Pathogenesis and Cell Biology of the Salmon Parasite Spironucleus salmonicida

ÁSGEIR ÁSTVALDSSON
Spironucleus species are classified as diplomonad organisms, diverse eukaryotic flagellates found in oxygen-deprived environments. Members of Spironucleus are parasitic and can infect a variety of hosts, such as mice and birds, while the majority are found to infect fish. Massive outbreaks of severe systemic infection caused by a Spironucleus member, Spironucleus salmonicida (salmonicida = salmon killer), have been reported in farmed salmonids resulting in large economic impacts for aquaculture.

In this thesis, the S. salmonicida genome was sequenced and compared to the genome of its diplomonad relative, the mammalian pathogen G. intestinalis (Paper I). Our analyses revealed large genomic differences between the two parasites that collectively suggests that S. salmonicida is more capable of adapting to different environments. As S. salmonicida can infiltrate different host tissues, we provide molecular evidence for how the parasite can tolerate oxygenated environments and suggest oxygen as a potential regulator of virulence factors (Paper III). To further investigate the molecular responses of the parasite and in addition, its host, during infection we set up an interaction system of S. salmonicida and ASK (Atlantic salmon kidney) cells (Paper VI).

To study the cell biology in S. salmonicida we optimized an enzymatic proximity labeling method using ascorbate peroxidase (APEX) as a reporter for transmission electron microscopy (TEM) (Paper IV). As the system is robust and versatile, we showed the localization and performed ultrastructural characterization of numerous proteins in S. salmonicida and G. intestinalis. We furthermore utilized the APEX system to study the annexin protein family in S. salmonicida (Paper II). Super resolution microscopy and TEM were applied to show that the annexins are mostly associated with cytoskeletal and membranous structures. In addition, we performed phylogenetic analyses concluding that the annexin gene family is expanded in diplomonads.

We performed experimental infection in Atlantic salmon and derived a potential model for the route of infection (Paper V). The results suggested multiple routes of transmission between hosts for the parasite.

To conclude, the comprehensive work in this thesis has provided valuable insights into the pathogenesis and cell biology of the highly adaptable diplomonad parasite S. salmonicida.

Keywords: Diplomonads, Spironucleus, Giardia, genome, annexin, oxygen stress, APEX, pathology

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To my children
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


*These authors contributed equally

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Publications not included in the thesis.

Contents

Introduction ................................................................................................... 11
Eukaryotes ................................................................................................ 11
Diplomonads ............................................................................................ 14
Spironucleus ............................................................................................. 15
  Taxonomy and host specificity of piscine Spironucleus spp. .............. 16
  Morphology .......................................................................................... 21
  Spironucleosis ...................................................................................... 23
  Life cycle and transmission ................................................................. 25
  Cell and Molecular biology ................................................................. 28
  Genomics ............................................................................................. 29
  Metabolism .......................................................................................... 30

Present Investigation ..................................................................................... 33
  The genome of S. salmonicida (Paper I) .................................................. 34
    Evidence of a cyst stage ..................................................................... 34
    Cysteine-rich protein families .............................................................. 35
    Metabolism .......................................................................................... 36
    Oxidative stress response ................................................................. 36
  Annexins in S. salmonicida (Paper II) ...................................................... 37
    Structure of annexins in S. salmonicida ............................................. 37
    Phylogeny of eukaryotic annexins ....................................................... 38
    Annexin localization in S. salmonicida ............................................... 38
  Oxygen response in S. salmonicida (Paper III) ........................................ 39
    Differentially expressed genes in oxidative stress response pathway . 40
    Virulence factors .............................................................................. 40
  Optimization of APEX protein tagging in diplomonads (Paper IV) .... 41
    Optimization of APX^{W41F}/APEX ......................................................... 41
    Activity of APEX in different cellular compartments of S. salmonicida ............................................................................. 42
    Ultrastructural localization .................................................................. 42
    Characterizations of two novel proteins .............................................. 42
  The infection cycle of S. salmonicida (Paper V) ..................................... 43
    Tools for parasite detection in infected fish ....................................... 43
    Infection cycle ..................................................................................... 43
    Transmission routes ........................................................................... 44
    Luciferase observations ................................................................. 44
Transcriptome analyses during interaction of S. salmonicida and salmon cells (Paper VI) ................................................................. 45
  Gene expression changes in S. salmonicida and salmon cells during interactions ........................................................................ 46
  Scanning electron microscopy ........................................................................... 47
  Transmission electron microscopy .................................................................... 47

Conclusions and future perspectives .................................................................. 48
  Genomics and transcriptomics ........................................................................ 48
  Cell biology ...................................................................................................... 49
  Infection biology .................................................................................................. 50

Svensk sammanfattning ......................................................................................... 51
  Genom- och genuttrycksstudier i S. salmonicida ........................................ 51
  S. salmonicida cellbiologi ...................................................................................... 52
  S. salmonicida infektionsprocess ........................................................................ 52

Ágrip á íslensku .................................................................................................. 54
  Rannsóknir á genamengi og genatjáningu í S. salmonicida ......................... 54
  Frumulíffræði S. salmonicida .................................................................................. 55
  Sýkingarferli S. salmoncida .............................................................................. 56

Acknowledgements .................................................................................................. 57

References ............................................................................................................ 61
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADH</td>
<td>Arginine dihydrolase</td>
</tr>
<tr>
<td>AF</td>
<td>Anterior flagella</td>
</tr>
<tr>
<td>APEX/APX</td>
<td>Ascorbate peroxidase</td>
</tr>
<tr>
<td>ASK</td>
<td>Atlantic salmon kidney cells</td>
</tr>
<tr>
<td>Bsr</td>
<td>Blasticidin S-deaminase</td>
</tr>
<tr>
<td>CR</td>
<td>Crescent-shape ridge</td>
</tr>
<tr>
<td>CRMP</td>
<td>Cysteine-rich membrane protein</td>
</tr>
<tr>
<td>CWP</td>
<td>Cyst wall protein</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
</tr>
<tr>
<td>ESV</td>
<td>Encystation specific vesicle</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>FLuc</td>
<td>Firefly luciferase</td>
</tr>
<tr>
<td>FP</td>
<td>Flagellar pocket</td>
</tr>
<tr>
<td>G3P</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GSS</td>
<td>Genome survey sequence</td>
</tr>
<tr>
<td>HCMP</td>
<td>High cysteine membrane protein</td>
</tr>
<tr>
<td>HITH disease</td>
<td>Hole-in-the-head disease</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>LGT</td>
<td>Lateral gene transfer</td>
</tr>
<tr>
<td>MRO</td>
<td>Mitochondria-related organelle</td>
</tr>
<tr>
<td>MT</td>
<td>Microtubules</td>
</tr>
<tr>
<td>PAC</td>
<td>Puromycin acetyltransferase</td>
</tr>
<tr>
<td>PC</td>
<td>Pyloric ceaca</td>
</tr>
<tr>
<td>PF</td>
<td>Posterior flagella</td>
</tr>
<tr>
<td>RER</td>
<td>Rough endoplasmic reticulum</td>
</tr>
<tr>
<td>RF</td>
<td>Recurrent flagella</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SIM</td>
<td>Structured illumination microscopy</td>
</tr>
<tr>
<td>SUF</td>
<td>Sulfur mobilization system</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
</tbody>
</table>
Introduction

Eukaryotes

The eukaryotes represent one of the three branches of the tree of life along with bacteria and archaea (Woese et al. 1990) with the line between archaea and eukaryotes becoming more and more obscure as current investigations are suggesting the phylogenetic position of the eukaryotes to potentially be within the archaea (Williams TA et al. 2012, Koonin 2015, Eme et al. 2017). Even though both archaea and bacteria contain a much higher number of organisms the eukaryotes exhibit greater variations, ranging from simple unicellular organisms to very complex, multicellular life forms such as animals and plants. Understandably, the spotlight has been on the macroscopic world with all its diversity visible to the curious human eye while the microscopic eukaryotic organisms have been somewhat neglected despite their astonishing diversity and complexity.

The main characteristic of the eukaryotic cell is the presence of the nucleus that is the DNA repository of the cell where genetic material is maintained, replicated and transcribed. The nucleus is enveloped by a double membrane that is a part of another characteristic feature of eukaryotes, the endomembrane system that is a complex system of membranes that divides the cell into different functional compartments called organelles. Organelles of the endomembrane system apart from the nucleus are for example the endoplasmic reticulum, the Golgi apparatus and various vesicles within the cell.

Another main feature of the eukaryotic cell are the mitochondria or the mitochondria-related organelles (MROs) such as mitosomes and hydrogenosomes that were first discovered in Entamoeba histolytica (Tovar et al. 1999) and Tritrichomonas foetus (Lindmark & Müller 1973), respectively. These organelles are not part of the endomembrane system and their origin have been debated with the most common hypothesis describing an endosymbiotic fusion event of a proto-eukaryote and an α-proteobacteria (Vellai & Vida 1999, Embley & Martin 2006). Current investigations indicate origins in an early branching proteobacterium that diverged from α-proteobacteria before the branching of all currently known α-proteobacteria (Martijn et al. 2018).

Recently it was shown that the oxymonad Monocercomonoides lacks any mitochondrion-derived organelle and the conserved iron-sulfur cluster assem-
bly, vital to the eukaryotic cell has been replaced by a cytosolic sulfur mobilization system (SUF) acquired from bacteria through lateral gene transfer (LGT) (Karnkowska et al. 2016).

The Eukaryota super-kingdom is formed by two domains, Amorphea and Diaphoretickes with several clades falling outside of these. One of these clades is the former super-group Excavata that is now considered as Incertae sedis in eukaryotes and have been given an informal name, the Excavates (Adl et al. 2019). The Excavates consists of the phylum Discoba, Malawimonadidae and Metamonada and includes several groups of heterotrophic unicellular eukaryotes. The excavates are grouped together based on a shared flagellar organization and a distinctive suspension-feeding groove though not all members share that feature, e.g. it is not existing in one of the best known members of the Excavata, *Giardia*. (Patterson 1999, Simpson 2003).

*Giardia* is a member of the Diplomonadida that forms the class Eopharyngea together with the Retortamonadida. (Figure 1) (Silberman et al. 2002, Cepicka et al. 2008). Eopharyngea, Carpediemonadina and Chilomastigida build the superclass Fornicata in the phylum Metamonada (Cavaliér-Smith 1993, Cavaliér-Smith 2013).

The representing genus of Carpediemonadina is *Carpediemonas* that are free-living heterotrophic cells found in oxygen-deprived environments (Bernard et al. 2000), are amitochondriate but contain a homologous organelle, two flagella and three basal bodies (Simpson & Patterson 1999, Simpson 2003). The Retortamonadida are represented by the genus *Retortamonas*, organisms that possess an endobiotic life cycle in the intestines of humans and other animals, have four basal bodies and two flagella (Brugerolle 1991, Simpson 2003).

Previously most members of Fornicata were considered to be early branching eukaryotes, simple eukaryotes known as Archezoans that had all features of a eukaryotic cell except were lacking the mitochondria and were therefore considered to have diverged before the endosymbiotic fusion event (Roger 1999). An early view held that the last common ancestor of eukaryotes was an amitochondriate Fornicata-like organism (Sogin et al. 1989). This view has been shown to be incorrect and the Archezoan clustering in phylogenies is due to a phylogenetic artifact called long-branch attraction where highly divergent lineages artificially cluster together (Stiller & Hall 1999). Moreover, the past 2 decades have seen the discovery of MROs in all Archezoan lineages as well as in members of the Fornicata and the once presumably primitive cells evolved rather by simplification (Roger et al. 1998, Embley & Hirt 1998, Simpson & Patterson 1999, Philippe Hervé et al. 2000, Tachezy et al. 2001, Tovar et al. 2003).
Figure 1. Maximum likelihood phylogeny of Eopharyngea. Based on SSU rRNA sequencing. Bootstrap values are indicated at each node. *: Unresolved nodes by bootstrap analyses. Reprint from Jørgensen & Sterud (2007) with permission from Elsevier.
Diplomonads

As all members of Fornicata, the diplomonads harbor MROs rather than traditional mitochondria and live in anaerobic or low oxygen environments such as the intestinal tracts of various animals or in sediments (Kulda & Nohynkova 1978, Bernard et al. 2000).

The main characteristics of most diplomonads are the double karyomastigont with cells containing two nuclei and two flagellar apparatuses though there are exceptions. The enteromonads are the only species among the diplomonads that is monokaryotic and were previously grouped as a sister-group to the diplomonads (Siddall et al. 1992, Brugerolle & Lee 2000). Based on molecular analyses this has been rejected and the enteromonads have been placed within the diplomonads (Kolisko et al. 2005, Kolisko et al. 2008). Based on ultrastructural studies the morphology of enteromonads and other diplomonads is very similar and only distinguishable by the double karyomastigont (Kulda & Nohynkova 1978, Siddall et al. 1992). The diplomonad cell could be described as two fused enteromonad cells while similarly could the enteromonad cell be described as a half of the diplomonad cell.

The ancestral stage of the diplomonads has been debated though the most common view is the one from Siddall et al. (1992) where they describe that a switch between the monokaryomastigont stage to the double karyomastigont happened several times independently. Other theories that have been proposed involve the opposite switch, from a double karyomastigont to the monokaryomastigont, and nuclear fusion in a double karyomastigont cell (Kolisko et al. 2008). While all theories are considered plausible more research is needed to be able to further investigate this important evolutionary event.

The majority of diplomonads are parasitic or commensals and have been recovered mostly from the intestinal tract of various hosts such as humans, birds, reptiles, amphibians and fish, though some species have been characterized as free-living (Sebesteny 1969, Poynton et al. 1995, Fenchel et al. 1995, Sterud et al. 1998, Brugerolle & Lee 2000, Adam 2001, Cooper et al. 2004, Fain et al. 2008). Based on morphological data the diplomonads are sub-divided into the Hexamitinae that contains both parasitic and free-living species and Giardiinae with only parasitic species (Kolisko et al. 2008).

Two genus are present in the Giardiinae, Giardia and Octomitus (Figure 1). Giardia is the most prominent member of the Diplomonads and has been proposed to be one of the first microorganisms to be discovered when the Dutch microscopist Antonie van Leewenhoek examined his own diarrheic stool. He described his findings in a letter to Robert Hooke of the Royal Society in 1681 (Dobell 1920).

Five species of Giardia are recognized to date infecting variety of host with Giardia intestinalis (synonyms: Giardia lamblia, Giardia duodenalis) as the only species known to infect humans (Adam 2001, Monis et al. 2009).
intestinalis comprises a species complex with different genetic groups, or assemblages. The different assemblages are found to infect canids (assemblage C and D), hoofed animals (assemblage E), cats (assemblage F), rats (assemblage G) and marine mammals (assemblage H). The two remaining assemblages, assemblages A and B, infect humans and other mammals (Monis et al. 2009).

The sister group of Giardia in the Giardiinae, Octomitus, contains one species, Octomitus intestinalis (syn. Octomitus muris) that inhabits the digestive tract of rodents and amphibians (Keeling & Brugerolle 2006). Morphologically is Octomitus thought to be very closely related to Giardia (Brugerolle et al. 1973) and SSU rRNA analyses has confirmed the placement of Octomitus in the Giardiinae as a sister group to Giardia (Keeling & Brugerolle 2006).

Hexamitinae contains five genera, Hexamita, Trepomonas, Spironucleus, Trimitus (will not be discussed further) and the previously mentioned monokaryomastigont Enteronads (Figure 1). Species of Hexamita and Spironucleus are morphologically related while Trepomonas can be identified by an ovoid cell shape. Trepomonas ssp. are mostly free-living but has also been reported in the intestinal tract of a variety of hosts (Bishop 1937).

Other species of Hexamitinae are parasites although some free-living species are found in the genus Hexamita (Siddall et al. 1993). One of the main features of the Hexamitinae is the usage of an alternative genetic code where UAA and UAG encode for glutamine rather than being stop codons and leaving UGA as the only stop codon in the genome (Keeling & Doolittle 1996, Keeling & Doolittle 1997).

In the next sections, I will further describe the Hexamitinae genus Spironucleus with the main focus on the piscine species of the genus and in particular Spironucleus salmonicida.

**Spironucleus**

To date there are nine species of Spironucleus that have been documented from a variety of hosts such as rodents, birds, amphibians and fish (Scullion & Scullion 2007, Jørgensen & Sterud 2007).

**Spironucleus muris** was previously known under the name Hexamita muris (Lavier 1936, Brugerolle et al. 1973) and can be found in the small intestine of healthy rodents where it can cause acute enteritis in young mice and chronic enteritis in adults (Meshoker 1969, Sebesteny 1969).

Two Spironucleus species are known to infect birds. Spironucleus meleagridis causes infectious catarrhal enteritis in many species of birds such as turkeys (Meleagris gallopavo), pheasant (Phasianus colchicus), demoiselle cranes (Anthropides virgo), sandhill cranes (Grus canadensis pratensis) and many psittacine species (Ippen et al. 1980, Spalding et al. 1994, Hussain 2001,

Spironucleus is most abundant in fish and five species have been described in various fish hosts (Table 1), mainly from northwest Europe and Canada. It is not implausible additional species will be described from other parts of the world with aquaculture constantly becoming progressively more important globally (Williams CF et al. 2011).

Taxonomy and host specificity of piscine Spironucleus spp.

For decades, species of piscine Spironucleus were characterized solely on light microscopy observations. This caused confusion in the taxonomy of the Hexamitinae with species designation between Hexamita, Spironucleus and Octomitus proving to be difficult. The morphological differences between these genera are very slim, making light microscopy inadequate to resolve the necessary details for correct species designation. To clarify the taxonomy Poynton & Morrison (1990) called for the use of electron microscopy to resolve morphological characteristics and Poynton & Sterud (2002) published much needed guidelines to allow for correct species designation between the three genera based on ultrastructural features (Table 2).

Reclassification based on these ultrastructural details have brought much needed clarity to the taxonomy of these genera, which has been further resolved using molecular methods (Poynton et al. 2004, Fard et al. 2007). Interestingly, all three genera have been described in fish based on light microscopy observation but based on ultrastructural characterization, only Spironucleus have been diagnosed in various fish hosts (Poynton & Sterud 2002).

The first reports of diplomonads in fish date back to the beginning of 20th century when Urophagus intestinalis was described in rainbow trout (Oncorhynchus mykiss) and around 1920 these flagellates were reported from brown trout (Salmo trutta) and classified as Octomitus intestinalis truttae (Poynton et al. 2004).

The first comprehensive cases were described by Moore (1923) and Davis (1926) in brook trout (Salvelinus fontinalis), brown trout, rainbow trout and lake trout (Salvelinus namaycush) where the parasite was referred to as Octomitus salmonis though shortly thereafter the name Hexamitus salmonis be-
The first use of TEM and SEM to validate ultrastructural details of *H. salmonis* were reported in 1979 in rainbow trout fry (*Salmo gairdneri* Richardson) (Ferguson 1979).

Poynton & Morrison (1990) published limited SEM data on *H. salmonis* from brook trout and in the same study introduced a new *Spironucleus* species, *Spironucleus torosa*, based on comprehensive EM studies, isolated from atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) and has in addition been reported from burbot (*Lota lota*) (Sterud 1998a).

**Table 1.** Host specificity of piscine *Spironucleus* species. Adapted from Williams et al. (2011).

<table>
<thead>
<tr>
<th>Species</th>
<th>Type host</th>
<th>Additional hosts</th>
<th>Location in host</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. barkhanus</em></td>
<td>Thymallus</td>
<td><em>Salvelinus alpinus</em></td>
<td>Lumen of gut and gall bladder</td>
<td>Sterud et al. (1997)</td>
</tr>
<tr>
<td></td>
<td>thymallus</td>
<td><em>Salmo trutta</em></td>
<td></td>
<td>Jørgensen et al. (2011)</td>
</tr>
<tr>
<td><em>S. elegans</em></td>
<td><em>Triturus alpestris</em></td>
<td><em>Pterophyllum scalare</em></td>
<td>Intestine</td>
<td>Lavier (1936)</td>
</tr>
<tr>
<td></td>
<td><em>Rana rana</em></td>
<td></td>
<td></td>
<td>Kulda &amp; Lom (1964a, 1964b)</td>
</tr>
<tr>
<td><em>S. salmonicida</em></td>
<td><em>Salvelinus alpinus</em></td>
<td><em>Oncorhynchus na-maykush</em></td>
<td>Systemic infection – in marine farms</td>
<td>Jørgensen &amp; Sterud (2006)</td>
</tr>
<tr>
<td></td>
<td><em>Salmo salar</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. salmonis</em></td>
<td><em>Salvelinus fontinalis</em></td>
<td><em>Oncorhynchus mykiss</em></td>
<td>Digestive tract, upper intestinal region</td>
<td>Moore (1923)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Davis (1926) Ferguson (1979)</td>
</tr>
<tr>
<td><em>S. torosa</em></td>
<td><em>Gadus morhua</em></td>
<td><em>Melanogrammus aeglefinus</em></td>
<td>Digestive tract, rectum in cod</td>
<td>Poynton &amp; Morrison (1990) Sterud (1998a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lota lota</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. vortens</em></td>
<td><em>Pterophyllum scalare</em></td>
<td><em>Leuciscus idus</em></td>
<td>Lumen of intestine</td>
<td>Poynton et al. (1995)</td>
</tr>
</tbody>
</table>
Kent et al. (1992) reported an uncharacterized diplomonad flagellate from Chinook salmon (Oncorhynchus tshawytscha) in seawater morphologically identical to *H. salmonis* but it was suggested the flagellate was a new, highly invasive strain of *H. salmonis* or a completely new species. The same year a new manifestation of severe systemic hexamitosis in Atlantic salmon (*Salmo salar*) was suggested to be caused by a new *Hexamita* marine species (Mo et al. 1990, Poppe T et al. 1992) and these findings were further elaborated by Poppe TT & Mo (1993).

In 1995, a new species of *Spironucleus* was reported in freshwater angelfish (*Pterophyllum scalare*) and described as *Spironucleus vortens* based on light microscopy, SEM and TEM. Furthermore, *S. vortens* has been described from ide (*Leuciscus idus*) and discus (*Symphysodon discus*) (Pau & Matthews 2001, Sterud & Poynton 2002).

Another species, *Spironucleus elegans*, has also been reported from angelfish and additionally it has been isolated from amphibians (Lavier 1936). Ultrastructural details have been described for the isolate from amphibians (Brugerolle et al. 1973, Brugerolle 1975) but only light microscopy descriptions are available for the angelfish isolate (Kulda & Lom 1964a, Kulda & Lom 1964b).

It has been suggested that *S. elegans* and *S. vortens* are in fact conspecific but due to the lack of SEM data available for *S. elegans* this has not been confirmed. Ultrastructural morphology of the amphibian isolate shows minor differences from *S. vortens* and in addition Kulda & Lom (1964a, 1964b) cross-infected angelfish with the isolate from newt (*Triturus alpestris*) and concluded that these isolates were identical. These findings further strengthen the idea that these are in fact the same species (Poynton et al. 1995, Poynton & Sterud 2002, Sterud & Poynton 2002, Jørgensen & Sterud 2007).

*H. salmonis* was reported from rainbow trout in 1996 though no EM studies were performed (Uldal & Buchmann 1996). Up to that point *H. salmonis* was the only described hexamitid flagellate from salmonids.

This changed in 1997 when *Spironucleus barkhanus* from grayling (*Thymallus thymallus*) and Atlantic salmon was described as a new species by light, scanning and transmission electron microscopy (Sterud et al. 1997). After the emergence of *S. barkhanus* as a causative agent for severe systemic infection in Atlantic salmon earlier reports of hexamitosis in Atlantic salmon were re-described as *S. barkhanus* and the disease termed spironucleosis (Mo et al. 1990, Poppe T et al. 1992, Poppe TT & Mo 1993, Sterud et al. 1998).

To confront existing taxonomical confusion specific guidelines for species descriptions of diplomonad flagellates from fish were published in 2002, along with the genus specific features previously mentioned (Poynton & Sterud 2002). Based on these guidelines *H. salmonis* (Ferguson 1979) and *O. salmonis* (Moore 1923, Davis 1926) were synonymised and renamed *Spiro-*
nucleus salmonis. Additionally, all published ultrastructural data for *S. barkhanus*, *S. torosa* and *S. vortens* was re-examined and the diagnostic guide for *Spironucleus* was expanded (Table 3) (Poynton et al. 2004).

These publications also raised the need for molecular characterizations for further species resolution of the genus. Molecular methods had not been used up to that point though Keeling & Doolittle (1996) and Keeling & Doolittle (1997) demonstrated genetic similarities between isolates from the Atlantic salmon and the Chinook salmon in the α-tubulin gene.

**Table 2.** Important ultrastructural features for identification of genus between *Spironucleus*, *Hexamita* and *Octomitus*. Adapted from Poynton & Sterud (2002) with permission from John Wiley & sons Ltd*

<table>
<thead>
<tr>
<th>Genus-level identification</th>
<th>Spironucleus</th>
<th>Hexamita</th>
<th>Octomitus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flagellar pockets (cytosomal canals)*</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Central axis formed by recurrent axonemes, microtubular bands, endoplasmic reticulum</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Two terminal spikes</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Shape of nuclei</td>
<td>S-shaped</td>
<td>Spherical</td>
<td>Reniform</td>
</tr>
<tr>
<td>Location of kinetosomes relative to nuclei</td>
<td>Sub-apical</td>
<td>External structure</td>
<td>Between</td>
</tr>
<tr>
<td>Position of recurrent flagella relative to nuclei</td>
<td>Medial</td>
<td>Lateral</td>
<td>Medial</td>
</tr>
<tr>
<td>Supra-nuclear microtubular band</td>
<td>+</td>
<td>+</td>
<td>Reduced</td>
</tr>
<tr>
<td>Infra-nuclear microtubular band</td>
<td>+</td>
<td>+</td>
<td>Reduced</td>
</tr>
</tbody>
</table>

*When present, the recurrent flagella are ensheathed

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Further molecular characterizations were not performed until Jørgensen & Sterud (2004) sequenced a 1154 bp part of the small subunit ribosomal RNA (SSU rRNA) gene of two populations of *S. barkhanus*, one isolated from the blood of farmed Arctic charr (*Salvelinus alpinus*) causing severe systemic spironucleosis (Sterud *et al.* 2003) and one from the gall bladed of asymptomatic wild Arctic charr. In addition, comparisons were made with sequences from *S. barkhanus* isolated in farmed Atlantic salmon and Chinook salmon. It had been previously demonstrated that all the isolates were morphologically identical in light microscopy, SEM and TEM but molecular analyses showed only 92.7% similarities between the farmed Arctic charr isolate versus the wild isolate while it showed 99.7% similarity with the isolates from Chinook and Atlantic salmon. It was proposed that the isolate causing severe systemic spironucleosis was a specific pathogenic strain of *S. barkhanus* (Jørgensen & Sterud 2004).

This was confirmed in a larger study where *S. barkhanus* was sampled from three different outbreaks of systemic spironucleosis in sea-water farmed fish (Atlantic salmon, Chinook salmon and Arctic charr), five wild grayling and five Arctic charr populations in Norway along with a *Spironucleus* isolate from Canadian Arctic grayling (*Thymallus arcticus* Pallas, 1776). In total 14 *Spironucleus* populations from five species of fish were studied molecularly and morphologically using light microscopy, SEM and TEM. Three genes were partially sequenced: SSU rRNA gene, gdh-1 and α-tubulin and sequencing data from *Giardia* was used for comparison of genetic distances between species and genotypes of closely related diplomonads. It was concluded that the differences between the isolates from farmed marine fish and wild fresh water fish was high enough for separation into two species. One species retained the name *S. barkhanus* and was considered a harmless commensal in the intestine and gall bladder of fresh water and anadromous wild salmonids (Jørgensen & Sterud 2006, Denikina *et al.* 2016). The other species was named *Spironucleus salmonicida* and considered to be the causative agent of severe systemic infection in sea-water farmed salmonids such as the Arctic charr, Atlantic salmon and the Chinook salmon (Jørgensen & Sterud 2006, Meseck *et al.* 2007).

In a later study it was shown that wild Arctic char and the brown trout can contain a dual infection by both *S. barkhanus* and *S. salmonicida* without showing signs of spironucleosis and it was suggested that these could serve as the potential wild reservoir hosts of *Spironucleus salmonicida* (Jørgensen *et al.* 2011).

Another comprehensive molecular study looked at the relationship between different isolates of *S. torosa*. In the study the SSU rRNA gene of 43 isolates of *S. torosa* from five species of marine gadoid fish and the burbot, the only freshwater gadoid. Phylogenetic analyses revealed two genotypes of *S. torosa*, one from marine fish and the other from the burbot, with genetic distances too
short for separation into two species (Jørgensen et al. 2007). Host specificity and location in the host are summarized in Table 1.

Morphology

Species of the genera *Spironucleus* are often morphologically similar and in some cases almost identical. *S. salmonicida* trophozoites are characterized by an oval to pear-shaped body, averaging 10.9 µm in length and 5.7 µm wide for cultivated trophozoites (Figure 2 and 3A) (Jørgensen & Sterud 2006) while in general *Spironucleus* species have a spherical to pyriform body ranging from 8-20 µm in length and 5-11 µm in width (Woo P 2006). In comparison *G. intestinalis* trophozoites have a body shaped of a flattened teardrop, 12-15 µm long and 5-9 µm wide (Luján & Svärd 2011).

All species of *Spironucleus* possess eight flagella originating from four basal bodies located between the two characteristic paired anterior nuclei (Figure 2 and 3A). The trophozoite ultrastructure displays binary axial symmetry about its long axis with each side consisting of a nucleus, four flagella and basal bodies. The nuclei are elongated and reduce towards the anterior end of the trophozoite coming into very close proximity and folding slightly around each other generating a characteristic S-shaped contact. Six of the flagella travel short distances intracellularly, emerge anteriorly in two groups of three and support the motility of the trophozoites (Figure 3B). The remaining two flagella pass from the anterior end through flagellar pockets within the body, emerge close to each other in the posterior end of the trophozoites and trail behind during swimming. The flagella are around 1.5 – 2 times the length of the trophozoites (Poynton & Sterud 2002, Woo P 2006, Williams CF et al. 2011). The flagellar pockets of the recurrent flagella (Figure 3C), additionally serve as conduits where nutrients pass through and are taken up by the cell via vacuoles.

*G. intestinalis* likewise has eight flagella but arranged into four bilaterally symmetrical flagellar pairs with the pairs emerging anteriorly, caudal, posterolateral and ventral (Simpson 2003). The beating of all flagellar pairs is used for orientation with complex movements (Dawson 2010). *Giardia* has no flagellar pockets or feeding groves and endocytosis can take place on most parts of the surface (Simpson 2003).

While the surface of *Spironucleus* trophozoites is mostly smooth, surface ornamentations are used to distinguish between the different species. *S. salmonicida* and the morphologically identical *S. barkhanus* are characterized by a crescent-shaped ridge or a barkhan around the opening of the flagellar pockets (Figure 3 A and C) (Sterud et al. 1997, Jørgensen & Sterud 2006) while *S. torosa* has a ring-shaped swelling or a torus where the recurrent flagella emerge from the cell (Poynton & Morrison 1990, Sterud 1998a). In *S. vortens* the recurrent flagella emerge from flagellar pockets with compound lateral
Table 3. Comparative ultrastructural elements for *Spiromastix* species recognition. Abbreviations: mt: microtubules; rf: recurrent flagella; a: adjacent to anterior kinetosomes; r: adjacent to kinetosomes of recurrent flagella; RER: rough endoplasmic reticulum; -: absent; +: present; +++: abundant; +/-: present or absent; ?: unknown. Adapted from Poynton et al. (2004).

<table>
<thead>
<tr>
<th>External surface and ornamentation</th>
<th>Cytoskeleton</th>
<th>Cytoplasm</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. barkhanus / salmonicida</strong></td>
<td>Unadorned body, rf emerge from barkhanus</td>
<td>3 radiate</td>
<td>None</td>
</tr>
<tr>
<td><strong>S. torosa</strong></td>
<td>Unadorned body, rf emerge from tori, caudal projection present</td>
<td>3 concentric</td>
<td>Mt and lamellae support tori and flanges</td>
</tr>
<tr>
<td><strong>S. salmonis</strong></td>
<td>Unadorned body, rf emergence unknown</td>
<td>3 radiate</td>
<td>None</td>
</tr>
<tr>
<td><strong>S. vortens</strong></td>
<td>Adorned body with compound lateral ridges counter-crossing at posterior end with papillae, rf emerge from counter-crossing ridges</td>
<td>3 staggered</td>
<td>Mt support peripheral ridges; structure supports central part of ridge</td>
</tr>
</tbody>
</table>

1Viewed in transverse section through middle of body; 2*Barkhans*: crescent-shaped ridges; 3*Tori*: salient (raised) round structures, in this case surrounded by flanges of body; 4*Compound lateral ridges*: a smooth broad central part and a rope like peripheral ridge narrower on left than on right, peripheral ridges bear tufts of microfibrillar material
ridges counter-crossing between the openings (Poynton et al. 1995) while S. salmonis lack surface ornamentations altogether (Poynton et al. 2004, Fard et al. 2007). S. salmonis is mainly characterized by cytoplasmic features, such as a posterior sac of dense free ribosomes, electron-dense bodies, bowl-shaped membranous and structures, all of which other piscine Spironucleus species lack (Poynton et al. 2004). It has been pointed out that these cytoplasmic features may differ between cells harvested directly from fish and ones grown in vitro (Poynton & Sterud 2002). Other ultrastructural features that are additionally used to distinguish between the piscine species of Spironucleus are described in Table 3.

Spironucleosis

As previously mentioned, the disease caused by Spironucleus species is termed spironucleosis and can range from being asymptomatic to a severe systemic infection causing major complication and death to the fish host. In general, the intestinal infections are chronic with a low number of parasites and the host shows no signs of symptoms. In fact, S. barkhanus and S. torosa have not been reported to cause severe clinical symptoms to their hosts and seems to be confined to the lumen of the gut and gall bladder and the rectum, respectively, in their hosts. This may suggests a commensal life style of the parasites (Woo PTK & Poynton 1995). Reports regarding H. salmonis suggests that the parasite is a commensal but can under certain conditions cause severe symptoms. Trophozoites are commonly found in the upper intestine and pyloric region though in heavy infections they can be found throughout the intestinal tract (Moore 1923, Woo PTK & Poynton 1995, Uldal & Buchmann 1996). Unusual swimming behavior have also been reported from fish infected with H. salmonis, such as corkscrew movements, swimming on the side and remaining in the corner of tanks (Tojo & Santamarina 1998). It should be noted that reports regarding H. salmonis should be reviewed carefully as previously most diplomonad flagellates from fish were assigned to H. salmonis. Many of the cases reported have not been re-investigated and it is implausible they refer to other Spironucleus species.

Symptoms caused by systemic spironucleosis seem to be rather non-specific and mainly caused by S. vortens and S. salmonicida. The former has been associated with the hole-in-the-head (HITH) disease in the cichlids angelfish and discus. The disease is described as pitting-type lesions on the head caused by the parasite migration into sensory pores where they cause decay of the tissue. In addition the parasite has been isolated from the lip tumors, intestine, kidneys, liver and spleen of infected fish and if not treated, can cause death of the host (Poynton et al. 1995, Woo PTK & Poynton 1995, Paull & Matthews
During moderate infections the parasite can only be seen in the intestine suggesting an intestinal origin of the parasite (Paull & Matthews 2001).

Severe systemic infections in salmonids was previously linked to *H. salmonicida* and *S. barkhanus* but is now considered to be caused only by *S. salmonicida*. The systemic disease seem to be confined to fish farms and has been described in various farmed fish such as the Arctic charr, the Chinook salmon and the Atlantic salmon (Kent *et al.* 1992, Poppe *T et al.* 1992, Sterud *et al.* 2003, Jørgensen & Sterud 2004, Jørgensen & Sterud 2006). In contrast, wild Arctic charr and brown trout has been suggested as the reservoir hosts as infections are asymptomatic (Jørgensen *et al.* 2011). It has also been shown that the parasite can cause infection in fresh water and is hence not considered to be strictly confined to marine conditions (Kent *et al.* 1992, Poppe *T et al.* 1992, Guo & Woo 2004a).

Figure 2. A schematic illustration of a *S. salmonicida* trophozoite. The mean size of cultivated trophozoites is 10.9 µm X 5.7 µm (Jørgensen & Sterud 2006). The two diploid nuclei are characterizing for the diplomonads. Four basal bodies are situated between the nuclei. Six flagella emerge anteriorly in two groups of three and two flagella run through the body and emerge posteriorly. Flagella are around 1.5 – 2 times the length of the trophozoite (Woo P 2006). The area posterior to the nuclei is dense and hydrogenosomes are scattered throughout the cytoplasm.
The symptoms of the disease seem to be host specific, reported as severe epicarditis and large caseonectrotic areas with granulomatous responses in the Atlantic salmon while infections Arctic charr and Chinook salmon do not show apparent granulomas formation (Kent et al. 1992, Poppe T et al. 1992, Poppe TT & Mo 1993, Sterud et al. 2003, Williams CF et al. 2011). Experimental infections in Atlantic salmon revealed high numbers of trophozoites in skin and muscle lesions in the tail, in lesions in internal organs such as the spleen and kidney and accumulation of trophozoites in the eye pocket bulging the eye outward. Two distinct phases were suggested, a blood phase and a tissue phase. The parasite was detected in the blood from week 1 until week 8 followed by a first wave of mortality during the later phases of the blood stage. The tissue phase followed the blood phase with lesions appearing on the skin, organs and in muscles and followed by a second wave of mortality. Interestingly, the parasite was never detected in the intestine which is considered to be the normal route of infection and the preferred location in the host for most Spironucleus species. Mortality in the experiment was close to 100% which is consistent with what Sterud et al. (2003) reported during infections in farmed Arctic charr (Guo & Woo 2004a, Guo & Woo 2004b).

In addition, an intracellular stage of the parasite was suggested by Sterud et al. (2003) in the Arctic charr. Intracellular stages have previously been described for S. salmonis (Moore 1923, Davis 1926) though it has been questioned and considered as misinterpretations (Becker 1977). The findings of Sterud et al. (2003) support the reports of an intracellular stage though more research is needed to confirm these findings.

It is evidential that S. salmonicida is asymptomatic in the intestine of wild Arctic charr and brown trout while causing severe systemic infection in farmed Arctic charr, Atlantic salmon and Chinook salmon. Specific triggers for infections in farmed fish are currently not known though host immune responses are considered to play a key role (Williams CF et al. 2011). It is feasible to consider that close proximity in the farms causes stress conditions in the fish, reducing the ability of the immune system to protect the host against S. salmonicida infections but these speculations need to be investigated further with experiments targeting the host response during infections.

Life cycle and transmission

Knowledge regarding the life cycle of Spironucleus sp. and especially how transmission occurs between hosts is currently not well described. The most documented form is the trophozoite, which is the actively swimming stage of the parasite. No sexual stages have been documented but asexual reproduction happens by longitudinal binary fission of the trophozoites as has been described for other diplomonad flagellates.

Generation times has been estimated in vitro and vary significantly between different species. Doubling time in vitro for S. barkhanus is considered to be
20 - 30 h (Sterud 1998b) while \textit{S. vortens} has been reported to have extremely rapid generation time of 1.79 h, suggesting it is one of the fastest dividing eukaryotes (Millet \textit{et al.} 2011b). Growth curve measurement of \textit{in vitro} growth of \textit{S. salmonicida} suggest average doubling time of 20 h with the fastest generation time being 16 h during mid-logarithmic phase (Figure 4) (Jerlström-Hultqvist \textit{n.d.}). In comparison \textit{G. intestinalis} has a generation time of around 6 h in \textit{in vitro} culture (Luján & Svärd 2011).

Very little information is available regarding the transmission of piscine \textit{Spironucleus} sp. A cyst form has been well defined in \textit{G. intestinalis} as the infectious form of the parasite. In \textit{Spironucleus} \textit{sp.}, cyst forms have only been described from \textit{S. muris} (Kunstýr 1977), \textit{S. melagridis} (Wood & Smith 2005) and \textit{S. salmonis} (Moore 1923, Davis 1926, Uldal & Buchmann 1996) which is the only piscine species where cysts have been described.

Comparison of the cysts of \textit{Giardia microti} and \textit{S. muris} revealed many similarities including 2-4 nuclei, flagellar axonemes, distinct cyst wall and same immunostainings when labeled with \textit{Giardia} cyst wall antibody. The cysts of \textit{S. muris} are significantly smaller with a thinner cyst wall and the flagella seem to be retained and wrapped up rather than disassembled as in the \textit{Giardia} cysts (Januschka \textit{et al.} 1988). The cysts of \textit{S. meleagridis} have a similar appearance as the \textit{S. muris} cysts and are found primarily within the intestinal mucus layer, often in clusters (Wood & Smith 2005).

The transmission of the cysts of \textit{S. muris} and \textit{S. meleagridis} follows the same route as \textit{Giardia cysts}, the fecal-oral route. The cysts are very resilient and can survive harsh conditions in the environment (Kunstýr & Ammerpohl 1978, Corliss 2001). Similar as in \textit{Giardia} the cysts are ingested by a new host and are not killed by the acidity of the stomach. When reaching the intestinal tract excystation occurs releasing trophozoites and beginning a new life cycle (Woo PTK & Poynton 1995, Luján & Svärd 2011).

Cysts have only been reported from one piscine \textit{Spironucleus} species, \textit{S. salmonis}, though the form is rarely seen (Tojo & Santamarina 1998). The cysts are oval or round, 7 x 10 µm, and retains the flagella at the early stages of encystment though it is lost in the advanced stages of the cysts. Each cyst is reported to contain two flagellates (Moore 1923, Davis 1926, Becker 1977, Woo PTK & Poynton 1995). Cysts of \textit{S. salmonis} have also been described in \textit{in vitro} cultures where the cysts are significantly bigger, measuring 12 +/- 1,1 x 8,5 +/- 0,9, and are often displayed in clusters (Uldal 1996).

Cysts have not been detected in other species of piscine \textit{Spironucleus} and other routes than the traditional fecal-oral route have been suggested such as transmission via skin lesions (Poppe T \textit{et al.} 1992) and through the rectal route (Kent \textit{et al.} 1992). A direct infection route has also been suggested where the trophozoite is the infectious form. This model has problems since the trophozoites normally do not survive for long periods in freshwater (Kent \textit{et al.} 1992) though they have been detected in fresh fecal material (Tojo & Santamarina

26
It has been reported that the trophozoites of *S. vortens* can survive for more than 36 days in the environment protected by the feces and it was speculated that the cysts are necessary for transmission of the parasite. However, no cysts were detected in the fecal material in the experiment suggesting the trophozoite as transmissive form of the parasite (Williams CF *et al.* 2013).

*Figure 3.* Scanning electron microscopy (SEM) images of *S. salmonicida*. A) A single trophozoite. The eight flagella are showing along with the crescent-shape ridge. B) Close up of one of the two pairs of three posterior flagella. C) Anterior side of the trophozoites clearly displaying the flagellar pocket of the recurrent flagella and the crescent-shape ridge. D) Swarms of trophozoites regularly seen with *S. salmonicida*. Abbreviations: af: anterior flagella; pf: posterior flagella; cr: crescent-shape ridge or a barkhan; fp: flagellar pocket. Scale bars indicated in images.
Genomic observations of \textit{S. salmonicida} reveal 11 proteins with high homology with the \textit{Giardia} cyst wall proteins suggesting transmission might occur through the formation of cysts of the parasite (Andersson \textit{et al.} 2007). The current knowledge available regarding the transmission of \textit{Spironucleus sp.} is very limited and further research is needed to establish the transmission process in piscine species.

**Cell and Molecular biology**


\textit{In vitro} studies of \textit{S. vortens} revealed three distinct swimming behaviors: 1) swarms, up to 200 µm in diameter, forming spontaneously and persisting for several minutes, 2) directional movement of the swarm and 3) independent swimming of the trophozoites. This behavior suggests interaction between the cells (Poynton \textit{et al.} 2018). This behavior has also been seen in \textit{in vitro} cultures of \textit{S. salmonicida} (Figure 3D).

Additionally, transfection systems have been developed for both \textit{S. vortens} and \textit{S. salmonicida}. In \textit{S. vortens} the histone H3 gene tagged with eGFP form a plasmid under its native promoter was successfully expressed (Dawson \textit{et al.} 2018).
Transfectants were selected using puromycin acetyltransferase (PAC) marker and the protein was demonstrated to localize to the chromatin in both nuclei. Furthermore, it was demonstrated by in situ hybridization (FISH) that the plasmid localized only to one of the nuclei, indicating no nuclear fusion between the nuclei during mitosis exactly as have been reported in Giardia. The expression levels varied between cells due to copy number ranging from 10-20 copies per cell, similar as seen in Giardia (Singer et al. 1998, Dawson et al. 2008).

A stable transfection system has been developed in *S. salmonicida* as well (Jerlström-Hultqvist et al. 2012). Vectors for epitope tagging and purification of proteins expressed using either native or constitutive promoters were developed using three different selection markers for selection of the transfectants. Site-specific integration has been achieved using the blasticidin S-deaminase (bsr) selection marker. Six proteins were successfully localized with their expression validated and double transfectants were generated for co-localization studies (Jerlström-Hultqvist et al. 2012).

These systems have since published been powerful tools for studying the cell and molecular biology of these organisms and have helped increasing our knowledge regarding the molecular aspects of *Spironucleus*.

**Genomics**

The genomes of *Spironucleus* species have been poorly studied. As other Hexamitinae, *Spironucleus* uses an alternative genetic code with UGA as the only stop codon and encoding UAA and UAG as glutamine (Keeling & Doolittle 1996, Keeling & Doolittle 1997). As Giardia, *Spironucleus* contain four copies of the chromosomes, two in each nuclei.

A genome project where 130 Mb of the *S. vortens* genome were sequenced along with two expressed sequence tag (EST) libraries was initiated by the Joint Genome Institute (JGI). The project was discontinued due to assembly problems as assembled contigs showed only 2x coverage and based on ESTs alignments only 18% of the gene space was assembled. Initially it was estimated that 130 Mb would give 8x coverage of the genome size was estimated to be 15 Mb in size. It was noted that that the genome contains large portions of repetitive sequences and did not assembly due extensive polymorphism between haploids, much larger genome size that initially expected and/or unclonable regions (Nordberg et al. 2014).

The first information about the genome of *S. salmonicida* came from a genome survey (GSS) and EST libraries. It is suggested a small genome around the same size as the Giardia genome (12 Mb). The genome was AT-rich (64%) with very few or any introns, had short intergenic regions and very short 3’ UTRs (Andersson et al. 2007). No TATA-boxes were reported in the promoter regions but A/T rich regions were found within 50 bp upstream of the
translational start sites reassembling the transcriptional machinery in *G. intestinalis* (Elmendorf et al. 2001, Andersson et al. 2007). Furthermore a large collection of cysteine-rich proteins were found containing CXXC motifs similar as the VSP gene family in *Giardia*. However, the characterized VSP motifs, CRGKA and GGCY (Nash 2002), were not found in the cysteine rich protein family in *S. salmonicida*. Interestingly, the majority of the proteins were found to be more similar to proteins in the ciliate *Tetrahymena thermophila* (Eisen et al. 2006).

A comparative genomics project based on ESTs from *S. barkhanus* and *S. salmonicida* indicated large genetic differences in the genomes of the morphologically identical species. The codon usage was reported to be altered in *S. barkhanus* compared to *S. salmonicida* and high allelic sequence variation was detected in *S. barkhanus* while *S. salmonicida* showed sequence homogeneity. The haploid genome size was estimated using flow cytometry and confirmed the *S. salmonicida* genome size to be similar as the *Giardia* genome (12 Mb), whereas the *S. barkhanus* genome was estimated to be larger (18 Mb) (Roxström-Lindquist et al. 2010).

In addition, many genes were detected in the *Spironucleus* genomes that are absent in the *Giardia* genome. For instance, an expanded metabolic repertoire was reported. Many of the metabolic proteins in *S. salmonicida* seem to have been acquired with LGT and have a prokaryotic origin (Andersson et al. 2003, Andersson et al. 2006, Andersson et al. 2007). Interestingly, it appears *Spironucleus* can potentially use all 64 codons to code for amino acids as it has the ability to incorporate selenocysteine into proteins, a feature missing in *Giardia* (Roxström-Lindquist et al. 2010).

**Metabolism**

Metabolism of *Spironucleus* species are in general poorly understood. During growth of *S. vortens* with glucose the end products are acetate, alanine, CO₂ and lactate though neither glucose or ethanol are considered to be the substrates of choice and amino acids are preferred over the two compounds (Millet et al. 2011a). No active arginine dihydrolase (ADH) pathway has been found in *S. vortens* and during in vitro growth no ornithine is produced, in contrast to *G. intestinalis* (Schofield et al. 1990, Schofield et al. 1992), *T. vaginalis* (Linstead & Cranshaw 1983) and *Hexamita inflata* (Biagini et al. 2003) where this pathway has been extensively characterized.

Members of the Fornicata do not possess oxidative mitochondria and harbor, as mentioned, MROs. The MROs of the last common anchestor of Fornicata has been suggested to be equipped with functional ATP synthesis and hydrogen production while the MROs of the last common ancestor of Diplomonadida has possibly lost the machinery for ATP synthesis while maintaining the ability to produce hydrogen (Leger et al. 2017). The first MRO to be discovered was the now well characterized hydrogenosome in *Trichomonas*.
vaginalis (Lindmark et al. 1975). It is functionally alike the MROs of the last common ancestor of Fornicata as it has retained the ATP production with concomitant production of hydrogen (Lindmark & Müller 1973, Lindmark et al. 1975, Müller et al. 2012, Stairs et al. 2015).

The first indication of hydrogenosomes in Spironucleus was the discovery of [FeFe]-hydrogenase gene in S. barkhanus (Horner et al. 2000). Another indication was the rapid hydrogen production of S. vortens that is in general a rare trade amongst eukaryotes (Millet et al. 2010). It was reported to have a higher hydrogen production than T. vaginalis and around 40 times higher production than Giardia (Lloyd D et al. 2002). An MRO has then been described in S. vortens but it remains to be fully characterized (Millet et al. 2013).

Additionally, a MRO has been described in one other piscine Spironucleus species as S. salmonicida harbors a hydrogenosome that has been well characterized (Jerlström-Hultqvist et al. 2013). The main function of the hydrogenosomes in S. salmonicida, along with Fe-S cluster biosynthesis, is the production of ATP through a substrate-level phosphorylation with hydrogen as a by-product as oxidative phosphorylation have been lost.

A comparison between the hydrogen production of S. salmonicida, S. barkhanus and S. vortens revealed eight- to tenfold higher production in S. vortens than in the other Spironucleus species (Jerlström-Hultqvist et al. 2013, Stairs et al. 2015).

Another example of a MRO is the mitosome in Giardia that is very functionally reduced and currently limited to the biogenesis of Fe-S cluster (Tovar et al. 2003) as ATP production in Giardia is strictly cytosolic using an extended glycolysis pathway and the arginine dihydrolase pathway (Schofield et al. 1990, Schofield et al. 1992, Sánchez et al. 2000, Tovar et al. 2003, Emelyanov & Goldberg 2011, Stairs et al. 2015).

Members of Spironucleus are described as anaerobes even though it is known they can cause systemic infection and hence must be able to tolerate fluctuations in the environmental O2 level as they can infect all parts of the fish. How the parasites cope with oxidative stress has not been extensively documented.

Close relatives of Spironucleus, G. intestinalis and H. inflata have been reclassified as microaerophilic based on their tolerance to low O2 concentrations (Paget et al. 1993a, Ellis et al. 1993, Paget et al. 1993b, Biagini et al. 1997). H. inflata has been shown to have a high affinity for oxygen suggesting a distinctive detoxification mechanism though this has not been well characterized (Biagini et al. 1997, Biagini et al. 2001). G. intestinalis is missing important elements from the traditional oxidative stress pathway of eukaryotes and uses a process similar to some bacteria where NADH oxidase is a key player in the generation of molecular hydrogen (Brown et al. 1995, Brown et al. 1996, Brown et al. 1998, Stanton et al. 1999, Biagini et al. 2001).
S. vortens lacks catalase and peroxidase activities similar as Giardia but possesses superoxide dismutase and NADH oxidase. Interestingly, glutathione has been revealed to be a major-protein thiol (Williams CF et al. 2014).

Our knowledge regarding oxidative stress response in S. salmonicida is currently even more limited. The parasite can be found in various different tissues in the fish host and is certainly exposed to high levels of oxygen throughout its life cycle but no efforts have been made to show how the parasite tolerates these oxygenated conditions.
Present Investigation

The diplomonads are a very diverse group of organisms and apart from *G. intestinalis* very few members have been extensively characterized. Many members are unculturable *in vitro* and the tools repertoire to study the cell and molecular biology is limited. An alternative model organism to *G. intestinalis* would be highly valuable to study these often complicated and diverse organisms. The establishment of a transfection system in *S. salmonicida* (Jerlström-Hultqvist et al. 2012) along with increased availability of genetic data (Roxström-Lindquist et al. 2010) made this fish parasite a potential candidate as later work characterizing the MROs revealed (Jerlström-Hultqvist et al. 2013).

The work represented in this thesis is focused on further characterizing *S. salmonicida* and establishing the parasite as an alternative model organism for the diplomonads. It investigates various aspects of the parasite and has a broad spectrum involving different biological fields such as genomics, cell biology, biochemistry and infection biology.

Paper I describes the genome of *S. salmonicida* and comparisons are made to the genome of *G. intestinalis*. Various features of the genome are identified and evaluated towards the life style of the parasite. This is further investigated in Paper III where the oxygen response of the parasite is described using transcriptome analyses of parasites exposed differently to oxygen.

Together, the genome and the transfection system compose powerful tools for further studies that were exploited in Paper II to look into the cell biology of the parasite. Characterization of the annexins is highly valuable from evolutionary aspects and provides deeper understanding of the structure of the diplomonad cell. Paper IV in addition utilizes the transfection system and describes the adaption of a proximity labeling system suitable for protein localizations and TEM imaging to obtain ultrastructural details of protein localizations. Furthermore, it is demonstrated that the system is transferable to *G. intestinalis* and potentially other diplomonads.

Experimental infections of Atlantic salmon were performed in Paper V to establish various elements of the infection cycle of the parasite. Detection and diagnostic tools were developed and employed to investigate different stages of infection and transmission of *S. salmonicida*. In Paper VI this is further elaborated as the molecular response of the parasite and the salmon is studied using an *in vitro* interaction system of *S. salmonicida* and Atlantic salmon.
kidney cells (ASK). To complement this, SEM and TEM images were acquired to visualize the interactions between the parasite and the ASK cells.

The genome of *S. salmonicida* (Paper I)

In this paper, we presented a high quality draft genome of *S. salmonicida* and compared it to its close relative *G. intestinalis*. Combined analyses of the genomic data and optical maps revealed an estimated genome size of ~12.9 Mb which is a similar in size as has been reported previously by flow cytometry measurements (Roxström-Lindquist *et al.* 2010). The genome is distributed in nine chromosomes and the assembly contains 233 scaffolds with 8067 coding genes that have a mean length of 373 aa. The G+C contents is 33.4%. Compared to *G. intestinalis* that has a smaller genome size of 11.7 Mb, the *S. salmonicida* genome contains more coding genes with a lower mean length (5901 genes with mean length 530 in *G. intestinalis*). The coding percentage is higher in *G. intestinalis* (78.2%) than *S. salmonicida* (72.1%) and the mean intergenic distance is longer. The G+C content is significantly higher in *G. intestinalis* (49%) and we found fewer canonical introns in *S. salmonicida*, three in *S. salmonicida* versus six in *G. intestinalis*. Furthermore, we could not detect any trans-spliced introns in the genome of *S. salmonicida* while *G. intestinalis* contains four (Kamikawa *et al.* 2011, Franzén *et al.* 2013).

Evidence of a cyst stage

Even though a cyst stage has been well characterized in *G. intestinalis* and cysts has been described for a few *Spironucleus* species, no cysts have been found for *S. salmonicida* and information regarding transmission of the parasite is lacking. Cyst-like objects have not been observed in *in vitro* cultures and encystation using traditional methods for *G. intestinalis* have been unsuccessful. Evidence of a cyst stage are hinted at in the genome as we identified 11 putative cyst wall proteins that show similarities to the *G. intestinalis* CWPs.

To further investigate this, we fused a CWP protein from *S. salmonicida* to the CWP-1 promoter, tagged it with a 3xHA epitope tag and transfected the construct into *G. intestinalis*. The expression of the construct was induced during encystation of the transfectants and localization studies showed that it localizes in the encystation-specific vesicles (ESVs) and gets incorporated into the cyst wall of the *G. intestinalis* cysts. These results indicate a potential cyst stage of *S. salmonicida*. 
Cysteine-rich protein families

*G. intestinalis* uses antigenic variation of cell surface proteins, known as the variable surface proteins (VSPs), to avoid the hosts immune system (Adam *et al.* 2010). The VSPs are cysteine-rich with numerous CXXC motifs, an N-terminal signal peptide and a conserved C-terminal transmembrane domain. Furthermore they are characterized by a hydrophilic cytoplasmic tail that contains a five amino acid sequence, CRGKA, which is conserved in almost all VSPs (Adam *et al.* 2010).

In *S. salmonicida*, we found hundreds of cysteine-rich proteins and it is the largest gene family in the genome. We further categorized these proteins into three subgroups; cysteine-rich membrane proteins 1 (CRMP-1), cysteine-rich membrane proteins 2 (CRMP-2) and cysteine-rich proteins (CRP). The CRMP-1 group contains 125 membrane proteins that showed the most similarities to the VSPs in *G. intestinalis*. They were characterized by numerous CXXC and CXC motifs, a transmembrane domain and a conserved five amino acid sequence, KKXKK, in the C-terminal that is reminiscent of the CRGKA sequence in *G. intestinalis*.

The CRMP-2 on the other hand contains 195 proteins that lacked the conserved five amino acid signature sequence of the CRMP-1 proteins but retained the numerous CXXC and CXC motifs along with a transmembrane domain. These are more similar to a group of high cysteine membrane proteins (HCMP) found in *G. intestinalis* than the VSPs as the HCMPs likewise retain the cysteine motifs and a transmembrane domain but lack the CRGKA tail (Davids *et al.* 2006).

The 52 remaining cysteine-rich proteins were grouped together as CRP. They lacked all the characteristics of the other groups but contained more than 10% cysteine residues.

To further establish the function of these proteins we tagged three CRMP-1 proteins with a 3xHA epitope tag and transfected the constructs into *S. salmonicida*. The constructs were expressed from their native promoter and showed three different localizations. SS50377_18013 localized to the cellular body and the flagella, showing similar characteristics as the VSPs in *G. intestinalis*. SS50377_18923 on the other hand localized strictly to the cell body while SS50377_17215 localized to structures in the ER.

It remains to be seen if these proteins show similar transcriptional features as the VSPs, where only one protein is expressed at a time and expression is switched every 6-13 generations (Nash *et al.* 1990). These findings suggest a similar mechanism can be found in *S. salmonicida* that would be highly valuable for the parasite to avoid the immune system of the host during infections.
Metabolism

Comparing the metabolism of *S. salmonicida* and *G. intestinalis* we detected a considerable difference in the metabolic gene repertoire as the former contained substantially more metabolic genes. In agreement with that, we also identified more transporter proteins for metabolite transport.

Even though *S. salmonicida* and *G. intestinalis* encode similar sets of glycolytic enzymes, the fish parasite is potentially able to feed five additional carbohydrates into glycolysis. In addition, *S. salmonicida* appears to have a greater capacity for amino acid metabolism than *G. intestinalis* and can potentially utilize a variety of amino acids as carbon sources. Furthermore, *S. salmonicida* is capable of incorporating selenocysteine into proteins, a feature absent in *G. intestinalis*.

Oxidative stress response

*S. salmonicida* can cause systemic infection and while it is infiltrating various different tissue of the host, it is exposed to different oxygen concentrations. The ability of the parasite to protect itself against reactive oxygen species (ROS) thus seems paramount for its infection cycle. In contrast to *G. intestinalis* who is limited to the intestinal lumen it is apparent that *S. salmonicida* needs a more competent protection system than its human infecting relative.

In the genome of *S. salmonicida* we indeed identified many more genes involved in the oxidative stress response than in *G. intestinalis*, especially there are multiple orthologs for many genes. In particular, we identified five orthologs of Rubreythrin that will be further discussed in Paper III. We did not find any enzymes for glutathione biosynthesis and recycling that suggests cysteine as the major intracellular thiol as in *G. intestinalis* (Babula et al. 2012) and we did not detect the enzyme flavohemoglobin that protects against nitric oxide in *G. intestinalis* (Mastronicoa et al. 2010).

While gene transcripts in *G. intestinalis* have been found to have fuzzy boundaries (Franzén et al. 2013) we noted much tighter transcriptional regulation in *S. salmonicida*. We identified several putative promoter elements including a C-rich motif, a TATA box motif and a putative transcription initiator element (Inr) upstream of many genes. The 11 nucleotide C-rich motif is found in the promoter region of 16.7% genes. RNASeq analyses revealed more tightly controlled expression over these genes supporting the hypothesis that these motifs are regulatory elements.

In addition, we identified 111 genes encoding for proteases including 50 cysteine proteases in the *S. salmonicida* genome, which is more than in *G. intestinalis*. This indicates that *S. salmonicida* may be more capable of degrading host tissue than its relative, which is congruent with the fact that *S. salmonicida* can cause systemic infection while *G. intestinalis* is restricted to non-invasive intestinal infections.
The comparative genomic analyses support that *S. salmonicida* is more capable than *G. intestinalis* to adapt to different environments with different levels of oxygen. The large functional differences between the parasites likely reflects the adaption to their respective host and habitats. In addition, the availability of a high-quality reference genome enables the development of *S. salmonicida* into a model system to aid diplomonad research.

**Annexins in *S. salmonicida* (Paper II)**

Annexins are multifunctional proteins that are associated with lipid binding in a calcium dependent fashion. They are found in organisms across all kingdoms and have been extensively studied in animals and plants. They all share a conserved fold, the annexin fold, and are important in diverse cellular processes such as vesicle trafficking, signaling and membrane-cytoskeleton interactions (Lizarbe et al. 2013).

Very little is known of protistan annexins, except for annexins in *G. intestinalis*, which are the best characterized annexins in unicellular eukaryotes. Twenty-one annexins have been identified in *G. intestinalis* where they are referred to as alpha-giardins. They are mainly associated with the cytoskeleton and some members have been shown to be immunodominant during *G. intestinalis* infections (Wenman et al. 1993, Weiland et al. 2003). Much less is known regarding the annexins in *S. salmonicida* although it has been reported that they are less derived at the sequence level than the alpha-giardins (Roxström-Lindquist et al. 2010). We identified 14 annexin homologs in *S. salmonicida* that displayed 23-41% sequence identity to the alpha-giardins. Two of these, annexin 3 and annexin 9 are present in two identical copies in the genome.

To establish these proteins as *bona fide* annexins we fractioned transfectants expressing the respective annexins to determine any evidence of membrane association. We detected annexin 4, 5, 9 and 12 in both the cytosolic and membranous fractions while annexin 2, 3, 10 and 13 showed much weaker membrane association. Annexin 1 and 8 weren’t detected in either fraction.

The phospholipid binding profiles of two annexins, Annexin 3 and 5, were investigated in greater detail. Both proteins were shown to be canonical annexins in that they bound phospholipids, in distinct patterns, in a calcium dependent fashion.

**Structure of annexins in *S. salmonicida***

To investigate the structure of the *S. salmonicida* annexins we performed comparative analyses to identify any signature motifs of the annexins. We revealed the presence of at least one intact AB loop (type II Ca$^{2+}$ coordination sites) in all annexins except Annexin 13. In *Giardia*, intact AB loops are only seen in
Alpha-14 giardin (Szkodowska et al. 2002). Annexin 13 is in addition unique as it is substantially larger than the other annexins and contains an additional domain that shows low similarities to DndD, a DNA sulfur modification protein (Zhou et al. 2005). The N-terminal sequence of *S. salmonicida* annexins are mostly short which is unusual in the annexin protein family but has previously been described in the alpha-giardins and in plant annexins (Clark et al. 2012).

We also noted that some members of the annexin family might be subjects to post-translational modifications such as lipid acylation as has been previously described for alpha-19 giardin (Šarić et al. 2009).

### Phylogeny of eukaryotic annexins

We were interested in the evolutionary relationship of annexins in eukaryotes. To investigate this we used maximum-likelihood phylogenetic analyses comparing sequences from selected eukaryotes together with the annexin homologs from *S. salmonicida* and other hexamitid flagellates (*S. vortens*, *S. barkhanus*, *Trepomonas* PC1) and the alpha-giardins from *G. intestinalis* and *G. muris*. As expected, the diplomonads formed a distinct but highly diverse sequence cluster compared to other annexins. The hexamitid annexins sit in a large cluster with the alpha-giardins nested within. However, the root position within the diplomonads is likely the result of a long-branch attraction indicating that the hexamitid annexin and alpha-giardins are possible sister clades suggesting independent expansion of the annexins in the two groups. *G. intestinalis* and *G. muris* homologs branch together in most cases indicating a functional conservation and similar observations are made for the closely related *S. salmonicida* and *S. barkhanus*. In *S. vortens* and *Trepomonas* PC1 the expansion of annexins is likely to be lineage-specific. Our analyses furthermore suggests that the annexins were probably present early in eukaryotic evolution.

### Annexin localization in *S. salmonicida*

To study the localization of the annexins in *S. salmonicida* we fused a C-terminal 3xHA tag to all the proteins and generated transfectant cell lines for each of them. We then labelled them with an anti-HA antibody and imaged them by either confocal laser scanning or structured illumination superresolution microscopy (SIM).

The annexins showed a variety of localizations with some showing very specific localizations within the cells. Annexin 1 had a spotty appearance in the plasma membrane with some labeling signal in the recurrent flagella or the striated lamina around it. Annexin 2 and 9 were localized to numerous foci in the cytosol. Annexin 3 and 12 showed a prominent signal in all flagellas, including the recurrent flagella. Annexin 4 and 10 were observed in the plasma
membrane while Annexin 4 also displayed localization in the flagella. Annexin 7 could not be expressed from its native promoter but expressed from the anx3 promoter it displayed a cytoplasmic localization. Annexin 8, 11 and 14 likewise displayed a mostly cytosolic localization.

Annexin 5 and 13 were localized to an unknown structure in the anterior of the cell roughly corresponding to the region of the cell that makes contact with the surface that the trophozoites attach to in vitro. Accordingly, this putative attachment region could be important in host cell contacts in a similar way as the Giardia ventral disc. Some labeling was also found in the in the plasma membrane as well as in a small structure in the middle portion of the cell body. This structure is potentially translocated to the anterior part of a newly formed daughter cell during division.

Annexin 6 had an interesting localization to eight, often paired, foci in the anterior part of the cells. Two of the foci were located posterior to the other six and the number of foci was doubled during division. Their location and numbers indicated that Annexin 6 could be associated with the basal bodies.

We further checked the association of the Annexins 3, 5, 6 and 13 to specific ultrastructural elements by exploiting the ascorbate peroxidase (APEX) proximity labeling system (discussed in Paper IV). We generated C-terminal APEX fusions and used 3,3'-diaminobenzidine (DAB) as a substrate to generate a dark precipitate at the location of the protein within the cell and used TEM to view the ultrastructural associations.

Annexin 5 showed a prominent staining of the flagella and the recurrent flagella, especially in the outer microtubule doublets and in the striated lamina around the recurrent flagella. As expected, Annexin 6 displayed a prominent signal near the basal bodies, in and around the centrioles. Annexin 5 and 13 both labeled a large area in the anterior of the cells, with the former being more prominent.

To further study the Annexins additional functional and phylogenetic investigations in other protists would be beneficial and providing deeper understanding of the roles of these proteins in animals and humans.

Oxygen response in \textit{S. salmonicida} (Paper III)

As \textit{S. salmonicida} can infiltrate the blood stream and colonize various host tissue we wanted to further investigate how \textit{S. salmonicida} can persist and cope with different levels of oxygen despite having a strictly anaerobic metabolism. We collected total RNA samples from trophozoites cultures that were grown in the presence of oxygen or in an antioxidant-free medium and compared to a control culture. We then sequenced poly(A) selected RNA using Illumina HiSeq2500.

Analyses of the RNASeq data revealed 1705 genes were differentially expressed when the cells were exposed to oxygen and 2280 genes in cells grown
in an antioxidant-free medium. This represents over 20% of the genome and suggest that *S. salmonicida* might deploy multiple oxygen defense mechanisms such as oxygen clearance, iron-sequestration and cysteine metabolism to tolerate oxygenated environments.

**Differentially expressed genes in oxidative stress response pathway**

A large portion of these differently regulated genes are hypothetical genes with unknown functions while others encode for known anaerobic metabolic proteins and cysteine and Fe-S cluster biosynthesis. We did not find any genes involved in oxygen metabolic pathways (catalase, superoxide dismutase, glutathione biosynthesis, oxidative phosphorylation) which is in line with what has been previously described in *S. salmonicida*. We detected upregulation of numerous genes, such as the bacterial-like oxidoreductases, that are involved in the oxygen response pathways. Many are potentially acquired laterally from prokaryotes (e.g. NADH oxidase, rubrerythrin, superoxide reductase) and are rarely found in the eukaryotic kingdom. Many of these genes were significantly upregulated in both our oxidative stress test conditions.

**Virulence factors**

Interestingly, we observed an upregulation of many host evasion- and invasion related genes. As previously described in Paper I, the *S. salmonicida* genome encodes at least 111 proteases that belong to mainly four classes: threonine, serine, metallo- and cysteine proteases. When the cells are exposed to oxygen, we detected a significant upregulation of more than half of the threonine and metalloproteases. Cysteine proteases are likewise upregulated in both test conditions and we speculate that *S. salmonicida* might use similar strategies as other pathogens when gaining access to the blood (Nikolskaia *et al.* 2006, Sumitomo *et al.* 2013). The parasite would secrete proteases to degrade the tight junctions and disrupt the integrity of the host epithelial layer. Additionally, other virulence factors such as the toxin hemolysin, efflux pumps and extracellular effector proteins were found to the upregulated.

Furthermore, we noted that in the antioxidant-free medium, the CRMPs (Paper I) are among the highest upregulated genes indicating their involvement in the stress response and raising the question if they might be coupled to antigenic variation in the parasite. This is further circumstantial evidence that the CRMPs have similarities to the VSPs in *Giardia* though additional experimental data is needed to determine if the CRMPs contribute to antigenic variation. These results suggest that different levels of oxygen might be an important gene induction signal for the invasion and colonization of *S. salmonicida* in host tissue.
Optimization of APEX protein tagging in diplomonads (Paper IV)

Function of many proteins is often correlated to their cellular localization and novel methods to investigate protein ultrastructural location have been introduced in recent years. Most methods have not been successfully implemented in protists, in parts due to their often highly derived cell biology.

Horseradish peroxidase (HRP) has been a popular tool previously to study the ultrastructural details of cells. However, a number of limitations to its use are sensitivity to the redox state of cellular compartments, necessity to coordinate calcium ions and inactivation by aldehyde-based fixatives (Laberge et al. 2003). Ascorbate peroxidase (APX) from pea have been reported to overcome these limitations (Martell et al. 2012).

To increase the method repertoire available in diplomonads we optimized an enzymatic proximity staining method based on two mutated versions of the ascorbate peroxidase (APXW41F and APEX) using S. salmonicida. This method has been previously used in G. intestinalis (Zumthor et al. 2016) and we furthermore exploited this method in the characterization of the annexins in S. salmonicida in Paper II. However, no systemic evaluation or optimization has been done in any diplomonad.

Optimization of APXW41F/APEX

We used the two APX variants as fusion partners to a well-characterized protein, Annexin 5 (Paper II) and optimized the conditions using two peroxidase substrates, Amplex UltraRed and DAB. APEX will catalyze the oxidation of the substrates in the presence of hydrogen peroxide (H2O2). Oxidized Amplex UltraRed generates a fluorescent precipitate (resurofin) detectable by fluorescent microscope while DAB oxidation will result in a dark brown precipitate visible by light microscopy and by TEM after osmification.

We did not detect any activity from the APEX fusion partners when the transfectant cell lines where grown in normal media due to the absent of hemin, an important peroxidase co-factor. With hemin added as a supplement to the media we were able detect activity from both fusion partners. However, to high hemin concentrations are detrimental to the cells and we performed titrations of the hemin concentrations with both substrates to establish a balance between cell viability and peroxidase activity. To further establish the optimal conditions to aid cell survival and integrity we did titrations of H2O2 with both substrates. The optimal conditions were established at 100 µM hemin and 200 µM H2O2.

We also noted that the activity of APEX was much higher than the activity of APXW41F under lower hemin and H2O2 concentrations. Additionally, the former showed more stable localization while APXW41F deposited label was more prone to be delocalized.
Activity of APEX in different cellular compartments of *S. salmonicida*

To validate the activity of APEX in different cellular compartments we fused APEX together with a V5 epitope tag to eight different proteins in *S. salmonicida* and used the Amplex UltraRed substrate to establish their localization using SIM. Additionally we tagged three proteins in *G. intestinalis* and used the optimized condition for *S. salmonicida* to demonstrate functionality for other diplomonads. The results showed that the resorufin and V5 co-localize in all transfectant strains confirming activity of APEX in different cellular compartments and its use in other diplomonads than *S. salmonicida*.

Ultrastructural localization

To display the full use of the APEX system we investigated the ultrastructural details of six APEX transgenic strains using the DAB substrate and TEM. Even though we could not detect the DAB precipitate in all transgenic strains strong DAB staining was observed for four of the *S. salmonicida* proteins and all three of the *G. intestinalis* transfectants. Furthermore, we were able to demonstrate the robustness of the DAB precipitate to withstand the harsh chemical preparations for the TEM.

Characterizations of two novel proteins

Furthermore, we determined the localization of two previously uncharacterized proteins, SS50377_12178 and SS50377_10316. The former has homology to NADH:Flavin oxidoreductases and was mentioned briefly in Paper III while the other has no recognizable homologs outside the diplomonads.

SIM localization data revealed that SS50377_12178 had a distinct localization in the perinuclear region around the nuclei and SS50377_10316 localized to two elongated structures in the anterior of the cell in close proximity to the nuclei. In TEM micrographs of SS50377_10316 we observed an intensive staining of the nucleus-abutting section of the basal body pockets while we were unable to find any DAB precipitate in the nuclear area for SS50377_12178.

It is our belief that the APEX proximity labeling system can be very useful in the unraveling of cell biological and cytoskeletal elements of the diplomonads. The system is robust and versatile and we anticipate its use can be extended in diplomonads to include spatially resolved enzymatic tagging and thereby enable compartment-resolved proteomics.
The infection cycle of *S. salmonicida* (Paper V)

While the life cycle of the human parasite *G. intestinalis* is well characterized much less is known about its piscine diplomonad relative, *S. salmonicida*. *S. salmonicida* has been reported to cause severe systemic infection in various farmed salmonids, e.g. the Atlantic salmon, the Chinook salmon and the Arctic charr while wild Arctic charr has been suggested as the reservoir for the parasite (Kent et al. 1992, Poppe TT & Mo 1993, Sterud et al. 2003, Jørgensen et al. 2011). However, key elements in the life cycle remain to be characterized as no transmission routes have been established nor has the initiation site of infection been defined.

In order to define the timeline of the infection cycle and look for possible transmission routes of *S. salmonicida* we performed experimental infections of Atlantic salmon from the Baltic Sea region with the parasite. We used the similar approaches as have been previously described for experimental infections with *S. salmonicida* in Atlantic salmon (Guo & Woo 2004a, Guo & Woo 2004b) in three separate trials. We furthermore developed several molecular tools for detection and diagnostics.

Tools for parasite detection in infected fish

To allow for easy detection of the parasite in blood we developed a highly sensitive nested PCR targeting the gene for the well-characterized cytoskeleton protein Annexin 3 (described in Paper II). Using this method, we could detect the parasite in the blood of all infected salmon from week 3 or 4 in trial 1.

Additionally, we produced two polyclonal antibodies, one raised against recombinant Annexin 3 protein produced in *E. coli* while the other was raised against a 14 aa peptide in the Annexin 5 protein (described in Paper II). As the anti-Annexin 3 antibody displayed much better specificity than the anti-Annexin 5 antibody we selected that one to use in the immunohistochemistry (IHC) stainings.

Infection cycle

In our study, we mainly infect the fish by oral intubation (OI) as we believe this will better reflect the natural infection route than intraperitoneal injection (IP) as has been done in previous experimental infections. To confirm that infection via IP injection is possible in our hands we included few fish in trial 1 that we injected IP.

In the OI fish, we detect the parasite using IHC in the intestinal region (pyloric caeca (PC)) and in the blood of infected fish one week after infection. For the IP injected fish the parasite was potentially stuck as it was colonizing on the outside of the intestine and internal organs such as the pancreas. In the
later stages, we note that the parasite has spread in OI infected fish as we can detect it in skin lesions and muscle tissue in week 5-7 after infection.

Transmission routes

One of the main elements missing from the life cycle of *S. salmonicida* is the transmission between hosts. Cyst have not been described for *S. salmonicida* though they have been reported for other *Spironucleus* species (Moore 1923, Davis 1926, Kunsty 1977, Wood & Smith 2005). During previous experimental infection with *S. salmonicida* no cysts could be detected (Kent *et al.* 1992, Guo & Woo 2004a). Likewise, we could not detect definite cyst forms in our experiments but pre-cyst like forms were detected in *ex vivo* cultures.

While genomic evidence strongly suggest a cyst stage of *S. salmonicida* (Paper I), the absence of the cysts from experimental infections and *in vivo* observations must raise speculations regarding alternative transmission routes for the parasite. Our data suggest the parasite will leave the intestine and potentially spread as trophozoites from skin lesions and epidermal mucus, possible through cannibalistic behavior (Amundsen 1994).

In addition, *S. vortens* trophozoites has been reported to survive within fecal material in water for long time periods while they are quickly killed outside of the feces (Williams CF *et al.* 2013). This could also be applicable for *S. salmonicida* and our data suggests that the fecal-oral route is one of the transmission routes of the parasite.

Luciferase observations

We constructed three transgenic strains carrying firefly luciferase (FLuc) expressed under three different promoters. One strain constitutively expressed FLuc under the glyceraldehyde 3-phosphate dehydrogenase (G3P) promoter while the others were fused to two CWP promoters, theoretically only being expressed during encystation. We then carried out two experimental trials here fish was infected with FLuc expressing *S. salmonicida* strains (Trial 2 and 3).

In trial 2 we only exploited the G3Pp-FLuc strain. At week 2 we could detect strong signals in the lower intestine and the anus while week signals were coming from skin lesions. At week 3 the signal had spread to the whole intestine with the strongest emission coming from the upper intestine. Additionally we could detect the parasite in the heart of the same fish. During the remaining weeks of the trial we could not detect signal from the intestine. However, in week 7 could we detect a strong signal from the head of a fish. The signal was defined to the head suggesting heavy colonization of *S. salmonicida*.

In trial 3 we included the other two strains, CWPp-FLuc and CWPEp-FLuc, in the experiments. The CWP strains were noted to have a leaky expression as signals were detected during normal growth of the parasite. We reasoned that during encystation we would see a dramatic signal increase during encystation.
of the parasite. However, no signal increase was detected for either strain during the trial suggesting that the parasite never entered encystation stages.

The data from trial 3 corresponded well to trial 2. During week 3 we detected signal in the whole intestine with the strongest signal in the lower intestine towards the anus. In addition, we detected signals from the gills and skin lesions on the fish. Further FLuc signals were not detected in the remaining weeks except for at week 7 when we detected strong emission coming from the head of a fish that had swollen eyes. Removal and further imaging of the eyes revealed a strong signal from the back of the eye while a signal was not detected in the eye sockets. The accumulation of the parasite in the head and under the eyes is in line with what has been previously described in experimental infections (Guo & Woo 2004a).

Our data strongly supports that the initial infection starts in the intestine. The parasite disrupts the integrity of the epithelial cell layer and enters the bloodstream from where it spreads to various tissues and organs of the fish. Furthermore, we suggest that the trophozoites are transmitted by multiple routes between hosts: from skin lesions, with epidermal mucus and with fecal material (Figure 5). However, we do not dismiss the cyst stage theory though we were not able to detect any evidence of cysts in our trials.

Transcriptome analyses during interaction of S. salmonicida and salmon cells (Paper VI)

S. salmonicida can cause massive outbreaks in farmed salmonids. Infected fish is unfit for consumption resulting in large economical impact for aquaculture. As no treatment is available, it is vital to gain more understanding of the infection process to effectively prevent and control the infections. Currently no information of important virulence factors of the parasite on a molecular level is available and furthermore, molecular host responses have not been studied.

Transcriptomic analyses during infection is a powerful tool to gain deeper understanding of the physiological pathways of the parasite and the host. As in vivo transcriptomic studies are considerably complicated and limited, we established an in vitro infection system with axenic S. salmonicida trophozoites and Atlantic salmon kidney (ASK) cells (ATCC CRL-2747). We collected total RNA samples for RNASeq at six different timepoints (0 – 1.5 – 3 – 6 – 12 – 24 h) and analyzed the transcriptome of both the parasite and the host cells during interactions (ASK:SS) and non-interacting (SS-CTRL and ASK-CTRL). Additionally, SEM and TEM imaging at four different timepoints (0.5 – 3 – 6 – 24 h) made visualization of the interactions possible.
Figure 5. Model of the infection cycle of *S. salmonicida*. During stressed condition the infection is initiated in the intestine and the parasite breaks up the epithelial layer to gain access to the blood stream. It spreads through the blood to various tissues and organs in the host. The parasite is transmitted between hosts as trophozoites through multiple transmission routes: from skin lesions, with epidermal mucus and with fecal material. A cyst form is possible during transmission though cysts were not detected in the experiment.

Gene expression changes in *S. salmonicida* and salmon cells during interactions

Our analyses revealed that the top 30 genes up-regulated genes in *S. salmonicida* during interactions with the ASK cells, are mainly hypothetical proteins, heat shock proteins, metabolic proteins, transporter and signaling proteins and cysteine rich membrane proteins. Genes encoding hypothetical proteins are furthermore among the the top up-regulated genes at all timepoints.

We do not see a large different in expression of the CRPs in *S. salmonicida*. Our analyses revealed that 49 CRPs were differentially expressed during interactions with ASK cells, many of which were up-regulated after 3 h. Additionally we did not detect up-regulation of many genes that are related to oxygen stress mechanisms. However, we did detect a up-regulation of a number of Myb-like transcription factors as well as other transcription factors at one or more timepoints.

As proteases are important virulence factors in *Giardia* (Amat et al. 2017, Liu et al. 2018) we expected to see an up-regulation in *S. salmonicida*. 

46
However, on the contrary we saw a decrease in expression of various cysteine proteases during the interactions.

The main up-regulated genes in the ASK cells during interactions are mainly immediate early responses genes, as expected, but there are also differentially expressed growth factors, transcription factors and transporters. Furthermore, we did not detect any up-regulation of apoptosis inducing genes that might suggest necrotic death of the host cells.

Scanning electron microscopy

During host-parasite interactions in vitro we observed that already after 0.5 h the parasites cluster together, similar behavior that has been described for S. vortens (Poynton et al. 2018). The parasites seem to use their flagella to associate to each other as we observed them intertwined in the clusters. The parasites bind to the host cells with its anterior and we noticed them already at 0.5 h penetrating the cells monolayer, specifically at the cellular junctions, which seems to be the main target of the parasite. We observed a heavy disruption of the ASK cell monolayer at 3 h and the parasites were clustering around host cells that started to show membrane blebbing, a common sign of cell death. At 6 h the parasite had potentially replicated while the ASK cells were rapidly decreasing. After 24 h we observed a total disruption of the host cell monolayer with parasites surrounding the remaining blebbed cells.

Transmission electron microscopy

We wanted to try to examine if the parasite could be detected inside the ASK cells, thus displaying an intracellular life stage. However, no intracellular parasites could be detected during our experiments. However, we observed a tight binding of the parasite with the anterior part of the cell in close contact to the host cells. That could suggest some type of adhesive structure in this region, anterior to the nuclei. We also noted condensed DNA in the host cell nuclei already at 0.5 h, further supporting cell death of the host cells.
Conclusions and future perspectives

The diplomonads are an understudied group of organisms and comprehensive characterization of additional members would provide valuable information on the evolution of eukaryotes and on eukaryotes in general. The work covered in this thesis provides extensive characterization of *S. salmonicida* and investigates various biological aspects of the parasite.

Genomics and transcriptomics

In Paper I, III and VI we focused on studying *S. salmonicida* on a molecular level. The sequencing of the genome in Paper I gave the first insights into the life cycle of the parasite and opened the path to study various different aspects of its biology. The key elements for a model organism consists of a high quality genome assembly and a functional transfection system, now both available in *S. salmonicida*. Further improvements to the genomic assembly would be highly beneficial to enable deeper understanding of the organism.

The comparative genomic analyses support that *S. salmonicida* is more capable than *G. intestinalis* in adapting to different environments with varying levels of oxygen. Large functional differences are found between the parasites that reflects the adaption to their respective host and habitats. Genomic comparison studies of *S. salmonicida* to other *Spironucleus* members would be intriguing as current evidence indicate high variations and large differences between the genomes of *Spironucleus* species.

As oxygenated environments are important parts of the *S. salmonicida* life cycle we investigated its oxygen defense pathways in Paper III. This gave us additional understanding on how the parasite survives in the different habitats and how it regulates genes in response to oxygen. One important realization in Paper III was that oxygen could act as a potential regulator for virulence factors. As the work in Paper III is strictly based on transcriptomic data further assessments of the data is necessary to confirm these findings. Localization assays of key proteins in the oxygen stress response pathways would be highly valuable for validation of the pathways. The multitude of hypothetical proteins amongst the oxygen stress-responsive genes are of particular interest as these could represent novel oxygen-response factors and be part of new *S. salmonicida* biology.
To gain deeper insight into the molecular aspects of the life cycle of *S. salmonicida* we investigated the gene expression profile of trophozoites during interaction with ASK cells in Paper VI. Additionally, we described the major transcriptional responses of the ASK cells and visualized the host-parasite interactions using SEM and TEM imaging.

In the parasites we observed large gene expression changes during the interactions compared to the controls. The major group of proteins up-regulated during the interactions are hypothetical proteins. These proteins are certainly interesting targets for further functional characterization as many of these have the potential of being important virulence elements for the parasite during infections. The majority of differentially express host genes are immediate early response genes as well as growth factors and transcription factors. Interestingly, we also did not detect up-regulation of genes inducing apoptosis which would suggest necrotic death of the host cells. That is complementing to what we detected in Paper V where infected tissue became necrotic.

During visualization of the parasite we observed clustering of the parasite on the ASK cell monolayer that could suggest some form of coordination system with the parasite. We did not detect an intracellular form and we suggest that the parasite targets the tight junctions to break up the cell layer. In addition, we noticed a potential adhesive structure in the anterior of the cell, potentially the same structure as described for Annexin 5 in Paper II.

As the data analyses are still in their infancy, further analyses will potentially make it possible to identify various important elements in the infection cycle of the parasite, e.g. virulence factors, immune system evasion and stress responses, and the host response.

**Cell biology**

The publication of the genome together with the transfection system gave us the opportunity to study the cell biology of *S. salmonicida* in Paper II and IV. The APEX proximity labeling method we presented in Paper IV has the potential to be a valuable tool to study the intricate cell biology and cytoskeletal elements of the diplomonads. Furthermore, this system has the potential to be adapted to other protists and enable studies of novel cell biology in diverse protists. The system is robust and versatile, and its use may additionally be extended in diplomonads to include spatially resolved enzymatic tagging thereby making compartment-resolved proteomics possible.

We took the advantage of the APEX system in our characterization of the annexins in Paper II. Characterization of important cellular elements is essential to gain full understanding of the functional capabilities of cells. In addition to obtaining localization data on all the *Spiroplasma* annexins we performed extensive phylogenetical analyses of this group of proteins. Further studies of
the annexins should include additional functional and phylogenetical investigations in other protists as this would provide deeper understanding of different functions and the evolutionary history of the annexins.

Infection biology

We performed experimental infections of Atlantic salmon in Paper V to investigate certain key elements in the infection cycle of *S. salmonicida*. The parasite has inflicted large economic losses in aquaculture in Norway and Canada due to fish becoming anorexic and losing weight. No efficient treatment options are available and current clinical diagnostic methods are slow and inaccurate. It is critical to understand the infection in order to take protective measures against the parasite and we describe a potential infection cycle of *S. salmonicida*.

We establish the likely initiation site of infection and suggest multiple potential transmission routes for the parasite as this information is valuable in identifying potential drug targets for treatment of infected fish.

Furthermore, we developed a nested PCR method for the detection of the parasite in blood and generated antibodies to allow for detection in various tissue. It is our belief that the nested PCR could potentially provide a quick and simple assay to detect the parasite sufficiently early in the infection cycle to contain the spread of the parasite in fish farms. The anti-Annexin 3 antibody could additionally be a useful tool in identification of the parasite from different tissues.

In conclusion, the diplomonad parasite *S. salmonicida* has been extensively studied in this thesis. As the work characterizes various different components of the parasite it has provided deeper insights into the pathogenesis and the intriguing biology of this highly adaptable salmon killer.
Livets träd är uppdelat i tre domäner, eukaryoter, bakterier och arkéer, även om linjen mellan eukaryoter och arkéer har blivit otydlig. Även om både bakterier och arkéer innehåller ett stort antal organismer uppvisar eukaryoter mycket större mångfald, allt från enkla encelliga former till mycket komplexa multicellulära organismer, såsom djur och växter. Som förväntat har intresset varit störst i organismer som människan kan upptäcka med egna ögon, medan enklare eukaryoter inte har studerats i stor skala trots sin stora mångfald.

En exempel på en grupp encelliga mikroorganismer är Diplomonader. Som namnet avslöjar så har de flesta Diplomonader två kärnor och totalt fyra kopior av kromosomerna, två i varje kärna. Den mest studerade diplomonaden är *Giardia intestinalis* som är en parasit i däggdjur och kan orsaka bl.a. allvarlig diarré. *G. intestinalis* är en av de vanligaste parasiterna hos människor och mer än 200 miljoner människor anses vara infekterade.


Genom- och genuttrycksstudier i *S. salmonicida*

Vi sekvenserade *S. salmonicidas* genom och jämförde det med *G. intestinalis* genomet. Skillnaden avslöjade en stor skillnad i genomen av dessa två organismer som kan förklaras av deras olika levnadsmiljöer. *G. intestinalis* kan endast hittas i tarmens syrefattiga miljö medan *S. salmonicida* kan infektera de flesta vävnader och organ, även om parasiten endast har anaerob metabolism. *S. salmonicida* är därför mycket mer anpassningsbar till olika syrekonzentrationer, vilket återspeglas i genomet av dessa två parasiter (*Artikel I*).

Nästa steg var att titta närmare på hur parasiten hanterar olika syrekonzentrationer genom att undersöka genuttrycket under olika skick. Vi visade hur *S.
**S. salmonicida cellbiologi**


**S. salmoncida infektionsprocess**

Vi karakteriserade tre processer som parasiten använder för att sprida sig mellan fiskar utan att ändra form. Parasiter som *G. intestinalis* sprids genom att ta på sig en annan form, så kallad cystform, men den livsformen kunde vi inte hitta under vår undersökning.

Dessutom gjorde vi en genetisk variant av parasiten som innehåller luciferase från eldfluga som gör det att parasiten glöder. Det gjorde det möjligt för oss att se var parasiten befinner i fisken med hjälp av speciellt kamerasystem (Artikel V).

Sammanfattningsvis då hoppas vi att innehållet i denna avhandling har bidragit till att öka kunskapen om viktiga aspekter i *S. salmonicida* och kommer att kunna användas vid fortsatt forskning kring parasiten tillsammans med andra encelliga eukaryoter.
Ágrip á íslensku

Tré lífsins skiptist í 3 lén, heilkjörnunga (eukaryotes), bakteriur og fornbakteriur (archaea) þótt lininn milli heilkjörnunga og fornbaktería virðist óskýr samkvæmt nýjust rannsóknum. Jafnvel þótt bæói bakteriur og fornbakteriur innihaldi mikinn fjölda lífvera þá er fjölbreytileiki heilkjörnunga miklu meiri, allt frá einföldum einkjörnungum til mjög flókinna fjölkjörnunga, eins og dýra og plantna. Eins og gefur að skilja hefur áhuginn verið mestur á lífverur sem mannanveran getur greint með eigin augum meðan heilkjarna örverur hafa ekki verið mikið skoðaðar, þratt fyrir mikinn fjölbreytileika.

Ein ætt heilkjarna örvera eru tvíkjörnungar (Diplomonads). Eins og nafnið gefur til kynna þá samanstandur sú átt af örverum sem flestar hafa tvo kjarna og tvöfalt erfðamengi, þ.e. samtals fjögur sett af líkiningum, tvö í hverum kjarna. Mest rannsakaða lífveran er Giardia intestinalis sem er sníkjudýr í spendýrum og getur valdið m.a. heiftarlegum niðurgangi. G. intestinalis er eitt algengasta sníkjudýr í mönnum og talið er að yfir 200 milljónir manna séu sýktir.


Rannsóknir á genamengi og genatjáningu í S. salmonicida

Við raðgreindum genamengið S. Salmonicida og bárum það saman við genamengi G. intestinalis. Raðgreiningin leiddi í ljós mikinn mun á genamengi þessa tveggja lífvera sem getur verið útskýrður af mismunandi lífsumhverfi þeirra. G. intestinalis er eingöngu að finna í súrefnissauðu umhverfi í þórmum meðan S. salmonicida getur sykt flesta vei og lífðar fiska þótt sníkjudýrið hafi eingöngu loftfirra öndun. Aðlögunarhæfni S.
**salmonicida** að mismunandi súrefnisstyrkjem er mun meiri sem endurspeglast í genemengjum þessara sníkjudýra (Grein I).

Næsta skref var að skoða betur hvernig sníkjudýrið meðhöndlar mismunandi súrefnisstyrk með því að skoða genajánungu þess við mismunandi aðstæður. Rannsóknir okkar sýnðu hvernig *S. salmonicida* getur varist súrefni án þess að það nái að valda skáda á frumunni, oft á tíðum með kerfum sem sníkjudýrið hefur fengið frá bakteríum í gegnum lárettan genaflutning. Einnig bentu niðurstöðurnar til að súrefni gæti verið mikilvægur þáttur í stýringu á tjáningu mismunandi gena sem eru mikilvæg í sýkingarferli sníkjudýrsins (Grein III).

Til að rannsaka frekar genajánungu *S. salmonicida* við sýkingu settum við upp *in vitro* kerfi sem innihélt nýrnafrumum frá Atlantshafslaxi ásamt sníkjudýrinu. Á ákveðnum tímapunktum voru ribósakjarnasýrur (RKS/RNA) einangraðar og raðgreindar. Með þessu gátum við skoðað hvaða prótíni eru mikilvæg fyrir smíterli dýrsins ásamt því að fá visbendingar um hvernig hýsilfrumur svara sýkingu af völdum þess. Auk þessa, var samspil þessara fruma metið sjónrænt í rafeindasmásjá (Grein VI).

**Frumulíffræði S. salmonicida**

Til að skoða frumulíffræði *S. salmonicida* var svokallað APEX kerfi aðlagað *S. salmonicida*. Kerfið er byggt upp á því að prótínið sem verið er að skoða er merkt með stökkbreytttri útgáfu af “ascorbate peroxidasa”. Þegar “peroxidasinn” kemst í snertingu við hvarfsemi sitt og vetnisperoxið, þá verður efnahvarf sem skilur eftir sig annað hvort flúrljómandi eða dókk brúna útforlingu eftir því hvaða hvarfsemi er notað. Flúrljómandi útforlingin er hægt að greina með flúrljómandi smásjá meðan dókk brúna útforlingin getur nýst sem merking við rafeindasmásjárskóðun. Með þessari aðferð gátum við synt fram á mögulega staðsetningu fjölda prótína í frumu sníkjudýrsins. Að lokum gátum við synt fram á virkni kerfisins í *G. intestinalis* sem gefur til kynna að mögulega megi yfirfæra það að fleiri heilkjarna einfrumunga (Grein IV).

Ennfremur notuðum við APEX kerfið til að hjálpa til við skilgreiningu á Annexin prótíni fjölskyldunni í *S. Salmonicida*, en þessi gerð prótína er mikilvæg fyrir uppbyggingu frumunnar og finnast í öllum hópum lífvera. Við sýndum fram á mögulega staðsetningu allra annexin prótína sem finnast í genemengi sníkjudýrsins og tengdum þau við ákveðna eiginlega í uppbyggingu frumunnar. Einnig skoðuðum við þróunarsögu þessara prótína í heilkjörningum með áherslu á tvíkjörninga (Grein II).
Sýkingarferli *S. salmonicida*


Við skilgreindum þrjá ferla sem dýrið notar til að dreifa sér á milli hýsla án þess að breyta um líftigform. Sníkjúguður eins og *G. intestinalis* dreifa sér með því að taka á sig annað form, svokallað hjúp form, en það við gátum ekki synt fram á hjúp form hjá *S. salmonicida* í þessari rannsókn.

Einnig erfðabreyttum við sníkjudýrinu með því að þæta inn í erfðamengi þess luciferasa úr eldflugu sem veldur því að sníkjudýrið glóir. Þetta gerði okkur kleift að skoða hvar sníkjudútíð var staðsett í fískinum með notkun sérstakar myndavélar.

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References


Sterud E. 1998a. Electron microscopical identification of the flagellate Spironucleus torosa (Hexamitidae) from burbot Lota lota (Gadidae) with comments upon its probable introduction to this freshwater host. The Journal of Parasitology 84: 947–953.


Williams CF, Yarlett N, Aon MA, Lloyd D. 2014. Antioxidant defences of *Spironucleus vortens*: Glutathione is the major non-protein thiol. Molecular and Biochemical Parasitology 196: 45–52.


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