One of the differences between myxozoa and protozoa organisms is that in myxozoans the sporoplasm is coated by several living cells which play protective role while in protistan organism the cyst or spore wall is a non-living protective coat (Feist and Longshaw 2006). Phylum Myxozoa are parasites in aquatic ecosystems and in terrestrial animals and they are regarded as causing disease in some commercially important fish (Kent *et al.* 2001).

Knowledge about the myxozoan biology in the fish host is still considerably limited although various studies have been carried out explaining their life cycles. It has been confirmed by Wolf and Markiw (1984) that myxozoan life cycle consists of 2 phases, myxosporean and actinosporean thus suppressing the class Actinosporea and integrates them into bigger Myxosporea class.

The impact of myxozoans in aquaculture is significant. They are the cause for important diseases such as whirling disease, proliferative kidney disease (PKD) and post-harvest myoliquefaction (Kent *et al.* 2001). In northern Europe for example, *H. zschokkei* infection is found in the freshwater and brackish water whitefish (*C. lavaretus*). Other fishes in which *H. zschokkei* has been observed include salmon, pike, perch, catfish, bream, and char (Lebbad and Willcox 1998).

C. lavaretus or whitefish is a commercially important fish in Finland. It is one of the important species in the commercial catches in sea along with cod (*Gadus morhua*) and smelt (*Osmerus eperlanus*) (Setälä *et al.* 1998). In 2011, whitefish production in Finland is 0.7 million kilograms, the second largest fish product in Finland after rainbow trout which is on top at 11.0 million kilograms. Production of whitefish fry in Finland is also significant with 25.4 million fries reared for aquaculture and stocking program (Finnish Game and Fisheries Research Institute 2011). As the myxozoan *H. zschokkei* is hampering both whitefish fishing and farming, the consequences of *H. zschokkei* parasitism are economically important.

2.2. Classification of the phylum Myxozoa

Myxozoa consists of two classes, the Myxosporea which alternate between fish and annelids and Malacosporea which alternates between fish and bryozoans (Canning & Okamura, 2004). Currently there are more than 2180 myxosporean species that have been described under class Myxosporea. There are also 4 malacosporean species that have been described under class Malacosporea. The species in both classes are assigned to total of 62 genera (Lom and Dyková 2006). General features of myxozoans are having three important structures; shell valves, sporoplasms and polar capsules (Lom and Dyková 2006). Myxozoan taxonomy is based on the number of shell valves, spore shape and position of the polar capsules but with the advent of molecular biology, more researches are using morphological and molecular approaches for identification of new species (Feist and Longshaw 2006). Molnar *et al.* (2010) showed that although some species share some similarities in spore characteristics, 18S rDNA sequences and phylogenetic analyses clearly showed significant differences among all species examined. The findings have demonstrated that morphologically similar Myxobolus spores of different species could only be correctly identified by considering the location of plasmodia in the fish and the genetic sequence of the myxozoan species.

Class Myxosporea consists of myxozoa with two phase life cycle myxospore and actinospore. According to Lom and Dyková (2006) the term myxosporean covers both life cycle phases. Until now myxospore life stages are relatively better known compared to enigmatic actinospore life stage. Interestingly the actinospore phase of these 2000 myxosporeans can be divided into 17 collective groups because they have similar key morphological features. Examples of the collective group are as follows: Antonactinomyxon, Aurantiactinomyxon, Echinactinomyxon, Endocapsa and Triactinomyxon (Feist and Longshaw 2006) along with 12 others.

Myxospores of Myxozoa can survive harsh conditions. Ruidisch *et al.* (1991) reported that Myxospores of *Myxobolus pavloskii* for example are able to infect tubificid

worms in the infection experiment even after being kept in the freezer at -20 °C for 12 months.

2.3. *Henneguya zschokkei*

H. zschokkei is commonly found in salmonids and coregonids (Woo *et al.* 2002). Other synonyms of *H. zschokkei* are *Myxobolus zschokkei* Gurley, 1894 and *H. kolesnikovi* Gurley, 1894 as indicated by Eiras (2002).

H. zschokkei is a histozoic myxozoan parasite which belongs to genus *Henneguya* from class Myxosporea. Currently there are 204 species assigned to this genus, making them second largest genus in Myxozoa. In Europe, *H. zschokkei* cysts occur in muscle tissue of the genus *Coregonus*, a freshwater whitefish (Lom and Dyková 2006). *H. zschokkei* has been observed in superficial intermuscular tissues of *Coregonus fera* and also in *C. schiuzii, C. hiemalis* and muscular tissue and gills of *C. watmanni* and *C. exiguous albellus*.

H. zschokkei has myxosporean stage in fish and actinosporean stage in annelids. Although they are not harmful to the fish, the heavy occurrence of *H. zschokkei* cysts on the muscle tissue makes them less appealing, unmarketable and gives negative impact to the pisciculture industry (Woo *et al.* 2002). *H. zschokkei* is not capable to cause disease to humans and they passed through human intestine intact (McClelland *et al.* 1997).

In myxospore phase the spore body of *H. zschokkei* is about 8-14 µm in length and 7-11 µm in width. The filamentous tail is about 26-40 µm long. The polar capsule size is approximately 3.7-5 x 2-3 µm (Lom and Dyková 1992). The actinospore of *H. zschokkei* is probably of triactinomyxon type. This suggestion is based on taxonomy notes from Lom and Dyková (2006). Actinospore is acknowledged as developmental stage in the life cycle of species *Myxobolus cerebralis* (El-Matbouli and Hoffmann 1989) and *H. nuesslini*. Actinospore is parasite in freshwater and marine oligochaetes. Total body length of *H. zschokkei* actinospore from anterior to posterior end is 120 µm (Lom and Dyková 2006).

The plasmodium cysts of *H. zschokkei* are very visible in the flesh. They are white in colour with creamy content, subspherical cysts up to 15 mm in diameter, occur in the muscle tissue and other tissues (Boyce *et al.* 1985). When the cysts grow and mature, they finally break through the top layer, releasing myxospores which transmit the parasite in the water. Open ulcers on the fish skin provide an excellent entry port for secondary pathogens. Boyce *et al.* (1985) have also reported that the highest prevalence of infection of *H. zschokkei* was detected in coho and sockeye salmon and has been associated with the length of time spent in juvenile fish in fresh water.

For *H. zschokkei*, the myxospore completes their phase in annelid worms and is released from worm as actinospore. Oumouna *et al.* (2003) reported various actinospores occurrence in the water throughout the year based on a research at a trout hatchery farm in Germany. The seasonal occurrence for *H. zschokkei* actinospore in Finland has not yet been ascertained.

2.3.1. Morphology

Myxozoans are characterized by the presence of multicellular spores. Spores have two or more rigid cell valves, polar capsules with an internal coiled polar filament, and a sporoplasm (the infectious stage of the parasite for the invertebrate host). Spores develop within large, multicellular plasmodia comprised of vegetative nuclei and generative cells. Generally myxospore size in fish hosts is between 10-20 µm.

According to Lom and Dyková (2006) *H. zschokkei* myxospore has ellipsoidal spores with spindle-shaped or rounded in valvular view and biconvex in sutural view. Each valve continues as a caudal projection, both projections may be opposing each other. Shell valves have smooth surface. Two polar capsules are as a rule very elongated. In the binucleate sporoplasm is usually a spherical polysaccharide inclusion. Polysporic plasmodium with pansporoblast formation is usually large, appearing like cysts. They also suggested that triactinomyxon type actinospore possess elongated spore body with extended tips of polar capsules and plasmodial sporoplasm bearing many infectious cells. Caudal projections continue as a common stem, eventually bifurcated into three slightly upwards curved, long arms attenuated as a rule into sharp tips (Lom and Dyková 2006).

In worms, myxospore generally will complete its development and released as actinospore. In general, actinospore has a spore axis with three polar capsules located in the apex, comprising a sporoplasm with germ cells and a style which divided into three upwardly curved and pointed caudal processes (Oumouna *et al.* 2003).

2.3.2. Life Cycle

At present the complete life cycle of *H. zschokkei* has not yet been elucidated. There are only three complete life cycles recorded from genus *Henneguya*, from *H. exilis*, *H. ictaluri* and *H. nuesslini* (Lom and Dyková 2006). According to Feist and Longshaw (2006) most actinospores were observed in oligochaetes, particularly in Tubificidae and Naididae. For example the invertebrate host for *M. cerebralis* is *Tubifex tubifex* but for *H. zschokkei* the specific invertebrate host has remained unknown. According to Kent *et al.* (2001), genus *Henneguya* may arise from genus *Myxobolus*. Therefore, it is possible to discuss the life cycle of *Myxobolus* as the reflection of the life cycle of *Henneguya*.

The following descriptions explain a typical freshwater myxozoan life cycle (Kent and Fournie 2007). Multicellular myxospores are released from infected fish following death, or are discharged in body fluids. Oligochaetes, such as *T. tubifex,* ingest the myxospores and eventually become the alternate host. In the alternate host particularly in worm intestine, asexual reproduction occurs and is followed by sporogony, resulting in the formation of actinospores. Actinospores are released from the oligochaete, and upon contacting the fish skin surface, the presence of chemical trigger in surface mucus stimulates the polar filaments to attach and penetrate to the skin and release sporoplasms (Kallert *et al.* 2010). Shortly thereafter, clusters of dividing myxosporean cells are found, sometimes intracellularly, within the epithelium. The parasite then migrates to its final target tissue, where development continues. With *M. cerebralis*, the extrasporogonic (vegetative) forms migrate to the cartilage via peripheral nerves. These extrasporogonic forms continue to divide during migration to the target tissue. Some species (e.g., *Sphaerospora* spp. and *Tetracapsuloides bryosalmonae*) exhibit prominent extrasporogonic multiplication in the circulatory system or vascular organs. The presporogonic organism becomes a multinucleated plasmodium containing free nuclei and internal generative cells. Sporogenesis is usually initiated by fusion of generative cells. The sporoblast divides and differentiates into the components of the spores.

The most common immune response to counter the presence of myxozoa in fish is the formation of cysts to encapsulate the myxospores and prevent its dispersal to neighbouring tissues (Sitja-Bobadilla 2008). The presence of cysts in *C. lavaretus* musculature is the example of immune response to the presence of *H. zschokkei* myxospores.

2.4. Effects of temperature and seasonal occurrence

Environmental parameters such as water temperature and seasonality have been considered as important factors affecting the development of myxospore particularly on the prevalence rate and time of development of actinospores in oligochaete host (Markiw 1986) and (Blazer *et al.* 2003). Infection experiment by Markiw (1986) indicated that development of *M. cerebralis* from initial exposure to myxospores to the release of triactinomyxon actinospores took at least 100 days in *T. tubifex* in temperature regime of 12.5 °C. In a more recent study, Blazer et al. (2003) investigated the effects of different temperature regimes in eastern *T. tubifex*. As temperature rose from 9.0 °C to 17.0 °C, development time from exposure to myxospores and release of triactinomyxons decreased, duration of triactinomyxon release declined, and infection prevalence increased. The total number of triactinomyxons released was highest at 13.0 °C. Furthermore, (Kerans *et al.* 2005) suggested that temperature-driven developmental rates of *M. cerebralis* were the primary determinant of the timing of triactinomyxon release, and that the timing did not change when strains of *T. tubifex* with different temperature requirements were exposed to myxospores under different temperature regimes.

Oumouna *et al.* (2003) reported that most actinospores were released from oligochaete hosts throughout the year particularly during summer. There are also seasonal and annual variations in prevalence of the host to myxospore due to some factors including abundance of the spores, availability of hosts and ambient temperature influencing parasite development (Feist and Longshaw 2006).

2.5. Whitefish

The whitefish (*C. lavaretus*) has the potential to become an important alternative to rainbow trout in fish farming in Finland and there is a long tradition of whitefish farming for stocking purposes in Finland. However, cultivation in net cages in the brackish water of the coastal area exposes fish to variety of bacterial infections such as vibriosis and furunculosis (Lönnström *et al.* 2001) as well as myxozoan infections and trematode infections (Dezfuli *et al.* 2005).

Increasing importance of disease problems in the fish farming industry and the impact disease may have on both wild and farmed fish in the Nordic countries makes monitoring and surveillance on diseases to be compulsory for socioeconomic interests. Regular monitoring for the parasite takes place in certain regions in Finland so they can be recognized to be free from the parasite in order to obtain additional guarantees within the EU (Hastein *et al.* 2001). As whitefish become more important if aquaculture industry, successful whitefish farming requires the efficient control of diseases as well as parasite infections (Lönnström *et al.* 2001).

2.6. Aims of the study

The aim of this study was to describe the life cycle of *H. zschokkei*. The first part of this study was done by observing the development of actinospores in oligochaete. More spesifically, the study recorded the effect of two different temperatures on the prevalence rate of infection by looking at number of actinospores shed by oligochaetes and the time when actinospores were shed. Secondly, seasonal occurrence of the actinospores was recorded in the laboratory from oligochaete samples from a fish farm to see the time when shedding of actinospores was the highest. Finally, morphological development of *H. zschokkei* plasmodium cysts in whitefish was observed by looking at prevalence and mean

intensity of infection of three types of cysts in two year classes of whitefish. The prevalence and intensity of melanisation of the cysts were also observed in this study.

3. MATERIALS AND METHODS

3.1. Study area

In this study, uninfected oligochaetes were collected at several sites for the infection experiment. The sites were Lohikoski (62° 15.755'N, 25° 45.463'E), Holsti (62° 15.499'N, 25° 45.332'E) and Myllyjärvi (62° 12.999'N, 25° 42.694'E) in the city of Jyväskylä. These sites were small ponds without whitefish *C. lavaretus*. Worms in those ponds were not infected by *H. zschokkei* as the fish host, whitefish, did not inhabit the ponds. All these sites served as the suppliers for uninfected worm stock for this study to be infected experimentally with *H. zschokkei* myxospores.

Another study area was RKTL Tervo fish farm (63° 1.500'N, 26° 39.387'E). It was located in the eastern of Finland. This study area served as sampling site for worms in the second experiment in which the seasonal occurrence of the actinospores shed by the oligochaetes was observed.

The final study area was the final study area was the fish farm which was located in the northern of Finland. This study site was the source of whitefish for the third experiment to measure *H. zschokkei* plasmodia development in whitefish of age one to four years old.

3.2. Worms sampling and maintenance for infection experiment

Worm stock for the study of the development of *H. zschokkei* actinospores in oligochaete was randomly collected from several small ponds at Lohikoski, Holsti and Myllyjärvi. They were collected along mud and detritus using kick net with mesh diameter of 200 µm. The worms were sorted manually in the lab by using pipette and transferred to plastic containers with 1 L of aged tap water and approximately 100 g of autoclaved mud.

Prior to infection experiment, worm stock was maintained in two versatile environmental test chambers of 8 °C and natural temperature. Versatile environmental test chamber with natural temperature means the temperature mimic the current environmental temperature. Aged tap water and salad were supplied once per month to maintain the worms.

3.3. Filleting and myxospore extraction from whitefish muscle for infection experiment

Two samples of whitefish were used for myxospores extraction. The first set of cysts was obtained from fish caught by fishermen from Lake Haukivesi (62° 4.14′N, 28° 34.6′E). The second set of cysts was obtained from whitefish of Lake Päijänne (61° 29′N, 25° 26′E).

Whitefish was hand filleted and skinned before examination. The fish was placed flat on the cutting board, and using a filleting knife, the skin behind the front dorsal fin was pierced. Knife was sliced transversely across the fish until it reached near the backbone. The knife was sliced from head to tail with a sawing motion to remove the fillet. Skin was removed from the fillet by fixing the fish on the cutting board with a peg before removing the skin using filleting knife. The procedure was repeated to remove fillet from the other side of fish. Fillet was deboned and stored on ice.

Plasmodium cysts were carefully separated from the muscle tissues using scalpel and thumb forceps. Collected plasmodia were homogenized to form liquid suspension and were examined under microscope to confirm the presence of *H. zschokkei* myxospores.

3.4. Infection experiment

For the infection experiment, 5 ml cysts suspension of myxospores of *H. zschokkei* was introduced to approximately 300 worms in a container with 1 L of aged tap water. Myxospore density was unaccounted. There were two treatments: infection of worms using fresh plasmodia cysts (FC) which was directly conducted after cysts were extracted from whitefish, and infection of worms with not fresh plasmodium cysts (NFC). NFC cysts were stored at 5°C for 80 day after extraction from whitefish. Control for this experiment was the worms without any infection given. The worms were kept in different containers containing autoclaved mud and aged tap water with weekly changes of water. All treatments were done at 2 different temperatures, 8°C, and natural temperature. Natural temperature was adjusted according to the seasonal temperature of the environment. There were 1 container with Lohikoski worms in FC; 1 container with Lohikoski worms in NFC; 1 container with Myllyjärvi worms in NFC; 4 containers with Lohikoski worms in Control; and 1 container with Myllyjärvi worm in Control. To observe the shedding of actinospores, the water in each container was filtered using 20 µm filter. Approximately 0.5 ml of filtered water was observed under light microscope (Olympus CH-2). Number of actinospores and type of actinospores in every observation were recorded. Actinospore production was monitored in infection experiment for the period of 38 weeks with weekly observation of the shedding of actinospores. The data was compared to see the difference in each treatment.

3.5. Observation of seasonal occurrence of actinospores from RTKL Tervo fish farm

To compare seasonal occurrence of actinospores between years, worms were randomly collected from lower stream of the river at RKTL Tervo fish farm. The same methods for worms sampling as described in chapter 3.2 were used to sort the oligochaetes in the laboratory. Worms were separated and placed in containers with 1 L of aged tap water. Experiment was carried out for the maximum duration of 38 weeks and data collection and observation methods were similar to methods of the infection experiment. The present data set of 2011 to 2012 was compared to data set of year 2010 to 2012 collected by Hanna Ahonen, University of Jyvaskyla, to see the difference in seasonal occurrence of the actinospores in term of number of actinospores observed and types of actinospore shed from lower stream of the river at RKTL Tervo fish farm.

3.6. Comparison of *H. zschokkei* cysts and its development in whitefish

A total number of 336 whitefish from a northern Finnish fish farm were collected from July 2011 to April 2012 and examined to observe *H. zschokkei* cysts development. Number of 1+ and 2+ year old fish were 151 and 185, respectively.

The whitefish were hand filleted and skinned before examination using the same method as mentioned in myxospores extraction method (3.3). Before inspection, fish fillet was sliced into small slices of 20 mm width. Fish muscles were observed under dissecting microscope (Leica MZ6) to observe the appearance of plasmodium cysts. Number, diameter and type of cysts were recorded and described, and possible melanisation was evaluated and compared between each year class. Cyst diameter was measured by using a digital caliper. The presence of *H. zschokkei* myxospores were confirmed by randomly examining the plasmodium cysts under microscope. Mean cysts size from several year

classes was compared using statistical analysis to see the difference between each year class.

3.7. Statistical analyses

All results were analysed with SPSS Version 15.0. One-way ANOVA was used to analyse the intensity of infection of *H. zschokkei* cysts in whitefish. The difference in fish size and seasonal variation of cysts size were also tested with ANOVA with Tukey post-hoc comparison.

Pearson Chi-Square Test was used to analyse differences in prevalence of infection between male and female whitefish. Pearson Chi-Square with Yates' continuity of correction was used to analyse prevalence of melanisation.

Non-parametric Kruskal-Wallis Tests and follow up Mann-Withney U Tests were used to analyse differences in the intensity of infection in different types of cysts.

4. RESULTS

4.1. Infection experiment

A total number of 300 worms in each group were exposed to myxospore extract from fresh plasmodium cysts (FC) and not fresh cysts (NFC) in the infection experiments which were carried out at 8 °C and natural temperature.

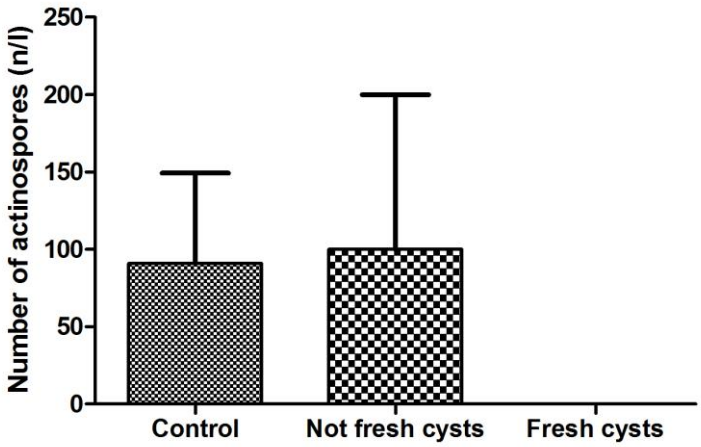

Figure 1. Number of actinospores from infection experiment using not fresh cysts and fresh cysts at 8 °C. No actinospore was observed from fresh cysts infection experiment. Error bars=s.d

In the infection experiment at 8 °C worms in not fresh cysts group produce slightly more actinospores compared to Control group. Infection experiment with FC did not release any actinospore. Types of actinospore in NFC group at 8 °C were actinospore type Neoactinomyxon and Hungactinomyxon. Actinospores from the Control group were released on week 2, 11 and 18 while actinospores from NFC group were observed three weeks after the experiment started. The actinospores were released from Lohikoski and Myllyjärvi containers in both groups.

The second infection experiment was done in natural temperature; worms from NFC, FC and Control group did not shed any actinospores.

4.2. Seasonal occurrence of actinospores from RTKL Tervo fish farm

Worms started to release actinospores in July of 2010 (Fig. 2). Worms shed 6 types of actinospores during the observation with the highest number of actinospore recorded was 3500 (Act. 5 type Aurantiactinomyxon) also in July of 2010.

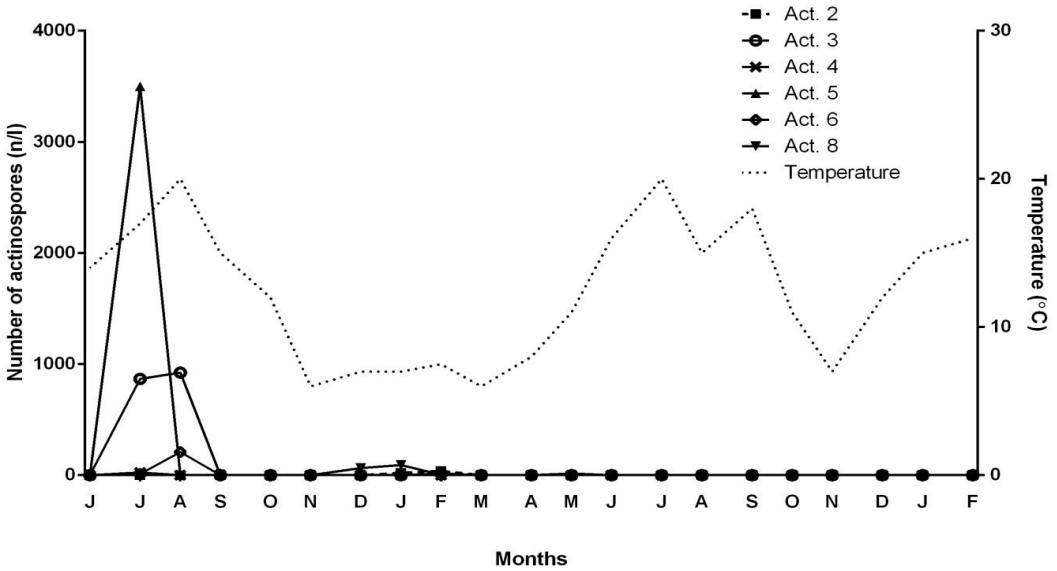

Figure 2. Number of actinospores observed from worms collected from Tervo in May 2010 and observed in laboratory at natural temperature. Observation was carried out for duration of 21 months until February 2012. Each type of actinospores possesses different morphological features but some belong to the same collective group and are described as follows: Act. 2, Act. 3 and Act. 6: Triactinomyxon; Act. 4: Raabeia; Act. 5 and Act. 8: Aurantiactinomyxon.

During the survey in July 2011 to February 2012, three types of actinospores belonging to the collective groups Triactinomyxon, Raabeia and Aurantiactinomyxon were observed (Fig. 3).

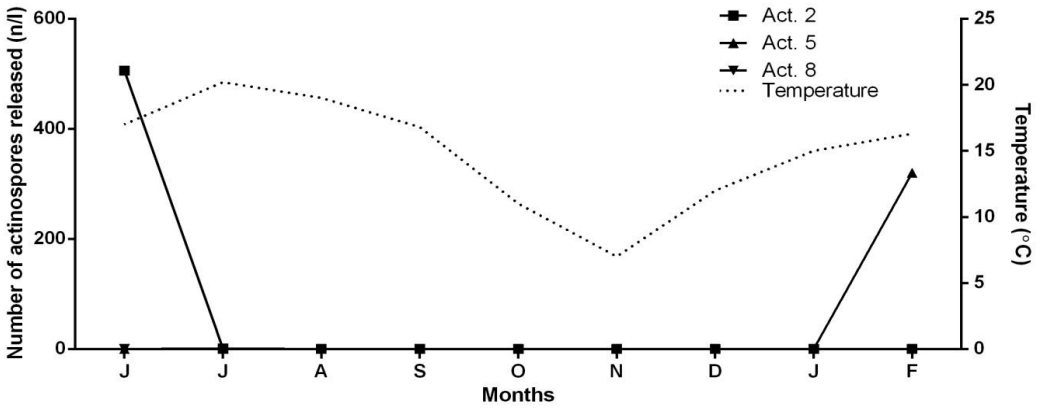

Figure 3. Number of actinospores released from worms collected from Tervo and observed in laboratory at natural temperature. Observation was carried out for duration of 9 months from June 2011 to February 2012. Each type of actinospores possesses different morphological features but some belong to the same collective group and are described as follows: Act. 2: Triactinomyxon; Act. 5 and Act. 8: Aurantiactinomyxon.

Worms started to release actinospores as early as the first week of observation. There was no actinospore detected from the worms sample after June of 2011 until it started to appear again in February of 2012.

The actinospores observed from Tervo samples during both observations were described (Fig. 4). There were eight types of actinospores and each actinospore possesses different morphological features but some belong to the same collective group.

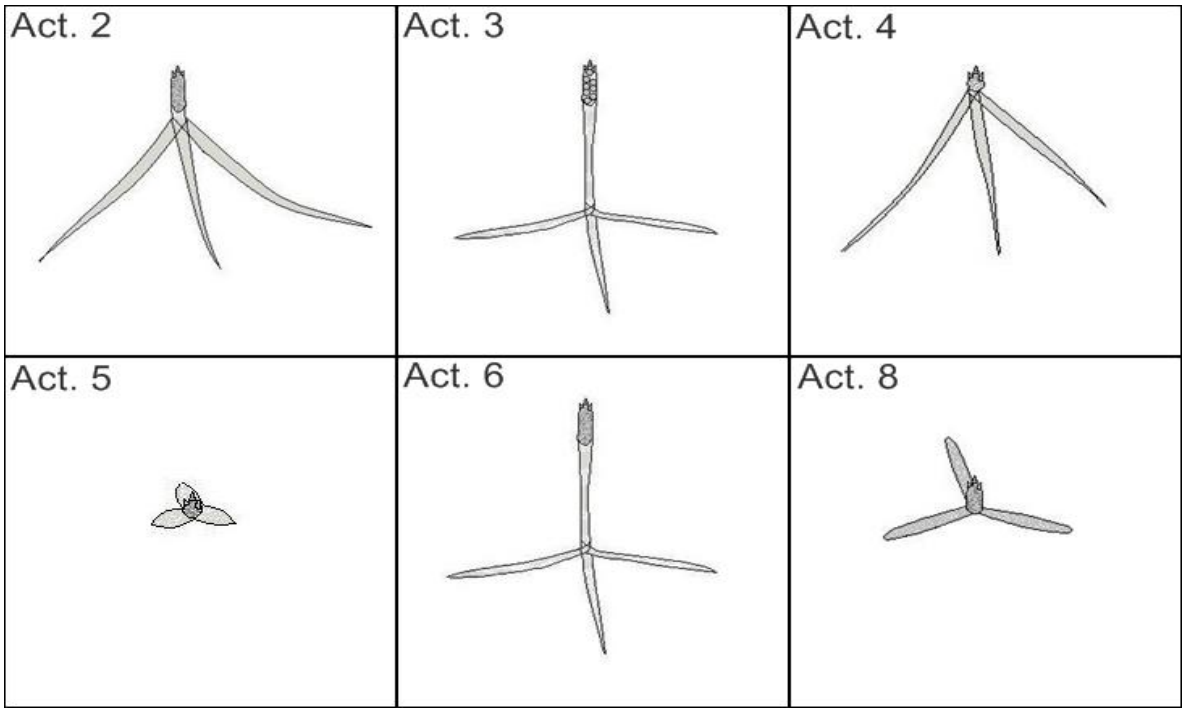

Figure 4. Act. 2, Act. 3 and Act. 6 belong to collective group Triactinomyxon. Act. 4 belongs to collective group Raabeia. Act. 5 and Act. 8 belong to collective group Aurantiactinomyxon.

4.3. Description of types of *H. zscokkei* cysts in whitefish

Fig. 5(A) shows *H. zschokkei* plasmodia detected in infected whitefish. Most of the detected plasmodium cysts were whitish in colour, with milky substance containing spindle-shaped myxospores with two tails (Fig. 5 (B). Some of the plasmodium cysts contained melanisation resulting in brown appearance of the cyst.

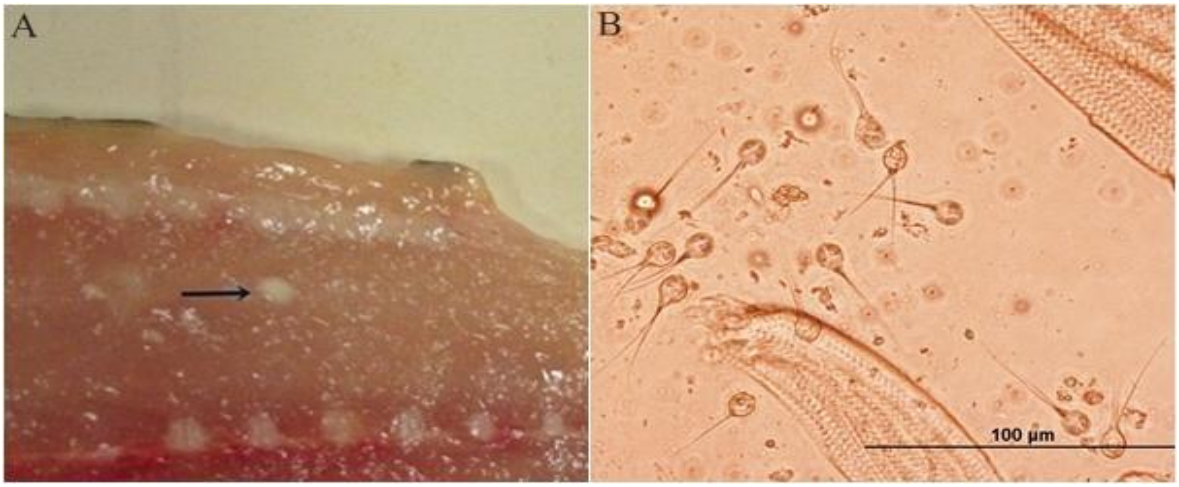

Figure 5(A). Cysts of *H. zschokkei* detected in whitefish muscle. Figure 5(B). *H. zschokkei* myxospores inside the cysts when observed under microscope.

Observation showed three types of cysts detected from the whitefish. They were mature plasmodium cysts with myxospore; plasmodium cysts with intermediate developmental myxospores; and cysts without myxospores. They were categorized as type-1, type-2 and type-3 cyst respectively (Table 1).

Table 1. Different types of *H. zschokkei* cyst and characteristics observed on infected whitefish

Cysts	**Characteristics**
Type-1	Mature plasmodium cyst with myxospores. It has a thick cyst-like wall. Being hard when pressed with needle.
Type-2	Intermediate plasmodium cyst with myxospores. Has a thin and flexible wall. Being elastic when pressed with needle.
Type-3	Immature cyst. It is small cyst without myxospores.

In this research all cysts were referred as type-1, type 2 and type 3 cyst according to the characteristics as in Table 1.

4.4. Prevalence and intensity of infection, cyst types and development of *H. zschokkei* in *C. lavaretus* of age 1+ and 2+ years old

A total number of 336 whitefish were collected from July 2011 to April 2012 from northern Finnish fish farm (Table 2). The whitefish were categorized as 1+ and 2+ years of age as they were collected when their age are more than 1 year and 2 years old.

Table 2. Total numbers (N), numbers of males (M), females (F) and immature in different age groups.

	1+ years					2+ years				
		Length (cm)	Sex				Length (cm)	Sex		
Season	N	Mean ± SD	M	F	I	N	Mean ± SD	M	F	I
July 2011	-	-	-	-	-	32	32.05 ± 1.63	14	15	0
Oct-Nov 2011	50	29.56 ± 1.56	29	18	0	55	32.82 ± 2.13	25	25	0
February 2012	50	28.59 ± 2.25	25	21	4	50	36.62 ± 1.66	27	23	0
April 2012	51	29.48 ± 2.06	35	16	0	50	36.14 ± 1.98	27	23	0

*SD=Standard deviation; Oct-Nov=October-November

There was a statistically significant difference in fish size between samples of 2+ year old whitefish (ANOVA, $F_{3,183}=65.569$, $p<0.001$). Tukey post-hoc comparisons indicated that July and October-November 2011 samples had a significantly smaller fish size than the February and April 2012 samples (Table 2).

In 1+ year old whitefish samples, there was a statistically significant difference in fish size (ANOVA, $F_{2,148}=3.713$, $p=0.027$). Tukey post-hoc comparisons showed that fish in February 2012 sample were shorter than in October-November 2011 and April 2012 samples (Table 2).

There was no statistically significant difference in sex-ratios between samples in 2+ years old whitefish (Pearson Chi-Square, χ^2=0.401, df=3, p=0.940). There was also no statistically significant difference in sex-ratios between samples in 1+ years old whitefish (Pearson Chi-Square, χ^2=2.089, df=2, p=0.352) (Table 1).

Table 3. Prevalence and intensity of infection by *H. zschokkei* in whitefish *C. lavaretus* of age 1+ and 2+ from a northern Finnish fish farm.

Fish age (year)	No. examined	No. infected	% prevalence	No. cysts / fish	Mean intensity
1+	151	1	0.7	0-1	1
2+	185	77	41.6	0-33	6.6

The prevalence of infection was significantly higher in 2+ whitefish, 42%, than in 1+ whitefish, 0.7 %, (Pearson Chi-Square, χ^2=78.25, df=1, p<0.001) (Table 3). The intensity of infection ranged from 1 to 33 cysts per infected fish. The first and only infected 1+ years old whitefish was found in April 2012 (in the last sample of the study). The prevalence of infection among 1+ years old whitefish in April 2012 was 2.0 %. All other 1+ years old fish, investigated in July 2011 and October-November 2011, as well as February 2012, were uninfected. The one infected individual of 1+ year old fish had one type-3 cyst (immature cyst). Size of the cyst was 0.60 mm. From 77 infected 2+ years old whitefish, only 1 whitefish was infected in July 2011 sample with one type-2 cyst (intermediate plasmodia cyst). Size of the cyst was 1.00 mm. It had melanisation on the cyst. The prevalence of infection in 2+ years old whitefish in July 2011 sample was 3.1 %. All other 2+ years old fish, examined in October-November 2011, February 2012 and April 2012 were infected with type-1 cysts (mature plasmodium cysts), type-2 cysts (intermediate plasmodium cysts) and type-3 cyst (immature cyst).

4.5. Seasonal variation in prevalence rate and mean intensity of infection

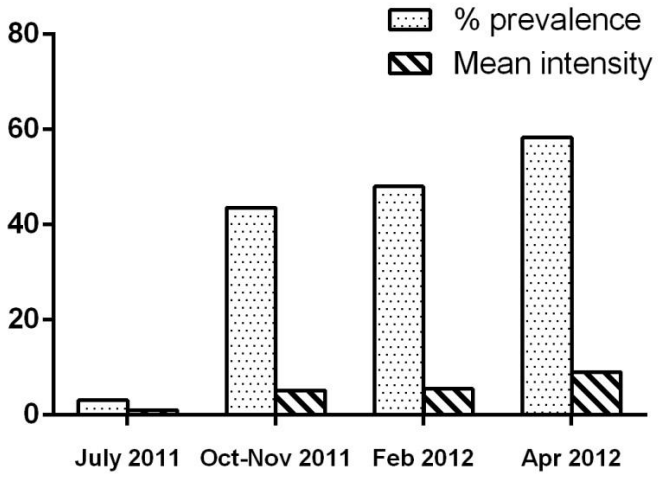

Figure 6. Seasonal prevalence rate (proportion of fish infected) and mean intensity (number of plasmodium cysts per infected fish) of infection in 2+ years old whitefish.

There was a statistically significant difference in seasonal prevalence of 2+ years old whitefish (Pearson Chi-Square, χ^2=16.213, df=1, p=0.000). The lowest prevalence was in July 2011 with only 1 fish infected (prevalence 3.1 %). The highest prevalence (58.3 %) was observed in April 2012. There was no statistical significant difference between months

in mean intensity of infection when tested using one-way ANOVA, $F_{3,73}$=1.648, p=0.200, although an increasing trend by time can be seen in Fig 6.

4.6. Seasonal variation in cyst sizes

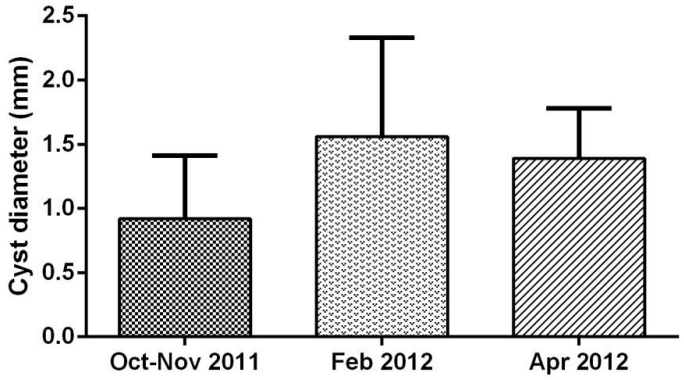

Figure 7. Seasonal variation in the mean cyst diameter (mm) of 2+ years old whitefish. Error bars=s.d.

A one-way ANOVA was conducted to compare the seasonal variation on plasmodium cyst sizes of 2+ years old whitefish. There was a significant different of cyst sizes across the three samples (ANOVA, $F_{2,69}$=7.70, p=0.001) (Fig. 7). Tukey post-hoc comparisons of the three samples indicated that the October-November 2011 sample had a significantly smaller cyst mean length than the February 2012 and April 2012 samples. However, the February 2012 sample did not significantly differ from April 2012 sample (Fig. 7).

4.7. Prevalence rate and mean intensity of infection by sex

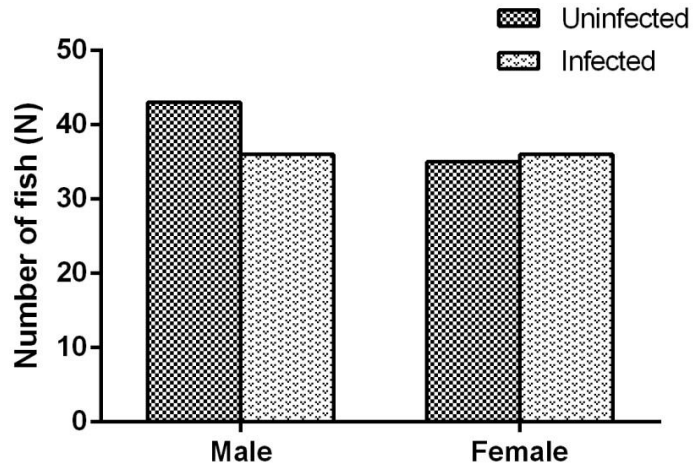

Figure 8. Number of uninfected and infected fish from sample of 2+ years old fish.

There was no statistically significant difference between male and female whitefish in prevalence rate of infection when tested using Pearson Chi-Square, χ^2=0.395, df=1, p=0.530) (Fig. 8).

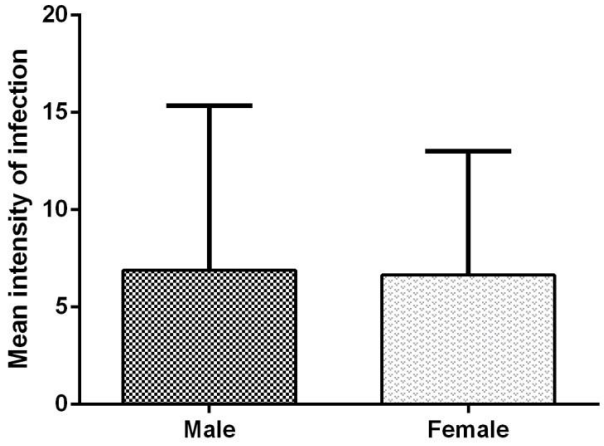

Figure 9. Mean intensity of infection in male and female whitefish of 2+ year old sample. Error bars=s.d.

There was also no statistically significant difference between male and female fish in mean intensity of infection when tested using one-way ANOVA, $F_{1,70}$=0.020, p=0.888 (Fig. 9).

4.8. Seasonal variation in prevalence of melanisation

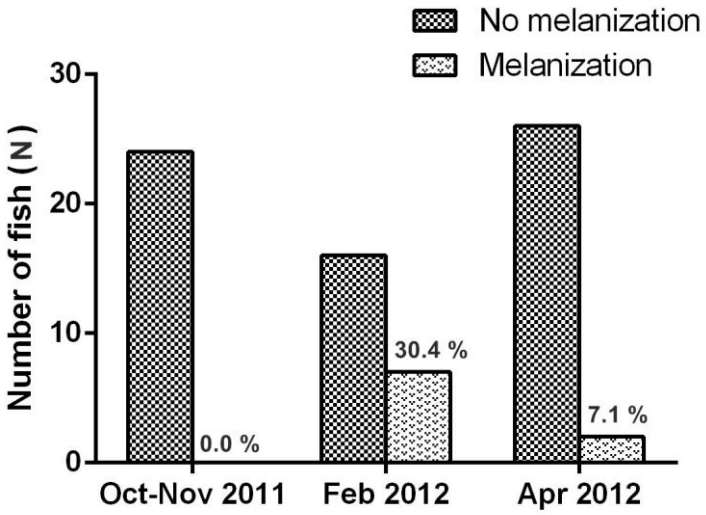

Figure 10. Melanisation rate of cysts in infected 2+ years old fish when compared to non-melanised cysts.

Statistical analysis showed that melanisation rate differs between months (Pearson Chi-squared, χ^2=11.300, df=2, p=0.004). The proportion of infected fish having melanised cysts was higher in February 2012 (30.4 %) when compared to October-November 2011 (0.0 %) (Pearson's Chi-squared test with Yates' continuity correction: χ^2=6.349, df=1, p=0.012) but not significantly different when compared to April 2012 (7.1 %) (Pearson's Chi-squared test with Yates' continuity correction: χ^2=3.247, df=1, p=0.072) (Fig. 10).

4.9 Variation of sex of the whitefish and cyst size in the prevalence of melanised cysts

From the result observed, the was no statistical difference between sexes in prevalence of melanisation (Pearson Chi-Square, χ^2=0.187, df=1, p=0.67). The result also

showed that there was no statistical significant difference in the size of melanised cysts when compared to non-melanised cysts (ANOVA, $F_{1,49}$=1.286, p=0.262).

4.10 Seasonal prevalence and intensity of infection of mature plasmodium cysts (cyst type-1)

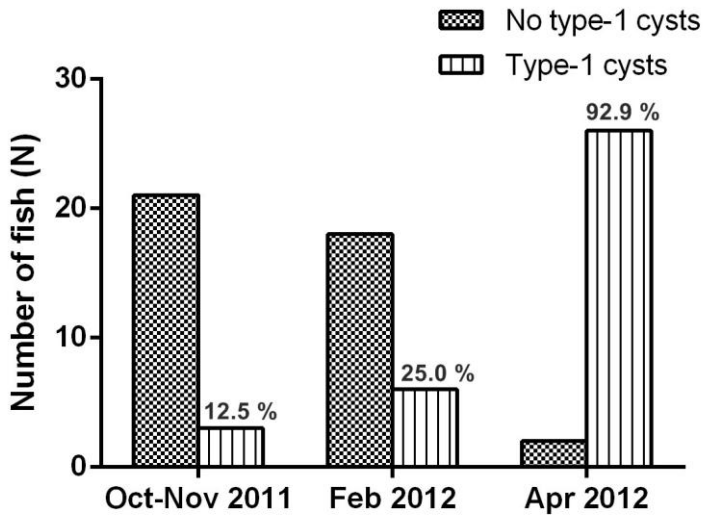

Figure 11. Seasonal occurrence of mature plasmodium cysts compared to no mature plasmodium cysts occurrence in 2+ years old whitefish samples.

There was a statistical difference in seasonal occurrence of type-1 cysts (mature plasmodium cysts) between months (Pearson Chi-Square, χ^2=39.846, df=2, p<0.001). Further analyses revealed that April 2012 sample had a higher prevalence (92.9 %) of mature plasmodium cysts when compared to October-November 2011 (12.5 %) (Pearson Chi-Square, χ^2=33.830, df=1, p=0.000) and February 2012 (25.0 %) (Pearson Chi-Square, χ^2=25.141, df=1, p=0.000). However, October-November 2011 sample was not statistically significantly different when compared to February 2012 sample (Pearson Chi-Square, χ^2=1,231, df=1, p=0.267) (Fig. 11). Thus proportion of mature plasmodia among plasmodium cysts increased significantly from October-November 2011 to April 2012.

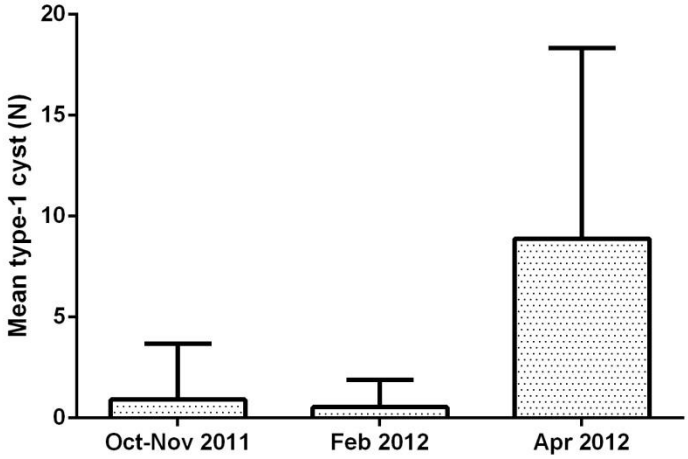

Figure 12. Mean number of type-1 cysts (mature plasmodium cysts) in three seasonal samples of infected whitefish of age 2+ year old. Error bars=s.d.

When studying the intensity of infection by mature plasmodium cysts, the result of Kruskal–Wallis test indicated significant difference ($\chi^2(2, N=76)=40.75$, $p<0.01$); the mean ranks of mature plasmodium cysts per infected host was significantly different among the three samples. Follow-up Mann-Whitney U-test tests were conducted to evaluate pairwise differences among the three samples. Thus, October-November 2011 sample was not statistically different compared to February 2012 sample (Mann-Whitney U=0.864, p=.388) but the mean number of type-1 cysts was higher in April 2012 (Mann-Whitney U=5.230, p<0.01) (Fig. 12).

4.11. Seasonal prevalence and intensity of infection of intermediate plasmodium cysts (cyst type-2)

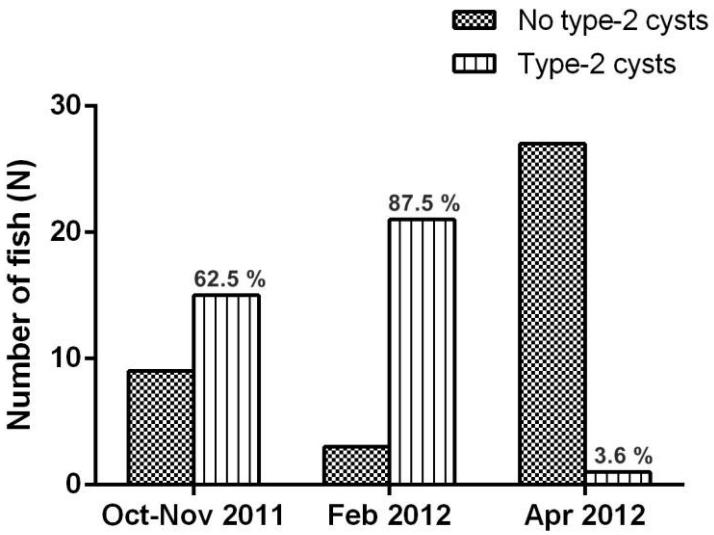

Figure 13. Number of infected whitefish with and without cyst type-2 (intermediate plasmodium cysts) in three samples.

There was a statistically significant difference in seasonal prevalence of type-2 cysts (intermediate plasmodium cysts) between months (Pearson Chi-Square, $\chi^2=39.117$, df=2, $p<0.001$). Pairwise comparisons also revealed that April 2012 sample had a lower prevalence of intermediate plasmodium cysts (3.6 %) when compared to October-November 2011 (62.5 %) (Pearson Chi-Square, $\chi^2=21.067$), df=1, $p<0.001$) and February 2012 sample (87.5 %) (Pearson Chi-Square, $\chi^2=37.295$, df=1, $p<0.001$). However, October-November 2011 sample showed no statistical different when compared to February 2012 sample (Fig 13).

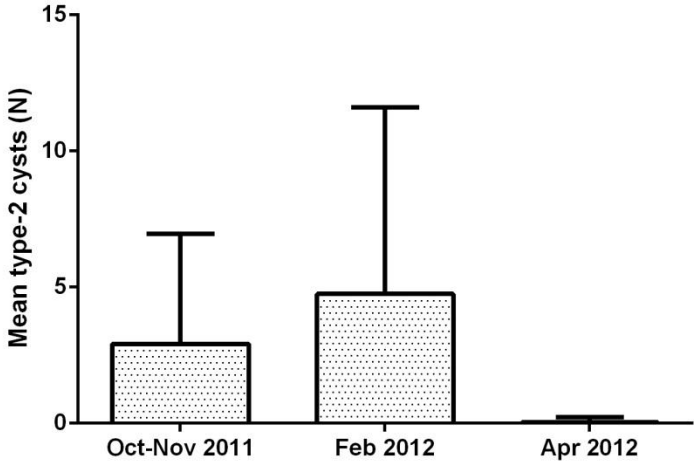

Figure 14. Mean number of type-2 cysts (intermediate plasmodium cysts) in infected 2+ years old whitefish from three samples. Error bars=s.d.

The result of Kruskal–Wallis test indicated significant difference ($\chi^2(2,N=76)=35.192$, $p<0.001$); the mean ranks of type-2 cysts per infected host was significantly different among the three samples. Follow-up Mann-Whitney U-test test was conducted to evaluate pairwise differences among the three samples. Thus, October-November 2011 sample was not statistically different compared to February 2012 (Mann-Whitney U=1.485, p=0.138) but was lower in April 2012 (Mann-Whitney U=-4.555, p<0.001). February 2012 sample also showed statistically significant different when compared to April 2012 sample (Mann-Whitney U=-5.911, p<0.001) (Fig. 14).

4.12. Seasonal prevalence and intensity of infection of immature cysts (cyst type-3)

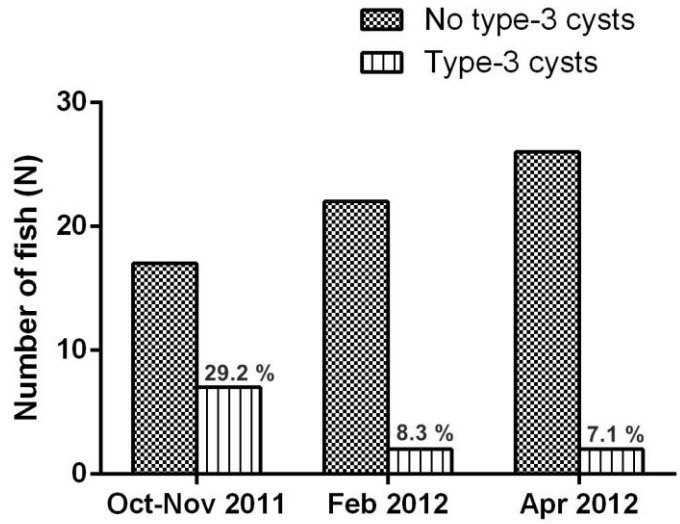

Figure 15. Number of fish with type-3 cysts (immature cysts) and number of fish not possessing type-3 cyst from infected whitefish samples.

There was a statistical significant difference in seasonal prevalence of the type-3 cysts (immature cysts) between months (Pearson Chi-Square, $\chi^2=6.132$, df=2, p=0.047). However, pairwise comparisons did not reveal significant differences in prevalence between months (Fig. 15).

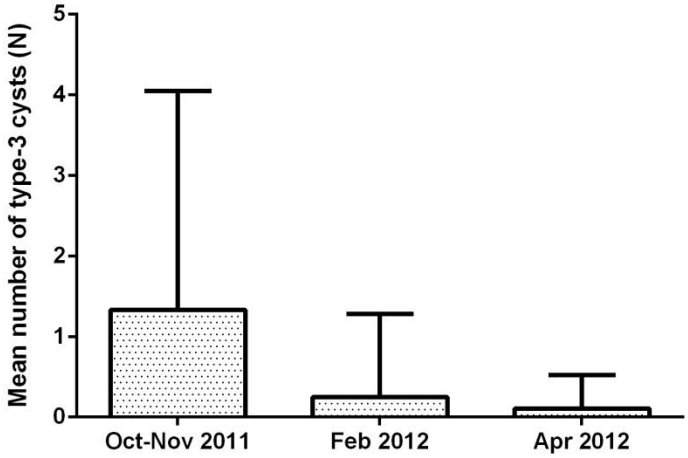

Figure 16. Mean number of type-3 cysts in infected 2+ years old whitefish from three samples. Error bar=s.d.

When studying the intensity of infection by immature cysts, the result of Kruskal–Wallis test indicated significant different ($\chi^2(2,N=76)=35.192$, $p<0.001$); the mean ranks of type-3 cysts (immature cysts) per infected host was significantly different among the three samples. Follow-up Mann-Whitney U-test tests were conducted to evaluate pairwise differences among the three samples. Thus, October-November 2011 sample was not statistically different compared to February 2012 (Mann-Whitney U=-1.878, p=0.060) but was lower in April 2012 (Mann-Whitney U=-2.200, p=0.028). However, February 2012 sample did not show statistically significant difference when compared to April 2012 sample (Mann-Whitney U=-1.179, p=0.858). Intensity of infection decreased from October-November 2011 to April 2012 (Fig. 16).

5. DISCUSSION

5.1. Infection experiment

Infection experiments in both natural temperature and in 8 °C were not successful in infecting oligochaete worms with *H. zschokkei* because during the period of observation, no actinospore resembling the characteristic of *H. zschokkei* was detected. The unsuccessful infection experiment may occur due to unsuccessful reproduction of similar environment parameters as in nature. Sitja-Bobadilla (2008) stated that research in myxozoa parasites often held back by the lack of in vitro cultures and difficulty to set up experimental transmission models. Moreover, the less aggressive phenotype of the actinospore may influence the capability of infection and their rate of proliferation. Since the discovery of myxozoa, there are only 33 life cycles for fresh water myxosporeans out of 2400 described species that have been successfully elucidated (Atkinson and Bartholomew 2009). To improve the efficiency of infection, there is a need to increase the ratio of myxospore concentration and number of worms to the volume of water.

5.2. Seasonal occurrence of actinospores in Tervo fish farm

In the monitoring of seasonal occurrence of the actinospore from RTKL Tervo fish farm, worms from year 2011 started to release actinospore as early as first week of observation (Fig. 3) which was in June 2011. Type of actinospores was Triactinomyxon and the same actinospore was observed in the second week of observation. Nonetheless

after the fourth week of observation, there was no actinospore shedding detected from the sample until Aurantiactinomyxon actinospores was released 30 weeks later in February 2012. One of the reasons for the early release of the actinospore may be due to the maturity stage of the actinospore. It was possible that the actinospores completed their development in worms and were immediately released to the environment. Furthermore, the temperature was at 15 °C in June during summer could also the optimum temperature for the shedding of actinospores. This observation was in line with observation by Oumouna *et al.* (2003) at a trout hatchery where shedding of actinospores peaked in summer.

In comparison with another set of data taken in 2010, similar pattern of actinospore occurrence was observed. High number of actinospores was released in the beginning of the experiment followed by several small occurrences weeks later. In contrast to the data collected in 2011, more types of actinospore were observed in 2010 (Fig. 2). None of the actinospores formed resembled *H. zschokkei* in morphology.

5.3. *H. zschokkei* cysts development in whitefish

Currently the complete life cycle of *H. zschokkei* have yet to be ascertained but it is known that the fish hosts of *H. zschokkei* myxospore are *C. lavaretus* (Woo *et al.* 2002) and vendace (*Coregonus albula*). The life cycle of *H. zschokkei* would involve two distinct morphological life stages: the actinospore and the myxospore, and two hosts, a coregonid fish and a freshwater oligochaete. *H. zschokkei* has intercellular histozoic vegetative stage as described in Kent *et al.* (2001). During the examination of *C. lavaretus* samples, *H. zschokkei* showed a tropism for the fish muscle (Fig. 5(A) but *H. zschokkei* infection may also take place in other organs in whitefish.

H. zschokkei plasmodium develops in the musculature of *C. lavaretus*. The fish will respond with the formation of cyst, which is a capsule of host connective tissue surrounding the plasmodia. These encapsulations of the plasmodia isolate the parasite and prevent its dispersal to neighboring tissues (Sitja-Bodabilla, 2008).

My study has shown that the prevalence rate was higher in the older fish (42.0 %) compared to the younger ones (0.7 %). This result was in line with the study of Work *et al.* (2008) so that *H. akule* also had higher prevalence in older big-eyed scad (*Selar crumenophthalmus*). This result also suggests the whitefish had developed the infection when they were young and the prevalence rate of infection increased as they grew older. However, it is also possible that the infection detected in some of the older fish had actually started when the fish was older. Unfortunately, it is not possible to differentiate the exact time when the infection had occurred.

Although Lom and Dyková (1992) reported that younger fish were generally more susceptible to myxosporean infections, this research nonetheless have shown the otherwise. Similar findings were found by Hallett *et al.* (1997) where heavier myxosporean *Kudoa ciliate* infections were detected in larger Indo-Pacific whiting *Sillago maculata*. This suggests either that fish have acquired more parasites with age or that parasites have proliferated with time within their hosts.

Fish age also has positive correlation with fish size. Older fish class tends to have bigger body size and may have the capacity to harbour higher prevalence of infection compared to smaller fish. This observation was in line with the work of Lester (1984). He reported that as the fish grow older, continuous acquisition of parasite in host will lead to an increase of parasite load and therefore high prevalence will be observed in large fish. Large fish generally have higher capacity to carry more parasites. However, Gbankoto *et al.* (2001) reported lower prevalence of infection in bigger host size due to a die-off of the

highly infected fishes. Gbankoto *et al.* (2001) also suggested myxozoa parasites located in internal host organs and not in contact with external environment except during host death need to wait for the death of its host or to induce it and therefore would have higher parasite load with fish host.

The seasonal pattern of prevalence of infection in whitefish of a northern Finnish fish farm varied significantly with a lower prevalence of infection in summer 2011 than in other months. This result suggests the infection started in young fish contributing to lower prevalence of infection in July 2011 and the prevalence became more intense in the later months as the fish grew older.

The seasonal pattern of mean intensity of infection did not vary significantly. However there was a trend towards an increase in mean intensity of infection in the older whitefish. The result suggests the development of *H. zschokkei* cysts in *C. lavaretus* and the parasite load depends on the age of the fish and the availability of actinospores in nature. It was in line with the exclusion of infection in the younger 1+ years old whitefish.

Smaller cyst mean length was observed in earlier sampling months. Observations on 2+ years old whitefish suggests the October-November 2011 sample was an early stage of spore development while February and April 2012 were later stages of spore development. According to Sitja-Bobadilla (2008) in her work with *Myxobolus ichkeulensis*, newly emerging cysts were single, circular, and smaller in the earlier stages of development. During the subsequent developmental stages, the size of each of the individual cysts increases. Similar trend was observed in my research.

Male hosts are generally more susceptible to the transmission of parasites compared to female hosts (Skorping and Jensen 2004). (Poulin 1996) reported parasite infections are more prevalent in males than females due to testosterone synthesis which is costly and therefore decreasing immune competency. However, in *C. lavaretus*, prevalence rate and mean intensity of *H. zschokkei* infection did not vary significantly between the sexes of the host, revealing that sex difference could be irrelevant. Similar result on myxosporean parasites was found by Gbankoto *et al.* (2001) in two tilapia species *Sarotherodon melanotheron melanotheron* and *Tilapia zillii*.

5.4. Melanisation of cysts

Fish has capability to produce immune response to myxozoan parasites (Sitja-Bobadilla 2008). According to Lom and Dyková (1992) the host responses to the parasites are involving cell and tissue reactions. It is difficult to observe host response aiming at destruction of the developing parasite except when there are changes in the appearance of the cyst. For example, the occurrence of melanised cyst which has brown appearance compared to normal white ones. Whipps and Diggles (2006) suggests the pseudocysts of *K. alliaria* were brown as a result of the accumulation of melanised breakdown products when the cysts were killed by the host immune response. Brown cysts in the musculature of *C. lavaretus* were probably due to the similar reaction between fish immune system and the *H. zschokkei* myxospores.

Melanisation may be a second stage of immunal response of the host to the parasites. The first stage is the formation of cyst to wrap the histozoic plasmodia and prevent them from spreading to nearby tissues. The second stage is the immune response to the cyst in order to eliminate the parasites resulting in the melanisation of the cysts.

According to Woo *et al.* (2002), host responses to the parasites only started after the sporogenesis of the myxospore is complete. This might explain the lower prevalence of

melanisation observed in October-November 2011 compared to the other months. It is suggested that *C. lavaretus* was still juvenile and presporogenic development of the myxospores had not yet completed. Moreover, immune system of the fish host which started to develop at juvenile stage will be fully developed only when the fish grow older, resulting in immune reaction and melanisation when fish was 2+ years old. Woo *et al.* (2002) also suggested that the host responses are usually towards mature plasmodium cysts. This condition was observed in this research as the rate of melanisation was more prevalent in older fish which have higher prevalence of mature plasmodium cysts.

According to Skorping and Jensen (2004), so-called Bateman principle suggest that females maximize their fitness by investing in longevity, whereas males invest more in mating success. Therefore, assuming that immunity is costly, females of any taxon should invest more than do males in their immune function and the result should show the prevalence of melanisation is higher in female *C. lavaretus*. However, there was no difference between sexes in the prevalence of melanisation found in this study.

Additionally, there was no difference between the size of melanized cysts and not melanized cysts. It is suggested the host immune response caused the melanisation and furthermore suppress the development rate of the *H. zschokkei* plasmodia inside the cysts. Further investigation should be done in regard of this matter to ascertain the cause of melanisation in some cysts.

5.5. Seasonal prevalence and intensity of infection of different types of cyst

Three different types of cysts were observed in this research namely cyst type-1 (mature plasmodium cyst), type-2 (intermediate plasmodium cyst) and type-3 (immature cyst). The cysts were classified according to the morphology and distinct characteristics possessed.

There was a high prevalence and high intensity of mature plasmodium cysts in April 2012, probably because of the development stage of cyst was completed when the fish grew older. The observation was in line with the lower prevalence and intensity of intermediate plasmodium cysts in April 2012 which was the opposite of mature plasmodium cysts. This could be explained by the development of cysts from type-2 cysts (intermediate plasmodium cysts) to type-1 cysts (mature plasmodium cysts).

There was no difference in the prevalence of small cysts between months but the intensity of small cysts was higher in October-November 2011 than in April 2012 sample. The decrease of intensity of small cysts suggests that type-3 cysts could be the early developmental stage of *H. zschokkei* and it is developed from type-3 to type-2 and finally type-1 cysts.

The prevalence of infection among 1+ year old fish in April 2012 was 2.0 % and prevalence of infection in 2+ years old fish in July 2011 was 3.1 %. Even though they were not the same group of fish, this may reflect the real development of prevalence in fish, with an increase from 2.0 % to 3.1 % although the increase was small. Therefore, it is suggested that the major increase in prevalence of infection took place after October-November 2011 (2+ years old whitefish).

The development of *H. zschokkei* plasmodia may require some time to mature based on observation that some of 1+ years old whitefish may got infection only in late summer 2011 and the development takes time so that the plasmodia were visible only next year in April 2012. However, as the major increase in prevalence of infection seems to take place not until the fish are 2+ year old (2+ years old whitefish in October-November 2011

sample) maybe majority of whitefish will not get infected during their 1+ years old summer, but in the beginning of their 2+ years old summer. In this research, it is suggested that *H. zschokkei* myxospore required six months to produce mature spores. This could explain the appearance of type-3 cysts (immature cysts) with high intensity in October-November 2011 followed by the high intensity of type-1 cysts (mature plasmodium cysts) six months later which was in April 2012. This was in line with our suggestion that type-3 cysts could be the early developmental stage of *H. zschokkei* and it is developed from type-3 to type-1 cysts. Woo *et al.* (2002) showed that *M. cerebralis* requires 4 months to produce mature spores.

5.6 Conclusion

In conclusion, the study has shown that the prevalence of infection was associated with host age and host size. Cyst development on the other hand was associated with three types of cysts, namely type-1, type-2 and type-3 cysts. Type-3 cyst (immature cyst) was the early developmental stage of the cyst and type-1 cyst (mature plasmodium cyst) was the final stage. Majority of whitefish got infection when they were 2+ years old. Melanisation of cyst was probably a host response to *H. zschokkei* infection and was prevalent in older *C. lavaretus*. The effort to describe myxozoa life cycle is often complicated due to no established methodology and it is challenging to mimic the reality of myxozoa environment. Therefore, there is a clear need for improvement in the experimental settings to be able to obtain more informative data to further elaborate the life cycle of *H. zschokkei*. Observations on the prevalence and the intensity of infection are complex and difficult to interpret on account of a number of variables that can shift the balance one way or the other. Factors such as host's age, size, sex, seasonal variation and host immunological response can play a role in the association. It is suggested that in future studies, investigating the development of *H. zschokkei* in its hosts should consider bigger sample size and include histological study to document a comprehensive development of the parasite. Nevertheless, it is important to acknowledge that this study has provided valuable preliminary information about the infection of *H. zschokkei* in whitefish, the type of cysts formed and developed as well as the process of melanisation in the cysts found.

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