Sphaerothecum destruens: Life history traits and host range.

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To my family and kindred spirit Miss Aria Johnson.

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Abstract

Sphaerothecum destruens is a multi-host parasite which can infect and cause mortality in a number of fish species including Chinook salmon *Oncorhynchus tshawytscha*, Atlantic salmon *S. salar* and the sunbleak *Leucaspius delineatus*. It has been hypothesised that *S. destruens* has been introduced to the UK with its invasive hosts; *L. delineatus* and topmouth gudgeon *Pseudorasbora parva*. As the effects of a novel parasite to naïve populations could be detrimental, this thesis aimed to better elucidate the life cycle of *S. destruens*, its prevalence in wild populations and the susceptibility of cyprinid species. *S. destruens* was able to infect multiple organs (kidney, liver, gill, gonad and intestine) with similar histopathology between *L. delineatus*, a cyprinid species, and the histopathology reported for salmonid species. Its spore and zoospore life stages displayed a wide temperature tolerance and zoosporulation occurred at temperatures between 4-30 °C. A survey of one UK location detected *S. destruens* in a wild *L. delineatus* population. A quantitative polymerase chain reaction was developed in order to quantify *S. destruens*' infection levels. Reproductive *L. delineatus* were more susceptible to *S. destruens* and experienced higher *S. destruens* prevalence and infection levels. The presence of a second host, *P. parva*, had no influence on *S. destruens*' prevalence and infection levels. However, presence of *P. parva* resulted in significantly lower somatic condition in parasitized female *L. delineatus*. Exposure to *S. destruens* through immersion in water containing *S. destruens* spores revealed that bream *Abramis brama* and carp *Cyprinus carpio* were susceptible to *S. destruens*. *A. brama* experienced high (53 %) mortalities when exposed to *S. destruens* whilst *C. carpio* experienced low (8 %) mortalities. The susceptibility of roach *Rutilus rutilus* and rudd *Scardinius erythrophthalmus* could not be excluded and needs to be further investigated.

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List of Abbreviations

bp: base pair

cm: centimetre

CEFAS: The Centre for Environment, Fisheries and Aquaculture Science

CEH: Centre for Ecology and Hydrology

DNA: Deoxyribonucleic acid

d.f.: degrees of freedom

fg: femtogram

H&E: Hematoxylin and Eosin

HO: Home Office

IMS: Industrialised Methylated Spirit

µg: microgram

µl: microlitre

µm: micrometers

µM: micromolar

m: meter

mg: milligrams

ml: millilitre

mm: millimetres

ml^{-1}: per millilitre

mM: millimolar

M: molar

ng: nanogram

nm: nanometre

no: number

PCR: Polymerase Chain Reaction

p.e.: post exposure

pg: pigogram

qPCR: quantitative Polymerase Chain Reaction

RA: Rosette Agent

RLA: Rosette Like Agent

RNA: ribonucleic acid

rRNA: ribosomal RNA

SE: standard error

S_{TD}: host specificity index

TM: trademark

°C: degree Celsius

&: and

Chapter 1: Introduction

The increase in the global trade of commercially important species provides many opportunities for the establishment of invasive species and new host-parasite associations (Gozlan *et al.*, 2006, Smith *et al.*, 2009). Over 150 aquatic non-native species introductions have been linked to the aquarium trade (Chang *et al.*, 2009) and over 50 percent of non-native fish introductions are attributed to aquaculture for food and associated ecosystem services (Gozlan, 2008, Gozlan and Newton, 2009). In the UK, species introductions for the ornamental and aquaculture sector have increased dramatically since the 1950s (Copp *et al.*, 2007) and are expected to continue to increase (Gozlan, 2008).

Biological invasions can impact on native species populations (Everett, 2000, Gurnell *et al.*, 2004), ecosystem function (Ellison *et al.*, 2005), and can be financially costly (Lodge *et al.*, 2000, Dunn, 2009, Hulme *et al.*, 2009). For example, the eastern hemlog *Tsuga canadensis*, a tree native to North America, plays an important role in the architecture and functional characteristics of hemlock groves (Ellison *et al.*, 2005). The invasive insect hemlock woolly adelgid *Adelges tsugnae* has caused high declines in *T. canadensis* (see Ellison *et al.*, 2005). These declines have been associated with local loss of uniquely associated ants and birds (Ellison *et al.*, 2005). Also, high population declines have been experienced by the native red squirrel *Sciurus vulgaris* (Linnaeus) in the UK, Ireland and Italy as the result of the alien grey squirrel *S. carolinensis* (Gmelin) introduction (Gurnell *et al.*, 2004). Gurnell *et al.* (2004) reported lower body mass and fecundity in *S. vulgaris* females that co-occurred with *S. carolinensis*. In Europe, the cost of invasive species have been estimated at €10 billion annually (Hulme *et al.*, 2009) This cost includes expenditure for eradicating

and controlling invasive species as well as documented costs of economic impact on commercially important species.

The role that parasites can play in biological invasions is of growing interest (Hudson and Greenman, 1998, Boots *et al.*, 2003, Drake, 2003, Prenter *et al.*, 2004, Hatcher *et al.*, 2006, Dunn, 2009). Parasites can alter host fitness (e.g. body condition, growth rate, feeding rate, anti-predator behaviour, reproductive status, reproductive output) and modify the interactions between native and non-native species, both of which can underpin invasion success. Parasites have been recognised as important in the invasion process when naïve host populations are infected with parasites transported by invasive species (Prenter *et al.*, 2004).

Many invasive species achieve higher population densities in their invasive range than in their native range (Marr *et al.*, 2008), facilitating the transmission of non-native parasites to new naïve hosts (Dunn, 2009). Well studied examples of the impact of non-native parasite introduction on native species include the crayfish plague *Aphanomyces astaci* (Schikora) that was introduced in the UK in the 1970s along with its host, the American signal crayfish *Pacifastacus leniusculus* (Dana). *A. astaci* is asymptomatic in *P. leniusculus* but has caused high mortalities in the UK native crayfish, *Austropotamobius pallipes* (Lareboullet), eliminating many of its populations (Phillips *et al.*, 2008, Dunn, 2009). Similarly, *Anguillicola crassus* (Kuwahara, Niimi and Itagaki), a parasitic nematode which is native to Japan, was introduced to Europe and America through the aquaculture trade and has since caused high mortalities in native eel populations (Kennedy, 2007).

Non-native species can be 'released' from their native parasites when moved into new ecosystems that do not contain their native parasites. This process is known as the enemy release hypothesis and it allows resources formerly used in defence to be redirected to invasion success (Torchin *et al.*, 2003, Colautti *et al.*, 2004). Torchin *et al.* (2003) compared the parasites of 26 invasive species (spanning a wide range of taxa from molluscs to mammals) in their invasive and native range. They found that introduced populations were parasitized by approximately 50 % less parasites in their introduced range. A number of factors can contribute to a reduction of parasite species in introduced population including: (a) a small subset of the population being introduced resulting in a small subset of parasites successfully being introduced and the part and (b) the parasite's life history traits.

A parasite's life history traits, such as its life cycle and its host specificity, can determine whether a parasite escapes the enemy release process and is introduced to new communities. Parasites which require a single host to complete their life cycle, i.e. they have a direct life cycle (Dunn, 2009) and low host specificity (Torchin and Mitchell, 2004) are more likely to be established in a new ecosystem along with their invasive hosts. Gozlan *et al.* (2006) reports ten introduced parasites causing negative impacts on native hosts in Europe; 70 % of which have direct life cycles. Parasites with indirect life cycles involving intermediate hosts have lower opportunities of becoming established since suitable intermediate hosts may not be present in their new environment (Torchin *et al.*, 2003, Poulin, 2007, Dunn, 2009). There are however, notable exceptions including *A. crassus* which has an indirect life cycle involving both invertebrates and other fish as intermediate hosts. Torchin and Mitchell (2004) suggest that generalist parasites will have more opportunities to be transported to

introduced ranges and use a new set of local hosts. Invasive species experience a higher release from specialist parasites (Marr *et al.*, 2008), for example, salmonid fish harbour fewer specialist parasites in their invasive range compared to their native range (Kennedy and Bush, 1994).

The effect of generalism on pathogenicity has been largely overlooked (Woolhouse *et al.*, 2001) but is of particular interest in the context of introduced parasites. Multi-host parasites do not have predictable associations with pathogenicity. Empirical examples offer evidence for both lower and higher virulence to a new host compared to existing host(s) (Woolhouse *et al.*, 2001). When a new host contributes little to the parasite's fitness, unusually high levels of virulence have been observed; as in the case of *Echinococcus multilocularis* which is highly virulent to its human 'dead end' host (Woolhouse *et al.*, 2001). The fitness of a generalist parasite can differ across its range of hosts, and such parasites usually have reservoir host(s), i.e., hosts acting as the source of infection (Thrusfield, 2007). In the presence of reservoir hosts, a generalist parasite can occur in new hosts as sporadic infections, minor outbreaks, or major epidemics (Woolhouse *et al.*, 2001). The epidemiological and evolutionary processes driving these dynamics are poorly understood, but are critical in the context of emerging diseases.

The recent discovery of the rosette-like-agent (RLA), an intracellular parasite, in two invasive fish species, sunbleak *Leucaspius delineatus* (Heckel) and topmouth gudgeon *Pseudorasbora parva* (Temminck and Schlegel) has caused concern for European fish biodiversity (Gozlan *et al.*, 2005). Gozlan *et al.* (2005) reported loss of spawning, emaciation and death in *L. delineatus* cohabited with *P. parva* following infection with RLA. Morphologically, RLA is similar to the rosette agent, *Sphaerothecum*

destruens, a multi-host parasite that has been found to cause disease and high mortalities in salmonid species in the United States (Harrell *et al.*, 1986, Hedrick *et al.*, 1989, Arkush *et al.*, 1998). Until its discovery by Gozlan *et al.*, (2005), *S. destruens* had not been reported in Europe (Gozlan *et al.*, 2009).

The potential introduction and dissemination (via its invasive hosts) of *S. destruens* in the UK could have important implications for aquatic biodiversity. In view of the scientific uncertainty pertaining to the susceptibility of native cyprinid species to *S. destruens*, further research is needed on the life-cycle, prevalence and native species susceptibility was required. Here, in Chapter 1, the discovery and classification of RLA is reviewed (section 1.1), followed by a review of the epidemiology, life cycle, and pathology of *S. destruens* (section 1.2). *S. destruens* classification, life cycle and detection of Rhinosporideacae members are described in sections 1.3, 1.4 and 1.5. Finally, the life history characteristics of the two invasive hosts, *L. delineatus* and *P. parva*, and aims of the thesis are summarised in sections 1.6, 1.7 and 1.8 respectively.

1.1 Rosette-like-agent

RLA was first reported in the UK in 2005 following the cohabitation of *P. parva* with *L. delineatus* (see Gozlan *et al.*, 2005). In this laboratory-controlled cohabitation, high mortalities (69 %) were observed for *L. delineatus*. RLA was associated with 67 % of these mortalities (Gozlan *et al.*, 2005). RLA infection in *L. delineatus* resulted in microscopic lesions (Figure 1.1) similar to the ones observed in Chinook salmon *Oncorhynchus tshawytscha* (Walbaum). Further work, revealed that fathead minnow *Pimephales promelas* (Rafinesque) was also positive for RLA DNA following immersion to *P. parva* holding water (Gozlan *et al.*, 2005).

Successful isolation, culture and propagation of RLA in *Epithelioma papulosum cyprini* (see Fijan *et al.*, 1983) and Chinook salmon embryo cell cultures (Lannan *et al.*, 1984) lines provided further support that RLA may be an European isolate of *S. destruens*. Partial sequencing of the 18S rRNA gene (1050 bp) of RLA revealed 100 % sequence homology with the 18S rRNA gene of the Bodega Marine Laboratory isolate (Genbank accession no: AY267345) (Dr David Stone, personal communication).

In 2009, Polymerase Chain Reaction (PCR) amplification of the ribosomal internal transcribed spacer (ITS) identified RLA as *S. destruens* (see Gozlan *et al.*, 2009). Comparison of the amplified ITS regions of the three US *S. destruens* isolates with the UK isolate revealed that the UK and US isolates are geographically isolated. The source of *S. destruens* in the UK remains unknown and the UK isolate has been identified as *S. detruens* (UK).

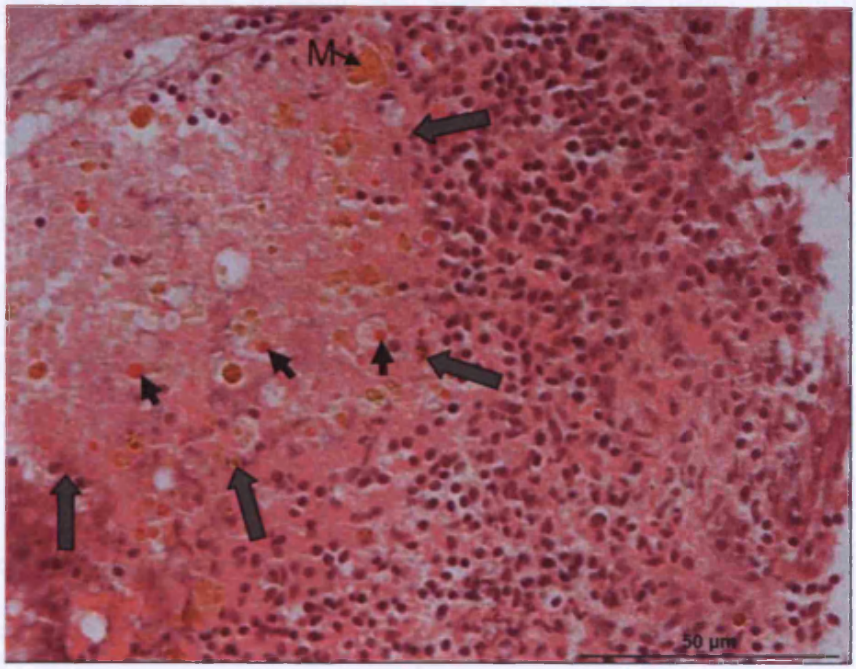


Figure 1.1: Light micrograph of connective tissue surrounding the liver of sunbleak *Leucaspius delineatus* stained with hematoxylin and eosin. *L. delineatus* were exposed to the rosette-agent *Sphaerothecum destruens* through cohabitation with topmouth gudgeon *Pseudorasbora parva*. *S. destruens* spores are eosinophilic (black arrows) and can be seen within necrotic tissues (grey arrows). Limited host immune response in the form of melanomacrophages (M) can be seen within the necrotic tissue. Picture by author.

1.2 *Sphaerothecum destruens*: epidemiology, life cycle and pathology

S. destruens is an obligate intracellular parasite belonging to the Rhinosporideacae family and the Mesomycetozoea class (Arkush *et al.*, 2003). *S. destruens* was first observed in Washington, USA, in net-pen reared *O. tshawytscha* where it caused 80% mortality in three year old fish (Harrell *et al.*, 1986). Laboratory and field observations led to the conclusion that *S. destruens* was a significant infectious disease of *O. tshawytscha* (see Kent, 2000). The parasite was later detected in sub-adult Atlantic salmon *Salmo salar* (Linnaeus) in a Northern Californian farm where it had caused chronic deaths (Hedrick *et al.*, 1989). The third reported occurrence of *S. destruens* was in winter run *O. tshawytscha* held at the Bodega Marine Laboratory in

California, where 40.1% of dead fish were found to be heavily parasitized with *S. destruens* (see Arkush *et al.*, 1998). In all three reported major outbreaks, the source of infection was unknown and the presence of an aquatic host reservoir was suspected. Screening of returning, hatchery-produced adult, late autumn run *O. tshawytscha* returning to the Upper Sacramento River of California revealed a 32% prevalence of *S. destruens* (see Arkush *et al.*, 1998).

Experimental infections are a powerful tool for determining whether new host-parasite associations are plausible (Poulin, 2007) and can be used to predict possible parasite impacts on naïve hosts. Following intraperitoneal injection with *S. destruens* spores, infection was achieved in coho salmon *O. kisutch* (Walbaum), rainbow *O. mykiss*, (Walbaum), brown *S. trutta* (Linnaeus) and brook trout *Salvelinus fontinalis* (Mitchill) (see Arkush *et al.*, 1998). Infection severity varied, with *O. kisutch* becoming heavily infected whilst the role of *O. mykiss*, *S. trutta*, and *S. fontinalis* as potential healthy carriers of the disease was highlighted (Arkush *et al.*, 1998). *S. destruens* prevalence was 98 % for *O. kisutch*, 42.5 % for *O. mykiss*, 43.3 % for *S. trutta* and 2.6 for *S. fontinalis* (see Arkush *et al.*, 1998). Natural infections in two salmonid species, *O. tshawytscha* and *S. salar* support the low host specificity of *S. destruens*. The wide range of host susceptibility revealed by the experimental infection using salmonids (Arkush *et al.*, 1998) suggests that in addition to *L. delineatus* other cyprinids could be potential hosts. Chapters 6 and 7 will be testing this suggestion by determining the susceptibility of carp *Cyprinus carpio* (Linnaeus), bream *Abramis brama* (Linnaeus), roach *Rutilus rutilus* (Linnaeus) and rudd *Scardinius erythrophthalmus* (Linnaeus).

S. destruens' life stages include spherical intra-cytoplasmic spore stages of two distinct morphotypes, 2-4 μm and 4-6 μm in diameter (Figure 1.2) (Arkush *et al.*, 2003). Spores replicate asexually through fission and can infect epithelial, mesenchymal, and hematopoietic cells, eventually causing cell death (Arkush *et al.*, 1998). *S. destruens* can infect the gills, heart, brain, kidney, liver, spleen, swim bladder, ovary, testis and the hind gut (Arkush *et al.*, 1998). It is most often detected in the kidney and upon release; the spores can infect further tissues or be excreted through the bile, urine, gut epithelium, and seminal and ovarian fluids (Arkush *et al.*, 2003). Fish infection is believed to occur through either ingestion and gut penetration, or attachment to the gills or skin, or both (Arkush *et al.*, 2003). When incubated in freshwater, spores undergo zoosporulation and release a minimum of five motile uniflagellate zoospores. Zoospores have an average body diameter and flagellum length of 2 μm and 10 μm, respectively (Arkush *et al.*, 2003). Although the spore stage of *S. destruens* is directly infectious, the zoospore stage has not yet been shown to be directly infectious (Arkush *et al.*, 2003). However, water-borne transmission of the zoospore stage has been demonstrated for the Rhinosporideacae member, *Dermocystidium salmonis* (see Olson *et al.*, 1991) and Arkush *et al.* (2003) proposed that the *S. destruens* zoospore stage could be infectious.

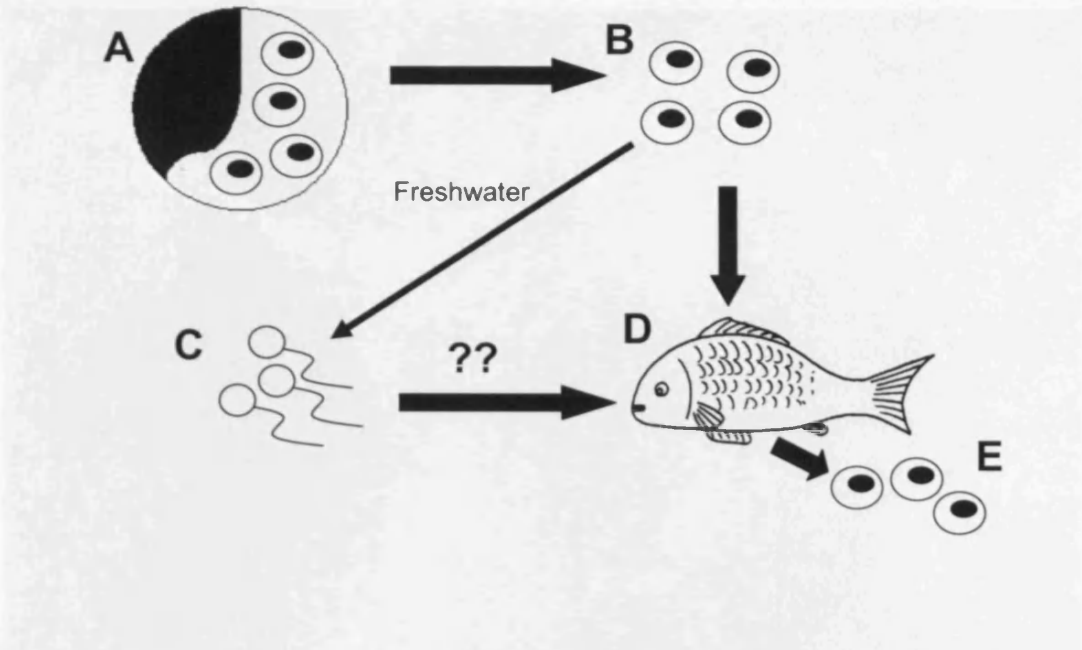


Figure 1.2: Proposed life cycle of *Sphaerothecum destruens* adapted from Arkush *et al*. (2003). (A) *S. destruens* spores infect cells and replicate sequentially through asexual division, eventually causing the host cell to erupt releasing spores (B). Released spores can release flagellated zoospores when incubated in sterile distilled water (C). Released spores can directly infect new host fish (D). Infection of new host fish by zoospores has not been demonstrated. Infected hosts can release spores via urine, bile, gut epithelium or reproductive fluids (E).

The cell wall of *S. destruens* is comprised of three well defined layers (Figure 1.3); an outer layer having a membraneous structure, a middle electron dense layer and an inner electro lucent layer (Elston *et al*., 1986). The outer cell wall layer has fibrogranular material adherent to its surface (Arkush *et al*., 1998).

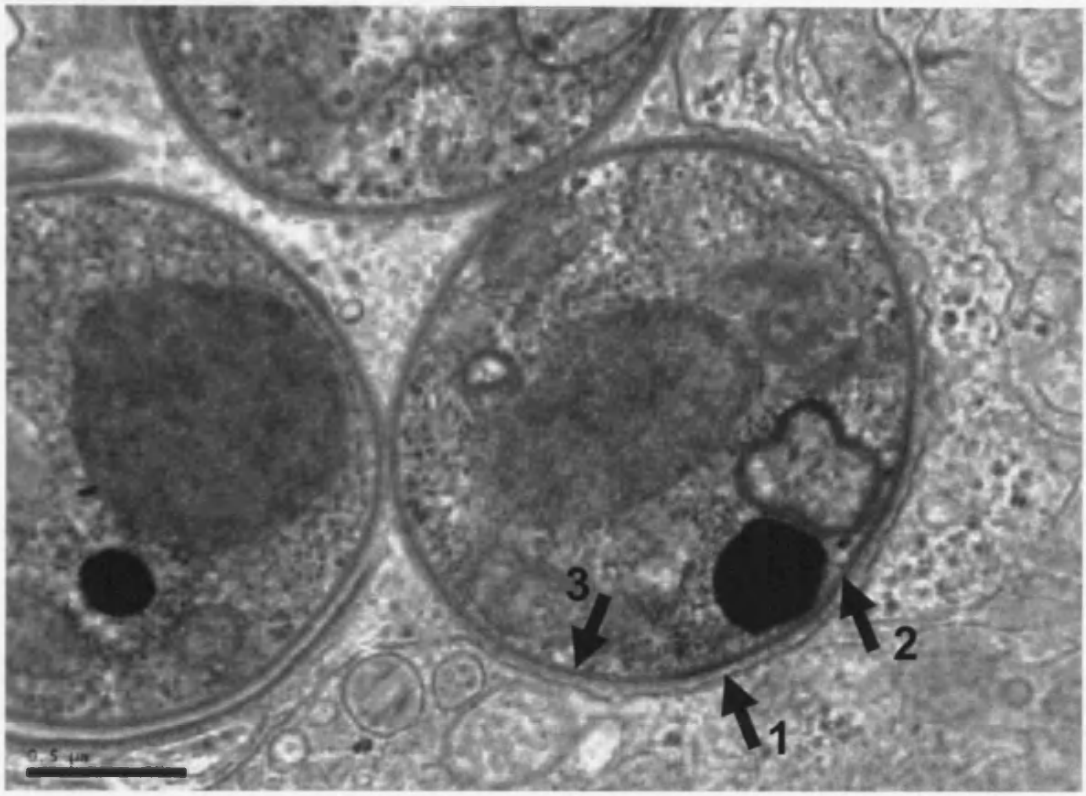


Figure 1.3: Transmission electron microscopy section of sunbleak *Leucaspius delineatus* renal tissue infected with *Sphaerothecum destruens*. The cell wall of *S. destruens* is comprised of three layers (indicated by arrows) (1) an outer layer with membraneous structure; (2) a middle electron dense layer and (3) an inner electrolucent layer. Scale bar: 0.5 µm. (Photo courtesy of Dr. Stephen W. Feist).

S. destruens pathology has been described for *O. tshawytscha* and *S. salar* (see Hedrick *et al.*, 1989, Arkush *et al.*, 1998) and it includes two types of microscopic lesions: disseminated and nodular (Arkush *et al.*, 1998). In the disseminated lesions, the agent is widely dispersed throughout the organism's organs with little host cell immune response. Where present, the host response includes edema and focal necrosis associated with developmental stages of the parasite (Elston *et al.*, 1986, Harrell *et al.*, 1986). In contrast, the nodular form of the disease is characterised by host cell immune response and the formation of distinct granulomas in visceral organs (Hedrick *et al.*, 1989, Arkush *et al.*, 1998). These disparate disease presentations may

reflect differences in host immune response, where fish with the disseminated presentation can be considered more susceptible to *S. destruens* while fish with granuloma formations might be considered more able to contain the infection (as a result of mounting an immune response). Although both microscopic lesions have been observed in the same species, species differences can also influence disease morphology (Arkush *et al.*, 1998). In Chapter 2, the pathology of naturally occurring *S. destruens* infections in *L. delineatus* is described and compared to the pathology observed in *O. tshawytscha.*

1.3 Classification history of *Sphaerothecum destruens* (Rhinosporideacae)

Species classification within the Rhinosporideacae has changed as a response to new taxonomical information. Phylogenetic analysis using nuclear-encoded small subunit rRNA gene sequences, has provided evidence for the existence of a clade of eukaryotic protists at the basal branch of the metazoa (Ragan *et al.*, 1996). This group of species has been referred to as the DRIPs clade (*Dermocystidium* spp; Rosette Agent; *Ichthyophonus hoferi* and *Psorospermium haeckelli*). As all DRIP members infected fish Cavalier-Smith (1998) placed them in the class Ichthyosporae. With the addition of *Rhinosporidium seeberi*, an agent that affects mammals and birds, the class Ichthyosporae became obsolete. Its members, the DRIPs and *R. seeberi*, were then grouped within the Mesomycetozoa to reflect their position between animals and fungi (Herr *et al.*, 1999). The class Mesomycetozoa was divided into two orders: Dermocystida and Ichthyophonida (Cavalier-Smith, 1998). Within the Dermocystida, *S. destruens*, *Dermocystidium* spp. and *R. seeberi* were then placed in the Rhinosporideaceae family (Mendoza *et al.*, 2001). The most recent addition to the Rhinosporideaceae family comes from the revision of the species *Dermocystidium*

ranae, which is a frog pathogen. *D. ranae* was renamed as *Amphibiocystidium ranae* to reflect its differences with the *Dermocystidium* genus (Pereira *et al.*, 2005).

The classification of protists, unicellular eukaryotes, has been changing in response to a deeper understanding of the relatedness between eukaryotic phylogenetic lineages (Adl *et al.*, 2005). In 2005, the Society of Protozoologists published a new level of classification for protists which consisted of a hierarchical system of nameless rank designations such as super-groups and numerically ordered ranks (Adl *et al.*, 2005). This new system replaced the traditional category designations of class, sub-class, super-order and order. For example, following the traditional classification system, *S. destruens*' systematics were: Kingdom Protozoa; Phylum Neomonada; Sub-phylum Mesomycetozoa; Class Mesomycetozoea; Order Dermocystida and Family Rhinosporidacea (Arkush *et al.*, 2003). Under the new proposed classification, *S. destruens* abides in the super-group Opisthokonta and is denoted as *S. destruens* (Mesomycetozoa: Ichthyosporea: Rhinosporideacae) (Adl *et al.*, 2005).

1.4 Rhinosporideacae life cycles

The complete life cycles of all members of the Rhinosporideacae have not been determined but current evidence suggests the absence of sexual development (Arkush *et al.*, 2003). However, most Rhinosporideacae members have only been studied during their parasitic stages and sexual development could potentially take place during their non-parasitic stages (Mendoza *et al.*, 2002). During their parasitic stages, *D. salmonis* and *R. seeberi* absorb nutrients through their cell wall (Mendoza *et al.*, 2002). Their putative life cycle indicates a direct life cycle with a free-living life stage (Figure 1.4) (Mendoza *et al.*, 2002). Different stages of the life cycle have been described with varying detail for the Rhinosporideacae members.

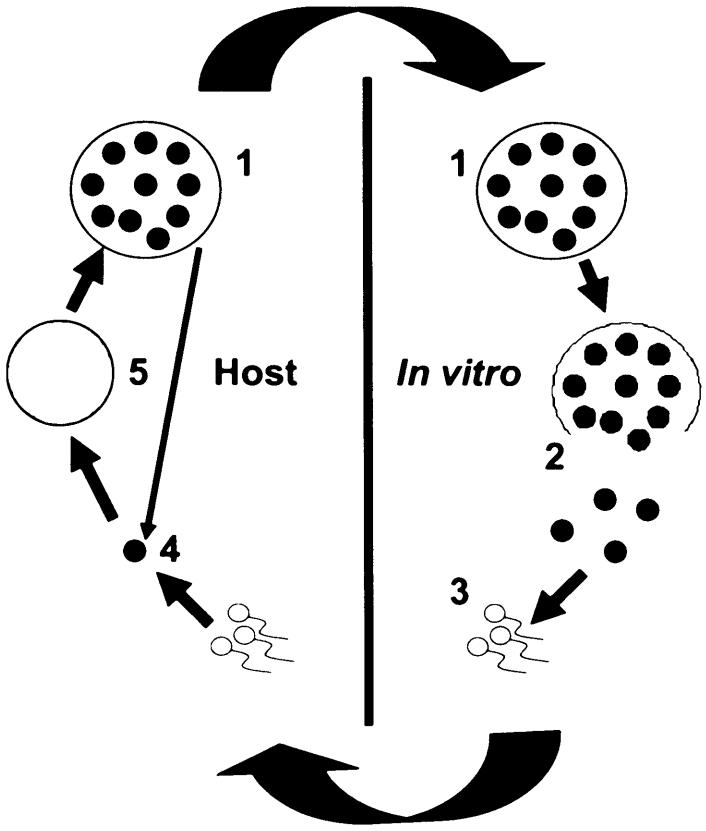


Figure 1.4: Putative life cycle of the Rhinosporideacae adapted from Mendoza *et al.* 2002. Members of the Rhinosporideacae develop spherical cells containing spores (stage 1). *In vitro* the spores can give rise to uniflagellated zoospores (stage 3). When the zoospores infect the host, they lose their flagella and encysts (stage 4), increase in size (stage 5) and undergo cleavage into spores (stage 1). Spores can be released within the host tissue and the life cycle is repeated within the host (stages 1, 4, 5, 1).

Members of Rhinosporideacae are hydrophilic parasites with global occurrence and low species specificity. This group can parasitize and cause disease in humans, birds, amphibians and fish (Table 1.1). *R. seeberi* has been reported to parasitize humans, dogs, cats and swans forming cutaneous and subcutaneous polyps mostly in the nasal cavity (Kennedy *et al.*, 1995, Herr et al., 1999, Mendoza *et al.*, 1999, Moisan and Baker, 2001, Nerurkar *et al.*, 2004, Hussein and Rashad, 2005, Silva *et al.*, 2005, Ghorpade, 2006, Harissi-Dagher *et al.*, 2006, Houreih *et al.*, 2006, Meier *et al.*, 2006, Mohanty *et al.*, 2006, Raveenthiran, 2006). A single disseminated form of the disease

has been reported in the literature (Raveenthiran 2006). Treatment involves surgical removal of the polyps and the geographical distribution of the parasite stretches from India to North America. *R. seeberi*'s life stages include spherical sporangia housing thousands of endospores (Herr *et al.*, 1999, Mendoza *et al.*, 1999). When mature, a single pore per sporangium is formed through which mature endospores are released several micrometers from the pore (Mendoza *et al.*, 1999). The endospores are then implanted and mature into sporangia carrying large numbers of endospores. Experiments have demonstrated the release of endospores in distilled water, saline solution and inactivated calf serum. No zooflagellate life stage has been observed (Mendoza *et al.*, 1999).

The most recent addition to the Rhinosporideacae, *A. ranae*, has been detected in wild green frog populations in Italy and Switzerland (Pascolini *et al.*, 2003, Pereira *et al.*, 2005). The agent has been detected under the skin where it forms spherical or U-shaped cysts containing endospores. An inflammatory response was observed in the infected tissue and a prevalence of 14.3-52.4 % has been reported in wild populations (Pascolini *et al.*, 2003).

The *Dermocystidium* spp. group includes both defined species such as *D. salmonis*, *D. cyprini*, *D. percae*, *D. fennicum*, *D. koi* and *D. anguilla*, as well as a large number of reported parasites only identified to genus level. They all infect freshwater fish belonging to a number of families (Table 1.1). All *Dermocystidium* members defined to a species level, except *D. koi* and *D. anguillae*, have a zooflagellate life stage. All except *D. percae* and *D. fennicum* infect the gills (Olson *et al.*, 1991, Olson and Holt, 1995, Lotman *et al.*, 2000, Pekkarinen *et al.*, 2003, Pekkarinen and Lotman, 2003). *D. percae* and *D. fennicum* infect the fins of perch *Perca fluviatilis* (Linnaeus)

(Pekkarinen *et al.*, 2003a, Pekkarinen *et al.*, 2003b). Where investigated, a temperature dependence of the zooflagellate stage has been reported, with higher temperatures promoting zoosporulation (Pekkarinen and Lotman, 2003).

Similar to *S. destruens*, the *Dermocystidium* spp. have been observed to cause disease in wild populations (Olson and Holt, 1995). However, most disease reports involve infections occurring in aquaculture facilities (Nash *et al.*, 1989, Cawthorn *et al.*, 1990, Hoglund *et al.*, 1997, Bruno, 2001, Zhang and Wang, 2005, Novotny and Smolova, 2006). Species within the *Dermocystidium* spp. complex have caused disease and mortality in species such as *O. tshawytscha*, *O. kisutch*, sockeye *O. nerka* (Walbaum) and pink *O. gorbuscha* (Walbaum) salmon, *O. mykiss* and *S. trutta*, *C. carpio*, *P. fluviatilis*, *R. rutilus*, bullheads *Cottus gobio* (Linnaeus) and catfish *Silurus meridionalis* (Chen) (see Cervinka *et al.*, 1974, Nash *et al.*, 1989, Cawthorn *et al.*, 1990, Olson *et al.*, 1991, Olson and Holt, 1995, Hoglund *et al.*, 1997, Athanassopoulou, 1998, Lotman *et al.*, 2000, Bruno, 2001, Pekkarinen *et al.*, 2003, Pekkarinen and Lotman, 2003, Feist *et al.*, 2004, Zhang and Wang, 2005, Novotny and Smolova, 2006). In most cases, the parasites form cysts on the gills, fins, skin, kidney, liver and heart. Histopathologically these parasites can cause a granulomatous response and systemic disease. No zooflagellate life stage has been identified for this group of organisms, however in the majority of cases the investigators did not look for this particular life stage. Overall, species of the genus *Dermocystidium* have been found to be distributed from Europe to China and North America (Cawthorn *et al.*, 1990, Zhang and Wang, 2005, Novotny and Smolova, 2006).

Table 1.1: Summary of life stages, disease morphology, affected organs and susceptible species of Rhinosporideacae members (only members classified to species level are included). All measurements in μm.

Species	Host	Geographical location	Spore and zoospore shape (D: diameter; FL: Flagellum length)	Disease morphology	Infection site	Reference [1]
Amphibiocystidium ranae	Frogs (*Rana esculenta*)	Italy, Switzerland	U-shaped or spherical cysts (D: 100-600) Cysts contain spherical encapsulated endospores (D: 2-6) No zoospore	Inflammatory response involving lymphocytes, macrophages & leukocytes	Ventral, toes	1, 2
Dermocystidium anguillae	Eel (*Anguilla anguilla*)	UK	Spherical parasite cells (D: 4-7) Parasite cells contained within elongated cysts. 3000 in length. No zoospore	Epithelial hyperplasia often causing fusion of secondary lamellae Inflammatory response in epithelial tissue.	Gills	3
Dermocystidium cyprini	Carp (*Cyprinus carpio*)	Former Czechoslovakia, Estonia	White, spherical cysts (D: 600 – 2000) Cysts contain spherical spores (D: 4 – 5) Zoospore (D: 0.2; FL: 8)	Hypertrophy	Gills	4; 5
Dermocystidium fennicum	Perch (*Perca fluviatilis*)	Finland	Plasmodium cysts; spherical, oval & dump-bell shape(D: ~ 360) Formation of sporoblasts which give rise to spores (D: not reported) Zoospore (D & FL: not reported).	Not reported	Skin of head region, fin bases (preferable dorsal fin)	6

Table 1.1 continued

Species	Host	Geographical location	Spore and zoospore shape (D: diameter; FL: Flagellum length)	Disease morphology	Infection site	Reference
Dermocystidium koi	Koi carp (*Cyprinus carpio* var *koi*)	UK	Spores formed in hyphae. Initially hyphae are narrow, thick walled cylinders (D: 10) Hyphae grow to thin walled tubes (D: 70) Spherical spores (D:6.5-15) No zoospore	Lesions with inflammatory response triggered by spore release	Skin	7
Dermocystidium percae	Perch (*Perca fluviatilis*) Ruff (*Gymnocephalus cernuus*)	Finland, Estonia	Elongated, cylindrical cysts (D: 580) Plasmodia segment into sporoblasts which mature into spores (D: 6-8) Zoospore (D: 2 μm; FL: 10-13)	Increased cellularity and pigmentation of fin tissue Inflammation	Abdominal fins	6,8
Dermocystidium salmonis	Chinook salmon (*Oncorhynchus. tschawytscha*) Coho salmon (*O. kisutch*) Sockeye salmon (*O. nerka*) Pink salmon (*O. gorbuscha*)	USA (Oregon)	White, spherical cysts (D: 500 – 1100) Cysts contain spherical spores (D: 5-8) Zoospore: (D: 1; FL: not reported)	Hyperplasia	Gills, epithelial tissues, visceral organs	9, 10
Rhinosporidium seeberi	Humans (*Homo sapiens*) Dogs (*Canis familiaris*) Swans (*Cygnus olor*)	Sri Lanka, USA (Florida, Georgia, North Carolina, Oklahoma) India, Egypt, Canada	Spherical sporangia (D: 60-450) Mature sporangia contain endospores (D: 7-15) No zoospore	Localised polyps	Nasal cavity, eyes, eyelids, thigh, knee	11-24

Table 1 continued

Species	Host	Geographical location	Spore and zoospore shape (D: diameter; FL: Flagellum length)	Disease morphology	Infection site	Reference [1]
Sphaerothecum destruens	Chinook salmon (*O. tshawytscha*) Atlantic salmon (*Salmo salar*) Coho salmon (*O. kisutch*) Brown trout (*S. trutta*) Rainbow trout (*O. mykiss*) Brook trout (*Salvelinus fontinalis*) Sunbleak (*Leucaspius delineatus*)	USA (California, Washington) UK	Spherical spores (D: 2-6) Zoospore: (D: 2; FL: 10)	Disseminated: parasites found intra- and extracellularly. If present, host response is in the form of edema and focal necrosis. Granulomatous: host cell immune response and granuloma formation in visceral organs.	Gills, heart, brain, kidney, liver, spleen, swim bladder, ovary, testis and hind gut	25-29

[1] (1) Pascolini *et al.* 2003 ; (2) Pereira *et al.* 2005; (3) Wooten and Vicar 1982; (4) Cervinka *et al.* 1974; (5) Lotman *et al.* 2000; (6) Pekkarinen *et al.* 2003; (7) Dykova and Lom 1992; (8) Pekkarinen and Lotman 2003; (9) Olson *et al.* 1991; (10) Olson and Holt 1995; (11) Fadl *et al.* 1995; (12) Kennedy *et al.* 1995; (13) Herr *et al.* 1999; (14) Mendoza *et al.* 1999; (15) Moisan and Baker 2001; (16) Nerurkar *et al.* 2004; (17) Hussein and Rashad 2005; (18) Silva *et al.* 2005; (19) Ghorpade 2006; (20) Harissi-Dagher *et al.* 2006; (21) Houreih *et al.* 2006) (22) Meier *et al.* 2006 (23) Mohanty *et al.* 2006; (24) Raveenthiran 2006; (25) Elston *et al.* 1986; (26) Harrell *et al.* 1986; (27) Hedrick *et al.* 1989; (28) Arkush *et al.* 1998; (29) Arkush *et al.* 2003

1.5 Rhinosporideacae detection

Detection and identification of Rhinosporideacae members mostly occur through microscopic examination of stained impression smears or histological sections of infected tissues. Owing to the highly similar nature of the organisms within this rank, such detection methods have low discriminatory power and can thus be problematic (Mendonca and Arkush, 2004). With the advent of molecular technologies, a number of diagnostic tests using PCR have been successfully developed for the detection of fish parasites and other pathogens (Mendonca and Arkush, 2004, De Andrade *et al.*, 2006, Whipps *et al.*, 2006).

PCR targets the pathogen's DNA and amplifies it to levels detectable through conventional DNA visualisation technologies (Mullis and Faloona, 1987). The benefits of PCR include a high degree of sensitivity, non-lethal sampling (where possible) and higher species discriminatory power (Mendonca and Arkush, 2004, Whipps *et al.*, 2006). Additionally, when the appropriate genetic markers are used, DNA regions amplified through PCR can be used in phylogenetic analysis to better discern the pathogen's taxonomy (Whipps *et al.*, 2006) and determine the presence of sexual or asexual reproduction (O'gorman *et al.*, 2009). Disadvantages of PCR include the inability to determine infection intensity, discriminate between viable and non-viable organisms and contamination risks leading to falsely assigned pathogen positive samples (Whipps *et al.*, 2006). Such drawbacks can be addressed through the use of quantitative PCR, histological inspection of PCR positive samples, and the use of appropriate controls to detect contamination events.

Ever since the work of Olsen and Woese (1993) on the use of ribosomal RNA sequences for determining molecular phylogenies, these molecules have been widely

used in phylogenetic studies (Rivas *et al.*, 2004). Ribosomal RNA consists of a large and small subunit. The eukaryotic small subunit (18S rRNA) is considered a single RNA species and has been used in phylogenetic studies of eukaryotes (Sogin *et al.*, 1986, Olsen and Woese, 1993). As a result, large databases of 18S rRNA sequences for a wide range of organisms are electronically available from public databases. The ease of access to such information favours the use of the molecule in the development of molecular diagnostic tools (Elwood *et al.*, 1985, Gargas and Depriest, 1996, Krieger and Fuerst, 2002, Bower *et al.*, 2004, Mendonca and Arkush, 2004, Rivas *et al.*, 2004, Whipps *et al.*, 2006).

The majority of reported infections with *Dermocystidium* spp. have not been classified to species level as discussed in section 1.4. In histological sections these species are morphologically similar and difficult to discriminate. Analysis of their 18S rRNA gene would result in a species increase within the Rhinosporideacea and add to epidemiological records of already established species. For example, *S. destruens* was known as the rosette agent or *Dermocystidium*-like parasite since its first reported record in 1986 until it was classified as *S. destruens* by Arkush *et al.* (2003).

1.6 *Leucaspius delineatus* and *Pseudorasbora parva* as *Sphaerothecum destruens* hosts

Today the only identified cyprinid hosts of *S. destruens* are *L. delineatus* and *P. parva*. These small cyprinids (up to 8 cm long) possess life-history traits and reproductive behaviours that would favour their establishment in new ecosystems. These include sexual maturation at one year of age, batch spawning and nest guarding by the males (Pinder and Gozlan, 2003). Their spawning season is typically between April and July during which the females lay several egg batches. Both fish species are invasive to the

UK and have been introduced through the ornamental and aquaculture trade (Gozlan *et al.*, 2002, Pinder and Gozlan, 2003).

L. delineatus originate from continental Europe and Russia and are distributed from the Caspian Sea to the North Sea and from the Volga to Britany, France. They are now considered rare or vulnerable across their native range under Appendix III of the Bern convention (Wcmc, 1996). *L. delineatus*, is the only representative of its genus and only nest guarder amongst European cyprinids (Gozlan *et al.*, 2009). They live in large shoals, feed on zooplankton and terrestrial insects and are sexually dimorphic with the females being larger. During spawning, males chose territories around the stems and leaves of water lilies and encourage a number of females to spawn (Gozlan *et al.*, 2003a).

Male *L. delineatus* sunbleak display parental care in the form of defending their nest until the eggs hatch (Gozlan *et al.*, 2003c) three days later (mean temperature 21.6 °C) (Pinder and Gozlan, 2004). Male *L. delineatus* do not display sexual ornamentation in the form of tubercles, or sexual colouration (Gozlan *et al.*, 2003c). However, high courtship and aggression in male *L. delineatus* has been associated with reproductive success (Gozlan *et al.*, 2003b). Such behaviours (i.e. courtship and aggression) are energy demanding (Knapp and Warner, 1991) and are used to signal to females that males are in good condition (Gozlan *et al.*, 2003b).

Cohabitation of *L. delineatus* with *P. parva* (an identified healthy carrier of *S. destruens*) during *L. delineatus*' reproductive season has shown that *L. delineatus* are highly susceptible to this parasite (Gozlan *et al.*, 2005). Infection with *S. destruens*, has been associated with total inhibition of spawning and significantly higher

mortalities in *L. delineatus* (Gozlan *et al.*, 2005). *S. destruens* prevalence ranged from 67 % in moribund or dead *L. delineatus* (n = 12) to 28 % in subclinical fish (n = 32) (Gozlan *et al.*, 2005). The work by Gozlan *et al.* (2005) identified *S. destruens* as a high risk to *L. delineatus* populations and a potential contributor to the species' decline.

P. parva is native to China, Korea, Japan and the River Amur basin (Gozlan *et al.*, 2002). They live in the bentho-pelagic zone and their diet consists of algae, benthic invertebrates, molluscs and zooplankton (Xie *et al.*, 2000). This species is also sexually dimorphic with males being larger than females. Secondary sexual characteristics during spawning have been observed including darker body colouration and tubercles around the mouth (Pinder and Gozlan, 2003). Females spawn with several different males on rock undersides and any flat-surfaced object. Males guard their nests until the eggs hatch seven days later.

There is a lack of thorough studies of the parasitic fauna of *L. delineatus* in its native range. One study on its parasitic fauna in its UK invasive range, reports seven parasite species in *L. delineatus* including *Neoergasilus japonicus* (Harada) and *Ergasilus briani* (Markewitsch), which are non-native to the UK (Beyer *et al.*, 2005). *P. parva* has been reported to harbour a number of parasites, both in its native (Sithithaworn and Haswell-Elkins, 2003, Parvathi *et al.*, 2007, Urabe *et al.*, 2007, Kim *et al.*, 2008, You *et al.*, 2008) and invasive range (Ahne and Thomsen, 1986, Cesco *et al.*, 2001, Galli *et al.*, 2007, Kakalova and Shonia, 2008), but has not been reported to be infected by *S. destruens* or a *Dermocystidium* –like species. However, the majority of studies targeted specific parasites and none focused on *Dermocystidium*-like parasites.

1.7 Aims and Objectives

This thesis aimed to further investigate the proposed life cycle of *S. destruens*, its prevalence in England and determine the susceptibility of four cyprinid species to the parasite. It is divided in two parts. In part A, the intracellular and free-living stages of *S. destruens* are addressed along with the parasite's prevalence in the wild. Part B investigates the influencing the susceptibility of cyprinid species to *S. destruens*.

1.8 Overview of chapters

The following sections give a brief overview of each chapter. Chapter 3 has been published and in this case the title of the chapter is the same as that of the published paper. Chapter 3 is referenced as both Chapter 3 and the paper in other chapters. Chapters that have not yet been submitted are referenced as the relevant chapter number and have been written in the form of papers. Where information in the materials and methods sections overlap between chapters, reference to the relevant section in a previous chapter is made.

Chapters 2 and 4-7 include histological analysis of *S. destruens* samples detected as positive by PCR. These samples were collected and fixed in 10 % neutral buffered formalin by the author. Embedding of the samples in paraffin wax blocks was performed by the author at the Cefas Laboratory, Weymouth. Sectioning and staining of the sections was performed by the Histology team at Cefas, Weymouth. Reading of the slides through light microscopy was performed by the author.

1.8.1 Part A: *Sphaerothecum destruens* life cycle - intracellular and free-living stages and prevalence in the wild.

Chapter 2: First occurrence and associated pathology of *Sphaerothecum destruens* in cyprinids.

This chapter describes the pathology associated with *S. destruens* in the cyprinid *L. delineatus* and compares the pathology to that reported for *O. tshawytscha* and *S. salar*. *S. destruens* life cycle characteristics such as tissue tropism and parasite over-dispersion are discussed and the implications for spread, detection and potential impact on naïve native cyprinid species are presented.

Chapter 3: Temperature influence on production and longevity of *Sphaerothecum destruens*' zoospores.

The temperature influence on longevity and production of *S. destruens*' free-living stages, i.e. the zoospores, is investigated in this chapter. The influence of a range of temperatures (4-30 °C) on the production of zoospores was determined. This work provides important information on the potential for environmental persistence of *S. destruens* and its life cycle with direct implications for *S. destruens*' risk assessment.

1.8.2 Part B: Susceptibility to *Sphaerothecum destruens*.

Chapter 4: Influence of sunbleak *Leucaspius delineatus*' reproductive state on *Sphaerothecum destruens* prevalence and infection level.

Chapter 4 investigates the influence of host reproductive state on *S. destruens* prevalence in *L. delineatus*. Increased investment in reproduction could decrease the energy invested in immune function and could facilitate parasite infection. Work by Gozlan *et al.* (2005) has indicated that reproductive *L. delineatus* may be highly susceptible to *S. destruens*. In this chapter, the influence of reproductive state is investigated further by comparing *S. destruens* prevalence and infection levels in

reproductive and non-reproductive *L. delineatus*. Infection levels are determined using a quantitative PCR which was developed and optimised for this work.

Chapter 5: Multi-host complexes: the effect of a second host, topmouth gudgeon *Pseudorasbora parva*, on the prevalence and infection level of *Sphaerothecum destruens* in the primary host, the sunbleak *Leucaspius delineatus*.

The prevalence of generalist parasites can be influenced by the number of available hosts. Work on the influence of multi-host complexes on the prevalence of generalist parasites is lacking form the literature. As a multi-host parasite, *S. destruens* is used to test the influence that the second host, *P. parva*, can have on the prevalence and infection level of *S. destruens* in *L. delineatus*.

Chapter 6: Susceptibility of cyprinid species to *Sphaerothecum destruens*.

The susceptibility of four economically important cyprinids, *Cyprinus carpio*, *Rutilus. rutilus*, *Abramis brama* and *Scardinius erythrophthalmus* is determined in this chapter. These four cyprinid species are exposed to *S. destruens* through water-borne immersion with *S. destruens* spores. Survival analysis of the treatment and control groups is performed and the level of susceptibility of each species is determined. The influence of phylogenetic distance on susceptibility to *S. destruens* is investigated for cyprinid and salmonid species and the level of host specificity displayed by *S. destruens* is determined.

Chapter 7: Susceptibility of cyprinid species exposed to *Sphaerothecum destruens* in semi-natural conditions.

The susceptibility of *C. carpio*, *R. rutilus*, *A. brama* and *S. erythrophthalmus* is further investigated under semi-natural conditions. This approach provides a setting that is more representative of the conditions in which these species exist in the wild. It allows for the determination of susceptibility under more natural conditions as well as provides information regarding the detection of mortalities in more natural settings. The implications for *S. destruens* detection are discussed.

Chapter 8: Discussion

The chapter examines the results and conclusions of the thesis, placing them in context of each other and the overall literature. It examines the overall conclusions that can be made and what further work is required.

Part A: *Sphaerothecum destruens* life cycle: intracellular, free-living stages and prevalence in the wild.

Chapter 2: First occurrence and associated pathology of *Sphaerothecum destruens* in cyprinids.

Abstract *Sphaerothecum destruens* is a novel obligate intracellular parasite of fish, which has caused disease and mortalities in North American *Oncorhynchus tshawytscha* and in *Salmo salar* aquaculture. In the UK, *Sphaerothecum destruens* has been associated with *Pseudorasbora prava* and *Leucaspius delineatus*. The association of *S. destruens* with the invasive species *L. delineatus* increases the risk of disease spread. Accurate disease identification based on thorough descriptions of clinical signs and histopathology is paramount to epidemiological studies. In this study, the first record of *S. destruens* in wild populations of *L. delineatus* is presented and its associated histopathology is described along with its pathogenesis. Histological examination of 100 *L. delineatus* from Stoneham Lakes, UK, revealed the presence of *S. destruens* infections with a prevalence of 5 %. *S. destruens* showed an over dispersed distribution and infections were observed in all vital organs (liver, kidney, spleen, intestine). Similar to *O. tshawytscha* and *S. salar*, the parasite was most prevalent in the kidney. Histological examination revealed the presence of two types of microscopic lesions similar to the disseminated and nodular lesions reported from *O. tshawytscha*.

2.1 Introduction

S. destruens is a novel obligate intracellular parasite of fish, which has caused disease and mortalities as high as 80 % in north American *O. tshawytscha* and chronic mortalities in *S. salar* aquaculture (Elston *et al.*, 1986, Harrell *et al.*, 1986, Hedrick *et al.*, 1989, Arkush *et al.*, 1998). Numerous potential hosts for *S. destruens* have been identified through experimental infections and they include *O. kisutch*, *O. mykiss*, *S. trutta*, *S. fontinalis* (see Arkush *et al.*, 1998) within the salmonidae family and *L. delineatus*, *P. parva* and *P. promelas* within the Cyprinidae family (Gozlan *et al.*, 2005). A detailed description of the life cycle and pathology of *S. destruens* in salmonids is provided in Chapter 1, section 1.2. *S. destruens*' life cycle and associated pathology in cyprinids have yet to be determined and could differ from salmonids.

Although *L. delineatus* has experienced high declines in its native range and is currently listed on the IUCN red list of vulnerable species (WCMC, 1996) it is invasive to the UK. *L. delineatus* was introduced to the UK in 1986 at a commercial fishery in southern England and has since spread to the entire Bridgewater canal and River Rue catchment (Gozlan *et al.*, 2003c). The rapid dispersal of *L. delineatus* from its points of introduction has been facilitated by its life history traits such as; its reproductive behaviour (batch spawning and male parental care), early sexual maturity and small adult size. Its association with the aquaculture trade has facilitated its dispersal from its initial introduction, with the species being reported in still water commercial fisheries as well as several unconnected ponds and lakes (Gozlan *et al.*, 2003c).

Under experimental conditions, i.e., cohabitation between *P. parva* and *L. delineatus*, *S. destruens* has been linked to emaciation and high mortalities in *L. delineatus* (Gozlan *et al.*, 2005). In this experiment, 67 % of the moribund *L. delineatus* and 28 % of non-moribund *L. delineatus* were positive for *S. destruens* (see Gozlan *et al.*, 2005). An association in the UK of *S. destruens*, a multi-host parasite, and the invasive *L. delineatus* would potentially increase the risk of *S. destruens* spread and would facilitate its introduction to new naïve native species.

Disease expression (i.e. pathology) can differ in hosts belonging to different families (Arkush *et al.*, 1998) and can be misidentified. Accurate disease identification based on thorough descriptions of clinical signs and histopathology is thus paramount to further support epidemiological studies. Here, the first record of *S. destruens* in wild populations of *L. delineatus* is presented and its associated histopathology is described along with its pathogenesis.

2.2 Materials and methods

2.2.1 Samples

The *L. delineatus* population in Stoneham lakes (SU 438 173) was sampled in spring 2006. A total of 100 individual fish were caught by seine netting of the lake. *L. delineatus* were transported live to the Cefas laboratory in 90 L bins supplied with oxygen with a maximum transport time of four hours. On arrival, the fish were kept in a flow through system for three months at 16°C. *L. delineatus* were subsequently euthanised with an overdose of methane tricaine sulfonate (MS- 222, Sigma-Aldrich).

2.2.2 Light microscopy

Tissues were fixed in 10% neutral buffered formalin (NBF) for 24 hours before being transferred to 70 % industrial methylated spirit (IMS). Fixed fish were cut with a sharp blade along their body (as illustrated in Figure 2.1) to obtain transverse sections. Samples were infiltrated with paraffin under vacuum using standard protocols (Bancroft and Stevens, 1996). Sections were cut to a thickness of 3-5 μm, mounted onto glass slides, and stained with haematoxylin and eosin (H&E) or Gram's stain. Five transverse stained sections were analysed by light microscopy (Nikon Eclipse E800); digital images and measurements were obtained using the Lucia™ Screen Measurement System (Nikon, UK). The severity of infection with *S. destruens* was determined for each individual at 4 × to 40 × objective magnification using five transverse sections. Scores of severity ranged from zero to three with zero representing no infection. Scores were based on parasite numbers present across organs and disease pathology (see Table 2.1). Disease prevalence was calculated as: (number of *S. destruens* positive fish / total number of fish tested) × 100.

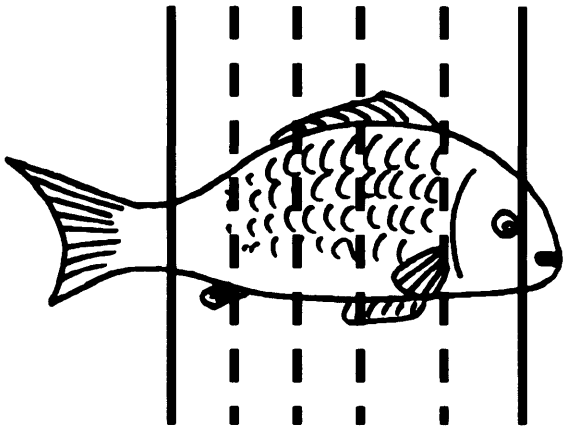


Figure 2.1: Illustration of fish dissection to obtain transverse sections following fixation with 10 % Neutral Buffered Formalin for 24 hours. First, the mouth and tail (just after the viscera) were cut and discarded (cut positions indicated with full lines). The remaining body was cut at four positions (indicated with dotted lines) to give five pieces from which the transverse sections were obtained.

Table 2.1:Description of severity grading for natural infections of *Sphaerothecum destruens*. Scores were obtained by estimating the total number of parasites in five transverse sections stained with haematoxylin & eosin and Gram's stain per fish at 40 × magnification using a light microscope. The total number of parasites in the five transverse sections was used to determine the severity grade.

Severity grade	Description
0	No infection present
1	1-5 parasites present in small focal areas of infection.
2	5-10 parasites present in multifocal areas of infection. Parasites intra- or extracellular.
3	> 10 parasites present in infections of multiple organs. Several parasites present within infected cells and disrupted and necrotic tissue may be present.

2.3 Results

2.3.1 *Sphaerothecum destruens* prevalence in Stoneham lakes.

Infection with *S. destruens* was detected in five fish (of the 100 captured), two of which were heavily infected with *S. destruens* with a severity grade of three (Table

2.2). Infected fish showed a range of disease severities with highest severity scores in males. Overall, *S. destruens* prevalence was determined to be 5 % and the kidney was parasitized in all five positive fish. Parasitized fish did not show any external and internal gross signs of disease and did not appear emaciated.

Table 2.2: Disease severity, infected organs, and gender of the five infected *Leucaspius delineatus* from Stoneham lakes. ♀ : female; ♂ : male

Individual	Disease severity	Organs infected	Gender
SB-35	1	Kidney	♀
SB-77	1	Kidney	♀
SB-7	2	Kidney, Spleen, Gonad	♂
SB-59	3	Kidney, Spleen, Liver, Intestine, Gonad, Eye, Adipose tissue	♂
SB-83	3	Kidney, Spleen, Liver, Intestine, Gonad, Eye, Adipose tissue, Muscle	♂

2.3.2 Light microscopy

S. destruens infection in *L. delineatus* was systemic with all vital organs being infected. Infection was observed in the kidney, spleen, liver, intestine, gonad, eye, adipose tissue (surrounding the intestinal tract) and skeletal muscle. Both the disseminated and nodular disease morphologies were observed with the former being most common. Intense inflammation was observed in the testis and kidney (Figure

2.2). Numerous stages of the organism were observed within the tissues with most stages appearing to be intracellular. *S. destruens* was deeply eosinophilic with H & E.

In the disseminated form, parasites were present both intra- and extracellularly within infected tissues and formed rosettes, i.e., aggregates of closely apposed spores as shown in Figure 2.3. Lesions varied in severity from intense (Figure 2.4A) to mild infections with minimal host cell response (Figures 2.4B,C). Hepatic lesions were associated with numerous *S. destruens* cells and inflammation (Figure 2.4A). Lesions appeared to infiltrate the hepatic parenchyma and parasites were present within macrophages. In the eye tissue, *S. destruens* cells were present within macrophages and giant cells (Figure 2.4B).

In the nodular form of the disease, multifocal granulomas of variable sizes were detected in the testis and liver (Figures 2.2A,B & 2.4D). A range of granuloma stages were observed; from enlarged macrophage aggregates surrounded by a single-cell layer of connective tissue (Figure 2.4D) to well demarcated ones surrounded by a thick fibroblast layer (Figure 2.2B). Granulomas replaced the normal parenchyma of the testis, and liver and were characterised by cellular debris, inflammation, and numerous macrophages. These granulomas contained numerous single parasites as well as parasite rosettes. Affected testis tissue was characterised by different sized granulomas, multifocal necrosis and intense inflammation (Figures 2.2A,B). Several 'ghost' (dead) parasites were present within these granulomas and were seen in lower numbers in other tissues (Figure 2.2B).

S. destruens infections in *L. delineatus* were caused by the 2-4 μm in diameter spore morphotype. Infection was often better discerned using Gram's stain instead of H & E (Figure 2.5 A, B). Gram stained sections clearly demonstrated Gram positive granules in the cytoplasm of *S. destruens*.

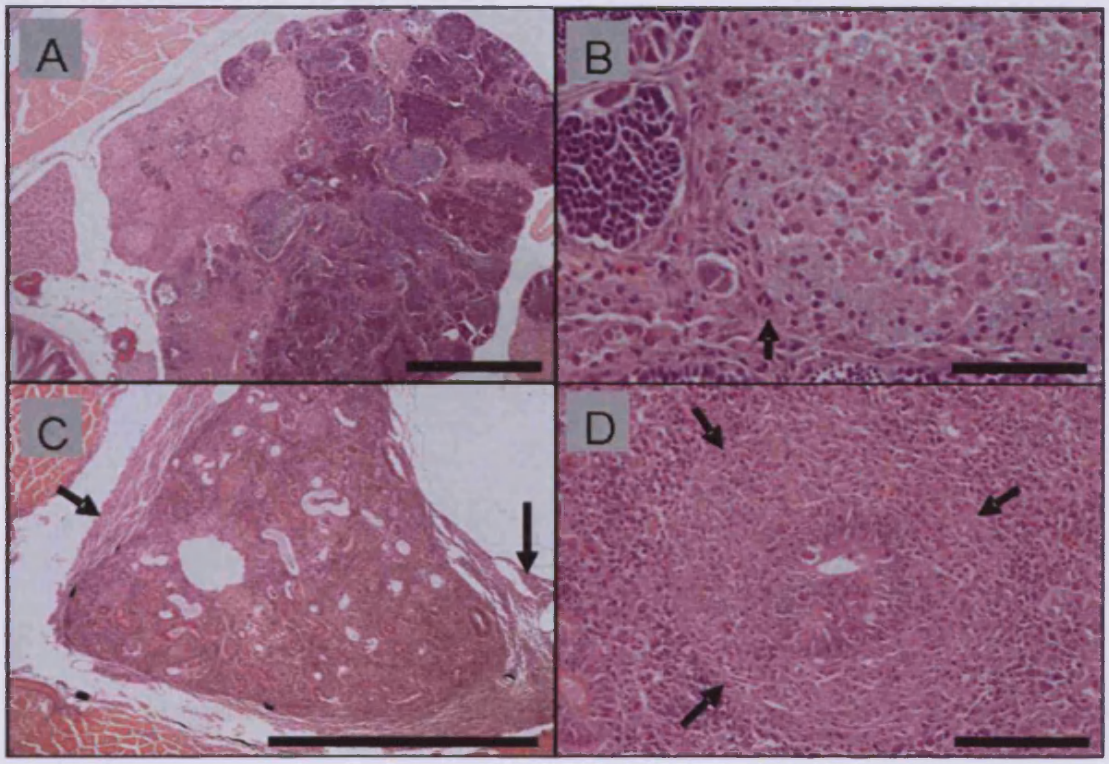


Figure 2.2 : Light micrographs of tissue sections stained with hematoxylin and eosin from naturally infected sunbleak *Leucaspius delineatus* with *Sphaerothecum. destruens*. (A) Low power view of testis showing localised, multiple, variably-sized, granulomas. Scale bar: 1 mm. (B) High power view of granuloma in the testis. Granuloma is surrounded by a thin fibroblast layer (arrow). Within the granuloma there are numerous stages of *S. destruens*, cell necrosis, numerous 'ghost' (dead) parasites, and macrophages. Scale bar: 50 μm. (C) Low power view of kidney. Note inflammation around the organ periphery (arrows). Scale bar: 1 mm. (D) Intense inflammation surrounding a kidney tubule (arrows). Clusters of *S. destruens* are present within tubular epithelial cells. Scale bar: 100 μm.

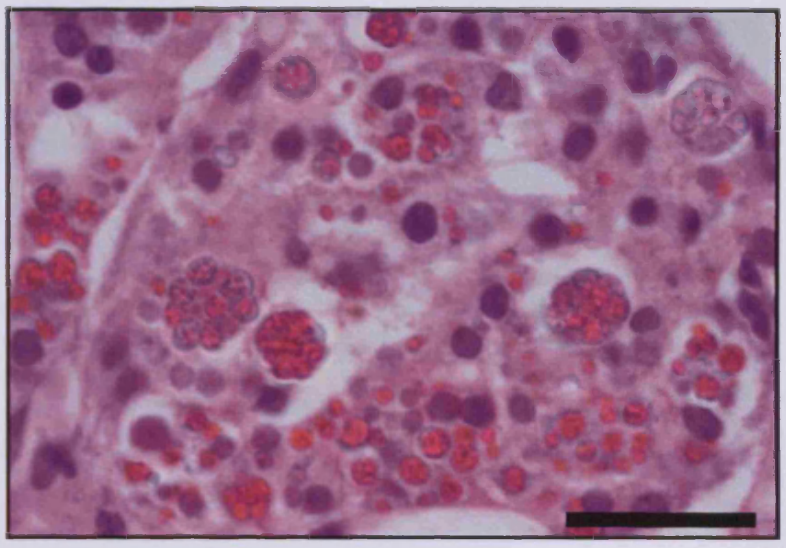


Figure 2.3: Light micrograph of the kidney stained with hematoxylin and eosin from naturally infected sunbleak *Leucaspius delineatus* with *Sphaerothecum destruens*. High power view showing variably-sized, intra- and extracellular *S. destruens* rosettes. Scale bar: 20 µm.

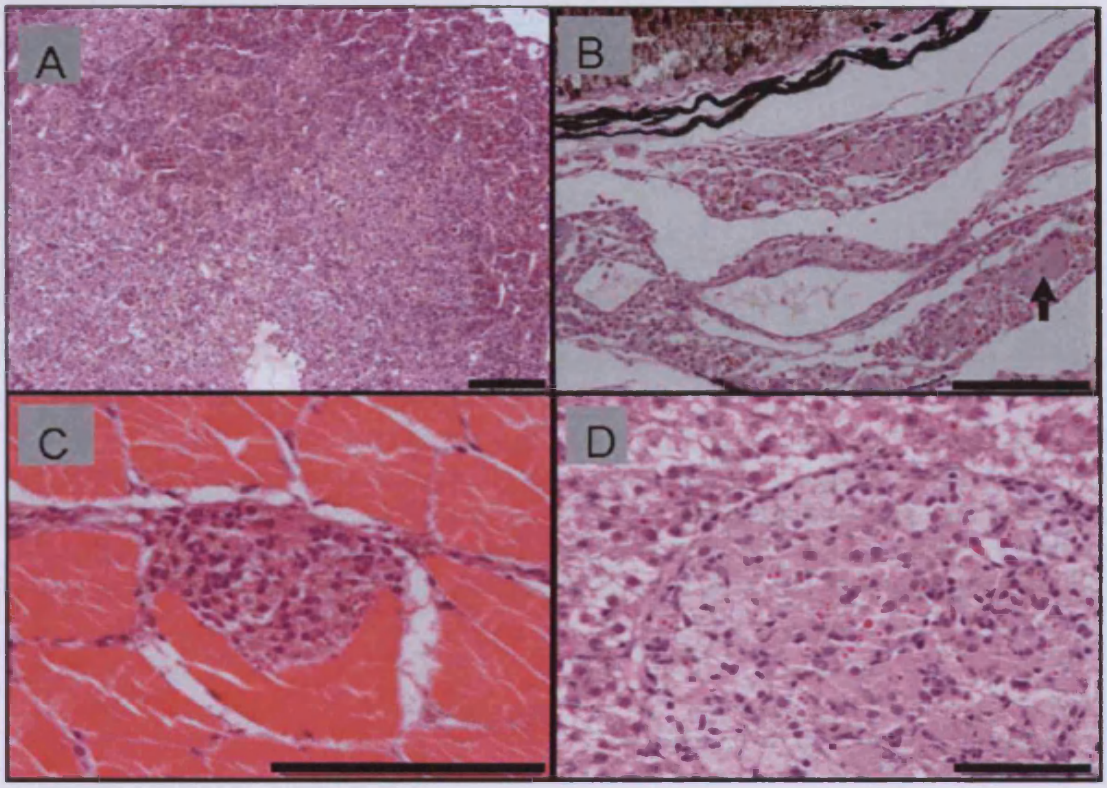


Figure 2.4: Light micrographs of tissue sections stained with hematoxylin and eosin. from naturally infected sunbleak *Leucaspius delineatus* with *Sphaerothecum destruens*. (A) Hepatic lesions associated with numerous *S. destruens* spores. There are extensive lesions associated with *S. destruens* infiltrating into the hepatic parenchyma. Scale bar: 100 µm. (B) *S. destruens* in the connective tissue and vessels posterior to the retina. *S. destruens* cells associated with melanomacrophages and giant cells (arrow). Scale bar: 100 µm. (C) Small focus of inflammatory tissue associated with *S. destruens* cells present between muscle fibres. Scale bar: 100 µm. (D) Enlarged macrophage aggregation in the liver containing moderate numbers of *S. destruens*. Scale bar: 50 µm.

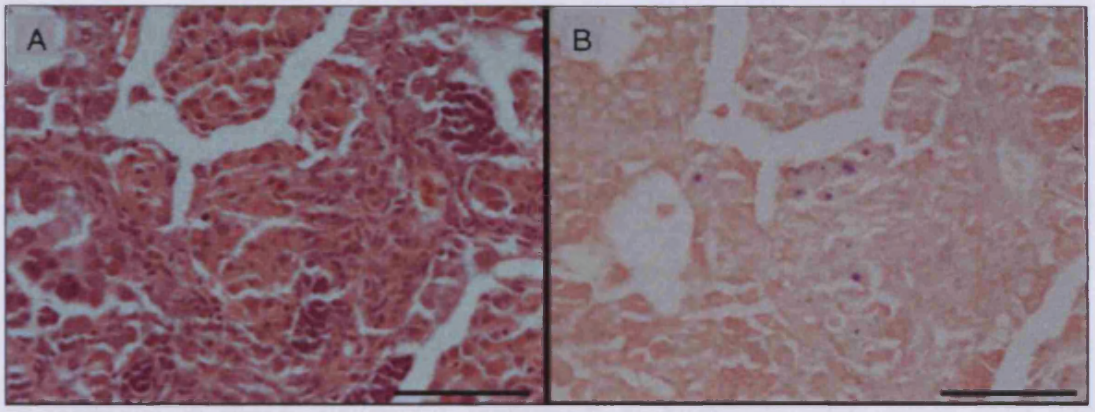


Figure 2.5: Light micrographs of the testis section, stained with (A) hematoxylin and eosin and (B) Gram's stain in naturally infected sunbleak *Leucaspius delineatus* with *Sphaerothecum destruens*. (A) There is extensive proliferation of connective tissue replacing the normal testis parenchyma. (B) Gram positive *S. destrues* spores (staining blue) are evident within the proliferation of connective tissue. Scale bar: 50 μm.

2.4 Discussion

This is the first description of an *S. destruens* infection in a wild cyprinid population. Histological examination of 100 *L. delineatus* from Stoneham lakes revealed the presence of *S. destruens* infections with a prevalence of 5 %. As the fish were kept in a flow through system for three months prior to being sampled, the natural prevalence as observed in the *L. delineatus* population at Stoneham lakes could have been overestimated. Housing the fish in experimental tanks for three months could have increased disease transmission between infected and uninfected leading to a potential overestimation of *S. destruens* prevalence. However, even with the use of Gram's stain low infections are difficult to detect (Mendonca and Arkush, 2004). Thus, the 'true' prevalence of the parasite would be expected to be higher.

A better understanding of the parasite's abundance in the wild and the factors influencing it would be gained by monitoring prevalence of *S. destruens* at different times during the year and across years. *S. destruens* prevalence in wild populations has only been reported from a single survey of the late autumn run of *O. tshawytscha* from the Sacramento River, California, USA where a 32 % *S. destruens* prevalence was detected (Arkush *et al.*, 1998). However, in captive *O. tshawytscha* broodstock, infection prevalence ranged from 0.7 to 40.1 % within three cohort (Arkush *et al.*, 1998). In laboratory experiments, infection with *S. destruens* in *L. delineatus* reached 28 % in non-moribund fish (Gozlan *et al.*, 2005). This is considerably higher than the 5 % prevalence observed in this study.

No internal or external gross signs of disease were present in examined *L. delineatus*. Emaciation was observed in moribund *L. delineatus* parasitized with *S. destruens*

following cohabitation with *P. parva* (see Gozlan *et al.*, 2005). Emaciation was not observed in the *S. destruens* positive fish in this study, however, it is highly probable that most emaciated fish would not be successfully sampled in the wild. Histological examination revealed the presence of disease forms similar to the disseminated and nodular morphologies reported from salmonids. The disseminated form appeared to be the most common disease form in *L. delineatus*; however, examination of more *S. destruens* positive fish could increase the number of reported nodular morphologies in this species. Differences in *S. destruens* pathology between *L. delineatus* and *O. tshawytscha* included the first report of *S. destruens* spores associated with giant cells (Figure 2.4B), and the presence of only the smaller spore morphotype (2-4 μm) in *S. destruens* infections of *L. delineatus* tissues.

In the disseminated form, *S. destruens* spores were often associated with the proliferation of connective tissue. This was observed in all organs. It has been suggested that the disseminated form of the disease and the lack of host response is indicative of fish which are more susceptible to the disease (Hedrick *et al.*, 1989, Arkush *et al.*, 1998). Individual variations in resistance to *S. destruens* could explain the two disease forms reported in *L. delineatus*. However, these two disease forms could also represent different stages of the disease. For example, in sample SB-83, both disease forms have been observed within the same individual. If the disease was allowed to progress, the formation of granulomas in organs displaying pathology similar to the disseminated form cannot be excluded.

The kidney was the most affected organ showing varying degrees of inflammation; from inflammatory tissue surrounding the organ (Figure 2.2C) to inflammation

surrounding parasitized tubules (Figure 2.2D). *S. destruens* is often found as focal aggregates, i.e. rosettes, in various tissues including the kidney (Figure 2.3). Presence of rosettes was usually associated with cell necrosis suggesting that host cell death results from intracellular parasitism (Arkush *et al.*, 1998). Detection of *S. destruens* within the lumina and epithelia of renal tubules and the intestinal tract, provide further support to the hypothesis that parasite excretion occurs through the urine and shedding of the gut epithelium (Arkush *et al.*, 2003).

Although the total number of intracellular parasites could not be accurately determined, the presence of only a few, heavily infected fish (Table 2.2) suggests that *S. destruens* infections are overdispersed , i.e., heavy *S. destruens* infections are only present in a small proportion of the *L. delineatus* population. This can have important implications for parasite detection using histology due to the technique's capability of only detecting moderately and heavily infected individuals. Gram's stain increases the detection of moderate infections (Figures 2.5A, B), however, low infections can still go undetected. Consequently, a large number of fish will need to be sampled in order to have a high probability of histologically detecting the disease.

Studies of *S. detruens*' prevalence in the wild would benefit from the use of molecular tools, such as PCR, which can detect lower infection levels compared to histology (Mendonca and Arkush, 2004). Even though PCR amplification of *S. destruens* DNA does not infer infection in the fish or parasite viability, it can be used alongside histology to facilitate epidemiological studies. PCR is more cost and time effective and can be used to screen large numbers of individuals (Cunningham, 2002).

Individuals with PCR amplification of *S. destruens* DNA can then be tested for clinical status and evidence for parasite viability using histology.

The origin and source of the infection in *L. delineatus* is currently unknown in the UK. This is largely due the lack of long term data on the parasite composition of different fish communities. As a result, it is very difficult to determine whether a parasite has been introduced to a new community or has simply added one more species to its range of hosts. Gozlan *et al.* (2005) have used *L. delineatus* originating from Stoneham lakes over a period of four years and have never reported *S. destruens* infections in their control fish, suggesting that the parasite is a recent colonizer of Stoneham lakes. Naturally occurring *S. destruens* infections in *L. delineatus*, adds this species to *S. destruens*' actual range of hosts and provides the first documented family switch for *S. destruens* in the wild. The parasite's range of potential hosts and susceptibility within the Cyprinidae are investigated in Chapters 6 and 7.

Chapter 3: Temperature influence on production and longevity of *Sphaerothecum destruens'* zoospores. [1]

Abstract *Sphaerothecum destrues* is an obligate intracellular parasite which has been shown to cause high mortalities in Chinook salmon *Oncorhynchus tschawytscha*, Atlantic salmon *Salmo salar* and the cyprinid sunbleak *Leucaspius delineatus*. When *S. destruens* spores are incubated in freshwater, the spores undergo zoosporulation and release zoospores (i.e. motile free-living stages). Understanding the influence of environmental factors, particularly temperature, on the production and survival of these free-living stages is key to predicting future spread and emergence of *S. destruens*. In this study, the influence of temperature on *S. destruens* zoospore production and longevity has been investigated at 4, 15, 25 and 30 °C. These temperatures were selected to represent the temperature range of the parasite's hosts. Highest numbers of zoospores were observed at 15 °C followed by 4, 25 and 30 °C. Zoosporulation had a later onset and longer lifespan at lower temperatures (4 and 15 °C). The opposite was observed for higher temperatures (25 and 30 °C). Overall, 15 °C appeared to be the optimal temperature for *S. destruens* zoosporulation.

[1] This chapter has been adapted from the paper: Andreou D., Gozlan. R. E. and Paley, R. (2009) Temperature influence on production and longevity of *Sphaerothecum destruens'* zoospores. *Journal of Parasitology* 95: (1539-1541).

3.1 Introduction

The majority of identified pathogens that cause significant human, domestic animal, and wildlife mortalities are capable of infecting more than one host species (Woolhouse *et al.*, 2001). Typical characteristics that enable infection of multiple hosts include high pathogen genetic variability, free-living infectious stages, and transport via vectors (Woolhouse *et al.*, 2001). Pathogens may produce vast numbers of free-living infectious stages that are then able to cause infection in new susceptible hosts. Understanding the influence of environmental factors, such as temperature, on the production and survival of free living stages can help make predictions regarding the potential of disease spread and emergence.

The life cycle of *S. destruens* includes a spherical intra-cytoplasmic spore stage, 2-6 µm in diameter (Arkush *et al.*, 2003; see Chapter 1, section 1.2). When incubated in freshwater, spores undergo zoosporulation and release five or more motile uniflagellate zoospores (Arkush *et al.*, 2003). Zoospores have an average body diameter and flagellum length of 2 µm and 10 µm, respectively (Figure 3.1). Although the spore stage of *S. destruens* is directly infectious, the zoospore stage has not yet been shown to be directly infectious (Arkush *et al.*, 2003). In contrast, water-borne transmission of the zoospores has been demonstrated for the Rhinosporidecae member, *Dermocystidium salmonis* (see Olson *et al.*, 1991) and Arkush *et al.* (2003), proposed that the *S. destruens* zoospores could be infectious.

Worldwide, natural infections of *S. destruens* have been seen in *O. tshawytscha* and *S. salar* in North America (Arkush *et al.*, 1998) and in *L. delineatus* (see Chapter 2) and *P. parva* (through detection of *S. destruens* in *L. delineatus* following cohabitation of

wild caught *P. parva* with naïve *L. delineatus*) in the UK (Gozlan *et al.*, 2005, 2009). In the UK, *L. delineatus* and *P. parva* are non-native invasive species. *P. parva* is currently considered the most invasive cyprinid in Europe with a range stretching from the west side of China to UK and North Africa (Gozlan *et al.*, 2002).

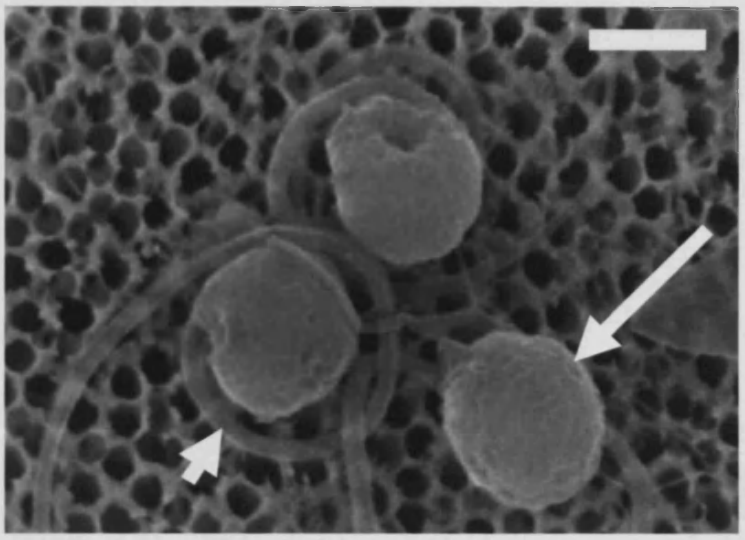


Figure 3.1: Scanning electron microscopy of *Sphaerothecum destruens* zoospores following incubation of culture derived *S. destruens* spores in sterile distilled water for one day at 15 °C. The head (long arrow) and flagellum (short arrow) can be seen for three zoospores. Scale bar: 1 μm. (Photo courtesy of Dr Stephen W. Feist).

Although the currently known host species of *S. destruens* have a wide geographical range (Gozlan *et al.*, 2002) and occupy a variety of habitats, it still remains unclear how *S. destruens,* and in particular its zooflagellate life cycle stage, would be affected by changes in temperature. Zoosporulation has been successfully induced from culture derived spores and from infected *O. tshawytscha* tissue incubated in distilled water at 15 °C (Arkush *et al.*, 2003). Harrel *et al.* (1986) reported seawater temperatures between 8 - 12.5 °C during an *S. destruens* epizootic in *O. tshawytscha* brood stock. The two invasive hosts, *L. delineatus* and *P. parva*, associated with *S. destruens* occupy environments with temperatures ranging from 2 °C in their North and Central

European range to temperatures over 30 °C in their southern European and North African ranges (Arnold and Längert, 1995). Here, we have investigated the influence of temperature on *S. destruens* zoospore production and longevity at four temperatures, 4, 15, 25, and 30 °C. These are representative of the temperature range at which *S. destruens* has been reported and that of its host species. Understanding how temperature affects zoospore production and viability will provide an insight into the potential persistence and spread of this parasite.

3.2 Materials and methods

3.2.1 Spore collection

The strain of *S. destruens* used in this study was originally isolated from sunbleak (Gozlan *et al.*, 2005, 2009). *S. destruens* was cultured in *Epithelioma papulosum cyprini* cells (Fijan *et al.*, 1983) maintained at 15 °C in minimal essential medium supplemented with 10% fetal bovine serum, 100 IU penicillin / ml, 100 µg streptomycin / ml, and 2 mM L-glutamine, and buffered with 7.5 % sodium bicarbonate.

Twenty days following the last passage, cell associated and cell free spores were collected and kept separately. Cell associated spores were obtained from the infected cell monolayer. Prior to collection, the monolayer was washed with 5 ml of MEM-7.5 (MEM Eagle; M2279 Sigma supplemented with 7.5 % fetal bovine serum) to remove any cell free spores. The infected cell layer was scraped with a cell scraper (Nunc, Roskilde, Denmark) and suspended in 10 ml of MEM -7.5 and transferred into 50 ml sterile tubes. The cells were centrifuged at 1,200 x *g* for 10 minutes at 10 °C and were re-suspended in 10 ml of MEM-7.5. The suspension of infected cells was then sonicated with a microprobe for 45 seconds at 30 % power to remove any host cells

(Soniprep 150 [MSE]). The released spores were washed twice with sterile water and centrifuged at 1,200 x *g* for 2 minutes at 10 °C between each wash step. The supernatant was discarded between steps and the spores were finally suspended in 10 ml of sterile double distilled water.

Cell associated spores were enumerated using a cell counting chamber (Improved Neubauer Depth 0.1 mm 1/400 mm^2; Weber Scientific International, Teddington, England) on an inverted phase light microscope (Olympus Inverted Microscope 102505). Cell associated spores were prepared in sterile distilled water at a concentration of 5.33 x 10^6 spores ml^{-1} and 2 ml were dispensed in eight sterile 12.5 - cm^2 flasks. Two flasks each were placed at 4, 15, 25, and 30 °C in incubators. As the flasks were not available over weekends, the two flasks were stacked by a two day gap to obtain a reading per day.

3.2.2 Spore and zoospore enumeration

Spores and zoospores counts were determined in a hemocytometer (Neubauer chamber) at 200 × magnification with an inverted phase light microscope. Zoospores were either classified as active (motile) or inactive (static). Daily counts were made until active zoospores were no longer observed.

Before counting, flasks were first gently swirled to evenly distribute zoospores prior to removal of 20 µl of solution from each replicate flask. For each sample, the mean zoospore count was taken from four squares on a standard hemocytometer. To facilitate the counting of zoospores the 20 µl pathogen containing solution was mixed with 20 µl of 2.5% glycerol solution (glycerol / water) to reduce zoospore motility. Active zoospore counts were made at a single time point for each small

hemocytometer square. Zoospore counts were averaged over three day intervals. Spore and zoospore concentrations (ml^{-1}) were calculated using the formula:

$$\text{Concentration } (ml^{-1}) = \text{mean count} \times 10^4 \times \text{dilution factor}$$

Close observation of zoospore behaviour revealed that zoospores were capable of having inactive periods between bursts of activity. As it was not possible to determine what proportion of inactive zoospores were 'resting' at any given time, all inactive zoospores were considered non-viable for the purpose of this work. Although this approach underestimates the number of active zoospores, it still allows (over a period of three days) the temporal characterisation of active zoospore production, i.e., an increase of active zoospore abundance is a true increase.

3.2.3 Statistical analysis

The Kruskall-Wallis test was used to test for significant differences between overall zoospore concentrations across the four temperatures. A significant Kruskall-Wallis test was complemented with pairwise Mann-Whitney *U*-tests to determine at which temperatures overall zoospore concentrations differed significantly. Presence of motile zoospores in time was used as a surrogate for longevity of overall zoospore production.

3.3 Results

Zoosporulation occurred across all temperatures, with later onset and longer production of zoospores at lower temperatures (Figures 3.2, 3.3). Zoospores were observed 3 days post incubation in distilled water at 4 °C and 1 day post incubation for 15, 25, and 30 °C. Active zoospores were present for 26 days at 4° C, 18 days at 15 °C, 7 days at 25 °C, and 5 days at 30 °C (Figure 2). Mean active zoospore

concentrations peaked on days 13 - 15 for 4 °C and 15 °C, and on days 1 - 3 for 25 °C and 30 °C, respectively (Figure 3.2). The mean active zoospore concentration reached a maximum of 26 x 10^4 active zoospores ml^{-1} at 4 °C, 24 x 10^4 active zoospores ml^{-1} at 15 °C, 21 x 10^4 active zoospores ml^{-1} at 25 °C, and 15 x 10^4 active zoospores ml^{-1} at 30 °C (Figure 3.2). There was no significant difference between overall active zoospore concentrations across all temperatures (P = 0.277; Kruskall-Wallis test).

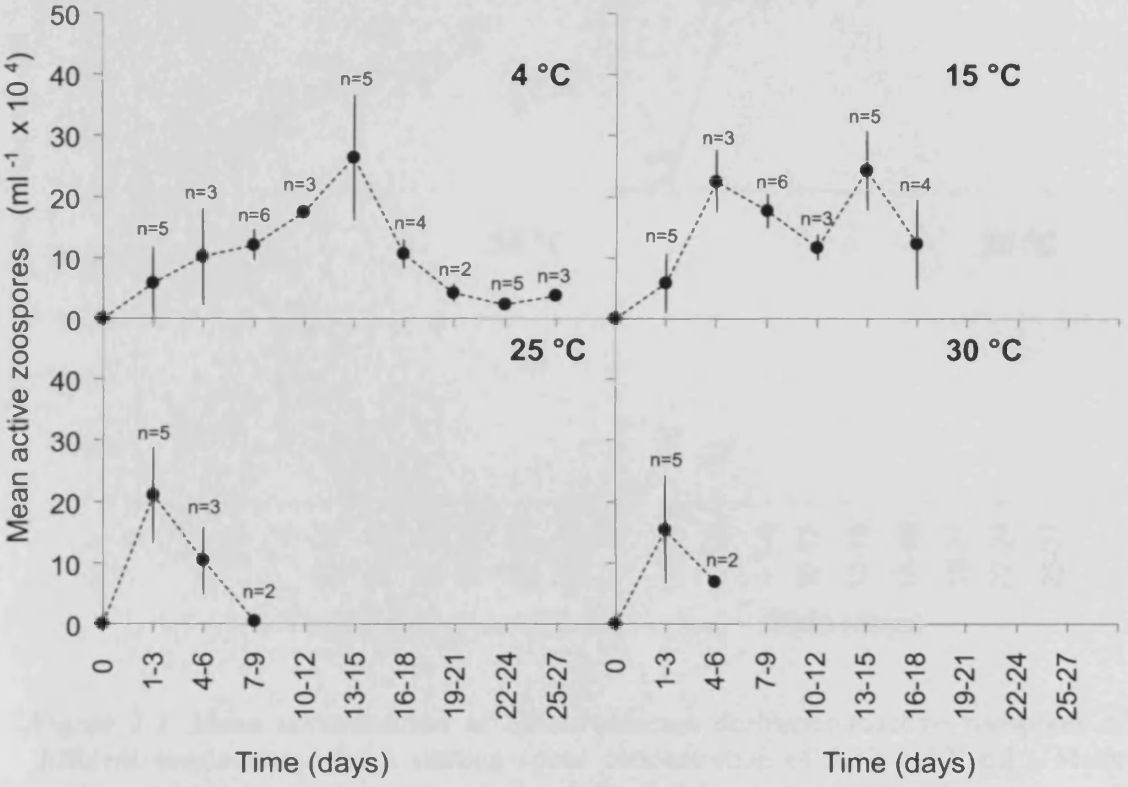


Figure 3.2: Mean concentration of *Sphaerothecum destruens* active zoospores at different temperatures for a starting spore concentration 5.33 x 10^6 ml^{-1}. Mean active zoospore counts were calculated for 3-day intervals. Error bars represent standard errors.

Inactive zoospore concentrations peaked on days 19 - 21, 10 - 12, 7, and 1 - 3 at temperatures 4, 15, 25, and 30 °C, respectively (Figure 3.3). Mean inactive zoospore concentration reached a maximum of 1,206 x 10^4 inactive zoospores ml^{-1} at 4 °C,

1,752 x 10^4 inactive zoospores ml^{-1} at 15 °C, 975 x 10^4 inactive zoospores ml^{-1} at 25 °C, and 378 x 10^4 inactive zoospores ml^{-1} at 30 °C (Figure 3.3). Overall mean inactive zoospore concentration was significantly different with highest concentration at 15 °C, followed by 4, 25, and 30 °C ($P < 0.01$; Mann-Whitney *U*-test; Figure 3.2).

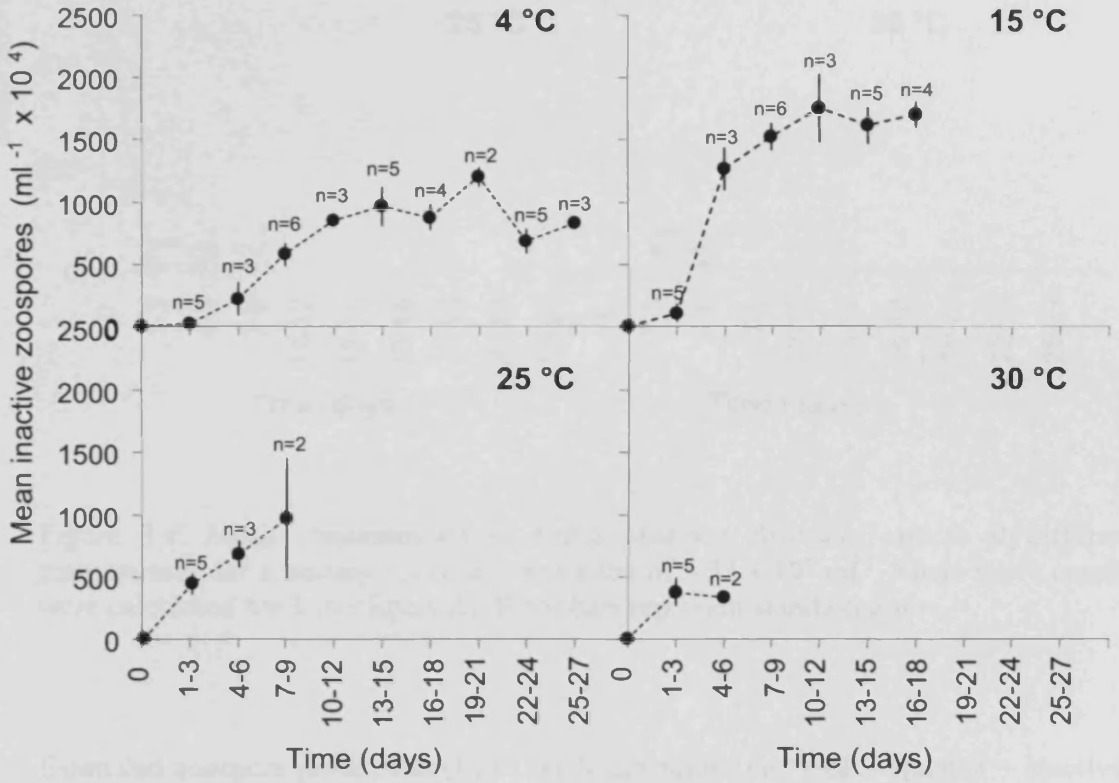


Figure 3.3: Mean concentration of *Sphaerothecum destruens* inactive zoospores at different temperatures for a starting spore concentration of 5.33 x 10^6 ml^{-1}. Mean inactive zoospore counts were calculated for 3-day intervals. Error bars represent standard errors.

Spore abundance markedly decreased by the third day post transfer across all temperatures (Figure 3.4); *S. destruens*' spores formed aggregates that were responsible for the sporadic increases in spore concentrations observed 3 days post incubation in sterile distilled water (Figure 3.4). These aggregates could not be separated since vigorous vortexing detached the flagella of the fragile zoospores.

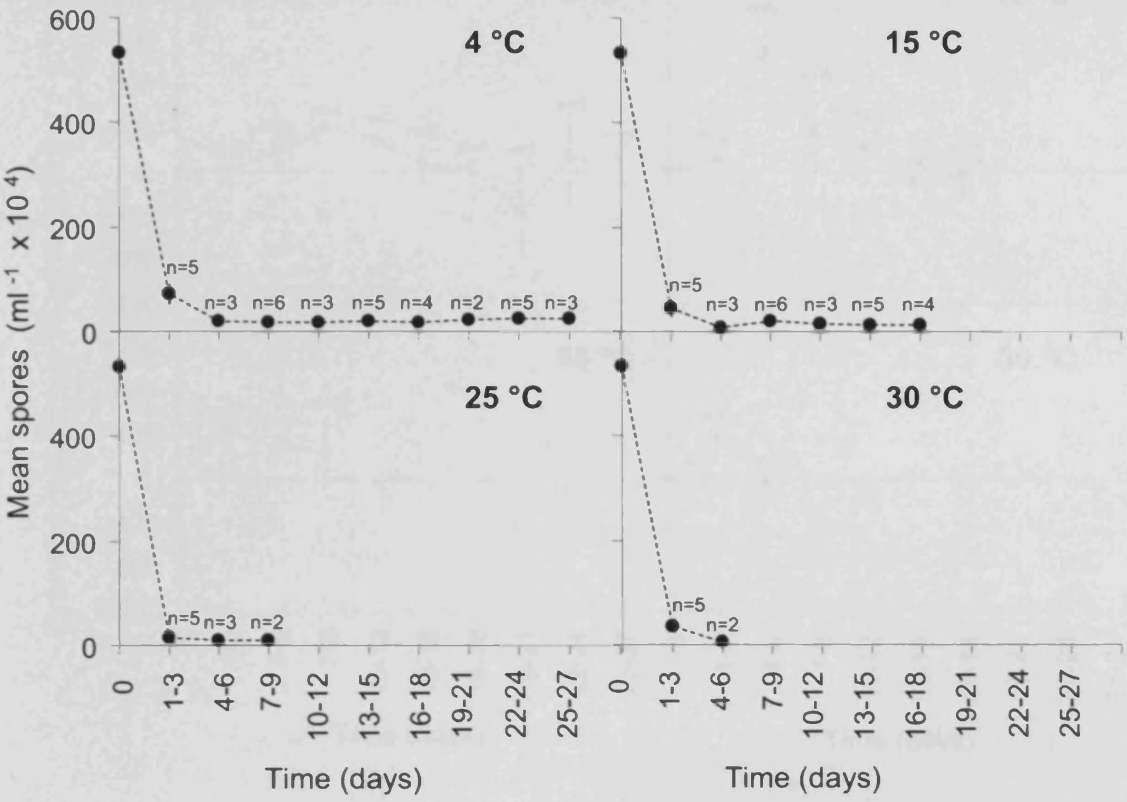


Figure 3.4: Mean concentration of *Sphaerothecum destruens* spores at different temperatures for a starting spore concentration of 5.33 x 10^6 ml^{-1}. Mean spore counts were calculated for 3-day intervals. Error bars represent standard errors.

Estimated zoospore production (EZP) for *S. destruens*, i.e., EZP = ([active + inactive] zoospores) $interval_{i+1}$ – ([active + inactive] zoospores) $interval_i$ reached a maximum of 399 x 10^4 zoospores ml^{-1} at 4 °C, 1,108 x 10^4 zoospores ml^{-1} at 15 °C, 457 x 10^4 zoospores ml^{-1} at 25 °C, and 386 x 10^4 zoospores ml^{-1} at 30 °C (Figure 3.5). Zoospore production fluctuated but was prolonged at 4 and 15 °C.

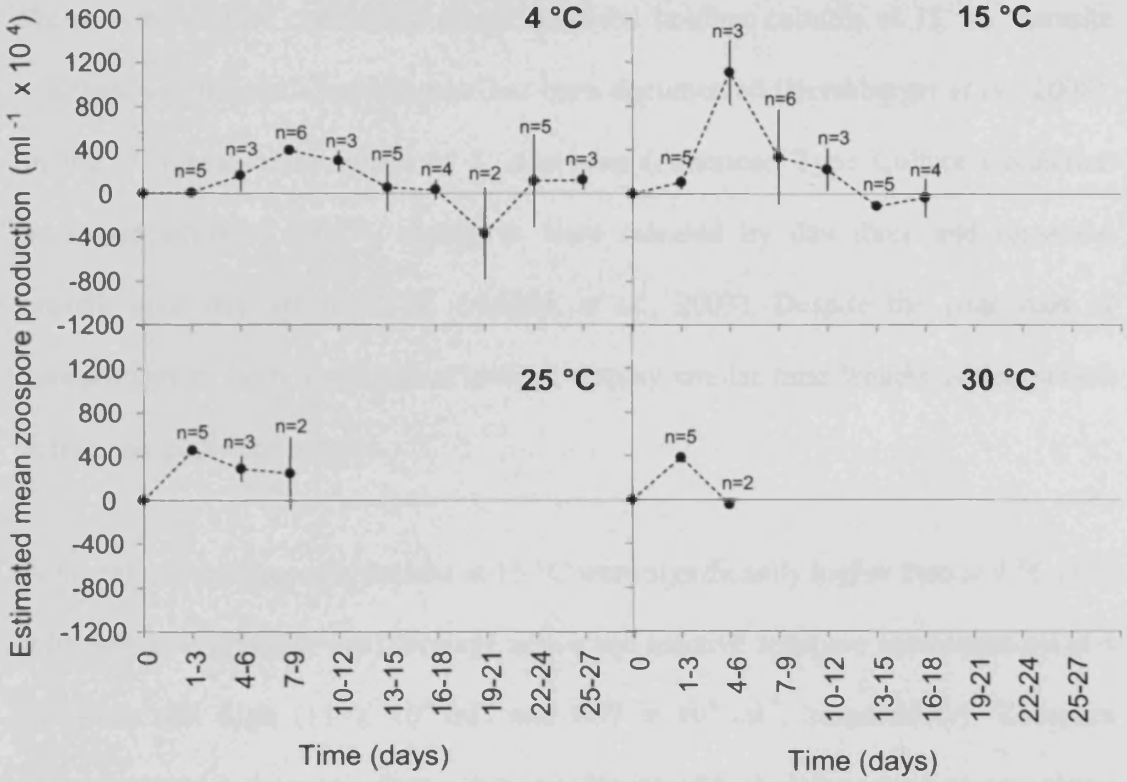


Figure 3.5: Estimated zoospore production (EZP) for *Sphaerothecum destruens*, i.e., EZP = ([active + inactive] zoospores) $interval_{i+1}$ – ([active + inactive] zoospores) $interval_i$ production at different temperatures for a starting spore concentration of 5.33 x 10^6 ml^{-1}. Error bars represent standard errors.

3.4 Discussion

High zoospore concentrations occurred at all the tested temperatures, indicating high temperature tolerance by *S. destruens* zoospores. Early onset of zoosporulation (day 1) was observed for 15, 25 and 30 °C, while later onset (day 3) was observed for 4 °C. A similar pattern has been observed for *D. percae* zoospores (Pekkarinen and Lotman, 2003), which also belongs to the Rhinosporidaceae.

Overall, 15 °C appears to be the optimum temperature for zoosporulation as the highest concentration of active and inactive zoospores was released at this temperature over a period of 18 days. This may in part be due to parasite adaptation to

the *in vitro* culture conditions, which involved holding cultures at 15 °C. Parasite adaptation to its host's environment has been documented (Hershberger *et al.*, 2008). In the *O. tshawytscha* isolate of *S. destruens* (American Type Culture Collection accession number: 50643), zoospores were released by day three and remained present until day 20 at 15 °C (Arkush *et al.*, 2003). Despite the later start of zoosporulation, both *S. destruens* isolates display similar time lengths during which active zoospores are present.

Although zoospore concentrations at 15 °C were significantly higher than at 4 °C ($P < 0.01$; Mann-Whitney *U*-test), average active and inactive zoospore concentrations at 4 °C were still high (11 x 10^4 ml^{-1} and 689 x 10^4 ml^{-1}, respectively). Zoospore concentrations and zoosporulation duration decreased with rising temperatures (above 15 °C). Despite the short duration of zoosporulation at 25 and 30 °C zoospore concentrations were high, potentially increasing infection pressure on thermally stressed hosts.

Estimated zoospore production fluctuated, particularly for 4 and 15 °C, but was prolonged at these temperatures. This prolonged release of zoospores by spores indicates that spores can remain viable in freshwater for long time periods prior to zoosporulation. Thus, although zoospores do not appear to remain motile for long periods of time, the presence of motile zoospores would be extended by this prolonged zoospore release by the spores. This could potentially increase the chances of host attachment and infection by the parasite.

Emerging wildlife diseases have been linked with anthropogenic factors such as climate change, biological invasions, land use change, and pollution (Gozlan *et al.*,

2006, Johnson *et al.*, 2007). *S. destruens* is a multi-host parasite that displays high temperature tolerance for two of its life stages, i.e., spores and zoospores, which could lead to environmental persistence of the parasite. High zoospore production, wide temperature tolerance, and the ability to infect and cause high mortalities in one invasive species (*L. delineatus*), while having another invasive species (*P. parva*) as a potential healthy carrier of *S. destruens* (Gozlan *et al.*, 2005), places this parasite as a strong candidate for range expansion, subsequent contact with naïve hosts and disease emergence.

Understanding the effect of temperature on spore viability and zoospore production provides important additional information on *S. destruens*' life cycle. This can be used to further investigate aspects of its life cycle and evaluate future risks to susceptible hosts. Temperature dependence of infectivity has been reported for other parasites (Pelizza *et al.*, 2008) and future work should include testing temperature influence on *S. detruens* spore and zoospore infectivity. In addition, future work should test how zoospore production is influenced by factors such as medium movement and molecular signals (for example fish pheromones).

PART B: Susceptibility to *Sphaerothecum destruens.*

Chapter 4: Influence of sunbleak *Leucaspius delineatus'* reproductive state on *Sphaerothecum destruens'* prevalence and infection level.

Abstract *Sphaerothecum destruens* is an obligate intracellular parasite with the potential to cause high mortalities and spawning inhibition in the endangered cyprinid *Leucaspius delineatus*. In this study, we investigate the influence of *L. delineatus*'s reproductive state on the prevalence and infection level of *S. destruens*. A novel real time quantitative polymerarse chain reaction (qPCR) was developed to determine *S. destruens*' prevalence and infection level. These parameters were quantified and compared in reproductive and non-reproductive *L. delineatus*. The detection limit of the *S. destruens* specific qPCR was determined to be 1pg ml^{-1}. Reproductive *L. delineatus* had a significantly higher *S. destruens* prevalence (41 %) and infection level (99 pg *S. destruens* DNA per ml *L. delineatus* DNA) compared to non-reproductive *L. delineatus* (19 % prevalence, 18 pg/ ml infection). The present work indicates that *S. destruens* infection in *L. delineatus* can be influenced by the latter's reproductive state and provides further support for the potential adverse impact of *S. destruens* on the conservation of *L. delineatus* populations.

4.1 Introduction

Every organism has limited energy resources which must be divided between key biological functions such as reproduction and growth (Simkova *et al.*, 2005). Commonly, parasites negatively impact the fitness of their host (e.g. body condition, growth rate, reproductive status, reproductive output), increasing the evolutionary pressure on hosts to select a performing immune system, which would act as an anti-parasite defence (Simkova *et al.*, 2008). This anti-parasite immune defence is costly in energy (Connors and Nickol, 1991, Lochmiller *et al.*, 1993, Sheldon and Verhulst, 1996) with expected knock-on effects on life history traits such as growth and reproduction (Simkova *et al.*, 2008). On the contrary, an increased investment in reproduction might decrease the energy invested in immune defence and thus facilitate parasite infection (Sheldon and Verhulst, 1996, Skarstein and Folstad, 1996, Skarstein *et al.*, 2001, Simkova *et al.*, 2005, 2008).

Seasonal changes in immunocompetence, i.e., the ability to produce a normal immune response, and parasitism have been observed across numerous taxa, including, birds and fish (Hamilton and Zuk, 1982, Nelson and Demas, 1996, Sheldon and Verhulst, 1996). Specifically, down regulation (decrease) of immunocompetence during the reproductive period has been well documented in fish such as *R. rutilus*, chub *Leuciscus cephalus* (Linneaus) and Arctic charr, *Salvelinus alpinus* (Linneaus) (Skarstein and Folstad, 1996, Skarstein *et al.*, 2001, Kortet *et al.*, 2003, Vainikka *et al.*, 2004, Simkova *et al.*, 2005, 2008, Lamkova *et al.*, 2007). Male and female fish usually invest differently in reproduction, with males placing a higher investment in mate attraction through the exaggeration of sexual ornamentation during spawning, while females invest more in gamete production (Simkova *et al.*, 2008). The energetic

cost of high investment in gametes (also known as the energetic hypothesis) is expected to create a trade off between reproduction and quality of the immune system in females (Simkova *et al.*, 2008). This has been confirmed for 17 species of cyprinid females including *A. brama, R. rutilus* and *P. parva* (see Simkova *et al.*, 2008). On the other hand, as females invest more in reproduction by producing larger gametes they are expected to be more susceptible to infection compared to males (i.e. sperm is energetically less costly) (Nordling *et al.*, 1998, Sanz *et al.*, 2001). For example, female stone loach, *Barbatula barbatula* (Linnaeus), were more heavily parasitized with *Gyrodactylus* spp. and *Raphidascaris acus* during their reproductive season compared to male stone loach (Simkova *et al.*, 2005).

Sexual ornamentation in males has been associated with increased steroid sexual hormones (such as testosterone) that can also negatively influence the immune system. Evidence for this includes the negative effect of high testosterone levels on spleen weight (the spleen plays an important role in immune defence of teleost fish and spleen size is widely used as an estimator of immunocompetence (Fange and Nilsson, 1985)). The impact of steroid sexual hormones on male immunity is known as the immunohandicap hypothesis (Hamilton and Zuk, 1982). However, support for the immunohandicap hypothesis in fish is equivocal. For example, Skarstein *et al.* (2001) has shown that reproductive male *S. alpinus* (Linnaeus), were more heavily infected with macroparasites compared to 'resting' (non-reproductive mature *S. alpinus*), suggesting a reproductive cost to immunity. On the other hand, Ottova *et al.* (2005) have shown no association between sexual ornamentation and parasite infection in male *A. brama.*

In addition, a high energetic cost to reproduction is also expected for the gender which provides parental care and nest defence (Simkova *et al.*, 2005). In the three-spine stickleback *Gasterosteus aculeatus* (Linnaeus), where males provide parental care and nest defence, males were more heavily parasitized with *Glugea anomala* cysts compared to females (Reimchen and Nosil, 2001, Arnold *et al.*, 2003). Arnold *et al.* (2003) suggest that the observed sex biased parasitism could be the result of intense nest defence and parental care. Sex-biased parasitism would thus be expected in the gender providing parental care and nest defence.

In *L. delineatus*, the cost of parental care, i.e., high courtship and aggression during reproduction in males and the high reproductive investment in females (batch spawning) could lead to reduced immunocompetence in reproductive individuals. *L. delineatus* has been found to be highly susceptible to *S. destruens* during its reproductive period with complete inhibition of *L. delineatus* spawning (Gozlan *et al.*, 2005). Infection of non-reproductive *L. delineatus* with *S. destruens* has not been investigated. Gozlan *et al.* (2005) has identified *S. destruens* as a high risk parasite for *L. delineatus* and a plausible cause for its decline through its native range. It is thus important to better understand this host-parasite association, and in particular the effect of reproduction, as it can have direct implications for the species' conservation.

As an intracellular, unicellular parasite the enumeration of individual *S. destruens* spores per host within fish tissues is particularly challenging. Currently, there is no established method to determine the parasite infection load. The only approach available includes an estimation of infection severity in organ impression smears (Arkush *et al.*, 2003) and histological sections (as applied in Chapter 2). Molecular tools such as real time quantitative PCR have been used to estimate infection load of

microparasites in aquatic organisms (Jones *et al.*, 2003, Hallett and Bartholomew, 2006, Funk *et al.*, 2007, Phelps and Goodwin, 2007). For example, Phelps and Goodwin (2007) have used quantitative PCR to detect and quantify the microsporidian *Ovipeistophora ovariae* in golden shiner *Notemigonus crysoleucas* (Mitchill) and have shown a synchronisation of spore release by *O. ovariae* with golden shiners' spawning.

The aims of this chapter were three fold. Firstly, a real time quantitative PCR was designed and optimised in order to detect and quantify infection levels of *S. destruens*. Secondly, the effect of reproduction on the prevalence and infection level of *S. destruens* in *L. delineatus* was investigated as this has never been studied From the work by Gozlan *et al.* (2005) and the energetic trade off between immunity and reproduction, it was predicted that the host's reproductive state should play an important role on the prevalence and infection level of *S. destruens* in *L. delineatus*. Higher prevalence and infection levels in reproductive *L. delineatus* were predicted. Thirdly, the difference in infection with *S. destruens* between the genders was also investigated. It was predicted there would be no significant difference in *S. destrues* prevalence and infection levels between male and female *L. delineatus* as both genders experience high energy costs during their reproductive period (nest guarding and batch spawning, respectively).

4.2 Materials and methods

4.2.1 Molecular analysis and light microscopy

DNA extraction. DNA was extracted using the Rodent tail protocol of the Qiagen DNeasy 96 Blood & Tissue kit. Extraction efficiency of each extraction was determined by including 50 µl of 10^5 and 10^3 *S. destruens* spores and 15 mg of *S.*

destruens-free common carp kidney spiked with 10^4 and 10^3 *S. destruens* spores. *S. destruens* spores were obtained from EPC cell cultures. Extracted DNA from fish tissues was quantified in a spectrophotometer at 260 nm (NanoDrop ND-1000; Labtech). The extracted DNA was then diluted to 125 ng μl^{-1} and was stored at -70 °C until further analysis with real time PCR.

Real time quantitative PCR primer selection. In order to detect the parasite's infection levels, a real time quantitative PCR was designed and optimised for *S. destruens*. Oligonucleotides for the qPCR were designed using small subunit ribosomal DNA (18S rRNA gene) sequences of the three *S. destruens* isolates (SK-AY267346; BML-AY267345; WA-AY267344), *Dermocystidium salmonis* (U21337), *Ichthyophonus hoferi* (U25637) and *Diaphanoeca grandis* (DQ059033). These sequences were obtained from GenBank and aligned with Clustal X (Thompson *et al.*, 1997). The 18S rRNA gene sequence of *Cyprinus carpio* (AF133089) was used as an outgroup and a cyprinid reference. Primers were visually selected to regions of 18-19 nucleotides showing 100 % complementarily to the three *S. destruens* isolate sequences. Primer sequences had a minimum of three mismatches with sequences of related organisms. The melting temperatures, percentage guanine and cytosine content and secondary structure of each primer were evaluated. A single primer pair was then subjected to a basic local-alignment-search-tool (BLAST) search against all the nucleotide sequences stored in GenBank to confirm its specificity to *S. destruens*. Two sets of primer pairs (Table 4.1) were tested in developing the real time PCR.

Table 4.1: Primer pairs (Set 1 and 2) used to develop a real time quantitative PCR (qPCR) for *Sphaerothecum destruens*. Primer sequence, PCR amplicon size and melting temperature of each amplicon are reported. Primers bind to the 18S rRNA gene of *S. destruens*.

Primer set	Primer designation	Primer sequence (5' → 3')	Amplicon size (bp)	Melting temperature °C
Set 1	Forward: Sd-RT-F	CAA TGT AAA AAC CTT AAC G	98	83
	Reverse: Sd-RT-R	AAG CTT ATC CCA AAA TCC		
Set 2	Forward: Sd-RT-F2	GGA TTT TGG GAT AAG CTT	83	86
	Reverse: Sd-RT-R2	GT AAA AGT CCC AAA CTC		

qPCR reaction conditions. The Platinum® SYBR® Green qPCR SuperMix-UDG kit (Invitrogen) was used for this assay. The reaction conditions were identical for both primer sets (Set 1 and 2) and consisted of 20 µl reaction volumes containing 10 µl of Platinum® SYBR® Green qPCR SuperMix-UDG kit mix, 1 × bovine serum albumin, 0.4 µM of each primer and 250 ng of template DNA.

Cycling conditions. All reactions were performed in a Rotor Gene™ 6000 (Corbett Research) and were analysed with Rotor Gene™ 6000 version 1.7.75 software. A standard curve was generated from a 1 in 10 serial dilution of purified *S. destruens* DNA (10 ng to 1 fg) obtained from cell culture. Cycling conditions for Set 1 consisted of an initial denaturation cycle at 95 °C for 7 minutes followed by 45 cycles of 95 °C for 30 seconds, 40 seconds at 50 °C and 20 seconds at 72 °C. A melting temperature curve was determined at the end of the run at a temperature range of 55-99 °C. Melting temperature analysis involved an initial pre-melt hold time of 90 seconds at 55 °C followed by 5 second hold for each one degree step until 99 °C. For Set 2

primers, cycling conditions consisted of an initial denaturation cycle at 95 °C for 2 minutes followed by 45 cycles of 95 °C for 30 seconds and 35 seconds at 53 °C. A melting temperature curve was calculated at the end of the run at a temperature range of 55-99 °C.

Limits of detection. In order to quantify the amount of genomic *S. destruens* in samples and determine the detection limit of the qPCR, a standard curve was generated using a ten-fold serial dilution of genomic *S. destruens* obtained from cell culture. Serial dilutions were prepared in sterile water to produce template concentrations of 10 ng to 1 fg. To simulate the screening of *S. destruens* infected samples, an additional standard curve was produced by spiking serial dilutions of genomic *S. destruens* DNA (10 ng to 1 fg) with 270 ng ml^{-1} common carp kidney genomic DNA. qPCR was also performed on 270 ng and 540 ng of common carp genomic DNA to detect possible non-specific amplification by the qPCR primers. The correlation coefficient was calculated (Rotor-Gene 6000 v 1.7) for both standard curves. Negative controls used in this assay included genomic common carp DNA and sterile water. The detection limit was defined as the lowest genomic *S. destruens* DNA concentration yielding a Cycle Threshold (C_T) value in the PCR reaction. The optimal fluorescent threshold was determined automatically by the Rotor Gene™ 6000 version 1.7.75 (Corbett research) with a set upper bound threshold of 0.38. This threshold varied for each reaction, (minimum value: 0.0056 and maximum value: 0.379) but did not alter the PCR efficiency and detection limits.

qPCR specificity. The specificity of the two qPCR primer pairs was tested using a PCR amplified *D. salmonis* DNA section inserted in the pGEM® –T (Promega) provided by Dr Richard Paley (CEFAS laboratory, Weymouth, UK). The *D. salmonis*

insert had a length of 830 bp and included the binding areas of the two primer sets designed for *S. destruens*. Cross amplification of the *S. destruens* specific real time primers was tested on 4 ng of *D. salmonis* DNA.

qPCR detection capacity. The detection capacity of the *S. destruens* specific qPCR was tested further through the analysis of genomic DNA samples from ten *S. destruens* positive organs confirmed as *S. destruens* positive through histological examination (Gram's and H&E stain). An additional five *S. destruens* samples negative by histology organ samples were also tested using the two qPCR primer pairs.

Light microscopy. All fish were also tested by light microscopy in order to (a) visually confirm the presence of the parasite and (b) determine the level of agreement between real time PCR and histology. Histological analysis consisted of staining with H& E and Gram's stain (as described in Chapter 2; section 2.2.2).

4.2.2 Determining the effect of *Leucaspius delineatus*' reproductive state on *Sphaerothecum destruens*.

Cohabitation experiments were carried out in 2006 for a total of 60 days each during *L. delineatus*'s reproductive (May 17th to July 16th) and non-reproductive season (October 19th to December 18th).

Fish source and collection. Three hundred and fifty *L. delineatus* were collected by seine netting from Stoneham lakes (Eastleigh, England: 50°57'14" N; 1°22'56" W) two occasions May 15th, 2006 and October 11th, 2006. *L. delineatus* were transferred to the laboratory in 90L bins of water with a supply of oxygen. The maximum

transport time was four hours. On arrival, *L. delineatus* were sorted by gender (through visual inspection: females are larger than males and have a prominent ovipositor) and were then placed in 70L aquaria (length × width × height = 90 × 30 × 30 cm). The sex ratio per aquaria was approximately 1:1 (this was confirmed through histology of the gonad at the end of the experiment; see section 4.3.2). Maximum stocking density per aquaria was 35 fish. The weight (measured to the nearest 0.1 grams) and fork length (measured to the nearest 0.1 cm) of 70 *L. delineatus* (35 females and 35 males) was recorded prior to the experiment's onset.

In order to determine the prevalence of *S. destruens* in Stoneham lakes, an additional 100 *L. delineatus* were randomly collected and euthanised straight after capture following Home Office (HO) guidelines using a lethal overdose of 2-phenoxyethanol anaesthetic (HO licence no. 80/1979) and severance of the spinal cord using a sterile blade. These samples were stored at -70 °C for further molecular analysis. Additional fish were euthanised following HO guidelines.

***Leucaspius delineatus* cohabitation set-up.** Two hundred *L. delineatus* from Stoneham lakes were cohabited in two three-tank re-circulating 70 L aquaria (see Figure 4.1) on two occasions; during the reproductive and non-reproductive season of *L. delineatus* (2 cohabitation groups per season). The water used in the re-circulating systems was dechlorinated tap water and fish were introduced into the system after being rinsed with clean water. The flow rate was approximately two litres per minute. Water was filtered through a biological filter with gravel substrate, and was then redistributed to the aquaria. In this experiment, fish in different tanks shared the same water supply but remained visually isolated from one another. Fish were fed twice a day with commercial flake food (Nutrafin MAX, Hagen) and kept under natural light

conditions at room temperature. Temperature was recorded hourly using a temperature recorder (Tinytag Splash and Aquatic, OmniInstruments, Dundee, UK). Each re-circulating system had its own nets and cleaning devices

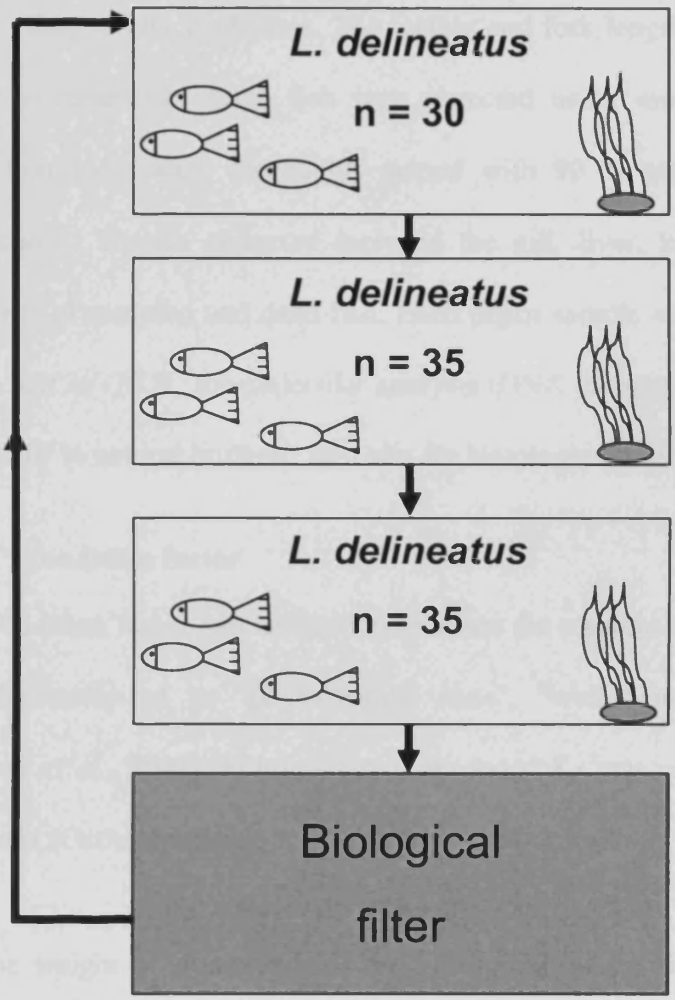


Figure 4.1: Diagrammatic overview of the cohabitation set up used. One hundred sunbleak *Leucaspius delineatus* were cohabited in each cohabitation system. Fish were cohabited during the species reproductive season (May 17th to July 16th) and non-reproductive season (October 19th to December 18th). Direction of water flow between the biological filter and water tanks is depicted by arrows (→).Water was pumped (using a Laguna 2000 water pump) from the biological filter to the top tank from which it flows through the bottom two tanks and back into the biological filter. Maximum water volume per cohabitation system was 280 L. Water temperature in the aquaria was monitored throughout the experiments' duration. Mean water temperature was 25 °C ± 1.5 during the reproductive season and 19 °C ± 1.3 during the non-reproductive period.

4.2.3 Sampling and sample processing

The fish were checked twice daily at which time any mortalities present were collected and dissected. At 14-day intervals, five non-moribund *L. delineatus* were randomly sampled across the three tanks of each re-circulating system. Tanks from which one fish was sampled were alternated with each sampling period. Fish were euthanised according to HO guidelines. The weight and fork length of each fish was recorded prior to dissection. Each fish was dissected using individual dissecting boards. Dissection tools were thoroughly wiped with 90 % ethanol and flamed between dissections. Tissues collected included the gill, liver, kidney, gonad and posterior intestine of sampled and dead fish. Each organ sample was divided in two, preserving one half at -70 °C for molecular analysis (DNA extraction and qPCR) and the other half in 10 % neutral buffered formalin for histological analysis.

4.2.4 Fulton's condition factor

The Fulton's condition factor can be used to estimate the somatic condition of a fish and give an indication of its "physiological state", "well being" and "fatness" (Ostlund-Nilsson *et al.*, 2005). Fulton's condition index K_F was calculated with the following formula (Ostlund-Nilsson *et al.*, 2005):

$$K_F = (W/ FL) * 10^5$$

where, W is the weight in grams and FL the fork length of the fish in millimeters. Increase weight of a fish at a given length would be reflected by an elevated condition factor. Reduced condition in fish has been associated with depletion of liver glycogen and body fat (Adams *et al.*, 1985) which has been shown to occur when stressed individuals experienced changes in their feeding patterns (Brown *et al.*, 1987). Fish condition was considered separately for each gender as they were expected to

naturally differ, especially during the reproductive season of *L. delineatus* when the females are gravid with eggs.

4.2.5 Statistical analysis

All statistical analyses were performed using SPSS 14.0 (SPSS Inc., Chicago, Illinois, USA). Data are given as means ± standard deviation (SD). The median and inter-quartile ranges are provided for *S. destruens*' prevalence, infection levels and condition factors. The Mann-Whitney *U* test was used to test for differences in *S. destruens*' prevalence, infection level and fish condition between *L. delineatus* in the reproductive season and *L. delineatus* in the non-reproductive season. Difference in prevalence and infection level between the sexes was also investigated. Spearman rank-order correlations were performed to look at the association between *S. destruens* infection levels and weight and length for parasitized female and male *L. delineatus*. Statistical significance was accepted when $P \leq 0.05$.

S. destruens prevalence was calculated as described in Chapter 2, section 2.2.3. Parasite infection levels were quantified using real time PCR as picograms (pg) of *S. destruens* DNA per microliter. The total infection level per fish was calculated as the sum of all infection levels across the five organs considered here.

4.3 Results

4.3.1 Real time PCR

Using a ten-fold serial dilution of genomic *S. destruens* DNA, the detection limit of the *S. destruens* specific qPCR was determined to be 1pg ml^{-1} both in the presence and absence of common carp genomic DNA for both primer sets. Correlation coefficients were high for both primer sets in the presence and absence of common

carp DNA (Table 4.2). The inclusion of common carp genomic DNA did not interfere with the amplification of *S. destruens* genomic DNA (Table 4.2). There was no non-specific amplification when the qPCR was performed on 270 ng and 540 ng of common carp genomic DNA (Table 4.2). There was however, non-specific amplification with *D. salmonis* DNA using primer Set 1.

Table 4.2: Summary of results for the two sets of qPCR primers. The correlation coefficient R^2 and standard curve equations (where y: C_T Cycle Threshold; x: DNA concentration) are provided per primer set in the presence and absence of fish DNA. Fish DNA here refers to common carp *Cyprinus carpio* DNA. NA: no cross amplification; CA: cross amplification.

Primer set	***S. destruens* DNA**		***S. destruens* DNA & 270 ng fish DNA**		**Fish DNA**		***D. salmonis* DNA (4 ng)**
	R^2	**Standard curve**	**R^2**	**Standard curve**	**270 ng**	**540 ng**	
Set 1	0.997	y =-3.406x + 19.154	0.998	y =-3.693x + 21.534	NA	NA	CA
Set 2	0.999	y = -0.361x + 18.972	0.973	y = -1.001x + 2.342	NA	NA	NA

In the detection capacity test, six of the ten *S. destruens*-positive samples were confirmed positive by qPCR. There was 100 % agreement between the two primer sets. Four of the five, *S. destruens*-negative (by histology) samples were detected positive by qPCR. Two of these samples tested positive by both qPCR primer sets.

Using primer set 1, real time PCR was successfully performed on the DNA of 460 organs, with a mean R^2 value of 0.993 ± 0.01. All extraction negative samples were detected as negative during PCR. The detection limit across all real time PCR runs was 10^3 *S. destruens* spores and 10^4 *S. destruens* spores spiked with 15 mg of common carp tissue.

4.3.2 Light microscopy

Not all *L. delineatus* that tested positive for *S. destruens* using qPCR also tested positive using histological methods (Table 4.3). qPCR detected three times more positive samples in non-reproductive *L. delineatus* and six times more in reproductive *L. delineatus* compared to histological methods. Fifteen percent of reproductive *L. delineatus* and 33 % of non-reproductive *L. delineatus* that have tested positive using qPCR were also found to be positive by light microscopy (Table 4.3). Histology of the gonad revealed approximately 1:1 ratio of male to female *L. delineatus* in both experiments.

Table 4.3: Number of sunbleak *Leucaspius delineatus* detected as positive by qPCR and histology and the percentage (%) agreement between the two detection methods.

Treatment	qPCR positive	Histology positive	% agreement
Non-reproductive	6	2	33
Reproductive	13	2	15

4.3.3 *Sphaerothecum destruens* prevalence in the wild source population, water temperature and mortalities.

In order to determine an increase in parasite prevalence as a result of the experimental treatment, *S. destruens* prevalence in *L. delineatus* (using the kidney as the organ of choice) was determined at the start of each experiment. *S. destruens* prevalence in *L. delineatus* was found to be 2 % during the reproductive season and 1 % during the non-reproductive season by real time PCR. Water temperatures in the cohabitation systems significantly differed during the reproductive and non-reproductive periods (Mann-Whitney U test; n = 2880, $P < 0.01$).

During the reproductive period, *L. delineatus* experienced an outbreak of white spot disease. All fish were immediately treated with Anti-White Spot Plus (Interpret) according to the manufacturer's treatment protocol. Although treatment successfully combated the disease, high mortalities were still experienced during this outbreak. It was thus not possible to investigate mortality differences due to infection with *S. destruens*. This has contributed to a sample size of 32 (20 and 12 fish from each cohabitation system) instead of 40 reproductive *L. delineatus*.

4.3.4 Does the reproductive state of *Leucaspius delineatus* influence *Sphaerothecum destruens*' prevalence and infection levels, and fish condition?

The reproductive state of *L. delineatus* influenced the prevalence and infection levels of *S. destruens*. The median and inter-quartile ranges for prevalence and infection levels for both reproductive and non-reproductive *L. delineatus* are provided in Table 4.4. Reproductive *L. delineatus* experienced statistically higher *S. destruens* prevalence (Mann-Whitney *U* test; n = 72, $P < 0.05$) and infection levels (Mann-Whitney *U* test; n = 72, $P < 0.01$) compared to non-reproductive *L. delineatus*. *S. destruens* prevalence was 15 % (n = 40) in non-reproductive *L. delineatus* and 41 % (n = 32) in reproductive *L. delineatus*. Mean *S. destruens* infection levels were 18 and 99 pg *S. destruens* DNA μl^{-1} in parasitized non-reproductive and reproductive *L. delineatus* respectively (Figure 4.2). There was no significant difference in *S. destruens* prevalence (Mann-Whitney *U* test; n = 13, $P = 0.13$) and infection level (Mann-Whitney *U* test; n = 13, $P = 1$) between reproductive female and male *L. delineatus*.

Overall, female and male *L. delineatus* in the reproductive season had significantly lower condition factors when compared to their non-reproductive counterparts (Females: Mann-Whitney *U* test; n = 38, $P = 0.01$. Males: Mann-Whitney *U* test; n =

34, $P < 0.05$). Table 4.5 summarises the mean weight, length and condition factor for each treatment and gender. There was no significant difference in the condition factor of parasitized reproductive male and reproductive female *L. delineatus* (Mann-Whitney *U* test; n = 13, $P = 0.23$). When the condition factor of parasitized reproductive female and male *L. delineatus* were compared to their respective non-infected reproductive counterparts there was no significant difference in condition (Reproductive females: Mann-Whitney *U* test; n = 14, $P = 1$. Reproductive males: Mann-Whitney *U* test; n = 18 , $P = 0.437$).

Table 4.4: Summary of the median and percentiles (25^{th} and 75^{th}) for *Sphaerothecum destruens* infection level and prevalence and sunbleak *Leucaspius delineatus* condition factor (K) for reproductive and non-reproductive *L. delineatus*.

	Reproductive *L. delineatus*			**Non-reproductive *L. delineatus***		
Variable	**Median**	**25^{th} percentile**	**75^{th} percentile**	**Median**	**25^{th} percentile**	**75^{th} percentile**
S. destruens infection level	0	0	0.43	0	0	0
S. destruens prevalence	0	0	1	0	0	0
L. delineatus condition factor	0.74	0.69	0.80	0.80	0.79	0.84

Table 4.5: Summary of fork length (mm), weight (g) and condition factors (K) for the sunbleak *Leucaspius delineatus* sampled in the non-reproductive and reproductive experiments. Sex (F: female, M: male). Mean and ±SD are shown.

Treatment	Sex	Sample no.	Fork length		Weight		K	
			Mean	±SD	Mean	±SD	Mean	±SD
Non-reproductive	F	24	61.1	5.3	1.9	0.5	0.8	0.1
	M	16	57.1	7.1	1.6	0.7	0.8	0.06
Reproductive	F	14	63.6	5.2	1.9	0.6	0.7	0.08
	M	18	58.1	3	1.4	0.2	0.7	0.08

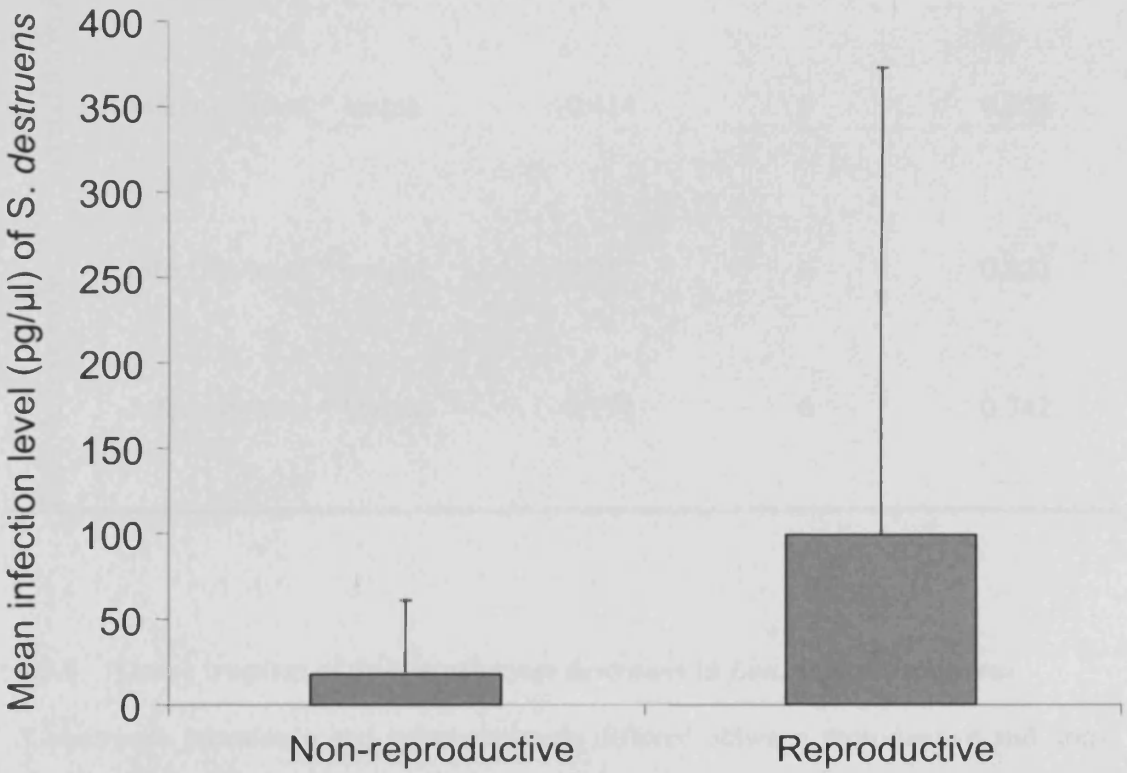


Figure 4.2: Infection level (pg/µl) of *Sphaerothecum destruens* for reproductive and non-reproductive parasitized sunbleak *Leucaspius delineatus*. Infection levels were determined by real time PCR of the 18S rRNA gene of *S. destruens*. Means ± SD are given (n = 6 and n = 13 for non-reproductive and reproductive *L. delineatus* respectively). Infection levels were significantly different (Mann-Whitney *U* test; n = 72, $P < 0.01$) with mean ranks of 31.9 and 42.2 for non-reproductive and reproductive *L. delineatus*, respectively.

There was no association between infection level and both weight and length of parasitized reproductive female and male *L. delineatus* (Table 4.6). The association between infection level and both weight and length for parasitized non-reproductive fish was not performed due to a very small sample size (n = 6).

Table 4.6: Association between infection level and both weight (g) and fork length (mm) in reproductive female and male sunbleak *Leucaspius delineatus* parasitized with *Sphaerothecum destruens*.

	Association	Spearman's rho	No. of parasitized fish	P – value
Reproductive female	Infection level * weight	-0.393	7	0.383
	Infection level * length	-0.414	7	0.355
Reproductive male	Infection level * weight	-0.257	6	0.623
	Infection level * length	-0.174	6	0.742

4.3.5 Tissue tropism of *Sphaerothecum destruens* in *Leucaspius delineatus*

S. destruens prevalence and infection levels differed between reproductive and non-reproductive *L. delineatus* (Tables 4.7 and 4.8). The parasite was present in all five organs in reproductive fish with varying prevalence whereas it was only present in three organs with equal prevalence in the non-reproductive fish (Table 4.7). The

highest infection level was in the gill for non-reproductive *L. delineatus* and the kidney for reproductive fish.

Table 4.7: Number of *Sphaerothecum destruens* infected organs in non-reproductive and reproductive sunbleak *Leucaspius delineatus*. n: number of *L. delineatus* which were positive for *S. destruens*.

	n	Gill	Kidney	Posterior intestine	Gonad	Liver
Non-reproductive	6	2	0	0	2	2
Reproductive	13	3	7	5	3	1

Table 4.8: Mean infection level (pg *Sphaerothecum destruens* DNA per µl) in non-reproductive and reproductive sunbleak *Leucaspius delineatus*' organs. Standard deviation from the mean is provided in brackets. *: Only one positive liver sample was present therefore mean infection level and standard deviation could not be calculated.

	Gill	Kidney	Posterior intestine	Gonad	Liver
Non-reproductive	52.9 (74.7)	0	0	0.19 (0.05)	0.13 (0.007)
Reproductive	0.63 (0.6)	166.8 (321.9)	22.7 (50)	0.26 (0.17)	5.9*

4.4 Discussion

4.4.1 Determining *Sphaerothecum destruens* infection levels

The benefits of using PCR to detect *S. destruens* have been discussed by Mendonca and Arkush (2004). In the absence of PCR, *S. destruens* can be detected through microscopic inspection of stained (e.g. H&E, Gram) impression smears or histology sections of affected organs. Low infection levels can often go undetected by use of microscopic methods as *S. destruens* can be hard to detect in the absence of lesions (Arkush *et al.*, 1998) and can be confused with related organisms with similar

morphological characteristics (Arkush *et al.*, 2003). A nested PCR has been developed by Mendonca and Arkush (2004) to allow detection of *S. destruens*. However, this method does not allow quantification of the parasite.

The real time quantitative PCR developed here was capable of detecting 1 pg ml $^{-1}$ of *S. destruens* DNA both in the presence and absence of common carp genomic DNA for both primer sets. This was equivalent to the detection limit of the nested PCR developed by Mendonca and Arkush (2004). The lack of non-specific amplification when the qPCR was performed on 270 ng and 540 ng of common *C. carpio* genomic DNA supported the high specificity of the reaction to *S. destruens* genomic DNA (Table 4.2). There was however, non-specific amplification with *D. salmonis* DNA using primer Set 1. Despite the cross amplification of primer Set 1 with *D. salmonis*, Set 1 was used in this study. *D. salmonis* has been reported to only affect salmonids such as *O. tshawytscha*, *O. kisutch*, *O. nerka*, *O. gorbuscha* (see Chapter 1, Table 1.1). As *L. delineatus* is a cyprinid and has not been reported to be affected by *D. salmonis* it was possible to use this primer set to detect *S. destruens* in *L. delineatus*. The presence of *S. destruens* was also confirmed microscopically to corroborate the qPCR results. As low infections are expected to go largely undetected by microscopy, it is expected that confirmation of *S. destruens* infection through histology will not occur for 100 % of all affected tissues. However, the detection of *S. destruens* in the studied group should suffice. Further work should test for cross reactivity of both primer sets to more Rhinosporideacae species.

In the detection capacity test, six of the ten *S. destruens* positive samples (by histology) were confirmed positive by qPCR with 100 % agreement between the two primer sets. The discrepancy between the histology and qPCR for four of the ten *S.*

destruens positive samples can be explained by (a) very little available fish tissue, as each organ of emaciated fish was halved for histology and qPCR analysis, (b) unequal distribution of the parasite within the organ, and (c) the possible misidentification of other *Dermocystidium* spp. as *S. destruens* in histological sections (Mendonca and Arkush, 2004). Small tissue samples, in some cases below 5 mg, showed non-homogeneous distribution of the parasite in the organ as well as low extraction efficiencies. This could lead to very low parasite genomic DNA available for PCR. As a result, such samples may test negative by PCR (i.e. false negative).

Discrepancies between histology positive samples and PCR analysis have also been reported in other studies involving aquatic organisms (Jones *et al.*, 2003, Kozubikova *et al.*, 2008, True *et al.*, 2009). Jones *et al.* (2003) and True *et al.* (2009) failed to detect the parasite *Parvicapsula minibicornis* in *O. tshawytscha* tissues with histological evidence of infection. This was attributed to either misidentification of the parasite in histological sections, mislabelling of samples or PCR false negatives due to the presence of PCR inhibitors. The difficulty of detecting low parasite numbers in histological sections (Mendonca and Arkush, 2004) is also evident in the detection of *S. destruens* in four of the five histologically negative samples by both primer sets. Only two qPCR positive samples were detected as positive by both primer sets. Discordance is typical for samples with low parasite quantities (True *et al.*, 2009).

Fifteen percent of reproductive *L. delineatus* and 33 % of non-reproductive *L. delineatus* detected as positive by qPCR were also found as positive by light microscopy (Table 4.3), reflecting the low agreement between these methods. In *O. tshawytscha* challenged with *S. destruens* by bath immersion, the percentage

agreement between nested PCR (with 1 pg μl^{-1} detection limit) and Gram stained kidney smears was 21 % (Mendonca and Arkush, 2004). Therefore, PCR (nested and quantitative) is better at detecting low infections and should thus be the preferred method for detecting *S. destruens*.

4.4.2 Influence of reproductive state on *Sphaerothecum destruens* prevalence and infection levels

In cohabitation studies, reproductive *L. delineatus* experienced higher prevalence and infection levels of *S. destruens* compared to non-reproductive *L. delineatus*. Overall, these results were in accordance to the predictions derived from the work of Gozlan *et al.* (2005) that reported high prevalence of *S. destruens* in moribund and sub-clinical *L. delineatus* during their reproductive season.

Although these results indicate that *L. delineatus*' reproductive state is influencing *S. destruens* prevalence and infection levels, other factors also need to be considered. The mean water temperature between the two experiments significantly differed with higher temperatures during the reproductive season (by 6 °C). Variation in temperature can affect fish metabolism (Wang *et al.*, 2009) and seasonality in temperature has also been associated with variation in immunocompetence (Bowden, 2008). Studies of the effect of temperature on the immunocompetence of fish indicate that lower temperatures have a negative impact on fish immunity (Bly and Clem, 1992, Zapata *et al.*, 1992, Le Morvan *et al.*, 1998, Alcorn *et al.*, 2002, Saha *et al.*, 2002, Cheng *et al.*, 2009). Therefore, the increase in temperature during the reproductive period of *L. delineatus* should favour *L. delineatus*'s immunity and can probably not account for the increase in *S. destruens* prevalence and infection level. Also, these differences in temperature are common during the reproductive and non-reproductive periods of *L. delineatus* in the wild (Pinder and Gozlan, 2004). The

lethal water temperature for adult *L. delineatus* has been reported to be 33.6 °C and they have been reported to show no signs of thermal stress in aquaria with temperatures 26-28 °C (Arnold and Längert, 1995). Furthermore, *S. destruens* prevalence in the current experiment were quite similar with the ones reported in *L. delineatus* cohabited in the presence of *S. destruens* in Gozlan *et al.* (2005) at a mean temperature of 20 °C.

Parasites have a range of permissive temperatures at which they complete their life cycle with some temperatures being more permissive (Kerans *et al.*, 2005, Fels and Kaltz, 2006, Wolinska and King, 2009). Temperature dependence in *S. destruens* spore survival and production of zoospores has been observed (Chapter 3; Andreou *et al.* 2009). Use of zoosporulation production as an indication of temperature preference by *S. destruens* suggested that 4 °C and 15 °C were more optimal (higher numbers of zoospores released and motility present for longer). This would suggest that infection pressure by *S. destruens* should be higher in the non-reproductive *L. delineatus* (19 °C± 0.03). However, temperature may have different effects on the proliferation of *S. destruens* within the host tissues.

Variation in the immune system (blood lymphocyte numbers and humoral immune responses) of vertebrates has been observed even under constant temperature and photoperiod (Zapata *et al.*, 1992). It has been suggested that this variation is the result of endogenous endocrine rhythms. Some parasites have been reported to increase in prevalence during their host's reproductive season (Simkova *et al.*, 2005). Such parasites can act as parasitic castrators ensuring that the hosts direct resources normally used for reproduction to somatic growth (Heins *et al.*, 2004). Other parasites have been shown to synchronise the shedding of infectious stages with reproduction.

Myxobolus fallax, a myxosporean, infects the testes of the dwarf green tree frog *Litoria fallax* and has been observed to shed its infectious spores during natural spawning (Browne *et al.*, 2006). The association between spore shedding and reproduction was supported further when spore shedding was induced in infected male hosts following the administration of reproductive hormones.

S. destruens spores have been reported from seminal fluids of reproductive *O. tshawytscha* and it has been hypothesised that release through seminal fluids is one possible route of the parasite's dissemination (Arkush *et al.*, 1998, 2003). The hypothesis that *S. destruens* synchronises its release with *O. tshawytscha* gametes is likely as the host's return to freshwater is marked with spawning followed by mortality (Quinn, 2004). Accordingly, the possibility that the observed increase in prevalence and infection level of *S. destruens* during *L. delineatus*'s reproduction is the result of the parasite's interaction with hormonal changes during reproduction cannot be excluded. Future work should include the monitoring of *S. destruens* infection level in pre-spawning, spawning and post-spawning wild *L. delineatus* while measuring reproductive hormone levels in each group. A similar approach to Browne *et al.* (2006) could also be applied; infected *L. delineatus* can be administered reproductive hormones whilst monitoring infection levels and parasite release through reproductive fluids.

The majority of studies investigating the effect of reproductive state on disease prevalence focus on sampling wild fish populations at different time intervals during their reproductive and non-reproductive seasons (Skarstein and Folstad, 1996, Skarstein et al., 2001, Kortet *et al.*, 2003, Simkova *et al.*, 2005, Lamkova *et al.*, 2007, Vainikka *et al.*, 2009). Although a sample of 100 fish was obtained during and out of

the reproductive season of *L. delineatus* these samples were only tested for *S. destruens* presence using the kidney. As each organ's prevalence varies in time with the highest prevalence being gills in non-reproductive *L. delineatus* and kidneys in reproductive ones, it was thus not possible to compare the disease prevalence using these samples.

The high reproductive investment experienced by both genders in *L. delineatus* (i.e. nest guarding and batch spawning) has led to the hypothesis that male and female *L. delineatus* would not differ in the level of parasitism by *S. destruens*. In line with this prediction, sex biased parasitism has not been observed here as reproductive female and male *L. delineatus* did not differ in the prevalence and infection level of *S. destruens*. This suggests that the species' high reproductive investment highly compromises its immunocompetence against infections such as the ones caused by *S. destruens*.

In addition to determining disease prevalence and abundance other studies have also quantified various aspect of the immune system, such as spleen somatic index, head kidney phagocytes, leucocyte concentration and plasma immunoglobin M, while investigating the effect that reproductive state can have on immunity (Skarstein *et al.*, 2001, Kortet *et al.*, 2003, Lamkova *et al.*, 2007, Vainikka *et al.*, 2009). Quantifying different aspects of the immune system represents a more direct way of addressing the association of reproduction and the immune system. Although using indirect methods such as disease prevalence and infection level to assess the impact of reproduction on immunocompetence has been previously used (Norris *et al.*, 1994), susceptibility to disease is influenced by a myriad of factors including temperature and host age.

Therefore, although host reproductive state appears to be associated, at least partially, with *S. destruens* infection it was not possible to exclude other factors.

The present investigation would benefit from including some measurements of the immune system as this would also allow an understanding of how the host's immune system operates. Immune parameters need to be carefully chosen as they vary in the manner in which they are affected by reproduction and by parasite species (Lee, 2006, Sitja-Bobadilla, 2008). Presently, there have been no studies on *L. delineatus*'s immune system but studies on other cyprinids such as *R. rutilus* are available and can be used as guidelines for selecting the appropriate immune parameters to study.

4.4.3 Reproduction, infection and fish condition

The reproductive state of *L. delineatus* appeared to influence fish condition. In particular, reproductive *L. delineatus* had significantly lower condition factors compared to non-reproductive *L. delineatus*. It was not possible to attribute this difference to either the reproductive state of the fish or to the higher S. *destruens* infection levels associated with reproductive *L. delineatus*. An investigation of differences in condition factors between reproductive and non-reproductive *L. delineatus* free from *S. destruens* infections would allow a better understanding of this relationship.

In the reproductive state, parasitized male and female *L. delineatus* did not have significantly different condition factors. This suggests that there was no apparent cost of parasitism, at least as measured by fish condition between the genders. Poor fish condition, specifically emaciation was reported for dead and moribund *L. delineatus* that were infected with *S. destruens* during *L. delineatus*' reproductive season (Gozlan

et al. 2005). Emaciation was also observed in *L. delineatus* mortalities during the reproductive period experiment (this study). However, when non-moribund parasitized fish were compared to their non-parasitized counterparts, there was no significant difference in their condition factors. Nevertheless, the possibility that advanced *S. destruens* infections could result in decreases in fish condition during spawning cannot be excluded. Emaciation was not observed in the non-reproductive period experiment.

There was no association between infection level and both weight and length of parasitized reproductive female and male *L. delineatus*. Associations between parasite infection intensity, weight and length have been reported in other parasite systems (Zelmer and Arai, 1998, Simkova *et al.*, 2005). For example, the parasite *R. acus* increased its abundance with host length in *P. fluviatilis* (see Zelmer and Arai, 1998) and host weight in *B. barbatula* (see Zelmer and Arai, 1998, Simkova *et al.*, 2005). Increased infection intensity with increasing age (and hence size) of *S. destruens* was reported for *O. tshawytscha* (see Arkush *et al.*, 1998).

Chapter 5: Multi-host complexes: the effect of a second host, topmouth gudgeon *Pseudorasbora parva*, on the prevalence and infection level of *Sphaerothecum destruens* in the primary host, the sunbleak *Leucaspius delineatus*.

Abstract The impact of increasing or decreasing host species diversity on disease prevalence has been extensively studied in specialist parasites. However, similar studies are scarce for generalist parasites. As a multi-host parasite, Sphaerothecum destruens can be used to test the impact of increasing host species diversity on its prevalence and infection intensity in its hosts. In this study, two S. destruens hosts, the sunbleak Leucaspius delineatus and topmouth gudgeon Pseudorasbora parva, were used to investigate the effect of increasing host number on the prevalence and infection levels of S. destruens. Individuals of L. delineatus, originating from a population with an established S. destruens infection, were cohabited on their own and in the presence of topmouth gudgeon Pseudorasbora parva. The influence of cohabitation with P. parva on the somatic condition of L. delineatus was also investigated. A quantitative PCR revealed no significant difference in infection level and prevalence of S. destriens in L. delineatus cohabited on their own and in the presence of P. parva suggesting that S. destruens infection in L. delineatus is mostly driven by within-species transmission instead of between species transmission (from P. parva to L. delienatus). However, L. delineatus cohabited in the presence of P. parva had a significantly lower somatic condition (by ~10 %) compared to individuals cohabited in the absence of P. parva suggesting that the presence of P. parva can have a negative impact on the somatic condition of L. delineatus. The importance of these findings for multi-host parasite systems are discussed.

5.1 Introduction

Parasites play an important role in the ecology of their host species through shaping community structure (Dobson and Hudson, 1986), influencing species interactions (Hatcher *et al.*, 2006) and regulating host population dynamics (Hudson *et al.*, 1998). These influences are the result of parasite virulence, i.e. the fitness reduction experienced by the host (Thrushfield 2007). Fitness reduction as a result of virulence has been quantified in a number of ways including, decrease in survival (Bull, 1994), decrease in fecundity (Perlman and Jaenike, 2003) and changes to body structure (Agnew and Koella, 1997). Parasite virulence and its evolution have been studied from both theoretical (Bull, 1994, Frank, 1996, Day, 2001, 2003) and empirical perspectives (Ebert, 1994, Messenger *et al.*, 1999, Benmayor *et al.*, 2009) and appear to be driven by transmission routes and host density (Bull, 1994).

The majority of theoretical and empirical studies on parasite virulence have focused on single host-parasite systems due to their relative simplicity (compared to multi-host systems) (Bull, 1994). However, the majority of parasites can infect and be transmitted by multiple hosts and exist in multi-host complexes (Woolhouse *et al.*, 2001, Gandon, 2004, Rutrecht and Brown, 2009). Well known examples of multi-host parasites include; *Gyrodactylus salaris* which can infect a number of salmonid fish and has caused high mortalities in Norwegian populations of *S. salar* (see Bakke *et al.*, 2007) and Rhinderpest virus in the Serengeti which can infect large vertebrates including the wildebeest *Connochaetes taurinus* (Burchell) and can cause high mortalities in African cattle (Dobson and Hudson, 1986).

A largely unconsidered aspect of generalism is its effect on pathogenicity (Woolhouse *et al.*, 2001). Regoes *et al.* (2000) modelled the evolution of virulence (defined as parasite-induced host mortality) in a two-host system and found that where generalist parasites evolved, their virulence was moderated by the presence of more than one host. Gandon (2004) however, suggests that generalists might evolve virulence levels depending on host quality, for example, high quality hosts could experience higher virulence and higher transmission rates. Woolhouse *et al.* (2001) proposed that the relationship of transmission and virulence between and within hosts will determine the pathogen's virulence in a second host compared to the first. Consequently, the introduction of a second host can increase or decrease the parasite's virulence in the first host. Where the second host contributes very little to the parasite's fitness, virulence will be unconstrained within that host. Under this scenario, unconstrained virulence in dead end hosts is predicted.

Generalism can also have important implications on the parasite's epidemiology (Woolhouse *et al.*, 2001). While single-host parasites must be able to persist in their hosts, multi-host parasites can infect hosts in which they do not persist indefinitely. These pathogens are maintained by infecting and maintaining infections in multiple species (also known as reservoir hosts) and can occasionally occur as localised outbreaks of variable sizes (Woolhouse *et al.*, 2001, Jokela *et al.*, 2005).

The number of empirical studies looking at parasite virulence against a multi-host background are very limited and demonstrate a large variance in virulence levels on different hosts (Perlman and Jaenike, 2003). For example, Perlman and Jaenike (2003) showed that the nematode *Howardula aoronympium* had higher virulence (measured as a decrease in female fecundity) in its common host species compared to novel

species. Their study thus provides support for the prediction of Gandon (2004) where higher virulence levels are predicted in high quality hosts. Rutrecht and Brown (2009) showed that the microsporidian *Nosema bombi* was most virulent (through castration) to its less susceptible host *Bombus terrestris* while it was less virulent (infected colonies produced queens and males capable of reproduction) to its more susceptible host *B. lucorum*. This work also supports the prediction of Woolhouse *et al.* (2001) that generalist parasites would display high virulence in non-suitable hosts (in this example *B. terrestris*). *N. bombi* is believed to be maintained through a multi-host system with varying virulence levels on different hosts. Rutrect and Brown (2009) however, did not investigate how virulence and parasite prevalence would vary when the two susceptible species shared resources and thus could transmit the disease to one another.

Empirical work on the simultaneous presence of more than one susceptible host and its influence on the parasite's virulence is limited. Riley *et al.* (2008) combined field studies and modelling of disease transmission to determine the multi-host transmission dynamics of *Schistosoma japonicum* in the Samar province of the Philippines. They determined parasite infection loads of humans, potential mammalian hosts (dogs, cats, domesticated water buffalo and rats) and snails and modelled varying transmission dynamics. Their results indicated that transmission dynamics tend to be location specific and that in the case of the Samar province the transmission intensity from snails to mammals appeared to be driving the difference in infection intensity between different locations within the province.

Although the impact of increasing or decreasing host species diversity on disease prevalence and severity has been extensively studied using specialist parasites

(Upatham and Sturrock, 1973, Mitchell *et al.*, 2002, Keesing *et al.*, 2006, Kopp and Jokela, 2007, Thieltges *et al.*, 2009), limited work exists for generalist parasites. Generalist parasites are an important part of communities and the presence and use of hosts (under different host combinations) is expected to influence the parasite's virulence in host populations (Fenton and Pedersen, 2005). More empirical studies investigating these relationships are therefore needed. As a multi-host parasite, *S. destruens* can be used to test the impact of increasing host diversity on its prevalence and infection intensity in different species.

In the work by Gozlan *et al.* (2005), *S. destruens* was transmitted from *P. parva* to *L. delineatus* with extensive loss in somatic condition and total spawning inhibition in the latter. A paradox was thus presented by these findings: how would *S. destruens* be maintained when it appeared to be driving its host to extinction? Possible explanations included that *S. destruens* only recently (in evolutionary terms) acquired the ability to infect *L. delineatus*, and thus had not evolved optimal virulence in that host, or that the disease would be maintained in a multi-host system, i.e. *P. parva* acted as a reservoir for *S. destruens*. *P. parva* has expanded its range throughout continental Europe resulting in *L. delineatus* and *P. parva* having overlapping ranges (Carpentier *et al.*, 2007). Due to the conservation status of *L. delineatus* and the adverse effects that the presence of *P. parva* and *S. destruens* appear to have on *L. delineatus*, it was important to determine whether the parasite's virulence (defined here as infection level and loss of somatic condition) was independent of the presence of a second host, *P. parva*.

This chapter aimed to elucidate the mechanisms by which *S. destruens*, a generalist parasite, is maintained in a multi-host complex. *L. delineatus*, originating from a

population with an established *S. destruens* infection were cohabited in the absence and presence of *P. parva*, investigating the effect that an additional *S. destruens* host had on the parasite's prevalence, infection level and somatic condition of *L. delineatus*.

5.2 Materials and methods

5.2.1 Fish source

P. parva were supplied by the Environment Agency and originated from a location in Cheshire, England (53°22' N; 3°08' W). A total of 100 fish were received in February 2006. *P. parva* were kept in aerated 70L aquaria until used in experiments. *L. delineatus* were obtained from Stoneham lakes on May 15th, 2006 as described in Chapter 4; section 4.2.2.

The prevalence of *S. destruens* in *L. delineatus* and *P. parva* was ascertained as follows. As described in Chapter 4; section 4.2.2, 100 *L. delineatus* were euthanised upon delivery and *S. destruens* prevalence was determined using their kidneys. For *P. parva*, however, 20 fish were euthanised with a 2-phenoxyethanol overdose upon arrival and were tested for *S. destruens* using real time PCR. Tissues from the kidney, liver and lower intestine were pooled for each *P. parva* individual for analysis.

5.2.2 Cohabitation experiments

Two cohabitation setups were used for these experiments. The first cohabitation consisted of *L. delineatus* (from Stoneham lakes) cohabited on their own (Figure 5.1). In the second cohabitation the identified healthy carrier of *S. destruens*, *P. parva*, (Gozlan *et al.*, 2005) was cohabited with *L. delineatus* (Figure 5.1). Both cohabitation

setups were duplicated. *P. parva* originated from a location in Cheshire, England whereas *L. delineatus* originated from Stoneham lakes (see Figure 5.1). A total of 100 fish were cohabited in a set of three-70L aquaria used in each of the two cohabitation experiments. In the first cohabitation, 100 *L. delineatus* were cohabited on their own (Figure 5.1). The cohabitation with *P. parva*, cohabited 30 *P. parva* with 70 *L. delineatus* (Figure 5.1). The sex ratio of both species used was approximately 1:1. The experiment was performed for 60 days during the *L. delineatus*'s reproductive season (from May 17th to July 16th).

The water used in the re-circulating systems was dechlorinated tap water and fish were introduced into the system after being rinsed with clean water. The flow rate was approximately two litres per minute. Water was filtered through a biological filter with gravel substrate, and was then redistributed to the aquaria. In this experiment, fish in different tanks shared the same water supply but remained visually isolated from one another. Fish were fed twice a day with commercial flake food (Nutrafin MAX, Hagen) and kept under natural photoperiod at room temperature. Temperature was recorded hourly using a temperature recorder (Mean water temperature was 25 °C ± 1.5; Tinytag Splash and Aquatic, OmniInstruments, Dundee, UK). Each re-circulating system had its own nets and cleaning devices.

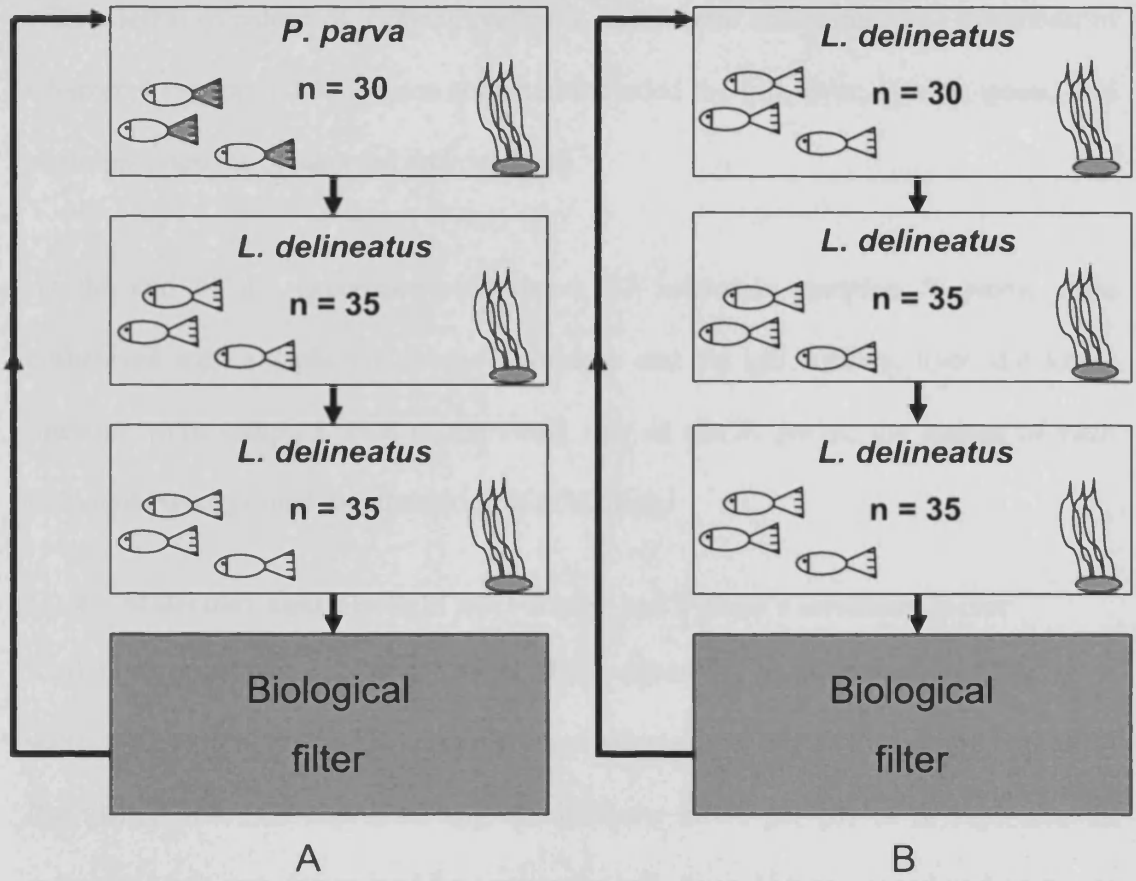


Figure 5.1: Diagrammatic overview of the cohabitation set up used. Water flow between the biological filter and water tanks is depicted by arrows (→). Overall, water is pumped (using a Laguna 2000 water pump) from the biological filter to the top tank from which it flows through the bottom two tanks and back into the biological filter. Maximum water volume per cohabitation system was 280L. (A) Thirty topmouth gudgeon *Pseudorasbora parva* were cohabited with 70 sunbleak *Leucaspius delineatus* (n = 2 cohabitation systems). (B) A total of 100 *L. delineatus* from the same source were cohabited (n = 2 cohabitation systems).

5.2.3 Sampling and sample processing

The fish were checked twice daily at which time any mortalities were collected and dissected. Five non-moribund *L. delineatus* were randomly sampled at 14-day intervals from each re-circulating system. In the cohabitation with *P. parva*, *L. delineatus* tanks from which three fish were sampled were alternated with each sampling period. Similarly, in the *L. delineatus* cohabitation the tanks from which one fish was sampled were alternated with each sampling period. Fish were euthanised

with a lethal overdose of 2-phenoxyethanol anaesthetic and sampled as described in Chapter 4 section 4.2.3. Tissues collected included the gill, liver, kidney, gonad and posterior intestine of sampled and dead fish.

At the end of the experiment (60 days), 23 randomly sampled *P. parva* were euthanised with a 2-phenoxyethanol overdose and the gill, kidney, liver and lower intestine were sampled. Due to the small size of the *P. parva*, the organs of each individual were pooled together prior to extraction.

5.2.4 Molecular analysis, light microscopy and Fulton's condition factor

Real time quantitative PCR followed DNA extraction as described in Chapter 4; section 4.2.1. Real time PCR was performed using primer Set 1 (Chapter 4, Table 4.1). Prevalence and infection level (pg *S. destruens* DNA per µl) of *S. destruens* in individual fish was determined by testing the gill, liver, kidney, gonad and posterior intestine. Total infection level per individual fish was calculated by summing the infection level across the organs tested. Tissue tropism in *L. delineatus* displayed by *S. destruens* was also investigated. Light microscopy and Fulton's condition were performed as described in Chapter 2; section 2.2.2 and Chapter 4, section 4.2.4 respectively.

5.2.5 Statistical analysis

All statistical analyses were performed using SPSS 14.0 (SPSS Inc. Chicago, Illinois, USA). Data are given as means ± standard deviation (SD) unless otherwise stated. The median and inter-quartile ranges are also provided for *S. destruens*' prevalence, infection levels and condition factors. The Mann-Whitney *U* test was used to test for differences in *S. destruens*' prevalence, infection level and fish condition between *L. delineatus* cohabited in the presence and absence of *P. parva*. Differences in condition

factors between the sexes were also investigated. *S. destruens* prevalence was calculated as described in Chapter 2; section 2.2.2. Statistical significance was accepted when $P \leq 0.05$.

5.3 Results

5.3.1 *Sphaerothecum destruens* prevalence in the wild source population, water temperature and mortalities.

S. destruens prevalence in wild *L. delineatus* population was found to be 2 % by real time PCR as described in Chapter 4; section 4.3.3. *S. destruens* was not detected in any of the 20 *P. parva* tested from the source population. Mean water temperature in the aquaria throughout the experiment was 25 ± 1.5°C. During the reproductive period, *L. delineatus* experienced an outbreak of white spot disease. This outbreak occurred in *L. delineatus* cohabited in the presence and absence of *P. parva*. It was thus not possible to investigate mortality differences due to infection with *S. destruens*.

5.3.2 Does the presence of a second host influence *Sphaerothecum destruens* prevalence, infection levels and fish condition?

Real time PCR was successfully performed on a total of 403 organs. A standard curve was generated using dilutions of genomic *S. destruens* DNA (10 ng to 1 pg) with a mean R^2 value of 0.993 ± 0.01. All extraction negative samples were detected as negative during PCR. The detection limit across all real time PCR runs was 10^3 *S. destruens* spores and 10^4 *S. destruens* spores spiked with 15 mg of common carp tissue. Infection with *S. destruens* was present in *L. delineatus* cohabited in the presence and absence of *P. parva*. The median and inter-quartile ranges for prevalence and infection levels for both reproductive and non-reproductive *L. delineatus* are provided in Table 5.1. Mean length, weight and condition factors by experimental treatment and gender are summarised in Table 5.2. No infection with *S. destruens* was detected in the 23 *P. parva* sampled at the end of the experiment.

Table 5.1: Summary of the median and percentiles (25^{th} and 75^{th}) for *Sphaerothecum destruens* infection level and prevalence and sunbleak *Leucaspius delineatus* condition factor (K) for *L. delineatus* in the absence and presence of topmouth gudgeon *Pseudorasbora parva*.

	L. delineatus			***L. delineatus & P. parva***		
Variable	**Median**	**25^{th} percentile**	**75^{th} percentile**	**Median**	**25^{th} percentile**	**75^{th} percentile**
S. destruens infection level	0	0	0.43	0	0	0.43
S. destruens prevalence	0	0	1	0	0	1
L. delineatus condition factor	0.74	0.69	0.80	0.65	0.59	0.71

Table 5.2: Summary data of the sunbleak *Leucaspius delineatus* sampled: *L. delineatus* (*L. delineatus* cohabited in absence of topmouth gudgeon *Pseudorasbora parva* (TMG); *L. delineatus* & *P. parva* (*L. delineatus* cohabited in presence of *P. parva*); Sex (F: female, M: male); Fork length in millimetres, Weight in grams; Condition factor (K). Mean and ±SD are shown.

Treatment	**Sex**	**Sample no.**	**Fork length**		**Weight**		**K**	
			Mean	**±SD**	**Mean**	**±SD**	**Mean**	**±SD**
L. delineatus	F	14	63.6	5.2	1.9	0.6	0.7	0.08
	M	18	58.1	3	1.4	0.2	0.7	0.08
L. delineatus & *P. parva*	F	17	60.9	2.6	1.5	0.3	0.7	0.1
	M	23	56.3	3	1.2	0.2	0.7	0.07

The prevalence of *S. destruens* did not differ significantly between reproductive *L. delineatus* cohabited with or without *P. parva* (38 %; n = 40 and 41 %; n =32

respectively; Mann-Whitney *U* test; n = 72; *P* = 0.78). Similarly, there was no significant difference in infection levels between reproductive *L. delineatus* cohabited with or without *P. parva* (Mann-Whitney *U* test; n = 72; *P* = 0.76; and Figure 5.2). There was no significant difference in infection level and disease prevalence between female *L. delineatus* cohabited in the absence and presence of *P. parva* (Infection level: Mann-Whitney *U* test; n = 31; *P* = 0.54; Prevalence: Mann-Whitney *U* test; n = 31; *P* = 0.49). Similarly, there was no significant difference in infection level and disease prevalence between male *L. delineatus* cohabited in the absence and presence of *P. parva* (Infection level: Mann-Whitney *U* test; n = 41; *P* = 0.85; Prevalence: Mann-Whitney *U* test; n = 41; *P* = 0.71).

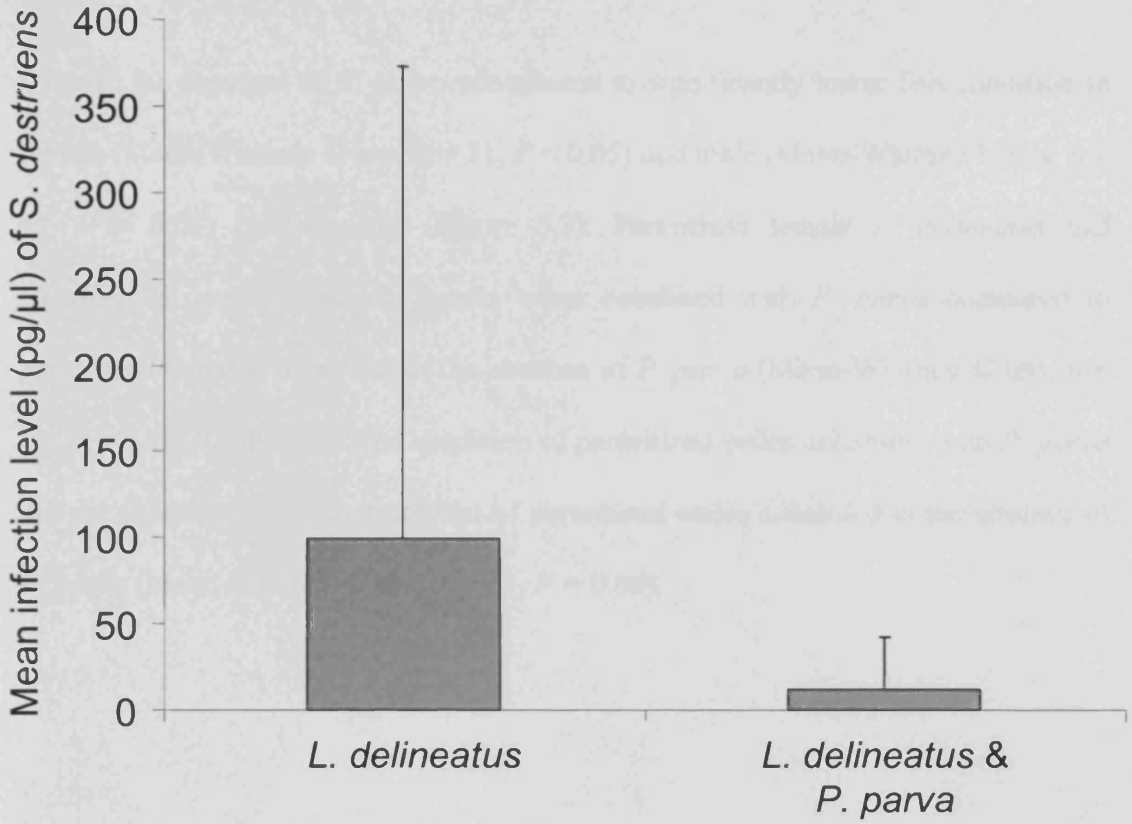


Figure 5.2: Infection level (pg/µl) of *Sphaerothecum destruens* in parasitized sunbleak *Leucaspius delineatus* cohabited either in the absence or presence of topmouth gudgeon *Pseudorasbora parva*. Infection levels were determined by real time quantitative PCR of the 18S rRNA gene of *S. destruens*. Means ± SD are given. *L. delineatus* (n = 13), *L. delineatus* & *P. parva* (n = 15). Infection levels were not statistically different (Mann-Whitney *U* test; n = 72; *P* = 0.76) with mean ranks of 37.2 and 35.9 for *L. delineatus* cohabited in the absence and presence of *P. parva*.

In the absence of *P. parva*, the somatic condition of parasitized female and male *L. delineatus* did not significantly differ (Mann-Whitney *U* test; n = 13, *P* = 0.23). The somatic condition of parasitized female *L. delineatus* did not significantly differ from the somatic condition of non-parasitized female *L. delineatus* (Mann-Whitney *U* test; n = 14, *P* = 0.71). Similarly, the somatic condition of parasitized male *L. delineatus* did not significantly differ from non-parasitized *L. delineatus* (Mann-Whitney *U* test; n = 18, *P* = 0.49).

Overall, the presence of *P. parva* contributed to significantly lower fish condition in female (Mann-Whitney *U* test; n = 31, $P < 0.05$) and male (Mann-Whitney *U* test; n = 41, $P < 0.05$) *L. delineatus* (Figure 5.3). Parasitized female *L. delineatus* had significantly lower condition factors when cohabited with *P. parva* compared to parasitized females cohabited in the absence of *P. parva* (Mann-Whitney *U* test, n = 13, $P < 0.05$). Conversely, the condition of parasitized males cohabited with *P. parva* did not significantly differ from that of parasitized males cohabited in the absence of *P. parva* (Mann-Whitney *U* test; n = 15, $P = 0.06$).

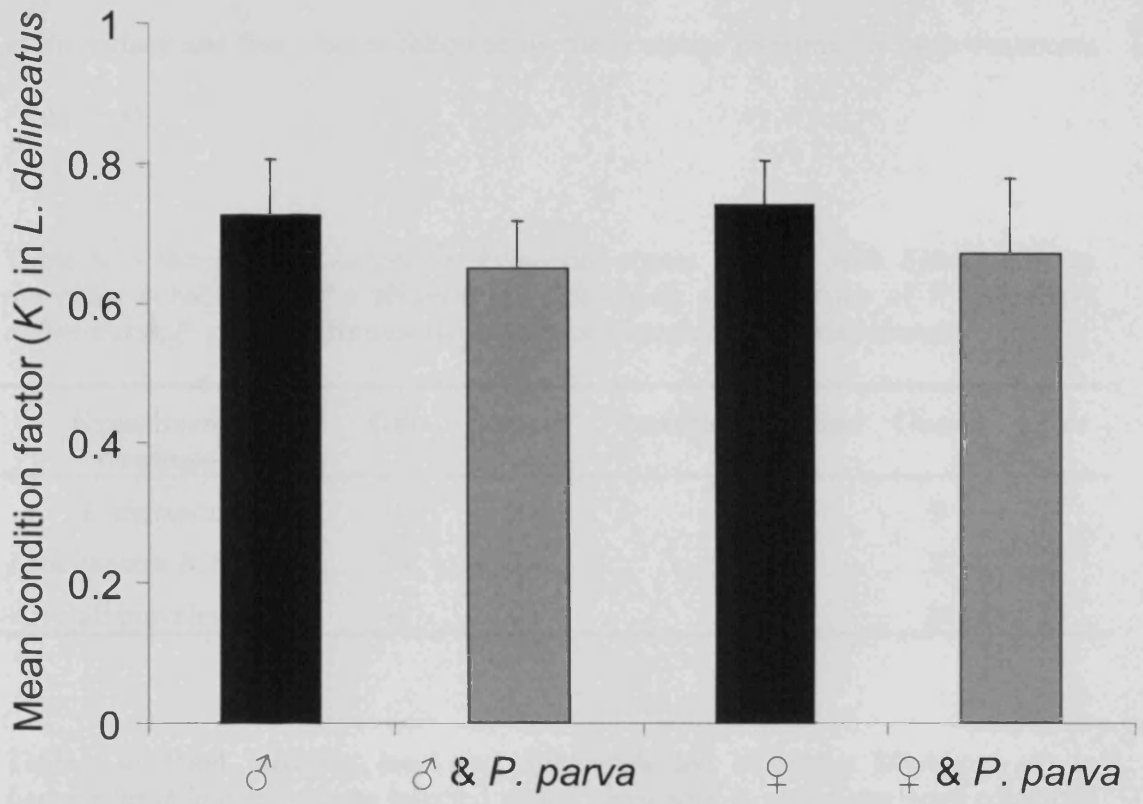


Figure 5.3: Mean condition factor in male (♂) and female (♀) sunbleak *Leucaspius delineatus* cohabited in the absence (black) and presence (grey) of topmouth gudgeon *Pseudorasbora parva*. Means ± SD are given. ♂ (n = 18), ♂ & *P. parva* (n = 14), ♀ (n = 23) and ♀ & *P. parva* (n = 17)

5.3.3 Tissue tropism of *Sphaerothecum destruens* in *Leucaspius delineatus*

The prevalence of *S. destruens* in *L. delineatus* differed according to the organ of interest (Table 5.3). Similar *S. destruens* prevalence was observed both in the absence and presence of *P. parva* for the posterior intestine, gonad and liver. Differences in *S. destruens* prevalence were present for the gill and kidney (Table 5.3). Overall organ prevalence (for both groups) shows that *S. destruens* was equally prevalent in the gill and kidney and was closely followed by the posterior intestine. The gonad and liver had lower overall *S. destruens* prevalence. Infection level in organs (pg *S. destruens* DNA per µl) did not greatly differ between the two treatments. Infection was highest

in the kidney and was closely followed by the posterior intestine for both treatments (Table 5.4).

Table 5.3: Number of *Leucaspius delineatus* organs infected with *Sphaerothecum destruens* cohabited in the absence (*L. delineatus*) and presence of *P. parva* (*L. delineatus* & *P. parva*). The overall prevalence in each organ is also shown.

Experimental treatment	Gill	Kidney	Posterior intestine	Gonad	Liver
L. delineatus	9	22	16	9	3
L. delineatus & *P. parva*	23	10	15	8	5
Overall prevalence (%)	44	44	43	24	11

Table 5.4: Total infection level (pg *Sphaerothecum destruens* DNA per µl) in *Leucaspius delineatus* organs infected with *S. destruens*. *L. delineatus* were cohabited in the absence (*L. delineatus*) and presence of topmouth gudgeon *Pseudorasbora parva* (*L. delineatus* & *P. parva*).

Experimental treatment	Gill	Kidney	Posterior intestine	Gonad	Liver
L. delineatus	1.89	1167.8	113.3	0.7	5.9
L. delineatus & *P. parva*	22.43	107.8	29.2	11.6	1.4

5.3.4 Light microscopy

Not all *L. delineatus* positive for *S. destruens* via qPCR were positive by histology (Table 5.5). qPCR detected six times more positive *L. delineatus* compared to histological analysis of tissue sections stained with H&E and Gram's stain. The percentage agreement between the two detection methods ranged from 13-15%.

Table 5.5: Number of sunbleak *Leucaspius delineatus* detected as positive by qPCR and histology (tissue sections stained with Haematoxylin & Eosin and Gram's stain) and the percentage (%) agreement between the two detection methods. *L. delineatus* were cohabited in the absence (*L. delineatus*) and presence of topmouth gudgeon *Pseudorasbora parva* (*L. delineatus* & *P. parva*).

Experimental treatment	qPCR positive	Histology positive	% agreement
L. delineatus	13	2	15
L. delineatus & *P. parva*	15	2	13

5.4 Discussion

As parasite prevalence and intensity can be influenced by the number of susceptible hosts present, it was important to study the epidemiology of *Sphaerothecum destruens* in the presence of more than one host. Cohabitation experiments here suggest that the presence of a second host, *P. parva*, does not influence the prevalence and infection levels of *S. destruens* in its susceptible host *L. delineatus*.This was also true when the genders were analysed separately. This could suggest that infection with *S. destruens* in *L. delineatus* is mostly driven by within-species transmission of the parasite as opposed to between-species transmission (from *P. parva* to *L. delineatus*).

Although the difference in infection levels between *L. delineatus* cohabited in the presence and absence of *P. parva* was not significantly different, *S. destruens* infection levels were higher in the absence (99 pg *S. destruens* DNA per µl) compared to the presence of *P. parva* (12 pg *S. destruens* DNA per µl). This difference was mainly driven by the presence of one heavily infected individual (978 pg *S. destruens* DNA per µl). As naturally infected *L. delineatus* were used in this cohabitation, this

high infection could be the result of a long-term infection and not necessarily the result of the treatment. This is a confounding factor of the experimental design.

S. destruens was not detected in *P. parva* in the source population at the beginning nor at the end of the experiment. This has also been observed by Gozlan *et al.* (2005) where in their experiments, *L. delineatus* were exposed to *S. destruens* through cohabitation with *P. parva*. *S. destruens* however, was still not detected in *P. parva* despite being characterised as a healthy host (Gozlan *et al.*, 2005). Molecular detection is more sensitive than light microscopy, however, the molecular tools available for *S. destruens* can still not detect very low infections, as would be the case of a healthy carrier. Where detection methods lack in sensitivity, healthy carriers are usually identified through cohabitation with susceptible species (St-Hilaire *et al.*, 2001).

Cohabitation studies of *P. parva* with *L. delineatus* replicated the initial work by Gozlan *et al.* (2005). Gozlan *et al.* (2005) determined *S. destruens* prevalence in both moribund and sub-clinical *L. delineatus* during cohabitation with *P. parva*. *S. destruens* had a prevalence of 28 % (n=32) in non-moribund *L. delineatus* whereas *S. destruens* prevalence in the current experiment was 38 % (n=40). This suggests that exposure to *S. destruens* either through cohabitation with *P. parva* or cohabitation with infected *L. delineatus* results in similar *S. destruens* prevalence in *L. delineatus*. This could be the result of very low between-species transmission and high within-species transmission. However, as the two species were kept in separate tanks and only shared the same water, the influence of contact rates between infected and uninfected *L. delineatus* on the observed results cannot be excluded. Investigating the influence of contact rates on *S. destruens* transmission would be an interesting future research avenue.

Gender differences in body condition as a result of parasitism have been reported for other species. In the eastern bluebird *Sialia sialis* (Linnaeus), infection with feather degrading bacteria led to lower body condition in females compared to males despite similar infection levels (Gunderson *et al.*, 2009). In the case of *S. destruens*, parasitism with *S. destruens* did not lead to significant differences in the somatic condition of female and male *L. delineatus*. However, cohabitation with *P. parva* contributed to lower (~ 10 %) condition factors in female and male *L. delineatus*. Infected females cohabited with *P. parva* had significantly lower condition (by 9 %) compared to infected females cohabited in the absence of *P. parva*. Infected male *L. delineatus* cohabited with *P. parva* did not have significant differences in their condition, however, this was a marginally non-significant difference ($P = 0.06$).

Although the fish were fed to satiation and the two species only shared the same water it appears that the presence of *P. parva* may have an adverse effect on *L. delineatus*'s somatic condition. Gozlan *et al.* (2005) reported emaciation in *L. delineatus* cohabited with *P. parva*. In these experiments, *P. parva* acted as the source of *S. destruens*, thus, it was not possible to decipher between the effect of *P. parva* and *S. destruens* infection on emaciation in *L. delineatus*. This work suggests that the presence of *P. parva* contributes to a worse somatic condition in *L. delineatus*. The processes leading to reduced condition in *L. delineatus* cohabited with *P. parva* are unknown. While the fish do not come in direct contact, chemical recognition of the presence of a different species is still possible (Burnard *et al.*, 2008). This could result in increased stress levels in *L. delineatus* leading to reduced condition. Future work should include a repetition of the experiment presented here while also including uninfected *L.*

delineatus as an additional control. At the time of this work, only *L. delineatus* from Stoneham lakes (where *S. destruens* is present) were readily available.

Chapter 6: Susceptibility of cyprinids to *Sphaerothecum destruens*.

Abstract *Sphaerothecum destruens* is a generalist parasite able to infect a number of salmonid species at varying levels, as well as, the cyprinids *Leucaspius delineatus* and *Pseudorasbora parva*.. Both *L. delineatus* and *P. parva* are invasive to the UK. As parasites carried by invasive species are brought in contact with a suite of potential hosts it is important to determine the susceptibility of UK native species to *S. destruens*. In this study, the susceptibility of four cyprinid species, *Abramis brama*, *Cyprinus carpio*, *Rutilus rutilus* and *Scardinius erythrophthalmus* was investigated. These species are of economical importance to coarse and sport fisheries and naturally co-exist with established populations of *L. delineatus* and *P. parva*. Each host species (n=60) were exposed to *S. destruens* via bath immersion in 8 litres of water containing 8.6×10^4 spores per ml. *S. destruens* presence was determined by nested PCR of a 434 bp amplicon of the 18S rRNA gene of the parasite. Exposure to *S. destruens* led to significantly higher mortalities in *S. erythrophthalmus* (60 % mean mortalities), *A. brama* (53 % mean mortalities), *C. carpio* (11 % mean mortalities) and *R. rutilus* (37 % mean mortlalities) compared to control conditions. Nested PCR revealed *S. destruens* in the tissues of *A. brama*, *C. carpio* and *R. rutilus* at a mean prevalence of 76 %, 11 % and 3 %, respectively. A meta analysis of the reported *S. destruens* prevalence (for salmonid and cyprinid species) and the genetic distance between these species revealed no phylogenetic influence on susceptibility to *S. destruens*; suggesting that a possible key life history characteristic of the parasite is its ability to exploit a broader phylogenetic range of hosts.

6.1 Introduction

Generalist parasites can infect a wide range of hosts. Some hosts can be infected but cannot support the reproduction of the parasite, others can support limited reproduction, whilst, in some hosts the parasite maximises its reproductive output (Holmes, 1979). The potential host range of a parasite is dictated by physiological, behavioural and ecological attributes of the host, that determine the ability of a particular parasite to infect and complete its life cycle (Solter and Maddox, 1998). A genetic basis for potential host suitability is suggested by parasites that are more likely to infect hosts phylogenetically close to their existing ones (Poulin, 2007). Due to existing barriers to dispersal, observed host ranges of parasites often represent only a subset of potential hosts (Perlman and Jaenike, 2003). The process of host translocation in new geographical areas or range expansion by an existing host, allows increased opportunities for parasites to expand their range of potential hosts (Poulin, 2007).

S. destruens is a multi-host parasite which experimental studies have shown to be able to infect a number of salmonid species at varying levels (Arkush *et al.*, 1998). Following inoculation with *S. destruens* spores (through intraperitoneal injection), *O. tshawytscha* and *O. kisutch* were highly susceptible to the parasite with infection of vital organs (kidney and liver) and disease prevalence of 100 % and 98 %, respectively (Arkush *et al.*, 1998). *O. mykiss* and *S. trutta* were found to be less susceptible, with limited parasite replication within their tissues, no mortalities and prevalence of 42.5 % and 43.3 %, respectively. *S. fontinalis* was found to be the least susceptible, with only 2.6 % prevalence with no detectable lesions in the infected tissues. The high susceptibility of *O. tshawytscha* was further supported when the

disease reached a prevalence of 71 % in fish infected with *S. destruens* via bath immersion with *S. destruens* spores (Mendonca and Arkush, 2004).

The discovery of *S. destruens* in *L. delineatus*, a cyprinid, increased the parasite's known species range (Gozlan *et al.*, 2005, Gozlan *et al.*, 2009). In the UK, *S. destruens* has been associated with two invasive cyprinids; *L. delineatus* and *P. parva*. The parasite has been found in established wild populations of *L. delineatus* (see Chapter 2) and has been indirectly (through cohabitation of *P. parva* with naïve *L. delineatus*) shown to be carried by the highly invasive *P. parva* (see Gozlan *et al.*, 2005). In experimental studies, infection with *S. destruens* has led to high mortalities in *L. delineatus* (Gozlan *et al.*, 2005). As parasites carried by invasive species are brought in contact with a suite of potential naïve hosts (Prenter *et al.*, 2004, Dunn, 2009) it is important to determine the susceptibility of native species to *S. destruens*.

Experimental studies investigating host specificity are valuable tools for determining the potential host range of a specific parasite (King and Cable, 2007, Poulin, 2007) and they are particularly important when investigating the potential impact of introduced parasites. Numerous types of experimental exposures can be used to determine susceptibility to a disease agent. For fish these can include the delivery of the agent through intraperitoneal injection, bath immersion with infectious stages of the parasite, infection via food pellets coded with the agent and through cohabitation with infected individuals. These processes range from highly invasive and unnatural methods (e.g. injection of the agent) to more natural methods such as bath immersion or cohabitation with infected individuals. Ideally the method which resembles the natural infection route of the parasite should be preferred. For example, if the parasite

infects new hosts by the consumption of parasite infected tissues, then hosts should be exposed to the agent via parasite coded food pellets.

In this study, the susceptibility of four cyprinid species, *A. brama*, *C. carpio*, *R. rutilus* and *S. erythrophthalmus* was investigated. These species are of economical importance to coarse and sport fisheries and they naturally co-exist with established populations of *L. delineatus* and *P. parva*. Population declines of these four cyprinids would have both significant economical and ecological implications. Experimental infection was performed via bath immersion with *S. destruens* spores. *S. destruens* is believed to infect hosts via attachment of zoospores on the gill, ingestion of spores and passive transfer of spores from the water through the opercula and into the intestine (Arkush *et al.*, 2003). Thus, bath immersion with the agent is closest to the natural infection route and has been previously shown to successfully cause infection in *O. tshawytscha* (see Mendonca and Arkush, 2004).

6.2 Materials and Methods

6.2.1 Source of *Sphaerothecum destruens*

S. destruens spores were cultured *in vitro* in *Epithelioma papulosum cyprini* cells (Fijan *et al.*, 1983) as described in Chapter 3; section 3.2.1.

6.2.2 *Sphaerothecum destruens* detection: DNA extraction and nested PCR

S. destruens was detected using nested PCR. DNA was extracted using the Rodent tail protocol of the Qiagen DNeasy 96 Blood & Tissue kit(Qiagen, Germany) (Chapter 4; section 4.2.1). Prior to extraction, the tissue was cut in fine pieces using a sterile scalpel blade. All steps were performed according to the manufacturer's guidelines, with an overnight incubation at 55 °C and elution volume of 150 µl.

Nested PCR was used to amplify a segment of the 18S rRNA gene of *S. destruens* using the oligonucleotide primers published in Mendonca and Arkush (2004). The sequence of the forward (Sd-1F) and reverse (Sd-1R) primers was 5' CGA CTT TTC GGA AGG GAT GTA TT 3' and 5' AGT CCC AAA CTC GAC GCA CAC T 3', respectively. The first round of amplification yielded a 550 bp long amplicon. The second round assay amplified a 434 bp segment of the 18S rRNA gene; using the forward primer (Sd-2F): 5' CCC TCG GTT TCT TGG TGA TTC ATA ATA ACT 3' and reverse primer (Sd-2R): 5' CTC GTC GGG GCA AAC ACC TC 3'

The reaction conditions for both amplifications were identical except in the amount of starting template. In the first round PCR, 5 µl (200-300 ng total DNA) of template was used whereas in the second 1 µl of first round product was used. The reaction conditions were as follows; a reaction volume of 30 µl contained 0.5 x Promega Flexi Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Bioline), 0.3 µM forward and reverse primer and 0.5 U Taq Polymerase (Promega). Cycling conditions consisted of an initial denaturation cycle at 95 °C for 5 minutes followed by 35 cycles of 45 seconds at 95 °C, 45 seconds at 60 °C and 45 seconds at 72 °C. A final elongation step at 72 °C was performed for 7 minutes. All reactions described here were performed using a Primus 96 thermocycler (MWG Biotech). Controls for each PCR assay included: (a) two *S. destruens* positive controls with 50 ng of pure *S. destruens* DNA from cell culture and (b) two PCR (no template) negative controls. Samples and mixes were handled in a PCR cabinet which was decontaminated using UV irradiation for 15 minutes prior and after each assay preparation.

6.2.3 DNA extraction efficiency and limits of detection of nested PCR

DNA extraction. A serial ten-fold dilution of cell-associated *S. destruens* spores was used to evaluate the extraction efficiency of the Qiagen DNeasy 96 Blood & Tissue kit. Cell-associated spores were selected to ensure a high viability rate since the viability percentage of cell-free spores was unknown. Ten-fold dilutions ranged from 10^6 spores ml^{-1} to 10 spores ml^{-1} in a total volume of 10 ml. Spores were thoroughly re-suspended between dilutions by vigorous vortexing for 30 seconds. Fifty microlitres of each dilution was then extracted giving an estimated total spore range of 50,000 to 0.5 spores per dilution.

A more natural situation for the detection of *S. destruens* was reproduced by adding 50 µl of the spore dilution to 15 mg of *S. destruens*-free common carp *C. carpio* kidney tissue. Although in this model the spores were not contained within the kidney cells, it was an accurate replication of the real life *S. destruens*-tissue model with quantifiable spores and fish tissue.

Duplicate samples of spore serial dilutions in the absence and presence of *C. carpio* kidney tissue were extracted. Mechanical disruption of all samples was achieved using a mixer mill (Mixer Mill MM 300; Qiagen) for 1.5 minutes at 20 Hz. Tissue digestion occurred for 12 hours or overnight at 55 °C and elution volumes were 100 µl for pure *S. destruens* spores and 150 µl for *S. destruens* plus fish tissue. Lysis success was evaluated by sampling the lysate and observing *S. destruens* spores at 40 × magnification under a phase contrast light microscope; quantification of extracted DNA by absorbance at 260 nm (NanoDrop ND-1000; Labtech) and by *S. destruens* specific nested PCR amplification. PCR products (10 µl) and molecular weigh ladder (HyperLadder I, Bioline; Figure 4.1) were migrated through a 1.5 % agarose gel. The

gel was post stained with ethidium bromide (0.5 µg / ml) for 30 minutes and destained in water for 15 minutes. Images were generated with transilluminator and a digital camera (Epi Chemi II Darkroom, UVP Laboratory Products).

Nested PCR. In order to assess the detection limit of the nested PCR assay, genomic *S. destruens* DNA at 50 ng / µl was diluted in 10-fold steps to 0.05 fg / µl in sterile UV irradiated water. Two microlitres of each dilution was used as starting template in the first round amplification giving a range of total *S. destruens* genomic DNA of 0.1 fg to 100 ng. One microliter of the first round PCR product was used as template in the second round reaction. PCR was performed both in the presence and absence of 270 ng total common *C. carpio* kidney genomic DNA. To test if greater amounts of fish DNA affected *S. destruens* detection by nested PCR, the reaction was performed using 1 pg and 10 pg of total genomic *S. destruens* DNA spiked with 270 ng, 540 ng, 810 ng and 1.08 mg total common *C. carpio* kidney genomic DNA.

6.2.4 Restriction enzyme digestion and DNA sequencing

The amplification of *S. destruens* DNA was confirmed by restriction enzyme digestion of the second round *S. destruens* amplicon. Suitable restriction sites for the amplified section of the 18S rRNA gene of *S. destruens* were determined using the REBsites tool from the Restriction Enzyme data BASE (REBASE; Roberts *et al.*,2007). The BML isolate 18S rRNA gene sequence (AY267345) was used. *Rsa*I was selected with restriction site 5'-GT^AC-3' (New England BioLabs Inc, USA). Reaction conditions were are follows; 50 µl reactions, with 1× NEBuffer 1 (10mM Bis Tris Propane-HCl, 10mM $MgCl_2$, 1mM dithiothreitol, pH 7.0 at 25 °C) 20 µl second round PCR product, and 1 U *Rsa*I. The digestion was performed at 37 °C for one hour. Restriction digests of the second round amplicons yielded two bands of 277

bp and 157 bp. Ten microlitres of the restriction digest and molecular weight ladder (HyperLadder I, Bioline) were migrated through a 2 % agarose gel at 80 volts for 1.5 hours. The gel was post stained with ethidium bromide (0.5 μg / ml) for 30 minutes and images were generated with transilluminator and a digital camera (Epi Chemi II Darkroom, UVP Laboratory Products).

In order to confirm that *S. destruens* specific DNA was amplified, a random selection of *S. destruens* positive samples was subjected to sequence analysis. Second round *S. destruens* amplicons were extracted from 1 % agarose gel using the Qiagen Gel Extraction kit (Qiagen, Germany). They were quantified using the spectrophotometer (NanoDrop ND-1000, Labtech) and were sent off for direct sequencing by the Qiagen sequencing services. Sequences were then aligned and checked for homology with the sequence of the three *S. destruens* isolates (AY267344, AY267345, AY267346) using BioEdit v7.0.8.

6.2.5 Fish source

A. brama, *R. rutilus* and *S. erythrophthalmus* were supplied by the Calverton fish farm operated by the Environment Agency (Claverton, Nottingham). *C. carpio* were bought from Water Lane fish farm (Burton Bradstock, Bridport). All fish were one years old (1+) at the time of exposure to *S. destruens* spores. Both farms have internal and external (Fish Inspectorate, CEFAS, Weymouth) fish health checks. There has been no report of *S. destruens* infection in any of the farms. Upon arrival to the Bournemouth University fish holding facilities, the fish were kept in 70 L aquaria (length × width × height = 90 × 30 × 30 cm) at a stocking density of 20 fish per aquarium. A total of 120 fish (60 exposed to *S. destruens* and 60 as controls) were used per species during the challenge experiments. The probability of at least one or

more fish being positive to *S. destruens* following exposure was calculated using the formula:

$$Pr(A) = 1-(1-P)^n$$

where, Pr(A) is the probability of one or more fish being positive to *S. destruens*, P *is S. destruens* prevalence and n is the sample size. Pr(A) was calculated for different sample sizes (n=20 to n=100) for a prevalence range of 1 to 14 % (Figure 6.1). From the pre-existing data on *S. destruens* prevalence in experimental challenges (obtained from Arkush *et al.* 1998, Gozlan *et al.* 2005 and Chapters 4 and 5) susceptible species could have a minimum prevalence in the range of 5 to 15%. A sample size of 60 fish was selected as the probability of detecting at least one positive fish was maximised for a hypothetical prevalence of 8 % (Figure 6.1). The weight and length of ten fish per species was recorded at the onset of the experiment. Fish were kept in quarantine for 30 days prior to challenge with *S. destruens*.

6.2.6 Fish husbandry

Fish were fed twice a day with 1 % of their body weight. *A. brama*, *R. rutilus* and *S. erythrophthalmus* were fed with CypriCo Crumble Astax (protein 53 %, fat 13 %, crude fibre 0.6 %, ash 10.7 %, astaxanthin 80 mg/kg; supplemented with vitamins A, D3, E and C) (Coppens, Netherlands). *C. carpio* was fed TetraPond floating pellets (protein 28 %, oil 3.5 %, fibre 2 %, ash 7 %; supplemented with vitamins A, D3 and E) (Tetra, Germany). All tanks had 25 % of their water exchanged weekly. All tanks were checked for mortalities three times per day. Dead fish were collected and dissected immediately and preserved for molecular analysis and light microscopy (Chapter 4; section 4.2.3) to test for the presence of *S. destruens*.

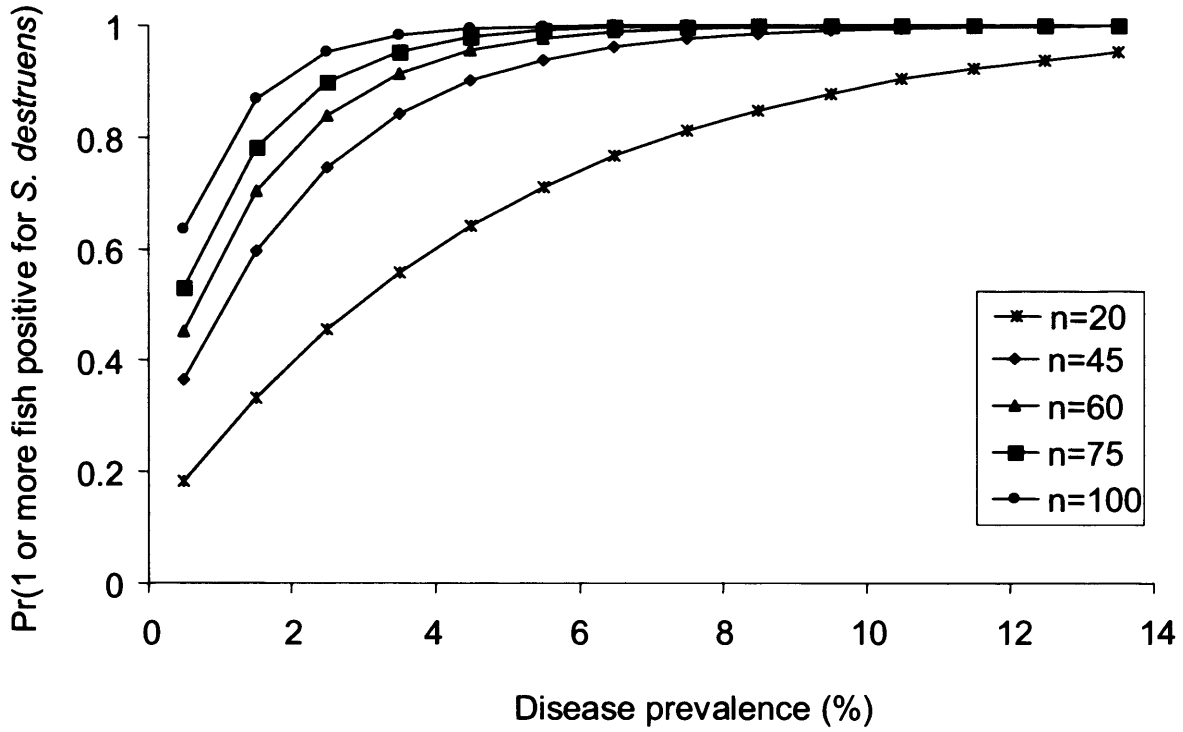


Figure 6.1: Plot of the probability of at least one or more fish being positive for *Sphaerothecum destruens* for a prevalence range of 1 to 14 % for a number of sample sizes.

6.2.7 Experimental infection of *Abramis brama*, *Cyprinus carpio*, *Rutilus rutilus* and *Scardinius erythrophthalmus* through immersion in water containing *Sphaerothecum destruens* spores

Fish were divided into six replicate 70 L tanks each containing 20 fish per tank. Each tank had its own biological filter and was aerated using an air pump. Water temperature was kept at constant of 20 °C and the photoperiod was maintained at 16 hours light and eight hours dark. The treatment group (n=60) was divided into three holding tanks (treatment group 1-3). Similarly, the control group (n=60) was dividied into three holding tanks (control group 1-3)

Prior to exposure with *S. destruens*, fish were netted out of the tanks and placed in a bucket with eight litres of de-chlorinated water aerated using an air pump. The maximum number of fish per bucket was 60. The exposure protocol used was similar

to the one used for exposing *O. tshawytscha* to *S. destruens* (see Mendonca and Arkush, 2004). A total of three exposures to *S. destruens* were performed at three-day intervals. Parasite spores were derived from cell cultures (UK isolate) and were purified as described in Chapter 3 section 3.2.1. The spores were suspended in distilled sterile water and were added to the 'treatment' buckets at each exposure period to achieve an average concentration of 8.6×10^4 spores ml^{-1}. Distilled water was added to the 'control' buckets. Exposures were maintained for four hours at the end of which the fish were returned to the tanks. The end of the third exposure was considered as Time 0 (i.e. start of the experiment).

Ten *C. carpio* were sampled 28 days post exposure (p.e.) (three *C. carpio* from treatment group 1; three from treatment group 2 and four from treatment group 3). Five fish of all species were sampled at six month p.e. from the treatment and control groups (two from group 1 and 2 and one from group 3). All surviving fish were euthanized and sampled at the end of the experiment (11 months). Fish were euthanized, weighed and their length was determined as described in Chapter 4, section 4.2.3. Fish condition (K) was calculated for all fish (see Chapter 4, section 4.2.4). Tissue samples were harvested and preserved for molecular analysis and light microscopy as described in Chapter 4, section 4.2.3.

6.2.8 Processing of fish tissues

The tissues collected from sampled experimental fish and mortalities included the kidney, liver, posterior intestine, gill and gonad (if present). DNA was extracted for each tissue separately (15 mg each) using the Qiagen DNeasy 96 Blood & Tissue kit (rodent tail protocol). Extracted DNA was quantified in a spectrophotometer at 260 nm (NanoDrop ND-1000; Labtech) and stored at -70 °C. Nested PCR was performed

on 200 to 300 ng of template (total DNA). PCR products were migrated on a 1.5 % agarose gel which was post stained with ethidium bromide (0.5 μg / ml). An individual was scored positive if *S. destruens*-specific DNA was amplified from any of its organs tested. Tissue sections (stained with H & E and Gram's stain) of PCR positive fish were examined under the light microscope (Chapter 2, section 2.2.2).

6.2.9 Statistical analysis

All statistical analyses were performed using SPSS 14.0 (SPSS Inc., Chicago, Illinois, USA) unless otherwise stated. Statistical significance was accepted when $P \leq 0.05$. Standard deviation of the mean was calculated. Disease prevalence was calculated as the number of individuals positive for *S. destruens* divided by the total number of individuals tested.

Survival analysis. Survival analysis (Kaplan–Meier survival curves, log rank tests and hazard function) were calculated for the four cyprinid species investigated. Survival curves were plotted using Stata (Version 10, StataCorp LP, Texas, USA). The Kaplan-Meier method was used to investigate the effect of exposure to *S. destruens* on fish survival. The method was used to estimate the proportion of fish surviving over the length of the experiment for each species and survival curves were produced for both treatment and control groups per species. Survival curves were compared using the log rank test with SPSS 14.0. The hazard function, also known as the risk of dying, was calculated for each species with SPSS 14.0.

Condition factors. The condition factors for the treatment and control groups of each species were compared using the Mann-Whitney *U* test.

6.2.10 Determining genetic and susceptibility distances

In order to investigate the phylogenetic influence of the host on the susceptibility to the parasite, genetic and susceptibility distances between susceptible host species were calculated. Genetic distance between susceptible species to *S. destruens* were calculated using the Tajima Nei genetic distance (Tajima and Nei, 1984) using the software MEGA version 4 (Tamura *et al.*, 2007). The Cytochrome b genetic marker was used to calculate genetic distances and sequences were obtained from NCBI GenBank (Table 6.1).

Susceptibility distance was defined as the difference in susceptibility to *S. destruens* between known susceptible species to the parasite. Susceptibility distance was calculated by subtracting *S. destruens* prevalence values for all possible pairs of fish species. Mean prevalence values were used for species with more than one reported *S. destruens* prevalence value. Prevalence values for the Salmonidae were obtained from Hedrick *et al.* (1989), Arkush *et al.* (1998) and Mendonca and Arkush (2004). For example, in the case of the *O. tshawytscha – O. mykiss* pair, *O. tshawytscha* had a mean *S. destruens* prevalence of 85.5 % and *O. mykiss* a 42.5 % prevalence giving a susceptibility distance of 43 % or 0.43. The genetic and susceptibility distance matrices were correlated using the Mantel test available from the Vegan package (Oksanen *et al.*, 2009) in R (R, 2009).

Table 6.1: GenBank sequences used in calculating genetic distances and *Sphaerothecum destruens* prevalence values used in calculating susceptibility distances. Where more than one prevalence value was present the mean prevalence was used for the analysis. The method of challenge to *S. destruens* is also provided.

Species	Cytochrome b	Prevalence	Method of challenge	Ref.[1]
Chinook salmon (*Oncorhynchus tshawytscha*)	AF392054	100	Injection	1, 2
		71	Water immersion	
Coho salmon (*O. kisutch*)	AF165079	98	Injection	1
Rainbow trout (*O. mykiss*)	L29771	42.5	Injection	1
Atlantic salmon (*Salmo salar*)	AF133701	75	Disease outbreak (in aquaculture)	3
Brown trout (*S. trutta*)	X77526	43.3	Injection	1
Brook trout (*Salvelinus fontinalis*)	AF154850	2.6	Injection	1
Carp (*Cyprinus carpio*)	X61010	11	Water immersion	4
Bream (*Abramis brama*)	Y10441	76	Water immersion	4
Roach (*Rutilus rutilus*)	Y10440	3	Water immersion	4
Sunbleak (*Leucaspius delineatus*)	Y10447	67	Cohabitation with *Pseudorasbora parva*	5, 6, 7
		40		
		38		
		15		
		2	Stoneham Lake survey	

[1](1) Arkush *et al.* (1998); (2) Mendonca and Arkush (2004); (3) Hedrick *et al.* (1989); (4) Chapter 6; (5) Gozlan *et al.* (2005); (6) Chapter 4; (7) Chapter 5

Intra-family variation of the relationship between genetic distance and *S. destruens* prevalence was investigated further for the Salmonidae. Genetic distance was calculated in comparison to *O. tshawytscha* as this species appears to be the most susceptible species to *S. destruens* within the Salmonidae. For this analysis, the values for *S. destruens* prevalence for *O. tshawytscha*, *O. kisutch*, *O. mykiss*, *S. trutta* and *S. fontinalis* were obtained from Arkush *et al.* (1998). Prevalence for *S. salar* was obtained from Hedrick *et al.* (1989). Genetic distance and *S. destruens* prevalence were correlated using Spearman rank order correlation.

6.2.11 Host specificity index for *Sphaerothecum destruens*

The specificity index (S_{TD}) proposed by Poulin and Mouillot (2003) measures the average taxonomic distinctness of a parasite's host species. The specificity index was calculated for *S. destruens* by performing the following steps: (a) *S. destruens* host species were placed within a taxonomic hierarchy using the Linnean classification; (b) the number of steps taken in order to reach a taxon common to two host species were calculated for all possible species pairs; (c) the number of steps was averaged across all species pairs. Step lengths between each hierarchical level were given the equal value of one. Specifically, the index was calculated using the formula by Poulin and Mouillot (2003):

$$S_{TD} = 2\frac{\sum\sum_{i<j}\omega_{ij}}{s(s-1)}$$

where s is the parasite's number of host species, the double summation is over the set ($i = 1, \ldots s; j = 1, \ldots s$, such that $i<j$), and ω_{ij} is the number of taxonomic steps needed to reach a common taxonomic node between host species i and j. The maximum value that the index S_{TD} can reach is five (when using the five taxonomic levels of genus, family, order, class and phylum). The lowest value S_{TD} can reach is one and this

occurs when all host species share the same genus. A measure of the taxonomic structure of the host species can be obtained by calculating the variance in taxonomic distinctness (Poulin and Mouillot, 2003):

$$VarS_{TD} = \frac{\sum\sum_{i \neq j} (\omega_{ij} - \varpi)^2}{s(s-1)}$$

where ϖ is the mean taxonomic distinctness or S_{TD}. The fish taxonomy proposed by Nelson (1994) was used in calculating the S_{TD} for *S. destruens*.

6.3 Results

6.3.1 DNA extraction efficiency and limits of detection of nested PCR

DNA extraction. In order to determine the extraction efficiency of the commercially available Qiagen DNeasy extraction method, ten-fold serial spore dilutions of pure *S. destruens* spores in the absence and presence of common *C. carpio* kidney tissue were extracted. Observation of lysate under the phase light microscope revealed intact *S. destruens* spores following overnight incubation for the Qiagen DNeasy extraction method. In addition, extraction of culture-derived *S. destruens* spores did not yield a spectrophotometer reading across all spore dilutions including concentrations of 10^6 spores ml^{-1}. *S. destruens* DNA was detected with nested PCR amplification that was subsequently used to assess extraction efficiency. By use of nested PCR, the extraction efficiency of the Qiagen DNeasy Tissue kit appeared to be 10^3 and 10^4 spores ml^{-1} in the absence and presence of 15 mg of *C. carpio* kidney tissue respectively (Figure 6.2). These results were successfully duplicated.

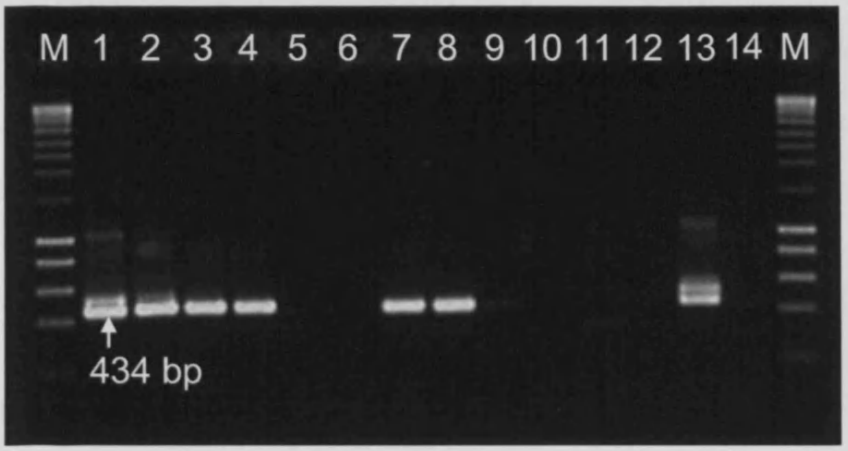


Figure 6.2: Extraction efficiency for the rodent tail protocol (Qiagen Dneasy kit) using ten-fold serial dilutions of *Sphaerothecum destruens* spores derived from cell culture in absence and presence of *S. destruens*-free common carp *Cyprinus carpio* kidney tissue. Extraction success was confirmed with *S. destruens* specific nested PCR. Second round amplicons (434 bp) were migrated trough a 1.5 % agarose gel and post-stained with ethidium bromide (0.5 µg / ml). Lanes 1-6: second round PCR products from serial pure spore dilutions 10^6 to 10 spores ml^{-1} respectively. Lanes 7-12: second round PCR products from serial spore dilutions, 10^6 to 10 spores ml^{-1}, in presence of 15 mg common *C. carpio* kidney tissue. Lane 13: PCR positive control containing 50 ng of purified total genomic *S. destruens* DNA, lane 14: no template negative control. M: molecular marker (Hyperladder I, Bioline).

Nested PCR. In order to determine and compare the detection limit of the *S. destruens* specific nested PCR, the reaction was performed on a ten-fold serial dilution of genomic *S. destruens* DNA in the presence and absence of genomic common *C. carpio* DNA. Using the ten-fold serial dilutions of genomic *S. destruens* DNA, the detection limit of the nested PCR was 10 pg and 1pg of total *S. destruens* DNA (Figure 6.3). There was no amplification at concentrations below 1 pg total *S. destruens* DNA(data not shown). Addition of 270 ng of total common *C. carpio* genomic DNA did not adversely affect the amplification of ≤ 10 pg total *S. destruens* DNA (Figure 6.4). Detection of 1 pg total *S. destruens* DNA appeared to be inhibited by the addition of 270 ng total common *C. carpio* DNA (Figure 6.4). With starting material greater than 10 pg total genomic *S. destruens* DNA, there was additional non-specific amplification (Figure 6.3: lanes 1-4; Figure 6.4: lanes 1-3). These included a

residual first round PCR product of 550 bp and faint products of 600 bp, 900 bp and 1100 bp. These products did not interfere with the amplification of the second round product specific to *S. destruens* and were not sequenced to determine their identity.

Addition of high quantities of total common *C. carpio* genomic DNA (270 ng, 540 ng, 810 ng and 1.08 mg) per reaction did not inhibit the amplification of either 1 pg or 10 pg total *S. destruens* DNA in each reaction (Figure 6.5). In the absence of genomic *S. destruens* DNA non-specific amplification by nested PCR was present at high quantities (> 540 ng) of total common *C. carpio* genomic DNA.

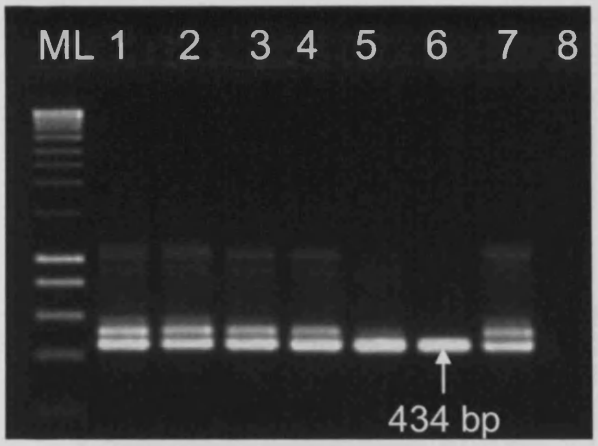


Figure 6.3: Detection limit of *Sphaerothecum destruens*-specific nested PCR using 10-fold serial dilutions of *S. destruens* genomic DNA derived from cell culture. Second round amplicons (434 bp) were migrated trough a 1.5 % agarose gel and post-stained with ethidium bromide (0.5 µg / ml). Lanes 1-6: second round PCR products from 100 ng, 10 ng, 1 ng, 0.1 ng, 10 pg and 1 pg total genomic *S. destruens* DNA respectively. Lane 7: positive control containing 50 ng of purified total genomic *S. destruens* DNA, lane 8: no template negative control. Lane M: molecular marker (HyperLadder I, Bioline).

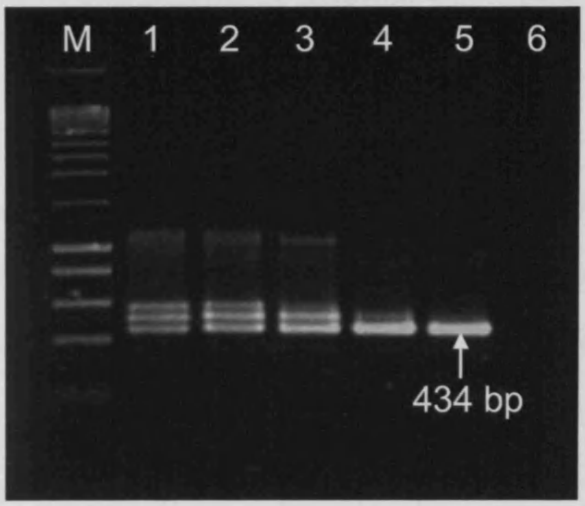


Figure 6.4: Detection limit of *Sphaerothecum destruens*-specific nested PCR in the presence of common carp *Cyprinus carpio* genomic DNA. Detection thresholds were determined using 10-fold serial dilutions of cell culture derived genomic *S. destruens* DNA spiked with 270 ng total common *C. carpio* genomic DNA. Second round amplicons (434 bp) were migrated through a 1.5 % agarose gel and post-stained with ethidium bromide (0.5 µg / ml). Lanes 1-6: second round PCR products from 100 ng, 10 ng, 1 ng, 0.1 ng, 10 pg and 1 pg total genomic *S. destruens* DNA spiked with 270 ng total common *C. carpio* genomic DNA respectively. Lane M: molecular marker (HyperLadder I, Bioline).

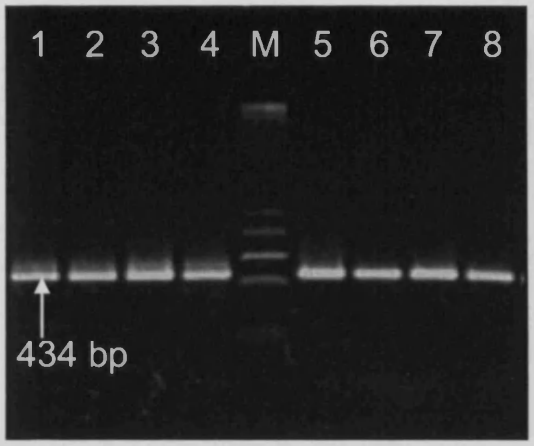


Figure 6.5: Test for inhibition to the *Sphaerothecum destruens*-specific nested PCR by addition of high quantities of common carp *Cyprinus carpio* genomic DNA to genomic *S. destruens* DNA. Total *S. destruens* genomic DNA (1 pg and 10 pg) was spiked with 270, 540, 810 & 1080 ng of total common *C. carpio* genomic DNA. Second round amplicons (434 bp) were migrated through a 1.5 % agarose gel and post-stained with ethidium bromide (0.5 µg / ml). Lanes 1-4 show second round amplicons of 1pg total genomic *S. destruens* DNA spiked with 270, 540, 810 & 1080 ng total common *C. carpio* genomic DNA, respectively. Lanes 5-8 show second round amplicons of 10pg total genomic *S. destruens* and DNA spiked with 270, 540, 810 & 1080 ng total common *C. carpio* genomic DNA, respectively. Lane M: molecular marker (HyperLadder I, Bioline).

6.3.2 Confirmation that *Sphaerothecum destruens* specific DNA was amplified

The amplification of *S. destruens* specific DNA was confirmed via restriction enzyme digestion of *S. destruens* positive samples. Digestion with the restriction enzyme *Rsa*I was successful and yielded two bands of 277 and 157 bp. In some cases, there was incomplete digestion of the PCR product (424 bp; Figure 6.6) and was most likely the result of high template quantities. Amplification of *S. destruens* specific DNA was further confirmed by sequencing of PCR amplicons. All PCR amplicons were homologous to the three *S. destruens* isolates published in GenBank.

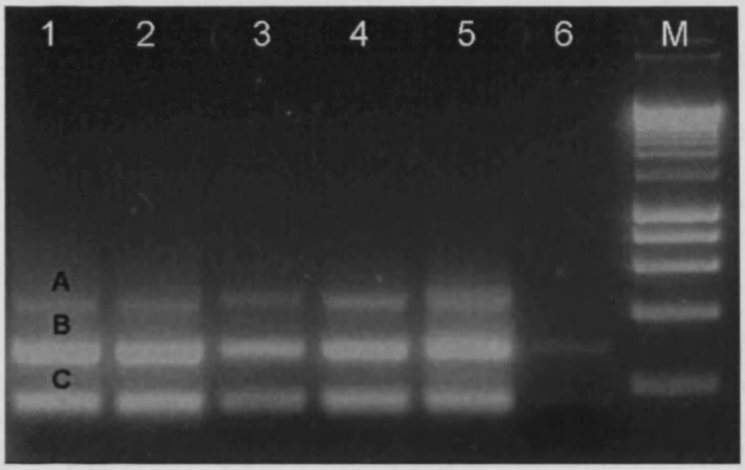


Figure 6.6: Restriction enzyme digest (*Rsa*I) of *Sphaerothecum destruens* positive samples (Lanes 1-2: *Abramis brama*; 3: *Rutilus rutilus*; 4: *Cyprinus carpio*; 5-6: PCR positive controls; ML: molecular marker (HyperLadder I, Bioline). Band A is the original second round PCR amplicon (434 bp). Bands B and C are the restriction digest bands of 277 bp and 157 bp, respectively. Restriction digests were migrated through a 2 % agarose gel and post-stained with ethidium bromide (0.5 µg / ml).

6.3.3 Experimental infection of *Abramis brama*, *Cyprinus carpio*, *Rutilus rutilus* and *Scardinius erythrophthalmus* by immersion in water containing *Sphaerothecum destruens* spores

A total of 120 fish per species were tested for the presence of *S. destruens* by sampling a combination of the kidney, liver, intestine, gills and gonads.

Treatment group (exposed to water containing *Sphaerothecum destruens* spores).

The tissues collected from sampled experimental fish and mortalities included the kidney, liver, posterior intestine, gill and gonad (if present). DNA was extracted from the kidney, liver and posterior intestine of all mortalities and fish sampled at 6-months p.e. and 11-months p.e. for all species (Table 6.2). Additional organs (gill and gonad) were extracted for 13 *R. rutilus* mortalities (five from treatment group 2 and six from treatment group 3). In the case of fish sampled 6-months p.e., the gill and gonad of *A. brama* and *R. rutilus* were also extracted (Table 6.2).

S. destruens DNA was detected in the treatment group of three species; *A. brama*, *C. carpio* and *R. rutilus* through nested PCR. Histological sections (stained with H & E and Gram's stain) were prepared for all samples testing positive for *S. destruens* through PCR. Examination of these sections did not reveal the presence of *S. destruens*.

Table 6.2: List of organs which were tested for the presence of *Sphaerothecum destruens* DNA in the treatment groups of bream *Abramis brama*, carp *Cyprinus carpio*, roach R*utilus rutilus* and rudd *Scardinius erythrophthalmus*. Organs tested included the kidney (K), liver (L), intestine (I), gill (Gi) and gonad (Go). n: number of fish sampled.

Species	**Mortalities**			**Sampled fish (6-months p.e.)**			**Surviving fish at 11 months p.e.**		
	K L I	**Gi Go**	**n**	**K L I**	**Gi Go**	**n**	**K L I**	**Gi Go**	**n**
A. brama	×		32	×	×	5	×		23
C. carpio	×		5	×		5	×		40
R. rutilus	×	×*	22	×	×	5	×		33
S. erythrophthalmus	×		36	×		5	×		19

*: only 13 of the 22 *R. rutilus* mortalities had their gill and gonad extracted

Control group (sham exposed through immersion in water in the absence of *Sphaerothecum destruens* spores).

S. destruens was not detected in the kidney, liver and intestine of any control (surviving and mortalities). All control mortalities of *A. brama* (n = 15) and *S. erythrophthalmus* (n = 23) were found negative for *S. destruens*.

Mortality and *Sphaerothecum destruens* prevalence in *Abramis brama*.

High mortalities occurred in all three holding treatment tanks for *A. brama* (mean percentage mortality: 53 % (±10); Table 6.3; Figure 6.8). All mortalities in the treatment group occurred over a period of 23 days p.e (mean mortality 53 %). Mortalities occurred in two of the three control holding tanks (mean % mortality: 25 % (±23)). Control mortalities occurred between days 10 and 20 p.e. (Figure 6.8) but were identified as *S. destruens* negative following nested PCR analysis. Exposure to *S. destruens* led to significantly less *A. brama* surviving compared to control *A. brama* (Log rank test; Chi-square = 10.6, d.f. = 1, $P < 0.05$). Treatment with *S. destruens* led to an increased risk of mortality by a factor of 2.9. The mean hazard's function for the treatment group was 0.90 (±0.27) compared to a hazard function of 0.31 (±0.28) for the control group.

Table 6.3: Percentage (%) mortalities and *Sphaerothecum destruens* prevalence in the treatment groups of bream *Abramis brama*, carp *Cyprinus carpio*, roach *Rutilus rutilus* and rudd *Scardinius erythrophthalmus*. Fish were divided into three treatment tanks (G1, G2, and G3). Mean percentage mortality and mean prevalence is also provided.

Species	**% mortalities**				***Sphaerothecum destruens* prevalence**			
	G1	**G2**	**G3**	**Mean**	**G1**	**G2**	**G3**	**Mean**
Abramis brama	65	45	50	53	69	89	70	76
Cyprinus carpio	0	15	10	8	0	33	0	11
Rutilus rutilus	55	0	55	37	0	0	9	3
Scardinius erythrophthalmus	80	40	60	60	0	0	0	0

The parasite was detected in the kidney, liver and intestine of *A. brama* mortalities in the treatment groups with a mean prevalence of 76 % (±11) (Table 6.3; Figure 6.7). The parasite was not detected in the sampled fish at six months p.e. and in surviving fish at the end of the experiment in both the treatment and control groups.

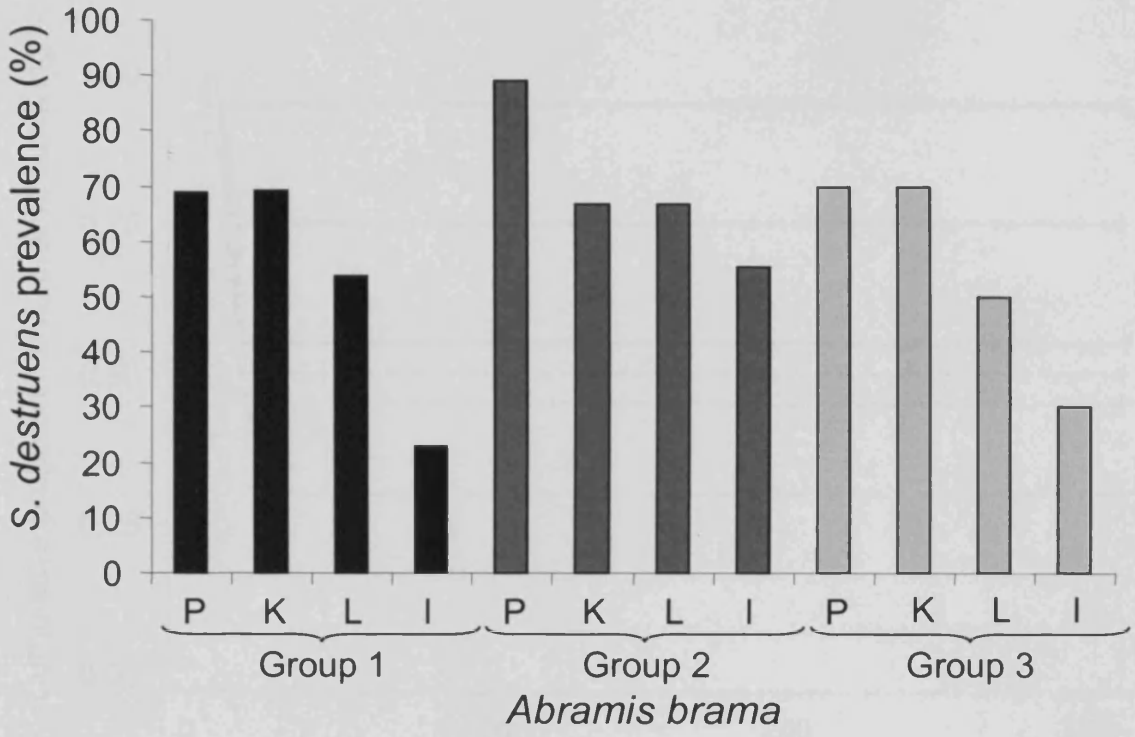


Figure 6.7: *Sphaerothecum destruens* prevalence in bream *Abramis brama* exposed to *S. destruens* by immersion in water containing *S. destruens* spores at a concentration of 8.6×10^4 spores ml^{-1}. A total of 60 *A. brama* were exposed to *S. destruens*. These were subsequently divided into three groups of 20 fish. Prevalence is provided per treatment group and per organ (P: overall prevalence in the group, K: kidney, L: Liver, I: Intestine) *S. destruens* was not detected in any of the three *A. brama* control tanks.

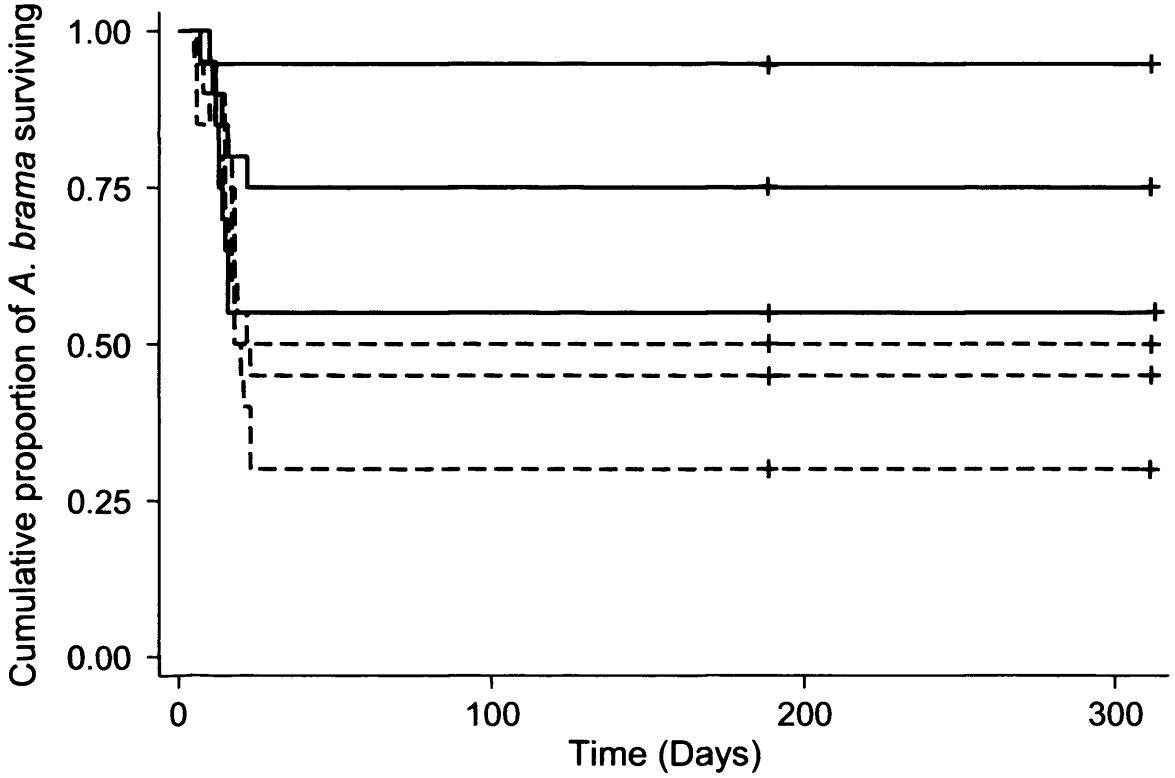


Figure 6.8: Cumulative proportion of bream *Abramis brama* surviving over 310 days post exposure to *Sphaerothecum destruens* via immersion in water containing *S. destruens* spores at a concentration of 8.6×10^4 spores ml^{-1}. The treatment group (---) consisted of 60 fish exposed to *S. destruens* which were subsequently divided into three groups of 20 fish each. The control group (—) consisted of 60 fish sham exposed to the parasite which were subsequently divided into three groups of 20 fish each. + : censored (sampled) fish.

Mortality and *Sphaerothecum destruens* prevalence in *Cyprinus carpio*.

Mortalities occurred in two of the three holding treatment tanks; with 15 % mortality in group 2 and 10 % mortality in group 3 (Table 6.3; Figure 6.9). Mean % mortality over the three treatment groups was 8 % (±7.6). Mortalities occurred between days 49 and 92 p.e. Exposure to *S. destruens* has lead to significantly higher mortalities in *C. carpio* (Log rank test; Chi-square = 5.18; d.f. = 1; $P < 0.05$). The mean hazard's function for the treatment group was 0.09 (±0.08) compared to a hazard function of 0 for the control group.

S. destruens DNA was detected in the kidney and intestine of *C. carpio* mortalities and sampled fish of the treatment group. Only one *C. carpio* mortality was positive for *S. destruens* DNA in group 2 giving a prevalence of 33 % for group 2 mortalities (Table 6.3). Mean % prevalence was 11 (±19) (Table 6.3). Parasite DNA was detected in the intestine of two *C. carpio* sampled at 28 p.e. resulting in 20 % prevalence in these individuals. *S. destruens* DNA was not detected in any fish sampled at six months p.e. or at the end of the experiment in both treatment and control groups.

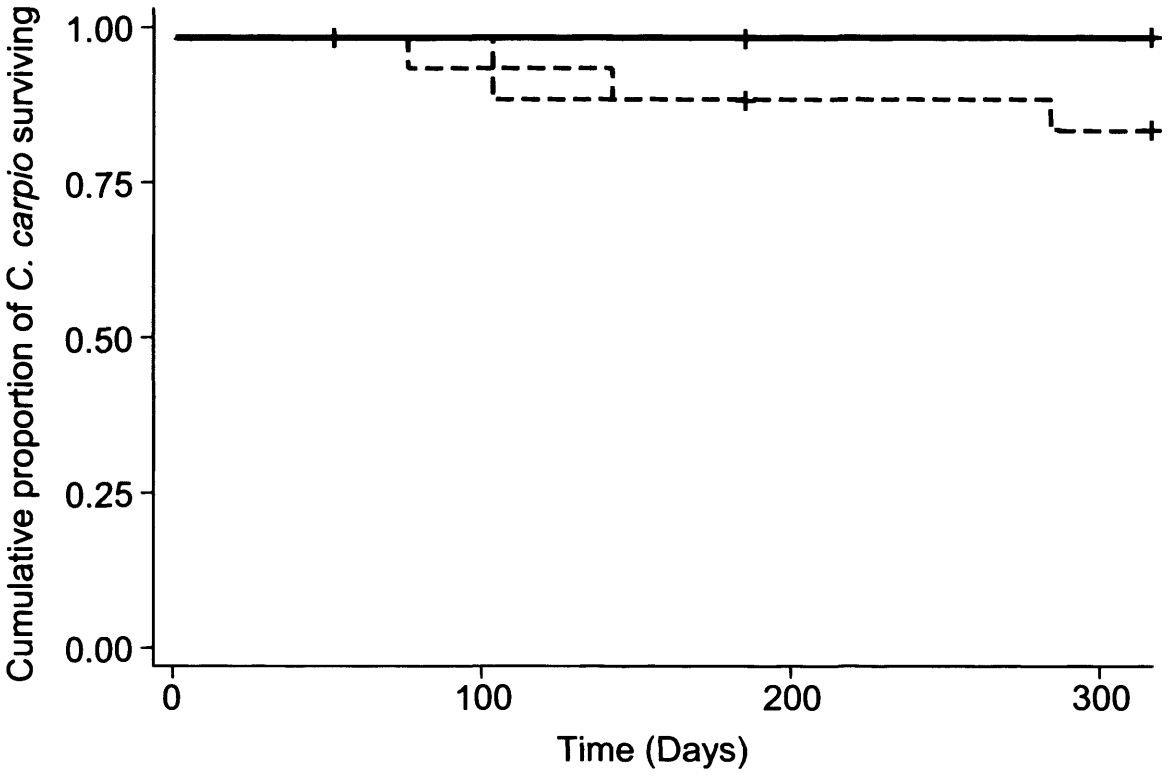


Figure 6.9: Cumulative proportion of carp *Cyprinus carpio* surviving over 310 days post exposure to *Sphaerothecum destruens* via immersion in water containing *S. destruens* spores at a concentration of 8.6×10^4 spores ml^{-1}. The treatment group (---) consisted of 60 fish exposed to *S. destruens* which were subsequently divided into three groups of 20 fish each. The control group (—) consisted of 60 fish sham exposed to the parasite which were subsequently divided into three groups of 20 fish each. + : censored (sampled) fish.

Mortality and *Sphaerothecum destruens* prevalence in *Rutilus rutilus*.

Similar to *C. carpio*, mortalities only occurred in two of the three holding treatment tanks with 55 % mortality in each (Table 6.3; Figure 6.10). Mean percentage mortality for treatment group was 37 % (±31.7) (Table 6.3). The majority of mortalities occurred between day 20 and 50 p.e. Challenge with *S. destruens* led to significantly higher mortalities in the treatment group (Log rank test; Chi-square = 26.96; d.f. = 1; $P < 0.05$). The mean hazard's function for the treatment group was 0.53 (±0.46) compared to a hazard function of 0 for the control group.

S. destruens DNA was detected in the kidney, liver and intestine of *R. rutilus* mortalities in the treatment group. No *S. destruens* DNA was detected in the gill and gonad of the 13 *R. rutilus* mortalities tested. *S. destruens* was detected 23 days p.e. in one treatment mortality of group 3 resulting in a parasite prevalence of 9 % (n = 11) (Table 6.3). Mean % prevalence for the treatment group was 3 (±5.2). *S. destruens* DNA was not detected at six months post exposure and at the end of the experiment in any both the treatment and control groups.

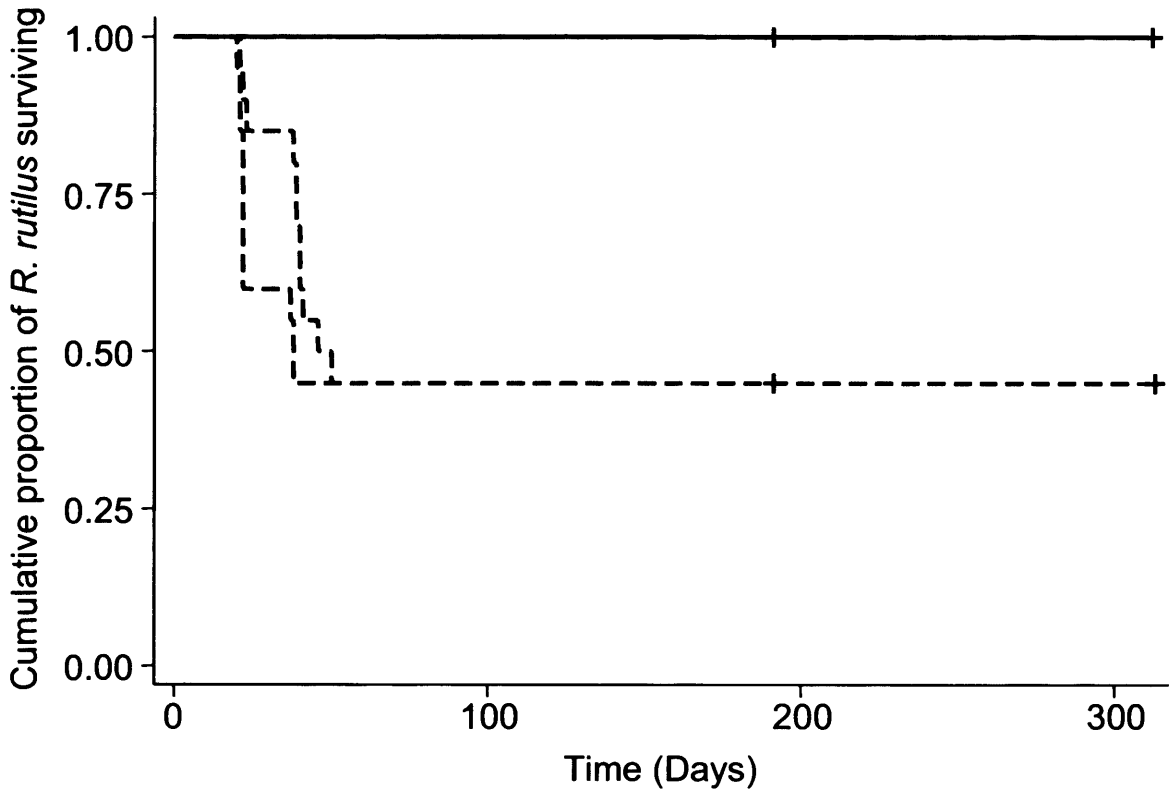


Figure 6.10: Cumulative proportion of roach *Rutilus rutilus* surviving over 310 days post exposure to *Sphaerothecum destruens* via immersion in water containing *S. destruens* spores at a concentration of 8.6×10^4 spores ml^{-1}. The treatment group (---) consisted of 60 fish exposed to *S. destruens* which were subsequently divided into three groups of 20 fish each. The control group (—) consisted of 60 fish sham exposed to the parasite which were subsequently divided into three groups of 20 fish each. + : censored (sampled) fish.

Mortality and *Sphaerothecum destruens* prevalence in *Scardinius erythrophthalmus.*

Exposure to *S. destruens* led to significantly higher mortalities in *S. erythrophthalmus* (Log rank test; Chi-square = 13.63; d.f. = 1, $P < 0.05$) and doubled the risk of mortality. The mean hazard's function for the treatment group was 1.01 (±0.55) compared to a hazard function of 0.51 (±0.26) for the control group. High mortalities were observed in both treatment and control groups. Mean % mortality was 60 % (±20) in the treatment group and 38.3 % (±15) in the control group (Figure 6.11). The majority of mortalities occurred between day 22 and 39 p.e. *S. destruens* DNA was not detected in any sampled and dead *S. erythrophthalmus* in both the treatment and control groups.

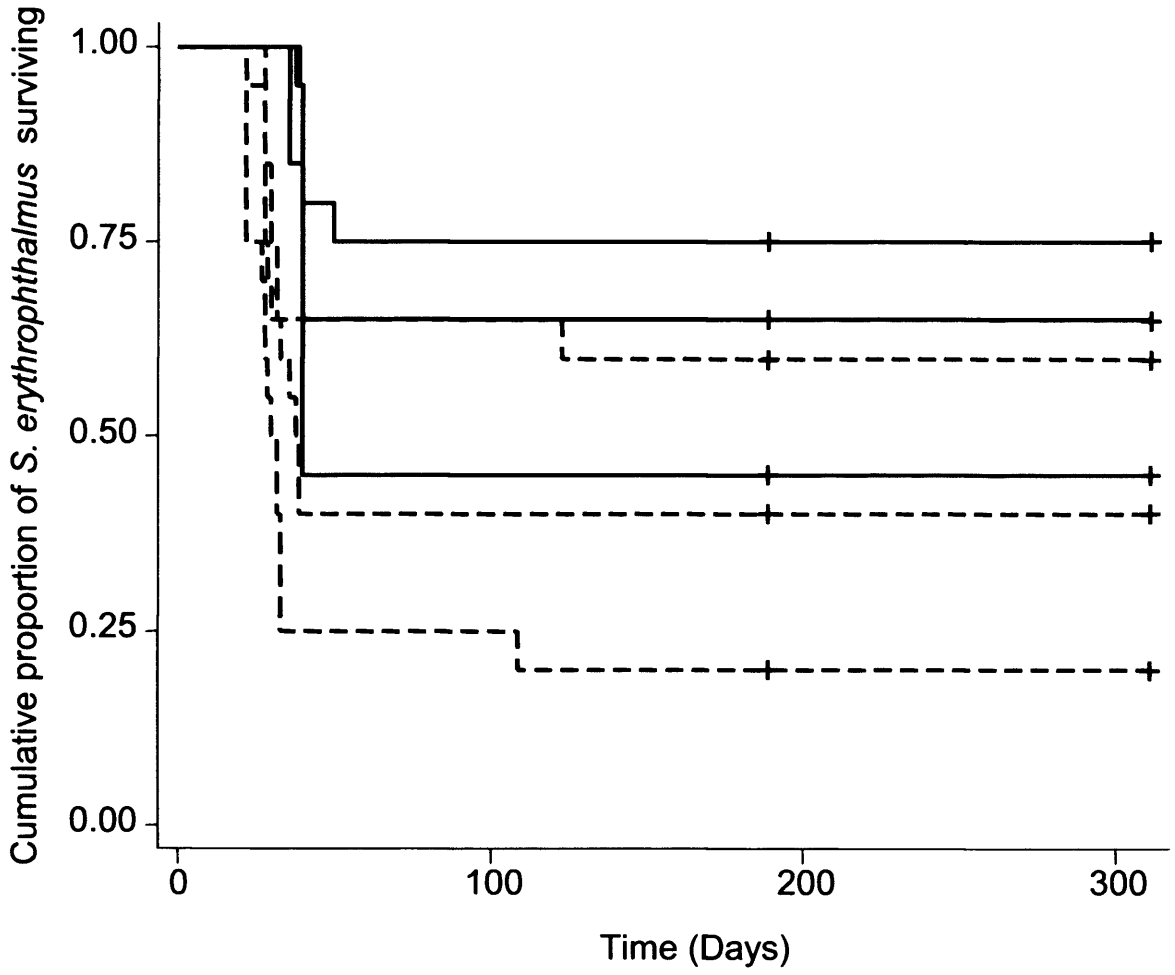


Figure 6.11: Cumulative proportion of rudd *Scardinius erythrophthalmus* surviving over 310 days post exposure to *Sphaerothecum destruens* via immersion in water containing *S. destruens* spores at a concentration of 8.6×10^4 spores ml^{-1}. The treatment group (---) consisted of 60 fish exposed to *S. destruens* which were subsequently divided into three groups of 20 fish each. The control group (—) consisted of 60 fish sham exposed to the parasite which were subsequently divided into three groups of 20 fish each. + : censored (sampled) fish.

Fish condition. Condition factor (K) was calculated for the treatment and control groups of each species at the end of the experiment (Table 6.4). There was no significant difference in condition between treatment and control for all species

Table 6.4: Mean (± SD) condition factor for the treatment and control group of bream *Abramis brama*, carp *Cyprinus carpio*, roach *Rutilus rutilus* and rudd *Scardinius erythrophthalmus* (n: total number of fish used in the analysis).

Species	Treatment	Control	Mann Whitney *U* test
A. brama (n = 56)	1.1 (0.1)	1.1 (0.1)	$P = 0.257$
C. carpio (n = 100)	1.9 (0.2)	1.9 (0.1)	$P = 0.457$
R. rutilus (n = 93)	1.1 (0.1)	1.1 (0.1)	$P = 0.511$
S. erythrophthalmus (n = 51)	1.3 (0.1)	1.3 (0.1)	$P = 0.716$

6.3.4 Investigating the relationship between genetic distance and susceptibility distance for *Sphaerothecum destruens*

The relationship between genetic distance and susceptibility distance between susceptible host species to *S. destruens* was calculated. For this analysis, both the susceptible species within the Salmonidae and Cyprinidae families were used. There was no significant correlation between the genetic distance and susceptibility matrices (Mantel statistic r = -0.0837, $P = 0.67$; Figure 6.12). Although not significant, a negative relationship between genetic distance and susceptibility appears to be present for the Cyprinidae.

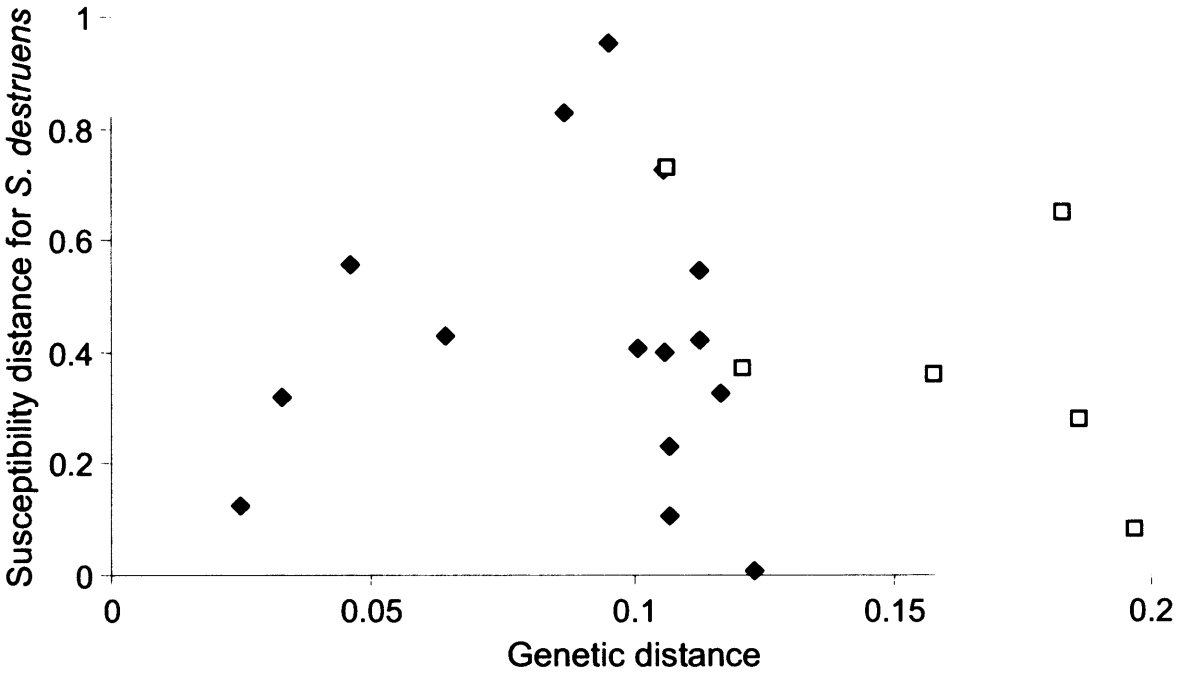


Figure 6.12: The genetic distance between all known susceptible species to *S. destruens* was plotted against the susceptibility distance to *Sphaerothecum destruens* for all the species combinations. The two families, Cyprinidae (□) and Salmonidae (♦) show different relationship patterns between genetic and susceptibility distances. Genetic distances were based in the pairwise analysis of ten Cytochrome b sequences. Analyses were conducted using the Tajima-Nei (Tajima and Nei, 1984) method in MEGA4 (Tamura *et al.*, 2007). All positions containing gaps and missing data were eliminated from the dataset. There were a total of 249 positions in the final dataset.

Intra-family variation of the relationship between genetic distance to *O. tshawytscha* and disease prevalence was investigated for the Salmonidae. A high variation in susceptibility between closely related genera was observed (Figure 6.13). There was a negative non-significant correlation between genetic distance to *O. tshawytscha* and *S. destruens* prevalence (Spearman's rho = -0.543, $P = 0.26$).

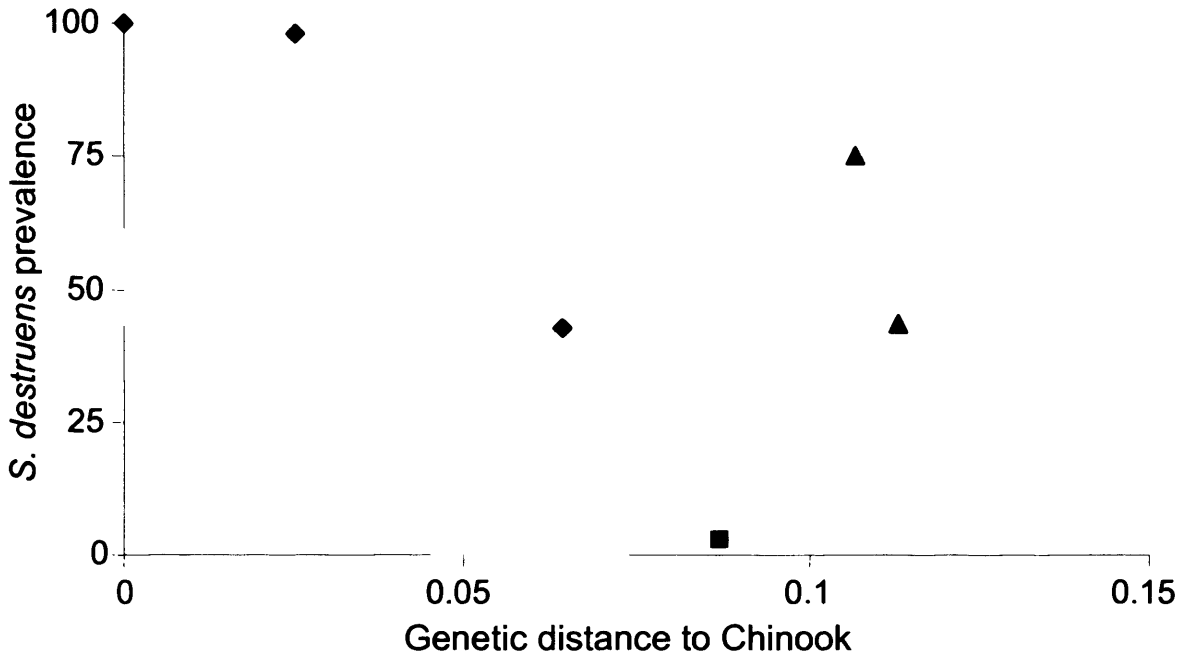


Figure 6.13: Genetic distance (Tajima-Nei) of Coho salmon *Oncorhynchus kisutch*, Atlantic salmon *Salmo salar*, Rainbow trout *O. mykiss*, Brook trout *Salvelinus fontinalis*, Brown trout *S. trutta* to Chinook salmon *O. tshawytscha* plotted against *Sphaerothecum destruens* prevalence. *S. destruens* prevalence values used for this analysis were obtained from Arkush *et al.* (1998) and Hedrick *et al.* (1989). Species are coded by genus: ♦: *Oncorhynchus*, ■: *Salvelinus* and ▲: *Salmo*.

6.3.5 Host specificity index for *Sphaerothecum destruens*

A tree representing the taxonomic hierarchy of *S. destruens* host species was constructed (Figure 6.14) using the Linnean classification. The specificity index (S_{TD}) for *S. destruens* was 2.98 with a variance in taxonomic distinctness ($VarS_{TD}$) of 0.633. When the five taxonomic levels above species (genus, family, order, class and phylum) are used, index values range form one to five. A parasite with an S_{TD} value of one colonises hosts which are congeners whereas a parasite with an S_{TD} value of 5 colonises hosts species belonging to different classes.

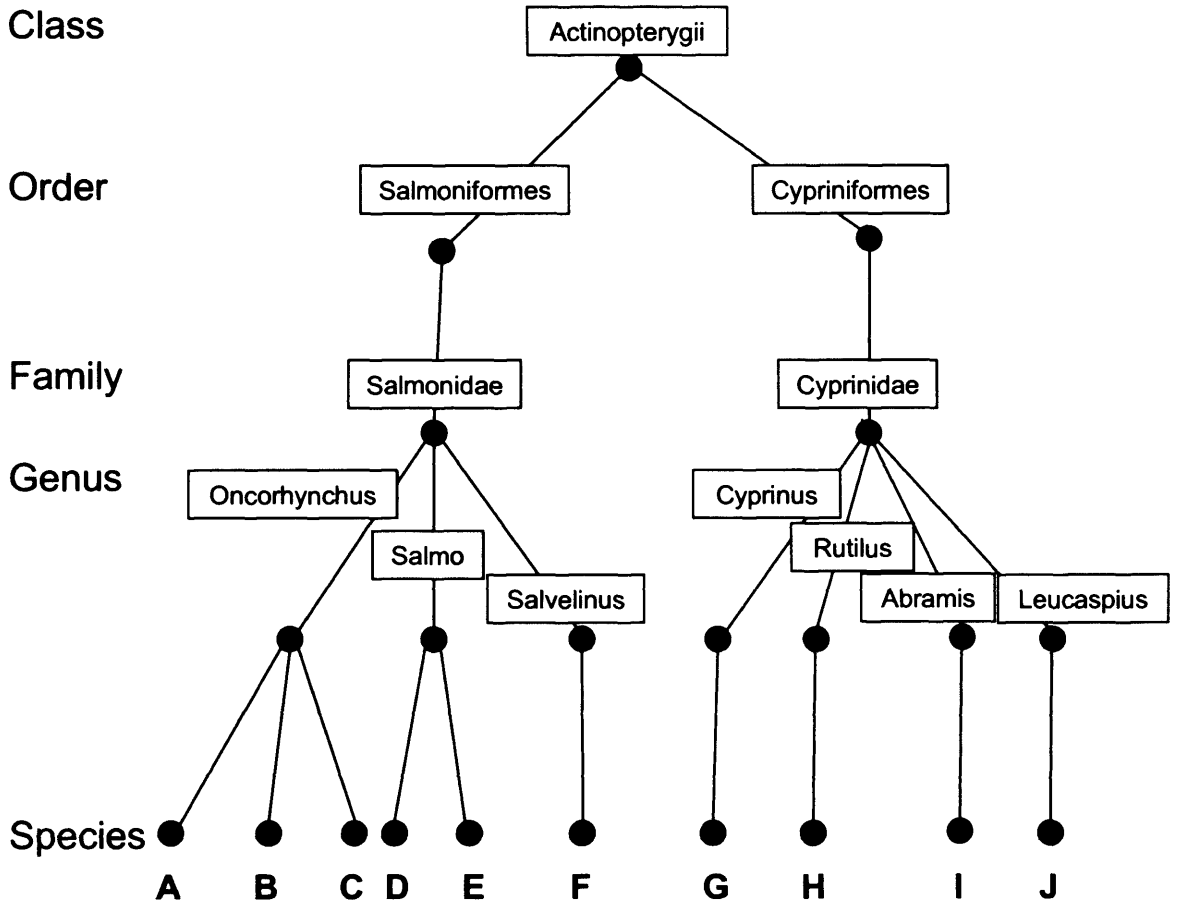


Figure 6.14: Hierarchical taxonomic tree for all currently known hosts (n = 10) of *Sphaerothecum destruens*. The tree is based on four taxonomic levels above the species level. *S. destruens* hosts belong to two different orders, Salmoniformes and Cypriniformes, and two families, Salmonidae and Cyprinidae. Within the Salmonidae, six species belonging to three genera are represented; A: *Oncorhynchus tshawytscha* (Chinook salmon), B: *O. kisutch* (Coho salmon), C: *O. mykiss* (rainbow trout), D: *Salmo trutta* (brown trout), E: *S. salar* (Atlantic salmon) and F: *Salvelinus fontinalis* (brook trout). The Cyprinidae is represented by four species belonging to four genera; G: *Cyprinus carpio* (carp), H: *Abramis brama* (bream), I: *Rutilus rutilus* (roach) and J: *Leucaspius delineatus* (sunbleak).

6.4 Discussion

6.4.1 DNA extraction efficiency and limits of detection of nested PCR

Quantifying nucleic acid extraction efficiencies of pathogens in the presence of host cellular material is essential in epidemiological studies. This allows a degree of confidence to be generated when assessing the absence and presence of pathogens in their associated hosts. In the absence of fish tissue 10^3 spores ml^{-1} ($\approx$ 50 spores) were extracted using the rodent tail protocol (Qiagen DNeasy kit) yielding sufficient DNA to be detected using nested PCR. Extraction efficiency was not compromised by the presence of host material between 10^6 and 10^4 spores ml^{-1} in fish tissue. For spore concentrations below 10^4 spores ml^{-1} the presence of fish tissue reduced the extraction of spores below the nested PCR detection limit. Mendonca and Arkush (2004) report a ten spore minimum DNA extraction efficiency for *S. destruens* (BML isolate) spores in the absence of fish tissue. However, extraction of dilutions containing ten *S. destruens* did not always yield enough *S. destruens* DNA for detection by nested PCR. Mendonca and Arkush (2004) did not report the spore quantity which was consistently extracted and did not test for inhibition of the extraction process by the presence of fish tissue. In this study, DNA extraction of 50 spores in the absence and 500 spores in the presence of fish tissue consistently yielded DNA quantities detectable by nested PCR. Thus, in the present study the minimum detection limit for *S. destruens* (using the Qiagen DNeasy 96 Blood & Tissue kit and the nested PCR developed by Mendonca and Arkush (2004) was between 50 and 500 spores.

The extraction efficiencies achieved for *S. destruens* were low compared to efficiencies equivalent to a single spore extraction reported for other pathogens (Andree *et al.*, 1998, Bell *et al.*, 1999, True *et al.*, 2009). This is likely due to

structural properties of the *S. destruens* cell wall which is apparently quite impenetrable to various extraction techniques (Mendonca and Arkush, 2004). As a result only a fraction of the total spores were successfully broken down and released their DNA during extraction and this was confirmed through direct observation of the lysate following overnight lysis as well as the lack of spectrophotometer readings even when genomic DNA from 10^6 spores ml^{-1} ($\approx$ 50K spores) was extracted. Elston *et al.* (1986) suggested the use of a French pressure cell press to disrupt the cell wall of *S. destruens*. This method is labour-intensive, not widely available in laboratories and prone to sample contamination and is thus not ideal for large sample numbers.

Successful amplification with comparable detection limits to those of *S. destruens* (BML isolate) (Mendonca and Arkush, 2004) was replicated in this thesis. Similar to Mendonca and Arkush (2004), nested PCR detected genomic *S. destruens* DNA at a quantity as low as one picogram total *S. destruens* DNA per reaction. Addition of purified common *C. carpio* genomic DNA did not inhibit amplification at equal and higher than 10 pg of genomic *S. destruens* DNA. In the presence of the tested concentrations of genomic common *C. carpio* DNA, the detection limit of *S. destruens* DNA was between 1 and 10 pg. Addition of fish DNA has been reported to inhibit PCR amplification of pathogen DNA (Gonzalez *et al.*, 2003).

The apparent inhibition of fish DNA on 1 pg of *S. destruens* DNA as seen in Figure 6.4 is likely a reflection of a pipetting error during the PCR set up. Amplification of 1 pg genomic *S. destruens* DNA was successful in the presence of common *C. carpio* genomic DNA concentrations exceeding 270 ng (Figure 6.5). Amplification inhibition of 1 pg *S. destruens* DNA was not detected when one milligram of fish DNA was added to the PCR reaction. These results were comparable to those of Mendonca and

Arkush (2004) who reported no inhibition and absence of non-specific amplification when adding 300 ng of *O. tshawytscha* genomic DNA to their dilutions of purified *S. destruens* genomic DNA. Absence of non-specific DNA amplification in the presence of high quantities of purified common *C. carpio* genomic DNA further supported the high specificity of the nested PCR reaction.

Mendonca and Arkush (2004) reported non-specific amplification when more than one nanogram of *S. destruens* genomic DNA was added per nested PCR reaction. These non-specific bands were 600 and 900 bp and have also been observed in the current study (Figures 6.2 and 6.3). Mendonca and Arkush (2004) reported that this non-specific amplicons did not interfere with the amplification of the second round *S. destruens* specific PCR product. Although the detection limit of this nested PCR is slightly inferior when compared to other similar studies (Andree *et al.*, 1998, Bell *et al.*, 1999, Palenzuela *et al.*, 1999, True *et al.*, 2009), the work presented here further supports the specificity, robustness and transferability of the nested PCR developed by Mendonca and Arkush (2004). This reaction provides a reliable, quantifiable and time efficient detection tool for *S. destruens*.

6.4.2 Susceptibility of *Abramis brama*, *Cyprinus carpio*, *Rutilus rutilus* and *Scardinius erythrophthalmus* to *Sphaerothecum destruens*

Experimental infections have been increasingly used to determine the potential host range of parasites (Poulin and Keeney, 2008). Here, *S. destruens* DNA was detected in *A. brama*, *C. carpio* and *R. rutilus* suggesting that these species are susceptible to the parasite. *A. brama* experienced mortalities exceeding 50 % when exposed to the parasite with 75 % of these being positive for *S. destruens* in at least two of the three organs tested. Exposure to *S. destruens* increased the risk of mortality by a factor of 2.9. These results suggest that *A. brama* is highly susceptible to *S. destruens*. Contrary

to *A. brama*, *C. carpio* experienced much lower mortalities (8.3 %) and had lower disease prevalence (11.1 %). *C. carpio* mortalities in the treatment group were considerably lower when compared to *A. brama*, suggesting that *C. carpio* is only lowly susceptible to the parasite.

R. rutilus and *S. erythrophthalmus* experienced high mortalities when exposed to *S. destruens*. However, the parasite was only detected at a prevalence of 9 % in *R. rutilus* and not detected in *S. erythrophthalmus*. Considering the observed mortality rates in isolation would classify *R. rutilus* and *S. erythrophthalmus* as highly susceptible to *S. destruens*. However, the low prevalence of the parasite in *R. rutilus* mortalities and its absence from *S. erythrophthalmus* mortalities led to equivocal conclusions regarding the susceptibility of these two species to *S. destruens*. The observed discrepancy between mortality and disease prevalence could be explained by: (a) the fish dying from a factor other than *S. destruens*, (b) parasite levels in the organs tested being lower that the nested PCR detection limit or (c) differences in parasite tropism in these species; with *S. destruens* being more prevalent in organs other than those tested.

The susceptibility of *R. rutilus* and *S. erythrophthalmus* will need to be further investigated by exposing these species to the agent and harvesting all organs for molecular and histological analyses. Exposure should be via intraperitoneal injection with the agent. Although unnatural, this method would ensure that sufficient *S. destruens* spores are administered to the fish increasing the chance of detecting histopathological changes in the fish. This will also reveal the organs of preference for each species. These organs can then be used in the detection of the parasite in wild and experimentally infected individuals.

Although there were significantly higher mortalities in the groups exposed to *S. destruens* compared to control groups, *A. brama* and *S. erythrophthalmus* also experienced high mortalities in their control groups. In the *A. brama* control, the majority of mortalities occurred during the first 15 days p.e. This could be due to stress following the sham exposure. Mortalities were also observed during this period for the treatment group, however, in the case of the treatment group mortalities continued to increase past day 15. In the *S. erythrophthalmus* control, the majority of mortalities occurred at day 40 p.e. and were possibly the result of a high nitrate and nitrite concentration. Nitrate and nitrite levels were checked on a weekly basis. However, on day 40 p.e. nitrate and nitrite levels were in the range lethal to fish. This was the only elevated nitrate and nitrite event for the entire duration of the experiment.

S. destruens was not detected in the sampled *A. brama*, *C. carpio*, *R. rutilus* and *S. erythrophthalmus* at six and 11 months p.e. Individual variation in susceptibility to disease is expected and could explain the disparity in disease susceptibility observed in each of the species tested. Surviving fish could have been naturally resistant to the parasite or recovered from infection. Recovered individuals can either have sterile immunity or have a carrier state or latent infection (Thrusfield, 2007). Sterile immunity occurs when the individual's immune system effectively clears all infective agents. A carrier state or latent infections involve the presence of the agent within the individual. In the carrier state, the agent is shed thus the carrier acts as a reservoir of disease, whereas in the latent state shedding may or may not occur (Thrusfield, 2007). The absence of *S. destruens* in the recovered fish suggests that either sterile immunity or resistance has occurred. However, the nested PCR used here lacks the sensitivity to

detect the carrier state. Thus, absence of carrier state should be confirmed via cohabitation of recovered fish with naïve individuals.

Pathology of the parasite within the infected tissues was not determined for any of the four species despite the high prevalence of the parasite in fish mortalities. Although tissue changes were observed in these fish, it was not possible to distinguish between disease pathology and autolysis (Hewlett *et al.*, 2009). None of the four species showed any external sign of disease and the majority of mortalities occurred in the first 40 days p.e. It is important to determine the pathology associated with the parasite in these species as it can facilitate disease detection and increase understanding of the host–parasite interaction. Future work can include challenging the species through intraperitoneal injection, or water-borne infection while sampling the fish in the early days p.e. In addition the use of *in situ* hybridisation techniques, where the parasite is detected by targeting its DNA in histological sections, could prove better at detecting *S. destruens* spores in autolytic tissues.

Conclusions on susceptibility vary greatly between studies and no clear cut criteria are available. Molecular detection of parasite DNA by PCR has been considered sufficient to determine susceptibility. Coelho *et al.* (2009) concluded that the shrimp *Farfantepenaeus subtilis* was susceptible to the infectious hypothermal and hematopoeitic necrosis virus after 10 % of the challenged animals tested positive for the agent's DNA. Histopathological changes were not evident in all shrimp and there was no evidence of virus-induced host mortalities. Also, Chinnadurai *et al.* (2009) classified the slimy salamander *Desmognathus orestes* as resistant to the chytrid fungus *Batrachochytrium dendrobatidis*. When challenged with *B. dendrobatidis*, *D.*

orestes was positive by PCR for the parasite but did not experience mortality or displayed any clinical signs of the disease.

Infection with an agent can occur in the absence of clinical signs (Chinnadurai *et al.*, 2009). In addition, disease severity as determined by histology does not always correlate with infection level (Tang and Lightner, 2001). Detection of disease pathology is heavily reliant on histopathological changes occurring within tissues and the pathologist's experience (Feist and Longshaw, 2008). However, such changes are the result of the host's response to the parasite and thus may not be representative of the parasite's exploitation strategies (Poulin, 2007). In the absence of a host response, it would be difficult to detect the parasite in histological sections. Amplification of the parasite's DNA does not infer viability of the parasite (Hiney and Smith, 1998, Mendonca and Arkush, 2004) and thus PCR detection of the parasite must be interpreted cautiously. Development of reverse transcriptase PCR targeting the pathogen messenger RNA of genes involved in DNA replication and cell division would enable the determination of viability (Gerard *et al.*, 2001).

The susceptibility and disease progression observed for the four cyprinids differed from the one reported for *O. tshawytscha* exposed to *S. destruens* via waterborne infection. Overall, the cyprinid species (except *C. carpio*) experienced high mortalities which in the case of *A. brama* mortalities were also associated with high *S. destruens* prevalence. *S. destruens* was not detected in any of the sampled fish at six months post exposure or at the end of the experiments (11 months p.e.). In *O. tshawytscha*, high disease prevalence (71.1 %) and no mortalities were observed (Mendonca and Arkush, 2004). In addition, infection severity (determined from Gram stained kidney smears) increased as time progressed, supporting the high

susceptibility of *O. tshawytscha* to *S. destruens*. Thus, although infection with *S. destruens* was slow in progression for *O. tshawytscha*, the parasite appeared to be more virulent in the cyprinid species.

Differences in observed susceptibility between *O. tshawytscha* and the four cyprinids challenged in the present study could be due to a number of factors. A different *S. destruens* isolate was used in the *O. tshawytscha* challenge compared the present work. Virulence differences between the two isolates could explain this discrepancy. This has been observed for a number of pathogens including the malaria parasites *Plasmodium falciparum* (see Chokejindachai and Conway, 2009) and parasitic protozoa (Biller *et al.*, 2009). Generalist parasites do not affect hosts equally showing preference for hosts on which they can maximise their reproductive output (Poulin, 2007). The difference in susceptibility observed here could be explained by difference in the parasite's host use. When novel host parasite combinations are created, dramatic impacts on populations can be observed due to the absence of co-evolutionary history between the host and parasite (Poulin and Keeney, 2008). This could be the case for the four cyprinid species. With sufficient time and host genetic diversity, hosts could evolve the ability to resist detrimental infections, while, the parasite can evolve mechanisms to avoid contact if such infections lower its own reproductive output (Poulin and Keeney, 2008). Trans-generational experiments would be extremely useful in determining the long term effects of the parasite's introduction on species populations.

6.4.3 *Sphaerothecum destruens* as a multi-host parasite: implications for disease emergence

Host specificity and cellular tropism is one of the most important characteristics in a parasite's life cycle (Poulin, 2007). Suitable hosts for a parasite are expected to be

similar to the hosts already used by the parasite. This can be the result of shared ancestry or convergent evolution (Poulin, 2007). A phylogenetic influence on host preference by generalists could thus be hypothesised. This was investigated here by correlating genetic distance between all known species susceptible to *S. destruens* with susceptibility distance to the parasite. Overall, there was no clear relationship between genetic and susceptibility distances. In addition, the phylogenetic influence on parasite prevalence does not appear to differ between families. A slightly negative relationship between genetic distance and susceptibility appears to be present for the Cyprinidae, however, this could be due to the smaller number of susceptible tested species tested within this family.

Among generalist parasites, some will preferentially exploit parasites from the same phylogenetic lineage whereas others appear to use a random set of locally available hosts (Krasnov *et al.*, 2008). The apparent lack of phylogenetic influence on host susceptibility for *S. destruens* suggests that this parasite belongs to the latter type of generalist parasites. Ecological distance (defined here as a combination of species' ecological niche and life history traits) between hosts can influence the type of hosts locally available. As two closely related hosts diverge with respect to ecological features necessary for parasite colonization, distantly related species may live in sympatry and thus share these ecological features (Elsheikha, 2009). In this case ecological distance could better explain observed patterns of host use. Investigating the relationship between ecological distance and susceptibility distance between the known hosts of *S. destruens* would help to better understand the driving force behind host use of the parasite.

By investigating the relationship between genetic distance (from *O. tshawytscha* - the most susceptible salmonid currently known) and *S. destruens* prevalence within the Salmonidae family, a high variation in susceptibility between closely related genera appears to exist. For example, the genera *Oncorhynchus* and *Salvelinus* are closely related but they vary greatly in their susceptibility to *S. destruens*. In addition, genera which include highly susceptible species to *S. destruens* appear more likely to have more species highly susceptible to the parasite. However, this could be an artefact of the species tested for susceptibility to *S. destruens*. In addition, this could not be tested with the data for the Cyprinidae as all four species susceptible to *S. destruens* belong to different genera. Therefore, this hypothesis will need to be further investigated by testing the susceptibility of more species within and across genera for both the Salmonidae and Cyprinidae.

Host specificity can be quantified by enumerating the number of species a parasite can infect (Lymbery, 1989). However, this does not provide information on the taxonomic distinctness of the species a parasite can infect. Poulin and Mouilot (2003) proposed a new index (S_{TD}) for determining specificity which was used here. The calculated value for S_{TD} confirms that *S. destruens* is a parasite which infects freshwater fish belonging to different orders. Using an index such as S_{TD} to quantify the level of host specificity is useful as it can be used to compare levels of host specificity between generalist parasites. *S. destruens*' observed host specificity is similar to helminths parasitic to Canadian freshwater fish (Poulin and Mouillot, 2003). The high variance around the S_{TD} index indicated that *S. destruens* is more likely to colonize new species and in doing so it is possible for the parasite to make bigger taxonomic jumps.

Overall, *S. destruens* does not appear to be limited to a phylogenetically narrow host spectrum and the current data suggest that *S. destruens* is a true generalist. It is possible that by exploiting a broader phylogenetic range of hosts, the parasite will use a number of locally available hosts and in doing so will maximise its survival and range expansion opportunities (Krasnov *et al.*, 2008). Similar life strategies have been reported for other generalist parasites, notably *Sarcocystis neurona* which is the cause of equine protozoal myeloencephalitis in horses (Elsheikha, 2009). *S. destruens*' association with two invasive cyprinid species further increases the possibility for range expansion by this parasite. *S. destruens*' generalist nature and the high mortalities it can cause in both salmonid and cyprinid species place it as high risk parasite for freshwater biodiversity.

Chapter 7: Susceptibility of cyprinid species exposed to *Sphaerothecum destruens* in semi-natural conditions.

Abstract Host-parasite interactions occur across a range of environmental conditions and as a result, interpretation of host-parasite interactions observed under laboratory settings must be treated with caution. In addition, for generalist parasites increasing the number of different host species available to a parasite could alter disease prevalence depending on the transmission dynamics between and within available hosts. *Abramis brama*, *Cyprinus carpio* and to some extend *Rutilus rutilus* have been shown to be susceptible to *Sphaerothecum destruens* when exposed to the parasite's spores through water-borne immersion (Chapter 6). These experiments however, were performed under controlled temperature and photoperiod and in the absence of cohabitation with other host species. In the current study, *C. carpio*, *A. brama*, *R. rutilus* and *Scardinius erythrophthalmus* were challenged with *S. destruens* through cohabitation with naturally infected *Leucaspius delineatus* and *Pseudorasbora parva* in semi-natural conditions (outdoor semi-natural ponds) for six months. High mortalities were observed for all species in both the treatment and control groups. However, *S. destruens* was only detected by nested polymerase chain reaction in *C. carpio* which also experienced reduced somatic condition in the presence of *S, destruens*. Despite high cumulative mortalities across all four species, only a small percentage of mortalities were collected. This was due to the chronic mortality pattern of *S. destruens*.

7.1 Introduction

Host-parasite interactions often occur across a range of environmental conditions (Wolinska and King, 2009). Environmental conditions have been shown to affect host-parasite interactions with direct implications on the outcome of infection. For example, Maddox and Cappuccino (1986), characterised the implication of contrasting environmental conditions on the strength of parasite interaction with hosts of different genotypes. Nevertheless, host-parasite interactions have primarily been investigated under constant laboratory conditions where the impact of the parasite on parameters, such as mortality, is more easily determined in the absence of other fluctuating parameters (e.g. temperature and food availability) (Wolinska and King, 2009).

Interpretation of host-parasite interactions observed under laboratory settings must be cautious as these are created outwith a natural ecological context (Poulin and Keeney, 2008). Parasites causing disease in an experimental setting can, for example, be excluded from parasitizing the host in nature due to inter-specific competition with other parasite species (Poulin and Keeney, 2008). Laboratory studies should thus be complemented with experiments performed in heterogeneous and fluctuating environments to provide a more complete understanding of the risk associated with particular parasites.

Prevalence and impact of generalist parasites can differ between single-host and multi-host systems (Keesing *et al.*, 2006). Increasing the number of different host species available to a parasite can also increase or decrease disease prevalence depending on the transmission dynamics between and within available hosts (Woolhouse *et al.*, 2001). In focal host populations with low intra-species

transmission, the presence of an additional host with high inter-species transmission of the parasite might increase the parasite's prevalence in the focal host (Keesing *et al.*, 2006). On the other hand, if the additional host is a poor reservoir for the parasite, the encounter rate between the pathogen and focal host will decrease, resulting in a decrease in parasite prevalence in the focal host (Mitchell *et al.*, 2002). Naturally occurring species complexes are multi-host systems. Thus, it is important to investigate disease susceptibility in multi-host systems (particularly for generalist parasites) and under natural conditions.

A. brama, *C. carpio* and to some extend *R. rutilus* have been shown to be susceptible to *S. destruens* when exposed to the parasite's spores through water-borne immersion (Chapter 6). These experiments however, were performed under controlled temperature and photoperiod and in the absence of cohabitation with other species. Since these conditions were not representative of the environmental fluctuations experienced by these species in their natural environments it was desirable to test the susceptibility of these cyprinids in more natural conditions. The use of artificial outdoor ponds allows for conditions resembling field studies whilst being more controlled and easily accessible. In this study, the susceptibility of *C. carpio*, *A. brama*, *R. rutilus* and *S. erythrophthalmus* was investigated in multi-host cohabitation with *L. delineatus* and *P. parva* under semi-natural environmental conditions. It was hypothesised that *S. destruens* prevalence and host mortalities could differ when these four cyprinid species were exposed to the parasite in a multi-host complex.

7.2 Materials and methods

7.2.1 Fish source

Two hundred, one-year-old *A. brama*, *R. rutilus* and *S. erythrophthalmus* were provided by the Calverton Fish Farm (Environment Agency, Nottingham). Two hundred and seventy-four one-year-old *C. carpio* were obtained from Water Lane Fish Farm (Bridport, Dorset). Six hundred *L. delineatus* (of mixed age) were collected from Stoneham Lakes (Eastleigh, England: 50°57'14" N; 1°22'56" W) on March 6th, 2007 by seine netting. *L. delineatus* were transferred to the facilities of the Centre for Ecology and Hydrology (CEH) at Winfrith, Dorset. During transport, *L. delineatus* were kept in 90L bins which were supplied with oxygen with a maximum transport time of four hours. Six hundred *P. parva* (of mixed age) from a location in Cheshire, England (53°22' N; 3°08' W) were provided by the Environment Agency in April 2007. No mortalities occurred during the transfer of *A. brama*, *R. rutilus*, *S. erythrophthalmus* and *C. carpio*. Some mortalities occurred during transport of *L. delineatus* (n = 52) and *P. parva* (n = 20).

A. brama, *R. rutilus* and *S. erythrophthalmus* originated from populations with no previous history of *S. destruens* infection. *S. destruens* has not been reported from fish originating from Calverton Fish Farm following regular health inspection tests performed by the Cefas Laboratory (Weymouth) and the Environment Agency. Seventy-five *C. carpio* from Water Lane Fish Farm were checked for infection with *S. destruens* by testing the kidney for *S. destruens* specific DNA using nested PCR.

7.2.2 Experimental set-up and exposure to *Sphaerothecum destruens*

Exposure to *Sphaerothecum destruens*. *A. brama*, *R. rutilus*, *C. carpio* and *S. erythrophthalmus* were exposed to *S. destruens* through cohabitation with *L.*

delineatus and *P. parva*. *L. delineatus* originated from Stoneham Lakes where the parasite is established in *L. delineatus* populations (Chapter 2, 4 and 5). *P. parva* was used as a possible additional source of *S. destruens*. The cohabitation set up consisted of two groups of four semi-natural ponds. Each pond had a surface area of 4 m^2 and contained water 1 m deep (Figure 7.1). Treatment group 1 consisted of a four species cohabitation of 250 *L. delineatus*, 250 *P. parva*, 80 *C. carpio* and 80 *S. erythrophthalmus*. Treatment group 2 consisted of a four species cohabitation of 250 *L. delineatus*, 250 *P. parva*, 80 *R. rutilus* and 80 *A. brama*. Each species was assigned to an individual pond. Fish within each pond were visually isolated from fish in different ponds. During the recirculation process the water was UV irradiated and then passed through a biological filter. All ponds were covered with a net to prevent avian predation.

Control group. For the control, 80 individuals of *C. carpio*, *R. rutilus*, *A. brama* and *S. erythrophthalmus* were kept in two circular ponds (diameter: 200 cm, depth 50 cm) in the absence of *L. delineatus* and *P. parva*. In one pond *C. carpio* was cohabited with *R. rutilus* and in the second pond *A. brama* was cohabited with *S. erythrophthalmus*.

The experiment started in March 2007 and lasted for six months. Temperatures were recorded using a temperature logger (Tinytag Splash and Aquatic, OmniInstruments, Dundee, UK). Fish were sampled at regular intervals (see section 8.2.4) and observed mortalities were collected. At the end of six months, surviving fish were euthanized with a 2-phenoxyl-ethanol overdose according to the guidelines by the Animals (Scientific Procedures) Act 1986.

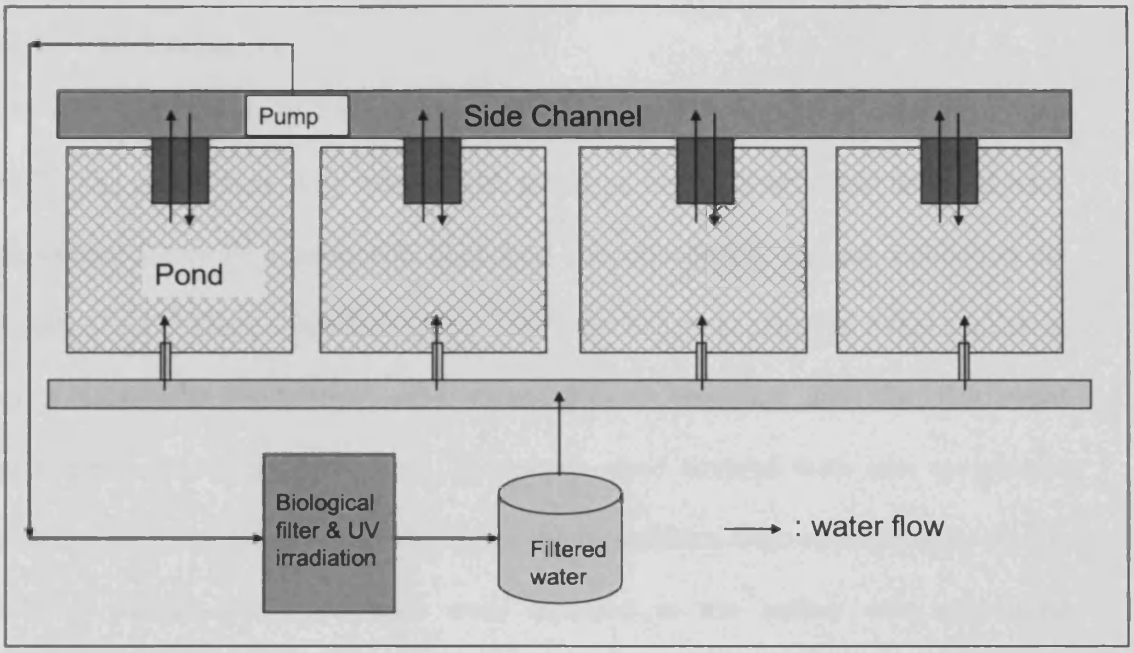


Figure 7.1: Diagram of water flow conditions in experimental semi-natural ponds. Each of two groups of four ponds was connected by a side channel and shared the same water. Each species was assigned to a specific pond and fish of different species did not come in direct contact. Water was pumped from the side channels to a treatment station where it was UV irradiated and passed through a biological filter. Water then collected in a reservoir tank from which it was redistributed to each of the four ponds. Arrows depict the direction of water movement.

7.2.3 Fish husbandry

Fish were checked daily for mortality and overall fish health (from March to May 2007). The ponds were fully mature with plenty of naturally available food but were supplement fed twice a week with flake food (Nutrafin MAX, Hagen). Following the closure of CEH Dorset, Winfrith (May 2007) the fish were checked twice a week at which time any mortalities were recorded and collected and the fish were supplemented with artificial food. The ponds were covered with nets to prevent predation by piscivorous birds. The fish holding facilities were checked daily by the building warden. Any problems were reported to the author who responded immediately with the appropriate course of action.

7.2.4 Sample collection

All detected mortalities were collected, recorded and preserved at -70 °C. Fish were sampled at 2, 3, 4 and 5 months post exposure to *S. destruens* through cohabitation. Ten *A. brama*, *R. rutilus*, *S. erythrophthalmus* and *C. carpio* from both treatment and control were sampled at each sampling event. Five *L. delineatus* were sampled from the treatment at each sampling time. Fish were euthanized by a 2-phenoxyl-ethanol overdose (following the guidelines of the Animals (Scientific Procedures) Act 1986). Weight (g) and fork length (mm) were recorded for each fish. The gills, liver, kidney, intestine and gonad (when present) were collected and stored at -70 °C and 10 % neutral buffered formalin for molecular and histological analysis respectively.

The overall mortality experienced per species was determined as the difference between the number fish present at the end of the experiment and the number of fish initially stocked. At the end of the experiment, all ponds containing *A. brama*, *R.*

rutilus, *S. erythrophthalmus* and *C. carpio* were thoroughly fished out by removing all water. All remaining fish were euthanized and sampled for molecular and histological analysis.

7.2.5 Detection of *Sphaerothecum destruens*

DNA extraction and nested PCR were performed as described in Chapter 4; section 4.2.1 and Chapter 6 section 6.2.2 respectively. *S. destruens* positive samples were processed for histology as described in Chapter 2 section 2.2.2.

7.2.6 Statistical analysis

All statistical analyses were performed using SPSS 14.0 (SPSS Ic. Chicago, Illinois, USA). Statistical significance was accepted when $P \leq 0.05$. Mortalities of *C. carpio*, *A. brama* and *S. erythrophthalmus* in the treatment and control groups were compared using the Mann-Whitney *U* test. Disease prevalence was calculated as: (number of *S. destruens* positive fish / number of fish tested for *S. destruens*) × 100.

7.3 Results

Mean water temperature was 20 °C ±0.3 SE. Minimum temperature was 16 °C and maximum temperature was 24 °C.

7.3.1 Mortalities

R. rutilus in the treatment group experienced an outbreak with *Saprolegnia* spp. within the first month of exposure. This led to high mortalities despite prompt detection and treatment of the disease with Medizin P (Waterlife, UK). Due to the high mortalities it was not possible to draw any conclusions regarding the

susceptibility of *R. rutilus* to *S. destruens* and this species was excluded from any analyses. Infection with Saprolegnia was not detected in any of the other species.

High mortalities occurred in both the treatment and control groups (Figure 7.2). Four months after the onset of cohabitation, only two *A. brama* had remained in the treatment pond. At the end of the experiment, ten *S. erythrophthalmus* and no *C. carpio* were present in the treatment ponds. Despite these high mortalities, only a few mortalities were detected and collected during the experiment duration (Table 7.1). Overall, there was no significant difference between mortalities in the treatment and control group for *C. carpio*, *A. brama* and *S. erythrophthalmus* (Mann Whitney *U* test, $P = 0.12$).Since not all mortalities were detected and collected, cumulative mortality could not be calculated.

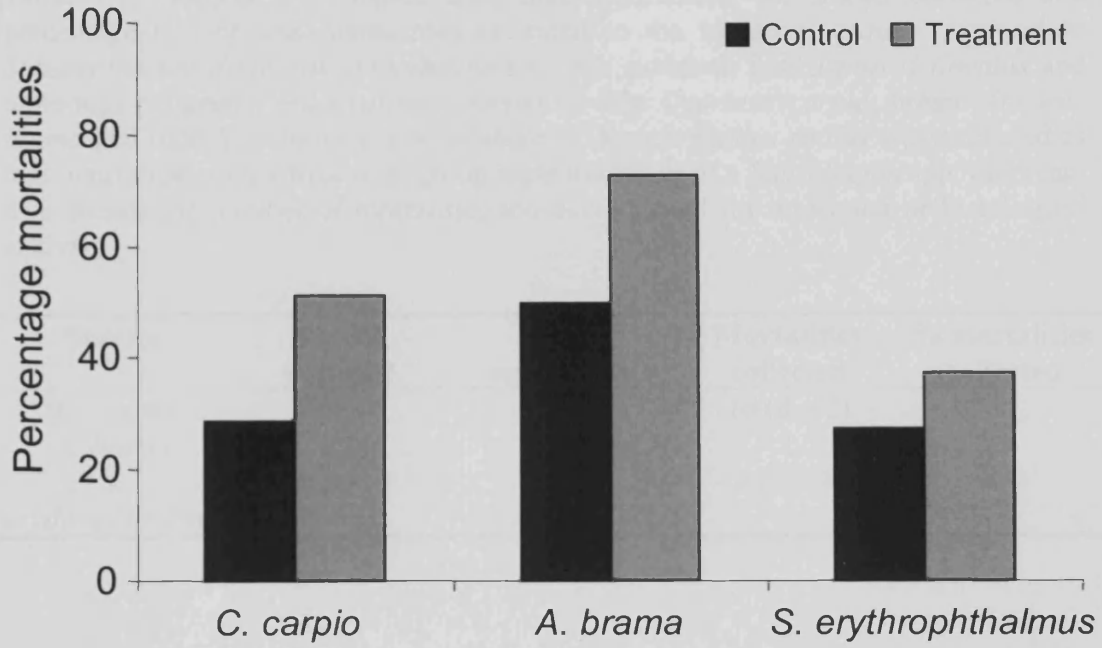


Figure 7.2: Total mortalities in the treatment groups (exposed to *Sphaerothecum destruens* via cohabitation with sunbleak *Leucaspius delineatus* and topmouth gudgeon *Pseudorasbora parva*) and control groups of carp *Cyprinus carpio*, bream *Abramis brama* and rudd *Scardinius erythrophthalmus*. Roach *Rutilus rutilus* was excluded as high mortalities in the treatment group were the result of a *Saprolegnia* spp. disease outbreak.

Table 7.1: Number of sampled fish, total mortalities, mortalities collected and percentage (%) of total mortalities collected in the treatment groups (exposed to *Sphaerothecum destruens* via cohabitation with sunbleak *Leucaspius delineatus* and topmouth gudgeon *Pseudorasbora parva*) of carp *Cyprinus carpio*, bream *Abramis brama* and rudd *Scardinius erythrophthalmus*. Roach *Rutilus rutilus* was excluded as high mortalities in the treatment group were the result of a *Saprolegnia* spp. outbreak. d: indicates the number of mortalities too decomposed for molecular or histological analysis.

Species	No. fish sampled	Total mortalities	Mortalities collected	% mortalities collected
C. carpio	39	41	10 (d = 2)	24
A. brama	22	58	1	2
S. erythrophthalmus	50	30	4 (d = 3)	13

Table 7.2: Number of sampled fish, total mortalities, mortalities collected and percentage (%) of total mortalities collected in the control groups of carp *Cyprinus carpio*, bream *Abramis brama*, rudd *Scardinius erythrophthalmus* and roach *Rutilus rutilus*. d: number of mortalities too decomposed for molecular or histological analysis.

Species	No. fish sampled	Total mortalities	Mortalities collected	% mortalities collected
C. carpio	57	23	9 (d=2)	39
A. brama	40	40	34	85
S. erythrophthalmus	58	22	22 (d=1)	100
R. rutilus	65	15	10	67

7.3.2 *Sphaerothecum destruens* prevalence in *Abramis brama*, *Cyprinus carpio*, *Rutilus rutilus*, *Scardinius erythrophthalmus* and *Leucaspius delineatus*

The kidney, liver, intestine, gill and gonad (when present) were tested for *S. destruens* specific DNA in all natural mortalities and sampled fish for all species. A total of 721 organs were tested.

***Abramis brama*, *Rutilus rutilus* and *Scardinius erythrophthalmus*.** *S. destruens* specific DNA was not detected in the treatment and control groups of *A. brama*, *R. rutilus* and *S. erythrophthalmus*.

***Cyprinus carpio*.** The kidney of 74 *C. carpio* from Water Lane fish farm tested negative for *S. destruens*. Following exposure to *S. destruens* through cohabitation with *L. delineatus* and *P. parva*, *C. carpio* were positive for *S. destruens* using nested PCR. *S. destruens* DNA was detected in mortalities with prevalence of 12.5 % (n = 8) and in sampled fish with a prevalence of 5 % (n = 39). *S. destruens* specific DNA was also detected in the mortalities and sampled *C. carpio* from the control group resulting in *S. destruens* prevalence of 28.5 % (n = 7) and 3.5 % (n =57), respectively. *S. destruens* DNA was detected in the kidney (71 % prevalence) and liver, intestine and gill (14 % prevalence) of infected *C. carpio* tissues. However, *S. destruens* was not detected in any histological sections of all *S. destruens* positive samples.

Emaciated *C. carpio* were collected from both the treatment and control groups two months post experimental onset. The condition factor of treatment and control *C. carpio* decreased during the experiment's duration. Control *C. carpio* had significantly higher condition factors at two months post exposure compared to *C. carpio* in the treatment group (Mann Whitney *U* tests, $P < 0.05$).

***Leucaspius delineatus*.** Forty *L. delineatus* (20 each from each treatment group) were tested for *S. destruens*. *S. destruens* specific DNA was not detected in any of these fish.

7.4 Discussion

The use of semi-natural outdoor ponds to test the susceptibility of *C. carpio*, *A. brama*, *R. rutilus* and *S. erythrophthalmus* to *S. destruens* further corroborated the susceptibility of *C. carpio* to *S. destruens* (in addition to the findings of Chapter 6). *S. destruens* was detected in *C. carpio* at prevalence levels similar to those observed in *C. carpio* exposed to *S. destruens* spores (Chapter 6). Total *C. carpio* mortalities differed between the two experiments with *C. carpio* in the pond experiments experiencing 51 % total mortalities compared to 8.3 % in the water-borne challenge (Chapter 6). Since a high proportion of mortalities were not collected, the higher mortalities could not be confidently attributed to infection with *S. destruens*.

S. destruens was also detected in the control group of *C. carpio*. Source *C. carpio* (from Water lane Fish Farm) tested negative for *S. destruens* DNA. Therefore, the presence of the parasite in the control *C. carpio* was likely the result of cross contamination from the treatment ponds. Cohabitation experiments between *P. parva* and naïve *L. delineatus* demonstrated that *S. destruens* can be transmitted through contaminated water in the absence of direct contact with infected hosts (Gozlan *et al.*, 2005). Due to the close proximity of the ponds airborne contamination of control groups was possible. Aerosolization of viable micro organisms and disease transfer has been documented (Angenent *et al.*, 2005). Treatment and control groups had similar *S. destruens* prevalence, however, lower mortalities (29 %) were observed in the latter which may be attributable to the different number of host species present in the control compared to the treatment ponds. In the control, *C. carpio* was cohabited in a two-host system with *R. rutilus* whereas in the treatment *C. carpio* was cohabited in a four-host system with *L. delineatus*, *P. parva* and *S. erythrophthalmus*.

Differences in species composition in the two groups could have caused changes in host-parasite interactions resulting in the observed dissimilarity in mortalities; however, this could not be determined from the current experimental design.

The somatic condition of *C. carpio* decreased over time in both the control and treatment experiments with somatic condition reaching significantly lower levels in treatment compared to control *C. carpio* after two months. Past two months, treatment and control *C. carpio* condition factors did not significantly differ. Emaciated *C. carpio* were also detected in both the treatment and control groups. Emaciation has been observed in *L. delineatus* and has been linked with *S. destruens* infection (Gozlan *et al.*, 2005). Reduced condition was not observed in *C. carpio* exposed to *S. destruens* through water borne exposure (Chapter 6). However, the presence of other hosts could influence the condition factor of *C. carpio*.

A large proportion of the mortalities were not detected in the treatment and control groups. The chronic mortality pattern caused by *S. destruens* is one where a low number of fish die between 1-3 day intervals over a period of time resulting in high cumulative mortalities (Harrell *et al.*, 1986). This mortality pattern is distinctly different from agents that cause single high mortality events and is one of the main reasons why S. *destruens* can go undetected in natural systems. As the majority of mortalities are undetected, *S. destruens* prevalence can be easily underestimated and in some cases the parasite can go undetected.

The susceptibility of *A. brama* was not confirmed in the current experiment. *A. brama* experienced high mortalities (73 %) in the treatment, of which, only 2% were

collected. The mortality rate in the present study is similar to the mortality in *A. brama* exposed to *S. destruens* spores (53.3 %; Chapter 6). In Chapter 6, 76 % of *A. brama* mortalities were positive for *S. destruens* whereas all sampled fish in the current study tested negative for *S. destruens* DNA. It could be expected that *S. destruens* is more likely to be found in dead hosts and thus the low percentage of mortalities collected from the treatment group of the current experiment could explain the absence of *S. destruens* detection. *S. destruens* was not detected in any of the sampled control *A. brama* or the 85 % of collected control mortalities.

S. destruens was not detected in *S. erythrophthalmus* and *R. rutilus* (treatment and control) despite high mortalities. *R. rutilus* mortalities in the treatment group were due to a *Saprolegnia* spp. infection which led to high mortalities within the first month of the experiment. Quarantine of the fish in their respective ponds for at least 30 days prior to cohabitation onset would have allowed for the outbreak to occur and new fish to be brought in. This procedure should be applied to any future study.

S. destruens was not detected in *L. delineatus*, however, only a small number of fish were tested (n = 40). The detection of *S. destruens* DNA in *C. carpio* indicates that *S. destruens* was present in at least one of the treatment ponds. Future work should include testing higher numbers of both *L. delineatus* and *P. parva* for *S. destruens* used in the cohabitation. Also, future studies should test for the presence of *S. destruens* in established populations of *A. brama* and *C. carpio* co-existing with *L. delineatus* and *P. parva*. This would enable us to determine whether these host-parasite associations can naturally exist. It is important to note that these associations will exist in a dynamic multi-host parasite association depending on the number and

type of available host species. Highly susceptible species such as *A. brama* might experience high mortalities therefore, to increase the probability of detecting the parasite large numbers of fish will need to be tested.

Despite the limitations in experimental design (due to the available resources and logistics involved in executing such a large scale outdoor experiment), the preliminary results of this study are still important. The current results suggest that against a multi-host background, *C. carpio* experienced higher mortalities and reduced somatic condition in the presence of *S. destruens*. This suggests that under the semi-natural conditions used here, *C. carpio* appear to be more susceptible to *S. destruens*. This further corroborates the need to perform such large scale outdoor experiments when trying to decipher the impact of parasites on natural populations. *A. brama* and *S. erythrophthalmus* experienced similar mortalities in a multi-host complex compared to exposure to *S. destruens* spores. However, the inability to collect the majority of mortalities possibly influenced the ability to detect the parasite in at least *A. brama*. Future work should further investigate the impact on parasite prevalence and perhaps even host susceptibility of at least *C. carpio* and *A. brama* within multi-host systems. These should primarily be conducted in indoor experiments where contamination of control groups can be better controlled.

Chapter 8: Discussion

8.1 Synthesis of principal results

***Sphaerothecum destruens* life cycle.** *S. destruens* can be best characterised as non-organ, non-temperature and non-species specific parasite. Similar to other members of the family Rhinosporideacae, *S. destruens* does not appear to have a particular tissue preference. The parasite was found in all organs tested (kidney, liver, gill, gonad and intestine) in both naturally infected (Chapter 2) and experimentally infected cyprinid fish (Chapters 4-6). Tissue tropism in susceptible species within the Cyprinidae and Salmonidae did not appear to differ, and *S. destruens* pathology was similar between *L. delineatus* and *O. tshawytscha* (Chapter 2). It would thus appear that *S. destruens* does not change its tissue preference even in hosts belonging to different families indicating that the parasite has maintained its generalist nature (i.e. it has not specialised for any particular host).

The presence of a second *S. destruens* host, in this work *P. parva*, did not influence the prevalence and infection levels of *S. destruens* in *L. delineatus* (Chapter 5). However, organ prevalence of *S. destruens* appeared to be influenced by the host's reproductive state with the kidney, liver, intestine, gill and gonad being infected in reproductive *L. delineatus* whereas only the gill, liver and gonad were infected in non-reproductive *L. delineatus* (Chapter 4). In addition, *S. destruens*' infection levels were influenced by the host's reproductive state with reproductive *L. delineatus* having significantly higher *S. destruens* infection levels compared to their non-reproductive counterparts (Chapter 4). The release of *S. destruens* in reproductive fluids of *O. tshawytscha* (Arkush *et al.*, 1998, 2003) and its documented high infection level in reproductive *L. delineatus* leads to the hypothesis that *S. destruens* could be synchronising its release with its hosts' reproductive activity.

S. destruens displayed high temperature tolerance in its extracellular life stage – the zoospore (Chapter 3). Zoosporulation, i.e., the release of motile zoospores from *S. destruens* spores, occurred at temperatures ranging from 4 to 30 °C. Fifteen degrees Celsius appeared to be the optimum temperature for zoospore release. Temperature dependence was observed for the onset and duration of zoosporulation with later onset and longer duration in lower temperatures. The estimated zoospore production was extended by the prolonged zoospore release of *S. destruens* spores. Overall, high zoospore production was observed across all temperatures increasing the chances of attachment to susceptible hosts. In *L. delineatus*, the high prevalence of *S. destruens* in the gill and the low infection levels (pg *S. destruens* DNA; Chapters 4 and 5) provided further support for the hypothesis that the gills could be a temporary site for attachment prior to migration to other organs (Arkush *et al.*, 2003, Mendonca and Arkush, 2004).

The persistence of both the spore and zoospore stage of *S. destruens* across a wide temperature range and *S. destruens*' lack of tissue tropism and host specialisation has inevitably contributed to the parasite's widely varied host range (Arkush *et al.*, 1998, Gozlan *et al.*, 2005). *S. destruens* hosts can live in widely different environments and under widely different temperature regimes (for example, *O. tshawytscha* and *L. delineatus*). *S. destruens* is present in at least one wild *L. delineatus* population in the UK (Chapter 2). Its non-specific nature and association with invasive fish (such as *L. delineatus* and *P. parva*) place *S. destruens* as a strong candidate for range expansion and subsequent contact with naïve hosts (cyprinids and salmonids). The susceptibility of a number of salmonids has been previously investigated (Hedrick *et al.*, 1989,

Arkush *et al.*, 1998) whereas the susceptibility of cyprinid species was investigated for the first time in this thesis.

***Sphaerothecum destruens* host specificity.** The investigation of cyprinid species susceptibility (Chapters 6 and 7), has shown for the first time that *S. destruens*' potential hosts include *A. brama*, *C. carpio* and *R. rutilus*. *A. brama* experienced high mortalities following exposure to *S. destruens* (mean mortality 53 %; Chapter 6) of which 76 % were positive for *S. destruens*. *R. rutilus* experienced moderate mean mortalities (37 %) and low *S. destruens* prevalence (3 %) whereas *C. carpio* experienced low mean mortalities (8 %) and relatively low disease prevalence (11 %) (Chapter 6). S. *destruens*' known host range as well as a qualitative estimation of their susceptibility is provided in Table 8.1. The known host range for *S. destruens* is likely to increase as the parasite's epidemiology is further investigated.

The lack of phylogenetic influence on susceptibility (Chapter 6) and the ability to infect species across families suggests that the parasite would use a number of locally available hosts and in doing so would maximise its survival and range opportunities (Krasnov *et al.*, 2008). This appears to be a key life history trait of *S. destruens* that could contribute to the parasite's persistence and one that is shared by other Rhinosporideacae members (Chapter 1). With the exception of *R. seeberi* (which can infect species belonging to different classes). *S. destruens* is the only Rhinosporideacae member which can infect species across families.

Table 8.1: *Sphaerothecum destruens* known hosts in the Salmonidae and Cyprinidae. Species were qualitatively classified into three prevalence categories: high, moderate and low. Qualitative scores were based on reported *S. destruens* prevalence (Low 0-33 %; Moderate 33-66 % and High: 66-99 % *S. destruens* prevalence). Prevalence values for the Salmonidae have been based on the work of Arkush *et al.* (1998) and Hedrick *et al.* (1989) and for the Cyprinidae they have been based on the work of Gozlan *et al.* (2005) and Chapters 6 and 7.

Salmonidae	**Prevalence**	**Cyprinidae**	**Prevalence**
Oncorhynchus tshawytscha	High	*Abramis brama*	High
Oncorhynchus kisutch	High	*Leucaspius delineatus*	Moderate
Salmo salar	High	*Cyprinus carpio*	Low
Oncorhynchus mykiss	Moderate	*Rutilus rutilus*	Low
Salmo trutta	Moderate	*Pimephales promelas*	Low
Salvelinus fontinalis	Low	*Pseudorasbora parva*	Low

The influence of multi-host systems on *S. destruens* was investigated in this thesis in two experiments. In the first experiment, *L. delineatus* was cohabited with *P. parva* (Chapter 5). In this two-host system, *S. destruens*' dynamics (in terms of parasite prevalence) were not influenced by the presence of *P. parva*. However, the somatic condition of infected female *L. delineatus* was adversely affected by the presence of *P. parva* suggesting that the presence of *P. parva* had an additional cost to parasitized female *L. delineatus*.

In the second experiment, *A. brama*, *C. carpio*, *R. rutilus* and *S. erythrophthalmus* were exposed to *S. destruens* in a multi-host cohabitation with *L. delineatus* and *P. parva* (Chapter 7). This cohabitation was performed in outdoor semi-natural ponds. Although preliminary, the results of this study suggest that *A. brama* and *S. erythrophthalmus* experience similar mortality levels in a multi-host system as in a single host system (Chapter 6 and 7). *C. carpio* however, experienced higher mortalities in the multi-host system despite similar *S. destruens* prevalence (Chapters 6 and 7). The results from both experiments indicate that in a multi-host system parameters such as mortality, somatic condition and perhaps *S. destruens*' prevalence might differ depending on species composition.

S. destruens' mortality pattern includes a low percentage of the population dying over a time period and a high cumulative mortality (Figure 8.1). This pattern has been previously described as chronic mortality (Hedrick *et al.*, 1989) and it has been observed in both *O. tshawytscha* and *S. salar* (see Harrell *et al.*, 1986, Hedrick *et al.*, 1989). The same mortality pattern has been observed in cyprinids (Chapters 6 and 7; Gozlan *et al.*, (2005)). Although only a small percentage (approximately 5 %) of fish die on a daily basis this still contributes to high cumulative mortality (Chapters 6 and 7; Harrell *et al.* (1989); Gozlan *et al.* (2005)).

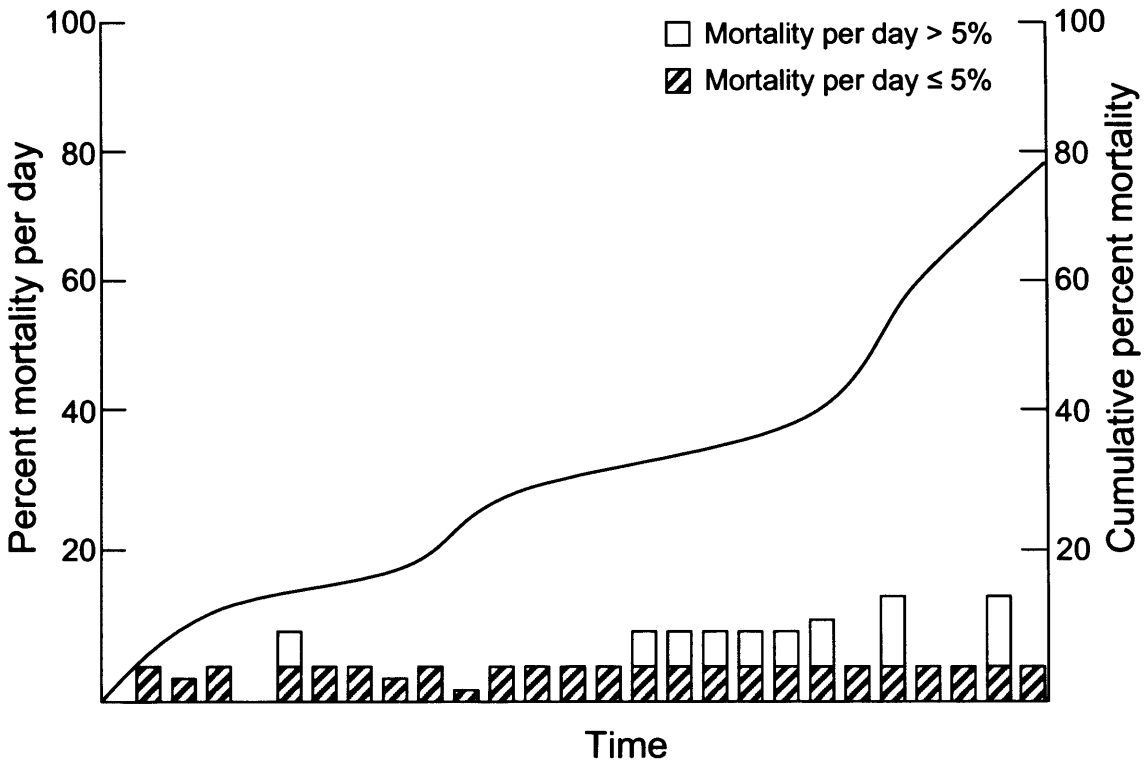


Figure 8.1: Hypothetical representation of the chronic mortality pattern observed for *Sphaerothecum destruens*. A small percentage of fish die per day leading to a high cumulative mortality.

This chronic mortality pattern is more likely to be detected in farmed populations where fish are checked on a regular basis. This is supported by the fact that reported mortalities from *S. destruens* have so far only been reported in aquaculture facilities (Harrell *et al.*, 1986, Hedrick *et al.*, 1989) and under more controlled experimental settings (Chapters 4 to 7 and Gozlan *et al.* 2005). This pattern is less likely to be detected in wild systems where diseased individuals have a high probability of being predated upon and dead fish are quickly scavenged (Copp *et al.*, 2009). Therefore, in the wild, only high mortalities and sharp population declines are likely to be reported and investigated. The exposure of cyprinid species to *S. destruens* in a semi-natural

system and the low numbers of mortalities collected corroborated the difficulty to detect and record all chronic mortalities (Chapter 7).

8.2 Conservation issues for *Leucaspius delineatus*

L. delineatus is invasive to the UK but has experienced high declines throughout its native range over the last 40 years (Gozlan *et al.*, 2009) and is currently listed on the IUCN red list of threatened species (WCMC, 1996). Inhibition of spawning in *L. delineatus*' has been associated with *S. destruens* (see Gozlan *et al.*, 2005). The increased *S. destruens* infection levels observed in reproductive *L. delineatus* (Chapter 4) provided further support that *S. destruens* could adversely impact the reproductive success of *L. delineatus* which in turn can have detrimental effects on its population size. Population declines in *L. delineatus*' native range have coincided with the spread of the invasive Asian cyprinid *P. parva*. Cohabitation with wild *P. parva* has been associated with transmission of *S. destruens* to *L. delineatus* (see Gozlan *et al.*, 2005) and has led to reduced somatic condition in female *L. delineatus* parasitized with *S. destruens* (Chapter 5).

Coexistence of *L. delineatus* with *P. parva* in a natural lake in France did not lead to declines in *L. delineatus* (see Carpentier *et al.*, 2007). This suggests that *L. delineatus* declines observed by Gozlan *et al.* (2005) following cohabitation with *P. parva* in a semi natural pond could have been the result of *S. destruens* infection. Carpentier *et al.* (2007) compared *L. delineatus*' abundance prior and after the introduction of *P. parva* but did not test for *S. destruens* infection nor reported somatic condition of the fish.

The European Union's Habitats and Species Directive requires that member states consider the reintroduction of certain regionally extinct native species. Species have been reintroduced in their native range using individuals originating from captive

breeding programs with varying success (Fischer and Lindenmayer, 2000). However, reintroductions from wild populations have been more successful (Fischer and Lindenmayer, 2000). Species like *L. delineatus* are locally extinct and threatened in parts of their native range while maintaining large populations in their invasive range. The use of established invasive populations in reintroduction and supplementation programs can be a potential conservation measure for *L. delineatus*. However, reintroduction success would depend on the identification and elimination of the factors that initially have caused the population decline. For *L. delineatus* one of these factors could be *S. destruens* or the presence of *P. parva*, or both.

The possibility that *S. destruens* has resulted in the decline on *L. delineatus* in its native range needs to be further investigated as an *S. destruens* free community could be a prerequisite to successful reintroduction. This could include surveys of communities from which *L. delineatus* has become extinct. Such surveys should sample and test cyprinid species such as *C. carpio*, *A. brama* and *P. parva* for *S. destruens*. Such surveys will also serve the role of further investigating *S. destruens*' proposed life history strategy i.e., that the parasite is maintained in communities in a multi-host dynamic. Thus, if *L. delineatus* declines have been caused by *S. destruens* it would be expected that the parasite would still be present within these communities.

Knowledge of the geographic distribution of a parasite can play an important role in risk analysis of fish transport (especially when fish are associated with the ornamental and aquaculture trade) and can be used in the design of targeted conservation efforts, for example, reintroduction efforts in parasite free waters (Edgerton, 2002).The use of molecular tools to perform systematic search and mapping of disease prevalence has

been increasing and their use to detect disease reservoirs has been proposed for making conservation decisions (Kozubikova *et al.*, 2009).

A quantitative PCR reaction has been developed (Chapter 4) to allow *S. destruens* detection and quantification of the agent and should be used when conducting a systematic regional survey for the parasite both in the UK and continental Europe. A systematic regional survey for *S. destruens* would allow: (a) the identification of *L. delineatus* populations threatened by *S. destruens*; (b) the identification of areas not suitable for *L. delineatus* reintroduction; and (c) identify high risk areas for disease transfer. In areas deemed as high risk for disease transfer, there should be increased education efforts on the risks of disease introduction to naïve fish populations accompanied by regulation of fisheries management practices and angling activities to reduce the risk of disease transfer to naïve locations.

8.3 Potential implications of *Sphaerothecum destruens* on aquaculture, commercial fisheries and wild fish populations

Predicting the impact of a parasite on wild and farmed host populations is particularly challenging as in most cases there is considerable lack of data on parameters such as the parasite's basic reproductive rate and thorough epidemiological maps indicating infected populations and animal movement maps (Peeler *et al.*, 2007). In addition, most of the studies on parasite-host dynamics are performed in controlled, artificial, indoor experiments which bear little resemblance to the conditions experienced in the field. Despite this however, there is still a need to assess the risk associated with a disease as this will allow better allocation of resources and inform public policy decisions (Peeler *et al.*, 2007). Qualitative and quantitative risk assessment methods are available and the choice between the two is dependent on the amount of available information and staff training (Peeler *et al.*, 2007).

Aquaculture facilities and commercial fisheries most often stock fish at high densities in order to respectively maximise their yield and achieve high angler satisfaction (Cowx and Gerdeaux, 2004, Hewlett *et al.*, 2009). High stocking densities can be extremely stressful to fish (Costas *et al.*, 2008, Caipang *et al.*, 2009) and can result in physiological changes including reduced immunocompetence (Caipang *et al.*, 2009). Such conditions increase the risk of disease outbreak and high mortalities.

Parasites with direct life cycles are usually more prevalent in farmed fish due to the lack of trophic transmission (since fish are fed) and the high contact rates due to high stocking densities (Nowak, 2007). Prevalent parasites in aquaculture facilities do not often occur in high prevalence in wild congeners of the farmed fish (Nowak, 2007) however this is not always the case. For example, *S. destruens* has been reported at high prevalence from both farmed and wild *O. tshawytscha* (see Arkush *et al.*, 1998). This makes the investigation of the life cycle and host range of *S. destruens* of particular importance.

S. destruens has been reported to cause disease outbreaks resulting in high mortalities in aquaculture facilities of *O. tshawytscha* and *S. salar* (see Harrell *et al.*, 1986, Hedrick *et al.*, 1989). The factors leading to the reported *S. destruens* outbreaks have not been investigated, however, stress as a result of fish handling appeared to exacerbate mortalities (Hedrick *et al.*, 1989). In the UK, *S. destruens* has been associated with *L. delineatus* and *P. parva* (see Gozlan *et al.*, 2005, 2009) which have been introduced to the UK through the aquaculture trade (Pinder and Gozlan, 2003, Pinder *et al.*, 2005). The possibility that *S. destruens* could be present in aquaculture facilities harbouring *L. delineatus* and *P. parva* cannot be excluded. In addition, the presence of *S. destruens* in fisheries which were stocked with farmed fish from

infected facilities cannot be excluded. The discovery of *S. destruens* in a wild *L. delineatus* population (Chapter 2) indicates that the parasite can be established in wild cyprinid populations increasing the risk of disease transfer between farmed and wild fish.

The unpredictability of *S. destruens* outbreaks as well as *S. destruens'* association with the aquaculture industry and the high mortalities documented for species such as *O. tshawytscha*, *S. salar*, *A. brama* and possibly *C. carpio* increase the risk of this parasite on both farmed fish and fisheries. Additionally, the repeated introduction of naïve hosts in an aquaculture setting usually leads to no costs of mortality to the parasite and can lead to increased virulence (Nowak, 2007); increasing the risk of high mortalities and financial impact on the aquaculture facilities.

In a commercial fishery, customer (angler) satisfaction is vital for business survival therefore fisheries' managers focus on their fishery's performance i.e. the number and size of fish caught by their customers (Hewlett *et al.*, 2009). The chronic mortality pattern caused by *S. destruens* would lead to underestimated high mortalities which would translate in reduced fishery performance. Stock enhancement is the principal method used by fisheries managers to rectify reduced catch rates and has a considerable cost (Cowx and Gerdeaux, 2004). The repeated introduction of naïve fish into a system with *S. destruens* will most likely contribute to continuous mortalities and reduced fisheries performance.

As open water fisheries and aquaculture facilities do not treat the water released to nearby streams, rivers and lakes there is a risk of disease transfer form farmed to wild populations. Transfer of parasites prevalent in farmed fish to wild fish has been reported (Valtonen and Koskivaara, 1994, Hilborn, 2006) and includes the notable

example of the parasitic fish lice (Krkosek *et al.*, 2005, Krkosek, 2008). The long survival period of *S. destruens* spores and zoospores at a wide temperature range (Chapter 3) and the possible continuous release of high parasite numbers from aquaculture facilities and fisheries into wild systems can potentially increase infection pressure on naïve wild fish and may lead to the successful establishment of the parasite within naïve wild fish populations.

The work presented in this thesis, reports high mortalities in *A. brama* and *R. rutilus*. It is particularly difficult to establish a formal link between mortality and *S. destruens* infection, however, a significantly higher mortality in the group treated with *S. destruens* and the high prevalence of the parasite associated with mortalities (at least for *A. brama*) is an initial indication that *S. destruens* can cause high mortalities in this species. Although preliminary, the cohabitation of naïve *A. brama*, *C. carpio*, *R. rutilus* and *S. erythrophthalmus* in outdoor ponds indicated that in a multi-host setting mortality and disease prevalence patterns can differ (Chapter 7). In addition, this work has also indicated the importance of performing disease challenges in semi-natural systems in order to better predict the potential impacts that parasites can have on wild populations. *S. destruens* transmission and mortalities have been observed in these semi-natural conditions.

S. destruens ability to infect multiple species, cause high mortalities, low temperature dependence and the high risk of not detecting the parasite due to its chronic mortality pattern place this parasite as potentially high risk to naïve wild fish populations of *A. brama*, *L. delineatus*, *O. tshawytscha*, *S. salar* and potentially *R. rutilus*, *S. erythrophthalmus* and *C. carpio*. The potential association of *S. destruens* and fish

reproductive state (Chapter 4) can potentially negatively impact susceptible fish species by reducing reproductive success.

8.4 What are the risks of *Sphaerothecum destruens* spread in the UK?

S. destruens' association with the aquaculture industry and its invasive hosts *L. delineatus* and *P. parva* increase the risk of *S. destruens* expansion to new locations in the UK. In England and Wales, fish movements and introductions within all inland waters are regulated by the Environment Agency. In order to prevent introduction of novel disease and prevent disease spread, certification of fish health is mandatory for fish stocked in open water sites (i.e. sites were water can flow from one water body to another). In the case of closed water sites, health checks can also be required by the Environment Agency consenting officer prior to any fish introduction.

Health checks are performed by the Environment Agency and fish movements are prohibited when: (a) more than 20 % of the fish are identified as clinically diseased (clinical disease includes heavy parasite, bacterial and viral disease as well as physical abnormalities such as spine deformities) and (b) when the presence of parasites listed on the Environment Agency's Category 2 and novel parasites list is detected (Environment Agency, 2008).

S. destruens has been identified as a potentially novel parasite (Gozlan *et al.*, 2005), however, it is currently not included in the novel disease list of the Environment Agency. During a fish health check, the Environment Agency requires that a minimum of 30 fish must be examined. This sample size is considered a sufficient for the detection of most pathogens. However, depending on the species and its status, it

is highly probable that *S. destruens* would not be detected in a sample of 30 sub-clinical fish.

8.5 Management implications

Careful consideration of the risks to the aquaculture industry, commercial fisheries and wild populations as well as the potential for disease spread (sections 8.5 and 8.6) has identified *S. destruens* as a high risk parasite. Since this risk classification is subjective to the author's interpretation of risk, a less subjective method of determining the risk associated with *S. destruens* was also applied (Box I). This semi-quantitative method has also identified *S. destruens* as a high risk parasite.

Following these evaluations, there are a number of recommendations that can be made to policy makers. The potential risk posed by *S. destruens* should be re-evaluated in light of the results presented here. In the event that *S. destruens* is placed on the Category 2 and novel parasite species of the Environment Agency, a specific sampling strategy will need to be adopted. As noted in section 8.5 the current sampling method employed by the Environment Agency would not have a high probability of detecting *S. destruens*. A sample size of at least 100 fish and the use of molecular techniques to detect the parasite using at least the kidney as the organ of choice are recommended. When a smaller sample size is preferred, the testing of at least three organs (kidney, liver and posterior intestine) is recommended. Similar to the present study, samples for both molecular analysis and histology should be collected for individual fish. PCR (nested or qPCR) should be used as the first step of detection, followed by histological analysis of fish determined positive by PCR.

To further assist the evaluation of risk associated with *S. destruens*, an extensive epidemiological survey should be performed. Such a survey should first focus on testing aquaculture facilities and fisheries where *L. delineatus* and *P. parva* have been reported. Where *S. destruens* is detected, wild populations in adjacent water bodies should also be tested for *S. destruens*. This will allow a more informed evaluation of the possibility that *S. destruens* spread can be controlled through fish movement restrictions and inform decisions on restricting fish movement.

8.6 Future work

As discussed in sections 8.3 and 8.6, the epidemiology of *S. destruens* in continental Europe and the UK needs to be further investigated. This should be complemented by an extensive review of the literature including technical reports on the health of wild populations of *S. salar*, *A. brama*, *C. carpio* and *L. delineatus*. The combination of the parasite's epidemiology as well as any population declines reported in the literature should better inform policy makers on the impact of the parasite as well as on management options. In addition, collection of *S. destruens* positive samples from continental Europe would allow the determination of *S. destruens*' invasive status in the UK. The ITS region can be used to determine geographic isolation between *S. destruens* populations from the UK and continental Europe and thus provide an indication as to whether the parasite is invasive to the UK. A similar approach has been applied to determine geographic isolation between *S. destruens* isolates from the USA and UK (Gozlan *et al.*, 2009).

An important outcome of this work has been the further support for *S. destruens*' most important life history trait, i.e., that the parasite persists in various communities by maintaining population in numerous hosts. The literature on host-parasite interactions

of multi-host parasites suggest that the existing host complex would influence the impact a generalist parasite would have on its host populations. The investigation of *S. destruens*' dynamics in multi-host systems (Chapters 5 and 7) has suggested that this could be true for *S. destruens*. This should be further investigated in controlled indoor experiments where various combinations of hosts should be cohabited while investigating *S. destruens*' prevalence and host mortalities. This work will need to be accompanied by the appropriate controls and should also incorporate experiments determining within and between species transmission of *S. destruens* as this will further support the results of the multi-host experiments and provide the necessary information for modelling the impacts of the parasite in a community.

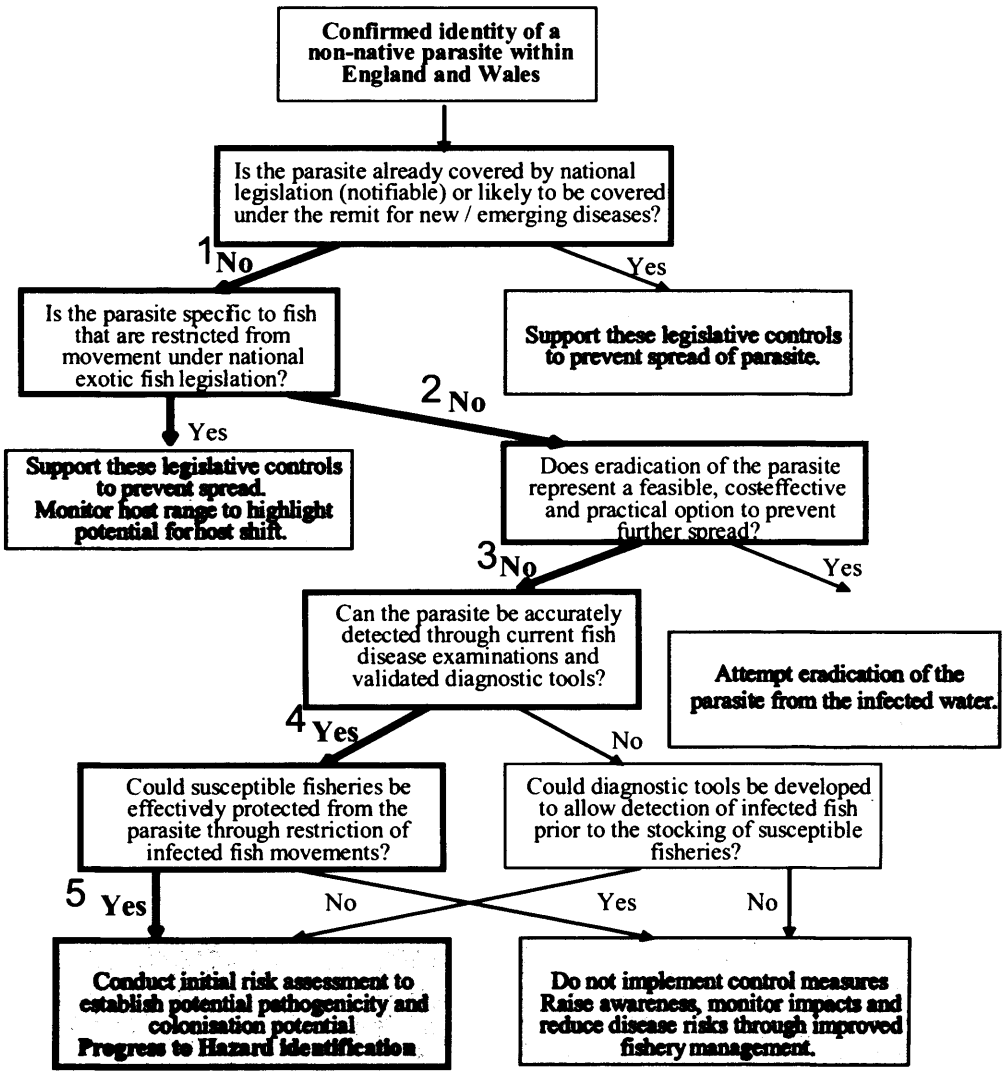
The potential that *S. destruens*' numbers increase with the reproductive state of its hosts is another research avenue which warrants further investigation. This should be investigated using *L. delineatus* as well as at least *A. brama* and *C. carpio* within the cyprinids and *S. salar* within the salmonids. A potential synchronisation of *S. destruens* numbers with the reproductive state of the fish can have important implications on host populations as well as help to better understand why certain hosts appear to be more susceptible to the parasite. Work could involve the administration of reproductive hormones whilst monitoring *S. destruens* numbers in *S. destruens* positive fish. Such work would also serve to help better understand the possible evolutionary history of *S. destruens*. For example, did *S. destruens* originally evolve with anandromous species such as *O. tshawytscha* which die following spawning?

A major limitation of the current work was the inability to detect histopathological changes of *S. destruens* infection in *A. brama*, *C. carpio* and *R. rutilus*. This has mostly been the result of autolysis within the tissues of *S. destruens* positive fish.

Challenging *A. brama*, *C. carpio, R. rutilus* and *S. erythrophthalmus* with *S. destruens* via intraperitoneal injection would serve to: (a) further test the susceptibility of *R. rutilus* and *S. erythrophthalmus* and (b) to determine the histopathology associated with these four species as well as determine species differences in tissue tropism. The infection protocol by Arkush *et al.* (1998) could be used.

Box I: Risk assessment for *Sphaerothecum destruens*. The decision diagrams have been adapted from Williams (2007). The risk assessment is divided into two parts. Part A determines whether it is possible to manage the spread of the introduced parasite. Part B determines the potential hazard posed by the parasite.

Part A Rationale supporting the decision made at each step. **1** *S. destruens* is not currently covered by any legislation in the UK. **2** *S. destruens* can infect *Salmo salar*, *Abramis brama* and *Cyprinus carpio*. The movement of these species is not restricted under the national exotic fish legislation. **3** Eradication of the parasite has been attempted by eradicating its healthy host *Pseudorasbora parva* using rotenone (Britton and Brazier, 2006) – this process is both ecologically and financially expensive. **4** The parasite can be detected using histology and molecular techniques. **5** Fish movement restrictions would be the only effective method to prevent the spread of the parasite to naïve fisheries.



Since susceptible fisheries could be protected through restrictions of infected fish, the risk associated with *S. destruens* should be determined.

Box I Part B Aims to provide a quick method for identifying the hazard risk associated with the parasite in question. Scoring criteria are: 0 = Very low or No; 1 = Low; 2 = Moderate; 3 = High; 4 = Very high or Yes. Scores were summed and an overall hazard score was calculated. The overall score was then translated to high (29-40), moderate (16-28) and or low (0-15) risk level.

The information used in determining scores has been divided between the available information at the time of *S. destruens*' first report (2005) and the information provided by the work conducted in this thesis.

Risk Query	Score	Reasons for scoring	
Value / susceptibility of native resources		**Knowledge at first report**	**Thesis contribution**
What is the economic value of the parasite's host(s) to fisheries in England and Wales?	3	*S. destruens* has a wide host range including economically and ecologically important species (*Salmo salar* Hedrick *et al.*,(1989).	The susceptibility of *Abramis brama*, *Cyprinus carpio* and to some extent *Rutilus rutilus* has been demonstrated in Chapters 6 & 7.
What is the ecological value of the parasite's host(s) to fisheries in England and Wales?	3		
Does the parasite infect a host that is endangered or threatened within England and Wales?	0	At present, none of the parasites hosts are endangered or threatened in England and Wales.	
Colonisation potential			
Based upon climatic conditions of donor and recipient localities, what is the likelihood that environmental conditions, or those reasonably expected through global warming, would favour colonisation of the parasite?	4	Low host specificity – *S. salar*, *Oncorhynchus tshawytscha*, *S. trutta*, *O. mykiss*, *Salvelinus fontinalis* (Hedrick *et al.*, 1989 & Arkush *et al.*, 1998).	Low host specificity -*A. brama*, *C. carpio*, *Leucaspius delineatus* (Chapter 2, 4, 6 & 7). Presence of temperature tolerant life stages (spores and zoospores) and environmental persistence through prolonged release (Chapter 3) increase colonisation potential.
Based upon the life-cycle development, host specificity, distribution of intermediate hosts (if needed) and reproductive potential of the parasite, what is the likelihood of successful colonisation of freshwater fisheries?	3		
How many legal fish movements take place annually within England and Wales comprising host species of the parasite (0-10 = very low; 10-50 = low; 50-250 = medium; 250-500 = high, >500 = very high)?	4	> 500	
What is the likelihood that viable life stages of the parasite would allow dissemination in the absence of the fish host?	3	*S. destruens* can be transmitted in the absence of direct contact between infected and naïve fish (Gozlan *et al.*, 2005).	Transmission to *C. carpio* was achieved in absence of direct contact (Chapter 7). Persistence of spore and zoospore stages (Chapter 3).
Potential disease risk			
Is the parasite known to impact upon wild or farmed fish populations in other geographical regions?	Yes	Losses have been recorded from the aquaculture facilities (Harrell *et al.*, 1986 & Hedrick *et al.*, 1989).	Losses have been recorded in experimental settings with *A. brama* and *C. carpio* (Chapters 6 & 7).
Do experimental observations or pathological descriptions suggest that the parasite may be an important pathogen to fish?	Yes	Damage at host level is well studied (Arkush *et al.*, 1998).	Damage for the cyprinid *L. delineatus* was described in Chapter 2.
Does the parasite have pathogenic congeners?	2	*Amphibiocystidium ranae* has caused high mortalities in frogs (Pacolini *et al.*, 2003)	

S. destruens hazard score = 30 (HIGH)

8.7 Conclusions

This thesis has provided new important information on the life cycle and susceptibility of four cyprinid species to *S. destruens*. Novel findings include the non-temperature dependence of the spore and zoospore stage (Chapter 3) which is very important in determining the colonisation potential of *S. destruens*. A quantitative PCR was designed and optimised and allowed the quantification of *S. destruens* infection levels (Chapter 4). This work highlighted the influence of the host's reproductive state on the susceptibility to *S. destruens* (Chapter 4) as well as the influence of multiple *S. destruens* hosts on parameters such as somatic condition (Chapter 5).

The susceptibility of *A. brama* and *C. carpio* to *S. destruens* was determined, further increasing the parasites repertoire of potential hosts (Chapter 6). The susceptibility of *R. rutilus* and *S. erythrophthalmus* could not be excluded from the current results (Chapter 6). Further support for *S. destruens*' chronic mortality pattern (Chapter 6 and 7) corroborated the risk of not detecting such mortalities particularly since long term monitoring of host populations is not common. Lastly, the importance of *S. destruens* generalist nature was emphasised both in terms of its evolutionary history and its potential for persistence and ability to cause host mortalities.

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