Review Article

Spironucleus species: Economically-Important Fish Pathogens and Enigmatic Single-Celled Eukaryotes

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Abstract

Diplomonads are aerotolerant anaerobic, binucleate flagellates, which are commonly found in the intestinal tract of wild and farmed fish. Of the diplomonad genera, *Spironucleus*, composed of opportunistic pathogens, poses the greatest threat to aquaculture. Immunocompromised hosts or fish without acquired immunity are thought to be more susceptible to parasitism by these otherwise commensal agents. Accumulation of flagellates along the intestinal tract often leads to systemic Spironucleosis causing high mortality of both ornamental and food fish in aquaculture. The life cycle of these piscine diplomonads is direct, consisting of a motile, parasitic trophozoite and a resilient encysted stage, which facilitates water-borne transmission. Confusion in the nomenclature, as well as numerous reassignments of taxa, hampers our understanding of host range and geographical distribution of fish diplomonads. Accurate identification requires transmission electron microscopy to characterise intricate ultrastructural features. Additionally, sequencing of the small subunit ribosomal RNA gene allows identification of cryptic *Spironucleus* spp. *In vitro* culture provides a convenient source of flagellates for biochemical and physiological research, allowing the identification of novel parasite-specific molecular pathways such as H_2 production within *Spironucleus* sp. This provides insight into the pathogenicity of these organisms and offers potential new targets for chemotherapy. Restrictions on the administration of the current drug of choice, metronidazole, in aquacultural settings, as well as reported cases of drug resistance, means that control of Spironucleosis is especially difficult. *Allium sativum* (garlic)-derived compounds have proven highly effective at inhibiting parasite growth *in vitro*, showing great potential as a novel alternative therapy in the treatment of Spironucleosis. Further characterisation of the biochemistry, pathogenicity and taxonomy of fish diplomonads is required in order to fully appreciate the true impact and economic consequences of *Spironucleus* spp. in aquaculture.

Keywords: Diplomonad; *Hexamita*; *Spironucleus*; Aquaculture; Taxonomy; Hydrogenosomes; Garlic

Introduction

The diplomonads (suborder Diplomonadida, family Hexamitidae) are a group of aerotolerant anaerobic flagellates, which possess a double set of cellular organelles. Amongst the diplomonad genera are *Hexamita*, *Octomitus*, *Giardia* and *Spironucleus* [1]. Species of *Hexamita* are mostly free-living organisms that reside in anaerobic water sediments [2], whereas the other taxa are almost exclusively parasites, which commonly occur in the intestinal tract of mammals, birds, reptiles, amphibians and fish [3-8].

Spironucleus species have attracted considerable attention from the veterinary and agricultural communities, due to the severe pathologies that they cause in companion and farmed animals [6,9-19]. Other *Spironucleus* spp. are of particular importance to aquaculture, as they can cause devastating outbreaks of systemic infections in both ornamental and food fish [6,20-27]. These ubiquitous flagellates occur in cold, temperate and tropical waters, and infect a wide range of freshwater [21,23,24] and marine fish [27-31], as well as shellfish and crustaceans [23,24,32-34]. Fish farmed in aquaculture, an industry of growing importance as wild fish stocks become increasingly depleted, are particularly susceptible to disease outbreaks caused by diplomonads. For instance, *S. salmonicida* (formerly *S. barkhanus*, see [27]), has caused massive outbreaks of systemic infections in farmed Norwegian *Salmo salar* (Atlantic salmon) [6,35-37] and British Columbian *Oncorhynchus tshawytscha* (Chinook salmon) [38]. Gross pathologies include internal haemorrhaging, splenomegaly and granulomatous lesions in the liver and spleen. The life cycle of these diplomonads is direct and involves a motile, parasitic trophozoite, typically followed by an encysted stage [39]. Transmission success is extremely high, especially in the crowded conditions of aquaculture, and results in significant host mortality [25,26].

In the ornamental fish industry, *Spironucleus* spp. cause severe infections in several economically important species including cichlids [5,24,40]. *S. vortens* has been proposed as the causative agent of hole-in-the-head (HITH) disease, a common condition generally associated with poor husbandry [24]. The disease is characterised by severe lesions on the head, lateral line and internally. HITH disease has been linked to systemic infection of *S. vortens*, with parasites recovered from internal organs and skin lesions of diseased fish [24].

It has been suggested that *Spironucleus* spp. are putative intestinal commensals, which become invasive in fish hosts with a compromised

immune system or those which lack acquired immunity, under aquacultural conditions [39,41,42]. This is reflected in the observation that *Spironucleus* infections in wild fish are generally non-pathogenic (e.g. *S. barkhanus* in wild grayling [43] and *S. torosa* in wild gadids [28]). Stressful conditions sometimes observed in aquaculture, including poor water quality, temperature fluctuations and overcrowding, have thus been implicated as triggers of *Spironucleus* pathogenesis [39,41,44,45].
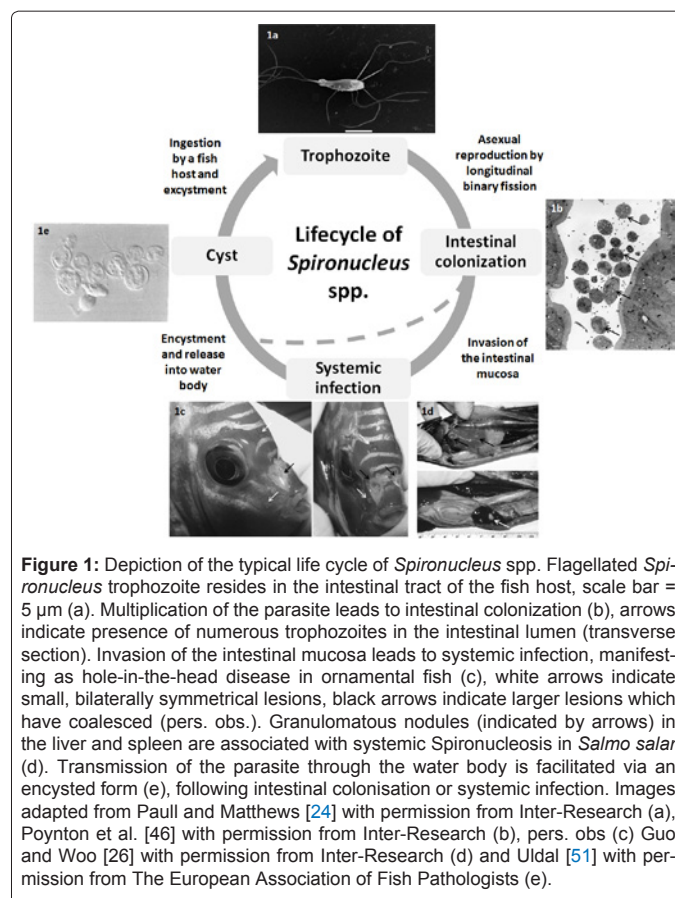
Despite the importance of *Spironucleus* spp. their host range, specificity and mechanisms of transmission are poorly understood, as are the geographical range and pathogenesis [1,46]. This lack of knowledge is particularly detrimental in disease management, as accurate diagnosis and identification of the source of infection are rendered difficult. Advances in that field are further hindered by the confusion occurring in the nomenclature of hexamitid parasites. Descriptions of piscine diplomonads are incomplete and comparisons difficult as few specimens were deposited in reference collections. Numerous isolates have been wrongly identified, sometimes even to the genus level [1,46], due to the use of limited techniques such as light microscopy [47-49]. However, even expert morphological investigations using scanning and transmission electron microscopy may fail to identify species accurately, as demonstrated by the reassignment of a highly pathogenic isolate of *S. barkhanus* to a new taxon, *S. salmonicida*, on the basis of small subunit ribosomal DNA (SSU rDNA) sequence analysis [27]. Such molecular-based techniques have provided considerable clarification of the taxonomy and phylogeny of *Spironucleus* parasites [50], however, diagnostic kits for the routine identification of specific pathogens, which would prove invaluable in disease management, remain to be developed.

Research of many fish microparasites is hampered by an inability to fulfil Koch's postulates. However, recent advances in our ability to identify cryptic species of *Spironucleus*, to culture the pathogen and accurately monitor its biochemistry paves the way for a better understanding of diplomonad biology. This review seeks to summarize current knowledge on life cycle, taxonomy and species identification, host-pathogen interactions, physiology and biochemistry and disease management of *Spironucleus* spp., whilst highlighting the need to focus on further characterisation of these aspects in order to efficiently control infection outbreaks in aquaculture.

Life cycle

Identification of piscine diplomonads relies on the characterisation of two distinct stages of the life cycle; the easily recognized and actively swimming trophozoite, and the non-motile cyst form (Figure 1). The trophozoite is the feeding stage of the parasite (see Section 4 for nutritional requirements), which also undergoes asexual reproduction by longitudinal binary fission, as is typical for flagellates. This stage is most familiar to fish health investigators, since it is commonly encountered in the intestinal lumen, and less commonly in the bile, blood, and organs [5,6,21,24,25,37].

Trophozoites typically do not survive for long periods outside the fish host [38], but have been detected in fresh faecal samples from *Oncorhynchus mykiss* (rainbow trout) infected with *S. salmonis* [23]. The same study failed to identify the presence of cysts in the faeces of heavily infected fish, highlighting the rarity of these forms. Despite this, *S. salmonis* cysts have been observed *in vivo* [47,48] as well as *in vitro* [51]. Cysts of other piscine *Spironucleus* spp. are poorly characterized. The cyst form facilitates direct transmission through the aquatic environment via the faecal-oral route [39], however transmission via

Figure 1: Depiction of the typical life cycle of *Spironucleus* spp. Flagellated *Spironucleus* trophozoite resides in the intestinal tract of the fish host, scale bar = 5 μm (a). Multiplication of the parasite leads to intestinal colonization (b), arrows indicate presence of numerous trophozoites in the intestinal lumen (transverse section). Invasion of the intestinal mucosa leads to systemic infection, manifesting as hole-in-the-head disease in ornamental fish (c), white arrows indicate small, bilaterally symmetrical lesions, black arrows indicate larger lesions which have coalesced (pers. obs.). Granulomatous nodules (indicated by arrows) in the liver and spleen are associated with systemic Spironucleosis in *Salmo salar* (d). Transmission of the parasite through the water body is facilitated via an encysted form (e), following intestinal colonisation or systemic infection. Images adapted from Paull and Matthews [24] with permission from Inter-Research (a), Poynton et al. [46] with permission from Inter-Research (b), pers. obs (c) Guo and Woo [26] with permission from Inter-Research (d) and Uldal [51] with permission from The European Association of Fish Pathologists (e).

skin lesions [36] as well as through the rectal route by both cyst and trophozoite [38] have also been suggested. The resilience of the cyst wall confers protection against harsh external conditions including osmotic changes, high O_2 tensions and temperature fluctuations [52]. After ingestion by a new host, the cyst passes along the digestive tract, and excysts [39]. Thus trophozoites are released, and the life cycle is completed. Details of the transformation from trophozoite to cyst (encystment), and from cyst to trophozoite (excystment) are poorly understood for piscine *Spironucleus*. As a result most details of the life cycle are inferred from avian [53] and mammalian [54, 55] *Spironucleus*, as well as the closely related genus *Giardia* in mammals [11-13,15]. Common features of encystment in diplomonads are the quiescence of the trophozoite, production of cyst wall material in the cytoplasm and its trafficking to the surface, formation of the cyst wall and doubling of the flagellate within the cyst [47,56,57]. *In vitro* encystment of *S. salmonis* has been induced by starvation of trophozoites in spent culture medium [51]. Furthermore, *in vitro* excystment of *S. muris* from mouse faeces was achieved by a low pH induction medium, followed by transfer to a neutral pH buffer or trypticase-yeast extract-iron (TY-I-S33) culture medium, mimicking passage of the cyst through the gastrointestinal tract [55]. Fundamental questions remain about the life cycle of *Spironucleus* in fish, including triggers for encystment, duration of infectivity, minimum infective dose and triggers for excystment.

Taxonomy and identification

Although taxonomic confusion in the literature regarding the genera of diplomonads that infect fish remains, ultrastructural

characterization has brought much needed clarity. Studies using only light microscopy have assigned the piscine diplomonads to *Hexamita*, *Octomitus* and *Spironucleus*. However, transmission electron microscopy has confirmed that all piscine diplomonads examined to date, belong unequivocally to the genus *Spironucleus*. Using this approach, 5 species of piscine diplomonads are currently recognized: *S. barkhanus, S. salmonicida, S. salmonis, S. torosa* and *S. vortens*. All of these species have only been comprehensively characterized from fishes in northwest Europe. However, given the global diversity of fish species, and the importance of aquaculture in Asia, it is likely that additional distinct species will be described from other parts of the world in future.

Reference specimens: Table 1 summarises the type host, type locality, type material and Accession Numbers of the best described *Spironucleus* spp. from fish, although even this is incomplete. This is exemplified in a recent study, which demonstrates that *S. salmonicida* can be found in the intestine of wild *Salmo trutta* (Brown trout) and *Salvelinus alpinus* (Arctic charr) [58]. However, farmed *Salmo salar* (Atlantic salmon) were designated the type host of *S. salmonicida* due to the lack of a wild host [27]. Based on current knowledge, Brown trout or Arctic charr should probably have been chosen as type host of *S. salmonicida*, however a formal redescription must then be undertaken. No reference material or sequence data are available from the original description of *Spironucleus elegans* (type host: amphibians) [59], although Brugerolle et al. [60] provided a valuable ultrastructural description. Based on morphology, Poynton et al. [5] considered a hexamitid isolated from angelfish to be different from *S. elegans* and thus established *S. vortens*. The conspecificity of *S. vortens* and *S. elegans* was later questioned due to the remarkable ultrastructural similarities between the two species [61], but has not been resolved. *S. vortens* (type host: *Pterophyllum scalare* (angelfish)) has been described from several species of aquarium fish and also from wild *Leuciscus idus* (ide) in Norway [61]. Subsequently, sequencing of the small subunit ribosomal RNA (SSU rRNA) gene revealed that the isolates from angelfish and ide were genetically more distant than *S. salmonicida* and *S. barkhanus* and therefore should be regarded as separate species [50]. However, again no formal description has been made.

Morphological identification: The genus *Spironucleus* have been subjected to considerable taxonomic confusion due to limited species descriptions based on light microscopy only. An example of this is illustrated by four Chinese studies which reported a total of 25 new species of *Spironucleus* and *Hexamita* from various fish species [62-65]. Most of these descriptions were based on light microscopy and/or poorly resolved ultrastructural data, thus these species should be regarded as insufficiently characterized. Detailed ultrastructural investigation, described below, using scanning and transmission electron microscopy is required in order to accurately distinguish between *Spironucleus* spp. Table 2 summarizes genus and species-specific morphological characteristics of piscine diplomonads.

Morphological characterization of *Spironucleus* is based upon the trophozoite form which is easily recognized, even by non-specialists. The cyst form has rarely been reported from fish, so detailed information on the ultrastructure of this form is lacking [1]. Trophozoites are characterised according to their distinct spherical to pyriform body, approximately 10–20 μm long and 5–10 μm wide, which is actively propelled through the water by the six anterior locomotory flagella. From the posterior of the body trail two additional flagella. Observation

Species	Type host	Additional hosts	Type locality	Location in host	Type material	Reference culture	Reference sequence (GenBank Acc.)
S. barkhanus [43]	*Thymallus thymallus*	*Salvelinus alpinus* *Salmo trutta*	River Glomma, Norway	Lumen of gut and gall bladder	B	ATCC 50467	DQ186581
S. elegans [59]	*Triturus alpestris* *Rana rana*	*Pterophyllum scalare*	NA	Intestine	NA	NA	NA
S. salmonicida [27]	*Salvelinus alpines*	*Oncorhychus namaykush* *Salmo salar*	Fiskfjorden, Norway (68°N, 15°E)	Systemic infection	C	ATCC 50377	DQ181595
S. salmonis [47]	*Salvelinus fontinalis*	*Oncorhynchus mykiss*	Bath, New York State, USA	Digestive tract, upper intestinal region	NA	NA	DQ394703
S. torosus [28]	*Gadus morhua*	*Melanogrammus aeglefinus* *Lota lota*	Chebucto Head approach to Halifax Harbour, Nova Scotia, Canada	Digestive tract, rectum in cod	D	NA	EF050055
S. vortens [5]	*Pterophyllum scalare*	*Leuciscus idus* *Symphysodon discus*	Archer, Florida, USA (29° 32'N, 82° 31W)	Lumen of intestine	A	ATCC 50386	U93085

[A] Holotype slide of protargol-impregnated specimens and TEM block in the International Protozoan Slide collection at the National Museum of Natural History, Smithsonian Institution, Washington D.C. Accession number USNM #47820.
[B] TEM blocks and SEM stubs kept at the Norwegian School of Veterinary Science, Oslo, Norway,
[C] SEM stub: 180-1 (hapantotype) deposited in the International Protozoan Slide collection at the National Museum of Natural History, Smithsonian Institution, Washington. D.C. Accession number USNM 1093603. TEM blocks: 2718-3, 2718-5, 2618-25 and 2618-30 representing paratypes are deposited at Norwegian School of Veterinary Science, Oslo.
[D] Holotype and numerous paratypes deposited in the Invertebrate Collection (Parasites), National Museum of Natural Sciences, Ottawa, Canada. Accession number NMCP 1988-0899.

Table 1 A summary of the type host, additional hosts, type locality, location in the host and type material, as well as reference culture and reference sequence acquisition numbers of the best described *Spironucleus* spp. from fish.

Genus-level Identification			
Genera	*Spironucleus*	*Hexamita*	*Octomitus*
Flagellar pockets (cytostomal canals)[a]	+	+	-
Central axis formed by recurrent axonemes, microtubular bands, endoplasmic reticulum	-	-	+
Two terminal spikes	-	-	+
Shape of nuclei	S-shaped	Spherical	Reniform
Location of kinetosomes relative to nuclei	Sub-apical	External structure	Between
Position of recurrent flagella relative to nuclei	Medial	Lateral	Medial
Supra-nuclear microtubular band	+	+	Reduced
Infra-nuclear microtubular band	+	+	Reduced
Species-level Identification			
Species	*S. barkhanus / S. salmonicida*	*S. salmonis*	*S. torosa*	*S. vortens*
Flagellar pocket ornamentation	Crescent-shaped (barkhan)	No ornamentation	Ring-shaped swellings (torus)	Counter-crossing ridges
Microtubular pattern around flagellar pocket	3 radiate bands	-	3 concentric bands	3 staggard bands
Distinctive organelles	-	Electron-dense bodies / membrane-bound sac of free ribosomes	-	Hydrogenosomes[b]

[a]When present, the recurrent flagella are ensheathed
[b]May also be present in other *Spironucleus* spp. but currently only documented in *S. vortens*

Table 2: Important ultrastructural aspects for genus and species level identification of diplomonad genera and *Spironucleus* spp. respectively. Adapted from Poynton and Sterud [1] with permission from John Wiley & Sons Ltd.

of actively swimming trophozoites shows that the six locomotory flagella are approximately 1.5 times the body length and emerge from the anterior body in two groups of three. The two trailing flagella are approximately twice the body length, and emerge from the posterior of the body close to each other. The six anterior flagella originate anterio-medially, near the meeting of the nuclei, and travel just a short distance before emerging anterio-laterally. In contrast, the two posterior flagella, which also originate near the meeting of the nuclei, travel within the body from the anterior to the posterior (through an invagination of the cell membrane called the flagellar pocket), and then emerge as trailing flagella. The flagella are revealed using protargol silver protein stain on whole organisms [1].

Light microscopy: All genera of diplomonads have paired anterior nuclei, the exact shape and location of which are diagnostic for genus. The nuclei of *Spironucleus* are elongate and taper anteriorly, lying very close to each other at the extreme anterior of the cell. Although the paired nuclei can be recognized by standard Hematoxylin and Eosin preparations, they are most effectively stained with the Feulgen reaction, in which the nuclei stain magenta pink and the cytoplasm stains turquoise [28]. Using DAPI (4'-6-diamidino-2-phenylindole), the nuclei appear blue/violet under UV illumination [1].

Both *S. torosa* and *S. vortens* have an accessory cytoskeleton, composed of microtubular bands supporting surface ornamentation, visualized using protargol silver protein stain. In *S. torosa* these microtubular bands appear as two loops at the posterior of the cell, each representing the microtubules in a torus (raised round structure) surrounding the opening of the flagellar pocket [28]. Conversely, in *S. vortens*, the accessory cytoskeleton is evident as a dark staining area at the extreme posterior of the cell, representing the complex of counter-crossing microtubular bands supporting the elaborate folds between the emerging posterior flagella [5].

Electron microscopy: The surface of a *Spironucleus* trophozoite is mostly smooth, although discharging vacuoles undergoing exocytosis may be seen. In 4 out of the 5 well described *Spironucleus* spp. from fish, surface ornamentation is distinctive. Its function is unknown, but is useful for species identification (Figure 2). *S. barkhanus* and *S. salmonicida* have a crescent-shaped ridge (barkhan) around the opening of each flagellar pocket [43], while *S. torosa* has a ring-shaped swelling (torus) around the flagellar pocket [28] and *S. vortens* is characterised by lateral ridges bearing tufts of microfibrils, which then form counter-crossing ridges between the opening of the flagellar pockets [5]. The presence of posterior papillae is also characteristic of *S. vortens* [5]. *S. salmonis* lacks ornamentation [46].

Internal ultrastructure reveals that the *Spironucleus* trophozoite is rotationally symmetrical about its long axis, with each cell having a double set of organelles [60]. The paired nuclei taper anteriorly and are wrapped around each other at their apices, forming an S-shape when viewed in transverse section of the anterior end of the cell. The bases of the flagella (kinetosomes) lie in two groups of four, each comprising three anterior kinetosomes and one recurrent (posterior) kinetosome. Other structural features of note are the three paired bands of microtubules: (i) supra-nuclear, passing anteriorly across from one nucleus to the other; (ii) infra-nuclear, originating near the kinetosome and passing posteriorly and then crossing to the other nucleus and then passing to the posterior of the cell close to the flagellar pocket; and (iii) direct, originating near the kinetosomes, and passing to the posterior of the cell close to the flagellar pocket. The flagellar pocket is also accompanied by an additional band, the striated lamella. Other
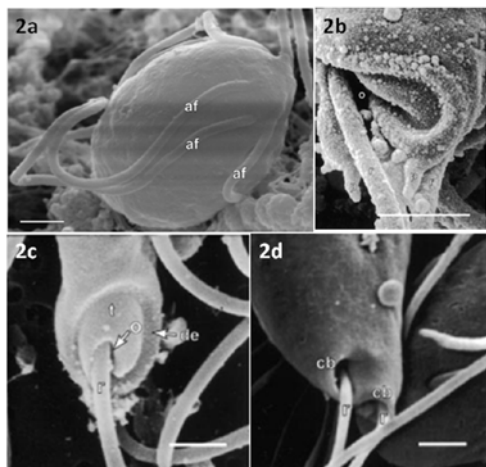
Figure 2: Distinctive surface ornamentation of piscine diplomonads, allowing identification to the species level. Anterior-lateral view of *Spironucleus salmonis* showing emergence of anterior flagella and unadorned surface (a). Ventral or dorsal view of *S. vortens* posterior end, with counter-crossing of right peripheral ridges around exits of recurrent flagella. Presence of papillae is also of diagnostic value (b). Lateral view of *S. torosa* which has ring-shaped swellings (tori) surrounding the exits of recurrent flagella (c). Oblique view of *S. barkhanus* recurrent flagella surrounded by crescent shaped ridges (barkhans), this is also found in the morphologically indistinguishable *S. salmonicida*. Abbreviations: (af) anterior flagella; (cb) crescent-shaped ridge (barkhan); (de) depression; (o) opening of flagellar pockets (cytostomal canals); (p) papilla (r) recurrent flagellum; (t) torus (scale bars = 1 µm). Adapted from Saghari-Fard et al. [66] with permission from Inter-Research (a), Poynton et al. [5] with permission from John Wiley & Sons Ltd (b), Sterud [29] with permission from American Society of Parasitologists (c) and Sterud et al. [6] with permission from Inter-Research (d).

diagnostic cytoskeletal features include the pattern of microtubules around the flagellar pocket (when viewed in transverse section though the middle of the body): *S. barkhanus* is characterised by 3 radiate, *S. torosa* 3 concentric and *S. vortens* 3 staggered bands [1].

The cytoplasm of piscine diplomonads typically comprises a homogenous area at the apex of the cell, but elsewhere it is heterogeneous and organelle-rich. The latter contains a variety of organelles, which may include (depending upon species) electron-dense bodies, endoplasmic reticulum, membranous structures and ribosomes. *S. salmonis* is characterised by the presence of electron-dense bodies and a membrane-bound sac of free ribosomes around the opening of the flagellar pockets [46,66]. Interestingly, the appearance of these cytoplasmic organelles can vary between *Spironucleus* harvested directly from the fish, and those maintained in culture [1]. Glycogen granules, vacuoles and bacteria may also be present in the cytoplasm [1,28]. There are no Golgi or traditional mitochondria present. However, a recent study suggests the presence of degenerate mitochondria, called hydrogenosomes, present within *S. vortens* [67]. Unlike mitochondria, these organelles lack DNA and cytochromes, thus oxidative phosphorylation does not take place. They do however retain some key mitochondrial features, including a double membrane, ATP production by substrate level phophorylation of pyruvate and some heat shock proteins (Hsp70, Hsp60 and Hsp10). As the name suggests, the main role of hydrogenosomes within the cell is to produce H_2 (discussed further in Section 4) [68].
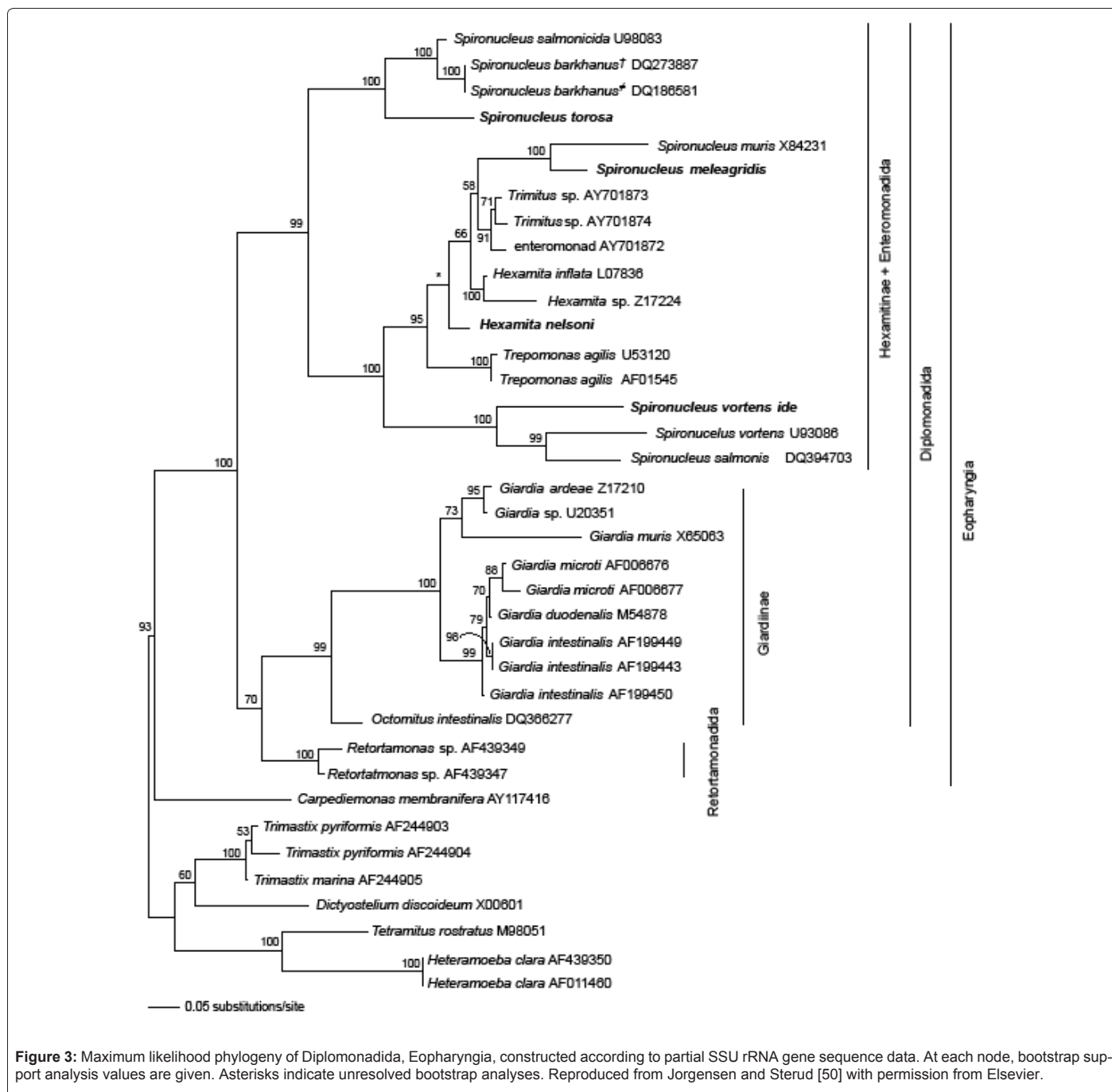
Molecular identification and genetics: Until recently ultrastructure was considered sufficient to differentiate species of piscine diplomonads, however this is not entirely the case. New species descriptions of *Spironucleus* spp. should include both ultrastructural and molecular data. Guidelines for ultrastructural description of diplomonad flagellates have been presented by Poynton and Sterud [1] and 18S PCR primers have now been developed for several taxa (Table 3). Sequence data of other genes, such as glutamate dehydrogenase (gdh) and alpha-tubulin (α-tubulin) are also available for *S. vortens*, *S. barkhanus* and *S. salmonicida* [27,31,50,69-72]. However, the SSU rRNA gene appears to be a suitable marker for species identification in the genus *Spironucleus*, even for morphologically cryptic species. This is exemplified by *S. salmonicida* and *S. barkhanus,* which are morphologically identical, but display only 92.7% similarity in 1154 bp of the SSU rRNA gene. For *S. torosa*, variation in the SSU rRNA gene separates the species into two genotypes that may be related to the two different morphs observed [73]. However this genetic variation was not considered sufficient to warrant species status for the two clades [73]. Phenotypic differences also exist between isolates of *S.*

Primer name	Sequence	Application	Reference
Spiro-1f	AAGATTAAGCCATGCATGCC	PCR/Seq	Jorgensen & Sterud [31]
Spiro-2r	GCAGCCTTGTTACGACTTCTC	PCR/Seq	
Spiro-3r	CATTGGGYAATYTYCCGCCCT	Sequencing	
Spiro-4f	GAYTCYGGAGAVTGRGCAYGAG	Sequencing	
Spiro-5r	STYTCCGTCAATMCYTTMAAGTTTC	Sequencing	
Spiro-6f	AAGRYTGAAACTTKAARGKATTGACGG	Sequencing	
Spironucleosis-1f	TCATTTATCAGTGGTTAGTACATGC	PCR/Seq	
Spironucleosis-2r	TTCAAGCCTAACCACGACAAG	PCR/Seq	Fard et al. [66]
Salmonis-1f	TTGTGTACGAGGCAGTGACG	PCR/Seq	
Salmonis-2r	TCCCCGGTTCGTTGGTCAAG	PCR/Seq	
Torosa 16sf	CTCTTGAGTGAGGTAGTCACCAGC	PCR/Seq	Jorgensen et al. [73]
Torosa 16sr	GTGTCCGAATAACTCACCAAAACC	PCR/Seq	

Table 3: Primer sequences for amplification of the small subunit ribosomal RNA (SSU rRNA) gene for identification of fish diplomonads to the genus or species level using PCR or sequencing approaches. Primers Spiro-1 and Spiro-2 will amplify most member of the genus *Spironucleus* and *Hexamita*. Spiro-3 to Spiro-6 may be used as sequencing primers for most species of *Spironucleus* and *Hexamita*. The Spironucleosis, Salmonis and Torosa primers are specific for *S. salmonicida*, *S. salmonis* and *S. torosa*, respectively

salmonicida. The isolate causing disease in Canadian *Oncorhynchus tshawytscha* (Chinook salmon) could not establish in Atlantic salmon [26,38]. Clinical signs and pathology in Chinook salmon also differed from those observed in *S. salmonicida* infections of Atlantic salmon in Norway. No sequence differences in the SSU rRNA, α-tubulin or gdh genes have been identified between these isolates. However, completion of ongoing genome projects of *S. barkhanus* and *S. vortens* will reveal a wide range of markers for diagnostic, taxonomic, systematic and for functional studies [42,71,74]. An episomal protein-tagging shuttle vector system has already been developed for *S. vortens*; enabling studies of protein expression, intracellular trafficking and cell signaling in *Spironucleus* [42].

The genome of *S. salmonicida* was the first in the genus to be sequenced [50]. The data revealed a reduced and compact genome with genes lacking introns, and several genes had apparently been acquired from different bacteria [70,71,75,76]. The genome of *S. salmonicida* has also been compared to more than 5000 expressed sequence tags (EST's) from the morphological indistinguishable species *S. barkhanus*. The genome size of *S. salmonicida* and *S. barkhanus* is ~12 Mb and 16 Mb, respectively. In addition, codon usage and frequency of allelic variation also differs in the two species. This indicates that at least parts of the *S. barkhanus* genome have been duplicated and that these morphological indistinguishable species have been subjected to extensive divergent evolution [74].

Figure 3: Maximum likelihood phylogeny of Diplomonadida, Eopharyngia, constructed according to partial SSU rRNA gene sequence data. At each node, bootstrap support analysis values are given. Asterisks indicate unresolved bootstrap analyses. Reproduced from Jorgensen and Sterud [50] with permission from Elsevier.

Phylogeny of *spironucleus:* The diplomonads have been considered to be among the earliest eukaryote lineages and true intermediates in the prokaryote-eukaryote transition [77]. The apparent lack of eukaryotic organelles within diplomonads suggested that they diverged from other eukaryotes prior to acquisition of the mitochondrial endosymbiont. As a result, these organisms were included as members of the paraphyletic Archezoa Kingdom [77]. However, it was later shown that the diplomonads contained remnants of mitochondrial symbiosis. Furthermore, several mitochondrial genes were discovered in the genomes of other Archezoa (including *Giardia*) [78-81] suggesting that ancestors of the diplomonads once harboured the α-proteobacteria endosymbiont. Later, mitochondrion-like organelles, hydrogenosomes and mitosomes, were found in several amitochondriate eukaryotes, including *G. duodenalis* and *S. vortens* [67,82-84]. As a result, the diplomonads have recently been re-classified into the Fornicata clade of the eukaryotic supergroup Excavata [85]**.**

Currently, the genus *Spironucleus* is a member of the Diplomonadida which also contains the genera *Giardia, Octomitus, Hexamita, Enteromonas, Trimitus* and *Trepomonas* [72]. Phylogenetic analysis of the genus *Spironucleus* has been based on SSU rRNA gene data [50,72,86-89]. *Spironucleus* constitutes three branches in the Hexamitinae sub-tree (Figure 3). The clade containing *S. salmonicida, S. barkhanus, S. torosa* and also the tortoise *Spironucleus* sp. isolate GEPA2H seems to be the most basal in *Spironucleus. S. salmonis* and *S. vortens* are sister taxa in all SSU analyses and branch off secondary to the clade containing marine *Spironucleus* and the tortoise isolate. Both these clades are well supported. The position of the clade consisting of *S. meleagridis* (type host: birds) and *S. muris* (type host: rodents) varies and is never well supported by any tree-building method [5,72,88].

Based on the habitat of their hosts, it was suggested by Jorgensen and Sterud [50] that the three *Spironucleus* clades may have evolved separately in the sea, in freshwater and on land. This was later refuted by Kolisko et al. [72], due to presence of the terrestrial GEPA2H tortoise isolate in the 'marine' *Spironucleus* clade. However, the phylogeny of the diplomonads and *Spironucleus* probably suffers from low taxon sampling with only 8 *Spironucleus* species included in the most recent studies [50,72]. This speculation is strongly supported by the previously mentioned Chinese studies, describing a total of 25 new species of *Spironucleus* and *Hexamita* from fish [62-65]. As with most fish pathogens, present records do not reveal the complexity of diplomonad diversity. More sampling of diplomonads from a broader geographical and host range is required in order to understand the evolutionary processes which led to the adaptation of these organisms to such different environments and resulted in the morphological and genetic diversity seen today.

Host-pathogen interactions

Distribution and host specificity: Piscine diplomonads infect both wild and farmed fishes globally, encompassing marine to freshwater habitats, and arctic to tropical climates. The diplomonad fauna of fishes is best known from regions of the world where fisheries and aquaculture provide key sources of protein for human consumption, such as Europe [6,23,90], North America [38] and increasingly China [62-65]. Most of the recent reports of *Spironucleus* are in commercially important species of fish, particularly those in aquaculture that are raised as food fish or as ornamentals. Thus, there is a sense that this genus of flagellates is primarily found in cichlids, cyprinids, gadids and salmonids. However a broader consideration of the literature, including numerous older publications focusing on ecological parasitology and

surveys, shows that diplomonads are commonly encountered in many more families of fish.

Since reliable species identification of *Spironucleus* is rare, a sound understanding of host range has not yet been achieved. Some species of *Spironucleus* have a broad host range, being reported from a variety of host species. For example, *S. salmonis* infects *Salvelinus fontinalis* (brook trout), rainbow trout, brown trout, Arctic charr and *Gasterosteus aculeatus* (three-spined stickleback) [47,48,58,66]. However, innate immunity may also play a role in host-susceptibility. Surveys show that not all species of fish in a given habitat are infected. In an infection trial conducted by Kent et al. [38], Atlantic salmon were not susceptible to experimental infection with an isolate of *Spironucleus salmonicida* from Chinook salmon. Conversely, Guo and Woo [26] found that 3 different families of Atlantic salmon remained fully susceptible to experimental infection with *S. salmonicida* isolated from Atlantic salmon. A genetic basis for susceptibility within host families has been suggested [26]. Cross infection is even feasible across animal groups; with *S. elegans* from newts infecting angelfish [90]. Moreover, individual fish may be co-infected, for instance with *S. salmonis* and *S. salmonicida* [58]. This implies that in previous studies, clinical disease and pathology may have actually been caused by multiple hexamitid infections. This underlines the need to use molecular methods, including cloning, for identification of *Spironucleus* spp.

Pathogenicity in wild versus farmed fish: *Spironucleus* spp. are found in high prevalence in both wild and farmed fish, however, the pathogenicity of isolates varies greatly between these two host habitats. For example, *S. barkhanus* and the morphologically indistinguishable *S. salmonicida* infect wild and farmed fish, respectively. However, systemic Spironucleosis has only ever been observed for *S. salmonicida* in aquaculture, whilst *S. barkhanus* infections in wild fish are generally commensal [27]. Furthermore, the *S. vortens* isolate recently characterized in wild Norwegian ide has not been associated with Spironucleosis in these fish, however farmed cichlids are extremely susceptible to this disease [24,61]. This difference in susceptibility between wild and farmed fish has led to the description of *Spironucleus* spp. as opportunistic pathogens, whereby pathogenesis is favoured over commensalism in an immunocompromised host. Factors such as poor water quality, malnutrition, temperature fluctuations, bad husbandry and overcrowding all have been associated with stress of the fish host, and subsequent reduction in host immunity [39,44,91,92]. Some of these factors are unavoidable in aquaculture, which explains why farmed fish are more susceptible to disease than wild fish. However, a recent study by Meseck et al. [93] has documented systemic Spironucleosis in Chinook salmon from Lake Ontario, meaning that disease outbreaks in wild fish cannot be ruled out.

As a result, it is plausible that wild fish may serve as a reservoir of infection for farmed fish. For example, following a series of systemic Spironucleosis outbreaks in Norwegian fish farms [35-37] it was hypothesised that wild feral Arctic charr were the source of parasite transmission to the sea-caged Atlantic salmon [6]. A more recent outbreak was observed in farmed Arctic charr, found in northern Norway [30]. The affected fish farm sourced their charr smolt from a hatchery which utilized water possibly infected with *S. barkhanus* from wild Arctic charr outside the farm. Despite the fact that no fish within the hatchery were found to harbour *S. barkhanus*, it was suggested that transmission of flagellates from wild to farmed char was the most likely source of the outbreak [30]. However, molecular analysis revealed large genetic differences in the isolates from wild and

farmed fish, resulting in species reassignment of the farmed isolate to *S. salmonicida* (see Section 2). This led to a further reappraisal of the likely transmission route from wild feral populations to farmed stocks [31]. Transmission of *S. vortens* from aquarium cichlids to wild ide has also been suggested, as a result of improper disposal of moribund or dead fish into domestic waste systems and/or dumping in lakes [61]. However, the large genetic differences between *S. vortens* isolated from wild and farmed fish implies a similar scenario to that of *S. salmonicida*, whereby species reassignment may be required [50].

Prevalence and intensity of *Spironucleus* infections have also been associated with fish age. This has particular importance for infections in aquaculture, where disease may only manifest at certain stages of the production cycle. For example the chronic disease associated with *S. salmonis* typically affects young fish, suggesting that there is acquired immunity to infection [94]. In contrast, *S. salmonicida* causes disease in adult fish [30,36,38].

Microhabitat preferences: In both wild and farmed fish, the intestinal tract is believed to be the natural habitat for piscine diplomonads. Most reports of *Spironucleus* in wild fish document the flagellates in the intestine, and less commonly in the gall bladder. Only in rare cases are wild fish reported to carry *Spironucleus* infections elsewhere; a particular case of note being the presence of *Spironucleus* spp. in abscess lesions in the somatic muscle adjacent to the dorsal fin of adult Chinook salmon from Lake Ontario [93]. However, in farmed fish, systemic infections (including the presence of the flagellates in the blood) are common (discussed further in Section 3.4).

Certain species of piscine *Spironucleus* have particular microhabitat preferences within the intestine. For example, *S. salmonis*, commonly infecting farmed rainbow trout, is concentrated in the pyloric region [66], while *S. torosa*, in wild gadids, clusters posterior to the ileo-rectal valve in the most posterior section of the intestine [28]. Different *Spironucleus* species may have different metabolic requirements, as based on differences in the organelles in the heterogeneous cytoplasm (see Section 4), it is likely that their metabolism varies. Along the length of the intestine, there are gradients in morphology, physiology and metabolic activity. In *S. salmonis*, the optimum and tolerable pH have been studied *in vivo* [95] and *in vitro* [96]. Flagellates survive within a pH range of 5.5-9.0, with optimal pH for population increase being between 7.5-8.0 [96]. I*n vivo*, however, parasite density within the intestinal tract did not correlate with optimal pH, suggesting that microhabitat preference is not dependent on this factor [95].

A focus for future studies should be determination of the factors that trigger unusual parasite-host interactions. For example, *S. torosa,* which usually swims free in the lumen, can attach to the intestinal microvilli, with apparent cytoplasmic connections between the flagellates and fish cells [28]. There are also marked differences in the cytoplasmic organelles of the free-swimming and attached forms [28]. In the case of *S. vortens*, another species that is usually found in the intestinal lumen, there is the capacity to become systemic, infecting kidney, liver, spleen and head lesions [24]. *Spironucleus* in farmed Arctic charr may even occur intracellularly within the capillaries and sinusoids of the liver, spleen and kidney [30]. Although, there may be numerous parasites, gross and histopathological lesions are rare and mortality is low [30]. One of the most challenging questions in understanding microhabitat preference of piscine diplomonads concerns identification of the factors involved in onset and establishment of systemic infections.

Pathology and clinical manifestation: Spironucleosis ranges from an asymptomatic to chronic disease, with the main clinical symptoms consisting of anorexia, weight loss, lethargy, enteritis, dark colouration, pale gills and egestion of stringy faeces [39,94]. Infected fish also exhibit unusual swimming behaviour such as corkscrew movements, a tendency to swim on their side and to linger in the corner of tanks [23, pers. obs.]. In addition to direct morphological and molecular identification from tissues (see Sections 2.2 and 2.3), microscopic analysis of the host's faeces provides a convenient method of non-invasively diagnosing Spironucleosis. Enumeration of parasites per field of view, allows an assessment of the severity of infection [23]. Although both cysts and trophozoites have been isolated from the faeces of infected fish [51,97], the motile trophozoite form is more easily identified, with the cyst form being exceptionally rare [23,38,90].

Two main forms of Spironucleosis are described causing coelozoic (intestinal) and histozoic (systemic) infections. In both cases, fish may appear asymptomatic; however, severe clinical signs and pathology are observed in relation to disease outbreaks.

Intestinal spironucleosis: The intestinal form of Spironucleosis (originally described as hexamitosis) in salmonid fingerlings and aquarium fish is considered to be benign [98,99]. There is no doubt, however, that the occurrence of vast numbers of the parasite in the gut may lead to severe enteritis with emaciation, exophthalmia, ascites and faecal casts [40,47,48]. Symptoms are non-specific, but often include locomotive disorders and increasing mortalities.

Systemic spironucleosis: Several diplomonad species may cause systemic infections in fish [6,24,35,37,38,40,100]. However, it is probable that all *Spironucleus* spp. have the potential of invading host tissue and inducing systemic infections. *S. vortens* infects a variety of fish species and is associated with hole-in-the-head (HITH) diseases in cichlids, e.g. angelfish and discus [5,24]. The disease is characterized by parasite-filled necrotic lesions in the head region, sometimes also extending posteriorly along the lateral line. Lesions are usually bilaterally symmetrical and may coalesce into larger lesions, discharging yellow mucus [24,39]. Large numbers of flagellates have been found in internal organs such as the heart, liver and kidney [35-37]. In addition, trophozoites of *S. vortens* were detected in the intestine of fish with systemic infections and it was suggested by Paull and Mathews [24] that the infection had an intestinal origin.

Systemic infections caused by *S. salmonicida* in Atlantic salmon, are marked by parasite filled abscesses and pustules in subcutaneous muscle tissue, in necrotic tissue of the heart, kidney, liver and spleen, but never in the intestine [6,27,31,35,37]. Typical histopathology of systemic infections with *S. salmonicida* in Atlantic salmon reveals severe epicarditis, large caseonecrotic areas with (at times) formation of granulomatous response in the kidney, liver and spleen. The characteristic paired anterior nuclei of the flagellates are usually observed centrally in the granulomas and foci of necrotic tissue [36,37]. *S. salmonicida* also causes systemic Spironucleosis in farmed Arctic charr and Chinook salmon. In these hosts the symptoms are somewhat different compared to the infection in Atlantic salmon. Granulomas in muscle tissues and internal organs are not apparent, but vast aggregates of parasites occur in the vasculature of most organs [30,38].

Specific triggers for pathogenicity and onset of systemic infection are currently unknown, and may vary greatly between *Spironucleus* spp. due to differences in microhabitat preferences. The host immune system is likely to play a key role in protecting against Spironucleosis, however further work is required in order to categorise the immune

response to *Spironucleus* infection, and understand subsequent methods of parasite evasion.

Physiology and biochemistry

Knowledge regarding the biochemistry and physiology of *Spironucleus* spp. is paramount in order to understand mechanisms of pathogenicity, and consequent exploitation of novel molecular pathways for chemotherapeutic control. This has been made possible as a result of optimisation of *in vitro* culture for several *Spironucleus* isolates (see Table 4).

Although detailed information on nutrient uptake, and pathways of carbon and nitrogen metabolism have not been investigated, a great deal on the physiology of fish diplomonads can be inferred from studies of nutritional and environmental conditions necessary for growth. This is especially true for *S. vortens* grown *in vitro* under aerobic conditions in stoppered tubes with ascorbate and cysteine to ensure removal of traces of O_2 [101]. Extremely rapid trophozoite multiplication corresponds to a mean generation time of 1.79 h at 25ºC [102]. Their copious production of H_2 (Figure 4), a metabolic product rare amongst eukaryotes, strongly suggests similarities to other flagellate parasitic protists that thrive in low O_2 environments, i.e. the parabasalid trichomonads and many diplomonads [103]. The progressive diminution of the aerobic life-style typical of their free-living ancestors, together with multiple lateral transfer of genes of bacterial origins have enabled these parasitic organisms to prosper energetically without the traits of their mitochondriate free-living counterparts [104]. Loss of a cytochrome-mediated respiratory chain driven electron transport system and its coupled proton driven ATP synthase, along with the acquisition of hydrogenases, characterizes a novel redox-active organelle, the hydrogenosome [105,106] recently found in *S. vortens* (see Section 2.2) [67]. Hydrogen production in *S. vortens* is even more rapid than in *Trichomonas vaginalis* and is similarly inhibited under microaerobic conditions. Cyanide- and CO-sensitivity in both organisms strongly suggests that the hydrogenase responsible is of the Fe-only category [107]. This conclusion has been validated for *T. vaginalis* by redox properties and furthermore by electron paramagnetic resonance spectra, a powerful technique frequently employed in the characterization of metal centres in iron-sulphur proteins, such as hydrogenase [108]. Identification of an Fe-hydrogenase-like sequence in the genome of *S. barkhanus* strongly supports these conclusions [103]. One of the mechanisms proposed for metronidazole reductive activation prior to growth inhibition and cell death in *S. vortens* is evident from the observation of its inhibition

of hydrogenase activity and H_2 production [103,109]. Hydrogenase inhibition occurs by competition by the drug for the reducing equivalents of metabolizable substrates.

As in the trichomonads, well-washed, non-proliferating *S. vortens* from anaerobic cultures avidly consume O_2 (Figure 4) over periods of several hours even in the absence of added substrates [110]. This denotes an exceptional O_2 scavenging ability for these parasites, which in turn suggests that they are well-equipped to cope with large fluctuations in O_2 levels during the course of infection or transmission. Their endogenous capacity must reflect a considerable reserve of reduced carbon analogous to the particulate (and thus easily sedimentable from disrupted cells) glycogen inclusions of *T. vaginalis*, although the biochemical identity remains to be established for *S. vortens*. As this O_2 consumption does not directly provide energy it should be regarded as an O_2 – scavenging function rather than as respiration. Similar remarks apply to the extra O_2 consumed on addition of C sources (e.g. glucose).

Both endogenous and glucose-driven metabolism in *S. vortens* yield CO_2, ethanol, acetate, alanine and lactate as revealed by membrane inlet mass spectrometry and ^1H NMR and ^{13}C NMR [111]. Ethanol can also be utilized, but like glucose is not as favoured as amino acids. In this respect, cultures of *S. vortens* show characteristics similar to those of some cultured mammalian cell lines where glucose uptake is slow and glutamate is the favoured substrate [111]. Cultures of *S. vortens* analysed by ion-exchange chromatography at intervals over a period of 6 days indicate that despite the presence of high concentrations of free amino acids, peptides and proteins become depleted [111]. Production of extracellular peptidases and proteases is likely to be an essential aspect of their invasiveness potential during pathogenesis. Free amino acids showing net gains during culture growth include alanine and aspartate, whereas lysine, arginine, leucine, cysteine and urea are extensively utilised. Unlike *T. vaginalis, G. intestinalis*, or *Hexamita inflata, S. vortens* appears not to have the arginine dihydrolases (ADH) pathway for energy production, as no ornithine has been detected. Addition of arginine to non-proliferating cells has resulted in no detectable ammonia or nitric oxide production. However, many homologues of the ADH pathway can be found in the *S. vortens* genome, thus the presence of such an energy generation pathway in this organism cannot be completely ruled out [111,112].

It should be emphasised that the physiology and underlying biochemical properties of *Spironucleus* species may vary widely, between and within species in relation to short time-scale changes of *in situ* microenvironments as well as the more profound changes

Species	Source	Culture medium	Temp. (ºC)	Reference
Spironucleus vortens	Lip lesion, angelfish, Florida (ATCC 50386)	Keister's modified TY-I-S33	25 (22-34)	Sangmaneedet & Smith [101]
	Intestine, internal organs and head lesions of cichlids with hole in the head disease, UK	Keister's modified TY-I-S33 supplemented with fresh *Oreochromis niloticus* liver	25	Paull & Matthews [24]
	Intestine of wild Ide, river Leira, Norway	Keister's modified, TY-I-S33	15 (5-15)	Sterud & Poynton [61]
Spironucleus barkhanus	Gall bladder of wild grayling, Norway	Keister's modified TY-I-S33	15 (5-20)	Sterud [22]
	Muscle abscess of farmed Atlantic salmon, Norway	Keister's modified TY-I-S33	10 (5-20)	Sterud [22]
	Blood, liver and kidney of farmed Atlantic char, Norway	Growth not supported by Keister's modified TY-I-S33	NA	Sterud et al. [30]
Spironucleus salmonis (previously *Hexamita salmonis*)	Intestine of wild rainbow trout, Dennmark	MEM with 10% bovine serum. Not supported by Keister's modified TY-I-S33	10 (5-10)	Buchmann & Uldal [96]

Table 4 *In vitro* culturing conditions reported for the different isolates of *Spironucleus* spp., including the source of the species and corresponding optimal culture medium composition and temperature (temperature range given in brackets). Reproduced from Millet [110].
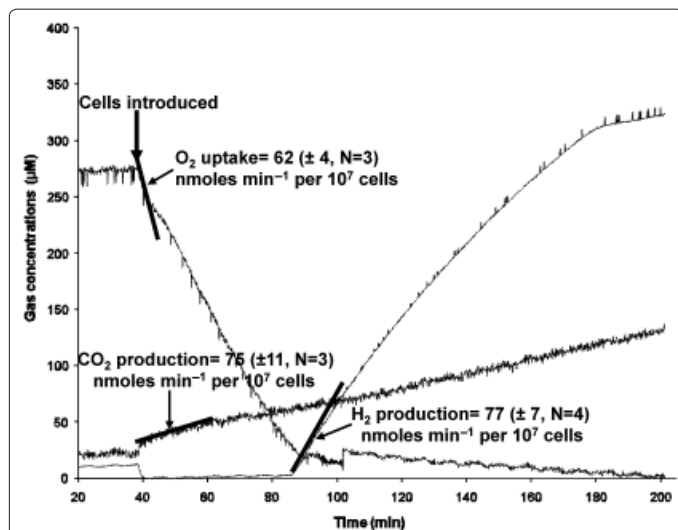
Figure 4: Gas metabolism of *Spironucleus vortens* in a stirred cell suspension of 7.4 x 10^5 cells/ml in air-saturated phosphate buffered saline at 20ºC, analysed by membrane-inlet mass spectrometry. Rates of O_2 consumption, H_2 production and CO_2 production were derived from linear regression at the indicated tangents (standard deviation and number of replicates are denoted in brackets). Reproduced from Millet et al. [103] with permission from John Wiley & Sons Ltd.

expected at different life cycle stages. Factors pivotal in these respects include nutrient supplies and environmental O_2 concentrations. Central to the extreme limits for parasite viability and persistence is the intracellular redox potential which must be maintained for normal metabolic function and redox signalling. This in turn depends on adequate supplies of host-derived reduced carbon compounds for growth and energy production. Further research in these areas will improve our knowledge of key parasite biochemical processes relating to pathogenicity and transmission, and will provide an insight into the phenotypic diversity of diplomonads.

Disease management

In vitro culture methods for routine maintenance of *Spironucleus* spp. also provides convenient means of screening new and existing chemotherapeutic agents. Furthermore, recent developments in our understanding of piscine diplomonad biochemistry show potential for the development of novel strategies of disease management.

Treatment: Treatment of salmonid infection is recommended when 15-20 trophozoites are counted microscopically per field of view (using 100 X total magnification) ([113] cited in Woo & Poynton [39]). The most common chemotherapeutic means of eradicating human and veterinary diplomonad infections is metronidazole (Flagyl, Fish-zole) [23,114]. This is an unique, limited spectrum antibiotic that is used to combat anaerobic or microaerophilic, pathogenic microorganisms. When administered, the metronidazole pro-drug acts as a high affinity electron acceptor and is activated by reduction at the drug nitro group by the low redox potential electron transfer systems present in anaerobic/microaerophilic organisms (see review by Freeman et al. [115]). This in turn results in cytotoxic reduction products including nitro-radical anions which are suggested to cause cell death via DNA damage, leaving the aerobic host unaffected [116].

Metronidazole has a minimum inhibitory concentration of 1 μg. ml^{-1} against *S. vortens in vitro*, with the recommended dose in ornamental fish being 10 mg per 1 g fish food [110]. For the treatment

of food fish, 2-5 g. kg^{-1} feed or 4 mg. L^{-1} bath treatment is recommended [23], with oral administration being more effective than bathing [45]. However in 1998, European Council Regulation 613/98/EEC [117] banned the use of metronidazole on outdoor European fish farms, due to its environmental side effects [118,119,120], cytotoxicity and genotoxicity in fish [121,122], and it being listed as a potential human carcinogen by the International Agency for Research on Cancer [123]. Administration of metronidazole to food animals is also banned in the US [124]. As a further complication, resistance to metronidazole has been documented on some fish farms [23].

Other effective nitroimidazoles include benznidazole, ronidazole, secnidazole [23] and dimetridazole [39], the latter, however, has severe toxic side effects in fish [23]. Some non-nitroimidazoles are also effective, including albendazole, aminosidine, diethylcarbamazine and nitroscanate, all at doses of 40 g. kg^{-1} feed for a 10 day period [23]. Furthermore, mebendazole, a highly active benzimidazole, has been shown to inhibit *S. vortens* growth *in vitro* at a concentration of 0.5 μg. ml^{-1} [110].

More recently, the inhibitory effect of *Allium sativum* (garlic) has been investigated as an alternative therapy for the treatment of Spironucleosis. *Allium*-derived compounds are broad-spectrum antimicrobial agents and thus are effective against a wide range of human and veterinary pathogens (see review by Williams and Lloyd [125]). *In vitro*, ajoene and a mixture of vinyl dithiins and thiosulphinates extracted from garlic are highly effective at inhibiting *S. vortens* growth, having minimum inhibitory concentrations of 107 and 83 μg. ml^{-1} respectively [126]. *In vivo* assays are required in order to determine the efficiency of garlic in fish. However, if successful, garlic-based therapy has the potential to provide a less toxic and more environmentally friendly alternative to metronidazole in the treatment of fish diplomonad infections.

Preventative measures: Due to our current lack of knowledge regarding the transmission of *Spironucleus* to fish farms, along with the possibility of sub-clinical infections, eliminating this parasite from aquaculture will not be possible in the short term. However, preventative measures can be employed in order to minimise the occurrence of an outbreak. These include, quarantine of new stock, use of appropriate under-gravel filters, frequent cleaning of tanks, good husbandry and healthy nutrition [39]. Furthermore, ozonization and UV irradiation of inlet water greatly reduces pathogen entry into tanks [127]. As outbreaks are associated with 'stress' of the host, care should be taken to ensure that water quality, aeration and temperature are maintained at an optimal level, especially when fish are under crowded conditions [39,41,45]. These are all factors which may contribute towards an outbreak (of this and other infectious diseases) by decreasing the efficiency of the fish immune system and thereby increasing susceptibility [44]. Identifying the source and method of transmission of *Spironucleus* into fish farms will lead to more specific measures to prevent outbreaks in aquaculture.

Economical impact: Failures in prevention and control of Spironucleosis may cause severe economic losses both to fish farmers and the aquarium industry. Systemic Spironucleosis caused by *S. salmonicida* in Norwegian farms of Atlantic salmon has resulted in near or complete loss of all stocks at some farms [35,36,43]. However, most infections reduced profits due to downgrading at slaughter and increased labor costs during the filleting process. *S. salmonis* may also have an economical impact on farming of rainbow trout due to reduced growth and increased mortality of young fry. *S. vortens* has a similar

effect in the production of ornamental fish, resulting in loss of highly valuable specimens, including angelfish and discus *Symphysodon discus*, the trade of which is a multi-billion dollar industry per annum [128].

Conclusions

Although piscine diplomonads have been described since the late 19th Century [129], our appreciation of these organisms remains in its infancy. However, our understanding of their taxonomy, biochemistry and pathogenicity has greatly increased in the last few years. Identification of morphologically cryptic *Spironucleus* spp. through SSU rDNA sequencing [50] has considerably clarified fish diplomonad taxonomy. Furthermore, optimisation of *in vitro Spironucleus* cultures has provided a convenient source of flagellates, allowing complete replacement of animal models for biochemical and physiological investigations of parasite pathogenicity. Such studies have revealed novel molecular pathways of gas metabolism, redox control and nutrition [102,103,111], which are essential for our understanding of parasite pathogenicity and consequent exploitation of novel molecular pathways for chemotherapeutic control. This is especially important in light of restrictions of the use of metronidazole in aquaculture. *Allium*-derived compounds show great potential as alternative antiparasitic agents, although *in vivo* investigations are required to confirm efficiency at eradicating Spironucleosis in fish [126].

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