Pathogenicity and infectivity of *Saprolegnia* species in Atlantic salmon (*Salmo salar* L.) and their eggs

Mwansa Mathilda Songe

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Veterinærinstituttet
National Veterinary Institute

CONTENTS

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2 SUMMARY

In the past decades the global population has becoming increasingly dependent on aquaculture due to over-fishing of oceans and rivers. Consequently, fish farming has become the fastest growing food sector in the world. Increased production intensity constituting intensive culturing of fish coupled with the ban in the use of malachite green, an effective remedy against *Saprolegnia* infections, accounts for the dramatic re-emergence of *Saprolegnia* infections in aquaculture which fish farmers are struggling to control. This precarious situation inevitably created a sense of urgency for researchers to explore the possibility of developing novel and sustainable treatments to control this devastating pathogen of fish. *Saprolegnia* infection in fish is characterized by white or grey patches of filamentous mycelia on the host. The infection initially appears on epidermal tissues of the head, tail and fins and subsequently spreads to the rest of the body. Lesion areas may be soft, necrotic and ulcerated and the surrounding areas may show oedema and necrosis. Fish eggs infected with *Saprolegnia* species appear like tufts of cotton wool, as the *Saprolegnia* hyphae form a white cotton wool or felt-like mat on the surfaces of the eggs.

Even though attenuation causes problems in various situations, from production of biocontrol agents to developing and testing of vaccine or drug efficacy, no data were available on attenuation of virulence in *S. parasitica*. We were therefore motivated to carry out a study to investigate how *in vitro* sub-culturing impacts on the virulence of different isolates of *S. parasitica*, assayed by infection rates in challenge experiments in Atlantic salmon. Our findings show that not all isolates of *S. parasitica* undergo attenuation of virulence to Atlantic salmon parr due to *in vitro* passages (paper I). This interesting observation inspired us to investigate whether the isolates prone to a loss of virulence would be the same ones that would show an increment in their morbidity rate upon passage through Atlantic salmon parr. The result was in the affirmative, but the opposite was true for *Saprolegnia* strains that did not lose their virulence upon successive *in vitro* sub-culturing. Upon histopathological examination of the diseased fish skin and muscle layers, we noted necrosis, oedema and haemorrhage of the epidermal layers extending into the dermis and very little inflammation. Severe karyorrhexis and oedema were observed in the kidneys and moderate congestion was detected in the liver. We concluded that not all isolates of

S. parasitica need to be passed through susceptible fish prior to challenge experiments. This is valuable knowledge in order to reduce unnecessary use of experimental animals in research.

In order to gain more knowledge on infection strategies and host/agent relationships, morphological studies of Atlantic salmon eggs infected with *S. parasitica* and *S. diclina* under natural conditions in the hatchery were carried out using light and scanning electron microscopy (SEM) (paper II). Challenge trials of eggs and characterization of cellular/tissue changes were also conducted. Both in naturally and experimentally infected fish eggs, light microscopy showed that *S. diclina* infection resulted in complete destruction of the chorion in some areas, whereas eggs infected with *S. parasitica* had an apparently intact chorion with hyphae growing within in or beneath the chorion. Scanning electron microscopy revealed that *S. parasitica* grew on the egg surface and hyphae were found penetrating the chorion of the egg, and re-emerging on the surface away from the infection site. Findings from our studies provide new knowledge about infection strategies and morphological changes that occur in Atlantic salmon eggs infected with *S. parasitica* and *S. diclina* (paper II). The findings also suggest the possibility that *S. diclina* employs a necrotrophic strategy whereas *S. parasitica* utilizes a facultative bio trophic mode of infection. As we did not observe any appressorium-like structures with the scanning electron microscope in this study, we were able to deduce that not all isolates of *S. diclina* strains form these specialized infection structures under hatchery or laboratory conditions.

Differences in susceptibility to *Saprolegnia* infection of Atlantic salmon eggs from different females under natural conditions in a hatchery prompted us to investigate the chorion, an important physical aspect of the egg which is very likely to be influenced by the female. We hypothesized that a thicker chorion plays a significant role in the resistance of Atlantic salmon eggs against saprolegniasis. Light and transmission electron microscopy (TEM) studies showed that the eggs suspected to be less prone to *Saprolegnia* infections had significantly thicker chorion than eggs more prone to such infections (paper III). With the aid of a TEM, we were able to establish that there were no structural defects in the eggs that seemed more prone to *Saprolegnia* infection. No morphological or structural differences were noted in the chorion (*zona externa* and *zona interna*) between the two groups of eggs. Our findings underscore and confirm the fact that the thickness of the chorion and its individual layers will play a very important role in the egg's susceptibility or resistance to disease. Our results indicate that a

thicker chorion gives more resistance against *Saprolegnia* infection than a thinner chorion (paper III).

Further studies to investigate and compare the hatching rates of the eggs in the two different resistance groups may be required. This would give an answer to the hypothesis that eggs with a thinner chorion (infection-prone) hatch earlier and have an increased survival rate than eggs with a thicker chorion, which are infection resistant. This effort could possibly lead to a very natural and environmentally friendly way of fighting off saprolegniasis in hatcheries.

3 SAMMENDRAG (Summary in Norwegian)

I de siste tiårene har overfiske av hav og elver gjort verdens befolkning stadig mer avhengig av havbruk. Fiskeoppdrett har derfor blitt den raskest voksende matsektoren i verden. Økt produksjonsintensitet kombinert med forbud mot bruk av malakittgrønt, et effektivt middel mot *Saprolegnia*, har ført til en dramatisk ny fremvekst av saprolegniainfeksjoner som oppdrettere sliter med å kontrollere. Denne situasjonen innebærer at det haster med å utvikle nye og bærekraftige behandlinger for å kontrollere denne ødeleggende fiskepatogene organismen. Saprolegniainfeksjon på fisk er karakterisert av hvite eller grå flekker av trådformet mycelium på verten. Infeksjonen vises innledningsvis på epidermalt vev på hode, hale og finner, som så sprer seg til resten av kroppen. Infiserte områder kan være myke, nekrotiske med sårdannelse og de omkringliggende områdene kan ha ødem og nekrose. Fiskeegg infisert med saprolegniaarter minner om vattkuler, fordi saprolegniahyfer danner en hvit vatt- eller filtlignende matte på eggenes overflaten.

Selv om attenuering av mikroorganismer kan føre til problemer i ulike situasjoner, fra produksjon av biokontrollsubstanser til utvikling og utprøving av vaksine eller effekt av medikamenter, fantes det ingen tilgjengelige data på attenuert virulens hos *S. parasitica*. Vi gjennomførte derfor en studie for å undersøke hvordan *in vitro* sub-kultivering virker på virulens av ulike isolater av *S. parasitica*, målt som infeksjonsrater i smitteforsøk på atlantisk laks. Våre funn viste at gjentatte dyrkninger *in vitro* ikke førte til attenuert virulens for alle undersøkte *S. parasitica*-isolater (artikkel I). Dette interessante funnet inspirerte oss til å undersøke hvordan *in vivo* passering i parr av atlantisk laks påvirket stammenes patogenitet. Resultatene viste at de stammene som så ut til å få redusert virulens ved gjentatt sub-kultivering ikke fikk økt virulens etter *in vivo* passering i parr. Derimot ble patogeniteten ikke påvirket av *in vivo* passering for isolatene som ikke ble attenuert ved *in vitro* sub-kultivering. Histopatologiske funn varierte fra nekrose, ødem og blødning av de epidermale lag som strekker seg inn i dermis med svært liten inflammasjon. Alvorlige karyorrhexis og ødem ble observert i nyrene. Moderat stuvning ble påvist i leveren. På bakgrunn av disse undersøkelsene konkluderte vi med at ikke alle *S. parasitica*-isolater må passere mottagelig fisk forut for smitteforsøk. Dette er viktig kunnskap for å redusere unødvendig bruk av forsøksdyr i forskningen.

For å få mer kunnskap om infeksjonstrategier og vert/agens-relasjoner utførte vi morfologiske studier av lakseegg infisert med *S. parasitica* og *S. diclina* ved hjelp av lys- og scanning elektronmikroskopi (SEM) (artikkel II). Både naturlig og eksperimentelt smittede egg ble undersøkt, og histopatologiske forandringer ble karakterisert. Både i naturlig og eksperimentelt infiserte egg var resultatet at *S. diclina* infeksjon førte til fullstendig destruksjon av deler av chorion, mens egg infiserte med *S. parasitica* tilsynelatende beholdt en intakt chorion, med hyfevekst i eller under chorion.

SEM-studier viste at *S. parasitica* generelt vokste på eggets overflate, men hyfene kunne så penetrerte chorion fra overflaten for siden å penetrere chorion igjen fra innsiden (artikkel II). Funn fra dette arbeidet gir ny kunnskap om infeksjonsstrategier og morfologiske endringer i lakseegg infisert med *S. parasitica* og *S. diclina*. Funnene tyder på at *S. diclina* benytter seg av en nekrotrofisk infeksjonsstrategi, mens *S. parasitica* strategi er fakultativt biotrofisk. Siden vi ikke observerte appressorium-lignende strukturer i våre studier, kan det tyde på at ikke alle *S. diclina*-isolater danner slike spesialiserte strukturer verken i klekkerier eller i laboratoriet.

På bakgrunn av observerte forskjeller i mottakelighet for saprolegniose på egg fra ulike mordyr av atlantisk laks, , under naturlige forhold i et klekkeri, studerte vi chorion, en viktig fysisk del av egget som svært sannsynlig blir påvirket av mordyret. Vår teori var at en tykkere chorion kan spille en betydelig rolle i forsvaret mot saprolegniose i egg av atlantisk laks. Ved hjelp av lysmikroskopi og transmisjonselektronmikroskopi (TEM) viste vi at egg som var mer motstandsdyktige mot saprolegniainfeksjon hadde signifikant tykkere chorion enn mindre motstandsdyktige egg (artikkel III). TEM-undersøkelser viste videre at det ikke var strukturelle defekter i eggene som var mer mottagelig for saprolegniainfeksjon. Det ble heller ikke påvist morfologiske eller strukturelle forskjeller i chorion (*zona externa* og *zona interna*) mellom de to gruppene av egg. Disse funnene bekrefter at chorions struktur og tykkelse spiller en viktig rolle i eggets motstandskraft mot sykdom. Våre undersøkelser indikerer at en tykkere chorion beskytter mot saprolegniose i laksegg (artikkel III).

Det er nødvendig med videre forskning for å sammenligne klekkeevnen for de to gruppene av egg. Det vil gi svar på hvorvidt egg med en tynnere chorion (mer mottagelig for infeksjon) klekker tidligere og har økt overlevelse sammenlignet med egg med tykkere chorion (mindre

mottagelig for infeksjon). Denne kunnskapen kan på sikt bidra til en naturlig og miljøvennlig måte å bekjempe saprolegniose i klekkerier.

4 LIST OF PAPERS

Paper I

M.M. Songe, E. Thoen, Øystein Evensen and Ida Skaar. 2014. In vitro passages impact on virulence of *Saprolegnia parasitica* to Atlantic salmon, *Salmo salar* L. Parr. Journal of Fish Diseases 2014, 37, 825–834, doi:10.1111/jfd.12175

Paper II

Mwansa M. Songe, Ariane Willems, Jannicke Wiik, Even Thoen, Kasi Rajan, Øystein Evensen, Pieter van West and Ida Skaar. 2015**.** *Saprolegnia diclina* IIIA and *S. parasitica* employ different infection strategies when colonising eggs of Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases

Paper III

Mwansa M. Songe*, Ariane Willems*, Nasif Sarowar, Kasi Rajan, Øystein Evensen, Keith Drynan, Ida Skaar and Pieter van West. 2015. Thicker chorion gives ova of Atlantic salmon (*Salmo salar* L.) an upper hand against *Saprolegnia* infections. Manuscript submitted to Journal of Fish Diseases

*Author contribution statement: MMS and AW contributed equally to this work

5 ABSTRACTS

Paper I

In vitro passages impact on virulence of _Saprolegnia parasitica_ to Atlantic salmon, _Salmo salar_ L. parr

M M Songe, E Thoen, Ø Evensen and I Skaar. 2014.

Journal of Fish Diseases 2014, 37, 825–834, doi:10.1111/jfd.12175

The effect of serial in vitro sub-culturing on three pathogenic strains of *Saprolegnia parasitica* was investigated. The isolates were passed through Atlantic salmon, *Salmo salar* L. parr, and then re-isolated as single spore colonies. All strains caused infection. The isolate obtained from diseased fish served as a virulent reference culture and was designated 'AP' ('activated through passage'). Successive sub-culturing was made by obtaining an inoculum from AP to produce the 2nd sub-culture and then passaged to the 3rd subculture (from the 2nd), until the 15th passage was obtained. Spores used to produce storage cultures were collected at passages 5, 10 and 15. The different passages of each strain were used to artificially infect Atlantic salmon parr. Morphological characterization of growth patterns was performed to observe differences occurring due to serial in vitro sub-culturing. Two of the strains declined in virulence after 15 successive in vitro subcultures, whereas one did not. This study is the first to investigate attenuation of virulence in *Saprolegnia* and whether or not isolates of *S. parasitica* should be passed through the fish host prior to challenge experiments. It reveals that some strains degenerate more rapidly than others when subjected to successive in vitro sub-culturing on glucose-yeast extract.

Paper II

Saprolegnia diclina IIIA and _S. parasitica_ employ different infection strategies when colonising eggs of Atlantic salmon, _Salmo salar_, L.

Mwansa M. Songe, Ariane Willems, Jannicke Wiik-Nielsen, Even Thoen, Øystein Evensen, Pieter van West and Ida Skaar

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Here we address the morphological changes of eyed eggs of Atlantic salmon, *Salmo salar* L. infected with *Saprolegnia* from a commercial hatchery and after experimental infection. Eyed eggs infected with *Saprolegnia* spp. from 10 Atlantic salmon females were obtained. Egg pathology was investigated by light and scanning electron microscopy. Eggs from six of ten females were infected with *S. parasitica*, and two females had infections with *S. diclina* clade IIIA, two *Saprolegnia* isolates remained unidentified. Light microscopy showed *S. diclina* infection resulted in the chorion in some areas being completely destroyed, whereas eggs infected with *S. parasitica* had an apparently intact chorion with hyphae growing within in or beneath the chorion. The same contrasting pathology was found in experimentally infected eggs. Scanning electron microscopy revealed that *S. parasitica* grew on the egg surface and hyphae were found penetrating the chorion of the egg, and re-emerging on the surface away from the infection site. The two *Saprolegnia* species employ different infection strategies when colonizing salmon eggs. *Saprolegnia diclina* infection results in chorion destruction while *S. parasitica* penetrates intact chorion. We discuss the possibility of these infection mechanisms representing a necrotrophic (*S. diclina*) versus a facultative biotrophic strategy (*S. parasitica*).

Paper III

Thicker chorion gives ova of Atlantic salmon, *Salmo salar* L., an upper hand against *Saprolegnia* infections

Mwansa M. Songe*, Ariane Willems*, Mohammed Nasif Sarowar, Kasi Rajan, Øystein Evensen, Keith Drynan, Ida Skaar and Pieter van West

*Author contribution statement: MMS and AW contributed equally to this work

Since the ban of malachite green in the fish farming industry, finding alternative ways of defeating or containing *Saprolegnia* infections has become of utmost importance. A better understanding of the infection mechanisms, perceived from both the host and the invader's side is mandatory to provide such consumption-safe alternatives. A lot of effort has been made to elucidate the mechanisms by which Oomycetes invade fish eggs however little is known about the defense mechanisms of the host, making some eggs more prone to infection than others as has been observed in fish farms for many years. One clue might lie in the composition of the eggs. As the immune system in the embryos is not developed yet, these differences could be explained by factors influenced by the mother herself, either by transferring passive immunity, by influencing the physical aspects of the eggs or both. One of the physical aspects that could be influenced by the female is the chorion, the extracellular coat surrounding the fish egg, which is in fact the first major barrier to be overcome by *Saprolegnia* spp. We therefore hypothesized that the chorion thickness in infection-resistant eggs is greater than the chorion thickness of infection-prone eggs. To test this hypothesis, the chorion thickness of uninfected eggs from females producing putative infection-resistant eggs were analysed, and compared to that of uninfected infection-prone eggs. Upon analysing the thickness and morphology of the chorion from different groups identified in a fish farm set-up, we were able to confirm the hypothesis that a thicker chorion is indeed a protection against infection with *Saprolegnia* spp. in Atlantic salmon eggs. In addition to the identification of differences in sensitivity of eggs in a fish farm set-up, we were able to confirm these results in a laboratory-controlled challenge experiment.

6 INTRODUCTION

6.1 GENERAL INTRODUCTION

Saprolegnia is the main genus of water moulds responsible for significant infections of freshwater fish and eggs worldwide, and species belonging to the genus are ubiquitous in freshwater ecosystems. The infection of fish with *Saprolegnia* is termed "saprolegniasis" (Beakes *et al*., 1994; Roberts, 1989) and it refers to any disease of fish or fish eggs caused by species of the family Saprolegniaceae (Oomycotina). This practical use of the term was suggested by Nolard-Tintiger as early as 1974. However, the history of saprolegniasis spans over a few centuries. Arderon (1748) reported what was clearly saprolegniasis of roach (*Rutilus rutilus* L.) in England, and there is even a possibility that the disease had been known long before his description. The epizootic in Atlantic salmon in the rivers of Great Britain between 1877 and 1882 in which Oomycetes were incriminated attracted a lot of attention. *Saprolegnia* was thought by some scientists to be the cause of the disease (Stirling, 1878; Stirling, 1880, 1881), which was later referred to as "salmon disease". Other observers believed that the presence of the fungal-like organisms was due to predisposing factors such as over-crowding of fish, pollution, low water levels, wounding of fish by anglers or from fighting on the spawning beds. It turned out that that "salmon disease" outbreak became the first documented epizootic of the disease now known as Ulcerative Dermal Necrosis (UDN) in salmonids.

Traditionally, saprolegniasis is known as "fungal infection" in fish, and is typically seen in the fresh water stage of salmonids. The earliest encounters with the disease were on wild fish populations. However, with the advent of aquaculture in the 20th century, the densely reared monocultures were soon seen to be suffering from *Saprolegnia* infections. This posed a potential obstacle to the young, fast-growing food sector. Ecological differences in different geographical locations play an important role in the species diversity of the *Saprolegnia* spp. that infect both fish and eggs (Alabi, 1971; Avtalion *et al*., 1973; Wood and Willoughby, 1986). Saprolegniasis is a widespread problem as losses due to *Saprolegnia* spp. have been reported worldwide in different fish species and their eggs in rivers and freshwater reservoirs. Some examples are given in Table 1.

Table 1. Some geographic locations and fish species in which outbreaks of saprolegniasis have been recorded worldwide.

Geographic location	Host	*Saprolegnia* species	Reference
OCEANIA			
Australia	Bony bream, *Nematalosa erebi*, (Günther)	*S. parasitica*	Puckridge *et al*., 1989
ASIA			
India	Indian major carps, climbing perch, *Anabas testudineus* & silver carp, *Hypophthalmichthys molitrix*	*S. parasitica*	Jha *et al*., 1984; Krishna *et al*., 1990; Mohanta and Patra, 1992
Iraq	Carp (Cyprinidae)	*S. ferax* and *S. terrestris*	Butty *et al*., 1989
Japan	Coho salmon, *Oncorhynchus kisutch*, Cultured rainbow trout, *Oncorhynchus mykiss*, & Ayu, *Plecoglossus altivelis*	*S. parasitica* *S. diclina*	Jha *et al*., 1984; Krishna *et al*., 1990; Mohanta and Patra, 1992
Taiwan	Rainbow trout, *Oncorhynchus mykiss*	*S. diclina*	Chien, 1980
EURASIA			
USSR (formerly)	Wild Carp (Cyprinidae) eggs	*Saprolegia* spp.	Gajdüsek and Rubcov, 1985
EUROPE			
England	Cultured rainbow trout, *Oncorhynchus mykiss,* eggs & Arctic char, *Salvelinus alpinus* L.	*S. parasitica*	Pickering, 1981
France	Cultured roach, *Rutilus rutilus*	*S. australis*	Papatheodoru, 1981
Ireland	Atlantic salmon*, Salmo salar L.*	*S. parasitica*	Smith, 1994
Norway	Atlantic salmon*, Salmo salar L.*	*S. parasitica*	Langvad, 1994
Scotland	Wild brown trout, *Salmo trutta* L., & Atlantic salmon, *Salmo salar*.L	*S. ferax*	Bruno and Stamps, 1987; Wood and Willoughby, 1986
Spain	Brown trout, *Salmo trutta* L.	*S. parasitica*	Diéguez-Uribeondo *et al.,* 1996
AFRICA			
Nigeria	*Oreochromis niloticus*	*Saprolegnia* spp.	Okaeme *et al*., 1988
South Africa	*Oreochromis mossambicus*	*Saprolegnia* spp.	Oldewage and van As, 1987
AMERICA			
Brazil	Silver mullet*, Mugil curema &* Lebranche mullet*, Mugil liza*	*Saprolegnia* spp.	Conroy *et al*., 1986
Chile	Atlantic salmon, *Salmo salar L.*	*S. parasitica*	Zaror *et al.,* 2004
USA	Channel catfish, *Ictalurus punctatus*, Atlantic menhanden, *Brevoortia tyrannus* ,and Atlantic sturgeon*, Acipenser oxyrhynchus*	*S. parasitica*	Bangyeekhun *et al*., 2001; Dykstra *et al*., 1986; Mueller and Whisler, 1994; Smith *et al.,* 1980; Xu and Rogers, 1991

6.2 SAPROLEGNIA – THE ORGANISM

Species belonging to the genus *Saprolegnia* are often referred to as "cotton mould" because of the characteristic white or grey fibrous patches they form. Current taxonomy puts *Saprolegnia* as a genus in the order Saprolegniales, belonging to the class Oomycetes. The Oomycetes are the largest group of Stramenopiles, and are found worldwide in fresh and salt-water habitats. Figure 1 shows the location of Oomycetes in the tree of life.

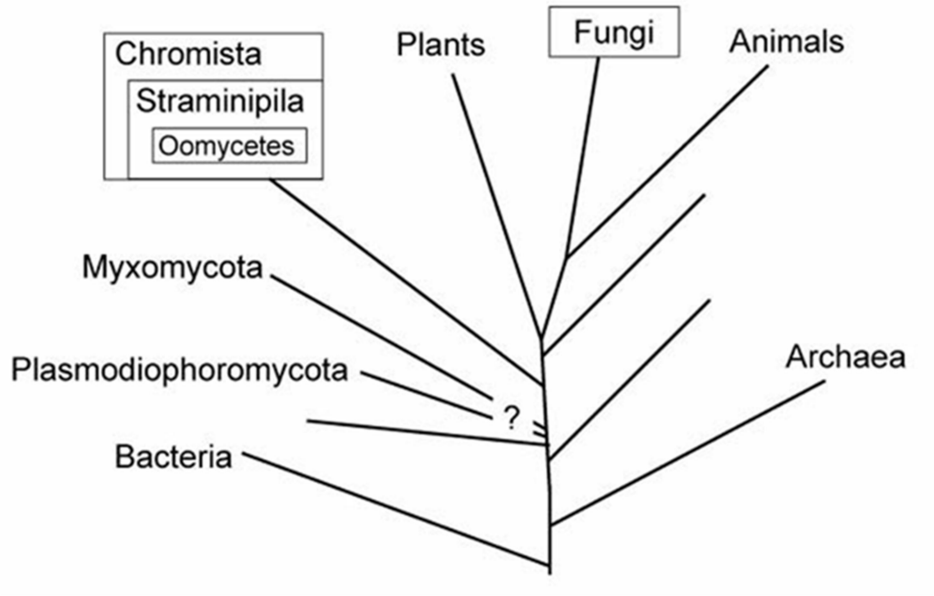

Figure 1. Phylogenetic tree illustrating the approximate relationship between Oomycetes and fungi (Link *et a*l., 2002).

CHARACTERISTICS OF THE OOMYCETES

Oomycetes are fungal-like organisms that are classified as heterokonts (stramenopiles) and are phylogenetically grouped together with diatoms and kelps (Baldauf *et al*., 2000). Oomycetes were long considered to be fungi because they obtain their nutrients via absorption and produce the filamentous threads known as mycelium, characteristic of most fungi. The Oomycetes are now classified as a distinct group based on a number of unique characteristics (Rossman and Palm, 2006). One important distinction is the cell wall composition. In the Oomycetes, the cell walls are composed of beta-glucans and cellulose rather than chitin found in the cell walls of true fungi (Söderhäll and Unestam, 1979). In addition, the Oomycetes produce motile zoospores with

two kinds of flagella. One is a whiplash flagellum oriented posteriorly, while the other tinsel flagellum has a fibrous, ciliated structure and is oriented anteriorly (Figure 2). The occurrence of two kinds of flagella places these organisms in a group known as heterokonts. Although some true fungi, namely the Chytridiomycota, produce stages with motile zoospores, their flagella are only of one kind; the posterior whiplash type (Dick, 1997).

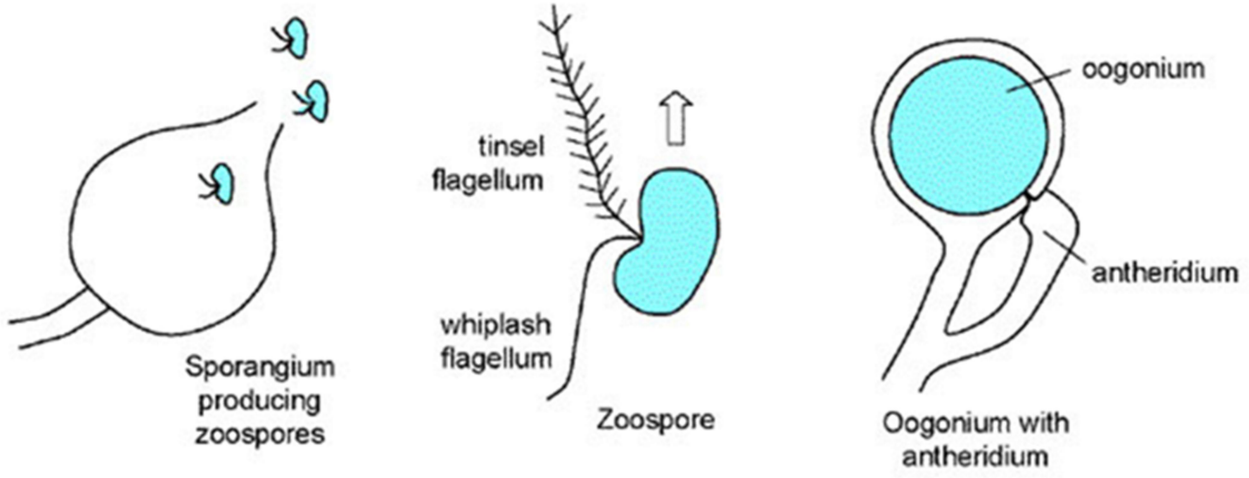

Figure 2. Reproductive structures of the Oomycota (Rossman and Palm, 2006)

Another major difference between the Oomycetes and the true fungi is that the vegetative cells of Oomycetes generally consist of coenocytic hyphae (hyphae without septa, i.e., without cross-walls), which contain diploid nuclei (these organisms exist primarily in a diploid state). This is unlike true fungi in which most of the mycelium is divided into cells by cross-walls, with each cell containing one, two, or more haploid nuclei.

Table 2. Summary of major distinctions between the Oomycetes in the Chromista and the true fungi. (Modified from Rossman and Palm (2006))

Character	Oomycota	True fungi
Nuclear state of vegetative mycelium	Diploid	Haploid or di-karyotic
Cell wall composition	Beta glucans, cellulose	Chitin. Cellulose rarely present
Type of flagella on zoospores, if produced	Heterokont, flagella of two types, one whiplash, directed posteriorly, the other tinsel, fibrous and ciliated, directed anteriorly	Flagella generally not produced, but if produced, usually only one type: whiplash directed posteriorly
Mitochondria	Tubular cristae	Flattened cristae

Transmission electron microscopic (TEM) studies have revealed that Oomycetes have mitochondria with tubular cristae and protoplasmic and nuclear-associated microtubules, while true fungi have flattened mitochondrial cristae. Through the use of TEM, a relationship has been hypothesized between the Oomycetes and the heterokont algae (Alexopoulos *et al*., 1997). Results from a number of studies using molecular sequence data, combined with the ultra-structural similarities, confirm unequivocally that the Oomycetes share a common ancestor with the other members of the heterokont algae or Chromista (Rossman and Palm, 2006).

SECRETORY BEHAVIOUR OF OOMYCETES

Fungi and Oomycetes, although phylogenetically very distantly related, are both osmotrophic microorganisms. This means that they live by secreting enzymes that degrade polymers such as cellulose, lipids, proteins, and lignin, into the external environment and transport the resulting simple sugars, amino acids, and fatty acids into the growing cell for use. Therefore, to a large degree the ecological niche that an Oomycete occupies is defined by the products that it secretes, be they small molecules such as toxins, proteinaceous effectors to perturb host signalling or metabolism, or the array of hydrolytic enzymes that digest extracellular macromolecules into smaller subunits that the fungus can use (Soanes *et al*., 2007). Due to their osmotrophic nature, plant pathogenic Oomycetes are able to breach intact surfaces of host plants, rapidly establishing infections that can have disastrous consequences for large-scale agricultural production. The virulence gene families of plant pathogenic Oomycetes encode numerous hydrolytic enzymes for degradation of plant carbohydrates, extracellular toxins such as NLP and PcF toxins, and at least three families of cell-entering effector proteins, RXLR effectors CHXC effectors and crinkler proteins (Kamoun, 2006; Schornack *et al*., 2009; Tyler, 2009; Tyler *et al*., 2006).

LIFE CYCLE OF *SAPROLEGNIA* SPECIES

Saprolegnia is homothallic, meaning one single individual contains both male and female sex organs, and has a diploid life cycle, which includes both sexual and asexual reproduction (Kanouse, 1932; Noga, 1993). In the sexual phase male and female gamentagium, antheridia and oogonia respectively, are produced, and these fuse to allow for fertilization. The zygote produced is named an oospore (Figure 3, acquired from Ali, 2014).

Asexual reproduction may occur by the production of chlamydospores or gemmae, but generally, zoospores are produced. Motile primary zoospores, produced by the zoosporangium encyst, and may germinate to produce a vegetative growth or a secondary motile zoospore. Germination produces a hypha, which may develop into a mycelium (Bruno and Wood, 1994). The secondary zoospore has the ability to remain motile for up to a few days, after which it encysts to produce a secondary cyst, which is also known as "cytospore", "encysted zoospore" or "zoospore cyst".

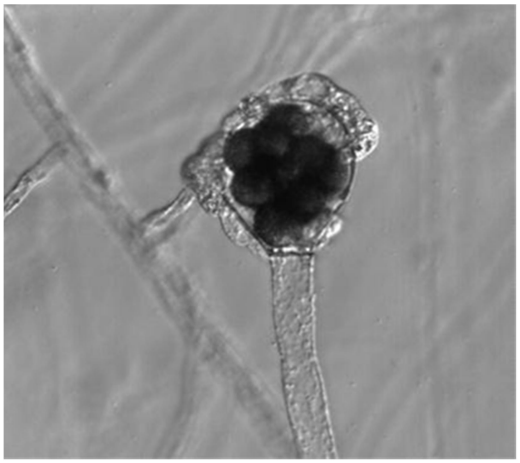

Figure 3. *Saprolegnia* oogonia containing oospores. Oogonium and antheridium morphology is among the criteria for identification (Ali, 2014)

The secondary cyst usually germinates into new mycelium on which sexual reproduction occurs, restarting the reproduction cycle. It may also release new secondary-like zoospores which are able to encyst again. The repeated cycles of zoospore encystment and release of secondary zoospores and cysts respectively are called polyplanetism or repeated zoospore emergence (RZE) (Diéguez-Uribeondo *et al*., 1994). By helping the Oomycete to make several attempts at locating a suitable culture medium to live on before settling down for good, polyplanetism contributes to the pathogenicity of *Saprolegnia* (Beakes, 1983). This and the fact that the secondary zoospores are more motile than the primary zoospores and also motile for a longer period, justify the premise that the secondary zoospores are the main dispersion phase in the life cycle of *Saprolegnia* (Pickering and Willoughby, 1982; van den Berg *et al*., 2013). For some species, the secondary zoospores develop boathooks that are presumed to aid in attachment to fish skin or promote floating (buoyancy) in water, to decrease sedimentation rate (Beakes *et al*., 1994) and for fungal-host recognition response (Beakes, 1983). van West (2006) illustrated the life cycle of *Saprolegnia* species very well, as shown in figure 4.

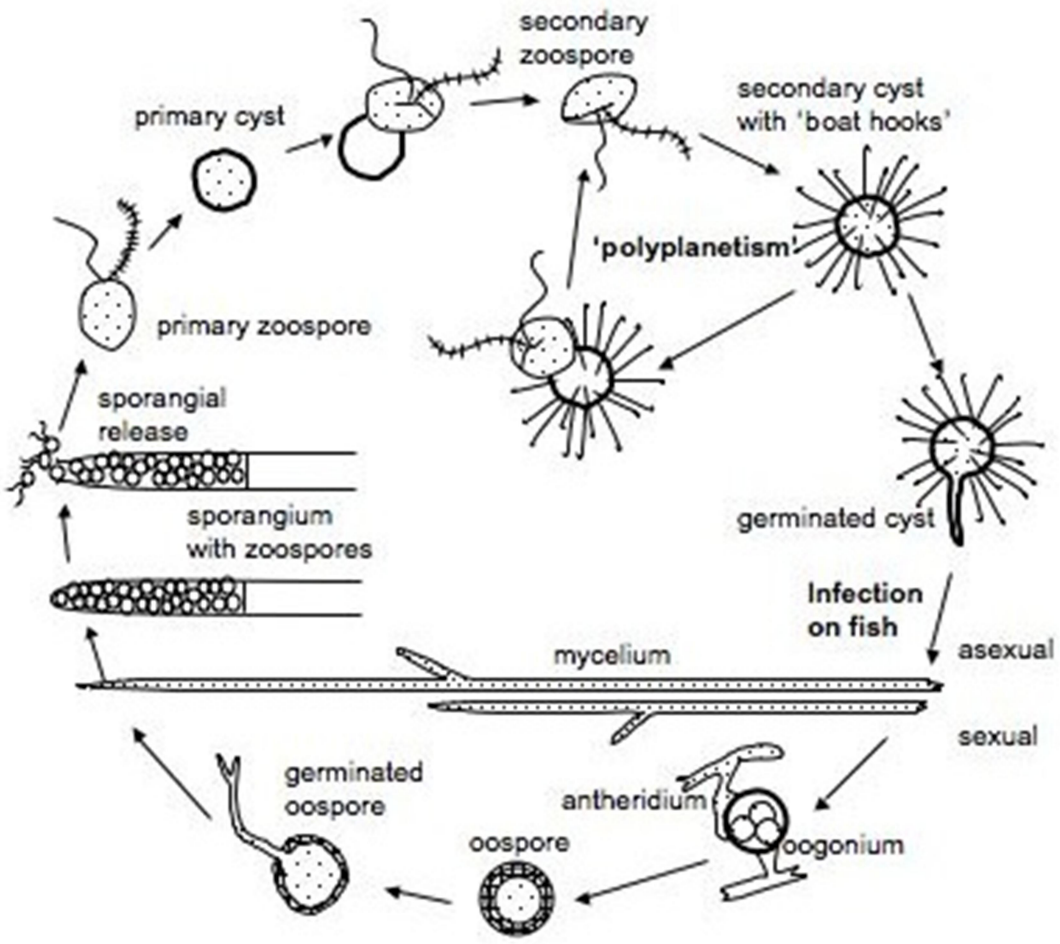

Figure 4. The life cycle of *Saprolegnia* spp. includes both sexual and asexual reproduction (van West 2006)

Classically, species of the genus *Saprolegnia* were differentiated on the basis of their morphological features, their sexual reproductive structures, oogonia and antheridia being the most important (Cocker, 1923; Seymour, 1970). Identification of isolates that do not produce sexual reproductive structures may thus be difficult. Lack of such structures is common in *S. parasitica*, the species considered most pathogenic to fish. Nowadays, several additional criteria have been introduced to help differentiate, especially the non-sexual isolates assigned to *S. parasitica* and *S. diclina*. Beakes *et al*. (1994) and Dieguez-Uribeono *et al.* (2007) asserted that differentiation of former members of the *S. parasitica*/*S. diclina* complex should be based on factors such as cyst coat ornamentation, germination pattern and the substrate from which the isolate originates. Hulvey *et al*. (2007), on the other hand, proposed that the differentiation should

be based on a multifactorial approach, including zoosporangial discharge, papillae morphology and sequencing of the internally transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA). There is presently no consensus on a standard method for resolving the *Saprolegniae* taxonomical problem in spite of the invaluable contribution of the afore-mentioned authors. However, scientists are engaging in research activities that will ultimately lead to a resolution of *Saprolegnia* taxonomy, which would make the definition of species less of a challenge (Beakes, personal communication). Table 3 shows taxonomic classification of *Saprolegnia* (Bruno and Wood, 1999).

Table 3. Taxonomic classification of *Saprolegnia* (Ali, 2014)

Taxonomic classification
Kingdom: Straminipila
Division: Oomycota
Class: Oomycetes
Order: Saprolegniales
Family: Saprolegniaceae
Genus: Saprolegnia

IMPORTANCE OF *SAPROLEGENIA* AND OTHER OOMYCETES

Few microorganisms match the impact that the Oomycetes have had on mankind, especially some plant pathogenic Oomycetes. Although different in their selection of host organisms, plant and fish pathogenic Oomycetes have many features in common (Banfield and Kamoun, 2013). Evidently, the formation of specialized spore structures including zoospores, sporangia and oospores are similar. Infection strategies are also comparable to some extent, involving encystment and attachment of zoospores on host surfaces, and penetration of host tissues.

Many Oomycetes are pathogenic to animals, from freshwater fish and crustaceans to mammals, such as livestock, pets, and humans (Phillips *et al*., 2008). Examples include pathogens from the

genera *Aphanomyces, Achlya, Saprolegnia, Phytophthora* and *Pythium*, (de Cock *et al*., 1987; Mendoza *et al*., 1993); (Cerenius and Söderhäll, 1996).

Phytophthora infestans is a species for which Oomycetes are very well known. This species is actually considered the most notorious Oomycete pathogen. *P. infestans* was responsible for nineteenth century Irish potato famine. In addition, it is a devastating pathogen of several other cultivated and wild plants (Lamour and Kamoun, 2009; Yoshida *et al*., 2013).

Pythium is a genus of parasitic Oomycetes. Most species are plant parasites, but *Pythium insidiosum* is an important pathogen of animals. Even if their host range tends to be rather unspecific, *Pythium* spp. have a devastating impact on crops of economic importance worldwide (Bala *et al*., 2010). *Pythium insidiosum* causes pythiosis, a life-threatening infectious disease. It is the only *Pythium* species of the kingdom Stramenopila known to infect mammals, such as humans, horses, dogs, cats, and cattle, in tropical and subtropical countries (Kaufman, 1998; Mendoza *et al*., 1996). *P. insidiosum* inhabits swampy areas, where it exists in two stages: perpendicular branching hyphae and biflagellate zoospores (Mendoza *et al*., 1993). Infection has been proposed to occur by invasion of the zoospores into host tissue after attachment and germination (Mendoza *et al*., 1993).

Aphanomyces astaci is destructive of crayfish populations in natural environments and is mentioned in the list of the 100 most unwanted alien invaders in the world (McGrath and Farlow, 2005).The pathogen invades the soft parts of the exoskeleton of freshwater crayfish, especially the soft cuticle between the segments or in the limb joints (Nyhlen and Unestam, 1980; Unestam and Weiss, 1970)

Aphanomyces invadans is implicated in mass mortalities of cultured and wild fish in many countries. The disease is referred to as epizootic ulcerative syndrome (EUS) (Baldock *et al*., 2005), but is also known as mycotic granulomatosis, red spot disease and ulcerative mycosis. Typical symptoms of EUS are significant ulceration of the skin and necrosis of muscle with extension to subjacent structures including abdominal cavity and cranium, and the disease is characterized by high mortality (Baldock *et al*., 2005; Callinan *et al.*, 1995; Lilley *et al*., 1998; Lilley and Roberts, 1997).

***Achlya** species* are major pathogens of many fish species (Jeney and Jeney, 1995). They can be found in most freshwater habitats and are responsible for significant infections of both living and dead fish as well as incubating eggs (Kales *et al*., 2007).

Saprolegnia species are, as already stated however, the most important Oomycetes when it comes to infections of fish and their eggs. Saprolegniasis is a major disease problem in different wild and farmed fish species, including salmonid species such as Atlantic salmon, rainbow and brown trout, and non-salmonid species including perch, eels and catfish (Bruno *et al*., 2010). Declines of natural salmonid populations have partly been attributed to *Saprolegnia* infections (Phillips *et al*., 2008). Saproleginasis of egg masses can have important ecological consequences. For example, Saprolegniaceae attacking amphibian eggs have been implicated in population declines of at least one toad species, the Western toad *Bufo boreas* Baird and Girard (Beakes *et al*., 1994). Rapid growth of *Saprolegnia* spp. can destroy a major part of incubated eggs (Lartseva, 1986; Lartseva and Altufiev, 1987) or devastate them completely (Dudka *et al*., 1989; Sati and Khulbe, 1981).

6.3 PATHOGENESIS OF *SAPROLEGNIA*

FACTORS PLAYING A ROLE IN PATHOGENICITY

Pathogenicity of *Saprolegnia* spp. cannot be attributed to one single factor, but is a result of a combination of several factors (Thoen *et al*., 2011). In addition to factors that predispose fish and fish eggs to disease, the outcome of infection can vary greatly owing to differences in the pathogenicity of individual strains of *Saprolegnia* (Stueland *et al.*, 2005). Environmental changes or seasonal variations are known to influence the growth, reproduction, and intensity of *Saprolegnia* infections. In addition, the occurrence of saprolegniasis may be related to water quality, temperature as well as physiological changes and the immune response of fish. The association of *Saprolegnia* infection with sexual maturation and a similar increase in susceptibility to some common skin parasites (e.g. *Ichthyophthirius* and *Trichodina*) are documented (Pickering and Christie, 1980). Furthermore, the increase in susceptibility of male salmonids to *Saprolegnia parasitica* during sexual maturation appears partly related to increased

levels of androgen which result in reduced numbers of mucous cells in the epidermis (Pickering, 1994). Other infections, mechanical damage, poor hygiene and social interaction are also well-known risk factors (Neish and Hughes, 1980; Noga, 1993; Pickering, 1994). *Saprolegnia* spores may be transmitted by hatchery fish, wild fish, eggs, water supplies, and equipment (Bruno and Wood, 1999).

Saprolegnia was previously often considered an opportunistic pathogen that was saprotrophic and necrotrophic (Bruno and Wood, 1999). However, it has become quite clear that some *S. parasitica* strains are highly virulent and able to cause primary infections of salmon (Neish, 1977; Whisler, 1996). Infections occur on both eggs and fish. On eggs, the infection is manifested by profuse mycelial growth on the egg surface resulting in rapid death. On fish, *Saprolegnia* invades epidermal tissues and can infect the entire surface of the body and gills (Willoughby, 1994). It causes cellular necrosis as well as dermal and epidermal damage, which ultimately leads to death by haemo-dilution (Hatai and Hoshiai, 1994; Pickering and Willoughby, 1982). Saprolegniasis is characterized by white or grey patches of filamentous mycelia on the host (Hatai and Hoshiai, 1992). The patches may consist of one or more species of *Saprolegnia* (Pickering and Willoughby, 1982; Whisler, 1996). The infection initially appears on epidermal tissues of the head, tail and fins (van West, 2006) and subsequently spreads to the rest of the body. Areas with lesions may be soft, necrotic and ulcerated, and the surrounding areas may show oedema and necrosis (Gieseker *et al.*, 2006). Severe *Saprolegnia* infections result in lethargic behaviour, loss of equilibrium and death of the fish (Bruno, 1996; Pickering and Willoughby, 1982). Respiratory difficulties may also feature when infection is associated with the gills (Bruno and Stamps, 1987).

Suppression of host defences is likely to play a critical role in *Saprolegnia* pathogenesis, just like in biotrophic plant pathogenic Oomycetes, such as *Peronospora* and several *Phytophthora* species (Torto-Alalibo *et al*., 2005). In fish, *Saprolegnia* causes cellular necrosis or dermal and epidermal damage (Pickering and Willoughby, 1982), involving the penetration of hyphae into the basement membrane (Bruno and Wood, 1999). This results in tissue destruction and loss of epithelial integrity (Bruno, 1996). However, *Saprolegnia* does not appear to be tissue specific (Neish, 1991). *Saprolegnia* triggers a strong inflammatory response in its host (i.e. induction of IL-1β1, IL-6 and TNF-α) while severely suppressing the expression of genes associated with

adaptive immunity in fish, through down-regulation of T-helper cell cytokines, antigen presentation machinery and immunoglobulins (Belmonte *et al.*, 2014). When *Saprolegnia* invades eggs, mycelial growth results in death of the embryo and spreads to the adjacent eggs (van West, 2006).

Saprolegniasis in an estuarine fish species, the Atlantic menhanden *(Brevoortia tyrannus)*, has been shown to be characterized by deep skin lesions which often involve the internal organs and include an intense inflammatory reaction (Dykstra *et al*., 1986; Noga *et al*., 1988).

Through analysis of small sets of Expressed Sequence Tags (EST) data of *S. parasitica* some researchers have revealed the presence of secreted protein families with potential roles in virulence such as glycosyl hydrolases, proteases, and protease inhibitors, as well as proteins involved in protection against oxidative stress (Torto-Alalibo *et al*., 2005; Van West *et a*l., 2010). The data set included a host-targeting protein SpHtp1 (*S. parasitica* host-targeting protein 1) that was subsequently demonstrated to enter fish cells through binding to a tyrosine-O-sulfated fish cell surface ligand (Wawra *et al*., 2012).

Willoughby (1989) asserted that fish have three types of defences against *Saprolegnia*. The first one involves the physical removal of attached spores by the continual renewal of mucous. Second, a powerful morphogen was detected in the external mucous, inhibiting the mycelium growing from the *Saprolegnia* spores but not killing it. A *morphogen* was described as a substance governing the pattern of tissue development in the process of morphogenesis, and Fevre (1977) had previously conducted research, which confirmed the role of these 'morphogen' enzymes in *Saprolegnia* hyphal morphogenesis. Third, a cellular immune response in the mucous is directed at growing mycelium. Therefore, the continuous replenishment of the mucous layer serves as a primary physical barrier (Bruno and Wood, 1999; Pickering, 1994; Pickering and Willoughby, 1982) although not for complete, i.e., 100%, removal of *Saprolegnia* spores (Murphy, 1981; Willoughby and Pickering, 1977). However, an intact epidermis is probably the best defence against saprolegniasis (Hatai and Hoshiai, 1994; Pickering, 1994).

Histological changes resulting from *Saprolegnia* infection in fish include loss of integrity of the integument, oedema and degenerative changes in the muscle mass. More severe lesions show deeper myofibrillar and focal cellular necrosis, spongiosis or intracellular oedema and sloughing

of the epidermis (Copland and Willoughby, 1982). It has been established that there is a relationship between the time necessary to cause the death of the fish and the time taken by the Oomycete to invade and destroy vital zones (Nolard-Tintiger 1973).

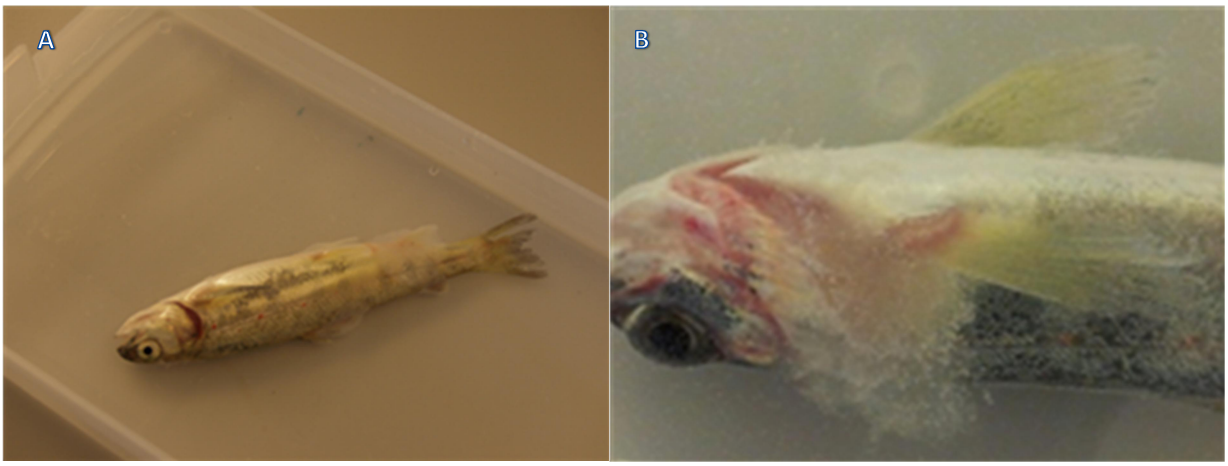

Figure 5. Atlantic salmon parr infected with *Saprolegnia parasitica*, showing cotton wool-like tufts of mycelium on: : A) the dorsal and ventral fins B) the head region, affecting the gills.

EGG SAPROLEGNIASIS

The susceptibility of fish eggs to Oomycetes and other infections is often dependent on water quality, water flow rates, and egg loading densities (Post, 1983). High density egg incubation systems promote *Saprolegnia* infections in several ways. Water flow through a culture system is often less than that required to prevent attachment of naturally occurring *Saprolegnia* zoospores to eggs. Over 10% of the salmonid eggs get infected with *Saprolegnia* spp. in hatcheries each year (Bruno *et al*., 2010). Studies of the pathogenesis of saprolegniasis in salmonid eggs are scarce (Kitancharoen and Hatai, 1996; Thoen *et al*., 2011)). The major *Saprolegnia* species infecting fish eggs is *S. diclina* (Hussein *et al*., 2001). Other *Saprolegnia* species that have been incriminated in saprolegniasis of fish eggs include *S. australis* (Czeczuga and Muszyńska, 1999*;* Hussein *et al., 2001)*, *S. ferax* (Cao *et al*., 2012) and *S. parasitica* (Czeczuga and Muszyńska, 1999; Songe *et al*., 2015).

Figure 6. Infected eggs entangled in cotton-wool like mesh (Courtesy of Shimaa Ali).

All vertebrate oocytes are surrounded by an acellular coat, an egg envelope, or chorion. This envelope is a relatively thick, proteinaceous, extracellular matrix that protects the egg and the developing embryo. The egg envelope is very useful during fertilization and in prevention of polyspermy (Wassarman, 1988).

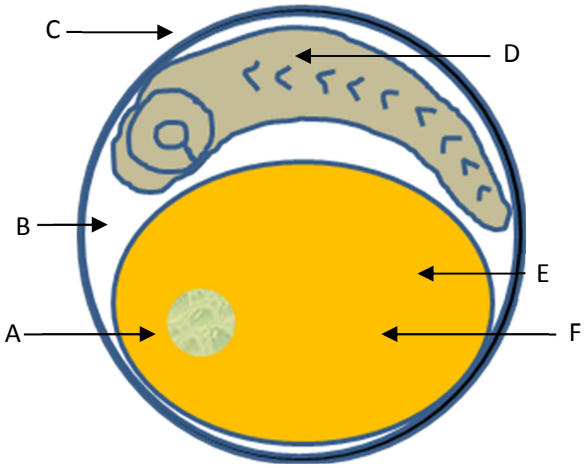

Figure 7. Diagram of a fish egg:

A) oil globule, B) perivitelline space, C) chorion, (D) embryo, E) vitelline membrane, F) yolk.

The chorion plays a very important role in protecting the egg, and hence the developing embryo against infection and mechanical injury. Therefore, according to the behaviour after ovulation, fish generate two kinds of eggs: a) pelagic highly hydrated eggs that float in seawater; b) demersal non-buoyant eggs, generally in freshwater, which attach to plants or substrate. The oocyte envelope structure is related to environmental conditions. Generally, fish that spawn pelagic eggs are non-adhesive and smooth, with poorly ornamented envelopes, whereas those that place their eggs over plants or on the bottom have sticky and ornamented eggs (Rizzo *et al.*, 2002).

Thoen *et al.* (2011) observed that live eggs were refractory to infection with *Saprolegnia* spores in suspension, and that infection of live eggs could only occur from an infection nucleus represented by dead eggs or debris. The chorion of live eggs thus seems to represent an effective barrier against the germinating spores of *Saprolegnia* spp. To what extent, and whether this is simply mechanical or a combination of mechanical and enzymatic defence in the egg envelope, as proposed by Kudo & Teshima (1991), remains unknown.

Different mechanisms of pathogenesis by *Saprolegnia* and other Oomycetes when they infect salmonids and their eggs have been proposed. Peduzzi & Bizzozero (1977) detected a chymotrypsin-like enzyme system in culture filtrate and mycelial extracts from watermolds associated with saprolegniasis in fish. They observed that an extracellular proteolytic enzyme produced by the Oomycete would favour the deep penetration by invading hyphae into the host tissue.

The fact that *Saprolegnia* spreads from dead to live eggs via positive chemotaxis (Bruno and Wood, 1999) means that some chemical signal from the live eggs causes the Oomycete to move towards them (Lawrence, 2000). When first established, *Saprolegnia* produces further zoospores which infect more eggs. Therefore, it is important to continuously remove dead eggs from the incubation units in the hatchery (Kitancharoen et al., 1997).

6.4 CONTROL AND TREATMENT OF SAPROLEGNIASIS

Over the years, a number of control measures have shown some promise in alleviating the economic burden caused by *Saprolegnia* infections, especially under experimental conditions. In practice none have proven to be anywhere near as efficacious as Malachite Green (MG), an antifungal agent whose biocidal effects were discovered by Foster and Woodbury (1936). MG proved very efficient in the control of all infectious stages of *Saprolegnia* spp. and was later on implemented as a standard treatment towards both saprolegniasis and external parasites. Therefore, *Saprolegnia* infections were manageable during the development of salmonid aquaculture until the 1990s, when evidence of potential risks to humans linked to the substance began to surface (Alderman and Clifton-Hadley, 1993; Fernandes *et al*., 1991; Rao, 1995). Malachite green was subsequently banned for use in production of fish for human consumption in

the European Union in 2000 (Hernando *et al*., 2006; Srivastava *et al*., 2004; Sudova *et al*., 2007) due to the risks it poses to the consumers of treated fish. In the United States, the use of malachite green is not approved for use by the Food and Drug Administration (Hernando *et al*., 2006).

As a result of the ban, there has been an increased incidence of *Saprolegnia* infections in aquaculture world-wide, causing huge economic losses (Hatai and Hoshiai, 1992; van West, 2006). Currently, there are no drugs available that are at the same time efficient and safe for the fish host and for human consumption of treated fish (van West, 2006). Consequently, scientists have intensified their efforts at the in-depth analysis of the biology of *Saprolegnia* and its host with a research focus towards the development of novel and sustainable control measures of *Saprolegnia* infections to support the aquaculture industry.

Conservative measures like removal of dead eggs and other debris have become more and more common as routine procedures and have proven effective in hatcheries, although laborious for large egg batches with some background mortality. However, this procedure is not applicable during the initial incubation in the closed, large incubation cylinders used in breeding stations, which still requires water treatment by bronopol or formalin.

In a bid to fight saprolegniasis, a wide array of control measures has been scrutinized, including the use of chemical additives, which is the most commonly employed method, and different mechanical, biological or physical filtration methods. Once a *Saprolegnia* infection has been established, central hyphae are protected by the resulting mycelium or even by the integument of the fish host. Also, the spore stages are more sensitive to chemical treatment than hyphae, probably mediated by the chitin and cellulose-containing cell walls (Willoughby and Roberts, 1992). Therefore, prophylactic treatment targeting the elimination of the spore stages has proved to be the most effective.

The use of sand filters; UV irradiation and ozonation have proved to be very effective systems of prevention of saprolegniasis. The latter two have been shown to significantly increase the hatching rate of fish eggs in experiments (Forneris *et al*., 2003). Although these alternatives are merely prophylactic and are not effective against established infections in eggs or fish, they are well appreciated by egg producers and hatcheries, as they are also relatively efficient at eliminating bacteria and viruses. Formaldehyde is currently one of the most frequently used

disinfectants for prophylaxis in the embryonic period and the first stages of larval development. The use of formaldehyde, however, does not eliminate the problem of environmental impact, if one considers the sensitivity of fluvial and lacustrine ecosystems that fish farms affect, where even minimum changes of the chemical characteristics of the waters can cause trauma for the ecosystem, destroying plants or bacterial filters (Stuart, 1983). Moreover, formaldehyde is suspected to have carcinogenic risk to humans as indicated by (Department of Health and Human Services, 1994).

Sodium chloride has been shown to reduce saprolegniasis in both experimental and practical conditions. It significantly reduces *Saprolegnia* growth at concentrations from 0.5 % (Bly *et al*., 1996; Edgell *et al*., 1993; Marking *et al*., 1994; Schreier *et al*., 1996; Waterstrat and L.L., 1995). Both parr and broodstock in the freshwater phase tolerate salinities up to approximately 1.5 % well, giving a therapeutic window ranging from 0.5-1.5 %.

Hydrogen peroxide has been shown efficient in treating eggs of rainbow trout, *Oncorhynchus mykiss*, infected with *Saprolegnia* spp. during incubation (Barnes *et al*., 1998).

Herbal products such as D-limonene, neem seed extract, tea tree oil, eugenol, hinokitiol, citral and allyl-isothiocyanate have been shown to have an antifungal activity against *Saprolegnia* and other Oomycete fish pathogens like *Aphanomyces* and *Achlya* (Campbell *et al*., 2001; Hussein *et al*., 2002; Mori *et al*., 2002).

The antimycotic activities of eugenol, a major essential oil of clove, against some fish pathogenic species of Saprolegniaceae, including *Saprolegnia* spp., was investigated by Hussein *et al* (2000). They confirmed that it was fungicidal against the pathogens in question but also highly toxic to salmonids.
Currently in Norway and the European Union, bronopol (Pyceze®) is one of the most frequently used remedies. In their study, Pottinger and Day (1999) found bronopol to be effective in reducing or preventing mycotic infection in challenged fish.

A few modified chitosans exert effective fungistatic action against *S. parasitica* (Muzzarelli *et al*., 2001).; thymoquinone from *Nigella sativa* has inhibitory effects on pathogenic *Saprolegnia* (Hussein *et al*., 2002). Thymoquinone was found to be toxic to salmonids.

Ali *et al.* (2014) showed that Boric Acid has a good potential as a prophylactic measure and also a curative intervention against *Saprolegnia* infection in fertilized eggs and yolk sac fry of Atlantic salmon.

In laboratory settings, probiotic strategies employing bacteria thought to have inhibitory effects on *Saprolegnia* (Bly *et al*., 1997; Hussein *et al*., 2001), and the application of invertebrates like *Gammarus pseudolimnaeus* and *Asellus militaris* Oseid (1977) have proven effective. However, they have not been adapted in commercial fish farming. More recently, Liu *et al.* (2014) conducted a study in which they demonstrated that even in the presence of virulent *Saprolegnia* isolates, salmon egg samples showed a low incidence of saprolegniasis which was strongly correlated with a high richness and abundance of specific commensal Actinobacteria. In particular, the genus *Frondihabitans* (*Microbacteriaceae*) was observed to effectively inhibit attachment of *Saprolegnia* to salmon eggs. These are valuable findings highlighting that fundamental insights into microbial landscapes of fish eggs may provide new sustainable means to mitigate emerging diseases.

6.5 METHODS FOR ISOLATION, PURIFICATION, IDENTIFICATION AND CHARACTERIZATION OF *SAPROLEGNIA* SPP.

One primary difficulty encountered when investigating Oomycete diseases of fish is isolation of the pathogen. Many *Saprolegnia* infections occur in the dermis and therefore more than one species may easily occur in lesions at the same time (Pickering and Willoughby, 1982). Numerous ways have been devised to isolate and culture members of the Saprolegniaceae. The methods have had one of two purposes, namely, the isolation of a single species of Oomycete, that is growth of a uni-fungal culture, or propagation of axenic (pure) cultures. It is of utmost importance that pure cultures are used for identification and storage.

ISOLATION OF *SAPROLEGNIA* SPECIES

In general, the various methods of propagation of *Saprolegnia* spp. involve either some mechanical manipulation or barrier, such as a sterile glass or plastic ring, or the incorporation of chemicals to suppress contaminants, or both.

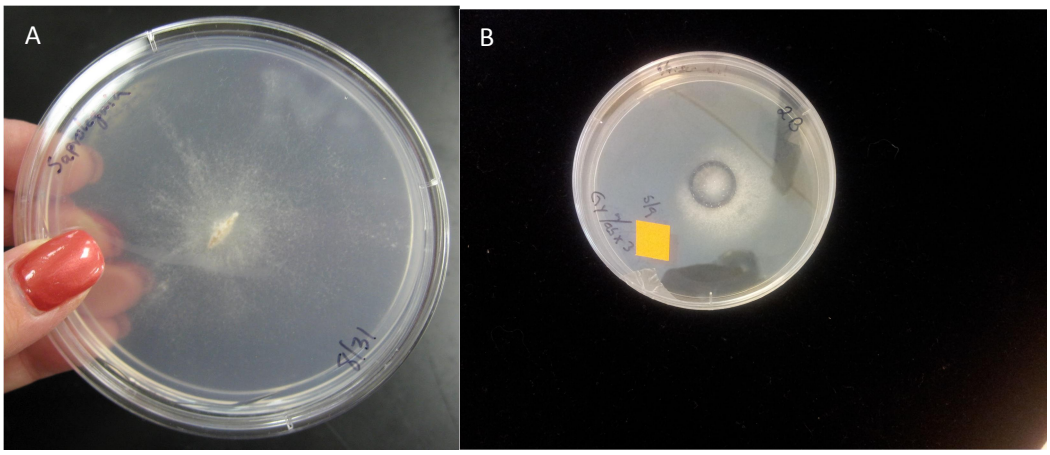

Figure 8. Isolation and purification: A) Isolation of *Saprolegnia* species by placing an infected small fish directly onto GY agar. B) Purification process of *Saprolegnia* isolate by placing a sterile glass ring, partially submerged in agar.

Isolation of *Saprolegnia* from fish

Saprolegnia spp. are usually isolated from infected organisms simply by gently rubbing sterile swabs into *Saprolegnia* infected areas on live or newly euthanized eggs or fish suffering from saprolegniasis. The swabs are incubated in a broth, e.g. Glucose-Yeast (GY) broth (Yuasa *et al*., 1997), at 21 ± 1°C for 1-2 days. Hyphae from the advancing edge of the growing colony are cut and sub-cultivated inside a glass or plastic ring, partially submerged in agar (Hatai and Egusa, 1979), and incubated at 21 ± 1°C for 2-4 days. When *Saprolegnia*-like hyphae appear outside the glass ring, they are removed with a small block of agar and then transferred to sterilized tap water (STW) for zoospore production. The isolate is free of bacteria if asepsis has been maintained, and single spore isolation can be performed on agar.

Another technique for isolation of *Saprolegnia* uses baits, i.e. hempseeds. However, this method has the same disadvantage as spore-filtration (see beneath). Isolation from infected fish skin, or in extreme cases, dissection of small parts of muscle from infected fish, and incubation on agar, is therefore often preferred by scientists in order to ensure isolation of pathogenic strains (Stuelend, 2009). Infected mucus transferred to GY-agar is another method to ensure the presence of pathogenic strains.

Isolation of *Saprolegnia* from eggs

Isolation of *Saprolegnia* from eggs is commonly performed by placing the whole infected egg on an agar (Potato dextrose agar (PDA) (Petrisko *et al*., 2008) or Glucose Yeast extract (GY) agar consisting of 1 % glucose, 0.25 % yeast extract and 1.5 % agar (Hatai and Egusa 1979) plate supplemented with antibiotics, and then leaving it to incubate for about a week. Thereafter, an agar-plug of 5 mm in diameter, colonized by *Saprolegnia* hyphae, is cut from the agar plate and transferred to another plate containing agar supplemented with antibiotics. The growing hyphae are then cut into small pieces and transferred to sterile aquarium water (SAW) for zoospore production.

PURIFICATION OF *SAPROLEGNIA* ISOLATES

Purification of cultures is performed from GY agar with antibiotics incubated at 21±1°C for 2-5 days. *Saprolegnia* hyphae are then cut from colonized GY agar plates and incubated in GY broth at 21 ± 1°C for 2 days for further hyphal growth. These procedures are repeated until pure cultures are obtained (Onions *et al*., 1981).

SPORE FILTRATION AND SINGLE SPORE ISOLATION

Bundles of young hyphae in pure culture are subsequently washed twice in sterilized distilled water (SDW), transferred to a glass bottle containing STW and then incubated at 21 ± 1°C for 24 hours for zoospore production. The zoospore suspensions are filtered through a sterilized tea filter (0.5 mm pores) into another glass bottle. This procedure mainly results in encysted zoospores. In order to make germlings, 100µL of the cyst suspension are inoculated on GY agar at 21 ± 1°C for 24 hours. The plates are then inspected on a stereo-microscope, and one single germling is cut out of the agar and transferred to a new agar plate to make a single-spore culture (Stueland *et al.*, 2005).

The disadvantage of isolation by use of spore filtration from water is the failure of segregation between saprophytic and parasitic species of *Saprolegnia.*

IDENTIFICATION AND STORAGE OF *SAPROLEGNIA* SPECIES FROM SALMONIDS

Most scientists store *Saprolegnia* strains on hemp seeds in STW at 4 °C, and then transfer isolates to new hempseeds and STW once a year. To prepare hempseeds for storage of the single-spore isolates, some researchers boil the seeds for 10-20 minutes to rupture the ovary and seed

coat, followed by sterilization (Emerson, 1958). Others find it sufficient to boil the seeds in distilled water for 1-2 minutes to soften the seed coat and then cut the seed transversely into two halves with a razor blade. They then air-dry the cut seeds for 30-60 minutes, place them on filter paper in a Petri dish and finally sterilize the dried halves by autoclaving at 121 °C for 8-12 minutes (Johnson, 1973). Neish (1976) noted that a whole hempseed, punctured with a needle seemed to enhance oogonal production in some isolates from fish.

Morphological identification methods

The type of zoospore discharge is the basis for morphological identification to the genus *Saprolegnia*. Characteristically, the primary zoospores emerge one by one in rapid succession through one exit pore in the sporangium. After a period of motility, the zoospores encyst (Fig 4). Under normal cultural conditions, this manner of discharge is typical for all *Saprolegnia* species (Seymour, 1970), and is the fundamental feature that serves to separate *Saprolegnia* from related genera.

The size and configuration of zoosporangia and asexual reproduction vary considerably within the same species, making identification to species level more complicated. Since vegetative and asexual reproduction are too variable to be considered as diagnostic for delimiting features, Seymour (1970) considered the female (oogonium) and male (antheridium) (Fig 4) reproductive elements to provide the only morphological character upon which one may hope to separate the various members of the genus. Sexual reproduction is accompanied by the fertilization of an oospore by the contents of the antheridial cell entering the oogonium through a fertilization tube. The number of oospores in the oogonium, ornamentation of the oogonial wall, and the type of antheridial attachment are some of the criteria used in the identification process. To induce sexual reproduction in *Saprolegnia*, hyphae are left in low nutrient STW for days, and sometimes several weeks, for oogonia and antheridial production. It is possible to characterize most *Saprolegnia* isolates to species level by this method. Due to low sexual reproduction in the laboratory, however, species identification of many fish-lesion isolates using traditional morphological criteria is difficult. This is one of the main reasons why taxonomy and specific identification of *Saprolegnia* species traditionally is considered to be complex and sometimes

confusing. Therefore, alternative morphological identification methods of fish-pathogenic isolates have been developed. The following are some of the characteristics of these isolates:

- Indirect germination
- Presence of repeated zoospore emergence
- Presence of long, hooked hairs in bundles on the secondary zoospore cysts
- Fast-running esterase iso-enzyme bands

The characterization of parasitic isolates of *Saprolegnia* using traditional taxonomic criteria has proven problematic (Diéguez-Uribeondo *et al*., 2009; Johnson Jr *et al*., 2002; Sandoval-Sierra *et al.*, 2013). In Saprolegniales, morphological, physiological, and pathological characters seem to provide little information for species determination (Dieguez-Uribeondo *et a*l., 1995; Sandoval-Sierra *et al*., 2013). Physiological characters, however, are sometimes used to differentiate individual degrees of pathogenicity and host specificity (Diéguez-Uribeondo *et al*., 1996; Dieguez-Uribeondo *et a*l., 1995; Holub *et al*., 1991; Neish, 1976; Wilioughby and Copland, 1984; Willoughby, 1978). Traditionally, the generic definition was mainly based on the asexual characters, especially the mode of zoospore discharge, and species differentiation was mainly on the features of sexual reproductive organs including oogonia, antheridia, antheridial origin and oospores. However, many of these characters may be variable in one species or similar in different species, and some strains either lose the ability to produce sexual reproduction or cannot be induced to form them under laboratory conditions. So, it is difficult to make definitive identification only using traditional morphological criteria to species level (Xiao-Li *et al*., 2010).

Molecular identification methods

DNA fingerprinting relies on detecting unique patterns in the DNA of a specific organism (Duncan and Cooke 2002) and several techniques and molecular methods have been developed in order to generate highly reproducible and detailed fingerprints. A number of these methods have also proved to be effective when describing Oomycetes. Random amplification of polymorphic DNA (RAPD) (Huang *et al* 1994), Restriction Fragment Length Polymorphism (RFLP) (Molina *et a*l., 1995) and sequencing of the Internal Transcribed Spacer (ITS) regions of rDNA (Lilley *et*

al 2003, Dieguez-Uribeondo *et al* 2007) are some techniques that have been used successfully on Oomycetes.

- **The Amplified Fragment Length Polymorphism (AFLP)** technique has the capability to detect various polymorphisms in different genomic regions simultaneously. It is also highly sensitive and reproducible. As a result, AFLP has become widely used for identification of genetic variation in strains or closely related species of plants, fungi, animals and bacteria. The AFLP technology has been used in criminal and paternity tests, population genetics to determine slight differences within populations, and in linkage studies to generate maps for quantitative trait locus (QTL) analysis. There are many advantages of AFLP when compared to other marker technologies including randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and microsatellites. AFLP not only has higher reproducibility, resolution and sensitivity at the whole genome level compared to other techniques (Mueller and Wolfenbarger, 1999), but it also has the capability to amplify 50 and 100 fragments at once (Stuelend, 2009). In addition, no prior sequence information is needed for amplification (Meudt and Clarke, 2007). As a result, AFLP has become extremely beneficial in the study of taxa including bacteria, fungi and plants, where much is still unknown about the genomic make-up of various organisms.

- DNA fingerprinting techniques appear to be useful to distinguish different clonal lineages within populations (Demeke *et al*., 1992; Williams *et al*., 1990). **Random amplified polymorphic DNA (RAPD)** has been used in the Oomycetes as well to distinguish different strains, species, and clones. In the case of the crayfish pathogen *Aphanomyces astaci*, a closely related species, genetic diversity among several isolates was studied by using this technique (Huang *et al*., 1994). The **RAPD polymerase chain reaction (PCR)** has also been applied successfully in some other species of Oomycetes, i.e. *Phytophthora* spp. (Ersek *et al*., 1994) and *Saprolegnia* spp. (Diéguez-Uribeondo *et al*., 1996). Random DNA markers have a wide application in genetic diversity studies. However, they are rarely used for species identification. The problem when applying random markers on population studies is that it is needed to know that all samples belong to the same species. In *Saprolegnia*, a recent molecular taxonomic study has validated a number of taxa, and

now allows the identification of isolates to species level using molecular techniques (Sandoval-Sierra *et al*., 2013).

- The **internal transcribed spacer (ITS) amplification and sequencing**, is another technique that has been employed for *Saprolegnia* identification (Hulvey *et al*., 2007). The ITS1 and ITS2 regions are non-conserved regions of ribosomal nuclear DNA and could be amplified with the PCR using universal primers ITS1 and ITS4 (Paul and Steciow, 2004). Using this method, before morphological analysis or DNA extraction is attempted; all isolates are processed to derive axenic, single spore or single hyphal tip cultures.

Other identification methods

Methods such as the use of monoclonal antibodies (MAB) (Fregeneda-Grandes *et al*., 2007) or SEM (Beakes *et al*., 1994; Stueland *et al*., 2005) provided information that led to the identification of zoospores of strains of *S. parasitica*. However, these techniques were found to be less suitable for the identification of strains of other *Saprolegnia* species such *S. diclina* that have much less pronounced cyst morphology (Beakes, 1983).

METHODS USED FOR PATHOGENICITY STUDIES OF *SAPROLEGNIA*

Enumeration of *Saprolegnia* for pathogenicity studies

In order to find the concentration of the zoospore cyst suspension, zoospores and cysts are counted using a counting chamber (Bürker chamber) and a microscope, and the spore suspensions are confirmed to contain both motile zoospores and sporocysts. The concentration in the challenge suspensions is often adjusted by dilution to 1.0 X 10^4 spores L-1 (Stueland *et al*., 2005; Thoen *et al*., 2011). Each calculation is performed at least twice. Another method is to inoculate a specific amount of zoospore suspension on growth agar, and then count the number of germlings. This method tends to be less accurate, and it takes longer. Thus, the use of the counting chamber is commonly preferred in pathogenicity testing, as the concentration would then be defined immediately (Stuelend, 2009).

Challenge models for experimental infections of fish

A lot of scientists have conducted research which depends on experimental infection of fish with Sapr*olegnia parasitica*. For the challenge experiments to successfully produce an infection, the fish involved need to be subjected to stress. Most forms of environmental stress (social, physical, chemical) can and do predispose fish to a wide variety of diseases, so as to cause suppression of their defence system. Different methods have been successfully used by researchers to induce stress in fish. These methods include: application of both abrasion and temperature stress before experimental challenge with zoospores (Howe and Stehly, 1998), causing social stress, based on conditions that are present in hatcheries or in the natural environment (DeWald and Wilzbach, 1992; Pickering, 1993), treatment of the fish with slow-release intraperitoneal implants containing cortisol, resulting in chronically elevated blood cortisol levels, rendering the fish susceptible to infection by *S. parasitica* (Pottinger and Day, 1999). Others have shown that 10 days exposure of rainbow trout to sub-lethal levels of ammonia and/or nitrite increased their susceptibility to an experimental challenge of *S. parasitica* spores (Carballo and Munoz, 1991).

For pathogenicity studies of *Saprolegnia* in fish, the challenge model frequently used is based on impairment of the natural protective mechanisms of fish against Oomycete infections, prior to exposure to *Saprolegnia* zoospores. The procedure, known as the "ami-momi" treatment, was first described by Hatai and Hoshiai (1993). It is generally achieved by dip-netting the fish and gently shaking them in a fine meshed net, out of water for a predetermined length of time. Ami-momi treatment induces an acute stress response and impairs the protective mucus layer of the fish, enhancing the susceptibility to *Saprolegnia* infection (Stueland *et al*., 2005).

The above-mentioned methods appear to be excellent choices for testing pathogenicity of *Saprolegnia* and other pathogens because they more accurately reflect the conditions fish actually experience in reality. Injection and loading the food with the infective agent are useful methods, but lack the potential sensitivity necessary to understand the infective process (Whisler, 1996).

Experimental infections of eggs

Experimental infections of fish eggs are based on the premise that an infection nucleus is necessary for healthy live eggs to get infected. So far, hemp seeds overgrown with the test isolate

of *Saprolegnia* (Pottinger and Day, 1999; Sarowar *et al*., 2013) and infected dead eggs Kitancharoen and Hatai, 1996; Thoen *et al*., 2011) have been used as foci of infection. Pottinger and Day (1999) aseptically placed 'bearded' hemp seed in coarse weaved muslin bags. Sarowar *et al* (2013) and Thoen *et al.* (2011) placed the infected hemp seeds and dead eggs respectively in between the eggs in Petri dishes serving as infection units. To ensure that the eggs are healthy before challenge experiments, they should be disinfected during incubation and before transport with buffodine (1:100, 10 min) and treated with formalin (Ali *et al*., 2014; Liu *et al*., 2014; Thoen *et al*., 2011). Other researchers disinfect the eggs by rinsing in 4 ppm malachite green (Kiesecker and Blaustein, 1995; Sarowar *et al*., 2013) for 40 min upon arrival in the lab.

MICROSCOPY

Both electron and light microscopes are technical devices which are used for visualizing structures that are too small to see with the unaided eye, and both types have relevant areas of applications in biology sciences. Therefore, the type of microscope a researcher chooses to use depends entirely on what type of specimen they would like to examine and what type of information they want to get out of it. The method of visualizing the structures is very different.

Light microscopy

The light microscope is used to observe small objects using visible light and lenses. It is highly used and well-recognized microscopes in the scientific community. The device can be used to view living or dead samples and can maximize these samples up to one thousand times (1,000x) their actual size. Light microscopes include almost all compound and stereo microscopes.

For preparation of samples for histopathological examinations Formalin (10% buffered) is the fixative of choice by most researchers. Histopathology sections are prepared by routine laboratory procedures, and most commonly stained with Hematoxylin and Eosin (Kiernan, 2008). Special stains Periodic Acid Schiff (PAS) and Grocott's modification of Gomori's methenamine silver (GMS) (Kiernan, 1999; Nassar *et al*., 2006) may also be employed to further demonstrate the presence of hyphae.

Electron microscopy

The electron microscope is a type of microscope that uses a beam of electrons to create an image of the specimen. It is capable of much higher magnifications and has a greater resolving power than a light microscope, allowing it to see much smaller objects in finer detail.

Transmission electron microscopy

Transmission electron microscopes (TEMs) offer the most powerful magnification, potentially over one million times or more. They produce high quality, detailed images and are therefore able to give information on element and compound structure. This microscopy technique is used to view the internal ultrastructure of cells and tissues.

Scanning electron microscopy

A scanning electron microscope (SEM) is a powerful magnification tool that utilizes focused beams of electrons to obtain information. In addition to topographical, morphological and compositional information, a scanning electron microscope can detect and analyse surface fractures, provide information in microstructures, examine surface contaminations, reveal spatial variations in chemical compositions, provide qualitative chemical analyses and identify crystalline structures. As it is used to view the surface or near-surface features of a sample, it is very useful for studies of morphology and host/agent interactions.

7 KNOWLEDGE GAPS

Since *Saprolegnia* species and other pathogens generally pose a global threat to biodiversity and food security (Liu *et al*., 2014) more rigorous research is necessary to clarify the morphological mechanisms involved in the pathogenesis of *S. parasitica* infections. Researchers testing control regimes of *Saprolegnia* spp. will obviously rely upon the use virulent strains of the Oomycete, particularly when the focus is *in vivo* experiments and infection rates. Strains of *Saprolegnia parasitica* that have previously been shown to be highly pathogenic under laboratory conditions are kept in storage for lengthy periods of time and, in certain cases, are passaged several times *in vitro* through media may undergo attenuation of virulence. Previously, such sub-culture changes in pathogenicity of *S. strains* had never been considered.

The reason why some organisms can peacefully co-exist with their hosts while others go on to produce disease lies in the nature of the interaction between microbe and host. There has been no documented scientific report comparing the infection strategies of *S. parasitica* and *S. diclina* when they infect eggs of Atlantic salmon. Designing novel treatments against an organism often requires knowledge about the pathogenicity of the organism. Thus, there was a need to explore the pathogenesis of *Saprolegnia* species in Atlantic salmon eggs, both under natural conditions in the hatchery and in experimentally infected eggs. A deeper understanding of the infection process will help develop sustainable control strategies against this devastating Oomycete infection.

In fish farm hatcheries, differences in susceptibility to *Saprolegnia* infection of Atlantic salmon eggs originating from different females were noted for many years. No research had been conducted before to offer an explanation, even just as a tip on the iceberg, for these differences that seem to be related to the ancestry of the eggs or the genetic makeup of the females. These differences may be key factors in order to develop strategies for decrease of *Saprolegnia* problems in hatcheries. Therefore, an undertaking to elucidate the mechanisms behind these differences was crucial.

8 OBJECTIVES AND AIMS OF THE STUDY

The overall objective of this work was to study the pathogenicity and infectivity of *Saprolegnia* species to Atlantic salmon and their eggs, in order to provide new knowledge of the biology of the pathogens and the host-pathogen interactions, with a motivation to better combat the disease. This was achieved through the following specific aims:

1. **Provide new knowledge on the stability of virulence in *S. parasitica* strains to Atlantic salmon**
    a. The impact of *in vitro* sub-culturing on the virulence of different strains of *S. parasitica* was assayed by determining infection rates in challenge experiments in Atlantic salmon (Paper I).

2. **Provide new knowledge on the pathogenesis of *Saprolegnia* infection of Atlantic salmon eggs**
    a. Infection strategies that *Saprolegnia diclina* IIIA and *S. parasitica* employ when they colonize eggs of Atlantic salmon were scrutinized and compared (Paper II).
    b. The hypothesis that chorion thickness of Atlantic salmon eggs correlates with higher resistant to *Saprolegnia* infection was tested (Paper III).

9 METHODOLOGICAL CONSIDERATIONS

SAPROLEGNIA STRAINS

In this work, our choice of *Saprolegnia* isolates used in the pathogenicity studies was influenced by the need to employ strains that have previously been proven to be highly virulent to Atlantic salmon and eggs from Atlantic salmon. Most of the isolates were obtained from the *Saprolegnia* collection library of the Norwegian Veterinary Institute (NVI) (stored on hemp seeds). For the egg challenge experiment that was carried out at the Aberdeen Oomycete Laboratory, the isolate used was obtained from their *Saprolegnia* library, but recently isolated from infected eggs. Production of challenge inocula for challenge experiments was achieved by inducing asexual spore reproduction in Glucose-Yeast (GY) agar and broth. The likelihood of bacterial contamination when this method is employed is low, it is reproducible and has previously been used by other scientists in the laboratory (Stueland *et al*., 2005; Thoen *et al*., 2011).

CHALLENGE MODELS

Fish

As described previously (6.2.9), different methods have been used to conduct experimental challenge in fish. Although different, a common requirement to successfully produce an infection is that the fish need to be subjected to stress. In our investigations of the effect of serial *in vitro* passaging of *S. parasitica* (Paper I), we opted for the "ami-momi" treatment (Fregenada-Grandes *et al.* 2001; Hussein and Hatai 2002 and Stueland *et al.* 2005). Our preference for this method was based on the fact that it mimics the stress from excessive handling of fish in aquaculture operations (Hussein and Hatai, 2002), and is a good method to characterize the virulence of *S. parasitica* strains (van West *et al.* 2010; Stueland *et al*. 2005). The external surface of salmonid fish is covered by a living epithelium capable of secreting copious amounts of mucus. In salmonids, the epidermal goblet cells are largely responsible for the production of this layer of slime, although secretions from other cell types (including the superficial Malpighian cells) may

also contribute (Whitear, 1986). Indeed, isolated fish mucus has been shown to be an effective growth medium for pathogenic strains of *S. parasitica* (Willoughby *et al*., 1983). Thus, the evidence to date indicates that the mucous layer acts primarily as a physical barrier to prevent colonisation. Most experimental studies of the transmission of saprolegniasis have necessitated abrasion of the surface of the fish to achieve successful infection, thus indicating the importance of an intact epidermis for the fish to resist colonisation by the Oomycete (Pickering, 1994). Evidence from studies of sexually mature salmonid fish also emphasises the relationship between physical damage and saprolegniasis (Richards and Pickering, 1978; White, 1975). Since the epidermal layer is damaged along with the mucus using the "ami-momi" method, it is fairly easy for an infection to be established once the fish are exposed to the *Saprolegnia* cyst suspensions. Mucus secretion by the epidermis of the fish acts as an important physical barrier preventing colonization but minor injuries to the skin can act as foci for fungal infection. Also, the "ami-momi" technique causes appropriate levels of internal stress in the fish, providing a basis for testing infectivity following physical damage to the epithelium.

Eggs

There are few documented, standardized techniques for experimentally inducing *Saprolegnia* infections in fish eggs in the literature. The two commonly used procedures differ in the manner by which *Saprolegnia* spores are administered. One method employs the use of hemp seeds overgrown with *Saprolegnia* hyphae (bearded hemp seeds) (Pottinger and Day, 1999; Sarowar *et al*., 2013) while the other uses infected dead eggs as the focus of infection (Kitancharoen and Hatai, 1996; Thoen *et al*., 2011). In the present study, we adopted both methods in two different laboratory settings. The choice of method in the different settings was based on the experience with use of the method in the two facilities where the experiments were performed. The 'bearded' hempseed method was convenient for the facilities at the Aberdeen Oomycete Laboratory, where we performed a laboratory-controlled challenge experiment on Atlantic salmon eggs to corroborate the differences that were observed in sensitivity of eggs to saprolegniasis in a fish farm set-up (Paper III). In order to investigate if the same contrasting pathology that was observed in Atlantic salmon eggs infected with *S. parasitica* and *S. diclina* IIIA under natural conditions would be reproduced in an experimental setting, we performed an experimental infection of eggs at the Norwegian Veterinary Institute (Paper II) with the "dead egg" technique. However, in both techniques ('bearded hemp seed' and 'dead egg' technique), we opted to use

petri dishes (Sarowar *et al*., 2013; Thoen *et al*., 2011) rather than plastic buckets or glass flasks (Pottinger and Day, 1999) as the experimental unit. Pottinger and Day (1999) administered the spore challenge by anchoring muslin bags containing 'bearded' hemp seeds within plastic buckets or glass flasks that served as challenge units. Our preferred method was the use of petri dishes where infected hemp seeds or dead eggs were placed in between the healthy test eggs. It is a very simple system which easily mimics the 'open shallow tray system' used in Norwegian hatcheries. The advantage of the 'open shallow tray system' in the hatcheries is that dead eggs, organic and other undesirable material can easily be accessed and removed.

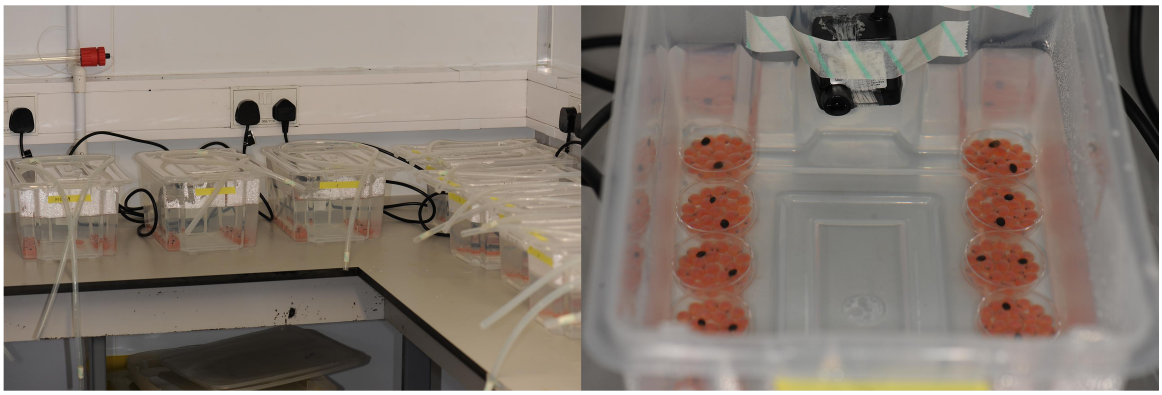

Figure 9. Experimental set-up of experimental infection of eggs with *Saprolegnia diclina* isolates.

HISTOLOGICAL PREPARATION AND MICROSCOPIC EXAMINATIONS

To fix fish and egg samples for histopathology, our choice fixative was 10% buffered formalin, by far the most popular fixing agent used for histopathology. Formalin penetrates tissues very well and quite rapidly and while the actual cross-linking is somewhat slower, it effectively arrests the post-mortem processes (Eltoum *et al.*, 2001). It thus preserves the morphology/integrity of the tissue.

Light microscopy was sufficient to reveal the pathological effects of *S. parasitica* on the skin, kidneys, liver and gills of Atlantic salmon parr. The use of this microscopic technique in combination with other non-microscopic methods enabled us to successfully answer our research question whether serial *in vitro* passaging impacts on virulence of *Saprolegnia parasitica* to Atlantic salmon parr (Paper I).

In order to study the infection strategies of *Saprolegnia* species and confirm differences in sensitivity of eggs to *Saprolegnia* infection (Papers II and III respectively), light microscopy was useful. Nevertheless, the technique was not sufficient to enable us to view and appreciate the morphological changes caused in Atlantic salmon eggs by infection with *Saprolegnia* species, or the detailed structures of the *zona externa* and *zona interna* of the egg chorion. Therefore, it was necessary to also use the electron microscope which has the advantage of showing smaller objects in finer detail.

For electron microscope sections, we used 4 % paraformaldehyde/1 % glutaraldehyde/phosphate buffer to fix the eggs. Glutaraldehyde fixes proteins and stabilises structures and its depth of penetration is 2 mm/hour. Paraformaldehyde has a depth of penetration of 10 mm/hour, but takes much longer to stabilise the tissue. The combination of formaldehyde with glutaraldehyde as a fixative for electron microscopy takes advantage of the rapid penetration of small formaldehyde molecules, which initiate the structural stabilization of the tissue. Rapid and thorough cross-linking is brought about by the more slowly penetrating glutaraldehyde oligomers (Kiernan, 2000).

In order to be able to achieve our aim of describing the behaviour of *Saprolegnia* species when they infect pre-eyed eggs of Atlantic salmon under natural conditions in the hatchery and investigate and compare the infection processes of *S. parasitica* to and *S. diclina* on eggs *in vitro* (Paper II), Scanning Electron Microscopy (SEM) was a useful technique. SEM can be used whenever information is required about the surface or near-surface region of a specimen and in the present study it helped us rule out the presence of appressorial-like structures.

For the purpose of examining chorion of *Saprolegnia* infected Atlantic salmon eggs assumed to be more versus less prone to infection, for structural defects or differences, there was need for a microscopic technique with high resolution. The use of Transmission Electron Microscopy (TEM) thus became imperative for the morphological examination of the *zona externa* and *zona interna* of both groups of eggs (Paper III).

ISOLATION OF *SAPROLEGNIA* SPECIES AND TESTING FOR GROWTH RATE ON SOLID AGAR

Isolation and cultivation of *Saprolegnia* species from fish lesions can be a challenge because a wide range of species may be isolated from an infection site, and the presence of multiple species often masks the primary pathogen (van den Berg *et al*., 2013). In the present study we took samples only from young and deep-seated tissue to limit contamination by opportunistic species. Because the composition of microbial communities on the skin dramatically changes after death of the host, dying and dead fish are an unreliable source for sampling to determine the primary cause of infection. We obtained pure cultures by growing cultures on plates using Glucose-Yeast extract media (Hatai and Egusa, 1979) and repeatedly transferring growing mycelia tips to new media. To exclude growth of other microbial species such as bacteria, specific antibiotics were used in the culture medium.

MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *SAPROLEGNIA* ISOLATES

Several techniques have been used for identification of *Saprolegnia* to species level. Until molecular techniques became available, methods adopted from Seymour (1970) and Willoughby (1985), which depend on microscopic examination of mycelia, sexual reproductive structures and oospores had been the main method by which species were distinguished. The afore-mentioned methods were used to identify purifies strains morphologically in this study. From a single-spore culture on GY agar, a 5-mm-diameter plug of the growing mycelium was placed in GY broth (Hatai & Egusa 1979; Kitancharoen *et al*., 1995) and incubated for 2–3 days at 15 ± 1 °C. Bundles of hyphae were washed with SAW and incubated with autoclaved hemp seeds in SAW. Examination of possible sexual structures on hemp seeds was performed at 5, 15 and 20 ± 1 °C, and the hemp seed cultures were examined for production of oogonia and antheridia twice a week over a 12-week period using an Olympus® inverted zoom stereo microscope (SZH-ILLD), with a bright field/dark field transmission light illumination base (Stueland *et al*. 2005). However, since many *Saprolegnia* isolates express very similar characteristics (Dieguez-Uribeondo *et al*., 2007) or fail to produce sexual stages under laboratory conditions or after long periods of being in storage, morphological identification is liable to cause confusion and uncertainties. The ease and

availability of sequencing technology led to the development of molecular identification methods that are providing new insights into Oomycete taxonomy. In the present study, we enhanced the morphological technique with molecular identification and opted for the most commonly used method of identification, which is the sequencing of the internal transcribed spacer (ITS) region. This highly conserved region is located between the 18S and the 28S rDNA and includes the 5.8S rDNA sequence. It is approximately 700 bp in length in *Saprolegnia* species, and is considered very suitable for intra-species analysis (Dieguez-Uribeondo *et al*., 2007). Variable sections from the 18S rDNA, 28S rDNA and cox2-gene can also be analysed to determine relationships and to identify Oomycete species (Levesque and De Cock, 2004).

10 GENERAL RESULTS AND DISCUSSION

The studies conducted as part of this thesis are motivated by the lack of efficient control and treatment methods against saprolegniasis in Atlantic salmon and their eggs. Our assertion has been that bridging some of the knowledge gaps would make a contribution to the industry in combating the disease and aid in establishing preventive measures that can reduce the occurrence or prevalence of saprolegniasis in salmon farming. We have focused on elucidating the pathogenicity and infectivity of *Saprolegnia* species to Atlantic salmon and their eggs and these studies have provided new and valuable knowledge of host-pathogen interactions and the biology of the pathogens. This is a first step towards improved disease control methods.

Successive *in vitro* sub-culturing has varying effects on different strains of *Saprolegnia parasitica*

Pathogenic organisms, fungi and fungal-like organisms in particular, are notorious for losing virulence and changing their morphology when successively cultured on artificial media (Butt *et al.*, 2006). Scientists in different fields have investigated attenuation of virulence or the absence of it in their organisms of interest, ranging from viruses, bacteria and fungi that affect a wide variety of host species (Brownbridge *et al.*, 2001; Hayden *et al.*, 1992; Nahar *et al.*, 2008; Shah *et al.*, 2007; Vandenberg and Cantone, 2004). However, the effect of successive *in vitro* sub-culturing on *Saprolegnia parasitica* had not yet been studied.

The importance of the development of novel solutions, which depend on in-depth analysis of host-pathogen interactions and the biology of the pathogen and its hosts to tackle saprolegniasis cannot be over-emphasized. Vaccine production and efficacy testing of drugs and control regimes are critical areas of scientific research as far as control and prevention of the spread of emerging pathogens is concerned (Liu *et al.*, 2014). To be achieved successfully, they require virulent isolates of the pathogenic organism in question. Our findings have enabled us to conclude that some strains of *S. parasitica* undergo attenuation of virulence following serial *in vitro* sub-culturing whereas others do not (paper I).

Researchers often obtain isolates for pathogenicity studies from stock cultures (Ali *et al*., 2014; Andersson and Lage Cerenius, 2002; Songe *et al*., 2015). It is essential that the most appropriate growth conditions and preservation techniques are used to ensure the viability, purity and stability of maintained microorganisms. Ideally, the methods used should retain all characteristics throughout storage (Smith, 1998). Virulent and stable strains of organisms are also essential for biological prevention, mitigation or control of certain diseases. Therefore, it is cardinal that studies are carried out to determine strain stability through successive sub-culturing before selecting a strain for commercial purposes (Ansari and Butt, 2011).

Armed with this knowledge, scientists concerned with *Saprolegnia* research should ensure that they study non-attenuated strains. Also in vaccine production and efficacy testing of drugs and control regimes as well as other procedures, which rely heavily upon virulent strains it is important to ensure that the strains used have not been in storage for too long or undergone several passages *in vitro*. Although this phenomenon has not yet been investigated in *S. diclina*, we applied the same principle when *S. diclina* strains were used for challenge studies herein. Consequently, when investigating the impact of chorion thickness in protection against *Saprolegnia* infections (paper III) we ensured that we used a *S. diclina* isolate that had recently been isolated from infected eggs in a hatchery.

The pattern of change in virulence of *Saprolegnia parasitica* isolates is strain-dependent

Very little information is available about why a fungus or Oomycete becomes attenuated; however, it is apparent that strains differ in their stability when maintained on artificial media, with some strains clearly attenuating more rapidly than others irrespective of whether the parent culture was derived from a single spore or multi-spore colony (Hutwimmer *et al*., 2008). Stable strains conserve virulence for several generations, whereas unstable strains usually become attenuated after a few subcultures (Butt *et al*., 2006; Losch *et al.*, 2010). Here we observed that the pattern of change in virulence was also strain dependent. The *S. parasitica* strains that underwent a loss of virulence as an effect of successive *in vitro* sub-culturing regained their virulence following passage through Atlantic salmon parr. Scientists have noted that single spore isolates of the unstable fungal cultures generally produce more phenotypic variants than stable cultures (Butt *et al*., 2006). In the present study, examination of morphological characteristics of

the different passages of the three strains of *S. parasitica* showed a variation in the production of sexual reproductive structures, which was not associated with virulence loss. We did not observe any significant differences in growth rates of the cultures of different passages, and the diameters of the vegetative colonies were in principle the same for all passages. Production of enlarged chlamydospores was abundant in all the cultures at the different passages. The most striking finding in our study was that the strain most attenuated by passage, was also the one that showed the lowest rates of sporangial release and proliferation.

The virulence of bacteria and fungi can be restored by passage through one or more series of susceptible hosts. Increase in virulence also occurs in obligate pathogens such as viruses. Generally, the virulence of a pathogen enhanced when passed through habitual or normal hosts. The virulence of a strain to a given host species may be altered by previous passage in another host species (Tanada *et al*., 1993). Serial infection of diverse host genotypes has been shown to rapidly impede pathogen fitness and virulence (Kubinak *et al*., 2014). In our study, the strains of *S. parasitica* prone to a loss of virulence upon serial *in vitro* sub-culturing showed an increase in their virulence when passaged through susceptible fish. Interestingly, the strain of *S. parasitica* that did not show a decline in virulence *in vitro* did not show an increase of virulence when passaged through fish.

***Saprolegnia diclina* and *Saprolegnia parasitica* employ different infection strategies when colonizing Atlantic salmon eggs**

The present study has presented us with very interesting findings about the mechanisms of infection that *S. parasitica* and *S. diclina* employ when they infect Atlantic salmon eggs (Paper II). Light microscopy reviewed that eggs infected with *S. parasitica* had an apparently intact chorion with hyphae growing within in or beneath the chorion, while *S. diclina* infection resulted in the chorion in some areas being completely destroyed/dissolved. This was evident in the eggs naturally infected with *Saprolegnia* in the hatchery as well as in the experimentally infected eggs. These results provide an understanding of pathological changes or the process of *Saprolegnia* infections of eggs. Moreover, they bridge the knowledge gap that has existed as regards differences in pathology caused by *S. parasitica* and *S. diclina* infection of eggs. The improved understanding of disparate infection strategies of the two *Saprolegnia* species will facilitate the

search for novel drugs or vaccines, which could significantly reduce losses in fish farms (Bruno *et al*., 2009; Phillips *et al*., 2008; Robertson *et al*., 2008; van West, 2006). The understanding has been that fish eggs are more prone to infection with *S. diclina* than *S. parasitica* (Hussein *et al*., 2001; Kitancharoen *et al*., 1997a; Kitancharoen *et al*., 1997b; Rand and Munden, 1993), hence scientists are often more inclined to use *S. diclina* isolates in experimental infections of Atlantic salmon eggs. However, more recent research has shown that in some instances *S. parasitica* is in fact the predominant species infecting fish eggs (Shahbazian *et al*., 2010; Songe *et al*., 2015). It is therefore, an imperative that extra effort is put into research on *S. parasitica* and its role in fish egg saprolegniasis.

Not all strains of *S. diclina* form appressorial structures under hatchery and laboratory conditions

Although appressorial infection structures have been reported earlier in some *Saprolegnia* species (Willoughby and Hasenjäger, 1987), we did not observe any appressoria-like structures by SEM in this study. Following detailed light and scanning electron microscopy of a wide array of infected hosts, Sarowar (2014) revealed that *Saprolegnia* species form appressorium-like structures only on selected hosts. Little is known about the developmental processes that lead to appressorium formation. In plant pathogenic Oomycetes, it has been proposed that appressorium formation may result from the difficulty that germlings experience in attempting to penetrate plant surfaces (Hardham, 2001).

A thicker chorion confers protection to Atlantic salmon eggs against *Saprolegnia* infection

Form our research we can correlate a thicker chorion with protection against *Saprolegnia diclina* infection in Atlantic salmon eggs (Paper III). Results from laboratory-controlled challenge authenticated findings from a fish farm, which adds even more credence to the observation. In hindsight, including a *S. parasitica* isolate in the experimental studies would have added additional information as it was later shown that *S. parasitica* and *S. diclina* infect Atlantic salmon eggs by different strategies (paper II). Together, these studies provide valuable information regarding factors of importance for resistance to saprolegniasis and could possibly

allow breeding for resistance. Chorion thickness has not yet been used as a measure of determining egg quality. Previous studies have examined a variety of parameters associated with egg quality in cultured fish and these include egg weight, chorion weight, egg iron levels, lipid composition and protein concentrations (Bobe and Labbe, 2010; Brooks *et al*., 1997; Kjorsvik *et al*., 1990; Lahnsteiner *et al*., 1999).

A number of studies have indicated that maternal genetics may have significant impact on egg quality in fish (Brauhn and Kincaid, 1982; Reinitz *et al*., 1979; Withler *et al*., 1987). For example female rainbow trout which produced high quality eggs during their first spawning season did so again during the subsequent season (Brooks *et al*., 1997). Our hypothesis that a thicker chorion provided some protection to the egg against *Saprolegnia* infection was backed by the fact that the two different groups of eggs in question were subjected to exactly the same conditions as far as husbandry practices, environmental factors, stress and nutrition are concerned.

11 CONCLUSIONS

The main conclusions that can be drawn from this work are:

(a) Successive *in vitro* sub-culturing may have varying effects on virulence of different strains of *Saprolegnia parasitica*

(i) The pattern of change in virulence is strain dependent. The *S. parasitica* strains that undergo a loss of virulence due to successive *in vitro* sub-culturing are the ones more likely to regain their virulence following passage through Atlantic salmon parr.

(ii) The ability to maintain virulence *in vivo* can vary between *S. parasitica* strains, and so not all isolates of *S. parasitica* need to be passed through susceptible fish prior to challenge experiments. With this knowledge, there would be a reduction in the unnecessary use of experimental animals in research.

(iii)The strain that does not show a decline in virulence after *in vitro* sub-culturing does not gain any incremental morbidity rate after *in vivo* passage through fish.

(b) *Saprolegnia diclina* and *S. parasitica* appear to have different infection strategies when colonizing Atlantic salmon eggs.

(i) *Saprolegnia diclina* appears to infect eggs by destroying the chorion in eyed eggs, whereas *S. parasitica* can penetrate the intact chorion and grow inside the eggs.

(ii) *Saprolegnia diclina* is capable of dissolving the chorion of salmon fish eggs.

(iii) *Saprolegnia diclina* strains do not form appressorial structures under hatchery and laboratory conditions.

(c) Chorion thickness plays a significant role in the resistance against saprolegniasis in eggs from Atlantic salmon

(i) The chorion of eggs assumed to be infection-resistant is thicker than the chorion of infection-prone eggs where both groups of eggs from females bred, maintained and spawned in the same water.

The practical significance of this study is that it has provided more insight into the fish and egg-*Saprolegnia* interactions and will thus impact basic understanding of pathogenicity and

infectivity of *Saprolegnia*. With this knowledge, fish breeders will be inspired to employ novel sustainable strategies such as the breeding of fish for resistance to infection with *Saprolegnia* species in hatcheries as well as natural fish populations. Apart from increasing profitability in aquaculture, the resulting reduction in use of chemicals will contribute to a healthier ecosystem.

12 FUTURE WORK

Although it is well established that fungi and Oomycetes become attenuated following *in vitro* passaging, very little information is available as to why these organisms lose their virulence. It will be important to carry out molecular studies to elucidate the virulence patterns of different strains of *S. parasitica* and thereby provide an understanding of the molecular basis for virulence. Knowledge of genetic diversity among strains is of importance in disease control (Ahmed *et al*., 2002).

The proposition that infection mechanism employed by *S. diclina* possibly represents a necrotrophic type whereas *S. parasitica* possibly uses a facultative biotrophic strategy (Paper II) is a fascinating thought. It is foreseen that the underlying molecular traits or virulence factors can best be studied through genome comparisons between *S. parasitica* (Jiang *et al*., 2013) and *S. diclina* could constitute a basis for understanding better the difference in infection strategies of the two species. And one might speculate if the two strains would have plasticity in their genomic constitution which would allow deploying (transcribing) batteries of virulence factors that best suit the various environmental conditions. These are topics for a multitude of studies in the future where combination of new genome sequencing methods and genome editing strategies might facilitate pin-pointing the underlying virulence factors. This could in turn allow for the development of effective treatment of prevention strategies that can reduce the impact of these Oomycete infections in the aquaculture industry.

Our results present the first documented record that a thicker chorion provides protection against *Saprolegnia* infection in Atlantic salmon eggs (Paper III). From this one might foresee a novel approach whereby *Saprolegnia* infections are controlled through selective breeding of resistant fish lineages, i.e. yielding eggs with thicker chorion. It is well documented that selective breeding has been successful in providing resistance against infectious diseases such as pancreas disease, infectious pancreatic necrosis (IPN) and Salmon Rickettsial Syndrome (SRS) (Houston *et al*., 2010; Storset *et al*., 2007; Zhan, 2006). Selecting brood fish (females) that produces eggs with thicker chorion has to be weighed against observations that that removal of the chorion before hatching results in increased movement and accelerated growth in rainbow trout (*Oncorhynchus*

mykiss) embryos (Ninness *et al*. 2006). These traits might therefore yield contradictory effects and a thicker chorion might delay or prevent hatching and thus increase mortality of developing embryos. Studies should be carried out to investigate hatching rates in groups with thicker chorion.

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PAPER I

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In vitro passages impact on virulence of *Saprolegnia parasitica* to Atlantic salmon, *Salmo salar* L. parr

M M Songe[1], E Thoen[1], Ø Evensen[2] and I Skaar[1]

1 Norwegian Veterinary Institute, Oslo, Norway
2 Norwegian School of Veterinary Science, Oslo, Norway

Abstract

The effect of serial *in vitro* subculturing on three pathogenic strains of *Saprolegnia parasitica* was investigated. The isolates were passed through Atlantic salmon, *Salmo salar* L. parr, and then re-isolated as single spore colonies. All strains caused infection. The isolate obtained from diseased fish served as a virulent reference culture and was designated 'AP' ('activated through passage'). Successive subculturing was made by obtaining an inoculum from AP to produce the 2nd subculture and then passaged to the 3rd subculture (from the 2nd), until the 15th passage was obtained. Spores used to produce storage cultures were collected at passages 5, 10 and 15. The different passages of each strain were used to artificially infect Atlantic salmon parr. Morphological characterization of growth patterns was performed to observe differences occurring due to serial *in vitro* subculturing. Two of the strains declined in virulence after 15 successive *in vitro* subcultures, whereas one did not. This study is the first to investigate attenuation of virulence in *Saprolegnia* and whether or not isolates of *S. parasitica* should be passed through the fish host prior to challenge experiments. It reveals that some strains degenerate more rapidly than others when subjected to successive *in vitro* subculturing on glucose–yeast extract.

Correspondence *I Skaar, Norwegian Veterinary Institute, PO Box 750 Sentrum, N-0106 Oslo, Norway (e-mail: ida.skaar@vetinst.no)*

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Introduction

Saprolegnia parasitica (Saprolegniaceae, Oomycota) is the causative agent of saprolegniasis, a well-known and increasing problem in aquaculture of salmonids and other fish species worldwide (Beakes, Wood & Burr 1994; Van West 2006). Scientific efforts related to *S. parasitica* and other *Saprolegnia* spp. have increased after malachite green, an efficient remedy, was banned from using in aquaculture in the European Union in 2000 (Sudova *et al.* 2007) because the general public may become exposed to malachite green through the consumption of treated fish. In the United States, the use of malachite green is not approved by the Food and Drug Administration (Hernando *et al.* 2006). Emphasis has been put on finding new ways to control the disease. In particular, a number of fungicides have been tested (Barnes, Sayler & Cordes 2001; Kashiwagi *et al.* 2002, 2003). Efficacy testing of drugs and control regimes rely upon virulent strains of the infective agent being available for testing, particularly when the focus is on *in vivo* experiments and infection rates. Loss of virulence, a well-known phenomenon observed in fungi and bacteria maintained in media and viruses grown in cell culture (Druelle *et al.* 2008; Almaguer-Chávez *et al.* 2011; Ansari & Butt 2011), can be a confounding factor in research studies. Various terms have been used to describe this phenomenon, including phenotypic degeneration, degeneration, phenotypic instability, phenotypic deterioration, dual phenomenon, saltation and attenuation (Kawakami 1960; Nagaichi

Journal of Fish Diseases

1973; Butt 2002; Ibrahim, Butt & Jenkinson 2002). Potential loss of virulence is highly relevant to the maintenance of strains in general and in the production of strains for comparative bioassays (Brownbridge, Costa & Jaronski 2001). Although attenuation causes problems in various situations, from production of biocontrol agents to testing of vaccine or drug efficacy, it has hitherto not been reported in oomycetes, even those of high economic importance such as *Pythium*, *Phytophthora* and *S. parasitica*. Therefore, this study is the first known to document the stability and/or instability of *S. parasitica* strains in continuous *in vitro* cultivation. The aim of this study was to explore whether *in vitro* subculturing impacts on the virulence of different strains of *S. parasitica,* assayed by infection rates in challenge experiments in Atlantic salmon.

Materials and methods

Saprolegnia strains and culture procedures

Three virulent strains of *S. parasitica* were included in this study, all originating from different salmonid species of fish. Table 1 summarizes their identities and sources. All three strains have previously been shown to be highly pathogenic to Atlantic salmon, *Salmo salar* L. All *S. parasitica* isolates had been stored in the culture collection of the Norwegian Veterinary Institute using standard culture and storage conditions (Stueland, Hatai & Skaar 2005), but to prevent any bias between strains at the start of *in vitro* culturing, all isolates were passed through Atlantic salmon (*Salmo salar* L.) parr and then re-isolated as single spore colonies. This principle is concordant with what has been used for entomopathogenic fungi (Fargues *et al.* 1997; Vidal, Lacey & Fargues 1997). All isolates VIO 2736, VIO 5337 and VIO 5708 produced >90% infection rate after challenge, and the isolate obtained from diseased fish served as a virulent reference culture and was referred to as the primary isolate and designated 'AP' ('activated through passage'). Successive subculturing was carried out by obtaining an inoculum from AP to produce the 2nd subculture and then passaged to the 3rd subculture (from the 2nd), until the 15th passage was obtained. This was achieved by inducing asexual spore reproduction in Glucose-Yeast (GY) agar and broth as described by Stueland *et al.* (2005). Spores used to produce storage cultures were collected at passages 5, 10 and 15. The cultures were stored at 4 °C in sterilized aquarium water (SAW) until use and no longer than 1 week for any of the passages. From the cultures obtained at the given intervals, we produced challenge inocula (spore suspensions) according to Stueland *et al.*, for the challenge experiments. Inoculum concentrations were assessed by counting the spores in a Bürker-türk haemocytometer (BT, Brand, Wertheim, Germany), and final exposure doses were adjusted by dilution, directly into the fish tanks.

Fish and experimental conditions

For the challenge experiments, this study used 315 Atlantic salmon parr of AquaGen strain with an average weight of 30 g (at start of the experiment). The fish were acclimatized for 1 week in a 1.0-m fibreglass flow-through tank before we moved them to 10-L challenge tanks at the time of the challenge. Water flow rate of 0.8 L kg min^{-} and temperature of 11 °C ± 1 were maintained throughout the study. The fish were fed a commercial pellet salmon feed (Biomar, 2 mm) during the study. The groups of fish were identified by the strain number of the *Saprolegnia* strain and then the passage number in upper case, AP, 5P and 15P. Thus, 2736^{AP} represented the primary passage of strain VIO 2736, 5337^{5P} the 5th passage of VIO 5337 and so on.

Table 1 *Saprolegnia parasitica* isolates included in the present study. All isolates were originally isolated from salmonids

Strain	NVI strain collection number[a]	GenBank accession number	Host or source of origin	Geographical location
A	VIO 2736	KC994645	Infected Atlantic salmon (*Salmo salar* L.) parr	Scotland
B	VIO 5337	KC994646	Infected rainbow trout (*Oncorhynchus mykiss*)	Norway
C	VIO 5708	KC994647	Infected spawned sea trout (*Salmo trutta* L.)	Norway

[a]Refers to the number in the strain collection of the Norwegian Veterinary Institute, Oslo.

Experimental challenge procedure

Prior to onset, the experiment was approved by the Norwegian Animal Research Authority and conducted in the Wet laboratory at the Norwegian Veterinary Institute/Norwegian School of Veterinary Science, Oslo, Norway.

The fish were stocked in groups of 7 or 8 per tank, in quadruple tanks per challenge inoculum. Two negative, non-challenged control tanks ($n = 7$ or 8) were also included. The latter groups were subjected to 'ami-momi' treatment, but not exposed to zoospores. The challenge was performed as follows. Water flow was turned off in all of the experimental tanks. The fish received 'ami-momi' treatment as described by Hatai & Hoshiai (1993) and later slightly modified by Stueland *et al.* (2005). After ami-momi treatment, they were transferred back to the tanks, followed by the addition of challenge inoculum to obtain an equal spore concentration of 1.0×10^4 spores L^{-1} for all tanks and isolates. The exposure lasted for 24 h before water flow was resumed, and during the exposure period, the water was aerated to keep an oxygen saturation of 80–90% in all tanks. Water quality parameters were checked every day in each tank.

Assessment of infection

After the challenge, the fish were maintained and monitored daily for signs of clinical infection, including tufts of cotton-wool-like growth on the surface of the fish, focal areas of haemorrhage, necrosis and ulceration, lethargy and loss of equilibrium over a period of 11–14 days. All fish that showed disease signs were promptly removed and killed in benzocaine. Saprolegniasis was confirmed by stereomicroscopic (Olympus) inspection, and the fish were photographed. The number of fish showing gross lesions of saprolegniasis was recorded.

In addition to macroscopic inspection, documentation of *Saprolegnia* infection was carried out by collecting a sample from the skin/muscle tissues from one infected fish from each tank and passage and testing for the presence of *Saprolegnia* hyphae (Stueland *et al.* 2005). In brief, the infected area (as observed macroscopically and by stereomicroscopy) was disinfected with 70% alcohol (cotton wool), and a small piece of skin and superficial muscle tissue were carefully excised. The sample was placed on GY agar supplemented with 200 mg L^- chloramphenicol and incubated at 21 ± 1 °C for 2–5 days. Growing mycelia were identified according to Seymour (1970) and Willoughby (1985).

Rechallenge with the passage 15 isolates

We also performed a final challenge experiment, in which we compared infection rates between passage 15 isolates (5337^{15P}, 2736^{15P} and 5708^{15P}), the primary passage isolates (AP) and an isolate obtained from diseased fish infected with the passage 15 isolates. The isolate obtained from these fish were termed '5337^{AP15P}', '2736^{AP15P}' and '5708^{AP15P}'.

Preparation of histopathology sections

Skin and muscle samples (<1 cm^3), including the leading edge of the lesion and the surrounding tissue, were collected for histopathological examination. Other tissues included samples of gills, liver, heart, spleen and posterior kidney. All samples were immediately fixed in 10% buffered formalin. Histopathology sections were prepared by routine laboratory procedures and stained with haematoxylin and eosin. Special stains periodic acid-Schiff (PAS) and Grocott's modification of Gomori's methenamine silver (GMS) stain were employed to further demonstrate the presence of hyphae.

Testing for growth rate on solid agar

Using a 5-mm cork borer, an agar block colonized by *Saprolegnia* hyphae from each passage was put in the centre of a 90-mm Petri dish containing GY agar and incubated at 21 ± 1 °C for 72 h, according to Stueland *et al.* (2005). Radial growth was measured every 24 h. Hyphae would be regarded as showing radial growth (>40 mm) when they reached the edge of the Petri dish. The test was performed in triplicate per generation per strain.

Morphological investigations of *Saprolegnia* isolates

Two millilitres of encysted zoospore suspension (equivalent to about 5×10^5 zoospores) from each passage under comparison was pipetted under aseptic conditions onto Petri dishes with sterilized aquarium water (SAW) and six sterilized hemp seeds (Ali 2004). This was done in triplicate per passage. Thereafter, the Petri dishes were incubated at 21 °C for 2 weeks. Morphological

characteristics of the developing colonies were evaluated by microscopic examination, starting from the second day and subsequently extended daily during the 2-week period of incubation. The cultures were thoroughly examined for abilities to produce oogonia on hemp seed at different generations (AP, 5, 15), zoospore release patterns, percentage of cysts with indirect germination after incubation in 25% GY broth at 21 ± 1 °C for 3 h, percentage of cysts germinating after incubation of *Saprolegnia* mycelium in SAW at 21 ± 1 °C for 20 h, formation of sexual reproductive structures and chlamydospores. The approximate total number of sporangia and sporangial releases and oogonia and chlamydospore production per colony (hemp seed) were assessed and scored. According to the scores, they were rated as follows: H = high rate, >9 sporangia per hemp seed, >16 oogonia per seed, >10 chlamydospores per seed; M = moderate rate, 4–9 sporangia per seed, 8–16 oogonia per seed, 5–10 chlamydospores per seed; L = low rate, 2–3 sporangia per seed, 4–7 oogonia per seed, 2–4 chlamydospores per seed; R = rarely present, <2 sporangia per seed, <4 oogonia per seed, <2 chlamydospores per seed; NP = not present at all.

Medium for physiological investigations

Glucose–yeast extract with 200 mg L^{-1} chloramphenicol was used in solidified form for purification and as an inoculum source of *S. parasitica.* Also, it was used as a liquid broth for physiological investigations into the oomycete at different passages.

Statistical analyses

Statistical analyses were performed by two-way ANOVA (Tukey's HSD) using the statistical package JMP®7.0.1 (SAS Institute Inc., Cary, NC). Fisher's exact test (two-sided) was used to analyse cumulative percentage infection (CPI) between groups at endpoint. A *P*-value <0.05 was considered to represent significant differences between groups/treatments.

Results

Challenge experiments

All strains and all passages of all strains resulted in an infection over a period of 11–14 days post-challenge. As fish, for ethical reasons and with consideration of humane endpoints, were collected and killed prior to death, an infection was considered as having been established when macroscopic signs of saprolegniasis were present. The infection was confirmed by stereomicroscopic examination and re-isolation from infected fish. We expressed the outcome of challenge as CPI, and the results are summarized in Table 2 and Figs 1–3.

Strain 5337^{AP} produced visible, typical lesions in a few fish within 48 h post-challenge, and the first moribund fish were collected and killed at this time point (48 h post-challenge) (Fig. 1). By day 3, some infected fish were observed in 5337^{5P} and 5337^{15P} tanks (Fig. 1). The CPI for 5337^{AP}

Table 2 Cumulative infection rate at the end of challenge with strains VIO 5337, 2736 and 5708 and different passage numbers

	Morbidity (%)		
	Strain		
Generation	VIO 5337	VIO 2736	VIO 5708
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
AP	77.2 ± 11.0[a]	23.3 ± 16.5[a]	67.4 ± 15.0
5P	47.3 ± 16.3	6.7 ± 0.0	46.9 ± 8.5
15P	49.6 ± 14.9	3.3 ± 0.0	79.3 ± 7.4[b]

The control was prepared in duplicate and values of the different strain passages represent four replicates.
[a]Denotes significantly different from other passages of the same strain.
[b]Denotes significantly different from the 5th passage of the same strain; PI, Post-infection.

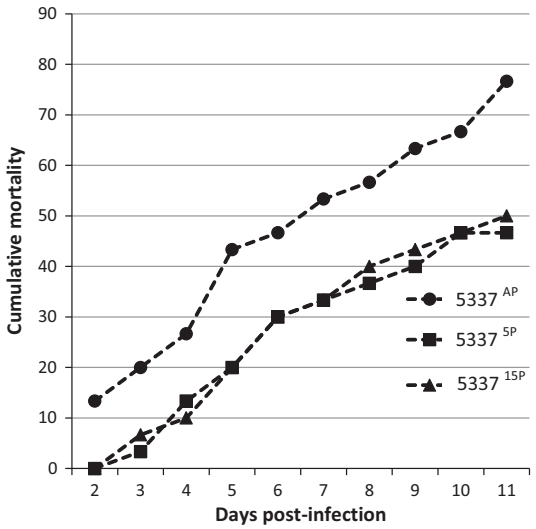

Figure 1 VIO 5337 Percentage of fish showing gross signs of infection over a period of 11 days.

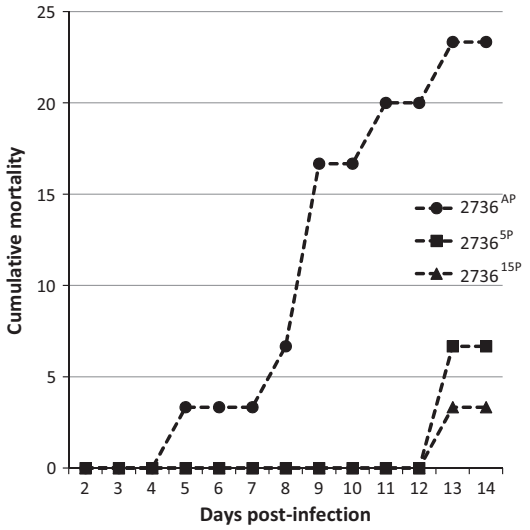

Figure 2 VIO 2736 Percentage of fish showing gross signs of infection over a period of 14 days.

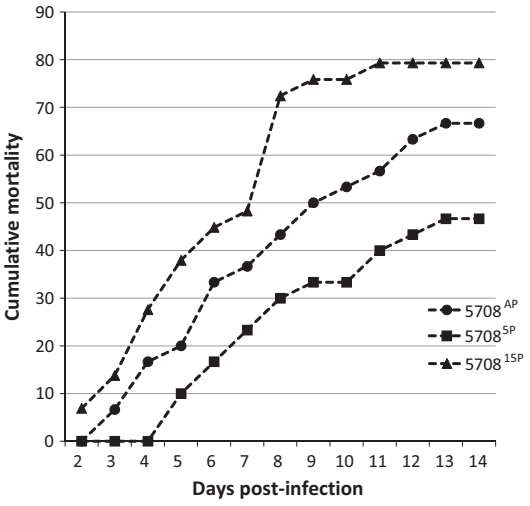

Figure 3 VIO 5708 Percentage of fish showing gross signs of infection over a period of 14 days.

was significantly higher than those in the 5337^{5P} and 5337^{15P} groups by day 11 (Fig. 1).

The 2736 strain gave low CPI for all passages tested (Fig. 2), with a CPI of 23.3 by day 14 post-challenge (Fig. 2; Table 2). The first infected fish were observed by day 5 post-infection for the 2736^{AP}-challenged fish (Fig. 2). Passage reduced the virulence of strain 2736, with a CPI at 14 days of 6.7 for 2736^{5P} and 3.3 for 2736^{15P}. There were significant differences in CPI between AP and 5P/15P challenges (Fig. 2).

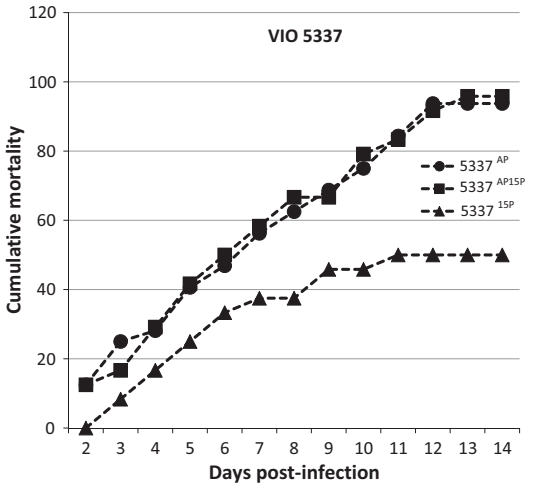

Figure 4 VIO 5337 Percentage of fish showing gross signs of *Saprolegniasis* over a period of 14 days after rechallenge with the 15th passage isolate.

For strain 5708, the infection rate was high, with CPI ending at 67.4, 46.9 and 79.5 for 5708^{A}, 5708^{5P} and 5708^{15P}, respectively. This shows that passage initially resulted in lower CPI ($P = 0.0065$), while it increased by 15P. 5708^{15P} gave a CPI not significantly different from that of the AP isolate ($P = 0.0792$) but was significantly more virulent than that of the 5P isolate ($P = 0.0001$). None of the control groups showed any signs of infection, and there were no fish dying in these tanks (Table 2).

As there was a trend that passage reduces the virulence of the tested strains (5337 and 2736), we were interested in finding out whether the passaged strains (15P) when re-isolated from dead fish would remain with reduced virulence or yield a successful infection. For strain 5337, all three passages (5337^{AP}, 5337^{15P} and 5337^{AP15P}) were significantly different from the control at 14 days post-infection. 5337^{15P} was also significantly different from the other two. However, there was no difference between 5337^{AP} and 5337^{AP15P} (Fig. 4).

For strain 2736, all three passages, 2736^{AP}, 2736^{15P} and 2736^{AP15P}, were significantly different from the control at 14 days post-infection. 2736^{15P} also gave significantly lower CPI compared with the other two passages. There was no difference between 2736^{AP} and 2736^{AP15P} (Fig. 5).

Strain 5708 was also included despite the fact that we did not observe a loss of virulence after passage. While we found that all passages resulted in significantly higher mortality than the controls

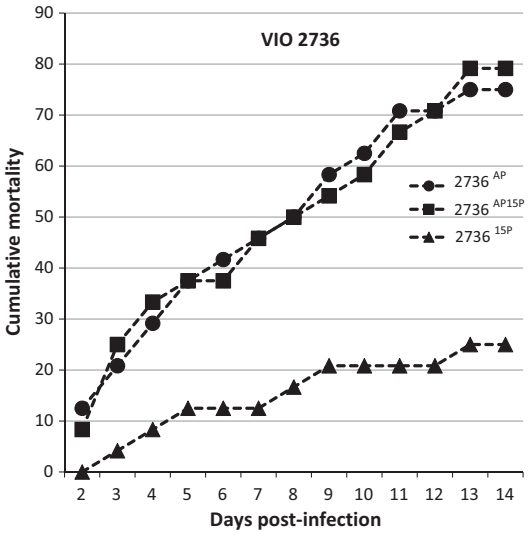

Figure 5 VIO 2736 Percentage of fish showing gross signs of *Saprolegniasis* over a period of 14 days after rechallenge with the 15th passage isolate.

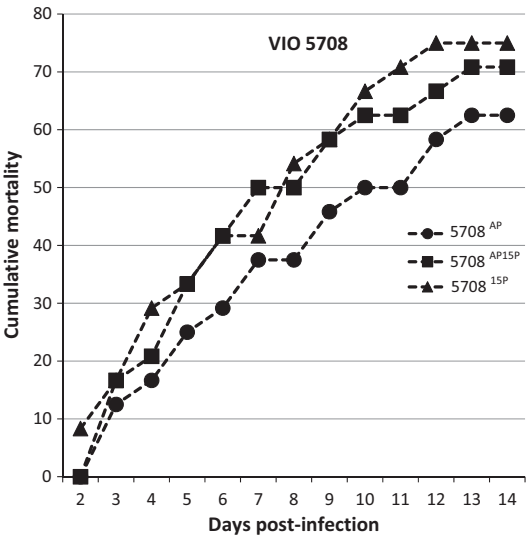

Figure 6 VIO 5708 Percentage of fish showing gross signs of *Saprolegniasis* over a period of 14 days after rechallenge with the 15th passage isolate.

14 days post-infection, again there were no significant differences (statistically) between 5708^{AP}, 5708^{15P} and 5708^{AP15P} (Fig. 6).

Gross and histopathology

Infection was confirmed by typical gross changes where signs varied from cotton-wool-like tufts on the fins, head, integument and gills (Fig. 7), to focal areas of haemorrhage, necrosis and ulceration. Clinical signs included lethargy and loss of equilibrium in cases where the fish was seen swimming in circles almost above the water surface.

By histopathology, a wide array of findings was evident in tissues infected with *S. parasitica* strains of different passages. One skin/muscle section from a fish infected with 5337^{AP} showed necrosis of the epidermal layers extending into the dermis. There was very little inflammatory reaction present, and only a few neutrophils and lymphocytes were found in infected areas. Oedema and mild haemorrhage were also observed, with wipeout of the epidermis (Fig. 8a). The kidney interstitium of the same fish (5337^{AP}) exhibited severe karyorrhexis and oedema (Fig. 8b). Mild pathological changes were detected in tissues collected from fish infected with 5337^{15}, characterized by moderate congestion of the liver (Fig. 8c) and mild degeneration on secondary lamellae of the gills (Fig. 8d). GMS staining did not show the *Saprolegnia* hyphae well, and the same was true for PAS, which showed very poorly defined hyphae (not shown).

Morphological characteristics of the *S. parasitica*

The impact of passage on the virulence of the strains included motivated us to examine in more detail the morphological characteristics of the different *S. parasitica* strains at different passages (summarized in Table 3).

While passage resulted in significant reduction in virulence for strain 5337, examination for sexual reproductive structures (oogonia and antheridia) of isolates by day 14 post-challenge did not show production of oogonia or antheridia. The samples from this time point were incubated for an extra 2 weeks in the laboratory, and still no sexual reproductive structures were observed for any of the cultures. For strains 2736 and 5708, oogonia were observed at very low rates (Fig. 9), but these findings did not associate with virulence/mortality figures.

The impact of *in vitro* passage on virulence for strains 5337 and 5708 was different (Figs 1 & 3), but we found a high rate of sporangia production by the different passages of both strains. The most striking finding was that 2736^{15P}, which was the most attenuated by passage, also showed the lowest rates of sporangial release and proliferation.

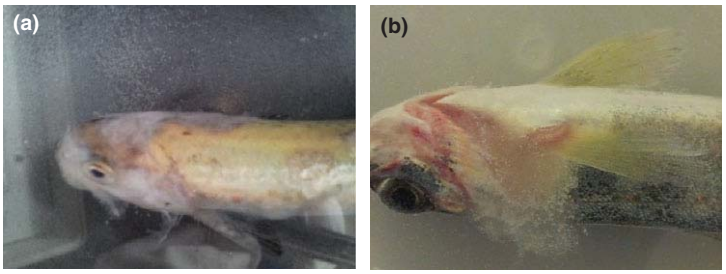

Figure 7 Gross lesions on the infected fish (a) an Atlantic salmon parr that got infected 3 days after being challenged with 5337AP. Note the cotton wool-like mycelial growth around the head. (b) 27365P primarily affecting the gills.

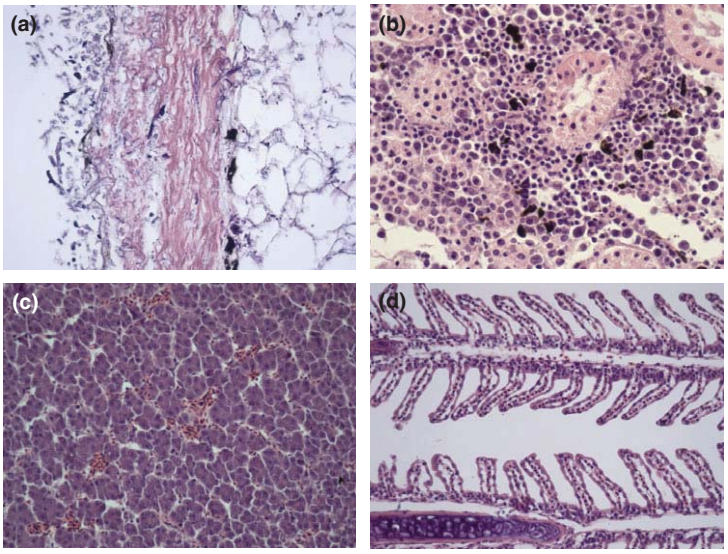

Figure 8 Histopathology of tissues from infected fish. (a) Skin, oedema and mild haemorrhage, with complete wipeout of the epidermis; (b) kidney, severe karyorrhexis and oedema; (c) liver, moderate congestion; and (d) gills, mild degeneration of secondary lamella.

Table 3 Morphological characteristics of the *S. parasitica* strains at different passages

	Saprolegnia parasitica strain/passage (VIO)								
Morphological features	5337^{AP}	5337^{5P}	53371^{5P}	2736^{AP}	2736^{5P}	27361^{5P}	5708^{AP}	5708^{5P}	57081^{5P}
Growth rate on agar (mm)	30.0	30.0	30.0	28	28	28	29	29	29
Sporangia formation	25	25	25	20	20	18	23	23	23
Sporangia release	25	25	25	20	12	10	23	23	23
Oogonia	NP	NP	NP	NP	R	NP	R	R	R
Chlamydospore formation	23	23	23	23	23	23	23	23	23

H: High rate; more than 9 sporangia per hemp seed, > 16 oogonia per seed, >10 chlamydospores per seed. M: Moderate rate; 4-9 sporangia per seed, 8-16 oogonia per seed, 5-10 chlamydospores per seed. L: Low rate; 2-3 sporangia per seed, 4-7 oogonia per seed, 2-4 chlamydospores per seed. R: Rarely present; less than 2 sporangia per seed, less than 4 oogonia per seed, less than 2 chlamydospores per seed. NP: Not present at all.

On solid GY, no significant differences were observed in growth rates of the cultures of different passages, and the diameters of the vegetative colonies were in principle the same at all passages for all three strains, <35 mm.

Finally, production of enlarged chlamydospores was abundant in all the cultures at the different passages (Fig. 10) and no differences were seen.

Discussion

The present study demonstrates that successive *in vitro* subculturing may have varying effects on different strains of *S. parasitica*. In this study, the pattern of change in virulence was strain dependent. Fifteen successive *in vitro* subcultures resulted in reduced virulence for strains 5337^{AP}

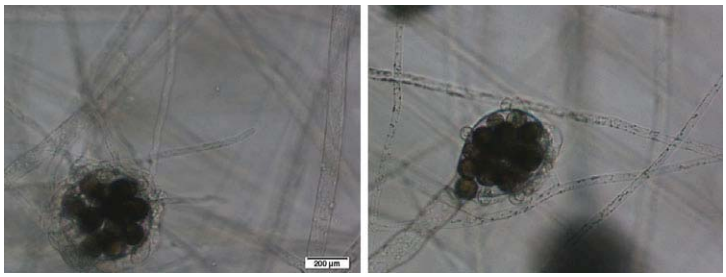

Figure 9 Oogonia observed in strains 2736 and 5708.

Figure 10 Chlamydospores of 5337AP at: (a) X4, (b) X10 and (c) X20 in a 4-week-old culture. Note the absence of reproductive structures.

and 2736^{AP}. Noteworthy among the results in this study was the fact that the strains that showed a loss of virulence due to successive *in vitro* subculturing were the ones that regained their virulence following passage through Atlantic salmon parr.

Strain 5708 produced the highest all-round morbidity and did not show a decline in virulence after 15 *in vitro* subcultures. A passage of this particular strain through the susceptible fish host did not cause any increment in its morbidity rate. So far, there is no single documented research report on attenuation of virulence in oomycetes, including those pathogenic to plants, but if reports from researchers working with other types of organisms are anything to go by, ours is not an isolated finding (Hayden, Bidochka & Khachatourians 1992; Brownbridge *et al.* 2001; Vandenberg & Cantone 2004; Shah, Wang & Butt 2007; Nahar *et al.* 2008). No obvious differences were found in the severity of lesions caused by the different passages of all three strains. 2736^{15} had an endpoint CPI of only 3%. We may attribute this to the fact that sporangia were very rarely observed in cultures of this isolate.

On the whole, our study shows that the ability to maintain virulence *in vivo* can vary between *S. parasitica* strains, and so not all isolates of *S. parasitica* need to be passed through susceptible fish prior to challenge experiments. Based on our findings, it would be advisable to be cautious and preserve several isolates of the same strain of *S. parasitica* on hemp seed. Until now, the sole possibility of overcoming attenuation in many different types of organisms is with a periodical passage through the target host (Ito *et al.* 2001; Ansari & Butt 2011), but this method is time-consuming and there is a risk of contamination (Butt & Goettel 2000). It may also raise questions of ethics and animal welfare.

The fact that the special stains were not able to demonstrate fungal hyphae in histopathology sections may be due to the fact that there is very little chitin in *Saprolegnia* (Yanong 2003). Most studies have indicated that in *Saprolegnia* infections, there is very little or no inflammatory response in the integument (Pickering & Richards 1980; Pickering & Willoughby 1982; Xu & Rogers 1991; Bly *et al.* 1992; Lopez-Doriga 1995) or inner infections (Bruno & Stamps 1987; Alvarez *et al.* 1988, 1995). These reports are consistent with our findings in the present study. The lack of inflammatory response seems to be related to immunosuppressive factors, because immune-competent catfish experimentally infected with *Saprolegnia* were able to produce inflammatory processes (Bly *et al.* 1994).

On hemp seed cultures, none of the isolates belonging to the strain VIO 5337 produced oogonia at 21 °C, but some of the passages of the other two strains (VIO 2736 and VIO 5708) did, albeit at very low rates of <4 per seed. In this particular study, there was no correlation between oogonia formation and the ability of an isolate to cause morbidity.

Production of enlarged *S. parasitica* chlamydospores was abundant all-round. The fact that chlamydospores were observed at high rates might not have much significance as far as pathogenicity is concerned. Yuasa & Hatai (1995) reported that highly pathogenic strains developed abundant chains of chlamydospores, but this was not confirmed by Fregeneda, Fernandez Diez & Aller Gancedo (2001). In the present study, the diameters of vegetative colonies of all the different generations of the three *Saprolegnia* strains in question were less than 35 mm after 72 h. This is in accordance with findings by Stueland *et al.* (2005) in which only *S. diclina* and S. *ferax*, but not *S. parasitica* strains, attained diameters above 40 mm over a similar time point and at a similar water temperature, 21 ± 1 °C.

Acknowledgements

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PAPER II

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Saprolegnia diclina IIIA and *S. parasitica* employ different infection strategies when colonizing eggs of Atlantic salmon, *Salmo salar* L.

M M Songe[1], A Willems[2], J Wiik-Nielsen[1], E Thoen[1], Ø Evensen[3], P van West[2] and I Skaar[1]

1 Norwegian Veterinary Institute, Oslo, Norway
2 Aberdeen Oomycete Laboratory, College of Life Sciences and Medicine, Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK
3 Faculty of Veterinary Medicine and Biosciences, Norwegian University of Life Sciences, Oslo, Norway

Abstract

Here, we address the morphological changes of eyed eggs of Atlantic salmon, *Salmo salar* L. infected with *Saprolegnia* from a commercial hatchery and after experimental infection. Eyed eggs infected with *Saprolegnia* spp. from 10 Atlantic salmon females were obtained. Egg pathology was investigated by light and scanning electron microscopy. Eggs from six of ten females were infected with *S. parasitica*, and two females had infections with *S. diclina* clade IIIA; two *Saprolegnia* isolates remained unidentified. Light microscopy showed *S. diclina* infection resulted in the chorion in some areas being completely destroyed, whereas eggs infected with *S. parasitica* had an apparently intact chorion with hyphae growing within or beneath the chorion. The same contrasting pathology was found in experimentally infected eggs. Scanning electron microscopy revealed that *S. parasitica* grew on the egg surface and hyphae were found penetrating the chorion of the egg, and re-emerging on the surface away from the infection site. The two Saprolegnia species employ different infection strategies when colonizing salmon eggs. *Saprolegnia diclina* infection results in chorion destruction, while *S. parasitica* penetrates intact chorion. We discuss the possibility these infection mechanisms representing a necrotrophic (*S. diclina*) vs. a facultative biotrophic strategy (*S. parasitica*).

Keywords: chorion disruption, egg infection, infection strategies, *Saprolegnia*.

Correspondence *I Skaar, Norwegian Veterinary Institute, PO Box 750 Sentrum, N-0106 Oslo, Norway (e-mail:* ida.skaar@vetinst.no*)*

Introduction

Aquaculture has become the world's fastest growing food sector. With increasing production intensity, the control and reduction of health problems in the entire production chain is paramount for future success of the aquaculture industry. It has been established that the greatest losses of fish eggs are caused by infection with *Saprolegnia* species (Willoughby 1970; Czeczuga & Kiziewicz 1999; Hussein, Hatai & Nomura 2001). The use of malachite green, a very effective treatment against *Saprolegnia* infections (Fitzpatrick *et al.* 1995; Kitancharoen, Yamamoto & Hatai 1997), was banned worldwide due to its carcinogenic and toxicological effects. At present, there is no equally efficient treatment available, and therefore, saprolegniasis has become an increasing problem worldwide. It has been estimated that over 10% of salmonid eggs become infected with oomycetes in hatcheries each year (Bruno, Van West & Beakes 2011). In Norwegian salmon farming, *Saprolegnia* infection is mainly a problem in incubating eggs and newly hatched fry (Thoen, Evensen & Skaar 2011). Outbreaks are also seen in fingerlings and parr throughout the freshwater stage,

Journal of Fish Diseases

although more sporadically. In other countries with large-scale salmonid farming such as Chile, Japan and Scotland, the problem is also present during the egg-incubation stage (Beakes, Wood & Burr 1994; Kitancharoen & Hatai 1996, 1997; Kitancharoen *et al.* 1997; Hussein *et al.* 2001; Van Den Berg *et al.* 2013). Numerous studies of *Saprolegnia* infection in immature and mature stages of salmonids have been conducted (Wood, Willoughby & Beakes 1988; Pottinger & Day 1999; Hussein & Hatai 2002; Stueland, Hatai & Skaar 2005; Thoen *et al.* 2011). However, only a few scientific reports of pathogenesis of *Saprolegnia* infections in salmonid eggs are available (Kitancharoen & Hatai 1996; Thoen *et al.* 2011). In particular, the role of *S. parasitica* as a cause of saprolegniasis in fish eggs is still unclear. Fish eggs are thought to be killed by hypha breaching the chorionic membrane regulating the osmosis of the embryo (Liu *et al.* 2014). A better understanding of the infection process would enable us to develop new sustainable control strategies against infection and can create the basis for the development of new therapeutic interventions. The aim of this study was to determine which species of *Saprolegnia* was the most prevalent cause of egg infection under natural conditions, and to better understand whether the two *Saprolegnia* species employ different infection strategies under these circumstances and following an experimental infection of eggs.

Materials and methods

Atlantic salmon eggs

Living eyed eggs, 280 degree-days old and visibly infected with *Saprolegnia* originating from 10 different Atlantic salmon, *Salmo salar* L. females were collected from Landcatch, a commercial hatchery located in Scotland. The different females from which the eggs were collected are treated as biological replicates and hence are kept in different units in the hatchery. The unit numbers were noted and recorded for possibility of lineage tracing. The eggs were considered infected if they were tightly clumped together in a tuft of mycelium. From each female, seven infected eggs were collected from each female ($n = 70$). One egg from each female ($n = 10$) was immediately placed on glucose–yeast extract (GY) agar consisting of 1% glucose, 0.25% yeast extract and 1.5% agar (Hatai & Egusa 1979) for cultivation, isolation and identification of the *Saprolegnia* isolates involved. Three of the 6 remaining eggs ($n = 60$) were fixed in 15 mL of 10% phosphate-buffered formalin (Bancroft & Stevens 1990) and kept at 4 ± 1 °C. The last three eggs ($n = 30$) were fixed in 15 mL of 4% paraformaldehyde/1% glutaraldehyde/phosphate buffer and kept at 4 ± 1 °C (Glauert & Lewis 1998). The fixatives were replaced with freshly prepared solutions of 10% phosphate-buffered formalin and 4% paraformaldehyde/1% glutaraldehyde/phosphate buffer after 24 h and stored until further processing. Additionally, one uninfected egg was collected from each female to serve as the negative control for the histology.

Purification of the *Saprolegnia* isolates

An agar plug of 5 mm in diameter, colonized by *Saprolegnia* hyphae, was cut from the GY plate and transferred to another plate containing GY agar (Hussein & Hatai 1999). To inhibit bacterial growth, the GY agar was supplemented with 200 μg mL^{-1} chloramphenicol (Fregeneda-Grandes, Rodríguez-Cadenas & Aller-Gancedo 2007). The growing hyphae were cut into small pieces and transferred to sterile aquarium water (SAW) for zoospore production. Single-spore isolations were performed on GY agar with chloramphenicol and incubated at 21 ± 1 °C for 2–5 days (Onions, Allsopp & Eggins 1981). These procedures were repeated until pure cultures were obtained. Pure cultures were stored on autoclaved hemp seeds at 4 ± 1 °C according to the procedure described earlier (Stueland *et al.* 2005).

Morphological identification

The purified strains were identified morphologically (Willoughby 1970, 1986). From a single-spore culture on GY agar, a 5-mm-diameter plug of the growing mycelium was placed in GY broth (Hatai & Egusa 1979; Kitancharoen *et al.* 1995) and incubated for 2–3 days at 15 ± 1 °C. Bundles of hyphae were washed with SAW and incubated with autoclaved hemp seeds in SAW. Examination of possible sexual structures on hemp seeds was performed at 5, 15 and 20 ± 1 °C, and the hemp seed cultures were examined for production of oogonia and antheridia twice a week over a 12-week period using an Olympus®

inverted zoom stereo microscope (SZH-ILLD), with a bright field/dark field transmission light illumination base (Stueland *et al.* 2005).

Molecular identification

The purified *Saprolegnia* isolates from infected Atlantic salmon eggs were subjected to molecular identification. Genomic DNA was extracted from 20 mg mycelia from each isolate using CTAB miniprep extraction protocol (Gardes & Bruns 1993). The ITS region was amplified using the universal fungal reverse primer ITS4 (White *et al.* 1990) and oomycete specific forward primer ITS1_Omyc. The 25 μL reaction mixture consisted of 1.7 μM of each primer, 2 μL of genomic DNA, puReTaq Ready-To-Go PCR Beads (Amersham Biosciences) and milliQ water. PCR was performed on a Bio-Rad (Biorad/MJR) DNA Engine Dyad thermal cycler. The PCR amplicons were visualized by gel electrophoresis on 1.0% agarose gel stained with Gelred (Huang *et al.* 2010). PCR products were purified with ExoSAP-IT (Amersham Bioscience) according to the protocol. The products were then sequenced in both directions with their respective primers, using the BigDye® Terminator v3.1 Ready Reaction Mix (Applied Biosystems, Life Technologies). The sequenced PCR products were purified with BigDye® XTerminator Purification kit (Applied Biosystems, Life Technologies) according to manufacturer's instructions and subsequently analysed on an ABI PRISM® 3100 – Avant Genetic Analyzer (Applied Biosystems). Sequence contigs were assembled and quality-controlled in BioEdit (Hall 1999). The sequences were compared to publically available sequences using the NCBI nucleotide Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1997) and identified on the basis 100% identity to well-annotated *Saprolegnia* reference strains.

Sample processing

For light microscopic examination, the infected and negative control eggs, fixed in 10% phosphate-buffered formalin, were treated as follows: dehydration through ascending alcohol grades, clearing in xylene, impregnation with wax, cutting at 5 μm, mounting on a glass slide, complete dewaxing and staining with haematoxylin and eosin. The microscopic slides were the examined with a light microscope.

The disruption of the chorion was the main finding, and the changes seen were categorized (subjectively) into three different grades: minor, moderate and severe. Minor changes were observed as single foci damage to the chorion with the loss of continuity of the chorion surface without these changes extending all the way through the chorion. These were found with hyphae on the surface of penetrating into the chorion. Moderate changes included loss of continuity over a larger area and typically with pore formations in the chorion. Hyphae were found on the surface and penetrating into the chorion. Severe changes were complete loss of continuity, disorganized chorion and exposure to underlying structures. Hyphae were located on the surface and growing into the layers beneath the chorion. Formation of cysts was also recorded. The degree of vacuolization of the egg cytoplasm was also included in the evaluation and was separated into minor (few vacuoles), moderate (increasing number and coalescing) and severe including vacuolation of large areas of the egg cytoplasm.

Scanning electron microscopy. The eggs were fixed in 4% paraformaldehyde/1% glutaraldehyde/phosphate buffer. Post-fixation was carried out with 1% buffered osmium tetroxide. Subsequent treatments included (i) dehydration in ascending grades of acetone and were dried in tetramethyl silane (TMS) following the method of Dey *et al.* (1989). The dried samples were mounted on brass stub (10 × 30 mm) using a double-sticky adhesive tape, connected via a patch of silver paint to ensure charge conduction. A thin conductive coating of gold was applied to the samples using JFC 1100 (Jeol) ion sputter at a relatively low vacuum of 10^{-3} torr, or (ii) air-drying and direct conductive gold coating as described. The coated samples were examined in a Zeiss EVO 50 EP Scanning Electron Microscope at an accelerating voltage of 20 kV in the secondary electron emission mode.

Salmon eggs for experimental infection

Eyed eggs from Atlantic salmon, *Salmo salar* L. of strain AquaGen Atlantic QTL-innOva IPN from AquaGen AS were used for the experimental infection. The eggs were 385 degree-days on the day of shipment. They were disinfected during incubation and before transport with buffodine (1:100, 10 min) and treated with formalin according to AquaGen's in-house protocols. The eggs

were gradually (approximately 24 h) brought to the laboratory water temperature used during the experiments, which varied from 8.7 to 9.6 °C (for the live egg experiments; described below). The eggs were then acclimatized for 3 days in the laboratory to avoid mortality from transportation damage, prior to the challenge experiments. Blank, pin-eyed and white eggs were removed if observed.

Saprolegnia strains used for experimental infections

Two strains of *Saprolegnia* spp (*S. parasitica* VIO 2741 and *S. diclina* clade IIIA, VIO 2739) previously shown to be pathogenic to Atlantic salmon eggs (Thoen *et al.* 2011) were used for the artificial infections. Cysts were produced according to the method described by Stueland *et al.* (2005). The cysts were counted using a haemocytometer (Bürker turk) and the cyst suspensions adjusted by dilution to obtain the required density 1×10^4 spores L^{-1} (Thoen *et al.* 2011).

Preparation of infected dead eggs (focus of infection)

Adopting methods used by Thoen and coworkers (Thoen *et al.* 2011), groups of live, eyed eggs were killed by immersion for 1 min in water bath at temperature of 60 °C. The dead eggs were incubated in *Saprolegnia* spore suspensions (1.0×10^4 spores L^{-1}) in 24-well microwell plates at 15 °C for 48 h. Incubated eggs were examined microscopically for the presence of *Saprolegnia* hyphae.

Challenge of live eggs by co-incubation with pre-infected dead eggs

Live eggs were assigned to duplicate groups of 100 ± 2 eggs each per *Saprolegnia* isolate and distributed in two separate compartments in small-scale hatching trays (one tray with two compartments of 100 eggs for each of the *Saprolegnia* species) with a flow-through system. The eggs were spread to form an even layer covering the bottom of the trays. In each compartment, groups of four eggs pre-infected with the respective isolates, as previously described, were placed on the layer of live eggs.

The four infected eggs per compartment were carefully placed in the corners of an imagined square on the layer of live eggs. This was carried out to let the hyphae from each of the infected dead eggs having the opportunity to infect an approximately equal number of the 100 live eggs. In addition to the groups with infected eggs, two groups of four dead eggs that were not exposed to *Saprolegnia* spores were introduced in compartments with 100 live eggs to serve as non-infected control groups. The experimental units were maintained and inspected daily for 10 days.

At termination of the experiment, the number of live eggs newly infected by hyphae from each of the introduced dead eggs was counted for each isolate. The live eggs were considered as infected and counted when they were entangled in hyphae and did not unfasten when the dead eggs were moved. At termination of the experiment (day 10 post-infection), representative samples of infected eggs were fixed in 10% phosphate-buffered formalin, embedded in paraffin and processed for examination by light microscopy, as described previously for the naturally infected eggs.

Results

Identification of *Saprolegnia* strains

The isolates infecting the collected eggs were identified by means of molecular and morphological methods. Two *S. diclina* and six *S. parasitica* isolates were collected and grown in pure culture from eggs from eight different fish (Table 1). Two of the *Saprolegnia* isolates could not be successfully purified due to recalcitrant contaminations with yeasts and bacteria.

Table 1 Molecular identification of *Saprolegnia* isolates

Female ID	*Saprolegnia* species	NVI culture collection number	GenBank accession number
1	*S. diclina clade IIIA*	VI0 6011	HG329742
2	*S. diclina clade IIIA*	VI0 6008	HG329735
3	*S. parasitica*	VI0 5977	HG329736
4	*S. parasitica*	VI0 5978	HG329737
5	*S. parasitica*	VI0 6009	HG329739
6	*S. parasitica*	VI0 5979	HG329738
7	*S. parasitica*	VI0 5980	HG329740
8	*S. parasitica*	VI0 5981	HG329741
9	Unidentified	–	–
10	Unidentified	–	–

Histopathological changes in field-collected eggs

Examination by light microscopy of eggs from the non-infected control group showed 2 intact layers of the chorion. The outer envelope is very thin and was stained dark pink, while the inner layer was pale pink and with thin radial lines (Fig. 1a). The cytoplasm was composed of yolk granules, and areas with blastomeres were also seen. The surface of the normal eggs was smooth and without any loss of integrity at the perimeter (Fig. 1a).

Examination of infected eggs showed clear differences between *S. parasitica*- and *S. diclina*-infected eggs. Eggs infected with *S. parasitica* varied and had an intact or minor-disrupted chorion, that is minor changes (Fig. 1b). Despite the chorion being intact, *Saprolegnia* hyphae could be observed inside the eggs from four females 6–9 (Fig. 1b, Table 2). Some eggs were found with numerous pores and cracks in their chorion (categorized as moderate changes) and with hyphae that had penetrated through the chorion and accumulated in the cytoplasm, where vacuolization was evident, that is moderate changes (Table 2, Fig. 1c).

Eggs of females (1 and 2) infected with *S. diclina* displayed severe changes with a chorion that was almost completely destroyed and in parts indiscernible (Fig. 1e,f). Germinated cysts were observed inside the egg, that is beneath the chorion or what was left of the chorion (Fig. 1e, Table 2) and with hyphae radiating out from the chorion (Fig. 1f). The chorion was discontinuous, only partly intact (Fig. 1f).

By scanning electron microscopy due to the eggs cracking during preparation (Fig. 2a), it was possible to show *Saprolegnia* hyphae on the inner side of the outer envelope (Fig. 2b). We also employed air-drying of the eggs, and on intact eggs, *Saprolegnia* hyphae were observed on the surface of the egg (Fig. 3b). Further, hyphae also

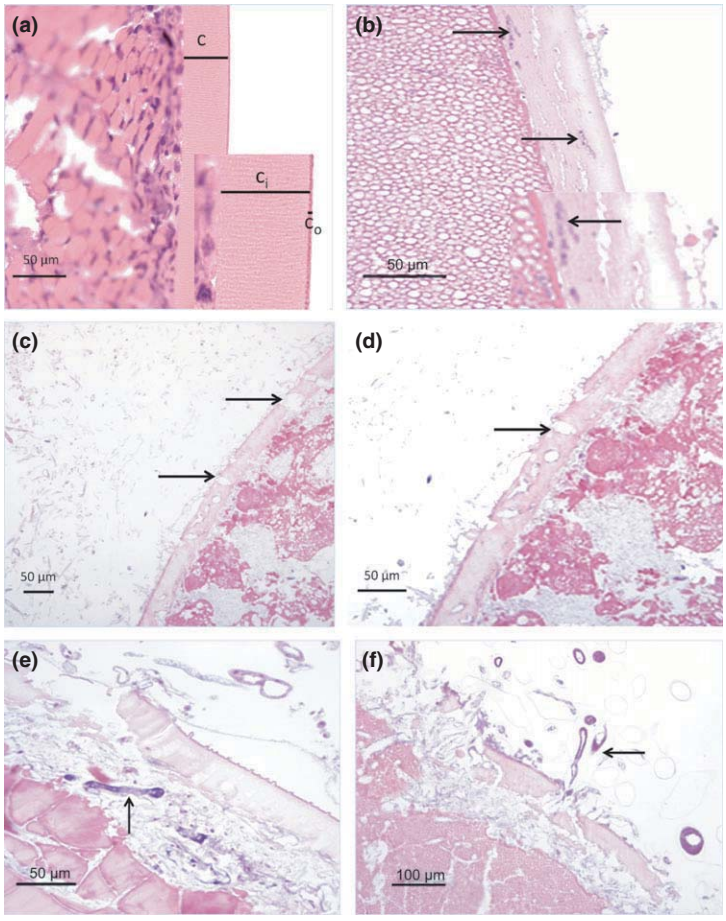

Figure 1 Histology of normal (a) and histopathology of infected eggs (b–f). (a) Healthy chorion separated into an outer thin layer (c_o; insert) and inner thicker layer (c_i). The cell-rich layer inside of the chorion is likely part of the blastoderm. (b) Hyphae of *S. parasitica* are located inside the chorion, which shows minor changes. The hyphae are located in the mid-part and towards the yolk granules (arrows). Details of the hyphae are shown in the insert (arrow). (c) *S. parasitica* infection with moderate chorion changes. Numerous hyphae on the outside of the egg, and there are several pores and vacuoles seen in the chorion wall (arrows). (d) Higher magnification of (c) detailing the vacuoles and the cracks in the chorion (arrow). Note numerous hyphae. (e) *S. diclina* infection, with moderate-to-severe changes of the chorion (and cytoplasm). Germinated cysts present below the cracked chorion and inside the egg (arrow). (f) *S. diclina* infection, severe chorion changes. Almost a complete wipeout of the chorion in some areas and with thinner chorion than normal in others. Chorion is also discontinuous and changes are associated with the presence of hyphae (arrow). Bars = 50 µm.

Table 2 Microscopic findings in infected ova of Atlantic salmon, *Salmo salar* L

			Light microscopy findings				Scanning electron microscopy (SEM) findings	
			Localization of hyphae				Localization of hyphae and/or germinated cysts	
Female number	*Saprolegnia* species involved	Grade of disruption of chorion	Egg surface	Inside	Vacuolation of cytoplasm	Germinated cysts present inside the egg	Egg surface	Penetration into chorion
1	*S. diclina*	Severe	+++	+++		++	Observed	Observed
2	*S. diclina*	Severe	+++	+++	Severe	++	No SEM micrograph	-
3	*S. parasitica*	Mild	++	++	Mild	+	Observed	Observed
4	*S. parasitica*	Moderate	+++	+++	Not observed	+	Observed	Not observed
5	*S. parasitica*	Mild	+++	+	Not observed	+	Not observed	Not observed
6	*S. parasitica*[a]	No	++	++	Not observed	Not observed	Observed	Not observed
7	*S. parasitica*[a]	No	++	++	Not observed	+	Observed	Not observed
8	*S. parasitica*[a]	No	++	++	Not observed	+	Observed	Observed
9	NI[a]	No	++	++	Moderate	+	Observed	Observed
10	NI	Mild	++	++	Not observed	Not observed	Observed	Observed

NI, not identified.
[a]Hyphae inside egg with intact chorion on histopathology.

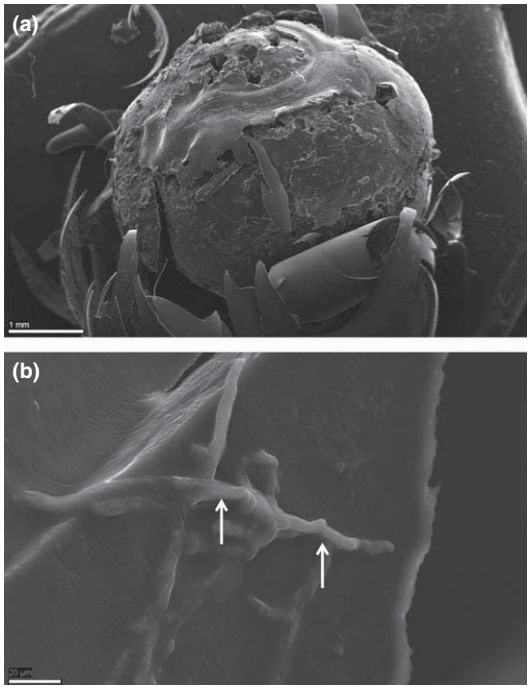

Figure 2 SEM of an infected egg. (a) Egg cracked open during processing, separating the outer and inner layer, thus exposing the inner surface. Bar = 1 mm. (b) *Saprolegnia parasitica* hyphae invading the inner surface of the outer layer of the chorion (arrows). Bar = 20 µm.

seemed to penetrate into the chorion of the egg and re-emerge on the surface (Fig. 3c). No appressoria-like structures were observed.

Experimental infection results

The high prevalence of *S. parasitica* infection in eyed eggs was a surprising finding, as was the contrast in pathology and infection dynamics observed between the two species under field conditions. For this reason and to ascertain that environmental factors in the hatchery had not selected for *S. parasitica* strains with particular virulence profiles, an infection experiment was carried out in the laboratory and included *S. parasitica* and *S. diclina* clade IIIA. The origin of these strains was different from what was observed in the field experiment.

Examination of the histopathological changes from experimentally infected eggs showed differences in egg pathology between *S. parasitica* and *S. diclina* that corroborated the findings from the field experiment. As it was difficult to demonstrate the early stages of infection from the field samples, particularly for *S. diclina*, we focused particularly on this stage for the experimental study.

Eggs infected with *S. parasitica* had an intact or moderately disrupted chorion and with hyphae located on the outside and/or on the inside of an intact chorion and chorion changes scored as 'mild' (Fig. 4a). Remnants of hyphae were seen on the outside of the chorion without the outer membrane loosing its continuity (Fig. 4a). At the early stage of *S. diclina* infection, the outer chorion membrane was found disrupted and also with disintegration of the inner chorion membrane (Fig. 4b). The radial orientation of the inner

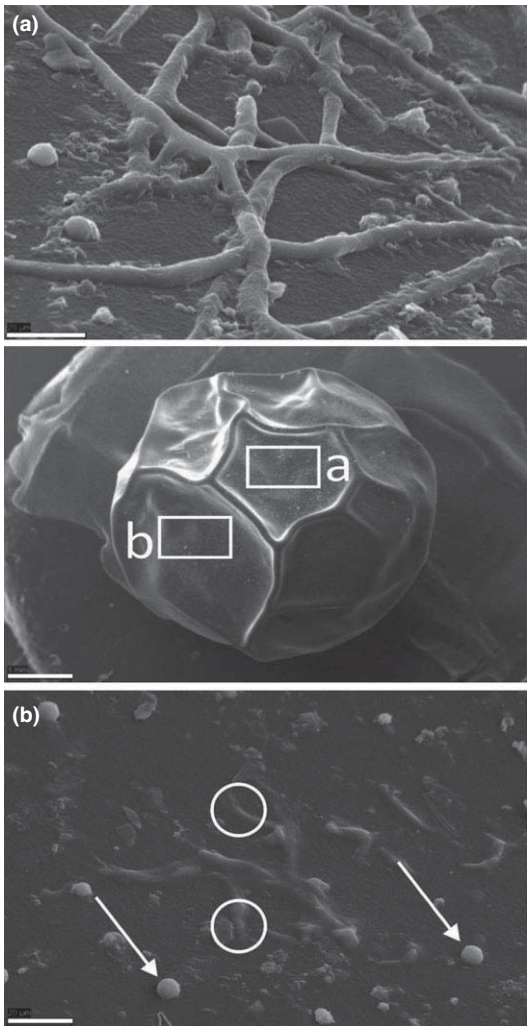

Figure 3 SEM of an air-dried infected egg (in the middle) Bar = 1 mm. A close-up of the areas marked a (upper) and b (lower) shows *Saprolegnia parasitica* cysts (arrows) and hyphae growing on the outer surface of the egg. Cysts (arrows) are also visible. Hyphae are seen penetrating into the chorion (circles). Bars a and b = 20 μm.

chorion membrane was distorted, and small cracks were seen in the inner membrane (Fig. 4b). Hyphae were found attached to the 'chorion wounds' and also extending down into the inner chorion membrane (Fig. 4b). More advanced stages appeared as for the field samples and are equivalent to what was shown in Fig. 1b–f.

Discussion

Two *Saprolegnia* species were isolated from infected eyed eggs in this study, *S. parasitica* and *S. diclina clade IIIA*. *Saprolegnia parasitica* was found more prevalent than *S. diclina* as a source of saprolegniasis in eyed eggs from field samples, in contrast to what has been considered the most frequent cause of egg infection (Kitancharoen *et al.* 1997; Fregeneda-Grandes *et al.* 2007; Van Den Berg *et al.* 2013). Furthermore, we noticed that *S. parasitica* hyphae penetrated the egg chorion without destroying it, while *S. diclina*-infected eggs had a completely disrupted or destroyed chorion. These findings were replicated following experimental challenge with eyed eggs. This would point towards the two species employ different infection strategies when colonizing and infecting eggs of Atlantic salmon (Van West *et al.* 2010; Wawra *et al.* 2012). We propose this might represent a necrotrophic strategy employed by *S. diclina,* which contrasts a possible facultative biotrophic mechanism by *S. parasitica*. Importantly, the *S. diclina* strain detected in field samples and used for experimental infection was of clade IIIA and, together with clade IIIB, these are the most prevalent variants found in Norwegian salmon hatcheries (unpublished results).

Despite differences in preferred hosts, *S. diclina* and *S. parasitica* are closely related (Dieguez-Uribeondo *et al.* 2007; Sandoval-Sierra, Martín & Diéguez-Uribeondo 2014) species, mostly reported from areas with a temperate climate, such as north-west Europe, Chile, Japan and Canada, where they have a large impact on salmon farming (Van Den Berg *et al.* 2013). Over the years, researchers have described different mechanisms of pathogenesis displayed by *Saprolegnia* and other oomycetes when they infect salmonids and their eggs. Peduzzi and Bozzozero (Peduzzi & Bizzozero 1977) detected a chymotrypsin-like enzyme system in culture filtrate and mycelial extracts from water moulds associated with saprolegniasis in fish. They observed that an extracellular proteolytic enzyme produced by the oomycete would favour the deep penetration by invading hyphae into the host tissue. Plant oomycetes are able to breach cuticles of host plants and establish infection rapidly (Soanes, Richards & Talbot 2007). First, the pathogen synthesizes the new cell wall to make infection-associated structures. Secondly, it breaks down the physical barrier of the plant cell wall by both enzymatic action and/or mechanical pressure, depending on the infection strategy of the pathogen (Mach 2008). As a consonance, the structure of the egg chorion and the thickness of the mucus

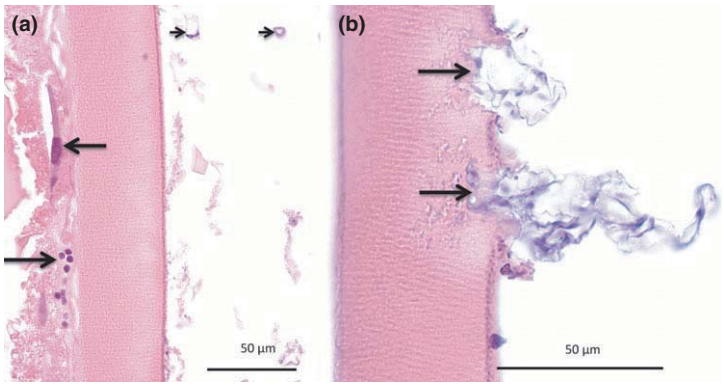

Figure 4 (a) *Saprolegnia parasitica*-infected egg showing an intact or moderately disrupted chorion and with hyphae located on the outside and/or on the inside of an intact chorion and chorion changes scored as 'mild'. Remnants of hyphae were seen on the outside of the chorion (arrowheads) without the outer membrane loosing its continuity. (b) *Saprolegnia diclina* infection with the outer chorion membrane disrupted and with disintegration of the inner chorion membrane. Hyphae were found attached to the 'chorion wounds' and also extending down into the inner chorion membrane (arrows). The radial orientation of the inner chorion membrane was distorted, and small cracks were seen in the inner membrane (arrow). Bar = 75 µm.

layer covering it play a role in the occurrence of different mycotic species of fish eggs (Lartseva & Altufiev 1987). Rand and Munden (Rand & Munden 1992) suggested that invasion of living fish eggs by pathogenic strains of *Saprolegnia* species is facilitated by a combination of mechanical pressure and enzymatic activities of their mycelia. They detected enzymes on *S. diclina*-infested brook char eggs and suggested that the enzymes may have altered the integrity of the chorionic membrane by solubilizing structural polymers and facilitating the penetration of the hyphae through the chorion membrane. Our findings concur with these observations for *S. diclina*-infected eggs, but the presence of *S. parasitica* hyphae beneath intact egg chorion is not easily understood. In concert with what Rand and Munden (Rand & Munden 1992) proposed, mechanical pressure might play a role in hyphae penetration but enzymes released from the hyphae might also facilitate penetration, or a combination of the two is also possible. Importantly, we observed the same phenomena from field infections as for experimental challenge with the two *Saprolegnia* species. While *S. diclina* infection results in distinct egg pathology and with severe necrosis of the chorion, *S. parasitica* obviously employ a different strategy. On this basis, it is tempting to speculate whether *S. diclina* possibly employ a necrotrophic strategy, while *S. parasitica* would represent a biotrophic lineage. This can possibly be used as guidance when searching for underlying biological traits, such as enzyme selections, as seen for certain lineages of necrotrophic fungal pathogens (Sprockett, Piontkivska & Blackwood 2011). *Saprolegnia diclina* might represent a lineage-specific expansion of a pathogen, particularly virulent for salmon eggs. These findings warrant additional studies into the pathogenesis of fish-egg saprolegniasis (Lartseva & Altufiev 1987).

Several researchers have reported *S. diclina* as the major species infecting fish eggs (Hussein *et al.* 2001). However, our findings are more in line with what was reported by Shahbazian *et al.* (2010), who found that *S. parasitica* was the most frequently detected species associated with egg infections. They also observed noticeable differences in the fungal and oomycete communities at the two hatcheries that they investigated. They asserted that ecological differences resulting from different hatchery conditions (chemical factors, age of broodstock, density of eggs) may have played a role in the type of fungi and oomycetes that developed on the rainbow trout eggs in their study (Willoughby 1986; Hussein *et al.* 2001). Khosravi and coworkers (Khosravi *et al.* 2012) also isolated a higher percentage of *S. parasitica* from infected eggs (54.3%), compared to 45% *Saprolegnia* spp and 0.7% *Fusarium solani* during their evaluation of antifungal activity of various essential oils for treatment of rainbow trout eggs infected with *Saprolegnia sp.* Therefore, it seems safe to state that *S. parasitica* is also an egg-pathogenic species and that local environmental factors may influence the prevalence of a particular species, but these remain to be identified.

Although the present study only yielded *S. parasitica* and *S. diclina* from the infected Atlantic salmon eggs from the commercial hatchery, other researchers have shown that other species may also be involved in infecting fish eggs. Rezinciuc and coworkers (Rezinciuc, Sandoval-Sierra & Dieguez-Uribeondo 2014) investigated the aetiology of chronic egg mortality events occurring in farmed brown trout, *Salmo trutta,* and identified the causative agent as *Saprolegnia australis.* Yet another study (Cao *et al.* 2012) isolated *Saprolegnia ferax* from infected fish eggs, showing the diversity of species infecting eggs of different fish species.

Appressorial infection structures have been reported before in some *Saprolegnia* species (Willoughby & Hasenjäger 1987). However, we did not observe any appressoria-like structures by SEM in this study.

In summary, we found that *S. parasitica* and *S. diclina* employ different mechanisms of infection of salmon eggs. For the former, hyphae are able to penetrate an apparently intact chorion, while *S. diclina* is capable of complete destruction of the chorion of salmon eggs. Further studies are needed to elucidate the mechanisms involved.

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PAPER III

A thicker chorion gives ova of Atlantic salmon (*Salmo salar* L.) the upper hand against *Saprolegnia* infections

Mwansa M. Songe[1*], Ariane Willems[2*], Mohammad Nasif Sarowar[2], Kasi Rajan[3], Øystein Evensen[4], Keith Drynan[3], Ida Skaar[1] and Pieter van West[2]

***Author contribution statement**:
MMS and AW contributed equally to this work.

[1]Norwegian Veterinary Institute, PO Box 750 Sentrum, N-0106 Oslo, Norway
[2]University of Aberdeen, Aberdeen Oomycete Laboratory, College of Life Sciences and Medicine, Institute of Medical Sciences, Foresterhill, Aberdeen, AB25 2ZD, Scotland UK.
[3]Landcatch Ltd.
[4]Norwegian University of Life Sciences, Faculty of Veterinary Medicine and Biosciences, PO Box 8146 Dep., N-0033 Oslo, Norway

Correspondence: Prof. Pieter van West
Email: p.vanwest@abdn.ac.uk
Tel: +44 (0) 1224 437327

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Abstract

Since the ban of malachite green in the fish farming industry, finding alternative ways of controlling *Saprolegnia* infections has become of utmost importance. A lot of effort has been made to elucidate the mechanisms by which *Saprolegnia* invade fish eggs. Little is known about the defence mechanisms of the hosts, making some eggs more prone to infection than others. One clue might lie in the composition of the eggs. As the immune system in the embryos is not developed yet, the difference in infection levels could be explained by factors influenced by the mother herself, either by transferring passive immunity, by influencing the physical aspects of the eggs or both. One of the physical aspects that could be influenced by the female is the chorion, the extracellular coat surrounding the fish egg, which is in fact the first major barrier to be overcome by *Saprolegnia* spp. Our results suggest that a thicker chorion in eggs from Atlantic salmon gives a better protection against *Saprolegnia* spp.. In addition to the identification of differences in sensitivity of eggs in a fish farm set-up, we were able to confirm these results in a laboratory-controlled challenge experiment.

Introduction

The development and commercialization of salmon farming has posed health-related challenges to the industry. Since the 1970s, salmonid farming has increased significantly and is now close to 2 million tons worldwide (SOFIA, FAO, 2014) with major farming countries being Norway, Chile, Canada, Ireland and Scotland. During this period, one important health problem has been that of freshwater fish and their eggs, arising from oomycete infections and more specifically oomycetes from the genus *Saprolegnia* (Ali et al., 2013). Infection with *Saprolegnia* spp. give rise to a disease called saprolegniosis. This disease became increasingly important after the ban of malachite green in the European Union in 2000 (Phillips et al., 2008; Sudova et al., 2007; van West, 2006) due to its potential carcinogenic effects (Rao, 1995). For salmonid eggs, a total infection rate of 10 % was estimated (Bruno et al., 2010), underscoring the million dollar losses this disease causes in salmon aquaculture (Phillips et al., 2008; van West, 2006). The main *Saprolegnia* species infecting salmon eggs is *S. diclina* (Hussein et al., 2001). However *Saprolegnia australis* (Czeczuga and Muszyńska, 1999), *S. ferax (Cao et al., 2012)* and *S. parasitic*a (Czeczuga and Muszyńska, 1999; Songe et al., 2015) are also known to infect fish eggs. Nonetheless, it is still unclear whether these oomycetes infect as primary pathogens or as opportunists.

Whereas a lot of research has focused on saprolegniosis in immature and mature stages of salmonids, only a few studies have been conducted on *Saprolegnia* infections in fish eggs. Most fish eggs, including those of Atlantic salmon, are spawned artificially and fertilized externally. The resulting embryos and larvae are therefore exposed to an aquatic environment full of potential pathogens capable of causing various types of diseases. At this stage fish embryos have little or only limited ability to synthesize immune-relevant molecules endogenously making them prone to external assaults (Ellis, 1988; Zapata et al., 2006).

Previous studies on several fish species have shown that maternal IgM is transferred from mother to offspring (Bly et al., 1986; Breuil et al., 1997; Castillo et al., 1993; Hanif et al., 2004; Mor and Avtalion, 1990; Olsen and Press, 1997; Picchietti et al., 2006; Picchietti et al., 2004; Swain et al., 2006; Van Loon et al., 1981), and likewise are innate immune factors including lectins (Bildfell et al., 1992; Jung et al., 2003; Tateno et al., 2002), protease inhibitors (Choi et al., 2002; Yamashita and Konagaya, 1996), lysozymes (Yousif et al., 1994; Yousif et al., 1991) and complement component C3 (Ellingsen et al., 2005; Huttenhuis et al., 2006; Lovoll et al., 2007; Lovoll et al., 2006; Wang et al., 2008) transferred to the offspring in different teleost species. Moreover several studies have reported that these transferred maternal molecules are indeed involved in the early defense against pathogens such as *Saprolegnia* spp. in developing fish embryos (Magnadottir et al., 2005; Manning and Nakanishi, 1996; Wang et al., 2008). Interestingly, very few studies have focused their attention to the relationship between the egg's physical characteristics and its susceptibility to *Saprolegnia* infections. Kuchnow and Scott (1977) have conducted studies on the ultrastructure of the chorion and its micropyle apparatus. They observed that an apparently homogenous population of a single species, the mummichog (*Fundulus heteroclitus*), displayed variability in thickness of the chorion. The difference in chorion thickness was suggested to reflect phenotypic variation in the population and variability in developmental maturity of extruded ova. On the other hand, differences in susceptibility of eggs originating from different females, in the same developmental stage, bred, maintained and spawned in the same water, were noted in the fish-farming industry for many years. These differences were also noted in a commercial hatchery.

The chorion of fish eggs plays an important role in the integrity of the eggs. It is the primary envelope and gives a biochemical and a morphological identity to the egg which is distinctive for each species. Moreover, it provides physical protection from the environment and plays a role in diffusive exchange of gases (Eddy, 1974; Grierson and Neville, 1981). However, it might also be involved in a more passive way protecting the embryo against intruders. The constituents of this chorion (mainly proteins) are synthesized in the liver and/or the ovary of the female fish in teleost species (Chang et al., 1997; Chang et al., 1996; Hyllner et al., 2001b; Modig et al., 2006; Mold et al., 2001), therefore the genetic make-up of the female fish could have a significant influence on the physical aspect of the egg chorion. This could explain why differences in the susceptibility to *Saprolegnia* infection in relation to the origin of the eggs are found in salmon hatcheries. Consequently, we hypothesized that the chorion thickness in infection-resistant eggs is greater than the chorion thickness of infection prone eggs. Furthermore, we asserted that some morphological differences on the chorion surface or the internal layers might promote *Saprolegnia* infection. To test these hypotheses, we compared the chorion thickness of uninfected eggs from females producing putative infection-resistant eggs with uninfected eggs from females producing putative infection-prone eggs by light and electron microscopy. Moreover, to further confirm our findings, we performed a challenge experiment further emphasizing our finding that the chorion thickness plays a major role in the ability of *Saprolegnia* to infect eggs.

Materials and methods

Collection and fixation of eggs

For the measurement of the chorion thickness a total of 200 eggs around 300 degree days old were collected from Landcatch Ltd., a commercial hatchery located in Ormsary, Scotland in following manner: 10 uninfected eggs per female from 10 different females that seem to be more resistant to *Saprolegnia* infection and 10 uninfected eggs per female from 10 different females that seem to be more prone to *Saprolegnia* infection. In the hatchery the fertilised eggs of each female were kept separately in individual incubators. *Saprolegnia* infection generally presents as a fluff of cotton wool on the top layer of eggs in the incubators. As we regularly checked the health status of the eggs, we observed that eggs in some specific incubators developed *Saprolegnia* infection much more frequently than others. This enabled us to make the distinction between putative infection prone and infection resistant eggs. Whole egg samples were fixed on site in 1 % glutaraldehyde (AGAR Scientific, Essex, UK) with 4 % paraformaldehyde (AGAR Scientific, Essex, UK) in phosphate buffered saline, and incubated for 3 days after which the fixative was replaced and the eggs were incubated for another 4 days

Measuring the thickness of the chorion

Of the 10 eggs, six whole eggs per female were processed for histology by dehydration in a series of graded ethanol solutions of increasing concentrations from 70% through 95% and then 100% (x4) until 100%, water-free alcohol was reached. They were then embedded in paraffin wax. Each block was first trimmed for 25-30 sections (1,250-1,500 µm) before a 5 µm section was obtained with a Motorized Leica RM2165 microtome. The sections were then collected with up to a total of ten numbered slides per paraffin block. The samples on the ten slides were at least 50 µm away from each other for each individual egg. This ensured that a measurement was obtained

over the entire egg, and not just a simple estimate of what would be assumed to be the middle of the egg. The sections were then stained with Haematoxyline (VWR PROLABO, Leuven, Belgium) and eosin (VWR PROLABO, Leuven, Belgium), according to Kiernan (2008). Images were captured at four points radial from each other, designated 1, 2, 3 and 4, using an EVOS®l transmitted light microscope (AMG, Washington, USA). Four pictures in total were taken from each egg section. There were ten sections per egg, adding up to 40 pictures per egg, giving a total of 480 pictures. For each picture taken, one measurement was performed using ImageJ 1.43u (http://rsbweb.nih.gov/ij.). Measurements were transferred to Excel and the average thickness of each egg on each slide was calculated. Since within one egg there was no significant difference between the measurements from the different slides, an average thickness was calculated over the 10 different slides.

Transmission Electron Microscopy (TEM)

Due to the fixation method used the chorion of the eggs detached from the yolk, making it easy to extract (peel off) the chorion from the remainder of the egg for proper processing steps for electron microscopy. Pieces of chorion were washed in phosphate-buffered saline three times for 15 minutes and then incubated in osmium tetroxide (OsO_4; aqueous solution; TAAB, England, UK) for one hour preceded by three five-minute washes with phosphate buffer, making a total of 15 minutes, and followed by three five-minute washes with distilled water and an extra wash step for 30 minutes with distilled water. Next, samples were dehydrated in increasing concentrations of ethanol (30 %, 70 %, 90 % and 100 %; 30 minutes each), followed by three incubations in acetone for one hour. Samples were then incubated in increasing concentrations of epoxy/acetone (1/1, 6/1 and 100 % epoxy) for one, six and 24 h respectively before embedding the samples in labelled capsules with freshly prepared resin, leaving the resin to polymerize for 48 h at 60^{o}C.

Resin was prepared by weighing out 0.83 g NSA (TAAB, England, UK), 0.33 g ERL 4221 (TAAB, England, UK) and 0.27 g DER 736 (TAAB, England, UK) per sample under continuous stirring. Then 12.53 µl/sample S1 accelerator (TAAB, England, UK) was added and the solution was mixed for 20 minutes before use.

Ultra-thin sections (70–80 nm) were cut with a Leica EM UC6 ultra microtome (Leica Microsystems, Vienna, Austria) and mounted on 200-mesh uncoated copper grids. Grids were stained with 2 % uranyl acetate and 0.5 % lead citrate on an automated contrasting instrument Leica EM AC20 (Leica Microsystems, Vienna, Austria). Finally the grids were analyzed at 80 kV using a Philips CM10 microscope and images captured using a 600-W camera (Gatan, Abingdon, UK).

Challenge experiment

To verify whether a thicker chorion gives better protection against saprolegniosis, we performed a challenge experiment with salmon eggs. Eggs of 330 degree-days from 10 different females were obtained from the hatchery. Five of the females produced eggs that seemed resistant to saprolegniosis while the other five females gave rise to saprolegniosis-prone eggs. The females were treated as biological replications and hence the eggs from each female were kept in different incubators. The eggs were washed two times with 0.1x Marc's modified Ringer's solution (MMR; 100 mM NaCl [Fisher Scientific, UK]; 2 mM KCl [VWR]; 2 mM CaCl2 [Fluka BioChemical]; 1 mM MgCl2 [SIGMA Life Science]; 5 mM HEPES [SIGMA Life Science]) according to Smith and Tata (1991) at 4°C. The eggs were then bathed in 4 ppm malachite green (Kiesecker and Blaustein, 1995) for 40 minutes (4°C) with occasional stirring. The latter was done in order to eliminate any accompanying oomycetes from the hatchery. Thereafter, they were washed with 0.1x MMR once again (4°C) to wash away excess malachite green. As a negative

control, three eggs were left untreated with malachite green. The eggs that appeared to be resistant to saprolegniosis from five different incubators were distributed in a monolayer in 90 mm petri dishes placed in five different 5 l plastic buckets at 4°C. The infection prone eggs from different females were kept separately and distributed into different buckets in a similar way. Each of the 5 l plastic buckets was filled with 3 l of 0.1x MMR and also fitted with a small water pump to increase aeration (50 L h^{-1}). The eggs were incubated for three days before being challenged with *S. diclina* -01 (SEI-01, Accession No. KF420267), an isolate that was originally obtained from Atlantic salmon eggs from a hatchery in Scotland. During those three days, the medium was changed 100 % every day to dilute out any effects of malachite green. After the challenge, medium was changed once every alternate day. Before the experiment was set up three eggs from each female were taken to verify the chorion thickness following the procedures described in "Light Microscopy" of Materials and Methods.

Molecular analysis of mycelia growing on (malachite green) untreated eggs

Total genomic DNA was extracted from the mycelia that grew on the three eggs that were not treated with malachite green (Zelaya-Molina et al., 2011). Amplification by polymerase chain reaction of the two internal transcribed spacers (ITS1 and ITS2), including the 5.8S region of the rRNA subunit, was performed using primers ITS4 and ITS5 (White et al., 1990). The PCR reaction mixture contained: 5 x colourless flexi buffer (Promega, UK), 5 mM MgCl2, 0.2 mM dNTPs, 0.3 µM of each of the primers and 1.25 units GoTaq Polymerase (Promega, UK), nuclease free water containing 1 µl extracted DNA (40-50 ng μl^{-1}) added to make the total reaction volume 25 µl. The PCR reactions were run in a thermal cycler which consisted of one cycle of initial denaturation at 95 ºC for 5 min followed by 30 cycles of each of 95 ºC for 30 s, 57 ºC for 1 min and 73 ºC for 1 min and 30 s and finally one cycle of final extension at 73 ºC for 7

min. Amplified products were separated by electrophoresis in 1 % agarose gels and run in 1 x TAE buffer for 30 min at 5 V cm $^{-1}$. The gel was stained with SYBR Green (SYBR Safe DNA Gel Stain - 10 000 x concentrate in DMSO Invitrogen®) and visualized under UV light. The lengths of amplification products were estimated by comparison to 10 kb Hyperladder I (Invitrogen®). The PCR products were sequenced by SourceBioscience® for further phylogenetic analysis. The sequences obtained were aligned by using Bioedit software (version 7.1.3.0) and compared with the homologous sequences of some of the *Saprolegnia* species retrieved from GenBank

For the challenge experiment, autoclaved hemp seeds overgrown with mycelium of *S. diclina* (SEI-01) were collected from Potato Dextrose Agar (PDA) plates aseptically (Sarowar et al., 2013). The agar plate had been inoculated five days earlier with the *S. diclina* agar plug. Four hemp seeds were added to each of the infection buckets. They were placed on about equal distance from each other on the plates full of eggs. The experiment was terminated after 18 days.

For each of the buckets the total number of eggs was counted, followed by a count of the number of eggs found entangled to the growing mycelia from the hemp seeds on day 18. As a control measure to monitor the efficacy of the malachite green treatment, three eggs from each experimental group were taken before and after the treatment and plated on PDA plates.

Statistical analysis

For both the measurements of the chorion thickness and the challenge experiment (thickness or number of infected eggs) statistical analysis was performed by Student's t-test (two-sided) using JMP, Version 7 (SAS Institute Inc., Cary, NC, 1989-2007).

Results

Comparison of chorion thickness

The average thickness of the chorion was measured in the collected eggs. We found that the infection-resistant group had a thickness of 42.43 µm ± 2.55 µm (mean ± SD; n = 10), whereas the average thickness of the chorion in the infection-prone group was 35.71 µm ± 2.74 µm (mean ± SD; n = 10), which makes a remarkable and statistically significant difference ($p \leq 0.05$) of 6.72 µm between the average thickness of the chorions between the two groups (Table 1).

TEM analysis of pieces of chorion did not reveal any differences in the morphology of the chorion regardless of whether the eggs were infection-prone or infection-resistant (Figure 2 panel A-D): the *zona externa* and the *zona interna* looked similar in morphology/integrity for both groups of eggs.

Challenge experiment

In the challenge experiment, the efficacy of the malachite green treatment was confirmed by the fact that the three eggs that were treated showed no mycelial growth on PDA incubated at 18 °C, even seven days post inoculation. This was in contrast with the three untreated eggs, which had mycelia growing on PDA after only two days. Molecular analysis of the mycelia growing on the latter eggs revealed the presence of *S. diclina*.

Eighteen days after experimental infection with a virulent strain of *S. diclina*, (SEI-01) , the number of eggs succumbing to infection in the group likely to be resistant to saprolegniosis was significantly ($p < 0.05$) lower than the number of eggs succumbing in the group likely to be prone to infection (Table 2). Afterwards the average chorion thickness of the group likely to be

infection-resistant was again confirmed to be bigger than the chorion of the eggs in the infection-prone group (43.90 µm ± 1.01 µm vs. 35.54 µm ± 1.11 µm, respectively; mean ± SD; $p < 0.05$; n = 15; Table 3).

Discussion

Our findings support our hypothesis that the thickness of the chorion of Atlantic salmon eggs plays a significant role in the resistance against saprolegniosis. An infection experiment confirms that a thicker chorion is an important treat in the protection against experimental saprolegniosis.

The egg envelope, which is often described as *zona pellucida*, vitelline membrane, chorion, egg shell, *zona radiata*, or vitelline envelope (Hyllner et al., 2001a), has several functions. The first and most relevant function in light of reproduction is that it serves to attract sperm. However, it also prevents polyspermy after fertilization. Concerning the development of the offspring, it protects the developing embryo and is involved in gas exchange and excretion in order to provide the embryo with the right amount of oxygen and to get rid of toxic waste products (Dumont and Brummet, 1980; Kunz, 2004). In viviparous fish, it also selects the transport of nutrients to the developing embryo (Groot and Alderdice, 1985). In some fish, the chorion also has an antibacterial action (Riehl, 1991), not only protecting the embryo from physical insults but also from microbial attacks. The chorion of most teleost fish consists of more than one layer. The structure and thickness of the chorion and its individual layers will therefore play a very important role in the egg's susceptibility or resistance to disease. Our findings in the present study underscore and confirm this phenomenon, as we found that Atlantic salmon eggs resistant to *Saprolegnia* infection had a thicker chorion compared to the infection-prone eggs.

In between species it is generally assumed that the chorion of pelagic eggs is much thinner than that of demersal eggs (Hirai, 1993; Loenning et al., 1988; Riehl, 1991; Stehr and Hawkes, 1979) because demersal eggs are subjected to more adverse conditions. However, some exceptions have been observed. For instance, the envelope of the pelagic egg of *Pleoronectes platessa* is exceptionally thick, about 15 µm in thickness, whereas the envelope of demersal, non-adhesive eggs of *Brachydanio rerio* is thin, approximately 1.5 - 2.0 µm thick (Kunz, 2004). Marked differences have been reported in chorion thickness among pelagic non-adhesive eggs belonging to the same species but different geographic locations e.g. European flounder, *Platichthys flesus* and Common dab, *Limanda limanda*. These variations may be correlated with differences in temperature, salinity and viscosity of sea water, which necessitate adjustments in the structure to secure adequate buoyancy (Kunz, 2004). However, an apparently homogenous population of a single species, *Fundulus heteroclitus*, which spawns demersal adhesive eggs, was shown to display variability in thickness of the chorion, which was suggested to reflect phenotypic variation in the population or variability in developmental maturity of extruded ova (Kuchnow and Scott, 1977).

In the present study we, like Kuchnow and Scott (1977), also found that fish eggs from the same species (*Salmo salar* L.), bred, maintained and spawned in the same water show statistically significant differences ($p \leq 0.05$) in chorion thickness amongst females in different resistance groups. These differences in resistance were first visually identified in the salmon hatchery itself upon evaluation of individual maturation incubators (one incubator contains the eggs of one female) and were subsequently confirmed to have differences in the thickness of their chorion with standard histological techniques confirming the hypothesis that a thicker chorion gives more resistance against *Saprolegnia* infection than a thinner chorion. On the other hand, Ninness *et al.* (2006) reported that removal of the chorion before hatching results in increased movement and

accelerated growth in rainbow trout (*Oncorhynchus mykiss*) embryos, which could be linked to a higher efficiency in gas/nutrient exchange and waste excretion. This observation suggests that a thicker chorion, provides protection against *Saprolegnia* infection, but could have negative impact on the maturation of the relevant embryos or delay or prevent hatching and therefore increase the mortality rate of developing embryos protected by a thicker chorion. It would be interesting to investigate and compare the hatching rates of the eggs in the two different resistance groups. This would give an answer to the hypothesis that eggs with a thinner chorion (infection-prone) hatch earlier and have an increased survival rate than eggs with a thicker chorion (infection-resistant).

Another two important questions to raise are: 'How many eggs can be lost to *Saprolegnia* infection due to a thinner chorion and how many embryos will be trapped in their egg, deprived from nutrients and oxygen or intoxicated due to a thicker chorion?' In times of *Saprolegnia* outbreaks, the more resistant eggs would save the yield for the coming generation. However, in years with low *Saprolegnia* infection, the population can really expand due to extra yield from the thinner eggs. This method of generating offspring could be beneficial in nature itself. However, in hatcheries, conditions are rather standardized so working with one type of chorion could be more beneficial than the other type. Selection on one specific characteristic needs targeted mating schemes and further genetic studies to reveal the genes involved in this process. All these measures could be beneficial for increasing the yield and lowering the cost of production. Further studies need to be performed to provide the answers on all these important questions not only for natural balance (or environmental preservation) but also for the fine-tuning of the efficiency in hatcheries. As the eggs used in this study come from a hatchery where the parents of these eggs are all tagged and monitored for weight, ancestry, DNA, etc. further genetic investigations can be carried out. Conducting these studies would take a considerable amount of

time. However, the effort would be worthwhile and could possibly lead to a very natural and environmental friendly way of fighting off saprolegniosis in hatcheries.

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Table 1 Comparison of the average chorion thickness of infection-resistant vs. infection-prone eggs and the individual measurements per female.

Infection-resistant		Infection-prone	
Female No.	**Chorion thickness (µm)**	**Female No.**	**Chorion thickness (µm)**
Female 1	41.34	Female 11	36.43
Female 2	37.66	Female 12	32.36
Female 3	47.54	Female 13	42.02
Female 4	43.95	Female 14	38.14
Female 5	42.79	Female 15	36.01
Female 6	41.25	Female 16	35.11
Female 7	40.85	Female 17	33.54
Female 8	43.24	Female 18	34.96
Female 9	43.56	Female 19	34.66
Female 10	42.12	Female 20	33.94
Mean (n=10)	**42.43**	**Mean (n=10)**	**35.71***
SD	**2.55**	**SD**	**2.74**

The chorion thickness in each individual female was measured as described in Materials and Methods. Each female value represents the average chorion thickness of six eggs from that specific female. Statistical analysis (Student's t-test) was performed on the average per female. This analysis revealed a statistical significant difference ($p \leq 0.05$) in the infection-resistant vs. the infection-prone group (42.43 ± 2.55 µm (mean ± SD) and 35.71 ± 2.74 µm respectively) and is indicated by an asterisk (*).

Table 2 The average number of infected eggs in infection-prone and infection-resistant eggs after experimental infection.

Infection-resistant				Infection-prone			
Bucket	**No. of eggs infected**	**No. of Eggs in bucket**	**% Infection**	**Bucket**	**No. of eggs infected**	**No. of eggs in bucket**	**% Infection**
1 [a]	0	220	0	6	39	225	17.3
2	15	215	7.0	7	37	208	17.8
3	36	224	16.1	8	69	222	31.1
4	28	217	12.9	9	34	210	16.2
5	24	234	10.3	10	46	214	21.5
		Mean (n=4)	**11.6**			**Mean (n=5)**	**20.8***
		SD	**3.9**			**SD**	**6.1**

[a] Control

Statistical analysis was performed by Student's t-test using JMP, Version 7, SAS Institute Inc., Cary, NC, 1989-2007. Asterisk (*) denotes significant difference at $p < 0.05$.

Table 3 Comparison of means of the chorion thickness of the Infection-prone and infection-resistant groups from the challenge experiment

Infection-resistant			Infection-prone		
Experiment bucket	**Egg no.**	**Chorion thickness (µm)**	**Experiment bucket**	**Egg no.**	**Chorion thickness (µm)**
1	1	44.31	1	1	33.69
	2	42.79		2	35.17
	3	42.97		3	34.78
2	1	44.69	2	1	36.65
	2	43.09		2	35.54
	3	43.99		3	37.11
3	1	43.41	3	1	0.00
	2	44.57		2	35.50
	3	43.31		3	33.82
4	1	44.57	4	1	34.57
	2	46.74		2	37.53
	3	42.85		3	35.07
5	1	44.04	5	1	34.96
	2	43.44		2	36.13
	3	43.72		3	36.50
Mean (n=15)		**43.90**	**Mean (n=15)**		**35.54***
SD		**1.01**	**SD**		**1.11**

Statistical analysis was performed by Student's t-test using JMP, Version 7, SAS Institute Inc., Cary, NC, 1989-2007. Asterisk (*) denotes significant difference at $p < 0.05$.

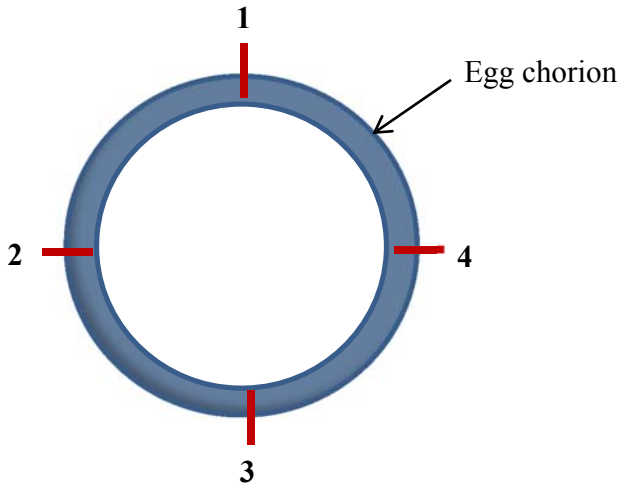

Figure 1 Diagram of chorion thickness measure points

Images for the measurement of the chorion thickness were captured at the four indicated points (see 1, 2, 3 and 4 in picture). For each picture taken one measurement was performed using ImageJ 1.43u (http: //rsbweb.nih.gov/ij.). Measurements were then transferred to Excel and the average thickness of each egg was calculated as described in M&M section.

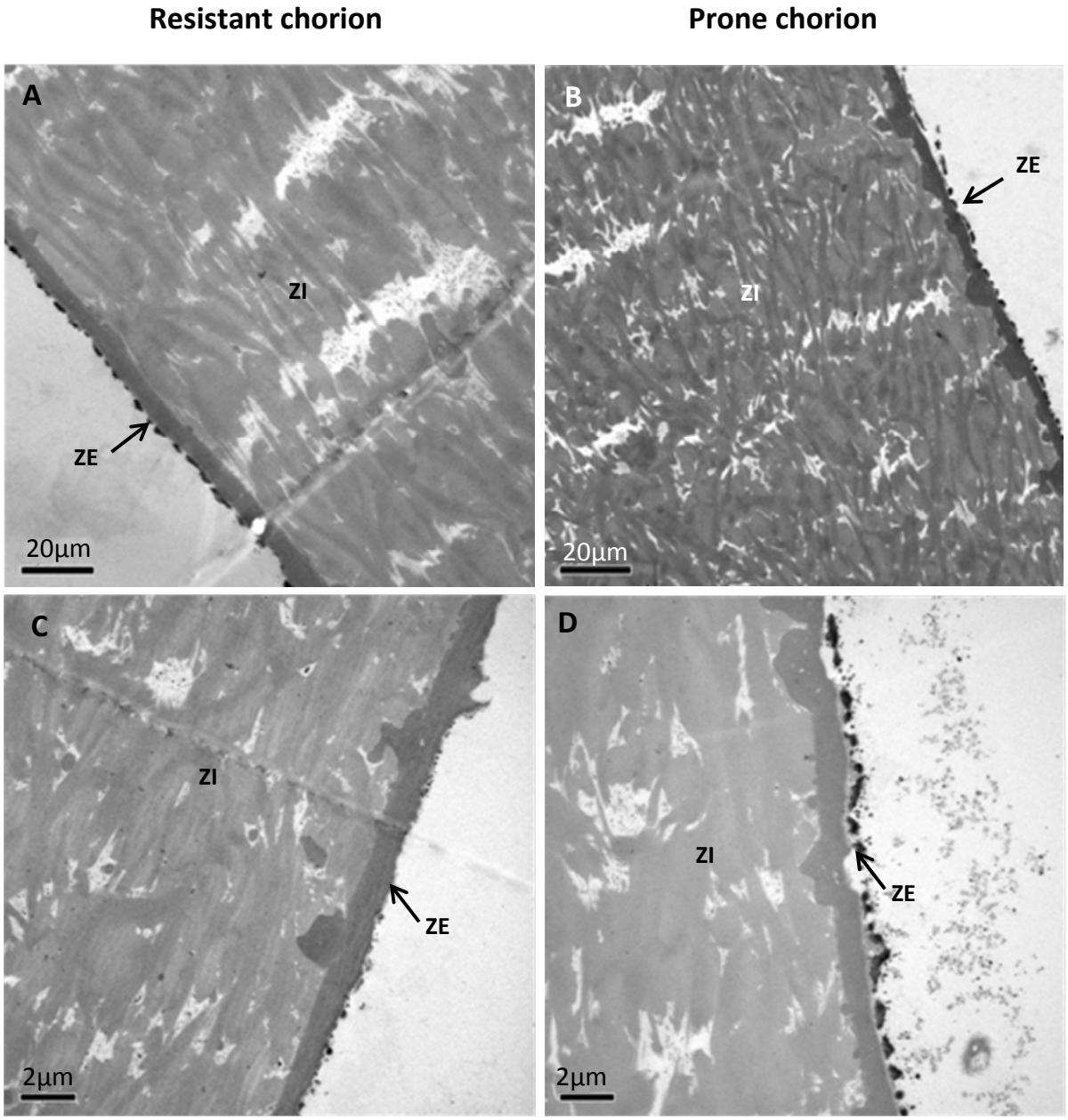

Figure 2 Transmission electron micrographs of the chorion of infection-resistant and infection-prone eggs

TEM images from the chorion of infection-resistant (panel A and C) and infection-prone (panel B and D) eggs from salmon (*Salmo salar* L.) were obtained as described in the Material and Methods. Indicated in the individual panels are the two major layers of the chorion: the zona interna (ZI) and zona externa (ZE). No differences were observed in morphology of zona externa and the zona interna. The length of each scale bar is indicated in the relevant panel.