Environmental factors impacting *Saprolegnia* infections in wild fish stocks

A thesis submitted to Cardiff University for the degree of Doctor of Philosophy (PhD) in the School of Biosciences

December 2019

Emily Matthews C1001277

Contents

List of Figures and Tables

Figures

Tables

Thesis Summary

Fish provide a primary source of protein for the rapidly expanding human population, and so it is imperative to both improve aquaculture production and protect wild fish stocks. Disease presents a major obstacle to this goal. Oomycetes of the genus *Saprolegnia* are endemic parasites of freshwater ecosystems that are responsible for staggering economic losses within aquaculture and have also been increasingly associated with wild salmonid declines across England and Wales. All 49 English rivers have previously experienced saprolegniasis, and moreover between 2010 and 2018, 11 of these experienced major epidemics (Chapter 2). These disease issues have exposed fundamental gaps in our knowledge of *Saprolegnia* epidemiology and the environmental risk factors associated with outbreaks.

For the first time, this thesis employed a landscape genomics approach to study the molecular epidemiology of *Saprolegnia parasitica* isolates collected from wild fish hosts across England and Wales (Chapter 3). All 46 isolates examined were genetically distinct and could be separated into two subpopulations. Adaptive genomic signatures of selection were detected within gene groups that likely underpin *S. parasitica* pathogenesis, including those that function in: sensing external stimuli, host invasion/colonisation, infective life stages and nutrient acquisition. Furthermore, genomic adaptation was linked with environmental temperatures.

The true extent of *S. parasitica* generalism was also investigated here (Chapter 4); among the four *S. parasitica* isolates examined, there was evidence of local host adaptation. An isolate displayed a preference for sea trout during *in vitro* induced zoospore encystment assays and also caused more sea trout than salmon mortalities in the first 48h of an experimental challenge infection. The influence of the abiotic factors, temperature and water flow, on *Saprolegnia* epidemics within English rivers and biological processes that contribute to transmission were assessed in Chapter 5. Records of disease outbreaks in 3 major English rivers between 2010 and 2018 could be linked with mean spring flow rates; lower flow rates in particular were found in years with increased levels of *Saprolegnia.* Water movement was also found to stimulate *S. parasitica* sporulation in a temperature dependent manner and low flows increased salmon (*Salmo salar)* susceptibility to saprolegniasis. Moreover, increased

temperatures were found to generally increase *Saprolegnia* spp. vegetative growth rates and decrease *S. parasitica* zoospore viability.

Lastly, this thesis examined the individual and combined effects of *S. parasitica* and various thermal regimes on the mortality of brown trout (*Salmo trutta)* alevins and fry (Chapter 6). Fish mortality only increased when warmer water temperatures and subsequent rapid temperature increases representative of a combination of climate change and thermopeaking were experienced at the time of *S. parasitica* exposure.

While future work is needed to predict the potential impact of climate change on this parasite, collectively, these results improve our understanding of *S. parasitica* adaptation to external pressures and provide insight into disease outbreaks. This data will ultimately aid the reduction of *Saprolegnia* infections in aquaculture and improve the protection of wild fish populations.

Collaborators

This PhD was funded by KESS 2 (Knowledge Economy Skills Scholarships) and the Environment Agency who partnered this project. Therefore key project aims, i.e. *Saprolegnia* sample collection and the subsequent landscape genomics investigation (Chapter 3) were outlined in the original project proposal conceived by my Cardiff University supervisors Jo Cable and Amy Ellison.

Chapter 2: EM led the writing of this review with input from all co-authors, Chris Williams, Joanna James and Jo Cable. *Saprolegnia* outbreak data in English rivers were provided by Joanna James.

Chapter 3: *Saprolegnia* sample collection was performed by area officers of the Environment Agency across England and Wales. Sample preparation for genomic sequencing was performed by EM with assistance from Angela Marchbank of the Cardiff University Genomics Hub. Data analysis was performed by EM with guidance from Amy Ellison and Pablo Orozco-terWengel. The chapter was written by EM with suggestions from Jo Cable and Amy Ellison.

Chapter 4: All experimental procedures, data analysis and writing were conducted by EM with suggestions from Jo Cable and Amy Ellison.

Chapter 5: The majority of experimental procedures were performed by EM with data collection for *Saprolegnia in vitro* growth rate aided by final year project students Victoria Thorne and Prema-Mohini Patel, and summer placement students Samuel Hillman and Dean Tulett. Analysis of environmental factors and *Saprolegnia* outbreaks in English rivers was conducted by Jo James. All other data analysis was performed by EM with writing suggestions from Jo Cable and Amy Ellison.

Chapter 6: Data collection for brown trout alevin growth and mortality at 4 different thermal regimes was performed by Roser Casas-Mulet and her final year project student Jessica Whitney. Subsequent *Saprolegnia parasitica* infections were conducted by EM. All data analysis and writing was performed by EM with additional writing suggestions provided by Jo Cable and Roser Casas-Mulet.

Acknowledgements

Firstly, I would like to thank my supervisors Jo Cable and Amy Ellison for the opportunity to undertake this PhD. Without their wisdom and encouragement over the past four years, this thesis would not have been possible. Also, special thanks to Pablo Orozco-terWengel for his boundless genetics knowledge; although our whiteboard sessions terrified me, they were very helpful!

To my collaborators Chris Williams, Joanna James and Amy Reading at the Environment Agency, you have been a fundamental part of this research. Thank you for rallying your excellent area officers and providing us with essential samples. Also, thank you for making me feel like one of the gang during my visits to Brampton.

Thank you to the bunch of misfits that are my research group; Numair Masud, Rhi Hunt, Alice Jones, Elissavet Arapi, Oluwaseun "Sam" Somoye, Mike Reynolds, Willow Smallbone and Rhidian Thomas for keeping me sane with gossip and laughter.

To the best people I know, my wonderful parents Keith and Carol, thank you for everything. I'm beyond grateful for your constant love and support (emotional and financial).

Lastly, to my loving partner and best friend Dave, thank you for being my biggest champion. I owe you a holiday… or ten.

Chapter 1 - General Introduction

Aquaculture is the world's fastest growing food industry with global fish production increasing from 3 million tonnes in 1970 to 80.1 million tonnes in 2017 (FAO 2019). The industry now accounts for 50% of the world's fish produced for human consumption (FAO 2019) and with the global population expected to reach 9 billion by 2050, this sector is under tremendous pressure to increase production and reduce waste (FAO 2013). To achieve high production yields, intensive farming practices are routinely employed, exposing fish to numerous stressors including high stocking densities and poor water quality. Such adverse conditions can not only impede the growth and quality of the fish produced, but also greatly promote the spread of infectious diseases. Indeed, disease is the key factor restricting aquaculture prosperity, with the major perpetrators: sea lice, bacteria, viruses, fungi and oomycetes, responsible for huge economic losses. Notably, mycotic infections are second only to bacterial diseases in terms of their economic impact (Ramaiah 2006; van West 2006; Bruno et al. 2011).

Oomycetes are fungal-like pathogens that cause infections in both terrestrial and aquatic plants and animals (Phillips et al. 2008). The lesser studied aquatic oomycetes of the genera *Saprolegnia* and *Aphanomyces* are responsible for substantial mortalities within aquaculture; *Saprolegnia parasitica* in particular is responsible for at least 10% of farmed salmonid deaths (Robertson et al. 2009). In addition to its impact on the fish farming industry, *S. parasitica* is endemic within freshwater ecosystems and has been implicated in worldwide declines of wild salmon (reviewed in Chapter 2). This parasite causes the disease saprolegniasis which presents as patches of white cotton-like mycelia on the fish body, predominantly on the head and fins during the early stages of infection (Figure 1.1). These mycelia largely act superficially via invasion of the epidermal tissues but can also penetrate muscle and blood vessels. Fish with severe infections often exhibit lethargic behaviour, respiratory distress and a loss of equilibrium (Bruno et al. 2013). Hosts commonly die as a result of impaired osmoregulation (Willoughby and Pickering 1977; van West 2006). Respiratory failure can also occur if the gills experience extensive mycelial coverage and in rarer cases organ failure has been reported (Pickering and Willoughby 1982).

Figure 1.1. Patches of Saprolegnia *mycelia on the head (B) and fins (A and C) of wild English salmon.*

Saprolegnia **lifecycle**

The *Saprolegnia* lifecycle is complex, involving both asexual and sexual stages (Figure 1.2). The asexual stage begins with the production of primary zoospores (10-20 µm in diameter) within hyphal tips known as sporangia. A decrease in available nutrients or sudden drop in environmental temperature are two factors known to trigger zoospore formation (Bly et al. 1992). Upon release, these primary zoospores swim by means of a tinsellated and whiplash flagellum (Figure 1.2). The tinsellated flagellum has numerous lateral filaments perpendicular to the main axis which create a large surface area, allowing it to steer the zoospore in a given direction. In contrast, the whiplash flagellum, which does not possess lateral filaments, functions to propel the zoospore. While primary zoospores can establish host infections, they are only able to swim for a short period before they encyst, offering a minimal level of independent dispersal. Primary cysts can subsequently release secondary zoospores (also 10-20 µm in diameter), which can swim for longer periods than their primary precursors and are considered the main infective unit of *Saprolegnia* (see Hatai and Hoshiai 1994; Willoughby 1994). For many *Saprolegnia* spp. secondary zoospores can undergo repeated cycles of zoospore encystment and release, a process known as Repeated Zoospore Emergence (RZE) (Beakes 1983; Diéguez-Uribeondo et al. 1994). *S. parasitica* in particular can achieve up to six consecutive rounds of RZE (Diéguez-Uribeondo et al. 1994). This process allows secondary zoospores several attempts to locate a host and establish an infection. Secondary cysts of *S. parasitica* also possess a set of hairs or boathooks which are thought to aid in the attachment to passing fish (Willoughby and Pickering 1977; Willoughby et al. 1983). Upon locating a host, cysts

can germinate and form new mycelial mats (van West 2006). The sexual stage of the lifecycle is employed to enhance survival during adverse environmental conditions (Beakes and Bartinicki-Garcia 1989). This stage begins with the formation of the male and female reproductive structures the antheridium and oogonium respectively, which subsequently fuse to produce a thick-walled oospore. Oospores (~15-30 µm in diameter) can withstand acutely stressful conditions including temperature extremes and defer germination until conditions become more favourable. However, certain *Saprolegnia* spp., including *S. parasitica*, do not appear to undergo sexual reproduction as they do not produce oospores, at least not under laboratory conditions (Coker 1923; van den Berg et al. 2013).

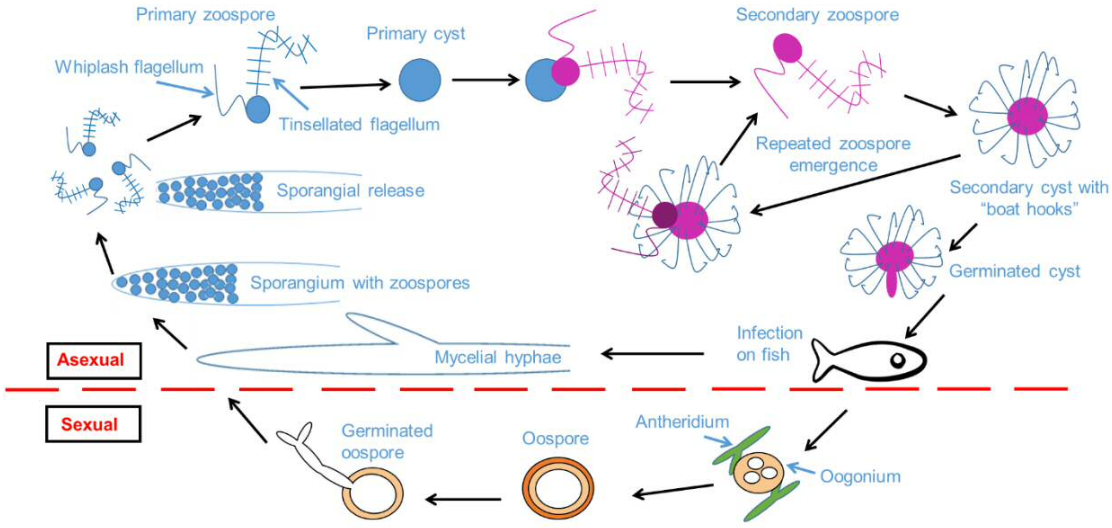

Figure 1.2. Schematic lifecycle of Saprolegnia *spp. (Adapted from van West 2006).*

Fish immune response to *Saprolegnia parasitica* infection

Upon infection with *S. parasitica*, fish upregulate the expression of several acute phase proteins together with the immunity-related ATP-binding cassette transporter TAP (vital to the MHC class I antigen presenting system), and the cytokine receptors CXCR4 and CD63 that are involved in humoral immunity and immune cell development/growth respectively (Roberge et al. 2007). The epidermal damage caused during host invasion triggers macrophage recruitment; Kales et al. (2007) showed macrophages are heavily involved in the initial immune response to *S. parasitica* and are able to target and destroy zoospores and mycelia. Typically this parasite elicits a strong host inflammatory response via induction of the proinflammatory cyto- and chemokines interleukin-1β1 (IL-1β1), IL-6, and tumour

necrosis factor alpha (TNF-α) alongside activation of innate cellular components such as cyclooxygenase-2 (COX-2), antimicrobial peptides and lectins (de Bruijn et al. 2012; Belmonte et al. 2014).

S. parasitica is also capable of host immunomodulation through downregulation of adaptive immune constituents including T-helper cell cytokines, antigen presentation machinery and immunoglobulins (Belmonte et al. 2014). Furthermore, *S. parasitica* produces the metabolite prostaglandin E_2 which suppresses the expression of interferon-γ (IFN-γ) and IFN-γ-inducible protein (γ-IP), key players in cellular immunity (Belmonte et al. 2014).

Saprolegniasis treatment

Effective control of saprolegniasis is one of the major challenges in aquaculture. The disease was previously kept under control by the organic dye malachite green, however its use in the production of fish for human consumption was banned worldwide in 2002 due its carcinogenic and toxicological effects (van West 2006). Consequently, *Saprolegnia* outbreaks have significantly increased in the last decade, posing a serious threat to food security (van West 2006; Phillips et al. 2008). A wide range of chemicals have been investigated as anti-*Saprolegnia* agents including formalin and formaldehyde formulations (Gieseker et al. 2006), sodium chloride (Ali 2009), boric acid (Ali et al. 2014), clotrimazole (Warrilow et al. 2014), ozone (Forneris et al. 2003), bronopol (Oono and Hatai 2007) and hydrogen peroxide (Barnes et al. 1998). However, none of these potential treatments provide sufficient control of this pathogenic oomycete and alternative strategies are urgently required.

Molecular tools have greatly expanded our understanding of *Saprolegnia* pathogenesis and are essential for developing an effective control agent. A subtilisin-like serine protease (SpSsp1) produced by *S. parasitica* has been shown to trigger an antibody response in rainbow trout (*Oncorhynchus mykiss*), moreover, trout that produced these antibodies could not be subsequently infected (Minor et al. 2014). These results indicate that the immunological recognition of SpSsp1 confers some resistance to this parasite and highlights the potential role of SpSsp1 in the development of a vaccine against *Saprolegnia* (see Minor et al. 2014).

PhD thesis aims

This thesis investigates the epidemiology of *S. parasitica*. Historic records of outbreaks within wild English and Welsh fish populations have been examined alongside a review of current surveillance and management practices (Chapter 2). Furthermore, a range of *Saprolegnia* spp. isolates have been collected from these wild fish populations to determine species diversity in this region; the collected *S. parasitica* isolates have been included in a landscape genomics study, examining whether environmental pressures have resulted in adaptive genetic variation between geographically distributed isolates (Chapter 3). The major host and environmental factors influencing *S. parasitica* outbreaks have been investigated, specifically; intra-specific differences in *S. parasitica* host-specificity (Chapter 4), the impact of temperature and water flow on biological functions involved in disease transmission/progression including; *in vitro* growth rate, zoospore viability and sporulation, and host susceptibility to infection (Chapter 5). Lastly, this thesis examines the combined effects of *S. parasitica* exposure and thermal regimes reflective of climate change and thermopeaking on early life stages of important fish species (Chapter 6). Collectively, this research will improve our understanding of *S. parasitica* epidemics and potentially lead to improved disease control within aquaculture and the wild.

Chapter 2 - *Saprolegnia* infections in wild salmonids: an increasing challenge for fisheries in England

Abstract

Members of the genus *Saprolegnia* are freshwater oomycetes that cause the disease saprolegniasis. While this disease is responsible for devastating losses of cultured fish, in the wild, *Saprolegnia* has long been considered a ubiquitous pathogen, responsible for natural losses of migratory salmonids. However, since 2010 the Environment Agency in England has received increased reports of this parasite. Of the 49 key salmonid rivers in England, 11 have experienced elevated levels of *Saprolegnia* between 2010 and 2018, although the severity of these incidences varied between rivers and years. These events have highlighted major gaps in our understanding of the diversity and pathogenicity of *Saprolegnia* in the UK and the role environmental factors might play in driving saprolegniasis outbreaks, hindering future predictions with regards to climate change. Here we summarise *Saprolegnia* diversity and molecular methods of *Saprolegnia* species identification, assess *Saprolegnia* surveillance and fish management approaches, and examine recent outbreaks in English rivers.

Introduction

Saprolegnia spp. are oomycete pathogens, ubiquitous in freshwater environments globally. Members of this genus cause the disease saprolegniasis, which is spread by the production of infective zoospores during asexual reproduction. Saprolegniasis is characterised by patches of pale, cotton-wool like mycelial growth (the appearance of 'fluffy fish') on the skin, fins and gills of infected fish (Figure 2.1). The mycelia subsequently invade the epidermis and underlying dermis, leading to disruption of osmoregulation and potential death via haemodilution (Richards and Pickering 1979; Shah 2010). *Saprolegnia* spp. were originally considered saprotrophic and necrotrophic opportunistic pathogens, only able to colonise immunocompromised or deceased hosts (Bruno and Wood 1999). However, it has become apparent that some isolates of *Saprolegnia parasitica* in particular, are extremely virulent and act as primary invaders (Neish 1977; Willoughby and Pickering 1977; Yuasa and Hatai 1995; Whisler 1996). The importance of this pathogen within the aquaculture industry

is reflected by a staggering 1 in 10 of all farmed raised salmon succumbing to saprolegniasis (van West 2006). Furthermore, *Saprolegnia* has been associated with increased mortalities in wild salmonid populations, which will form the focus of the current review chapter.

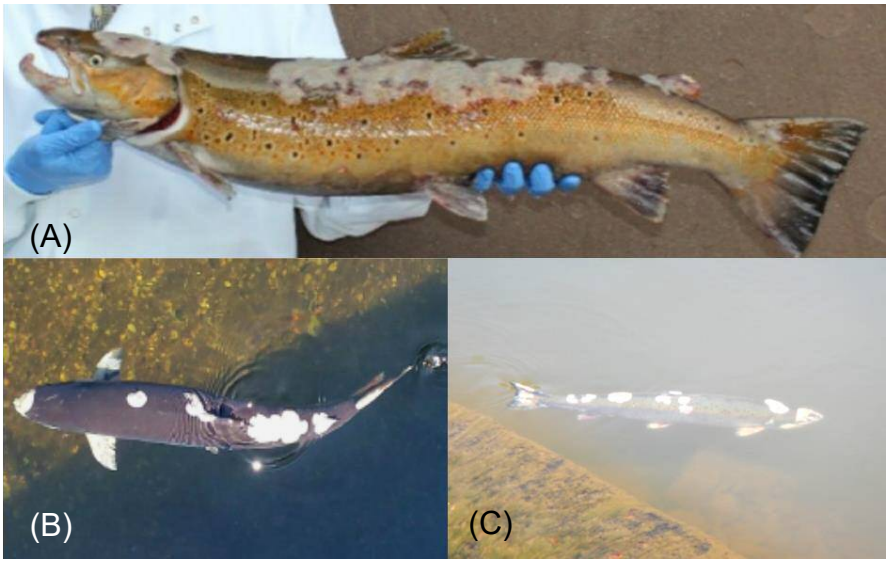

Figure 2.1. (A), (B) and (C) Visible signs of saprolegniasis on wild fish.

Saprolegnia-related losses of wild salmonids as a consequence of migration, marine survival, reproduction, physiological stress and barriers to migration are common, however historic records of this parasite have been complicated by the concomitant emergence of the cryptic disease Ulcerative Dermal Necrosis (UDN). This disease is characterised by pale lesions on and around the head of salmon that are frequently confused with, or later infected with, the mycelial growths of *Saprolegnia*. Consequently, UDN is often mis-reported by fishery owners/anglers and has overshadowed our understanding of the diversity and characterisation of *Saprolegnia* in wild aquatic environments.

The detection and management of *Saprolegnia* infections in wild migratory salmonids raises a number of challenges. First, accurate surveillance of infections in wild transient salmonid populations makes the assessment of prevalence difficult, particularly when the size of the overall population is unknown and can change over relatively short timescales. Second, there is no standardised approach for the consistent reporting of *Saprolegnia* severity in either farmed or wild salmonids. Third, despite extensive literature on *Saprolegnia* infections in both wild and farmed fish populations, there remains a poor understanding of the environmental drivers

associated with infections, which hinders our ability to predict future disease risk in relation to climate change and anthropogenic influences. Finally, there remains the challenge for fishery managers and policy makers of what can actually be done to help mitigate disease and protect wild salmon stocks from what is a natural and ubiquitous aquatic pathogen.

Here, we review but also quantify our current understanding of the recent outbreaks of saprolegniasis in wild salmonids from English rivers. Specifically, we outline: a) *Saprolegnia* diversity; b) methods for *Saprolegnia* species identification; c) historic records of *Saprolegnia* in the UK and confusion with UDN; d) *Saprolegnia* surveillance and fishery management; and e) recent outbreaks and potential drivers (pathogen/host/environmental) of disease.

Saprolegnia **diversity**

Oomycetes are a class of eukaryotic microorganisms that cause disease in a wide range of host species including plants, algae, protists, arthropods and vertebrates (Kamoun 2003; Phillips et al. 2008). They form part of the Kingdom Stramenopila together with diatoms and brown algae and possess some fungal-like characteristics including production of filamentous hyphae. The majority of animal pathogenic oomycetes are grouped within the Saprolegniomycetidae subclass, which is split into two orders: Saprolegniales and Leptomitales. Within the Saprolegniales there are three main genera, *Saprolegnia, Achlya* and *Aphanomyces*. While most oomycetes are saprophytic, several parasitic species cause major economic and environmental damage. Terrestrial oomycetes have been more intensively studied compared to their aquatic equivalents; the most notable example being *Phytophtora infestans*, the oomycete responsible for the potato blight that devastated Ireland in the 1800s (van West and Vleehouwers 2004). Of the aquatic oomycetes, *Aphanomyces astaci*, the causative agent of crayfish plague, has caused mass mortalities of native European crayfish populations for decades (Alderman 1996; Holdich 2003), while members of the genus *Saprolegnia* have detrimentally affected populations of fish, amphibians, and their respective eggs (Kiesecker et al. 2001; Pounds 2001). Despite such an overwhelming impact on a wide range of aquatic organisms, very little is known about differences in virulence both within and between *Saprolegnia* species. Although, laboratory-based challenge experiments of rainbow trout (*Oncorhynchus mykiss*) with 24 isolates (15 *S. parasitica* and 9 *S. diclina*) confirmed that *S. parasitica* isolates

caused higher mortality than *S. diclina,* both species exhibited marked differences in virulence at an isolate level (Yuasa and Hatai 1995). Meanwhile, our knowledge of the diversity and distribution of *Saprolegnia* in UK Rivers is unexplored. A handful of isolates could be responsible for the majority of disease. Alternatively, a multitude of isolates could exist within a single river, each exhibiting varying levels of pathogenicity.

Saprolegnia species identification

Within the *Saprolegnia* genus, the total number of species is unclear due to taxonomic problems. The first comprehensive descriptions of saprolegniales presented by de Bari (1852) and Coker (1923) described the 'water mould' *Saprolegnia* as a fungus. Historically, species identification relied on morphological characteristics of sexual structures (oospores, antheridium and oogonia), defining between 19 and 24 species (Coker 1923; Seymour 1970; Johnson et al. 2002). This, however, proved problematic as several species possessed similar morphological traits and the production of sexual structures can be difficult to achieve *in vitro* (Coker 1923; van den Berg et al. 2013). Furthermore, as several species within this genus were described solely on morphological data, this resulted in species synonyms, with identical taxonomic descriptions for separate species, for example: *S. salmonis* and *S. parasitica*, *S. longicaulis* and *S. ferax, S. multispora* and *S. australis,* and *S. bulbosa* and *S. oliviae* (see Diéguez-Uribeondo et al. 2007). The reliance on morphological criteria particularly complicated the identification of fish-pathogenic isolates. Based on the original species descriptions by Coker (1923) cultures were designated as *S. parasitica* if they were obtained from fish hosts and failed to produce sexual structures *in vitro.* This resulted in many isolates being mis-identified as *S. parasitica* simply due to their establishment on fish hosts (Beakes and Ford 1983; Diéguez-Uribeondo et al. 2007). Also, there has been a long-standing debate as to whether *S. parasitica* should comprise part of a species complex alongside the egg-pathogen *S. diclina* (see Seymour 1970; Neish 1976; Willoughby 1978; Johnson et al. 2002). Neish (1976) proposed that the morphological criteria for this complex should include oogonia that possess diclinous antheridial branches alongside thin, unpitted oogonial walls. However, prospective new species of *Saprolegnia* have been consistent with this ambiguous criterion so it is still unclear which species should be included within the

proposed species complex (Hussein and Hatai 1999; Johnson et al. 2002; Paul and Steciow 2004).

The delineation of fish-pathogenic *Saprolegnia* isolates was further complicated by Willoughby (1978) who performed a detailed analysis of cultures obtained from infected fish and the environment. After examining oogonial morphology and the optimum temperature for oogonium formation, Willoughby (1978) proposed 3 sub-groups of *S. diclina*, Types 1, 2 and 3. Type 1 isolates were obtained from lacerations on salmonids and were generally compliant with Coker's original description of *S. parasitica*. A small number of isolates derived from coarse fish were designated as *S. diclina* Type 2, while characteristic saprobiotic isolates were assigned to Type 3 (Willoughby 1978). Consequently, different studies have embraced varying taxonomic criteria for identifying fish-pathogenic *Saprolegnia* isolates, resulting in a perplexing body of literature.

DNA-based methods are now recognised as essential for *Saprolegnia* species discrimination, and the availability of sequencing technology has shed new light on oomycete taxonomy. The most common method for *Saprolegnia* species identification is sequencing of the internal transcribed spacer (ITS) region. This is a highly conserved region located between the 18S and 28S nuclear ribosomal DNA (nrDNA) and includes the 5.8S gene, approximately 700 bp in length in *Saprolegnia* spp. (see Diéguez-Uribeondo et al. 2007). The high level of sequence variability within this region makes it suitable for species identification, at least within the genus. One of the first large scale molecular phylogenetic studies of the proposed *S. diclina*-*S. parasitica* complex examined 128 isolates from diverse geographical locations and hosts using ITS sequence, morphological (cyst ornamentation) and physiological data (retracted germination and ability to undergo repeated zoospore emergence) (Diéguez-Uribeondo et al. 2007). This supported the separation of *S. parasitica* and *S. diclina* as distinct taxa and resulted in the identification of 5 phylogenetically distinct clades (Diéguez-Uribeondo et al. 2007). More recently, molecular operational taxonomic units (MOTUs as suggested by Blaxter et al. 2005) have been proposed to resolve the taxonomic issues of this genus. Analysis of 961 *Saprolegnia* ITS sequences (461 from culture collections and 500 from Genbank) identified 29 DNA-based MOTUs; 18 of which coincided with previously described species, while the remaining 11 either represented new *Saprolegnia* species or previously described species that lacked ITS

sequence data (Sandoval-Sierra et al. 2014). This method of species identification is both rapid and repeatable and permits taxonomic clarity within this genus.

While identification to the species level via ITS sequencing has proved successful for the *Saprolegnia* genus, very few studies have attempted to genotype individual isolates of a species. In 2018, a multi-locus sequence typing scheme was developed using seven housekeeping genes of *S. parasitica* in an attempt to genotype 77 isolates collected from wild fish across Switzerland and France (Ravasi et al. 2018). The study uncovered ten distinct genotypes; two having a wide distribution across the geographic area investigated, while the remaining eight were exclusive to a specific region or river (Ravasi et al. 2018). Similar molecular epidemiology studies need to be performed for UK Rivers in order to assess the diversity and prevalence of *S. parasitica* isolates across our landscape.

Historic records of *Saprolegnia* in the UK and confusion with Ulcerative Dermal Necrosis

The first case of saprolegniasis in the UK was reported from roach (*Rutilus rutilus*) in 1748 (Hughes 1994). It was not, however, until the late 19th century that this disease, then referred to as the "salmon disease" was associated with losses of wild salmon (Hughes 1994). In 1879, observations of large numbers of diseased salmon in rivers across England and Scotland prompted the formation of the Salmon Disease Commission (Hughes 1994). This was the first organised body created to manage losses of fish from a disease. In 1880, the commission published a report of their investigation into the "salmon disease" epizootic occurring in British rivers, concluding that the 'fungus' involved in the losses was likely to be *S. ferax* (Buckland et al. 1880; Hughes 1994). However, the concomitant emergence of the disease now known as Ulcerative Dermal Necrosis (UDN) made it difficult to determine whether these losses were in fact attributable to *S. ferax* (see Roberts 1993).

UDN is a chronic dermatological condition of unknown aetiology that gained most attention in the 1960s and 1970s with outbreaks reported across the UK and subsequently in parts of North-West Europe (Munro 1970; Roberts 2012). Only diagnosable by histopathology; the disease manifests as rounded lesions occurring predominantly on the head of affected fish (Bruno et al. 2011). In early stages, lesions are patches of focal necrosis characterised by degeneration of the malpighian cells of the epidermis (Roberts 2012). As the lesion develops, layers of the epidermis begin to

slough off providing a route for secondary pathogens, such as *Saprolegnia* (see Roberts 2012). This complicates UDN diagnosis as *Saprolegnia* spp. also colonise the host epidermis and cause cellular changes. Hence early lesions of live hosts are necessary to mitigate *Saprolegnia* interference and confirm UDN cases.

Several studies have attempted to uncover the aetiological agent behind UDN; Jensen (1965) suggested that the symptoms resembled that of the myxobacterial columnaris disease. White (1965) endeavoured to substantiate Jensen's observations and although able to cultivate a range of bacteria from affected fish, found no evidence of myxobacteria. Further bacteriological research ultimately refuted the involvement of a myxobacterium and found no evidence that bacteria act as more than a secondary contaminant in the condition (Carbery and Strickland 1968; Ordal 1968). The possibility of a viral agent was also investigated, however efforts to isolate a virus from affected fish via tissue culture, ultracentrifugation and electron microscopy were unsuccessful (Roberts 1993). Further studies have concluded that there is a premycotic phase during UDN infections, hence fungal/oomycete pathogens such as *Saprolegnia* are not the underlying cause of the disease (Carbery 1968; Stuart and Fuller 1968; Willoughby 1969).

The uncertain aetiology of UDN has heightened interest in this condition as a mysterious and cryptic cause for mortality, elevating concern far above that of *Saprolegnia*. In 2012, an 'outbreak' of 'UDN' was reported in the media following losses of salmon in the River Spey, Scotland (*BBC News* 2012). This was based on observations from a single fish and came at a time where elevated mortalities attributed to *Saprolegnia* infections were being observed in rivers across the UK, but these incidents went largely unreported by the media. Notwithstanding, UDN continues to be reported frequently by anglers and there remains some political pressure to investigate reports of UDN in wild salmonids due to the history of the disease and cryptic diagnosis. This raises a number of challenges, primarily the lack of clear and robust diagnostic characteristics and the need to sacrifice fish to obtain the necessary tissue samples for histological assessments. As UDN is considered a natural disease of adult salmon entering freshwater and lesions can heal without any long-term implications on fish health, it may be questioned why fish are being removed from the spawning population to confirm this condition, particularly when it is quite feasible that the cellular changes within the skin are not seen.

In 2013, elevated losses of salmon on the River Dart, Devon, prompted investigations into the cause of mortality. Due to ongoing reports of UDN from anglers, samples of salmon with suspected UDN lesions were submitted to the Environment Agency National Fisheries Laboratory for post-mortem examination. Images of salmon exhibiting suspected UDN lesions were taken to enable visual assessment. Despite the absence of secondary infections in any of these cases, and detailed histological assessments carried out of the skin, no cases of UDN were confirmed. Subsequent investigations of mortalities in these rivers, which involved broad spectrum viral and bacterial screening and examinations for parasites and histopathology, confirmed *Saprolegnia* as the predominant cause for the observed losses (see Figure 2.2).

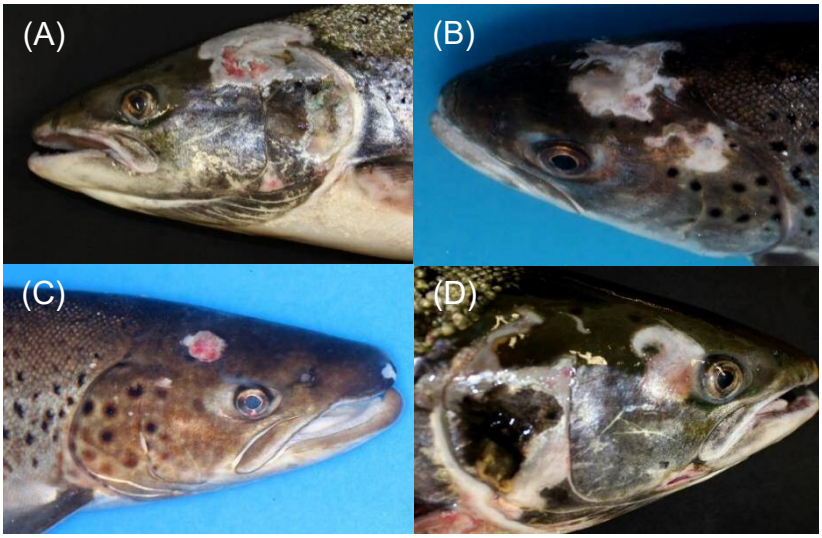

Figure 2.2. Head lesions of wild Atlantic salmon (A), (C), (D); and sea trout (B) from rivers in England showing gross pathological changes of Saprolegnia *infections that are often misreported as Ulcerative Dermal Necrosis (UDN).*

Saprolegnia **surveillance and management in wild fish populations**

In the context of aquatic animal disease, surveillance is defined as the ongoing collection, validation, interpretation and dissemination of data with which to inform stakeholders and implement measures to reduce or mitigate a disease (Oidtmann et al. 2013). It is fundamental in enabling early detection, communication to interested parties and management measures to either restrict pathogen spread or protect susceptible host populations. Disease surveillance of wild fish populations, however, represents a complex challenge due to difficulties with capture and scrutiny of the population. This is exacerbated with migratory species where observations of fish are transient and restricted by access to different reaches of the river and ability to observe

infected fish. As such, monitoring populations, reliably assessing thresholds of infection above which action should be taken and then managing such outbreaks becomes a significant challenge. Whilst greatest attention has been given to surveillance of notifiable diseases in farmed populations, surveillance of disease in wild animals is becoming increasingly important, particularly in the case of new and emerging diseases and for pathogens that can have significant effects on wildlife ecology (Mörner et al. 2002). According to Häsler et al. (2011) and later Oidtmann et al. (2013), the use of surveillance for mitigating disease has three phases: 1) 'sustainment' where the objective is to sustain a low prevalence and ensure this has not been exceeded; 2) 'investigation' where surveillance is used to obtain epidemiological information to inform decisions or interventions and; 3) lastly, 'implementation' to action measures to reduce the occurrence of a disease or pathogen.

Saprolegnia infections often generate a binary response that greatly influences the likelihood of reporting. Light infections will often go undetected, ignored or considered inconsequential based on the location of infection or size of the host. Conversely, heavy infections of *Saprolegnia* can prompt considerable concern due to the unpleasant appearance of infected fish and associated mortality. Hence, the need for standardised reporting, defined descriptions of disease severity and clear avenues for reporting to relevant authorities.

Current approaches to surveillance of Saprolegnia *in England*

In response to increased reports of wild salmonid mortalities in some rivers, a standardised responding system has been developed by the Environment Agency, which aims to provide a consistent approach for the monitoring of *Saprolegnia* infections in the 49 main salmon rivers in England. Variation may exist due to river size or the number of salmonids present at any time, but the following thresholds enable an approximate assessment of severity and a means to compare the status of different rivers. If passive surveillance conducted by anglers, stakeholder groups and members of the public yields <5 reports of *Saprolegnia*-infected fish in a month, that river has experienced "normal" levels of the oomycete. If reports of 6-20 infected fish occur, the disease status of the river changes to "elevated". At this point, more active surveillance may be employed; with Environment Agency staff walking stretches of the affected river and scoring the number of visibly infected and uninfected fish. A river is designated "potentially problematic" when the number of diseased fish

exceeds 20 in a month; at this stage more frequent active surveillance is implemented. A diagnostic investigation may be conducted, along with discussions for mitigation methods (see Figure 2.3).

Whilst this framework provides a useful standardised methodology for *Saprolegnia* surveillance there are several limitations, namely the risk of missing and/or duplicating animals, inability to access remote stretches of the river and dependency on light conditions. Bank side surveillance is labour intensive, time consuming, and therefore costly. Moreover, a major caveat is that natural levels of *Saprolegnia* infection vary between rivers i.e. what may be "elevated" for one river may be "normal" for another, hence it is important to consult area officers with local knowledge of a river. The timing of reporting is also an important consideration given that levels of infection are always higher post-spawning season. Overall, infection reports must be treated on a case specific basis.

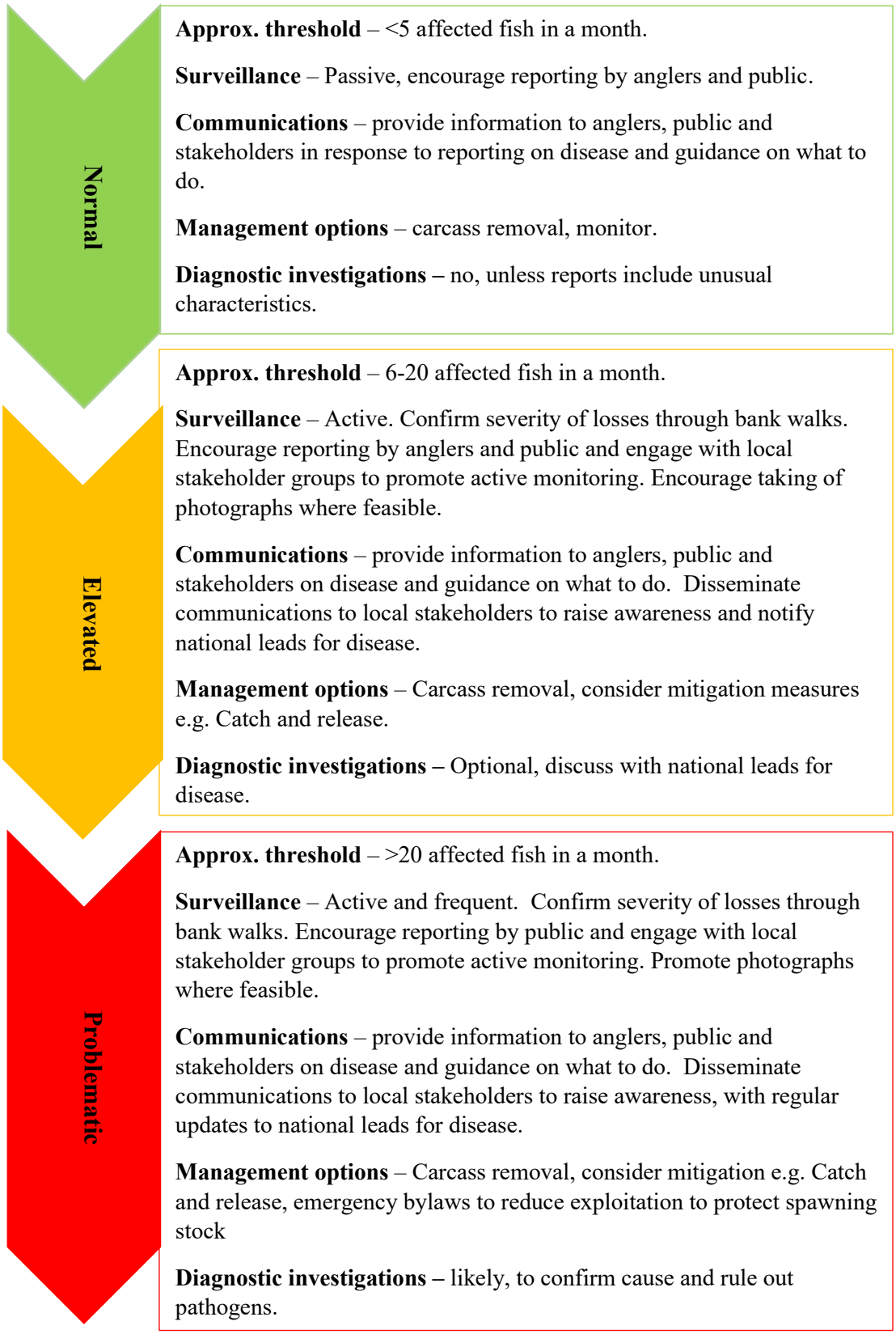

Figure 2.3. Standardised responding procedure for suspected Saprolegnia *infections employed by the Environment Agency.*

Future surveillance of wild salmonid stocks, including assessment of *Saprolegnia* presence, could be conducted through the use of unmanned aerial vehicles

(UAVs). UAVs are increasingly used for biological conservation and ecological research, facilitating collection of real time, high resolution spatial data (Fornace et al. 2014). UAVs are already being used to monitor populations of large, threatened species (Koh and Wich 2012; Hodgson et al. 2013) and their potential use in disease surveillance is being explored. In Thailand, UAVs are being used to successfully distinguish infected from uninfected plants in coffee plantations affected by *Hemileia vastatrix*, the causative agent of coffee rust (Dunning 2019). As the white mycelial patches caused by *Saprolegnia* infections are easily visible, it might be feasible to detect infected animals using UAVs. These are relatively inexpensive and may have several long-term benefits including improved accuracy, reduced man power and increased ability to access more remote locations. As the use of UAVs for biodiversity monitoring is, however, still in the relatively early stages, several challenges are likely to be encountered when trialling these for *Saprolegnia* surveillance, including: achieving suitable spatial resolution, weather restrictions and flight permissions (Fornace et al. 2014).

In addition to visual methods, molecular techniques testing for environmental DNA (eDNA) are being used increasingly for species surveillance. eDNA refers to any DNA that can be extracted from environmental samples such as water, soil or sediments (Rees et al. 2014). Such methods are un-intrusive, sensitive and cost-effective (Rees et al. 2014). For oomycetes, eDNA detection also circumvents the need for culturing which can be challenging, time consuming and species specific (Rocchi et al. 2017). eDNA has already been successfully used to detect and monitor the oomycete *Aphanomyces astaci* which causes crayfish plague (Strand et al. 2014; Robinson et al. 2018; Wittwer et al. 2018; Wittwer et al. 2019). Furthermore, a qPCR assay for detecting *Saprolegnia parasitica* in water samples has been developed (Rocchi et al. 2017), although not yet widely tested.

Management measures to protect wild salmon stocks

Importance of salmon, economy and protection

Atlantic salmon (*Salmo salar*) is one of the most economically valuable freshwater fish species in the UK, supporting both commercial net fisheries and generating an estimated £100 million revenue from angling (EA personal communication). As an iconic fish species, salmon also have considerable societal value and they are a key

indicator species of good environmental quality (Helfman 2007). However, wild salmon stocks have declined significantly across the North Atlantic, with many river populations now threatened or at risk (Parrish et al. 2011). This has increased scrutiny on the number and health of salmon returning to our rivers and placed growing pressure on authorities responsible for the management of these fisheries.

Management of salmon stocks and health

The management of disease in wild fish populations represents a considerable challenge (Bakke and Harris 1998), particularly when the pathogen is widespread and the host migratory and ecologically under threat. Efforts to manage the recovery of wild salmon stocks have focussed on five generic objectives: 1) improving marine survival; 2) reducing exploitation; 3) removing barriers to migration and improving habitat; 4) safeguarding sufficient flows; and 5) maximising spawning success through improved water quality (EA 2018). Whilst these measures do not specifically reference disease, they serve to promote health and survival, and include activities to reduce anthropogenic and environmental stressors that can influence susceptibility to disease and act as important precursors for *Saprolegnia* infection (Anderson 1990; Bruno and Wood 1999; Miller et al. 2014)

Efforts to promote the recovery of wild salmon stocks in England and Wales are also being delivered through a number of regulatory frameworks. These include the EU Water Framework Directive and the EU Habitats Directive 92/43/EEC, the latter placing requirements on managers to maintain river habitats in a favourable condition for Atlantic salmon (ICES 2018). Additional measures in the form of national bylaws have been imposed to limit exploitation and promote salmon survival in rivers where salmon stocks are deemed to be 'at risk'. These include closure of commercial net fisheries, specific protection of larger spring salmon and mandatory catch and release by anglers.

Catch and release is an effective management tool for the protection of migratory salmonids, particularly where stocks are threatened (Thorstad et al. 2003). It also serves to reduce handling, stress and mechanical damage, which are known to increase susceptibility and are recognized precursors for *Saprolegnia* spp. infection (Bruno and Wood 1999). Current standards for catch and release are well embedded within the UK angling sector to ensure fish are retained in the water when unhooked, handled as little as possible and supported during recovery and release (EA 2017).

This also provides a short window for fish to be visually inspected for disease and anglers are encouraged to report any signs of fish with disease to the relevant authorities across the UK (Atlantic Salmon Trust 2019).

Recent status of *Saprolegnia* in the UK

A survey conducted by the Environment Agency revealed that *Saprolegnia* spp. have historically been reported in all 49 main salmonid rivers in the England. Since 2010, when the EA started receiving increased reports of *Saprolegnia,* 11 of these rivers have been identified as having "elevated" or "problematic" levels (see Figure 2.4), although the severity of these incidences has varied between rivers and years. The worst outbreaks occurred in 2013 on the River Lyn (Somerset) and in 2015 on the River Dart (Devon), where a large proportion of the salmonid run exhibited *Saprolegnia* infections, many of which were severe and resulted in mortality. In both incidences, the Environment Agency implemented an emergency bylaw closing the rod and line fishery to facilitate stock recovery. Similar high levels of *Saprolegnia* were observed in the River Dart in 2017, and this river appears to be one of the worst affected. Recent outbreaks of *Saprolegnia*, however, have not been confined to rivers in the South-West of England. The River Esk, Yorkshire, has experienced moderate or high levels of the oomycete in 2011, 2014 and 2015. The River Coquet, Northumberland, experienced a severe outbreak of saprolegniasis in 2015 with several hundreds of fish being lost due to the disease. Across England, *Saprolegnia* reports were most frequent in 2015.

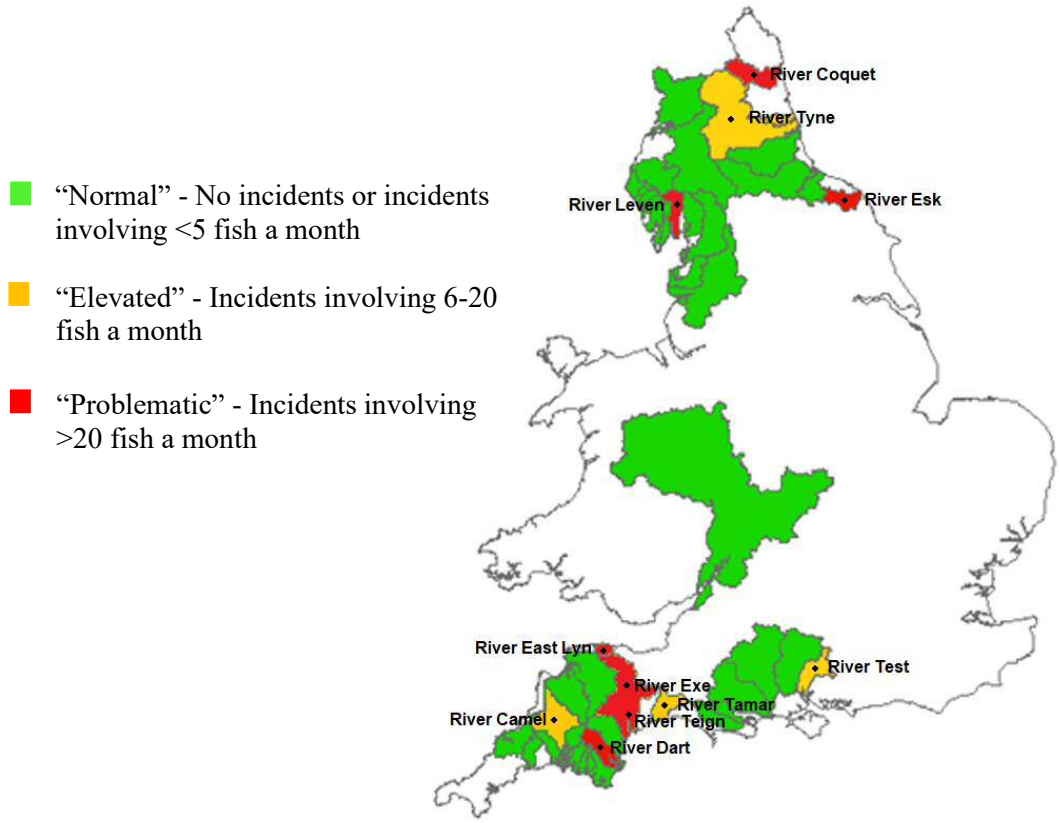

Figure 2.4. Distribution of Saprolegnia *incidences in the principal salmon rivers in England between 2010 and 2018.*

Potential drivers of disease

Pathogen

The virulence of individual *S. parasitica* isolates is diverse (Neish 1977; Whisler 1996). Analysis of expressed sequence tags (ESTs) from a mycelial cDNA library of *S. parasitica* uncovered several putative secreted protein families including: cellulose binding domain (CBD) proteins, glycosyl hydrolases, proteases and protease inhibitors that have potential roles in pathogen virulence (Torto-Alalibo et al. 2005). One of these secreted proteins, *S. parasitica* host targeting protein 1 (SpHtp1), is able to translocate specifically into fish cells via tyrosine-O-sulfate-modified cell-surface molecules (Wawra et al. 2012). Compilation of the *S. parasitica* draft genome revealed this oomycete possesses one of the largest repertoires of proteases found in any organism; these virulence factors were shown to bombard the host at various points during an infection (Jiang et al. 2013) causing damage to host tissues and enabling colonisation.

Environment

Our understanding of the environmental factors that drive saprolegniasis epidemics is limited. *Saprolegnia* can tolerate a wide range of temperatures; laboratory studies have demonstrated that vegetative growth and zoospore production occur between 5-30°C (Kitancharoen et al. 1996; Koeypudsa et al. 2005). Species, however, do prefer neutral to weak alkaline conditions between pH 7-10 and are only able to tolerate low levels of salinity, the optimum being between 0-0.5% (w/v) NaCl (Koeypudsa et al. 2005). There are also anecdotal associations with low water flow that require further study. The effects of key environmental factors need to be assessed on both the vegetative and asexual stages of geographically diverse *Saprolegnia* isolates to better understand the abiotic drivers of this pathogen.

Host

As with many other infectious diseases, host stress enhances the proliferation of *Saprolegnia* infections. Water temperatures outside of the host's optimal thermal range can lead to thermal stress that negatively impacts immune function (Le Morvan et al. 1998). Indeed, incidences of saprolegniasis in the wild are frequently observed during the winter and spring months when temperatures decrease and increase respectively. Furthermore, migratory salmonids face trauma and stress when returning to freshwater rivers to spawn. During the transition from a marine to freshwater environment, salmon stop feeding and utilise their energy reserves for the extensive upstream migration and defending spawning territories (Fleming 1996). These metabolically taxing processes compromise immune function and increase disease susceptibility (Dolan et al. 2016). Moreover, the journey can render severe damage to the fish body, impairing the initial line of pathogen defence and providing direct entry points for *Saprolegnia* zoospores to attach and enable host colonisation.

Conclusion

Saprolegnia is one of the most common and widespread diseases of freshwater fish globally. In the UK, *Saprolegnia* has caused significant losses as a secondary opportunist for centuries. This is still observed every year in migratory salmonids with weak or stressed individuals succumbing to saprolegniasis after long and stressful spawning migrations. *Saprolegnia* is a ubiquitous freshwater pathogen so a certain

level of ‘natural’ infections are to be expected. However, increasing numbers of *Saprolegnia*-induced salmonid deaths in some rivers pose a significant threat to these spawning populations. This warrants further attention to better understand the drivers for infection, the genetic characteristics of the pathogen and future disease risks in context of climate change and anthropogenic pressures.

Chapter 3 – Landscape genomics of *Saprolegnia parasitica*

Abstract

Advances in genomic technologies are key to expanding our understanding of *Saprolegnia parasitica*; a fish-pathogenic oomycete responsible for considerable losses in aquaculture and damage to freshwater ecosystems. Here for the first time, we use landscape genomics to investigate the whole genome data of 46 *S. parasitica* isolates collected from fish hosts within 12 waterbodies across England and Wales. All isolates were genetically distinct, indicating high species genetic diversity; however, population structure analysis categorised the isolates into just two subpopulations. Genomic signatures of selection were identified within important gene groups relating to: sensing external stimuli, host invasion/colonisation, infective life stages and nutrient acquisition. Moreover, we were able to link adaptive changes in ubiquitin-ligase activity with minimum environmental temperatures and ATPase activity with mean/maximum temperatures. These findings provide insight into *S. parasitica* epidemiology and reveal targets of adaptive selection that likely play essential roles in the pathogenesis of this species.

Introduction

Identifying environmental conditions associated with parasite outbreaks greatly aids our understanding of disease emergence and allows us to predict future epidemics. Parasite species can be distributed over a wide geographic range, typically exposed to different environmental and host pressures; this can cause local adaptation and drive genetic diversity between individuals across a landscape. Landscape genomics explores the interaction between the environment and genome using next-generation sequencing and powerful computational technologies. It is an expansion of landscape genetics, which examines how landscape features influence variation in a small number of neutral genetic loci (genetic markers, often microsatellites, which have not undergone selective pressures); such studies enable identification of patterns of gene flow between geographically diverse individuals or populations. In contrast, landscape genomics focuses on the detection of genome-wide genetic variation, generally single nucleotide polymorphisms (SNPs), under selective environmental pressures. Until

now only applied to non-parasitic plants and animals (see Abebe et al. 2015; Berg et al. 2015; Vangestel et al. 2016; Li et al. 2017), landscape genomics has the potential to revolutionise epidemiology.

Oomycetes are fungal-like eukaryotes responsible for substantial economic losses and serious damage to natural ecosystems. Despite their widespread impact, aquatic oomycetes remain understudied; this is especially true for members of the animal pathogenic genus, *Saprolegnia*. Causative agent of the disease saprolegniasis, the species *Saprolegnia parasitica* is most commonly associated with infections of freshwater fish and poses a serious threat to both the aquaculture industry and wild fish populations. The environmental and ecological significance of the *Saprolegnia* taxon means robust species identification is essential. Traditional methods based on morphological characteristics of the sexual structures oogonia, oospores and antheridia (Coker 1923; Seymour 1970; Johnson et al. 2002; see Chapter 2) have resulted in taxonomic confusion within the genus. Several species possess very similar morphologies and many animal parasitic isolates do not produce sexual structures *in vitro* (Coker 1923; van den Berg et al. 2013). Molecular methods enable rapid species identification via PCR amplification of highly conserved regions and subsequent DNA sequencing. The suitability of the nuclear ribosomal DNA internal transcribed spacer (ITS) region for oomycete species resolution has been well demonstrated (Cooke et al. 2000; Robideau et al. 2011). Diéguez-Uribeondo et al. (2007) identified five major clades of *Saprolegnia* using ITS sequence data in combination with morphological and physiological data. Subsequently, Sandoval-Sierra et al. (2014) proposed a taxonomic clustering system that used ITS sequence data to define molecular operational taxonomic units (MOTUs), an approach used to resolve species delimitation in difficult organisms (Blaxter et al. 2005). Sandoval-Sierra et al. (2014) identified 18 molecular clusters consistent with previously described *Saprolegnia* species, 11 potentially new species, and corrected the species names of several mis-assigned GenBank sequences. A subsequent phylogenetic study by de la Bastide et al. (2015) generally supported the suggested molecular taxonomic cluster system by Sandoval-Sierra et al. (2014) making it a widely adopted method for establishing species boundaries within this genus.

Genomic resources have made it possible to explore *S. parasitica* pathogenesis. The analysis of small sets of expressed sequence tag (EST) data

uncovered secreted protein families predicted to function in virulence including: proteases, glycosyl hydrolases and protease inhibitors (Torto-Alalibo et al. 2005) alongside an effector protein, *S. parasitica* host-targeting protein 1 (SpHtp1) that was found to be highly expressed in *S. parasitica* zoospores/cysts during the early stages of infection (van West et al. 2010) and able to translocate specifically into fish cells (Wawra et al. 2012). Further advances were provided by Jiang et al. (2013) upon sequencing of the *S. parasitica* genome in combination with transcriptomic analyses. The study found *S. parasitica* has one of the largest numbers of proteases found in any organism; these virulence factors were shown to attack the host at different stages during an infection and at least one actively suppressed the initial immune response via degradation of Immunoglobulin M (Jiang et al. 2013). Moreover, adaptation to an animal-pathogenic lifestyle was evidenced by the loss of large gene families that are hallmarks of plant pathogenic oomycetes (e.g. RXLR effectors, Crinkler's and Necrosis Inducing-Like Proteins (NLP)) and acquisition of genes typical of animal-pathogens (e.g. disintegrins and galactose-binding lectins) via horizontal gene transfer (Jiang et al. 2013). Two-thirds of the genome displayed a high rate of polymorphism (peak of 2.6%) indicating *S. parasitica* may adapt rapidly to environmental pressures (Jiang et al. 2013); this could result in genetically diverse isolates across the geographical landscape.

Here, we utilise whole genome data of 46 *S. parasitica* isolates collected from fish in 12 waterbodies across England and Wales to assess: the phylogenetic relationships between isolates, establish whether there is an underlying population structure, identify genomic regions under adaptive selection and assess whether these can be linked to particular environmental and/or host traits using a landscape genomics approach. We hypothesise that genomic variation may be associated with geographic location (i.e. North vs South sampling points) and host species. This data will provide an insight into *S. parasitica* genomic variation within this region and expand our currently limited understanding of the adaptive mechanisms employed by this destructive oomycete species.

Materials and Methods

Saprolegnia *sample collection and maintenance*

The Environment Agency monitors disease outbreaks in the 49 main salmon rivers in England and are closely linked with Natural Resources Wales (NRW) that are responsible for the 32 in Wales. Area officers collected 133 samples of suspected *Saprolegnia* from 15 rivers and 6 smaller waterbodies (i.e. lakes, fisheries, brooks) between 2015 and 2017 (Total of 133 samples from 94 fish and 21 sites; see Figure 3.1 and Appendix 1). Multiple patches of suspected *Saprolegnia* were sampled from some fish to examine whether a single host can be infected with multiple isolates. From live fish caught by area officers, forceps were used to extract mycelia from the affected tissue before culturing on potato dextrose agar (PDA, 39g L^{-1}) plates. These plates were sealed with parafilm and sent to Cardiff University. As samples were obtained directly from fish hosts in the field, contamination by bacterial/fungal sources was common and complicated the culturing process. Consequently, species identification via ITS sequencing (see below) was only achieved for 77 samples that established contamination-free stock cultures. Sub-culturing onto fresh PDA plates was performed monthly as described by Stewart et al. (2017).

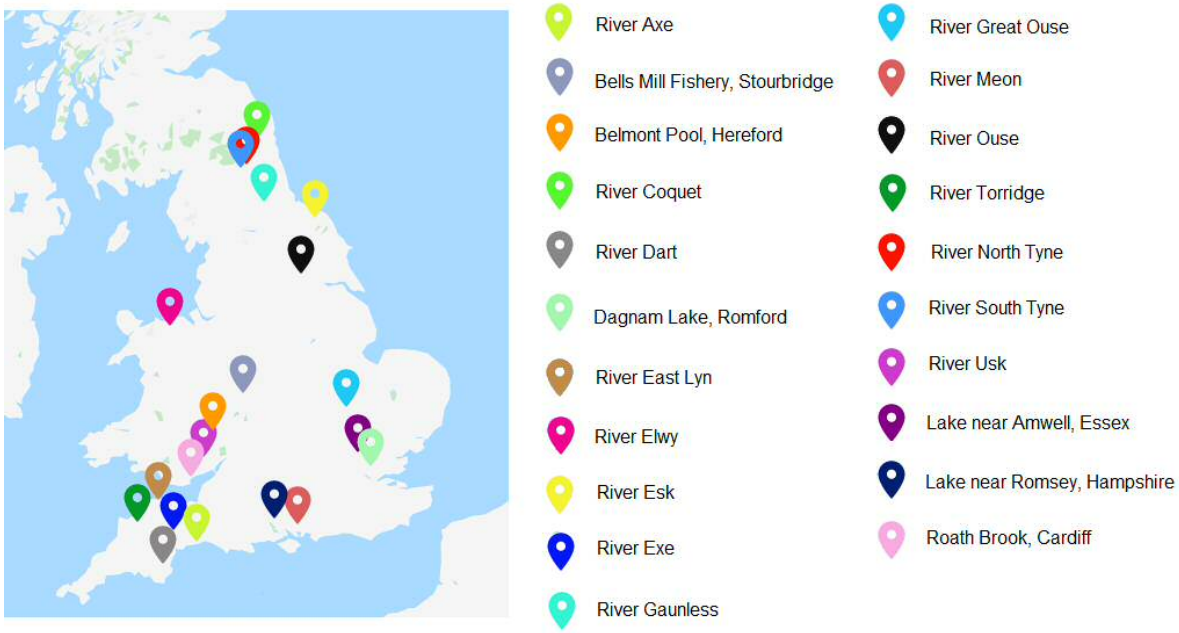

Figure 3.1. Map of Saprolegnia *sampling sites.*

Extraction of genomic DNA and quality assessment

Genomic DNA was extracted from each suspected *Saprolegnia* sample using a modified protocol outlined by Vilgalys and Hester (1990). Briefly, the extraction

procedure involved suspending ~0.3g of mycelia from the stock culture (ground under liquid nitrogen) in 500 µl of 2X (w/v) CTAB extraction buffer (100mM Tris, 20mM Na_2EDTA, 1.4M NaCl, pH 8.0) before freeze-thawing (first they were placed at -80°C for 10 min and then 65°C for 30 min). Samples were extracted twice using equal volumes of chloroform-isoamyl alcohol (24:1). DNA precipitation was achieved via the addition of 2 volumes of isopropyl alcohol and placing the samples at -20°C for 24 h. The resulting genomic DNA pellets were washed once with 70% EtOH, dried under a laminar flow hood and re-suspended in 50 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Extracted DNA was quantified using the Qubit Broad Range double-stranded DNA assay (Life Technologies). The quality of the DNA samples was assessed by Genomic DNA Screen Tapes using an Agilent 4200 TapeStation.

Species identification

Samples were identified to species level via sequencing ITS regions 1 and 2 of nuclear ribosomal DNA. This is a highly conserved region located between the 18S and 28S ribosomal subunits and includes the 5.8S gene. ITS PCR amplification was performed using the universal fungal primers of White et al. (1990); 5'-GGAAGTAAAAGTCGTAACAAGG-3' (ITS 5-Forward) and 5'-TCCTCCGCTTATTGATATGC-3' (ITS 4-Reverse). PCR was performed with 15 µl of Taq PCR Master Mix (Qiagen), 1.5µl of each forward and reverse primer, 20-50 ng genomic DNA, with nuclease-free water to give a total reaction volume of 30 µl. Following initial denaturation at 94°C for 5 min, amplification was achieved using 5 cycles of denaturation at 94°C for 30s, annealing at 58°C for 30s and extension at 72°C for 1 min followed by a further 33 cycles, where the annealing temperature was reduced to 48°C, and a final extension at 72°C for 10 min. PCR products were subject to electrophoresis on a 1% agarose gel and visualised using UV trans-illumination to confirm they were the correct size (approx. 700 bp). The forward and reverse PCR products were then sequenced via Sanger sequencing. The resulting sequence data was identified to the species level via an NCBI BLAST search for related sequences. Only samples identified as *Saprolegnia parasitica* (samples with >98% sequence identity to *S. parasitica* GenBank sequences) were considered for the landscape genomics investigation.

Whole genome library preparation

Genomic DNA was re-extracted for whole genome library preparation (January 2018) from 46 isolates of *S. parasitica* (Table 3.2) that were of high genomic quality and free from contaminants. The extracted genomic DNA was fragmented using the NEBNext dsDNA Fragmentase kit. The reaction mixture comprised: ~100 ng of genomic DNA, 200 mM $MgCl_2$, Fragmentase buffer v2 (1X) and dsDNA fragmentase (1X). This mixture was then exposed to the following thermal cycle; 4°C for 6 min, 37°C for 15 min. The resulting DNA fragments were subject to size selection via SPRI bead clean-up. Following the fragmentase step, the concentration of fragmented gDNA was quantified via the Qubit dsDNA High Sensitivity Kit (Life Technologies) according to the manufacturer's instructions and diluted to achieve a concentration of ~15 ng/µl for use in the NEBNext® Ultra™ DNA Library Prep Kit. The method was slightly modified such that at every step in the protocol the recommended volume of reagents was halved. Fragment size distribution for each resulting library was checked via Agilent D5000 Screen Tapes on an Agilent 4200 TapeStation. The libraries were then pooled and sequenced using the Illumina NextSeq500 Sequencer.

Phylogenetic analysis

ITS sequence data for the 46 *S. parasitica* isolates included in the whole genome re-sequencing were compared with homologous sequences of *Saprolegnia* spp. obtained from GenBank that have been previously classified into phylogenetic clusters by Sandoval-Sierra et al. (2014). ITS sequence data of an isolate of *Aphanomyces astaci* was also included in the phylogenetic analysis as an outgroup (see Table 3.1). The Molecular Evolutionary Genetics Analysis (MEGA) software v10.0.2 was used to construct a phylogenetic tree based on the ITS sequence data. Sequences were first aligned using the ClustalW algorithm using default settings, subsequently the tree was constructed using the Maximum Likelihood method based on the Jukes-Cantor model. To estimate the relative branch support of the tree, bootstrap analysis with 1000 replicates was performed, all other settings were set to default. The resulting tree was converted into a Newick file and imported into FigTree v1.4.4 to construct a circular phylogenetic tree.

GenBank Accession No.	Origin	Species (designated by Sandoval-Sierra et al. 2014)	Cluster (designated by Sandoval-Sierra et al. 2014)
DQ393507	USA	*S. diclina*	1
AM228818	Spain	*S. diclina*	1
FN186016	Norway	*Saprolegnia* sp. 1	2
AM947036	Norway	*Saprolegnia* sp. 1	2
AM228782	Spain	*Saprolegnia* sp. 1	2
AM228724	Spain	*S. parasitica*	3
AY455776	Japan	*S. parasitica*	3
AY455771	Japan	*S. parasitica*	3
AY267011	Argentina	*S. ferax*	4
AM228850	Australia	*S. ferax*	4
AB219390	France	*S. ferax*	4
KF717961	Poland	*S. australis*	5
KF717960	Poland	*S. australis*	5
AB219394	Japan	*S. australis*	5
AM228811	Spain	*S. delica*	6
AM228812	Spain	*S. delica*	6
AM228813	Spain	*S. delica*	6
KF718048	UK	*S. litoralis*	7
DQ393558	UK	*S. litoralis*	7
KF718105	Ecuador	*Saprolegnia* sp. 2	8
KF718083	Ecuador	*Saprolegnia* sp. 2	8
KF718102	Ecuador	*Saprolegnia* sp. 2	8
KF718122	Spain	*Saprolegnia* sp. 3	9
KF718123	Spain	*Saprolegnia* sp. 3	9
AB219380	Japan	*S. subterranea*	10
KF718124	USA	*S. subterranea*	10
AY270033	Argentina	*S. subterranea*	10
KF718125	Argentina	*S. torulosa*	11
KF718126	Argentina	*S. torulosa*	11
DQ393567	Japan	*S. torulosa*	11
KF718131	Ecuador	*S. monilifera*	12
KF718132	Ecuador	*S. monilifera*	12
KF718133	UK	*S. monilifera*	12
KF718134	USA	*S. terrestris*	13
KF718135	Ecuador	*S. terrestris*	13
KF718136	Ecuador	*S. terrestris*	13
KF718140	UK	*S. eccentrica*	14
KF718141	Japan	*S. eccentrica*	14
AB219395	Japan	*S. eccentrica*	14
KF718142	South Africa	*Saprolegnia* sp. 4	15
KF718143	UK	*S. furcata*	16
DQ393561	UK	*S. furcata*	16
KF718144	Poland	*Saprolegnia* sp. 5	17
KF718145	Poland	*Saprolegnia* sp. 5	17
KF718146	Spain	*Saprolegnia* sp. 5	17
KF718174	Argentina	*Saprolegnia* sp. 6	18
KF718175	Argentina	*Saprolegnia* sp. 6	18
KF718176	Argentina	*Saprolegnia* sp. 6	18
KF718178	UK	*S. asterophora*	19
KF718179	Poland	*Saprolegnia* sp. 7	20

KF718180	Poland	*Saprolegnia* sp. 7	20
KF718181	Ecuador	*Saprolegnia* sp. 7	20
KF718186	Ecuador	*S. megasperma*	21
KF718187	Ecuador	*S. megasperma*	21
KF718188	Ecuador	*S. megasperma*	21
KF718190	Germany	*S. turfosa*	22
HQ644012	UK	*S. turfosa*	22
KF718192	Poland	*S. anisospora*	23
KF718193	Netherlands	*S. anisospora*	23
KF718194	Argentina	*S. anisospora*	23
EU292729	China	*S. brachydanis*	24
AB219392	Japan	*Saprolegnia* sp. 8	25
AM228847	Sweden	*Saprolegnia* sp. 9	26
DQ393540	USA	*Saprolegnia* sp. 9	26
DQ393550	USA	*Saprolegnia* sp. 9	26
HQ643992	Japan	*Saprolegnia* sp. 10	27
HQ438029	Antarctica	*Saprolegnia* sp. 11	28
HQ643998	UK	*S. monoica*	29
KF718204	Spain	*Aphanomyces astaci*	Outgroup

Table 3.1. Genbank sequences used for phylogenetic analysis based on internal transcribed spacer (ITS) sequence data, their accession numbers, origin, species and the cluster designation described by Sandoval-Sierra et al. (2014).

SNP Calling

The Illumina NextSeq sequence reads were checked for quality using FastQC v0.11.5. Adaptor sequences were removed from paired-end fragment reads via Trimmomatic v0.36 using default settings (Bolger et al. 2014). Paired end fragment reads were aligned to the *S. parasitica* CBS223.65 reference genome assembly using the Burrows-Wheeler Aligner (BWA) MEM algorithm with default settings (Li 2013). Properly-paired read alignments for each isolate were sorted by supercontig and position along the reference genome assembly and subsequently separated into mitochondrial and nuclear reads using samtools v1.3.1. Details of the number of properly-paired reads, their percentage alignment to the reference genome, and the depth of coverage for each *S. parasitica* isolate are outlined in Table 3.2.

The resulting nuclear reads for each isolate were compiled into a single binary variant call format (BCF) file using samtools and SNP calling was performed using bcftools v1.5. The resulting variant call format (VCF) file was filtered using bcftools to include only biallelic variants and remove low quality variants using the following criteria: minimum base call quality 30, minimum depth of coverage 2, maximum depth of coverage 1121 (mean depth of coverage + 3x √mean depth of coverage, as suggested by Li 2014) and a minimum allele count of 2. A minor allele frequency

report of the filtered VCF file was obtained via PLINK v1.9 (Chang et al. 2015) and a histogram was generated using R v3.5.1 (Figure 3.2). Consequently, the SNPs with a minor allele frequency of <0.05 were excluded from the data using PLINK v1.9 (Chang et al. 2015) leaving a dataset of 1,030,764 SNPs.

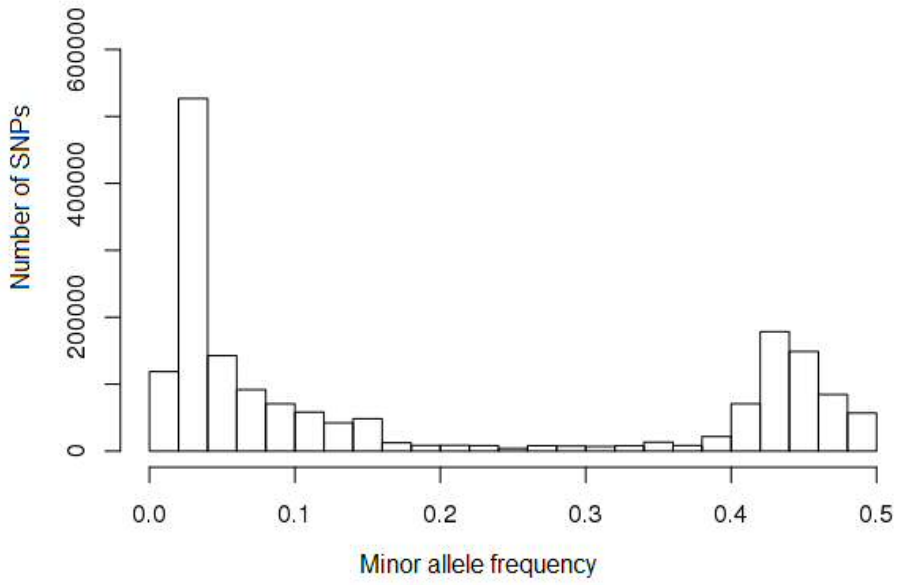

Figure 3.2. Minor allele frequencies of SNP data obtained from 46 Saprolegnia parasitica *isolates.*

Identity by descent and isolation by distance analysis

The dataset of 1,030,764 SNPs was subject to identity by descent analysis using PLINK v1.9 with default settings (Chang et al. 2015) to assess whether any of the *S. parasitica* samples were duplicates. Isolation by distance analysis was also performed using a partial-mantel test included in the R package vegan (Oksanen et al. 2012). Nei's genetic difference was compared between isolates obtained from the same host and isolates collected from different hosts while controlling for the effect of geographic distance.

Isolate ID and location	Host species	Date isolated	Properly paired reads		Coverage			
			No.	% aligned to reference	Depth (X)	Base pairs with ≥X1	Nuclear (X)	Mitochondrial (X)
EA001 - River Esk, Yorkshire, England (54°26'59.1"N, 0°48'12.42"W)	Atlantic salmon (*Salmo salar*)	10/01/2015	5422186	83.3	14.3	44119063	13.8	754.1
EA054 - River Gaunless, County Durham, England (54°37'32.3"N, 1°46'13.6"W)	Sea trout *(Salmo trutta)*	12/09/2016	2775544	24.6	5.9	42516021	5.9	56.2
EA095 - Belmont Pool, Hereford, England (52°02'18.4"N, 2°44'55.3"W)	Mirror carp (*Cyprinus carpio*)	11/04/2017	3363746	50	8.7	46344872	8.4	414.9
EA055[1] - River Coquet, Northumberland, England (55°18'25.8"N, 1°55'20.5"W)	Atlantic salmon	18/12/2016	5719150	58	14.9	41413716	14.2	990.1
EA056[1] - River Coquet	Atlantic salmon	18/12/2016	3416348	50.7	8.9	40888125	8.7	311.1
EA057[1] - River Coquet	Atlantic salmon	18/12/2016	3765064	58.3	9.9	41034178	9.7	292.2
EA096[2] - River Coquet	Atlantic salmon	19/04/2017	7835466	92.1	20.6	44608169	20.2	480.8
EA097[2] - River Coquet	Atlantic salmon	19/04/2017	8626620	92.2	22.6	44693393	22.3	412.6
EA098[2] - River Coquet	Atlantic salmon	19/04/2017	9860230	94.9	25.9	44730614	25.4	684.2
EA099[2] - River Coquet	Atlantic salmon	19/04/2017	11110136	95.1	29.1	44815548	28.6	735.3
EA058[3] - River North Tyne, England. (55°01'52.4"N, 2°07'10.5"W)	Atlantic salmon	19/12/2016	6797190	92.8	17.9	44446586	17.5	555.2
EA059[3] - River North Tyne	Atlantic salmon	19/12/2016	6663992	94.2	17.5	44308107	16.7	1064.2
EA063[4] - River North Tyne	Atlantic salmon	19/12/2016	9008778	83	23.6	44665789	23.1	714.9
EA064[4] - River North Tyne	Atlantic salmon	19/12/2016	8407210	90.9	22	44618257	21.7	445.7
EA065[4] - River North Tyne	Atlantic salmon	19/12/2016	7428630	84.6	19.5	44544221	19.2	416.3
EA009 - River North Tyne	Sea trout	24/11/2015	3211726	30.4	7.3	42892728	6.9	588.8
EA105[5] - River North Tyne	Atlantic salmon	01/11/2017	10479284	92.5	27.4	44732393	26.3	1613.2
EA106[5] - River North Tyne	Atlantic salmon	01/11/2017	6855578	46.3	17.2	44313102	16.7	653.9
EA109[6] - River North Tyne	Atlantic salmon	01/11/2017	9113116	79.6	23.7	44684797	23.3	581.8
EA110[6] - River North Tyne	Atlantic salmon	01/11/2017	7887610	84.5	20.6	44592320	20.2	509.4
EA112 - River North Tyne	Atlantic salmon	01/11/2017	20643508	90.4	53.4	45145082	52.6	1155.5
EA114 - River North Tyne	Sea trout	01/11/2017	9929950	75.7	25.8	44696288	24.9	1262
EA118 - River North Tyne	Sea trout	01/11/2017	6359386	51.7	16.3	44374267	16	422

EA120 - River North Tyne	Atlantic salmon	01/11/2017	10950070	83	28.6	44723343	27.9	867.9
EA068[7] - River South Tyne, England (54°59'29.6"N, 2°12'31.3"W)	Atlantic salmon	19/12/2016	9308086	94.6	24.2	44596218	23.2	1378.3
EA069[7] - River South Tyne	Atlantic salmon	19/12/2016	8541244	94.1	22.2	44533623	21.6	852.3
EA070[7] - River South Tyne	Atlantic salmon	19/12/2016	13342138	95.2	34.6	44828282	33	2155.6
EA071[7] - River South Tyne	Atlantic salmon	19/12/2016	10473416	94.6	27.1	44665239	25.7	2007.1
EA072[8] - River South Tyne	Atlantic salmon	19/12/2016	9678146	95.9	25.4	44665940	24.1	1728.2
EA077[8] - River South Tyne	Atlantic salmon	19/12/2016	10472608	90.2	27.5	44775449	26.4	1497.4
EA025 - River South Tyne	Atlantic salmon	01/11/2016	8721854	83.7	22.8	44639195	22	1087.6
EA026 - River South Tyne	Atlantic salmon	01/11/2016	8629636	67	22.3	44570264	21.6	972.3
EA123[9] - River South Tyne	Atlantic salmon	16/11/2017	10512318	79.5	27.4	44709073	26.4	1376.4
EA124[9] - River South Tyne	Atlantic salmon	16/11/2017	8401436	87.4	22	44598761	21.7	462
EA125[9] - River South Tyne	Atlantic salmon	16/11/2017	4680186	46.9	11.8	44056898	11.6	248.2
EA126[10] - River South Tyne	Atlantic salmon	16/11/2017	9402402	79.8	24.5	44695888	24	702.8
EA127[10] - River South Tyne	Atlantic salmon	16/11/2017	7154352	73.8	18.6	44487484	18.5	252.1
EA128[10] - River South Tyne	Atlantic salmon	16/11/2017	9187460	70.4	23.8	44622671	23.1	877.6
EA129 - River South Tyne	Atlantic salmon	16/11/2017	9739338	72.9	25.1	44661875	24.9	336.8
CF009 - Roath Brook, Cardiff, Wales. (51°29'54.1572"N, 3°9'54.2484"W)	Three-spined stickleback (*Gasterosteus aculeatus)*	09/08/2016	7986162	78.5	21.1	41727841	21	264.6
CF010 - Roath Brook	Three-spined stickleback	15/08/2016	6781884	80	17.9	41565325	17.5	563.7
EA016 - River Dart, Devon, England. (50°27'36.432"N, 3°41'42.144"W)	Sea trout	03/06/2016	12757970	94.9	33.6	44821792	33.1	716.8
EA014 - River Exe, Exeter, England. (50°51'56.1"N, 3°30'01.3"W)	Atlantic salmon	10/05/2016	2118078	16.9	5.1	41200667	4.7	455.4
EA043 - River Usk, Wales. (51°43'47.5"N, 2°56'55.9"W)	Atlantic salmon	25/11/2016	8369300	94.4	21.9	44608666	21.4	759.9
EA046 - River Torridge, Devon, England. (50°57'25.1"N, 4°10'00.4"W)	Atlantic salmon	03/12/2016	10559206	95.3	27.7	44758259	26.7	1479.6
EA007 - River Ouse, Yorkshire, England. (53°50'08.2"N, 1°04'33.4"W)	European eel (*Anguilla anguilla*)	25/11/2015	3255982	21.5	7.3	45542357	7.2	225.4

Table 3.2. Saprolegnia parasitica *isolates included in landscape genomics study. Isolates marked with the same superscript number (e.g. EA055[1], EA056[1], EA057[1]) were obtained from the same fish host.*

Population Structure and genetic relationships

PLINK v1.9 was used to calculate the pairwise Linkage Disequilibrium (LD) between SNPs within a 1 million kb window based on their allele frequency correlations (r^2) (Chang et al. 2015). The distribution of LD as a function of distance in kilobase pairs in the genome was derived from the PLINK results using in-house scripts in Python v2.7.15 (Figure 3.3). For population structure analysis, PLINK v1.9 was used to prune SNPs from the total dataset with an LD higher than 0.1 to avoid using markers that largely reflected duplicated data points due to their correlation. For this pruning, a sliding window approach was used with each window consisting of 50 SNPs, a step size of 10 SNPs and an $r^2 \geq 0.1$. Pruning resulted in 50,889 SNPs left for this analysis.

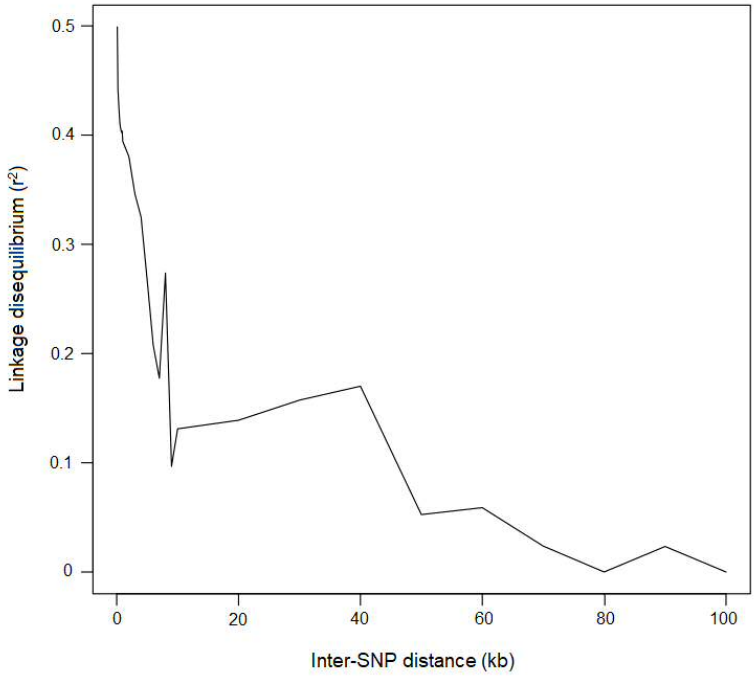

Figure 3.3. Linkage Disequilibrium (LD) decay for 46 isolates of Saprolegnia parasitica*. LD estimates are reported as squared correlations of allele frequencies (r^2) and have been averaged within 100kb windows.*

The software Admixture v1.3 (Alexander et al. 2009) was used to estimate the most likely number of clusters (K) in the data. For each specified value of K, Admixture estimates the proportion of each individual's genotype that derives from each cluster. The values of K tested in this analysis ranged between 1 and 10 and the cross validation (CV) statistic was used to select the most suitable number of clusters. To compliment this analysis, principal component analysis (PCA) was carried out using PLINK v1.07

(Purcell et al. 2007) with default settings and Splitstree v4.16.6 was used to construct a NeighbourNet network (Huson and Bryant 2006).

Treemix v1.13 (Pickrell and Pritchard 2012) was used to determine the historical relationships between *S. parasitica* isolates. The program uses genome-wide allele frequency data to identify splits and migration events (allele mixing) between individuals. Treemix was run iteratively with migration edges between 0 and 10. The f index, which represents the fraction of variance in the sample covariance matrix that is accounted for by the model covariance matrix, was used to identify the number of migration events that best fitted the data (Pickrell and Pritchard 2012).

Genome-wide signatures of adaptive selection

The Bayesian based method implemented by BayeScan v2.1 (Foll and Gaggiotti 2008) was used to identify candidate loci under selection. BayeScan uses differences in allele frequencies between populations to calculate a fixation index (F_{ST}) at each SNP. The F_{ST} parameter is split into two components; the population-specific component (beta) that is shared by all loci and the locus-specific component (alpha) that is shared by all populations (Foll and Gaggiotti 2008). When the locus-specific component is necessary to explain the observed pattern of genetic diversity (i.e. the alpha value differs significantly from 0) it indicates a locus has undergone selection. An alpha value can either be positive, indicating diversifying selection, or negative indicating balancing selection (Foll and Gaggiotti 2008). Consequently, there are two alternate models at each locus, one including and one excluding the alpha component, to model selection. BayeScan uses a reversible-jump Markov-chain Monte Carlo (MCMC) algorithm to estimate the posterior odds ratio; this represents the ratio of posterior probabilities for both models and is a measure of support for the model of local adaptation relative to the neutral demography model. BayeScan requires the user to set the prior-odds, these indicate how much more likely the neutral model is versus the model with selection. Prior-odds generally increase with the number of genetic markers included in the analysis.

Before performing the outlier analysis, the 46 *S. parasitica* samples were split into the two subpopulations determined by the population structure analysis. The following BayeScan settings were implemented; a prior-odds of 100 (i.e. the neutral model is 100 times more likely than the model including selection), a sample size of

10,000, thinning interval of 10, 20 pilot runs of length 5,000 and a burn-in of 50,000 resulting in a total of 150,000 iterations following the initial burn-in. Model convergence was verified using the R package CODA (Plummer et al. 2006) and two runs were performed to ensure consistency in the number of loci identified. Loci with q-values <0.05 were considered outliers; the q-value of a given locus is the minimum false discovery rate (FDR) at which the locus may become significant. Hence a q-value threshold of 0.05 means that 5% of the outlier markers (that have a q-value <0.05) are false positives. Pairwise F_{ST} was also calculated for each SNP using PLINK v1.9 (Chang et al. 2015) with subpopulations defined as previously in the BayeScan analysis. SNPs with the highest 5% of F_{ST} values were compared with the BayeScan results.

Gene enrichment analysis

If an outlier determined by BayeScan was located within a gene, that gene was presumed to be the target of selection. For the remaining outliers, genes within 10kb upstream and downstream were considered to be the most likely targets of selection, with a ~20% chance of being linked with a respective SNP (see Linkage Disequilibrium plot in Figure 3.3; r^2=0.2 at ~10kb). However, we did also consider genes within a 50kb region as potentially under selection (~5% chance of linkage at 50kb, see Figure 3.3). Gene enrichment analysis was conducted separately for genes containing a SNP and for genes within 50kb of a SNP; these genes were further divided into 10kb sections (i.e. 0-10kb, 10-20kb, 20-30kb, 30-40kb, 40-50kb). The analysis was performed using a classic Fisher test within the R package topGO (Alexa and Rahnenfuhrer 2019) with the threshold for significance set at P<0.05. All *S. parasitica* genes and annotated gene ontology (GO) terms were downloaded from UniProt (UniProt 2019).

Genome-environment association analysis

The association analysis of SNP markers and environmental variables was performed using SamBada v0.8.1 (Stucki et al. 2017). The multivariate approach of this program uses logistic regression models to estimate the probability of genotype presence/absence in an individual, given the environmental variables that characterise its sampling site and the underlying population structure.

The host and environmental variables included in the analysis comprised: host species, longitude, latitude, nitrate concentration (mg L^{-1}), water temperature (°C) and pH. Spearman and Kendall rank correlation tests were used to confirm variable independence. For the sampling year of each isolate, annual mean, min and max values of the latter three parameters were calculated for their respective sampling sites. Furthermore, ten-year (2008-2017) mean, min and max values were included for each sampling site. These data were collected from the Environment Agency Water Quality Archive (Environment Agency 2019) for the English rivers (Coquet, Dart, Esk, Exe, Gaunless, North and South Tyne, Ouse and Torridge) while for the Welsh River Usk, the data were obtained from the Natural Resources Wales (NRW) UK Water Quality Sampling Harmonised Monitoring Scheme Detailed Data for the period 2008-2013 (Natural Resources Wales 2019). Data for the period 2014-2017 were requested directly from NRW. As environmental data was not available for Belmont Pool, Hereford and Roath Brook, Cardiff, isolates EA095, CF009 and CF010 were excluded from this analysis. Population structure was included as an environmental variable, with isolates separated into the two subpopulations determined by the population structure analysis. A model was considered significant if $P<0.05$ following Bonferroni correction for both a log-likelihood ratio (G-score) and Wald test (Joost et al. 2008). If the SNPs identified were located within a gene, that gene was assumed to be associated with the environmental variable; for SNPs not located within a gene, genes within 10kb upstream and downstream of the SNP were considered potentially associated with the environmental variable.

Results

Saprolegnia *species distribution*

S. parasitica was the predominant species found in English and Welsh rivers and water bodies. Of the 77 samples that were ITS sequenced, 87% were identified as *S. parasitica* while only 6.5%, 3.9% and 2.6% were identified as *S. ferax, S. australis* and *S. declina* respectively (see Appendix 1). The *S. parasitica* isolates were obtained from 5 fish species; the majority of which (71.6%) were acquired from Atlantic salmon. Isolates were also obtained from sea trout (11.9%), carp (9%), three-spined stickleback (6%) and European eel (1.5%). Most of these isolates were collected from

the fins (50.7%), while 20.9% and 19.4% were collected from the skin of the head and body respectively. The remaining samples (3%) were collected from the gills.

Phylogenetic analysis

All 46 *S. parasitica* isolates included in the whole genome re-sequencing analysis fell within Cluster 3 of the taxonomic system proposed by Sandoval-Sierra et al. (2014) alongside other isolates designated as *S. parasitica* (Figure 3.4).

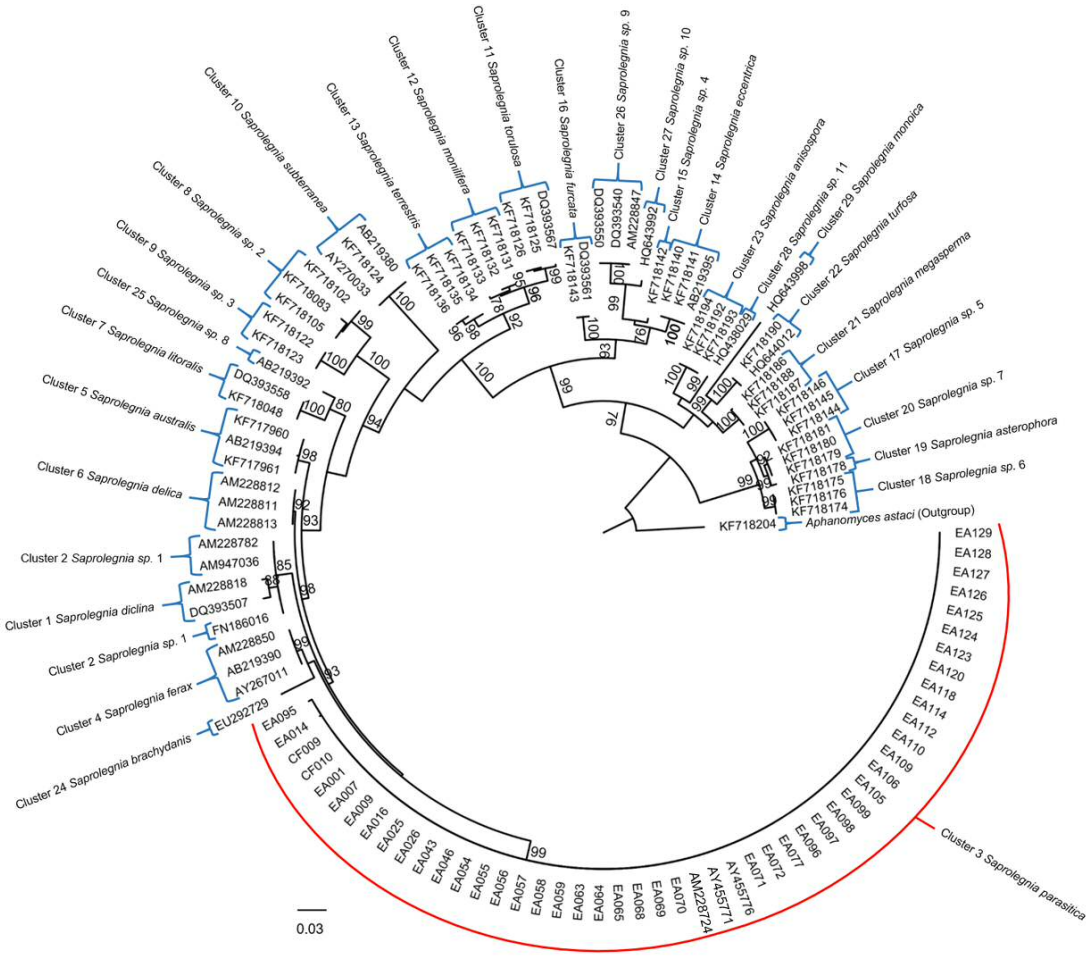

Figure 3.4. Phylogenetic tree showing relationships of the 46 Saprolegnia parasitica *isolates within the* Saprolegnia *genus (highlighted by red bracket) inferred from maximum likelihood analysis using the Jukes-Cantor model. Bootstrap support values >75% based on 1000 replicates are indicated next to the branches. The scale bar indicates the number of nucleotide substitutions per site.*

Identity by descent and isolation by distance analysis

The proportion identity by descent values were all below 1 (Figure 3.5A) allowing us to conclude that there were no duplicated samples in the dataset. The histograms in

Figures 3.5B and C represent comparisons between isolates obtained from different hosts and isolates obtained from the same host respectively; they display similar peaks at 0.8 and have a maximum proportion of just below 0.9. As isolates from the same fish have comparable proportions of identity by descent to those obtained from different fish, we can conclude that all 46 isolates are distinct. Furthermore, the partial-mantel test of the isolation by distance analysis revealed no significant difference in genetic variation between isolates obtained from the same host and isolates obtained from different hosts (Mantel r statistic= -0.0068, *P*= 0.537).

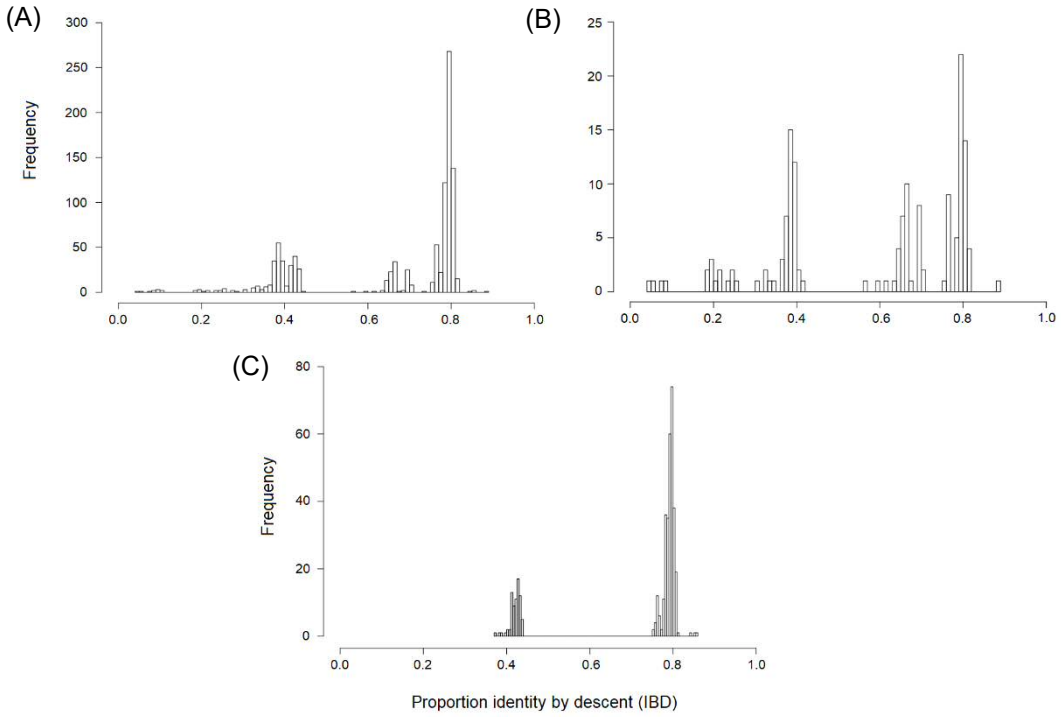

*Figure 3.5. Histograms displaying proportion identity by descent comparisons between (A) All 46 Saprolegnia parasitica isolates, (B) Isolates obtained from different fish hosts, (C) Isolates obtained from the same fish host. Proportion identity by descent represents P(IBD=2) + 0.5*P(IBD=1).*

Population structure and genetic relationships

Admixture analysis suggested that all 46 English and Welsh *S. parasitica* isolates belong to a single population (Figure 3.6A) with the lowest CV statistic (0.254) obtained at K=1 (Figure 3.6B).

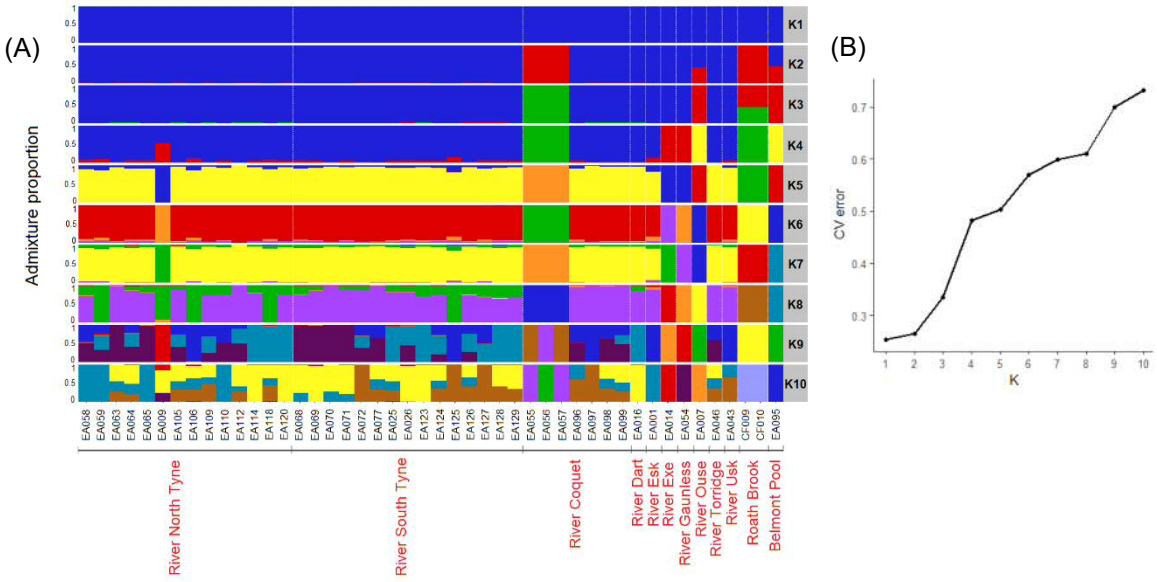

Figure 3.6. (A) Admixture analysis of 46 Saprolegnia parasitica *isolates collected from 12 locations across England and Wales. Each vertical bar represents an isolate and is separated into different colours. The colour proportion reflects the proportion of genetic variation originating from the cluster of that colour. The number of clusters (K) tested ranged from 1-10. The sampling location for each respective isolate is listed below the isolate ID. (B) Cross-validation error values for clusters (K) 1-10. The minimum CV error was obtained at K=1.*

While Admixture attempts to estimate the best way of dividing all individuals in the dataset in clusters (such that each cluster deviates the least possible from Hardy-Weinberg Equilibrium and is as close as possible to Linkage Equilibrium) and uses the inferred allele frequencies in each cluster to estimate the ancestry of each individual separately, PCA groups individuals on the basis of the similarity of the allele frequencies without the need of inferring the admixture proportions of each sample, and thus, it is a simpler way of identifying groups of similar samples. Here, PC1 explained 21.6% of the total variation in the data, whereas PC2 only explained 2.6% (Figure 3.7). Hence, PCA2 and the remaining components (which explained <2% of the total variance) were not used to determine population structure. PC1 separated the data into two subpopulations; (i) isolates obtained from Rivers North Tyne, South Tyne, Esk, Usk, Exe, Dart, Torridge, Gaunless and 4 isolates from the Coquet (EA096, EA097, EA098, EA099), and (ii) those from Roath Brook, Belmont Pool, River Ouse and 3 isolates from the River Coquet (EA055, EA056, EA057). The NeighbourNet network supported separation of isolates into the same two subpopulations (Figure 3.8).

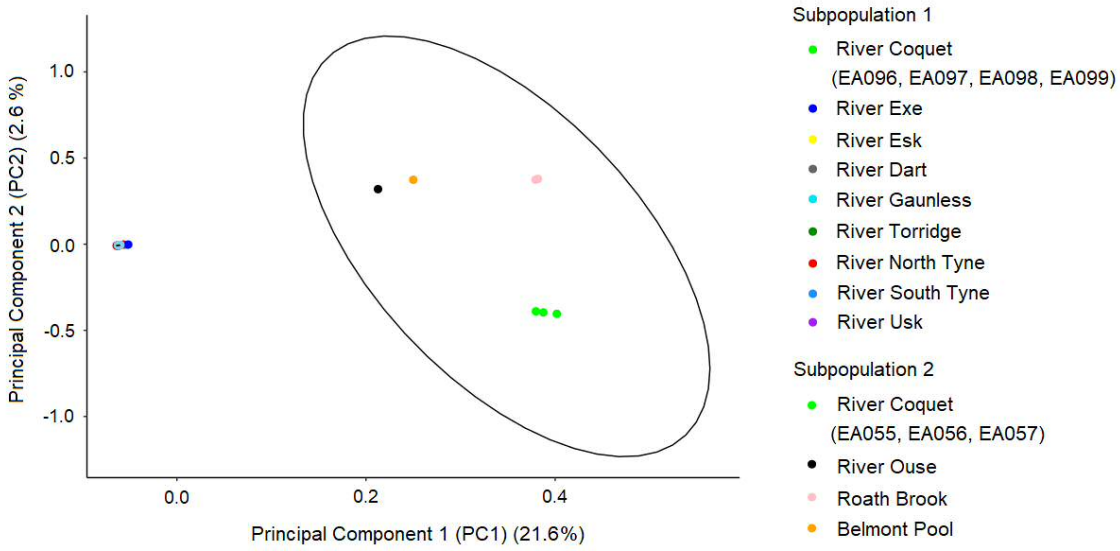

Figure 3.7. Principal Component analysis showing PC1 and PC2 accounting for 21.6% and 2.6% of the total variance respectively. 95% confidence ellipses are outlined in black. PC1 accounts for the most substantial proportion of the total variance within the data and separates the 46 Saprolegnia parasitica *isolates into two subpopulations. Isolates are coloured according to sampling location.*

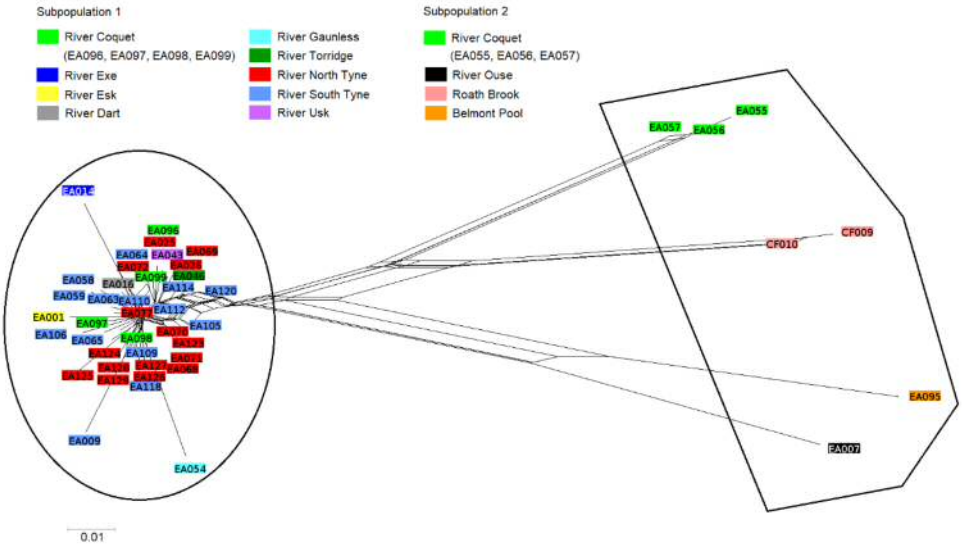

Figure 3.8. NeighbourNet depicting the relationship between the 46 Saprolegnia parasitica *isolates. Isolates are coloured according to sampling location. The isolates diverge into two main subpopulations which are outlined in black. The scale bar represents distances estimated using the uncorrected p-distance.*

The Treemix output supported the general population structure outlined in the PCA and NeighborNet analysis. The addition of three migration edges improved the amount of the variance explained by the phylogenetic model (model without migration edges; f index = 0.088, model with three migration edges; f index = 0.25) and suggests a migration event occurred between the River South Tyne (isolates EA069 and EA127

of the main subpopulation) and the River Coquet (isolates EA055, EA056, EA057 of the second subpopulation; see Figure 3.9).

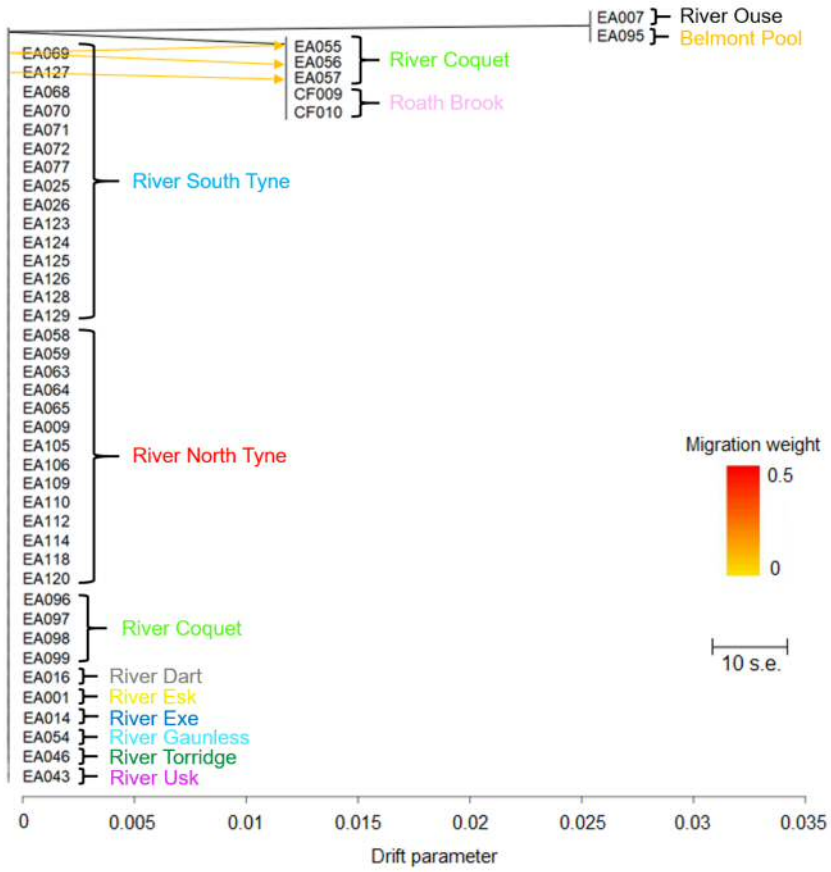

Figure 3.9. The phylogenetic network inferred by Treemix of the relationships between the 46 Saprolegnia parasitica *isolates. Three migration edges between isolates are shown with arrows pointing in the direction toward the recipient; coloured according to the percent ancestry received from the donor. The scale bar shows ten times the average standard error of the entries in the sample covariance matrix.*

Genome-wide signatures of adaptive selection

Both runs of BayeScan detected 133 outlier loci (FDR=0.05, prior odds 100:1) (Figure 3.10), all of which displayed positive alpha values, indicative of adaptive selection. These outlier loci were well dispersed throughout the genome (Figure 3.11) and were also within the upper 5% of SNP F_{ST} values identified by PLINK.

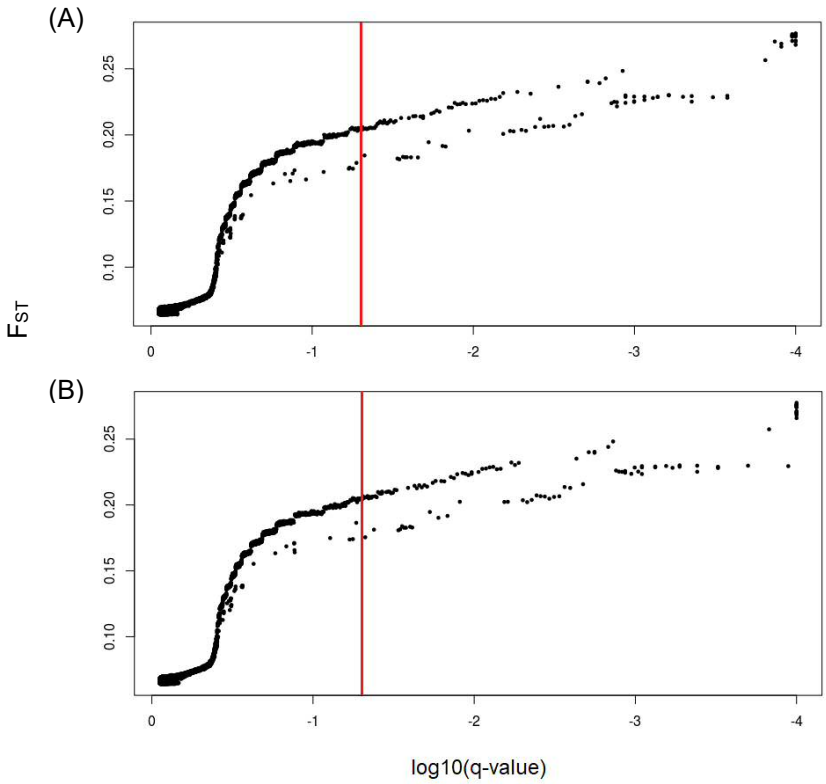

Figure 3.10. F_{ST} outlier analysis of 50,889 SNP markers from 46 Saprolegnia parasitica *isolates by BayeScan. F_{ST} values are plotted against log10-transformed q-values (the minimum false discovery rate at which a locus becomes significant). Two runs of BayeScan were performed (A) and (B); both identifying 133 loci as under adaptive selection (False discovery rate (FDR) threshold = 0.05, vertical red line).*

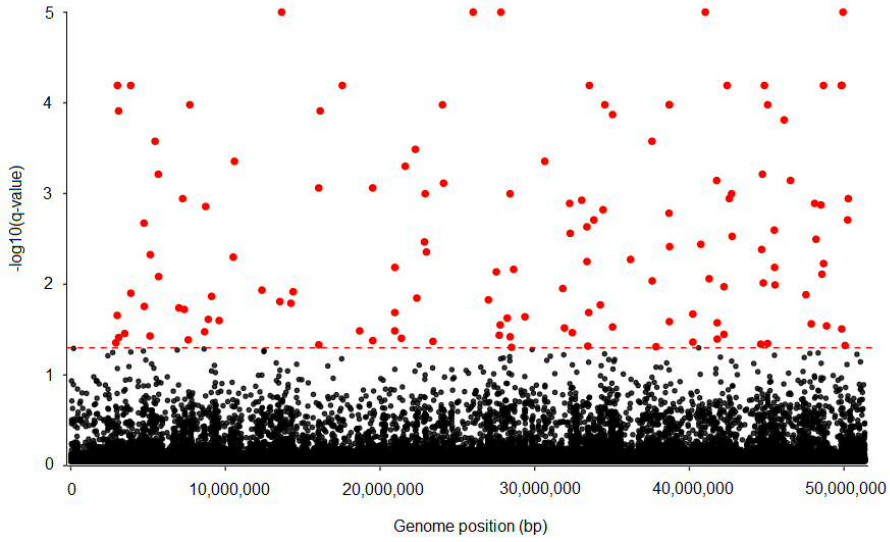

Figure 3.11. Manhattan plot showing the negative log10-transformed q-values (the minimum false discovery rate at which a locus becomes significant) for 50,889 genome-wide SNPs from 46 Saprolegnia parasitica *isolates. The 133 red dots represent SNPs targeted by positive selection as identified by BayeScan. The dotted red line indicates a false discovery threshold (FDR) of 0.05.*

Of the 133 outlier loci detected by BayeScan, 54 were located within genes (40.6%) and 24 of which had assigned gene ontology terms (Table 3.3). The following gene functions were among those that were over-represented in the gene enrichment analysis (P<0.05): positive regulation of kinase activity, purine ribonucleoside salvage and biosynthesis, and functions involved in flagella formation/cell movement including: cilium assembly, axonemal dynein complex assembly and microtubule based movement.

For the remaining 79 outlier loci not positioned within a gene, gene enrichment analysis of genes within 10kb up- and downstream highlighted the following: fibroblast growth factor binding and various post-translational protein modifications including protein acetylation/methylation, histone regulation/acetylation and the regulation of chromatin structure. Gene enrichment analysis of genes between 10-50kb also yielded: protein homoligomerisation (10-20kb); cell wall organisation and chitin catabolism (20-30kb); regulation of the cell cycle (30-40kb) and cell killing in other organisms (40-50kb).

SNP position		Gene	Gene ontology annotation (UniProt)
Supercontig no.	Position on supercontig (bp)		
2.6	677326	SPRG_02579	ATP binding
2.6	679264	SPRG_02579	ATP binding
2.14	6855	SPRG_04987	Protein kinase binding Protein kinase regulator activity Ribosome binding Positive regulation of kinase activity Regulation of translation
2.17	251235	SPRG_05566	Ion channel activity Integral component of membrane
2.27	409742	SPRG_07418	Symporter activity Integral component of membrane
2.35	96405	SPRG_07940	Integral component of membrane
2.43	287916	SPRG_20695	Integral component of membrane
2.41	94611	SPRG_09016	Calcium ion binding
2.56	233724	SPRG_09936	ATP binding Protein kinase activity Integral component of membrane
2.69	18695	SPRG_09939	Integral component of membrane
2.68	69038	SPRG_11168	IMP 5'-nucleotidase activity Magnesium ion binding Inosine salvage

			Nucleotide metabolic process
2.73	32801	SPRG_11980	ATP binding Protein serine/threonine kinase activity
2.88	10712	SPRG_21104	ATP binding Protein kinase activity
2.88	75995	SPRG_12239	Integral component of membrane
2.92	2982	SPRG_12598	Oxidoreductase activity
2.106	126345	SPRG_12951	ATP binding ATPase activity
2.126	506	SPRG_13747	ATP binding Protein kinase activity Integral component of membrane
2.110	92798	SPRG_13894	ATP binding Microtubule binding Microtubule motor activity Microtubule-based movement
2.191	6462	SPRG_15776	Integral component of membrane
2.264	4245	SPRG_16270	ATP binding Protein kinase activity Integral component of membrane
2.361	3892	SPRG_16737	Ion channel activity Integral component of membrane
2.722	640	SPRG_17691	Zinc ion binding
2.799	2882	SPRG_17828	Axonemal dynein complex assembly Cilium movement
2.842	3017	SPRG_17895	Beta-amyrin synthase activity Lanosterol synthase activity Triterpenoid biosynthetic process

Table 3.3. Gene ontology terms for outlier SNPs identified by BayeScan analysis of 50,889 SNP markers from 46 Saprolegnia parasitica *isolates that were located within genes.*

Genome-environment association analysis

SamBada identified three SNPs significantly associated with temperature related environmental factors. Two SNPs associated with minimum and mean temperature of the sampling year were located in genes that function in ubiquitin-protein ligase activity and ATPase activity respectively. The third SNP was connected with maximum temperature of the sampling year; again ATPase activity was among the most likely targets of selection (see Table 3.4).

SNP position		Associated environmental variable	Within gene?	Genes within 10kb	Gene ontology annotation (UniProt)
Supercon-tig no.	Position on supercon -tig (bp)				
2.59	56640	Minimum temperature – Year of sampling	Yes SPRG_22263	N/A	Ubiquitin protein ligase activity
2.192	44598	Mean temperature – Year of sampling	Yes SPRG_14806	N/A	ATPase activity
2.101	21527	Maximum temperature – Year of sampling	No	SPRG_13661	Integral component of membrane
				SPRG_13663	Integral component of membrane
				SPRG_13664	• ATP binding • Protein serine/threonine kinase activity
				SPRG_13665	• mRNA (nucleoside-2'-O-)-methyltransferase activity • 7-methylguanosine mRNA capping
				SPRG_13666	• Heme binding • Oxygen binding
				SPRG_13668	• DNA-binding transcription factor activity • Sequence-specific DNA binding

Table 3.4. SNPs and target genes significantly associated with environmental variables by SamBada analysis of 50,889 SNP markers from 43 Saprolegnia parasitica *isolates.*

Discussion

The current study identifies *S. parasitica* as the main *Saprolegnia* species infecting wild fish populations in England and Wales and is the first to assess the genetic diversity of this parasite across this region. The *S. parasitica* isolates examined could be separated into two subpopulations and a large number of adaptive polymorphisms were identified between isolates, some of which were linked with environmental factors.

Opportunistic sampling was employed here as it was the only available approach; this meant we were unable to obtain a comparable number of *S. parasitica* isolates from each of the locations or from a range of host species within a given location. Consequently, the level of genetic variation within certain waterbodies (i.e. those where only one isolate was collected) may be not be representative, which limits our ability to correlate genomic adaptations with host or environmental factors. Nonetheless, this study is the first to identify genome-wide signatures of selection within this devastating oomycete species and reveal important gene groups likely to be critical to *S. parasitica* local adaption.

A previous molecular epidemiology study by Ravasi et al. (2018) employed a multi-locus sequence typing (MLST) approach of 7 housekeeping genes to characterise 77 *S. parasitica* isolates collected from wild and farmed fish across 25 locations in Switzerland and France. They identified 10 different genotypes; 8 of which were unique to a particular geographic region or river basin, while the remaining 2 were fairly widely distributed (Ravasi et al. 2018). More recently, a phylogenetic analysis of 132 *Saprolegnia* spp. isolates from 9 fish farming facilities across Nova Scotia, Canada was conducted based on ITS and cytochrome c oxidase subunit 1 (*Cox1*) sequences (Sarowar et al. 2019). Of the 4 *S. parasitica* strains uncovered, 2 were widely distributed and constituted 79.5% of the isolates collected (Sarowar et al. 2019). The whole-genome sequencing approach employed in the current study offers a much higher genetic resolution than the aforementioned studies and found all isolates examined were genetically distinct, indicating a relatively high level of diversity. Both here and in the study by Ravasi et al. (2018), mixed isolate colonisation of the same host was evident, indicating potential intra-specific competition within *S. parasitica*.

Of the two subpopulations identified here; 84.8% of isolates formed the main subpopulation while only 15.2% fell within a second subpopulation. This division of isolates was not correlated with geographic location, nor reflected in our single-gene phylogenetic analysis in which all isolates fell within Cluster 3 of the taxonomic clustering system proposed by Sandoval-Sierra et al. (2014). Notably, the four *S. parasitica* isolates that were not obtained from salmon or sea trout hosts (CF009, CF010 – three spined stickleback, EA007 – European eel, EA095 – mirror carp) all fell within the smaller second population. However, host species was not associated

with any adaptive genomic changes in the genome-environment analysis presented here, likely as a result of the aforementioned consequences of opportunistic sampling.

Isolates collected from the River Coquet were split between the two subpopulations; those isolates within the second subpopulation were shown to have migrated from the River South Tyne, potentially as a result of environmental cross-contamination by anglers. The introduction of isolates into a new area may have a devastating effect on local fish populations; particularly if the area does not have naturally high levels of *S. parasitica* and the residing fish have not been previously been exposed to the oomycete. Furthermore, as *Saprolegnia* spp. can reproduce sexually, a newly introduced isolate may recombine with those in the local area, potentially resulting in more highly virulent isolates. This emphasises the importance of schemes promoting good angling practices such as the 'check, clean, dry' campaign aimed at stopping the spread of invasive species in aquatic environments.

We find *S. parasitica* is a highly polymorphic species with the potential to show adaptive genomic changes in response to selective pressures. Genes targeted by adaptive selection had a wide range of functions, including the positive regulation of protein kinase activity. Eukaryotic protein kinases catalyse the phosphorylation of target proteins and play a major role in many cellular functions. *S. parasitica* has a very large kinome comprising 543 predicted protein kinases, 131 of which were found to contain predicted transmembrane helices and thus may function as cell surface receptors that recognise environmental and host stimuli (Jiang et al. 2013). Positive regulation of these kinases may enable *S. parasitica* to sense and respond rapidly to external pressures; our data suggests that these kinases likely play a major role in *S. parasitica* adaptation to specific environments/localities.

Adaptive changes in *S. parasitica* purine ribonucleoside salvage/ biosynthesis were also uncovered here and likely resulted from host pressures. Fungi use purines from their surrounding environment/host as a source of nitrogen, they play diverse roles in energy metabolism, signal transduction and DNA/RNA biosynthesis (Chitty and Fraser 2017). The host immune response functions to prevent *S. parasitica* cell invasion, impeding purine uptake from host cells and consequently mycelial growth. Enhancing the ability of *S. parasitica* to both salvage host purines and synthesise them from precursor molecules may aid continued disease proliferation. Moreover, we

report selective pressures on fibroblast growth factor (FGF) binding that may play an important role in host invasion. The fungal pathogen *Candida albicans* induces FGF-2 in mammalian hosts to promote angiogenesis; this is predicted to improve hyphal penetration into deeper host tissues (Vellanki et al. 2019). Conversely, *Aspergillus fumigatus* produces secondary metabolites that supress host angiogenesis (Ben-Ami et al. 2009). The inhibition of new blood vessel formation is thought to isolate *Aspergillus*-infected tissue, limiting access of immune effector cells and antifungal drugs to the infected site (Paterson et al. 2003; Ben-Ami et al. 2009). *S. parasitica* may also influence FGF induction and thus angiogenesis in fish hosts as part of its pathogenesis. Thus, adaptation in *S. parasitica* FGF binding ability may advance hyphal penetration of host cells and augment disease progression. To test this theory, future work could assess the extent of *S. parasitica* hyphal penetration and damage in fish epithelial cells with normal and inhibited FGF activity.

Adaptive selection upon genes relating to flagella formation were also identified; zoospores are the flagellated infective units of *S. parasitica* and so these adaptive changes may denote important modifications in zoospore production rates, the number of repeated zoospore emergence (RZE) cycles that a zoospore can undergo and/or zoospore structure to facilitate optimal transmission in different aquatic environments. *S. parasitica* zoospore production in particular can vary greatly (see Chapter 5) and may be extremely sensitive to external factors in order to maximise successful disease transmission. The potential effects of flagella formation related genes on sporulation could be investigated *in vitro* using *S. parasitica* isolates with mutated versions of these genes.

Other potential gene targets of selection had functions related to cell wall organisation and chitin catabolism. The *S. parasitica* hyphal cell wall is in direct contact with host epidermal cells during invasion/infection and is therefore subject to constant host pressure. Oomycete cell walls are generally comprised of an inner layer of cellulose microfibrils overlaid by a layer of glucans containing (1→3)-β/ (1→6)-β linkages (Bartnicki-Garcia 1968). While the major component of typical fungal cell walls (Wessels and Sietsma 1981) - chitin - is largely absent from oomycetes, this compound has been shown to play an essential role in *Saprolegnia* hyphal tip growth (Guerriero et al. 2010). Consequently, adaptation within genes related to chitin metabolism may enable faster hyphal growth and enhance host colonisation. Selective

pressures on *S. parasitica* cell killing genes were also indicated here; necrosis of the host epidermal layer underpins saprolegniasis pathogenesis (Pickering and Willoughby 1982; Bruno and Wood 1999), hence adaptive selection within cell killing related genes could improve *S. parasitica* virulence and facilitate host invasion.

Temperature was the only environmental factor studied here that could be linked with *S. parasitica* genomic adaptation. Minimum temperatures were correlated with ubiquitin-ligase activity which has been shown to mediate the degradation of proteins involved in cold responses in both *Arabidopsis* (Dong et al. 2006) and yeast (Isasa et al. 2016). In *Arabidopsis* in particular, a mutated ubiquitin-ligase protein was found to improve plant-freezing tolerance (Dong et al. 2006); hence this adaptive response may improve *S. parasitica* survival at low temperatures. Conversely, mean and maximum temperatures were both connected with ATPase activity. *S. parasitica* produces energy in the form of Adenosine triphosphate (ATP) through aerobic cellular respiration. ATPase enzymes catalyse the hydrolysis of ATP into ADP and a free phosphate ion to release energy needed for various cellular functions. At increased temperatures, more ATP is needed to power processes driven by higher cellular kinetic energy (Clarke and Fraser 2004). Indeed, in Chapter 5 it is apparent that *S. parasitica* vegetative growth generally increases as temperatures increase. Consequently, ATPase adaptations likely optimise *S. parasitica* energy production to cope with the more intense metabolic pressures at high temperatures.

Here, we find evidence of high genetic variation among *S. parasitica* isolates indicative of their adaptive potential in the wild. Also, a wide range of gene groups have undergone adaptation in this species including those involved in: temperature tolerance, sensing external stimuli, infective life stages, host invasion and nutrient acquisition. These data should form the focus of future functional studies to fully understand their impact on *S. parasitica* survival and host interaction; such research should allow us to fully understand the parasitic success of this destructive organism. Moreover to overcome the issues associated with opportunistic sampling, future efforts should focus on obtaining a comparible number of *S. parasitica* isolates from each sampling location and a variety of host species within each location; this would help us to fully comprehend the selective influence of host and environmental factors on this parasite.

Chapter 4 – Host specificity of *Saprolegnia parasitica* isolates

Abstract

The ubiquitous freshwater pathogen *Saprolegnia parasitica* has long been considered a true generalist, capable of infecting a wide range of fish species. It remains unclear, however, whether different isolates of this pathogen, obtained from distinct geographic locations and host species display differences in host preference. The current study assessed the host-specificity of four *S. parasitica* isolates by testing their induced zoospore encystment responses towards the skin of four fish species. While three of the isolates displayed 'specialist' responses, one appeared to be more of a 'generalist'. Salmon and sea trout *in vivo* challenge infections involving a 'generalist' (salmon isolate EA001) and 'specialist' (sea trout isolate EA016) pathogen isolate did not support the *in vitro* findings, with no apparent host preference reflected in infection outcomes. Survival of sea trout and salmon, however, was significantly different during the challenge infection with the sea trout (EA016) isolate. These results indicate that while *S. parasitica* isolates can be considered true generalists, they may target hosts to which they have been more frequently exposed (potential local adaptation). Understanding host preference of this pathogen could aid our understanding of infection epidemics and help with the development of fish management procedures.

Introduction

Host specificity is an important parasite trait; providing an accurate depiction of a parasite's ecological niche (Poulin and Mouillot 2003). It is determined by the number of host species that a parasite can successfully invade and the taxonomic relationship between these host species. It is generally accepted that parasites trade off their virulence (the severity of infection) against transmissibility (ability to spread infection from host to host); with optimum parasite fitness striking a balance between the production of transmission stages and damage to the host (May and Anderson 1990). A given parasite species may infect a wide range of phylogenetically distinct host taxa, with parasite fitness varying from host to host. Thus, the composition of the host population presents a selective pressure that contributes to evolution of parasite generalism or specialism (Futuyma and Moreno 1988). Parasites with low host

specificity are considered generalists, capable of switching between distantly related host species, moreover they tend to exhibit a similar level of virulence across their broad host range (Poulin and Mouillot 2003; Leggett et al. 2013). More specialised parasites may possess a high specificity for certain host species or taxonomic group for which they exhibit an optimal level of virulence.

Fungal and fungal-like parasites are thought to possess the broadest host range of any parasite group (Fisher et al. 2012). Perhaps the most notorious example being the aquatic chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) that infects approximately 700 amphibian species (Olson et al. 2013) and is also capable of infecting fish (Liew et al. 2017). Furthermore, several fish pathogenic oomycetes are considered true generalists with many species able to infect several different families (Gozlan et al. 2014). Members of the *Saprolegnia* genus are particularly destructive; *Saprolegnia diclina* is a virulent pathogen of fish eggs (Kitancharoen et al. 1997; Fregeneda-Grandes et al. 2007; van den Berg et al. 2013) and *S. ferax* is believed to be partly responsible for declines in amphibian populations (Kiesecker et al. 2001; Pounds 2001). *S. parasitica* is arguably the most important animal pathogenic oomycete with a reported 1 in 10 of all farmed raised salmon succumbing to saprolegniasis and frequent associations with declining natural wild fish populations (van West 2006). Previously, *S. parasitica* was considered opportunistic, only able to infect fish hosts as a secondary pathogen. Several salmonid studies, however, have highlighted that certain isolates of *S. parasitica* are primary invaders and highly virulent (Neish 1977; Willoughby and Pickering 1977). Despite this, marked differences in virulence have been observed between *S. parasitica* isolates (Yuasa and Hatai 1995) and the host range of individual isolates remains unexplored.

S. parasitica produces free-swimming zoospores during the infective stage of its life cycle. These zoospores are unicellular, single nucleated cells that are able to swim freely via two flagella; one tinsellated and one whiplash flagellum (Burr and Beakes 1994). They are responsible for the first essential step in establishing an infection, namely locating and attaching to a host. They employ a range of strategies to achieve their principal function of pathogen transmission; releasing a secondary zoospore from an encysted primary precursor, and subsequently undergoing repeated rounds of encystment and release via a process of Repeated Zoospore Emergence (RZE) (Diéguez-Uribeondo et al. 1994). This revival of the infective agent increases

its likelihood of locating a host (Bruno and Wood 1999). The secondary cysts of *S. parasitica* also possess long hairs with hooks on their outer surface, which are thought to aid host attachment and potentially enhance their floatation in water (Beakes 1983; Hallett and Dick 1986; Burr and Beakes 1994). If isolates of *S. parasitica* are specialised for particular hosts, this could be reflected in their induced zoospore encystment responses towards different fish species.

A key aim of the current study was to examine whether levels of induced zoospore encystment towards different fish hosts varied between isolates of *S. parasitica*. This study combines *in vitro* induced zoospore encystment data with targeted *in vivo* challenge experiments to uncover the extent of specialism/generalism within this species. The *in vitro* investigations assessed the host preference of four *S. parasitica* isolates that are both geographically distinct and originally isolated from different host species. The *in vivo* studies aimed to determine whether the *in vitro* findings were reflected in challenge infection outcomes. We hypothesise that while the isolates investigated may not be highly specialised to a single host species, they do display preferences towards a limited number of host species. A phylogenetic analysis of the four *S. parasitica* isolates based on nuclear ribosomal internal transcribed spacer (nrITS) sequence data has also been included to examine their position within the *Saprolegnia* taxonomic system proposed by Sandoval-Sierra et al. (2014). This data could potentially expand our understanding of *S. parasitica* infections and inform future aquaculture practices.

Materials and Methods

Host origin and maintenance

Atlantic salmon (*Salmo salar),* sea trout (*Salmo trutta)*, common carp (*Cyprinus carpio)* and three-spined stickleback (*Gasterosteus aculeatus*) (n=6 per species) were net caught from hatcheries or the wild and delivered to our aquarium facilities at Cardiff University for use in the induced encystment assays. Moreover, further Atlantic salmon and sea trout (n=60 per species) were obtained for use in the experimental challenge infections (see Table 4.1 for details). Prior to experimental procedures, fish were maintained in 90 L tanks at a density of 1 fish L^{-1}. Both prior to and during experimental procedures, fish were exposed to: a water temperature of

12±0.5°C, oxygen saturation of >91%, 12 h light: 12 h dark cycle and fed trout pellets daily unless otherwise stated.

Experiment	Fish Species	Life stage	Mean weight (g) + range	Mean standard length (mm) + range	Source	Date of arrival at Cardiff University
Induced zoospore encystment assays	Atlantic salmon (*Salmo salar*)	Juvenile	17.4 (14.8-20.4)	111.0 (104.6-117.8)	Kielder Salmon Hatchery, Hexham, Northumberland	June 2016
	Sea trout (*Salmo trutta*)	Juvenile	9.2 (7.8-10.2)	92.3 (88.5-96.2)		
	Common carp (*Cyprinus carpio*)	Juvenile	41.41 (23.5-63.5)	123.9 (103.8-138.2)	DC Freshwater Fish, Brookwood, Surrey	
	Three-spined stickleback (*Gasteroste-us aculeatus*)*	Adult	1.7 (0.6-2.8)	48.0 (43.9-57.2)	Roath Brook, Cardiff	
Challenge infections	Atlantic salmon	Juvenile	1.8 (0.6-4.7)	45.2 (34.9-64.9)	Kielder Salmon Hatchery, Hexham, Northumberland	July 2017
	Sea trout	Juvenile	1.3 (0.6-2.1)	41.5 (32.9-48.2)		

Table 4.1. Origin of fish used for induced zoospore encystment assays and challenge infections.

Saprolegnia *culture and zoospore production*

Four *Saprolegnia parasitica* isolates were obtained directly from four naturally infected fish hosts collected during routine sampling by the Environment Agency (see Table 4.2). On the river bank, a small mycelial clump (approx. 4-5 cm^2) was extracted from the affected tissue of a live, recently caught fish using forceps and placed immediately onto a potato dextrose agar (PDA, 39g L^{-1}) plate. The plate was sealed using parafilm and sent to our facilities at Cardiff. Cultures were sub-cultured monthly onto fresh PDA plates according to Stewart et al. (2017). For zoospore production, petri dishes containing ~40 ml glucose-yeast broth (Glucose 10g L^{-1}, Yeast Extract 2.5g L^{-1}) were inoculated with three 5 mm diameter plugs of heathy white mycelia from the stock culture. The *S. parasitica* mycelia were left to grow for 72 h at 20°C,

then washed with dechlorinated water in order to remove excess glucose-yeast broth. To induce zoospore production, the mycelia were placed in a 50/50 mixture of dechlorinated water and aquarium water at 10°C for 72 h. The resulting zoospore suspension was concentrated via centrifugation at 4600 rpm for 10 min at room temperature. Zoospores in the concentrated suspension were enumerated using a haemocytometer.

Isolate	Host species	River/waterbody	Date isolated
EA001*	Atlantic Salmon (*Salmo salar*)	River Esk, Yorkshire, England. (54°26'59.1"N, 0°48'12.42"W)	10/01/2015
EA016*	Sea trout (*Salmo trutta*)	River Dart, Devon, England. (50°27'36.432"N, 3°41'42.144"W)	03/06/2016
EA012	Common carp (*Cyprinus carpio*)	Lake near Romsey, Hampshire, England. (50°59'40.6"N, 1°34'46.7"W)	22/03/2016
CF006	Three-spined stickleback (*Gasterosteus aculeatus*)	Roath Brook, Cardiff, Wales. (51°29'54.1572"N, 3°9'54.2484"W)	15/07/2016

Table 4.2. Origin of Saprolegnia parasitica *isolates used in the* in vitro *induced zoospore encystment assays. *Denotes isolates used in subsequent induced zoospore encystment assays and challenge infections.*

DNA extraction and sequencing of internal transcribed spacer (ITS) region

DNA was extracted using the following modified protocol outlined by Vilgalys and Hester (1990); ~0.3g of mycelia from each of the respective *S. parasitica* isolates was ground under liquid nitrogen and suspended in 500 µl of 2X (w/v) CTAB extraction buffer (100 mM Tris, 20 mM Na_2EDTA, 1.4M NaCl, pH 8.0). The samples were then subject to a freeze-thaw step in which they were placed at -80°C for 10 min and subsequently incubated at 65°C for 30 min. Samples were extracted twice using equal volumes of chloroform-isoamyl alcohol (24:1); DNA was precipitated by adding 2 volumes of isopropyl alcohol and placing the samples at -20°C for 24 h. The resulting genomic DNA pellets were washed once with 70% EtOH, dried under a laminar flow hood and re-suspended in 50 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

PCR amplification of the ITS region was performed using the universal fungal primers of White et al. (1990): 5'-GGAAGTAAAAGTCGTAACAAGG-3' (ITS 5-Forward) and 5'-TCCTCCGCTTATTGATATGC-3' (ITS 4-Reverse). The PCR reaction mix consisted of: 15 µl of Taq PCR Master Mix (Qiagen), 1.5 µl of each forward and reverse primer, 20-50 ng genomic DNA and nuclease-free water to give a total reaction volume of 30 µl. The PCR protocol was as follows: initial denaturation at 94°C for 5 min, 5 amplification cycles of; denaturation at 94°C for 30s, annealing

at 58°C for 30s and extension at 72°C for 1 min; subsequently a further 33 cycles were included where the annealing temperature was changed to 48°C. A final extension at 72°C for 10 min concluded the PCR. In order to check the PCR products were the correct size (approx. 700 bp), they were run on a 1% agarose gel and visualised using UV-transillumination. PCR products were then sequenced via Sanger sequencing and a NCBI BLAST search for related sequences was used to confirm each sample as *S. parasitica* (>98% sequence identity to *S. parasitica*).

Phylogenetic analysis

ITS sequence data for the four *S. parasitica* isolates investigated here were included in a phylogenetic analysis alongside *Saprolegnia* spp. sequences from GenBank that have been previously designated into phylogenetic clusters by Sandoval-Sierra et al. (2014) and an isolate of *Aphanomyces astaci* which acted as an outgroup (included the same sequences as in Chapter 3, see Table 3.1). The Molecular Evolutionary Genetics Analysis (MEGA) software v10.0.2 was used to first align the ITS sequences using the ClustalW algorithm with default settings, and subsequently construct a phylogenetic tree using the Maximum Likelihood method based on the Jukes-Cantor model. Relative branch support of the tree was estimated using a bootstrap analysis with 1000 replicates, all other settings were set to default. The tree was converted into Newick format and imported into FigTree v1.4.4 to produce a circular phylogenetic tree.

In vitro *induced zoospore encystment assays*

To obtain skin samples for the induced zoospore encystment assays, Atlantic salmon, sea trout, common carp and three-spined stickleback (n=6 per species; details in Table 4.1) were euthanised with an overdose of MS-222 and subsequently pithed to destroy the brain (Home Office Schedule 1 method). Skin was subsequently removed from each fish and samples from each respective fish species were pooled together and homogenised in phosphate buffered saline (PBS 1X) at a concentration of 0.1g skin ml^{-1}. The homogenised solution was centrifuged at 1000 rpm for 5 min. The resulting pellet was discarded and the supernatant was aliquoted and stored at -20°C until required.

A modified capillary root model (Halsall 1976) was used to assess the induced zoospore encystment responses of the four *S. parasitica* isolates listed in Table 4.2.

The assay was performed in a plastic petri dish (48 mm base diameter x 12 mm depth) containing 5 ml of zoospore suspension prepared as described previously and adjusted to a concentration of ~300 zoospores ml^{-1}. A cell scraper was used to ensure no spores were encysted on the sides or base of the petri dishes and that the spores were evenly dispersed throughout the suspension. Two micro-capillary tubes (Drummond Scientific, 2.9 cm length, 20 µl volume) were introduced into the petri dish, containing either the fish skin solution ('test' solution) or PBS (1X 'control' solution) and positioned at specific distances apart using forceps (see Figure 4.1A). The test and control tubes were left in the zoospore solution for 10 min, then removed and the contents of each expelled into an Eppendorf tube. These tubes were vortexed for 45 s, causing any zoospores to encyst. The number of encysted zoospores in each tube was then counted using a haemocytometer.

To account for potential differences between batches of zoospores, the assays were conducted in the following manner; for each *S. parasitica* isolate 2 batches of zoospores were produced. Per batch, 10 replicates of the assay were performed against each fish species. Hence, across the 2 batches of spores per isolate, a total of 20 replicates were achieved against each fish species. To control for potential side bias, the position of the test and control tubes was alternated. Room lighting (constant overhead LED lighting) and temperature (20±1°C) were kept constant throughout the assays. The induced zoospore encystment responses were expressed by a ratio that was calculated using the following equation:

$$\text{Induced zoospore encystment ratio (IZER)} = \frac{\text{Mean no. of zoospores in 'test' tubes}}{\text{Mean no. of zoospores in 'control' tubes}}$$

The assay was subsequently modified to assess the induced zoospore encystment responses of the salmon (EA001) and sea trout (EA016) *S. parasitica* isolates (see Table 4.2) when presented with a direct choice between the skins of two fish species. Micro-capillary tubes containing salmon skin solution ('salmon test') and sea trout skin solution ('sea trout test') were used in the same assay alongside a PBS control (Figure 4.1B). All other experimental conditions were consistent with those described above. The induced zoospore encystment responses of these isolates towards salmon and sea trout were calculated using the induced zoospore encystment ratio (IZER) equation above.

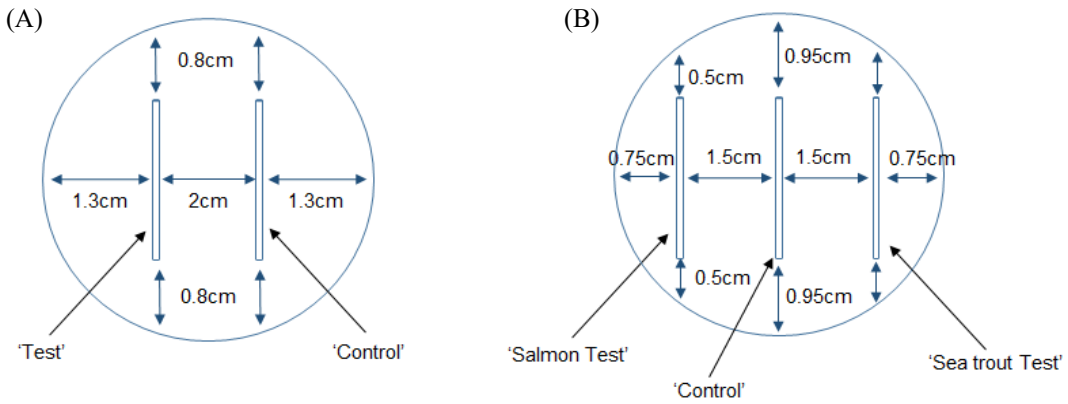

Figure 4.1. Induced zoospore encystment assays - experimental set-up. (A) Assay used to assess the induced zoospore encystment of four Saprolegnia parasitica *isolates: EA001 (salmon), EA016 (sea trout), EA012 (common carp) and CF006 (three-spined stickleback) against a fish skin extract (from salmon, sea trout, common carp or three-spined stickleback) and PBS control. (B) Modified assay used to compare the induced zoospore encystment of isolates EA001 (salmon) and EA016 (sea trout) when simultaneously exposed to salmon and sea trout skin extracts alongside a PBS control.*

In vivo Saprolegnia parasitica *challenge infections*

Challenge infections were conducted to assess whether the induced zoospore encystment ratios (IZERs) obtained for the salmon (EA001) and sea trout (EA016) *S. parasitica* isolates were reflected in infection outcomes. The experimental procedure described here was performed separately for these isolates in order to avoid cross contamination. Zoospore suspensions were prepared as described above and a concentration of ~3x10^5 L^{-1} used for all of the experimental infections. Juvenile salmon and sea trout (n=60 per species, see Table 4.1) were subjected to an adjusted 'ami-momi' technique (Hatai and Hoshiai, 1994) in which they were individually shaken in a net for 30 s to introduce abrasions to the fish body and remove protective mucus. Of these fish, n=50 per species were assigned to the 'treatment' condition and placed into glass aquaria (31 W x 61 D x 31 H cm) separated by species, containing a well-oxygenated zoospore suspension at a density of 1 fish L^{-1} for 24 h without food. Following zoospore exposure, fish were transferred into individual transparent plastic 1 L containers of dechlorinated water and daily feeding was resumed. The remaining fish acted as the 'controls' (n=10 per species) and were handled in exactly the same manner without exposure to zoospores, before transfer to individual containers. Water in both the treatment and control containers was changed every 24 h. Fish were checked hourly for signs of saprolegniasis over the duration of the experimental period of 168 h. Fish were categorised as either symptomatic (mild and cleared or severe) or

asymptomatic according to the severity of their symptoms (Table 4.3). Any fish displaying severe signs of infection were euthanised via overdose with MS-222 and pithing.

	Symptomatic	
Asymptomatic	Mild and cleared infection	Severe infection
• No signs of saprolegniasis	• Small tufts of mycelial growth on the body which were no longer present upon conclusion of the experiment	• Extensive mycelial body coverage • Lethargy • Respiratory distress • Loss of equilibrium

Table 4.3. Fish in the Saprolegnia parasitica *experimental infections were categorised as either asymptomatic, symptomatic (mild and cleared) or symptomatic (severe) of saprolegniasis according to these symptoms.*

This experiment was subsequently repeated using two different groups of juvenile salmon from the River Tyne: one from the North Tyne and another from the South Tyne. While there is no available data to confirm whether these were infact genetically distinct populations of fish, the literature on within-river genetic structure of salmon suggests that river tributaries generally contain distinct populations (Vähä et al. 2007). Hence these will be referred to as different populations from here onwards. The fish were kept separated according to population and all other experimental procedures were the same as those described previously.

Animal Ethics

All procedures and protocols were approved by the Cardiff University Animal Ethics Committee and conducted under UK Home Office license PPL 30/3424.

Statistical Analyses

Analyses were conducted using R statistical software (version 3.5.1, R Core Team 2018) with the significance threshold $P<0.05$ used for all models. Non-significant terms were removed during model refinement based on Analysis of Variance (Crawley 2007) while model robustness was assessed using residual plots (Pinheiro and Bates 2000).

A Generalised Linear Model (GLM) fitted with a Gaussian error family and identity link function was used to examine the induced zoospore encystment of four *S. parasitica* isolates towards the skin of four fish species. Induced zoospore

encystment ratio (IZER) was the dependent term in the model, fixed terms included: *S. parasitica* isolate (EA001, EA016, EA012, CF006), fish skin (salmon, sea trout, common carp, three-spined stickleback - herein referred to as stickleback) and the interaction between these two terms. Zoospore batch (1 vs 2) was included as a fixed factor in the original model but was removed during model refinement due to non-significance (P>0.05). *Post-hoc* analysis (lsmeans package; Lenth 2016) was conducted to compare the IZERs of each *S. parasitica* isolate.

A second GLM fitted with a Gaussian error family and identity link function was used to examine the induced zoospore encystment of the salmon (EA001) and sea trout (EA016) isolates when presented with salmon and sea trout skin in a single assay. The dependent and independent terms were the same as those outlined in the previous model and again, zoospore batch (1 vs 2) was included in the original model but removed due to non-significance (P>0.05). Comparisons between the IZERs of the two *S. parasitica* isolates were assessed using *post-hoc* analysis (lsmeans package; Lenth 2016).

Proportional odds logistic regression (POLR) models (MASS package; Venables and Ripley 2002) were used to examine the infection outcomes of the *S. parasitica* experimental challenge infections. Infection outcome of each fish (asymptomatic, mild and cleared, severe) was the dependent term in the models, fixed effects included; *S. parasitica* isolate (salmon isolate EA001 vs sea trout isolate EA016), fish species/population (model 1 - salmon vs sea trout, model 2 – North Tyne vs South Tyne salmon) and fish standard length (mm).

Kaplan-Meier survival curves (survival package; Therneau 2015) were plotted for challenge infections involving the sea trout isolate (EA016) only, log-rank tests were used to compare the survival curves. Survival analysis was not possible for the salmon isolate (EA001) as no mortalities occurred during the challenge infections with this isolate.

Results

Phylogenetic analysis

All four isolates fell within Cluster 3 of the taxonomic system proposed by Sandoval-Sierra et al. (2014) alongside other *S. parasitica* isolates (Figure 4.2).

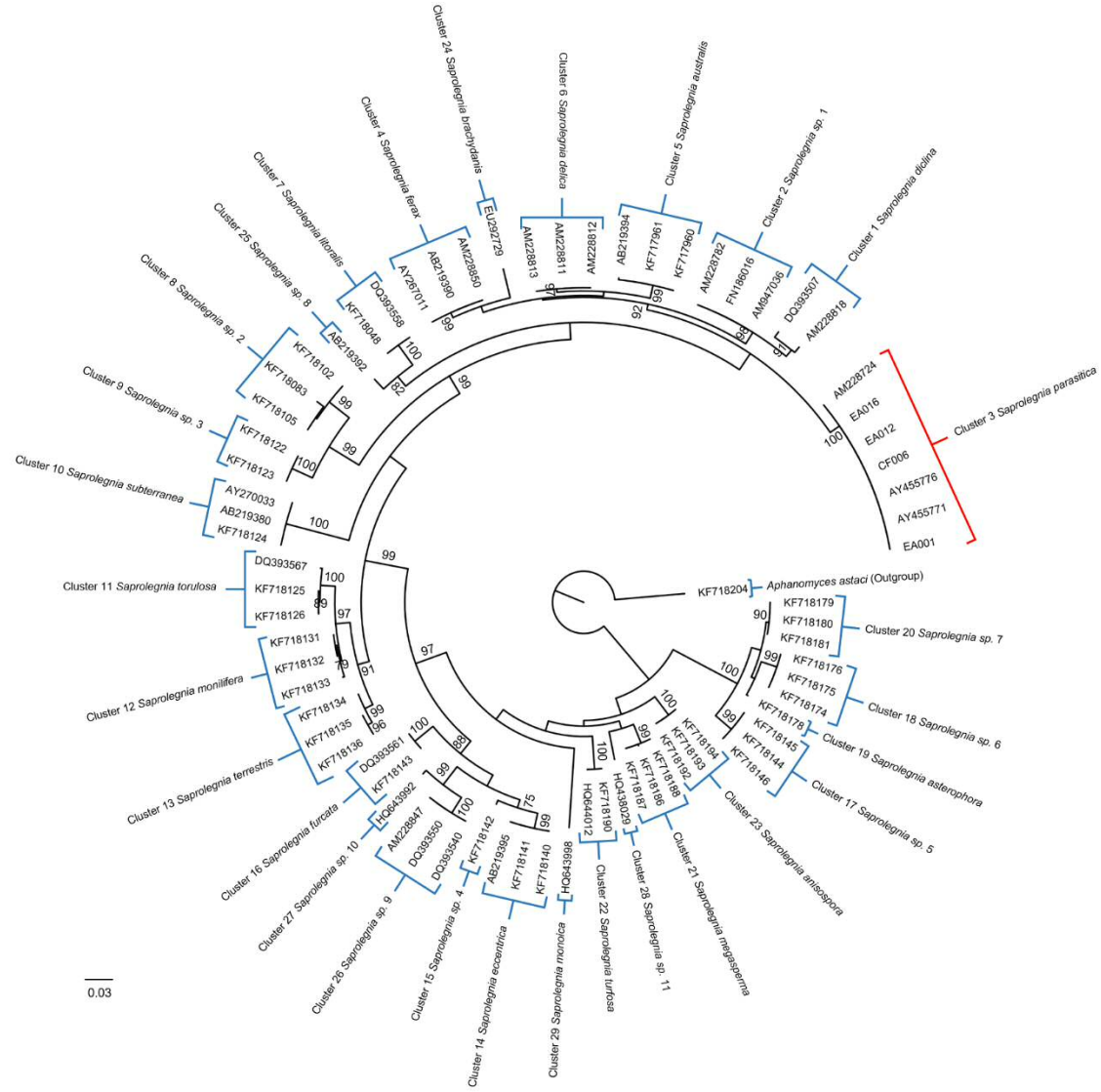

Figure 4.2. Circular phylogenetic tree showing the relationships of; the salmon (EA001), sea trout (EA016), common carp (EA012) and three-spined stickleback (CF006) isolates within the Saprolegnia *genus (highlighted by red bracket) inferred from maximum likelihood analysis using the Jukes-Cantor model. Bootstrap support values >75% based on 1000 replicates are indicated next to the branches. The scale bar indicates the number of nucleotide substitutions per site.*

In vitro *induced zoospore encystment assays*

Induced zoospore encystment ratios (IZERs) displayed by the *S. parasitica* isolates (EA001, EA016, EA012, CF006) were dependent on fish skin (salmon, sea trout, common carp, stickleback) (GLM; df=9, P<0.0001), but there was no clear trend in specificity. The salmon isolate (EA001) showed a significant preference for salmonid and carp over stickleback (Figure 4.3A). The sea trout isolate (EA016) IZERs were significantly higher for sea trout and common carp compared to salmon or stickleback skin (Figure 4.3B). The common carp (EA012) isolate displayed a preference for common carp and salmon over sea trout and stickleback skin (Figure 4.3C). Lastly, the IZERs of the stickleback isolate (CF006) were significantly higher for salmon compared to sea trout or stickleback skin (Figure 4.3D). In light of these results, the salmon EA001 and the sea trout EA016 isolates were selected for further *in vitro* induced zoospore encystment testing and *in vivo* challenge experiments (EA001 showing no preference for sea trout or salmon, compared to EA016 preferring sea trout skin).

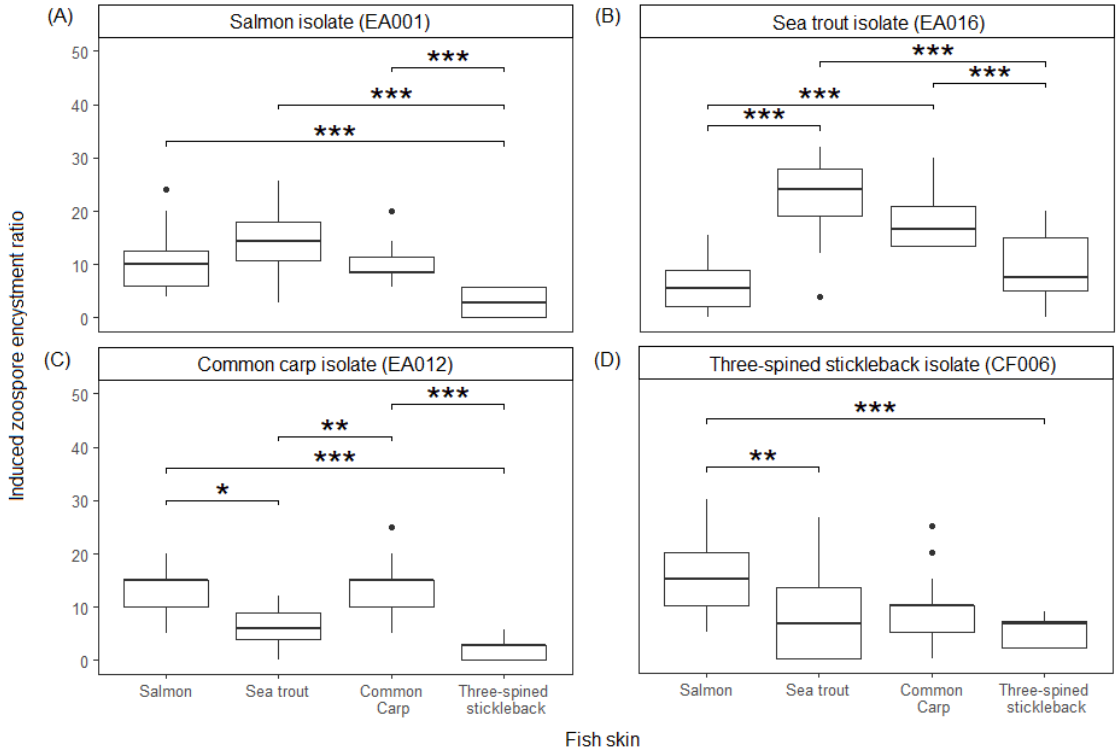

Figure 4.3. Induced zoospore encystment ratios of four Saprolegnia parasitica *isolates: (A) Salmon isolate (EA001), (B) Sea trout isolate (EA016), (C) Common carp isolate (EA012) and (D) Three-spined stickleback isolate (CF006) against skin extracts from four fish species (salmon, sea trout, common carp and three-spined stickleback). Statistical significance displayed; P<0.05(*), <0.01 (**), <0.001 (***).*

When simultaneously presented with salmon and sea trout skin, zoospores of the salmon isolate (EA001) and the sea trout isolate (EA016) displayed consistently generalist and specialist induced zoospore encystment responses, respectively. For EA001, there were no significant differences in IZER for salmon and sea trout skin (P>0.05) (Figure 4.4). Conversely, we confirmed the preference of EA016 for sea trout compared to salmon skin (P<0.0001) (Figure 4.4).

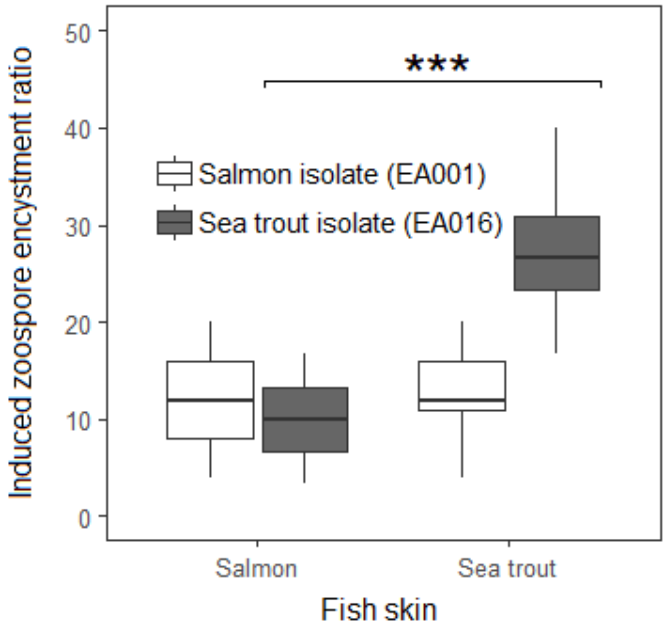

Figure 4.4. Induced zoospore encystment ratios of the Saprolegnia parasitica *zoospore isolates; salmon isolate (EA001) and sea trout isolate (EA016) when presented with a direct choice between salmon and sea trout skin extracts. Statistical significance displayed; P<0.0001 (***).*

In vivo Saprolegnia parasitica *challenge infections*

Infection outcomes (asymptomatic, mild and cleared, severe) from the *S. parasitica* challenge infections revealed no evidence of isolate-specific host specificity. Neither fish species (salmon vs sea trout; Figure 4.5A and B), fish population (North vs South Tyne salmon; Figure 4.5C and D) nor fish standard length significantly affected fish infection outcomes for either parasite isolate (salmon isolate EA001, sea trout isolate EA016; GLMs; df=1, P>0.05). Infection outcomes were, however, significantly different between the two *S. parasitica* isolates (GLMs; df=1, P<0.0001); EA001 established infections in only 34% and 24% of salmon and sea trout respectively (Figure 4.5A) in addition to 20% and 24% of North and South Tyne salmon (Figure 4.5C). Conversely, EA016 successfully infected 100% of challenged fish (Figure 4.5B and D).

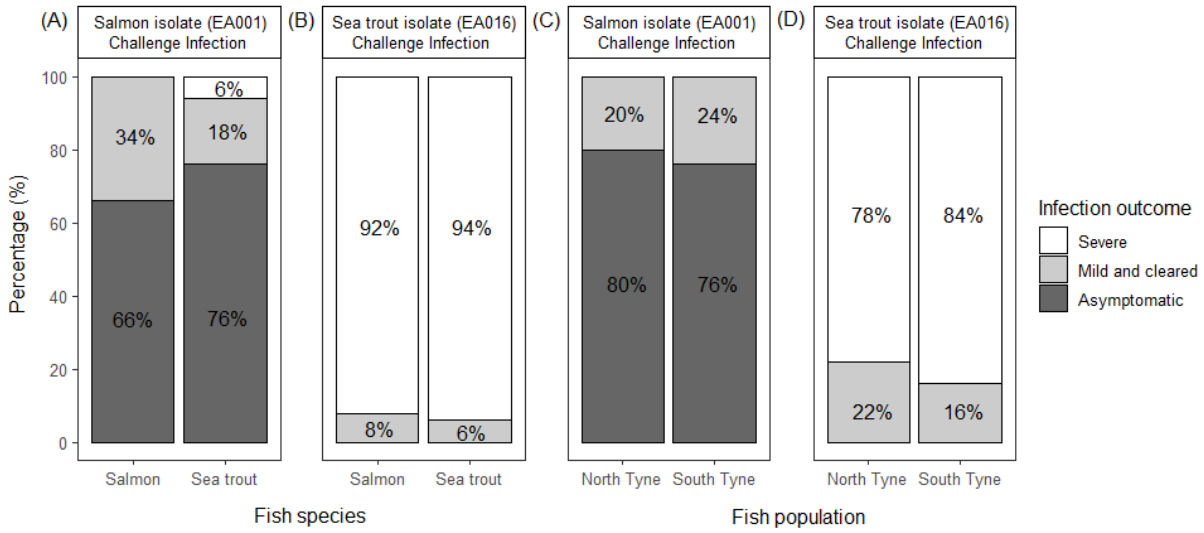

Figure 4.5. Percentage of salmon and sea trout, and North and South Tyne salmon that were asymptomatic, mild and cleared and severely infected with saprolegniasis when challenged with the Saprolegnia parasitica *salmon isolate (EA001; (A) and (C) respectively), and the sea trout isolate (EA016; (B) and (D) respectively).*

Indications of host-preference were observed from the survival analysis; while there was no overall significant difference in the survival of salmon and sea trout challenged with the sea trout isolate (EA016) (log-rank test; $P>0.05$), there was a significant difference during the initial 48 h of the infection (Figure 4.6A). At 24 h, sea trout survival was reduced to 84%, whereas none of the salmon died during this period (log-rank test; $P<0.05$; Figure 4.6A). At 48 h, 60% of the sea trout were alive compared to 96% of the salmon (log-rank test; $P<0.0001$; Figure 4.6A). There was no significant difference in survival for North and South Tyne salmon challenged with the sea trout isolate (EA016) (log-rank test; $P>0.05$; Figure 4.6B).

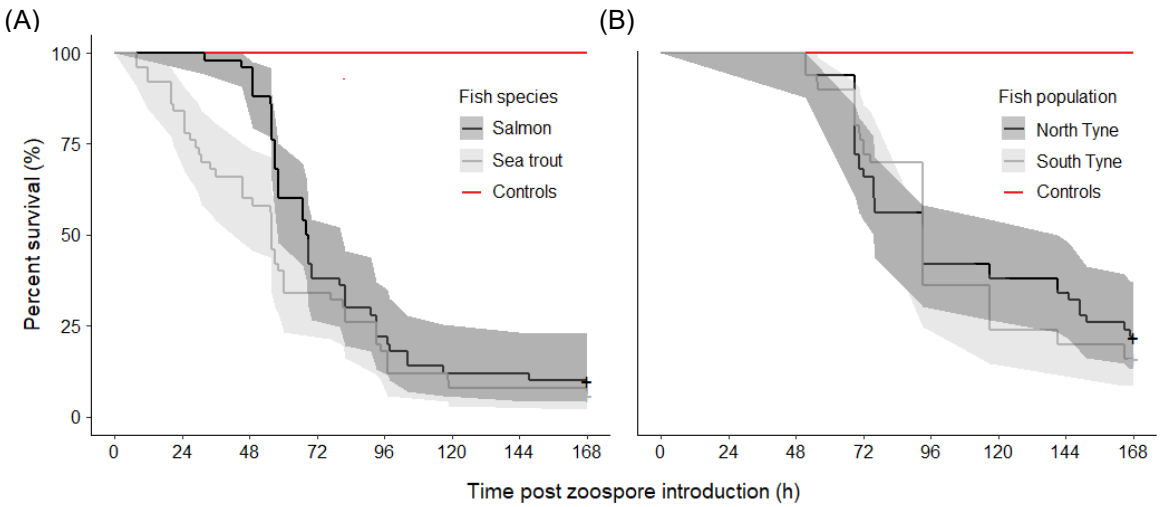

Figure 4.6. Survival curves showing percent survival (+95% confidence intervals) of (A) Salmon and sea trout, and (B) North and South Tyne salmon challenged with Saprolegnia parasitica *sea trout isolate (EA016). Control fish (both species and populations) not challenged with* S. parasitica *displayed 100% survival.*

Discussion

The current study is the first to investigate the host-specificity of *S. parasitica* at an isolate level. The *in vitro* induced zoospore encystment assays indicated differences in host specificity between isolates; the salmon isolate (EA001) appeared to be more of a generalist, exhibiting a similar level of preference for three fish species, whereas the sea trout (EA016), carp (EA012) and stickleback (CF006) isolates appeared more specialist, showing a higher preference for one or two fish species. These *in vitro* results, however, were not reflected in the *in vivo* challenge infections; no differences between salmon and sea trout infection outcomes were observed within the salmon isolate (EA001) or sea trout isolate (EA016) challenges. Infection outcomes between salmon populations (North and South Tyne) were also not significantly different for either isolate, suggesting specificity does not occur at a population level. Despite this, survival analysis revealed a significantly higher number of sea trout mortalities compared to salmon within 48 h of the sea trout isolate (EA016) challenge infection, which could be a potential indication of host preference.

The phylogenetic analysis presented here offered no separation between the four isolates as they all fell within Cluster 3 of the taxonomic system proposed by Sandoval-Sierra et al. (2014) alongside other isolates classified as *S. parasitica*. Interestingly, all isolates examined here yielded a low induced zoospore encystment response to sticklebacks. Moreover, even the more 'specialist' isolates demonstrated

a similar level of induced zoospore encystment for fish from different families. The induced zoospore encystment response of the sea trout isolate (EA016), for instance, was higher for sea trout and carp (families Salmonidae and Cyprinidae, respectively). Hence, reaffirming the postulation that *Saprolegnia* spp. are able to target a wide range of phylogenetically distant species. A potential criticism of the induced zoospore encystment assay is that homogenisation of the fish skin in solution would have destroyed its structure and biological components. However, El-Feki *et al.* (2003) employed the same methodology in their chemotaxis assay and found that fish skin induced the highest chemotactic response compared to other fish tissue extracts including mucus, blood, and gills.

Notably, the levels of virulence displayed by the salmon (EA001) and sea trout (EA016) isolates in the challenge infections were drastically different; this may be a reflection of different time in culture as EA001 was isolated 510 days before EA016. Maintaining pathogens (fungi, bacteria and viruses) on/in artificial culture media for extended periods can cause an attenuation of virulence (Druelle et al. 2008; Almaguer-Chávez et al. 2011; Ansari and Butt 2011). Passage through a susceptible fish host and subsequent re-isolation can restore virulence in *S. parasitica* cultures (Songe et al. 2014), there are, however contamination risks associated with re-culturing which could confound results.

The disparity between sea trout and salmon survival during the initial 48 h of the sea trout isolate (EA016) challenge infection could reflect the different induced zoospore encystment responses displayed by this isolate; if its zoospores are less attracted to salmon skin, as our induced zoospore encystment results would suggest, it may have taken longer to locate the salmon in comparison to the sea trout. It may also reflect subtle differences in the early stages of host-pathogen interaction. The initial attachment of *S. parasitica* zoospores to host cells is purportedly achieved via cell-binding proteins such as lectins (Jiang et al. 2013). In salmonids, this triggers a strong inflammatory response via the induction of proinflammatory cytokines and antimicrobial peptides (AMPs), in particular interleukin-1β1 (IL-1β1), IL-6 and tumour necrosis factor-α (TNF-α) (de Bruijn et al. 2012; Belmonte et al. 2014). Effector proteins and/or proteases then suppresses several constituents of the adaptive immune system by downregulating T-helper cell cytokines, antigen presentation machinery and immunoglobulins (Jiang et al. 2013; Belmonte et al. 2014).

Subsequently host cells are attacked by a multitude of virulence factors in the form of proteases, lipases and lysing enzymes (Jiang et al. 2013). It may be that the initial salmon immune-suppression by this isolate was not sufficient to establish an infection, consequently triggering upregulation of pathogen virulence factors to overcome the host immune response. This would explain the sudden drop in salmon survival between the 48 and 60 h period from 96 to 60%. Sequencing of the *S. parasitica* genome revealed the arsenal of virulence proteins employed by this pathogen are rapidly evolving due to co-evolution with the host (Jiang et al. 2013). Hence, isolates of *S. parasitica* could potentially target host species to which they have been more frequently exposed and consequently adapted to (Williams 1966; Kawecki and Ebert 2004; Savolainen et al. 2013). The sea trout isolate (EA016) examined here may be better adapted to sea trout, which is reflected in its induced zoospore encystment responses and ability to initiate rapid virulence and host mortality during the challenge experiment.

Overall, this study indicates that *S. parasitica* is a generalist with isolate variation in host preference. Uncovering the host preference of the key isolates within the UK could aid our understanding of disease outbreaks in the wild and fish management practises within aquaculture. For example, if the sea trout isolate (EA016) investigated here was present in a salmon aquaculture facility, knowledge of the 48 h lag in salmon mortality would prompt the application of treatments to boost immune function and potentially reduce mortalities.

Chapter 5 - Temperature and water flow impact *Saprolegnia* infections

Abstract

Despite *Saprolegnia* species being amongst the most important pathogens of salmon, the impact of environmental stressors on these oomycetes remains poorly understood. The current study examines the effects of temperature and water flow on *Saprolegnia* epidemics within English rivers and biological processes that contribute to transmission. Between 2010 and 2018, there was a significant relationship between mean spring flow rates and *Saprolegnia* levels in three English rivers, with lower flow rates observed in years with elevated *Saprolegnia*. Mean annual nor seasonal (summer, autumn, winter) flow rates, nor mean minimum/maximum temperatures, were significantly correlated with *Saprolegnia*. In the laboratory, vegetative growth rates of *Saprolegnia parasitica* and *S. australis* isolates generally increased with temperature but were not dependent on species or disease status (normal vs. elevated) of the river from which the isolates originated. Zoospore viability decreased at higher temperatures while the physical stimulation of water movement increased zoospore production in a temperature dependent manner. Lastly, salmon exposed to *S. parasitica* zoospores and low water flow experienced elevated saprolegniasis when compared with static (no flow) conditions. Such data contributes to our ability to predict disease outbreaks and inform fish management practices in both aquaculture and the wild.

Introduction

Infectious diseases caused by fungal and fungal-like pathogens are a global threat to biodiversity and food security (Fisher et al. 2012; Gozlan et al. 2014). This is particularly true for freshwater ecosystems which have experienced extensive biodiversity losses in recent decades as a result of anthropogenic factors, including pollution, invasive species, habitat destruction and water exploitation (Dudgeon et al. 2006). Oomycetes, commonly known as water moulds, are ubiquitous in freshwater environments and one of the most prominent groups of fungal-like organisms impacting both wild and cultured fish. The most problematic species belong to the genera *Aphanomyces, Achlya* and *Saprolegnia*. *Aphanomyces invadans* for example,

the causative agent of Epizootic Ulcerative Syndrome (EUS), reportedly infects over a hundred species of fish (Vishwanath et al. 1998; Blazer et al. 1999). Members of the *Saprolegnia* genus, are perhaps even more devastating, with *Saprolegnia parasitica* often described as the most important pathogenic oomycete (van West 2006). *Saprolegnia* spp. are reportedly responsible for 10% of annual global economic losses in salmonid aquaculture (Hussein and Hatai 2002; van West 2006; Phillips et al. 2008; Robertson et al. 2009; van den Berg et al. 2013) and this may in fact be an under estimation of impact, with actual losses as high as 50% (Bly et al. 1992; Hatai and Hoshiai 1992; van West 2006; Bruno et al. 2011). In addition to their adverse economic impact, *Saprolegnia* spp. are connected with worldwide declines of wild salmonid populations (van West 2006; Phillips et al. 2008). Generally, infections in wild salmonids occur during the spawning season when the immune system of sexually mature individuals is compromised and defensive fights over spawning beds can impair their protective mucus layer (Richards and Pickering 1978; Willoughby 1994). Incidences of *Saprolegnia* may be linked with environmental conditions, however, outbreaks in a given river are often sporadic, varying in severity from year to year (Environment Agency (EA), personal communication). A comprehensive assessment of the environmental factors that contribute to *Saprolegnia* proliferation is essential to identify potential predictors of epidemics and ultimately control disease spread.

Temperature is the abiotic factor most frequently linked to *Saprolegnia* infections; sudden temperature changes during the spring and winter months can elicit immunomodulatory effects that render fish more susceptible to infection (Bly and Clem 1991; Le Morvan et al. 1998). These effects are well demonstrated on catfish farms in the United States where mass *Saprolegnia*-induced mortalities occur during the winter months, hence the common name “winter kill syndrome” (Bly et al. 1992). Furthermore, Stewart et al. (2018) demonstrated that the temperature at the time of *Saprolegnia* exposure influenced infection success, but not previous thermal conditions experienced by the fish. It is important, however, to untangle the effects of temperature on the host and the pathogen itself. Moreover, *Saprolegnia* spp. have both sexual and asexual life stages which may be impacted differently. During the asexual stage of the life-cycle, free-living zoospores are produced which are responsible for locating and attaching to potential hosts. Sudden decreases in ambient water

temperatures are known to trigger this process (Bly et al. 1992); however, optimal temperatures for zoospore production and viability have not been thoroughly investigated. Once zoospores have successfully attached to a host, it is the vegetative stage that is responsible for host colonisation, with hyphal branches penetrating the host epidermis. The impact of temperature on *Saprolegnia* vegetative growth rate has been previously assessed (Kitancharoen et al. 1996; Koeypudsa et al. 2005), though it has not been examined in a real-world context and linked to outbreak data from sampling sites. This combined with zoospore production and viability data for multiple *Saprolegnia* isolates across the geographic landscape will help us to fully understand the influence of this abiotic factor and identify potential trends in epidemics.

River water temperature is influenced by a range of factors, both climatic and hydrological. A clear relationship exists between water temperature and flow rate in particular, with temperature increases often observed during low flow events (van Vliet et al. 2011). Anecdotally, increased levels of saprolegniasis are correlated with these low flow, warm temperature occurrences in temperate ecosystems (van West 2006). Indeed, low flow rates led to increased levels of saprolegniasis and reduced hatching success in rainbow trout (*Oncorhynchus mykiss*) eggs (Rach et al. 1995). However, the effect of water flow on *Saprolegnia* zoospore production remains poorly understood.

The current study combines field and laboratory data to examine the individual and combined effects of temperature and water flow on *Saprolegnia* infections. According to historic data collected by the EA, three English rivers; the Coquet, Dart and Esk, have experienced severe but intermittent outbreaks of *Saprolegnia* in recent years (see Chapter 2). Here, we attempted to correlate infection records for these severely affected rivers with temperature and flow data. We predict that increased disease incidences will be correlated with temperature extremes (low and high) and low water flow. Then, a series of laboratory-based experiments examined the impact of these abiotic factors on *Saprolegnia parasitica* and *S. australis* isolates; primarily focusing on those collected from the aforementioned severely affected rivers, but also including 11 isolates from less severely impacted rivers/waterbodies. Specifically, we assessed key processes that underpin *Saprolegnia* transmission: the effects of temperature on vegetative growth rate and zoospore viability, the combined effects of temperature and flow on zoospore production, and the effect of flow on salmonid

susceptibility to infection. Taken together, this information will help to predict future saprolegniasis outbreaks.

Materials and Methods

Host origin and maintenance

Juvenile salmon (n=140, age 1+, mean standard length = 61.9 mm, range = 45.2 to 94.6 mm; mean mass = 3.5 g, range = 1.7 to 10.7 g) were net caught from the Kielder Salmon Centre, Northumberland, and transported to the aquatic facilities at Cardiff University in July 2017. Prior to experimental procedures, fish were maintained in 90 L tanks with a water temperature of 12±0.5°C and oxygen saturation of >91%. Unless otherwise stated, fish were fed trout pellets daily for the duration of experimental proceedings. All experiments were performed under a 12 h light: 12 h dark cycle.

Saprolegnia *culture and zoospore production*

The *Saprolegnia parasitica* reference isolate CBS223.65, originally obtained from a Northern Pike (*Esox lucius*) in Centraal Bureau voor Schimmelcultures (CBS), the Netherlands in 1965 was kindly provided by Prof. van West (University of Aberdeen). All other samples were obtained directly from net caught fish hosts around England and Wales by the EA. A small tuft (approx. 4-5 cm^2) of mycelia was extracted from the affected tissue and placed immediately onto a potato dextrose agar (PDA, 39g L^{-1}) plate. Once sealed with parafilm, the plate was sent to our facilities at Cardiff University. Cultures were sub-cultured monthly onto fresh PDA plates as detailed by Stewart et al. (2017).

For the production of zoospores, petri dishes containing ~40 ml glucose-yeast broth (Glucose 10g L^{-1}, Yeast Extract 2.5g L^{-1}) were inoculated with three 5 mm diameter plugs of heathy white mycelium from the stock culture. The mycelia were left to grow for 72 h at room temperature (~20°C). To induce sporulation, the mycelial mats were washed with dechlorinated water and placed in a 50/50 mixture of dechlorinated water and aquarium water at 10°C for 72 h. Two 10 ml samples of the resulting zoospore solution were centrifuged at 4500 rpm for 10 min; the top 9 ml of this solution was then aspirated and the zoospores in the remaining 1 ml were re-suspended via pipetting. Zoospore number in the concentrated solution was subsequently enumerated using a haemocytometer.

English rivers experiencing Saprolegnia *outbreaks*

The Rivers Coquet (Northumberland, England), Dart (Devon, England) and Esk (Yorkshire, England) experienced elevated incidences of *Saprolegnia* infections between 2010 and 2018 (see Chapter 2). For each of these rivers, individual years were categorised in accordance to whether or not elevated levels of *Saprolegnia* were observed above long-term baselines monitored by local fishery officers with detailed knowledge of each river (Table 5.1).

	Year								
Elevated levels of *Saprolegnia* in River:	2010	2011	2012	2013	2014	2015	2016	2017	2018
Coquet	No	No	No	No	No	Yes	No	No	No
Dart	No	No	No	No	No	Yes	No	Yes	No
Esk	No	Yes	No	No	Yes	Yes	No	No	No

Table 5.1. Incidences of elevated Saprolegnia *occurrences in English rivers; Coquet, Dart and Esk from 2010-2018 (data from Environment Agency local fishery officers).*

Environmental data

For the period 2010-2018, air temperature (°C) data were obtained from the Met Office station (Camborne, 50°13'4.8"N, 5°19'37.2"W; Whitby Coast 54°28'51.6"N, 0°37'26.4"W; and Eskdalemuir (55°18'39.6"N, 3°12'21.6"W) geographically closest to each river (River Dart, Esk and Coquet, respectively) (Met Office 2019). These data were used to calculate annual, spring (March-May), summer (June-August), autumn (September-November) and winter (December-February) mean minimum and mean maximum temperatures for each year. When calculating winter means, data were used from December the previous year.

Daily flow rate data (m^3 s^{-1}) were obtained from the EA monitoring stations for the period 2010-2018 (data obtained directly from EA hydrometry and telemetry officers). For the River Dart, flow rate data were collated from the Bellever (50°34'55.2"N, 3°53'52.8"W), Dunnabridge (50°33'7.2"N, 3°55'1.2"W) and Austins Bridge (50°28'44.4"N, 3°45'39.6"W) monitoring stations, whereas for the Rivers Esk and Coquet, flow rate monitoring stations were situated at Briggswath (54°27'43.2"N, -0°39'14.4"W) and Morwick (55°19'58.8"N, 1°37'51.6"W), respectively. These data were used to calculate mean annual, spring (March-May), summer (June-August), autumn (September-November) and winter (December-February) flow rate for each

year. Again, data from December the previous year were used to calculate winter means.

Growth rate of Saprolegnia parasitica *and* Saprolegnia australis *isolates*

The growth rates of 11 *S. parasitica* and 2 *S. australis* isolates from sites experiencing either normal or elevated levels of saprolegniasis across England and Wales (Table 5.2) were assessed over a broad temperature range. A reference *S. parasitica* strain, CBS223.65, from the Netherlands was also included to examine the effect of serial sub-culturing. The experiment was performed in two separate batches. During batch 1, growth rates were obtained at 15, 25 and 30°C. During batch 2, the growth rates were recorded at 5 and 10 °C, but also repeating the 15°C trials to account for the elapsed time period (~ 35 weeks) between batches. Subsequently, isolates from the Rivers Coquet, Dart and Esk (those which had experienced elevated levels of *Saprolegnia*) underwent further *in vitro* growth rate analyses at 4, 6, 8, 10, 12 and 15°C. These reflect the typical range of UK river temperatures throughout the year.

Isolate	Location	Host species	Date isolated	*Saprolegnia* species
EA008	River Ouse, Yorkshire, England. (53°50'08.2"N, 1°04'33.4"W)	European eel (*Anguilla anguilla)*	25/11/2015	*S. australis*
EA021	Bells Mill Fishery, Stourbridge, England. (52°28'17.1"N, 2°10'56.9"W)	Mirror carp (*Cyprinus carpio*)	31/08/2016	*S. australis*
EA097*	River Coquet, Northumberland, England. (55°18'25.8"N, 1°55'20.5"W)	Atlantic salmon (*Salmo salar*)	19/04/2017	*S. parasitica*
EA016*	River Dart, Devon, England. (50°27'36.432' N, 3°41'42.144"W)	Sea trout (*Salmo trutta)*	03/06/2016	*S. parasitica*
EA001*	River Esk, Yorkshire, England. (54°26'59.1"N, 0°48'12.42"W)	Atlantic salmon	10/01/2015	*S. parasitica*
EA054	River Gaunless, County Durham, England (54°37'32.3"N, 1°46'13.6"W)	Sea trout	12/09/2016	*S. parasitica*
EA046	River Torridge, Devon, England. (50°57'25.1"N, 4°10'00.4"W)	Atlantic salmon	03/12/2016	*S. parasitica*
EA007	River Ouse, Yorkshire, England.	European eel	25/11/2015	*S. parasitica*
EA012	Lake near Romsey, Hampshire, England. (50°59'40.6"N, 1°34'46.7"W)	Common carp	22/03/2016	*S. parasitica*

EA020	Bells Mill Fishery, Stourbridge, England.	Mirror carp	31/08/2016	*S. parasitica*
CF006	Roath Brook, Cardiff, Wales. (51°29'54.1572"N, 3°9'54.2484"W)	Three-spined stickleback (*Gasterosteus aculeatus*)	15/07/2016	*S. parasitica*
CF009	Roath Brook, Cardiff, Wales.	Three-spined stickleback	09/08/2016	*S. parasitica*
CF010	Roath Brook, Cardiff, Wales.	Three-spined stickleback	15/08/2016	*S. parasitica*
CBS223.65 Reference isolate	Centraal Bureau voor Schimmelcultures (CBS), the Netherlands	Pike (*Esox lucius*)	1965	*S. parasitica*

Table 5.2. Saprolegnia parasitica *and* S. australis *isolates examined in the* in vitro *growth rate assay. *Denotes isolates from the Rivers Coquet, Dart and Esk that have experienced elevated levels of* Saprolegnia *and were used in further experiments.*

Petri dishes (145 mm x 20 mm, Greiner Bio-One) filled with ~50 ml of PDA (39g L^{-1}) were inoculated in the centre with an agar plug (5 mm diameter) of healthy white mycelia taken from the stock *Saprolegnia* culture. At 12 h intervals, the inoculated agar plates were photographed using a Samsung Galaxy S7 camera against a black background with overhead strip lights (24 cm above the plate) to provide standard lighting conditions and minimise reflections. When the mycelia reached the edge of the agar plate, photographs were no longer taken. A total of 10 replicates were produced for each isolate, at each of five temperatures (5, 10, 15, 25 and 30°C). Photographs were analysed by measuring the area of culture growth using the imaging software ImageJ v1.51j8.

Effect of temperature on Saprolegnia parasitica *zoospore viability*

Zoospore viability assays were conducted on *S. parasitica* isolates from the Rivers Coquet, Dart and Esk (Table 5.2). A stock zoospore solution was diluted to achieve a concentration of ~ 2 x10^4 zoospores ml^{-1} and subsequently divided into 1 ml aliquots. Of these aliquots, 40 were placed into each water bath at 5, 10, 15 or 25°C. Every 24 h, 10 aliquots were removed and zoospore viability assessed with a propidium iodide (PI) assay. To each 1 ml aliquot, 1 µl of PI (conc. 1mg ml^{-1}, Sigma-Aldrich) was added and left to incubate in the dark for 5 min. The proportion of live and dead zoospores was then counted immediately in a haemocytometer using an Olympus BX40 fluorescence microscope (400X magnification). This protocol was repeated for a second batch of zoospores, resulting in 20 replicates for each temperature and time point.

Effect of flow and temperature on Saprolegnia parasitica *sporulation*

To determine how flow and temperature impact sporulation of *S. parasitica*, mycelial mats of the River Coquet, Dart and Esk isolates (Table 5.2) were produced as described above. The mycelia were washed with dechlorinated water to remove excess broth, and approximately 10 g of mycelia were then placed into a porous bag and submerged in 1.5 L dechlorinated water within a small tank (dimensions H 17 x W 13 x D 13 cm). To generate 'flow' conditions, a water pump (Blagdon MiniPond Feature Pump 275l) was placed directly opposite the porous bag containing the mycelia (constant flow rate of 7.64 $x10^{-5}$ m^3 s^{-1}, or 0 m^3 s^{-1} for the control). At 24, 48, 72 and 96 h, two 10 ml samples of water were removed and the number of spores enumerated using a haemocytometer. The 20 ml of water removed at each sampling time point was immediately replaced with dechlorinated water to maintain a constant volume. The experiment was conducted at 5, 10, 15 and 25°C with 10 replicates per temperature for both flow and control treatments.

Effect of flow and Saprolegnia parasitica *exposure on juvenile salmon survival*

To determine whether the presence of flow impacts fish infection rates, this experiment was conducted in open channel re-circulatory flumes with and without flow. Briefly, each flume (L 150 x W 20 x D 16 cm) possessed a 10 cm diameter impeller attached to a 1 hp three-phase 4-pole motor capable of a maximum shaft speed of 1500 rpm (Machine Mart) wired to a 1.1 kW inverter (RS Components) to control the speed of the motor (see Hockley et al. 2014). At either end of the flume was a 2 cm aluminium honeycomb flow straightener with a 6.4 mm cell diameter, which ensured laminar flow throughout the flume and prevented fish from approaching the impeller region. The presence of these flow straighteners restricted the fish to a 100 cm length region, equivalent of 300 m^3. The flumes were filled to a depth of 15 cm with either a dilute *S. parasitica* zoospore solution (River Dart isolate - EA016), ~$3x10^5$ zoospores L^{-1}, or dechlorinated water depending on the treatment group. The flumes were well aerated and maintained at 10±1°C throughout the trials.

Salmon (n=70) were divided into 4 treatment groups: *S. parasitica* with and without flow (n=25 each), and controls with and without flow (n=10 each). A second replicate of this experiment was then performed with a different batch of *S. parasitica* zoospores and salmon. All other experimental proceedings including sample sizes

remained the same and the re-cirulatory flumes were cleaned thoroughly with Virkon in between each replicate. For the flow treatments, the continuous flow rate of 0.26 m^3 s^{-1} was used as it induced rheotaxis without exhausting the fish. Prior to placement in their respective treatments, all salmon were subjected to an adjusted ami-momi net shaking procedure (Stewart et al. 2017) for 30 seconds. This removes protective mucus and introduces abrasions to the fish body and is the standard method for inducing saprolegniasis. After 24 h of zoospore exposure, during which the fish were not fed, fish were temporarily removed and kept in holding tanks for a few minutes while the flumes were drained, cleaned with Virkon and re-filled with dechlorinated water. The process of draining and refilling the flumes with dechlorinated water was repeated daily to ensure high water quality. The fish were maintained in their respective treatment groups for 168 h and monitored hourly for signs of saprolegniasis. Any fish displaying severe signs of infection were euthanised via overdose with MS-222 and pithing.

Animal Ethics

All procedures and protocols were conducted under UK Home Office license PPL 30/3424 and approved by the Cardiff University Animal Ethics Committee.

Statistical analyses

Analyses were conducted using R statistical software version 3.5.1 (R Core Team 2018) with a $P<0.05$ significance threshold. Model refinement involved removing non-significant terms based on Analysis of Variance (Crawley 2007) and model robustness was assessed using residual plots (Pinheiro and Bates 2000).

Generalised Linear Mixed Models fitted with binomial error structures and identity link functions (GLMMs, lme4 library; Bates et al. 2014) were used to investigate whether there was a correlation between elevated *Saprolegnia* levels and mean minimum air temperature, mean maximum air temperature and/or mean flow rate. Separate models were used for annual, spring, summer, autumn and winter data. In all models, river ID (Coquet, Dart Esk) and year (2010-2018) were included as random effects.

The growth rates of the 11 *S. parasitica* and 2 *S. australis* isolates alongside the CBS223.65 reference isolate at 15°C were first assessed for both batches (batch 1 – 15, 25 and 30°C; batch 2 – 5, 10 and 15°C) and it was confirmed that they were not

significantly different (P>0.05). For this, a Gaussian GLMM with identity link function was fitted; the dependent term was growth rate (mm h^{-1}), the fixed terms included: isolate, batch (1, 2) and the interaction between these terms, while the random terms were time and plate ID. Plate ID identified the isolate, temperature and replicate number. Subsequently, the growth rates at all temperatures were assessed in a single Gaussian GLMM, using the values from batch 1 for 15°C. The model was fitted with an identity link function, the dependent term was growth rate (mm h^{-1}) and the fixed effects included: temperature (5, 10, 15, 25 and 30°C), isolate (EA001, EA002 etc.) and the interaction between these two terms. As previously, time and plate ID were included as random terms. *Post-hoc* analysis (lsmeans package; Lenth 2016) was used to compare the growth rates between isolates. A similar model (same fixed and random terms) was used to investigate the growth rates of *S. parasitica* isolates from the Rivers Coquet, Esk and Dart at 4, 6, 8, 10, 12 and 15°C. *Post-hoc* analysis (lsmeans package; Lenth 2016) was conducted to compare the growth rates of the isolates at these temperatures.

The effect of temperature on *S. parasitica* zoospore viability was analysed using a Generalised Linear Model (GLM) fitted with a binomial error family and logit link function. The dependent term in the model was the percentage of live zoospores, fixed effects included: *S. parasitica* isolate (Coquet, Dart, Esk), temperature (5, 10, 15, 25°C), the interaction between these two terms, and experimental time point (24, 48, 72, 96 h). Zoospore batch (1, 2), included as a fixed effect in the starting model, was non-significant (P>0.05) and therefore removed during model refinement. *Post-hoc* analysis (lsmeans package; Lenth 2016) was conducted to compare the proportion of live zoospores for each *S. parasitica* isolate at the different temperatures.

A negative binomial GLMM ("glmmTMB" library; Brooks et al. 2017) fitted with an nbinom2 error family and log link function was used to investigate the effects of temperature and flow conditions on *S. parasitica* sporulation. Number of zoospores was the dependent term in the model, fixed effects included treatment (flow, no flow), temperature (5, 10, 15 and 25°C), and *S. parasitica* isolate (Coquet, Dart and Esk). The interaction term between treatment, temperature and isolate was also included in the model. Time of the zoospore count post-sporulation induction (24, 48, 72 and 96 h) and replicate ID were included as random terms in the model. Replicate ID uniquely represented the replicate (1-10) for each isolate at each treatment and temperature. To

compare the number of zoospores produced by each isolate at each temperature and treatment, *post-hoc* analysis was performed using the lsmeans package (Lenth 2016).

A GLM fitted with a binomial error family and a logit link function was used to examine the effect of flow vs. no flow on salmon mortality during a *S. parasitica* challenge infection. Experimental replicate (1, 2) and fish standard length (mm) were also included as fixed effects in the model.

Results

Environmental factors influencing Saprolegnia *incidences in English rivers*

There was a significant negative relationship between mean spring flow rates and *Saprolegnia* levels (GLMM; df=1, P<0.05), with lower flow rates observed in years with elevated *Saprolegnia* (P<0.05). Mean annual, summer, autumn and winter flow rates were not, however, significantly correlated with *Saprolegnia* increases (P>0.05). Also, there was no significant correlation between elevated *Saprolegnia* levels and mean minimum or mean maximum temperatures (in annual or seasonal models; both P>0.05).

Effect of temperature on Saprolegnia parasitica *and* Saprolegnia australis *growth rate*

Temperature significantly affected the vegetative growth rate of *S. parasitica* and *S. australis* isolates; responses were isolate specific (GLMM; df=52, P<0.0001; Figure 5.1 A, B and C). Comparisons between isolates revealed no specific trends according to the disease status (normal vs. elevated levels of saprolegniasis) of the respective waterbody, or *Saprolegnia* species (Figure 5.1A, B and C; see Table 5.2 for isolate details). For all isolates, growth rates generally increased as the temperature increased, peaking at 25°C before declining at 30°C (Figure 5.1A, B and C). Furthermore, the growth rates exhibited by the reference isolate CBS223.65 were similar to the other isolates (Figure 5.1B), despite an extended time spent in culture (CBS223.65 isolated in 1965, other isolates obtained between 2015-2017).

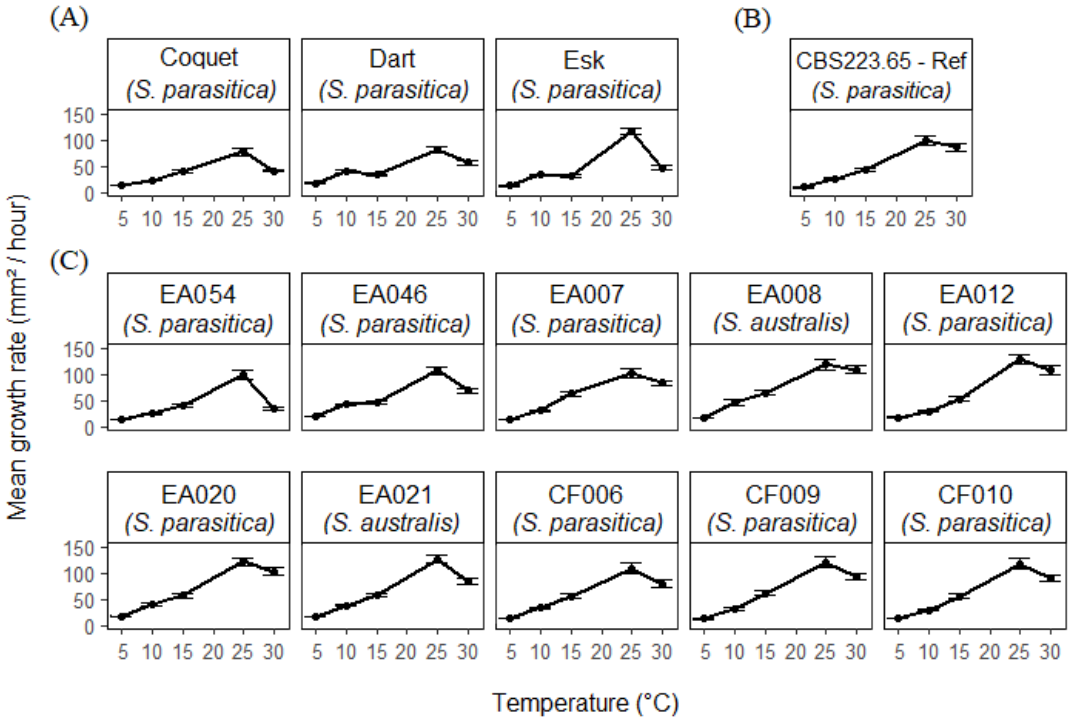

Figure 5.1. Growth rates of Saprolegnia parasitica *and* S. australis *isolates (±SE) at 5, 10, 15, 25 and 30°C. (A) Isolates from rivers that have experienced elevated saprolegniasis outbreaks (Rivers Coquet, Dart and Esk); (B) The reference isolate CBS223.65; (C) Isolates from sites that have experienced normal levels of saprolegniasis (River Gaunless (EA054); River Torridge (EA046); River Ouse (EA007, EA008); Lake near Romsey, Hampshire (EA012); Bells Mill Fishery, Stourbridge (EA020, EA021); and Roath Brook, Cardiff (CF006, CF009, CF010). Potato dextrose agar plates were inoculated with these isolates, a total of 10 replicates were obtained for each isolate at each temperature.*

Growth rate of Dart, Coquet and Esk Saprolegnia parasitica *isolates*

As in the previous growth rate experiment, temperature significantly affected growth rate and this was dependent on isolate (GLMM; df=10, P<0.0001; Figure 5.2). Growth rate generally increased with higher temperatures, but the isolates showed variable responses. The Coquet isolate grew significantly faster than the other two isolates at 6 and 8°C, the Dart isolate grew significantly faster at 12 and 15°C but slower at 8°C, while the Esk isolate grew significantly slower at 10 and 12°C (Figure 5.2).

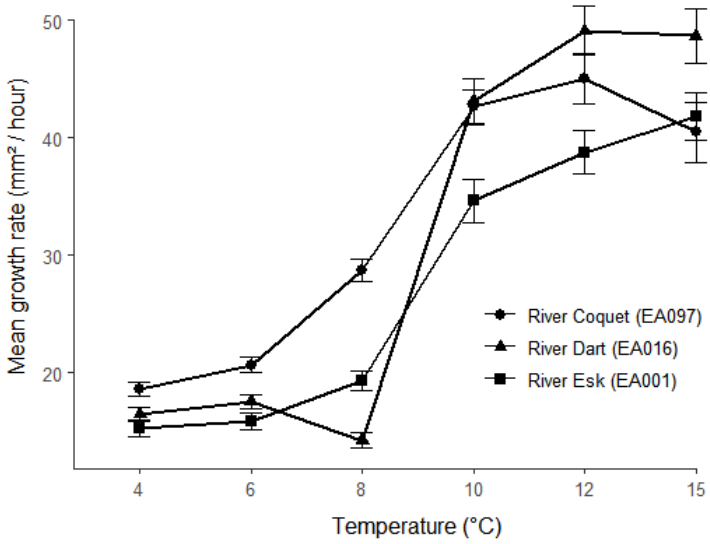

Figure 5.2. Growth rates of Saprolegnia parasitica *isolates (±SE) from Rivers Coquet (EA097), Dart (EA016) and Esk (EA001) at 4, 6, 8, 10, 12 and 15°C. Potato dextrose agar plates were inoculated with these isolates, a total of 10 replicates were obtained for each isolate at each temperature.*

Effect of temperature on Saprolegnia parasitica *zoospore viability*

Zoospore viability generally declined over time (GLM; df=3, P<0.0001) and was significantly affected by temperature and isolate (GLM; df=6, P<0.0001). For the River Coquet and Esk isolates, zoospore viability was significantly higher at 10°C compared to 5, 15 or 25°C (P<0.05; Figure 5.3). For the River Dart, the percentage of live zoospores at 5 and 10°C was significantly higher than at 15 or 25°C (P<0.0001; Figure 5.3). For all isolates 25°C yielded a significantly lower percentage of live zoospores than all other temperatures (P<0.0001; Figure 5.3). The River Coquet isolate was the most resilient, with zoospore survival significantly higher than both of the other isolates at 10 and 15°C (P<0.05), and higher than the River Esk isolate only at 25°C (P<0.0001).

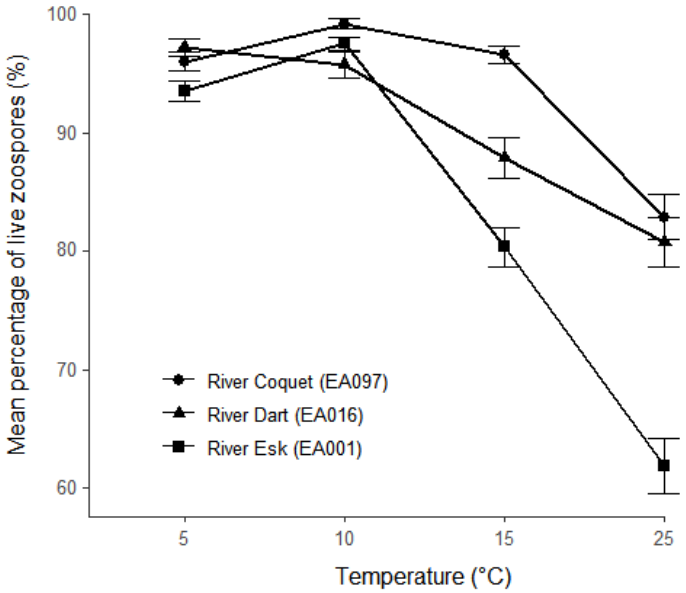

Figure 5.3. Mean percentage of live zoospores (±SE) of Saprolegnia parasitica *isolates from Rivers Coquet (EA097), Dart (EA016) and Esk (EA001) at 5, 10, 15 and 25°C.*

Effect of flow and temperature on Saprolegnia parasitica *sporulation*

Flow significantly affected *S. parasitica* sporulation and was dependent on temperature and isolate (GLMM, df=6, P<0.05). All isolates produced significantly more zoospores in the flow treatment compared to the control at all temperatures, except 25°C (P<0.05; Figure 5.4A, B and C). Under flow conditions, there was a clear optimal temperature for the River Coquet isolate with a significantly higher number of zoospores produced at 10°C (P<0.0001; Figure 5.4A). For the River Dart isolate, the number of zoospores produced at 15°C was significantly higher than at 5 and 25°C (P<0.05; Figure 5.4B). In contrast, a similar number of spores were produced by the River Esk isolate at 5, 10 and 15°C (P<0.05; Figure 5.4C).

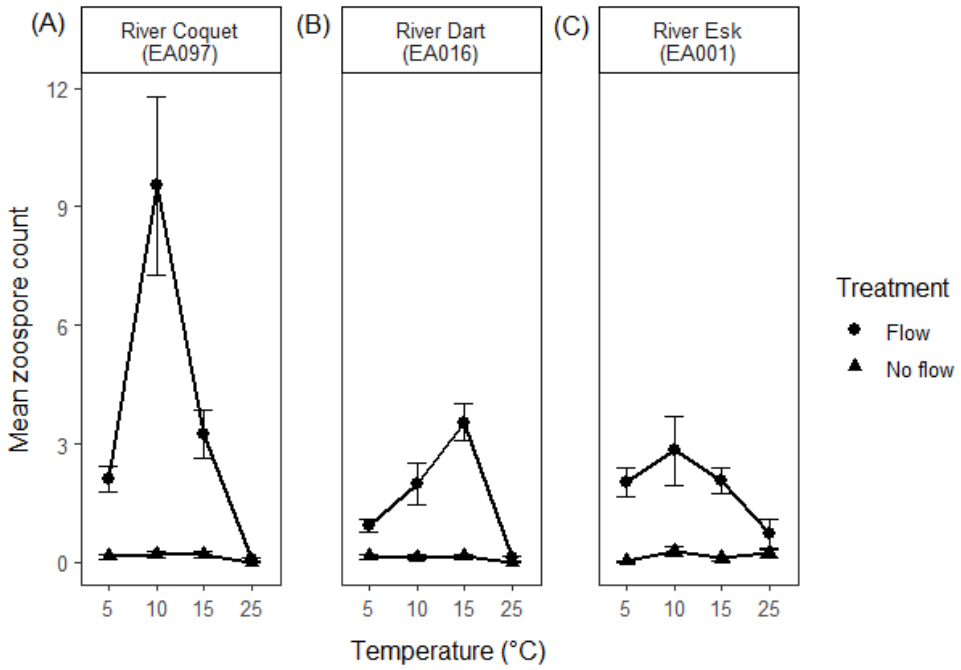

Figure 5.4. Mean zoospore count (±SE) of Saprolegnia parasitica *isolates from the River (A) Coquet (EA097), (B) Dart (EA016) and (C) Esk (EA001) in flow and no flow conditions at 5, 10, 15 and 25°C.*

Effect of flow and Saprolegnia parasitica *on juvenile salmon mortality*

Flow conditions significantly reduced salmon survival during the *S. parasitica* challenge infection (GLM; df=1, P<0.0001), with significantly more mortalities in the flow compared to no flow treatment (P<0.05; Figure 5.5A and B). Neither experimental replicate (1 vs 2) nor fish standard length (mm) was related to fish survival (GLM; df=1, P>0.05).

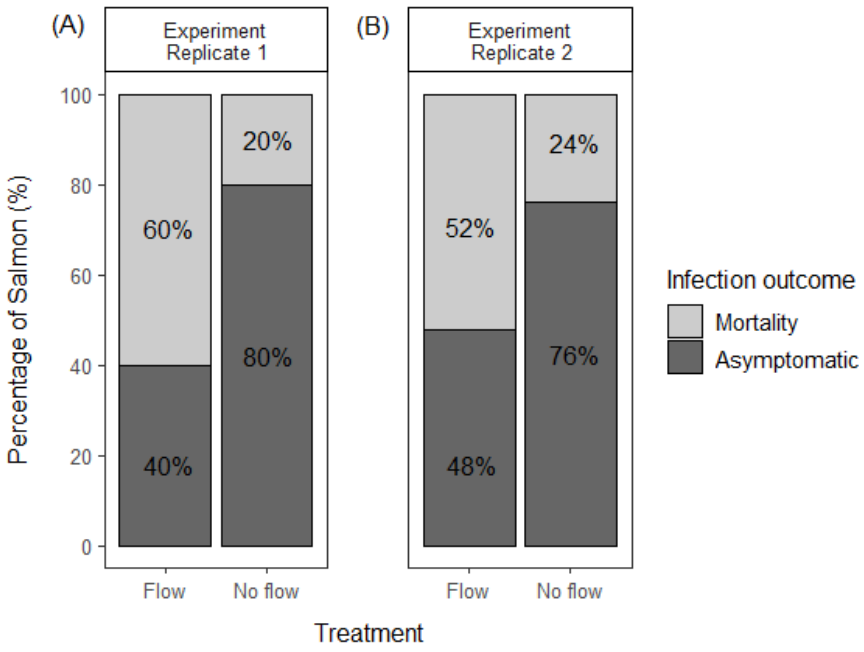

Figure 5.5. Percentage of salmon during; (A) Experiment replicate 1 and (B) Experiment replicate 2 that experienced mortality or were asymptomatic of saprolegniasis during Saprolegnia parasitica *challenge infections with and without flow.*

Discussion

The current study is the first to show increased *Saprolegnia* infections are associated with low water flow conditions in natural systems. Incidences of this parasite within the English Rivers Coquet, Dart and Esk were correlated with average spring water flow, with an elevated incidence in years with lower flow rates. In our laboratory experiments, *Saprolegnia* isolates displayed variable responses to temperature and water flow in terms of their vegetative growth, zoospore viability and sporulation indicative of their respective optimal conditions. Moreover, salmon experimentally exposed to *S. parasitica* zoospores under low water flow experienced increased disease incidences in comparison to no flow conditions.

The lack of association between temperature and historic incidences of *Saprolegnia* in the current study was surprising due to the well-established effects of temperature on fish immunity (Bly and Clem 1991; Le Morvan et al. 1998) and *Saprolegnia* sporulation (Bly et al. 1992; Kitancharoen et al. 1996). However, the importance of this abiotic driver is probably overridden due to the variable and complex conditions within the wild. The impact of temperature on *S. parasitica* and *S. australis* vegetative growth rate, however, was evident and generally consistent with

Koeypudsa et al. (2005). Moreover, the growth rates reported were repeatable; the experiment was performed twice at 15°C in separate batches and despite an extended period of time elapsed between them, there were no batch-specific differences. The variable responses to the temperatures examined were specific to isolate not disease status (normal vs. elevated) of their respective rivers/water bodies, or *Saprolegnia* species, conforming with Kitancharoen et al. (1996) who examined the growth rates of *S. parasitica* and *S. diclina* isolates. While differences in vegetative growth may not contribute to increased *Saprolegnia* disease incidences, the general increase in growth rates at higher temperatures suggest saprophytic growth within the environment is likely to intensify with climate change. Conversely, zoospore viability was generally higher at lower temperatures, with some isolates more sensitive to temperature increases. Hence climate change may have a negative impact on the abundance of infectious propagules produced by this parasite in temperate regions.

The combined effects of temperature and water flow greatly influenced *S. parasitica* sporulation; temperature alone, had little impact with few zoospores produced at any of the temperatures within the 'no flow' treatment. This is inconsistent with the known effects of temperature decreases below ~10°C on *Saprolegnia* sporulation (Bly et al. 1992); indeed Kitancharoen et al. (1996) demonstrated that *Saprolegnia* zoospore production was elevated at 10°C in comparison to 20 and 30°C. Water flow generally increased *S. parasitica* sporulation, but was dependent on temperature and isolate. This supports an observation by Thoen et al. (2016); when examining risk factors affecting zoospore numbers in salmon hatcheries, the authors noted that higher flow rates appeared to increase zoospore production but were unable to state this conclusively due to a lack of sites with comparable water temperatures. For general zoospore production presented in this thesis, mycelia were grown at room temperature and placed in a 50:50 mix of 10°C dechlorinated water and aquaria water, however sporulation was often sporadic and we were unable to establish conditions that led to consistent zoospore production across batches (see Appendix 2). Hence, while we may be able to state that water flow increases *S. parasitica* sporulation, the reliability of the isolate-specific responses to temperature and flow presented here may be questionable.

The physical stimulation of water movement appears to trigger sporulation, however, Diéguez-Uribeondo et al. (1994) demonstrated that mechanical agitation can

elicit *S. parasitica* zoospore encystment and subsequent release of a secondary zoospore. This process, known as repeated zoospore emergence (RZE), provides a brief resting stage during unfavourable zoospore swimming conditions and revives the infectious agent when conditions are more advantageous. So although the number of zoospores may increase with water flow, this may not result in higher infection levels since a large proportion of zoospores may be in the encysted stage and not actively seeking hosts. Consequently, it may be desirable to investigate the maximum flow rate that induces motile zoospores for respective isolates.

The low water flow implemented during the *in vivo S. parasitica* challenge infection was representative of late spring/summer UK riverine flow rates (EA water flow data). These flow conditions induced rheotaxis and forced the salmon to congregate together; although direct contact between hosts is not required for *Saprolegnia* transmission, the sustained close proximity between fish likely increased transmission between hosts and was notably absent from the no flow treatment. Moreover, the presence of flow may have increased the contact rate between zoospores and fish, thus aiding host establishment. Trinidadian guppies (*Poecilia reticulata*) exposed to continually flowing water acquired much higher *Gyrodactylus turnbulli* parasite burdens as oppose to those held in interrupted or no flow conditions (Reynolds et al. 2019). The energy demand placed on these fish under the continuous flow conditions likely compromised their immunity and enabled parasite proliferation (Reynolds et al. 2019). In contrast to *G. turnbulli* which has a direct life cycle and reproduces *in situ* on its host, *Saprolegnia* transmission is dependent on free living zoospores. Within the wild, periods of high water flow dilute the number of infectious zoospores within a river; this is likely to account for the anecdotal reduction in saprolegniasis at high water flow.

This research highlights the importance of uncovering the environmental factors impacting *Saprolegnia* infections. Further work focussed on the impact of temperature and water flow in light of future climate change is necessary to predict how this parasite will impact fish populations and aid the implementation effective fish management schemes.

Chapter 6 - Combined effects of variable thermal regimes and parasite exposure (*Saprolegnia parasitica*) on brown trout (*Salmo trutta*)

Abstract

A multitude of environmental stressors threaten the future prosperity and biodiversity of freshwater ecosystems. Moreover, interactions between these stressors can confound their effect on freshwater species and their overall impact remains poorly understood. Here, in a multi-staged experiment, we examined non-lethal thermal effects and pathogen exposure on the early life of an environmentally and economically important freshwater species, the brown trout (*Salmo trutta*). First, we examined the baseline effects of four different thermal pre-conditions on alevin mortality and growth. These thermal pre-conditions reflected: current environmental temperatures (CURR), climate change (CC), thermopeaking (TP) and climate change/thermopeaking combination (CC/TP). While there were no differences in alevin mortality between these thermal regimes, alevin growth was significantly higher following CC, TP and CC/TP pre-treatments compared to those within CURR. The second and third experimental stages studied the combined stress of the aforementioned thermal regimes and *Saprolegnia parasitica* exposure on brown trout mortality at two periods of early life: alevins and fry. In both cases, *S. parasitica* only increased mortality when fish were exposed to both warmer water temperatures and subsequent rapid temperature increases at the time of infection. These findings indicate the combined effects of climate change, thermopeaking and *S. parasitica* exposure pose a serious threat to already fragile brown trout populations and emphasise the need for effective river management strategies.

Introduction

Average global temperatures have increased 0.2°C per decade over the last 30 years (Hansen et al. 2010; Papalexiou et al. 2018) and further increases of up to 3°C are expected by 2050 (Rowlands et al. 2012; van Vliet et al. 2013). Climate change has been identified as the key contributor and its effects are already felt in UK freshwaters, with long-term studies reporting 0.03°C average annual increases in rivers (Orr et al. 2015). Freshwater environments are particularly vulnerable to the effects of climate change; their isolated nature, which has been exacerbated by anthropogenic-induced

connectivity loss (Geist and Hawkins 2016; Wohl 2017), restricts the opportunity for inhabitants to migrate in response to temperature fluctuations (Dudgeon et al. 2006; Strayer and Dudgeon 2010). Anthropogenic barriers, including hydropower installations (Moran and Dann 2008; Sample et al. 2015), have further eroded connectively in many riverine ecosystems. While renewable hydropower aims to reduce the effects of climate change, variations in energy demand often lead to fluctuating operational regimes, which cause sporadic water releases into rivers. Such hydropeaking may promote sub-daily fluctuations in natural river temperatures, resulting in thermopeaking (Toffolon et al. 2010; Zolezzi et al. 2011). Downstream water temperatures are generally lowered by this process in the spring and summer (Feng et al. 2018), and increased in the winter (Olden and Naiman 2010; Casas-Mulet et al. 2015). Thermopeaking has ecosystem-wide implications, including macroinvertebrate drift (Bruno et al. 2015), reduced aquatic plant abundance (Mjelde et al. 2013) and repercussions for fish as their immunology is greatly influenced by temperature (Le Morvan et al. 1998) affecting growth, spawning (Nelson 1986; Finch et al. 2015; Casas-Mulet et al. 2016) and other behavioural changes (Vollset et al. 2016).

Salmonids are particularly vulnerable to changing conditions; this includes the brown trout (*Salmo trutta*), a prized angling resource (reviewed by Mawle and Peirson 2009) which contributes to freshwater ecosystem balance by controlling invertebrate abundance and distribution (Jensen et al. 2008). In recent years, catch declines of European brown trout of up to 50% have been attributed to climate change (Poulet et al. 2011), with temperature known to impact their development (Ojanguren et al. 2001), behaviour (Breau et al. 2011), fecundity (Vladiĉ and Jätrvi 1997; Warren et al. 2012) and distribution (Taylor 2008; Santiago et al. 2016). The optimal temperature range for ontogenetic development is 8 - 10°C (Ojanguren and Braña 2003) with a lower lethal limit for embryonic development of ~1°C (Maddock 1974; Lahnsteiner 2012) and upper limit of 14 - 16°C (Elliott 1991; Elliott and Elliott 1995; Lahnsteiner 2012). Moreover, deformities occur more frequently in embryos incubated below 3°C and above 11°C (Lahnsteiner 2012; Réalis-Doyelle et al. 2016). Many studies have highlighted the potential effects of climate change on salmonids (Hari et al. 2006; Graham and Harrod 2009; Jonsson and Jonsson 2009; Elliott and Elliott 2010; Poesch

et al. 2016; Santiago et al. 2016), but our understanding of the combined impact of climate change and thermopeaking on *S. trutta* during early life is limited.

Disease presents another major stressor to salmonids. The freshwater oomycete *Saprolegnia parasitica* is a particularly devastating pathogen; responsible for an estimated 10% of deaths in all farmed salmon and has been increasingly associated with declines of wild salmon populations across England and Wales (Chapter 2). This pathogen causes the disease saprolegniasis, characterised by patches of grey/white cotton-like mycelia on epidermal tissues, predominantly on the head or fins during the initial stages of infection. These mycelia can subsequently spread to cover the entire body and cause impaired osmoregulation; the most frequent cause of death associated with this disease (Willoughby and Pickering 1977; van West 2006). In the wild, saprolegniasis is most prevalent during the winter and spring when temperature changes expose fish to thermal stress resulting in an impaired immune response and increased disease susceptibility (Pickering and Willoughby 1982; Pickering and Duston 1983; Bly et al. 1992). Climate change may intensify *S. parasitica* incidences in the wild due to the increased thermal stress on fish hosts.

While the optimal thermal thresholds for growth and survival of brown trout are known and *S. parasitica* infections on salmonids have been well documented, the combined effects of climate change and thermopeaking alongside *S. parasitica* exposure remains unexplored. The current study presents a multi-stage experiment which assesses the combined effects of varying non-lethal thermal regimes and parasite exposure on the mortality and growth rates of brown trout alevins and fry. The thermal regimes examined here reflect: i) our current climate, ii) predicted future climate, iii) thermopeaking in our current climate, and iv) thermopeaking in the predicted future climate. Given the association between brown trout declines and climate change, we predict the climate change and thermopeaking conditions examined here will cause an increase in brown trout mortality.

Materials and Methods

All practical work was conducted using a combination of indoor and outdoor facilities at Cardiff University. The first stage of the experiment, examining the effect of varying thermal regimes on the growth and survival of brown trout (*Salmo trutta*) alevins, took place between 24/01/18 -11/02/18. The thermal conditions of this experimental stage

formed the pre-conditions for the subsequent experimental stages. In the second stage, we assessed the combined effect of temperature and parasite exposure on the mortality of alevins between 13/02/18 - 19/02/18, and then finally these combined stressors were repeated on fry 12/03/18 - 18/03/18 during the third stage of the experiment.

Host origin and maintenance

Brown trout eggs, from Northern Trout (Brow Well Fisheries Limited, UK), were transferred to our aquatic facilities in January 2018. Specimens were all female diploid – this ploidy state is generally preferred as individuals are of a higher quality and more suitable for experimental use (O'Flynn et al. 1997). Eggs maintained within the appropriate temperature range for brown trout development (6-10°C) hatched into alevins ready for the start of the experiment on 24/01/18.

Parasite origin, maintenance and zoospore production

The parasite, *Saprolegnia parasitica* isolate EA016, originated from a sea trout (*S. trutta*) in the River Dart, UK on 03/06/2016. Mycelia (approx. 4-5 cm^2) were extracted from the affected tissue using forceps and placed directly onto a potato dextrose agar (PDA, 39g L^{-1}) plate, which was sealed with parafilm and transported to our facilities at Cardiff. A stock culture was then plated onto fresh PDA plates monthly according to Stewart et al. (2017). To produce zoospores, petri dishes containing ~40 ml glucose-yeast broth (Glucose 10g L^{-1}, Yeast Extract 2.5g L^{-1}) were inoculated with three 5 mm diameter plugs of heathy white mycelia from the stock culture and were left to grow for 72 h at room temperature (~20°C). The resulting mycelial mats were washed with dechlorinated water in order to remove excess glucose-yeast broth and placed in a 50/50 mixture of dechlorinated water and aquarium water at 10°C for 72 h to induce sporulation. Two 10 ml samples of the resulting zoospore solution were centrifuged at 4500 rpm for 10 min. The top 9 ml of solution was aspirated, zoospores in the remaining 1 ml were re-suspended via pipetting and enumerated using a haemocytometer. The concentration of the zoospore solution was then calculated and diluted to $3x10^5$ L^{-1} with dechlorinated water for use in the challenge infections.

Experimental setup

Four thermal conditions were examined during all three stages of this study. The current condition (CURR) represented current natural environmental water

temperatures (months Jan-Feb). In the climate change condition (CC), an increase of 3-4°C in environmental water temperature reflected the predicted climate change-induced temperature increase within the UK by 2050 (Webb and Walsh 2004; Hannah and Garner 2015). The third, thermopeaking (TP), condition involved intermittent increases of 2-3°C (Vanzo et al. 2016), typical of those currently occurring in the UK for 1 h daily (1600-1700) (Olden and Naiman 2010; Gavin 2014). The final condition examined the combined effect of climate change and thermopeaking (CC/TP) where conditions in the CC and TP treatments were implemented concurrently.

To establish these different conditions, four large lidded aquaria (64 L capacity, dimensions 37W x 60.5D x 28H cm) filled with 50 L of aerated dechlorinated water were installed in our outdoor facilities (Figure 6.1). Lids on top of the aquaria at a ~45° angle enabled air flow with the external environment but prevented entry of cool rain water. Aquarium heaters maintained the desired temperatures of the four thermal conditions which were monitored by water temperature loggers (HOBO Pro v2 onset®) at 15 min intervals throughout all experimental stages. Water changes were performed twice weekly and water quality checks (pH, % dissolved O_2, µs) were conducted at least weekly. There were no significant differences in water quality within each thermal condition throughout the experimental proceedings (One-way ANOVAs; $P > 0.05$ for all).

Stage 1: Thermal pre-conditioning

Into each of the four aquaria representing the thermal pre-conditions CURR, CC, TP and CC/TP (average temperatures; Table 6.1) four replicate containers (400 ml capacity, 12W x 12D x 4H cm) punctured with small holes (approx. 2 mm) were introduced, each containing brown trout alevins, no more than 4 days old (n=62-85 per replicate container, ~290 per thermal pre-condition, 1177 total). A Styrofoam tray weighted with stones served to submerge the boxes and a mesh layer was then overlaid along with the aquarium lid (Figure 6.1).

During this thermal pre-conditioning stage, we assessed mortality and growth of alevins over 18 days. Dead individuals were recorded daily and removed upon detection. For the growth analysis, a sub-set of 60 individuals per thermal pre-condition (15 from each replicate container) were removed on day 18 of experimental proceedings, humanely euthanised via clove oil immersion (Davis et al. 2015) and

photographed on millimetric paper to determine fork lengths. All photographs were subsequently analysed using the software ImageJ v1.51j8.

Stage 2: Interacting stressors: the impact of thermal stress and Saprolegnia parasitica *on brown trout alevins*

The remaining alevins from Stage 1 were kept separated into the four respective thermal pre-conditions but were further divided into two experimental thermal regimes; 'constant' and 'variable' temperature (average temperatures; Table 6.1). Alevins within the 'constant temperature' experimental thermal regime were housed inside our aquaria facilities while those within the 'variable temperature' were returned to their respective outdoor aquaria from the thermal pre-conditioning stage (CURR, CC, TP, CC/TP). These experimental thermal regimes were further subdivided into 'parasite exposed' and 'control' treatments which were housed in small lidded containers (1.5 L capacity, dimensions 18.8W x 18.8D x 11.7H cm) (n=45 per container, 360 per treatment, 720 total, see Figure 6.1). On day 20, the 'parasite exposed' alevins were challenged with 1 L of *S. parasitica* zoospores at a concentration of $3x10^5$ L^{-1} for 24 h (Stewart et al. 2017). After this initial exposure period, the zoospore solution was removed and replaced with 1 L of dechlorinated water. 'Parasite control' alevins were maintained in the same conditions but without the introduction of *S. parasitica* zoospores. The mortality rate of the alevins was subsequently monitored every 2 h from the point of initial parasite exposure until cessation of the experiment at midnight on day 26. Any dead alevins were removed from their respective containers upon discovery. Water changes were conducted every 24 h on both 'parasite exposed' and 'control' treatments.

Stage 3: Interacting stressors: the impact of thermal stress and Saprolegnia parasitica *on brown trout fry*

Upon conclusion of Stage 2, the remaining alevins in the 'constant temperature' experimental thermal regime were maintained under the same conditions until their yolk sacs were totally absorbed. These fry were then were fed ground trout pellets twice daily. Containers were checked daily for any dead individuals which were removed. On day 47, fry within the 'parasite exposed' treatment (n=30-42 per container, 142 total) were re-challenged with a new batch of EA016 *S. parasitica* zoospores (concentration $3x10^5$ L^{-1}). The fry within the 'parasite control' group (n=30-

42 per container, 163 total) remained unexposed to *S. parasitica* zoospores. The 'parasite exposed/control' treatment groups were returned to the thermal pre-condition (CURR, CC, TP, CC/TP) that they were originally allocated to in Experimental Stage 1 (Figure 6.1, average temperatures; Table 6.1). Mortality of the fry was monitored at 9:00, 12:00, 15:00 and 18:00 daily until completion of experimental procedures at midnight on day 53. All other experimental procedures were kept the same as those from the initial challenge infection except for the additional feeding twice daily due to the absence of the yolk sac.

All procedures and protocols were conducted under UK Home Office license (PPL licence 30/3424) with approval by the Cardiff University Animal Ethics Committee.

Exp. Stage	Experimental Thermal Regime				
	Variable temperature (°C)				Constant temperature (°C)
	CURR	CC	TP	CC/TP	
Stage 1	6.7±0.04	10.3±0.03	7.7±0.04 9.9±0.3*	10.6±0.04 12.7±0.3*	N/A
Stage 2	6.6±0.1	10.3±0.1	8.1 ±0.1 10.8±0.3*	11.2±0.1 13.8±0.3*	13.8±0.1
Stage 3	9±0.02	12.1±0.02	10.3±0.1 13.1±0.1*	13.4±0.1 16.1±0.1*	N/A

Table 6.1. Average temperatures (±SE) of the experimental thermal regimes (constant vs. variable temperature; CURR (current environmental temperature), CC (current environmental temperature +3-4°C), and TP (current environmental temperature + intermittent temperature increases of 2-3°C for 1 h daily) CC/TP (combination of CC and TP conditions)) employed during the thermal preconditioning stage (Stage 1) and the Saprolegnia parasitica *challenge infections with brown trout alevins and fry (Stages 2 and 3 respectively). Average peaking temperatures (±SE) for the TP and CC/TP thermal regimes are highlighted with an *.*

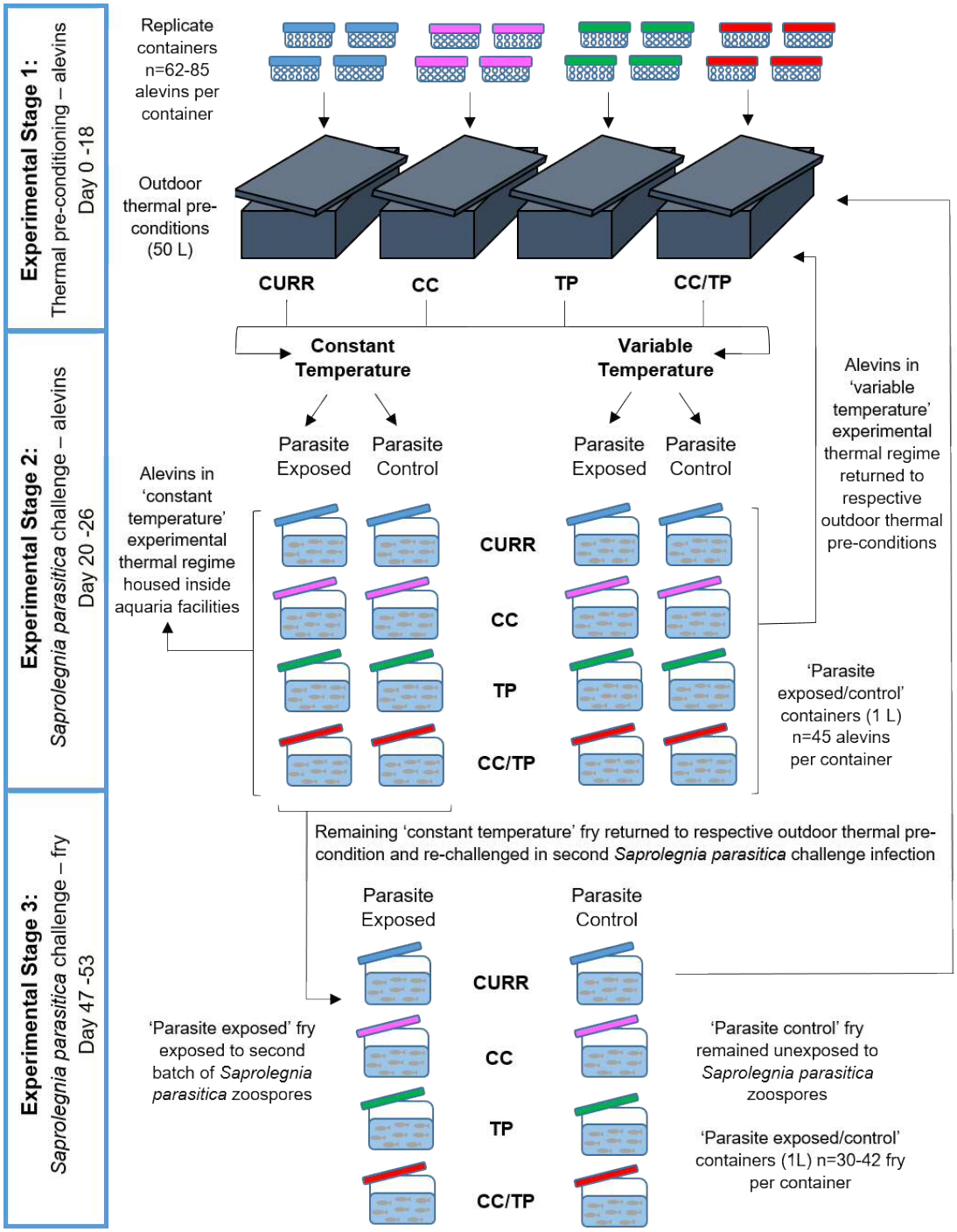

Figure 6.1. Summary of set-up for all experimental stages. Stage 1 (thermal pre-conditioning): four replicate containers containing brown trout alevins (n=62-85) were introduced into four aquaria representing the thermal pre-conditions; CURR (current environmental temperature), CC (current environmental temperature +3-4°C), TP (current environmental temperature + intermittent temperature increases of 2-3°C for 1 h daily) and CC/TP (combination of CC and TP conditions). Alevin mortality was recorded from day 0-18. On day 18 of experimental proceedings, a subset of 60 individuals per thermal pre-condition were humanely euthanised and photographed to determine fork lengths. Stage 2: the remaining alevins from Stage 1 of the experiment were kept separated according to thermal pre-condition and were

further divided into two experimental thermal regimes; 'constant' and 'variable' temperature. Alevins within the 'constant temperature' experimental thermal regime were housed inside our aquaria facilities while those within the 'variable temperature' were returned to their respective outdoor aquaria from the thermal pre-conditioning stage (CURR, CC, TP, CC/TP). These experimental thermal regimes were further subdivided into 'parasite exposed' and 'control' treatments (n=45). On day 20, the 'parasite exposed' alevins were challenged with Saprolegnia parasitica *zoospores while 'parasite control' alevins were not. The mortality rate of the alevins was subsequently monitored every 2 h from the point of initial exposure to the parasite until the cessation of the experiment stage at midnight on day 26. Stage 3: remaining alevins in the 'constant temperature' experimental thermal regime from Stage 2 were maintained under the same conditions until their yolk sacs were totally absorbed. On day 47, the fry within the 'parasite exposed' treatment were re-challenged with a new batch of* S. parasitica *zoospores. The fry within the 'parasite control' treatment remained unexposed to* S. parasitica *zoospores. The 'parasite exposed/control' treatments were returned to outdoor aquaria that they were originally allocated during thermal pre-conditioning (Stage 1) (CURR, CC, TP, CC/TP). Mortality of the fry was monitored at hours; 9:00, 12:00, 15:00 and 18:00 daily until the completion of all experimental procedures at midnight on day 53.*

Statistical analyses

Analyses were conducted using R statistical software v3.5.1 (R Core Team 2018) with the threshold for significance being $P<0.05$. Model robustness was assessed using residual plots (after Pinheiro and Bates 2000).

To investigate the effect of pre-thermal condition on alevin percentage mortality (Stage 1) we used a Generalised Linear Model (GLM) fitted with a binomial error family and logit link function. Percentage mortality was the dependent term in the model while thermal pre-condition (CURR, CC, TP, and CC/TP) was the independent term. Using a GLM fitted with a Gaussian error family and identity link function, we also investigated whether thermal pre-condition had a significant effect on alevin growth (Stage 1). Fork length was the dependant term in the model and thermal pre-condition was the independent variable. For both of these models *post-hoc* analysis (lsmeans package; Lenth 2016) was conducted to compare the mortality and fork lengths of the alevins from the respective thermal pre-conditions.

Binomial GLMs fitted with logit link functions were used to assess the impact of *S. parasitica* exposure on brown trout alevin and fry survival. For the primary *S. parasitica* infection (Stage 2), percentage mortality was the dependant term in the

model; the fixed effects included; thermal pre-condition (CURR, CC, TP and CC/TP) nested within experimental thermal regime (constant temperature vs variable temperature), parasite exposure (exposed vs control) and the interaction between these variables. When modelling the second *S. parasitica* infection (Stage 3), percentage mortality was the dependant term while fixed effects included; experimental thermal regime (CURR, CC, TP and CC/TP), parasite exposure and the interaction between these effects. For both of these models, *post-hoc* analysis (lsmeans package; Lenth 2016) was used to compare treatment groups.

Results

Stage 1: Thermal pre-conditioning

While thermal pre-condition (CURR, CC, CC/TP, TP) did not affect alevin mortality (GLM; df= 3, P>0.05, % mortality, CURR= 9.3; CC= 7.4; TP= 10.2; CC/TP=6), it did significantly affect alevin growth (fork length; GLM; df=3, P<0.0001) (Figure 6.2A and B respectively). Alevins within the CC, TP and CC/TP thermal pre-conditions were significantly longer than that those in the CURR treatment (P<0.0001, mean fork lengths (mm) ±SE, CURR= 207.6±1.8; CC= 226.6±1.6; TP= 221.9±1.3; CC/TP= 235.7±1.5).

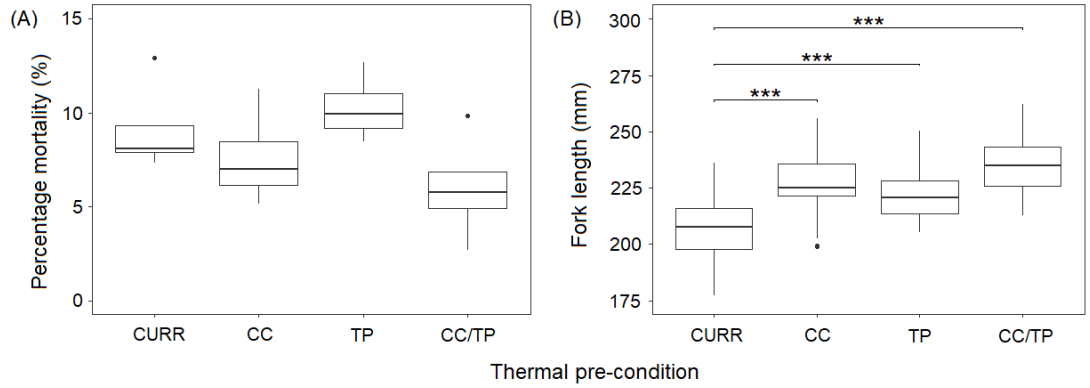

Figure 6.2. (A) Percentage mortality and (B) Final fork length (mm) of alevins within the thermal pre-conditions (Stage 1) CURR (current environmental temperature), CC (current environmental temperature +3-4°C), TP (current environmental temperature + intermittent temperature increases of 2-3°C for 1 h daily) and CC/TP (combination of CC and TP conditions).

Stage 2: Primary Saprolegnia parasitica *challenge on brown trout alevins*

For the 'constant temperature' experimental thermal regime, only alevins within the CURR pre-thermal condition experienced significantly higher mortality in the 'parasite exposed' treatment compared to the 'parasite control' (P<0.05; Figure 6.3A). This was also true for the 'variable temperature' CC/TP thermal regime (P<0.05; Figure 6.3B).

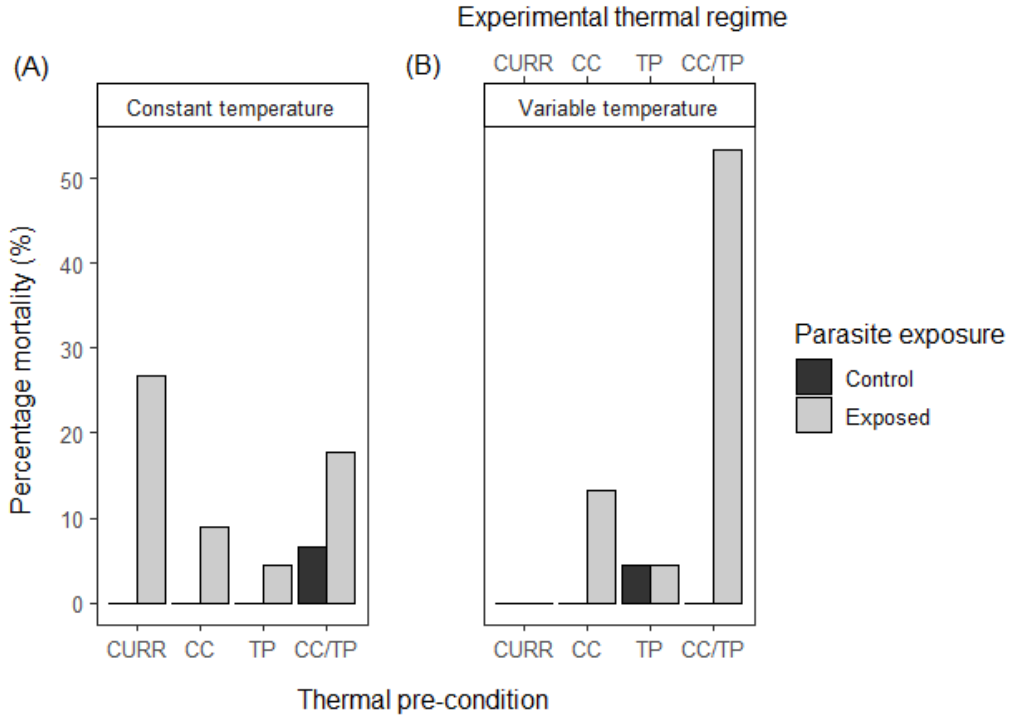

Figure 6.3. Percentage mortality of alevins from the primary Saprolegnia parasitica *challenge (Stage 2) within the (A) Constant temperature and (B) Variable temperature experimental thermal regimes. Percentage mortality is displayed for the thermal conditions CURR (current environmental temperature), CC (current environmental temperature +3-4°C), TP (current environmental temperature + intermittent temperature increases of 2-3°C for 1 h daily) and CC/TP (combination of CC and TP conditions) when exposed ('parasite exposed') and unexposed to this parasite ('parasite control').*

Focussing on the 'parasite exposed' treatments; for the 'variable temperature' thermal regime, those within CC/TP experienced significantly higher mortality (53.3%) compared to CURR (0%; P<0.0001), CC (13.3%; P<0.05) and TP (4.4%; P<0.0001) (Figure 6.3B). In contrast, within the 'constant temperature' thermal regime, there were no significant differences in mortality between 'parasite exposed' CURR (26.7%), CC (8.9%), TP (4.4%) and CC/TP (17.8%) (P>0.05; Figure 6.3A).

Stage 3: Secondary Saprolegnia parasitica *challenge on brown trout fry*

Only fry within the CC/TP thermal regime experienced a significantly higher mortality within the 'parasite exposed' treatment compared to the 'parasite control' treatment (P<0.0001; Figure 6.4). Within each treatment ('parasite exposed' and 'parasite control') fry mortality was significantly higher within the CC/TP thermal regime compared to the other regimes (P<0.05; 'parasite exposed' mortality; CURR=20%, CC=25.6%, TP=21.1%, CC/TP=85.7%; 'parasite control' mortality; CURR=0%, CC=5%, TP=5%, CC/TP=28.6%; Figure 6.4).

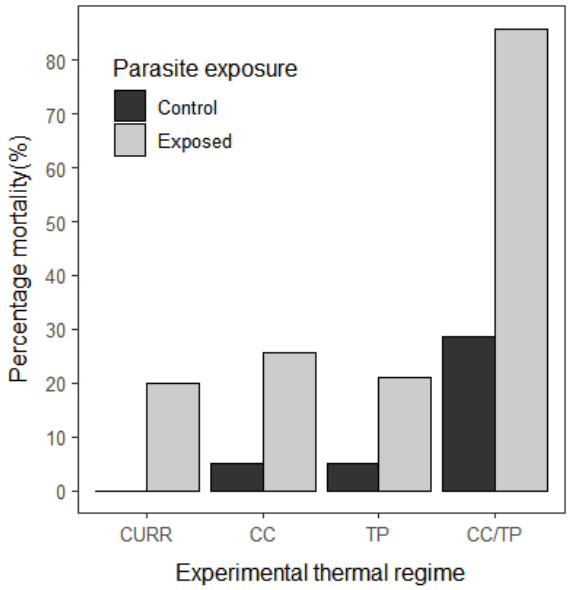

Figure 6.4. Mortality of fry exposed to the secondary Saprolegnia parasitica *challenge experiment (Stage 3). Remaining fry from the 'constant temperature' treatment of the primary* S. parasitica challenge *(Stage 2) were placed back into the aquaria that they were originally allocated in the thermal pre-conditioning stage (Stage 1) (CURR (current environmental temperature), CC (current environmental temperature +3-4°C), TP (current environmental temperature + intermittent temperature increases of 2-3°C for 1 h daily) and CC/TP (combination of CC and TP conditions). Fry within the 'parasite exposed' group were re-challenged with a second batch of the zoospores of* S. parasitica *isolate EA016 while the 'parasite control' group remained unchallenged.*

Discussion

The current study highlights the importance of contextualising the impact of multiple environmental stressors, in this case temperature and parasite exposure. During Stage 2 within the variable temperature climate change with thermopeaking condition, brown trout alevins experienced a high level of mortalities when exposed to *S. parasitica*, despite the average peaking temperature being equivalent to that within the

constant temperature condition (13.8°C, see Table 6.1). This indicates that the thermal stress of warmer temperatures alone was not enough to increase disease susceptibility, only when combined with rapid daily thermopeaking did this increase saprolegniasis.

Our findings also support Stewart et al.'s (2018) observations that the temperature at the time of *Saprolegnia* exposure substantially affects fish (three-spined stickleback*, Gasterosteus aculeatus*) infection and immunity, while generally, prior thermal conditions do not. During our pre-thermal conditioning (Stage 1) alevins within the climate change with thermopeaking thermal condition were exposed to an arguably higher level of thermal stress for 18 days. Despite this, upon exposure to *S. parasitica* at a constant temperature (13.8°C), these alevins did not experience an increase in mortality compared to the other thermal pre-conditions (Stage 2). Only when alevins (Stage 2) and fry (Stage 3) remained within the climate change with thermopeaking condition at the time of *S. parasitica* exposure did mass infections occur. Notably, temperature increases within the climate change with thermopeaking thermal regime between Stage 2 (average temperature 11.2±0.1°C with peaking temperature 13.8±0.3°C; Table 6.1) and Stage 3 (average temperature 13.4±0.1°C with peaking temperature 16.1±0.1°C; Table 6.1) meant that fry within this condition experienced increased mortalities compared to the other thermal regimes even when the parasite was not introduced, indicating that the thermal stress of this regime alone was lethal.

During thermal pre-conditioning (Stage 1), alevins grew larger within the climate change, thermopeaking and climate change with thermopeaking regimes in comparison to those held at current environmental temperatures. If fast development at higher temperatures reported here occurred in the wild, there could be a mismatch between fry emergence and optimal conditions for survival. Earlier spring fry emergence may result in increased mortalities due to a lack of food resources (Jensen et al. 1991; Casas-Mulet et al. 2016) or high levels of predation (Brännäs 1995; Rooke et al. 2019).

Multiple stressor research allows us to realistically assess the threats facing key species within the wild; the current study demonstrates that climate change-induced declines in brown trout populations are likely to intensify with the increased implementation of renewable energy sources such as hydropower and the endemic

presence of destructive pathogens such as *S. parasitica.* These results highlight the need for effective river management strategies aimed at protecting economically and environmentally valuable species and maintaining freshwater biodiversity.

Chapter 7 - General Discussion

The oomycete *Saprolegnia parasitica* poses a huge threat to cultured fish production and the prosperity of wild fish populations. This thesis aimed to expand our limited understanding of the epidemiology of this problematic freshwater pathogen and identify environmental and host factors that propagate epidemics. This chapter collates findings from all previous chapters and presents ideas for future research on freshwater oomycetes.

Saprolegnia parasitica diversity across England and Wales

This thesis identifies *S. parasitica* as the principal species infecting Atlantic salmon (*Salmo salar)*, sea trout (*Salmo trutta)*, carp (*Cyprinus carpio*), European eel (*Anguilla anguilla*) and three-spined stickleback (*Gasterosteus aculeatus)* in English and Welsh waterbodies. Furthermore, the first *S. parasitica* landscape genomics study presented in Chapter 3 uncovered a high level of genetic diversity within this species, with all 46 isolates examined identified as genetically distinct. The study also highlighted the impact of selective host and environmental pressures as a large number of adaptive changes were detected within genes relating to: temperature tolerance, sensing external stimuli, infective life stages, host invasion and nutrient procurement. Moreover, isolates could be separated in two subpopulations, although the underlying cause of this separation (i.e. geographic or host factors) could not be identified here. Further selection tests may help explain this divide, for example cross-population extended haplotype homozygosity (xp-EHH) could be used to identify the signatures of selection that are specific to each subpopulation.

While *S. parasitica* isolates collected from wild fish hosts in England and Wales have been the focus here, future research should also assess the genetic diversity of isolates from aquaculture facilities; this would greatly aid our understanding of outbreaks within this industry, potential spillover and spillback between wild and farmed stocks, and could ultimately reduce losses.

Saprolegnia parasitica host adaptation

While host species was not linked with adaptive genomic variation in the landscape genomics investigation (Chapter 3), there was a notable separation of host species

between the two identified subpopulations with all non-salmonid species falling within the smaller, second subpopulation. However this separation was not absolute with three salmon isolates also comprising the second subpopulation. Adaptive changes among isolates were also detected within genes involved in: fibroblast growth factor binding (FGF) binding that could potentially modulate host angiogenesis, host nutrient acquisition and cell killing. Moreover, Chapter 4 presented evidence of *S. parasitica* host preference; the sea trout isolate (EA016) preferred sea trout over salmon in the induced zoospore encystment assay and caused greater sea trout mortalities than salmon during the first 48 h of an experimental challenge infection. Collectively, these results may be indicative of some degree of parasite 'specialism' with host species driving *S. parasitica* local adaption to overcome specific host defences. However, these adaptive changes may also be related to *S. parasitica* plasticity, enabling this oomycete to rapidly tailor its pathogenesis to any host encountered. Indeed, while salmon experienced significantly fewer mortalities than sea trout in the first 48 h of the EA016 challenge infection (Chapter 4), there was a sudden drop in salmon mortality between 48 and 60 h and overall survival was not significantly different between the two species.

To help clarify the influence of selective host pressures on *S. parasitica* genomic adaptation, future work should focus on the genomics of isolates collected from a range of host species within the same region. Furthermore, challenge experiments similar to those in Chapter 4 could be combined with genomic data to identify adaptive changes within a single isolate following exposure to different host species.

Influence of abiotic factors on *Saprolegnia parasitica* epidemics

This thesis has primarily focussed on the influence of temperature and water flow on *Saprolegnia* infections; historic records of outbreaks in key English Rivers suggest a link with mean spring flow rates; in particular, *Saprolegnia* levels were found to increase in years with lower flow rates (Chapter 5). Furthermore, low water flows increased *S. parasitica*-induced salmonid mortality and enhanced the production of infectious zoospores in a temperature dependent manner (Chapter 5). Temperature was found to impact the vegetative and asexual life stages of this oomycete (Chapter 5) and significantly affected host susceptibility to infection at the time of *S. parasitica*

exposure (Chapter 6). Moreover, temperature was the only environmental factor included in our landscape genomics study that was linked with *S. parasitica* adaptive selection, other factors examined: host species, longitude, latitude, nitrate concentration and pH were not significant (Chapter 3).

Undoubtedly the environmental conditions in the wild are complex, making it difficult to pinpoint the major contributors to disease incidences. To provide further insight into *S. parasitica* genome-environment associations, future work should prioritise obtaining genomic data from a comparable number of isolates from each location, however, this will always be difficult to achieve via opportunistic sampling. Moreover, further multi-stressor experiments focussed on the combined effects of several environmental factors will be necessary to fully understand the risk factors associated with *S. parasitica* epidemics.

Potential effects of climate change on *Saprolegnia parasitica* outbreaks

Climate change poses a serious threat to environmental prosperity; global average temperatures are increasing at an alarming rate and have been accompanied by extreme weather events; UK summers are predicted to become hotter and drier while winters will likely be milder and wetter (Met Office 2018). These phenomena will undoubtedly cause further disease problems for both wild and farmed fish due to their poikilothermic nature. For *S. parasitica* in particular, saprophytic growth is likely to intensify during the summer periods of low water flow and high temperatures, subsequent sudden water cooling due to large volumes of rainfall in the winter may induce *S. parasitica* zoospore production while concurrently impacting fish immune defences, causing an increase in disease epidemics. Furthermore, as demonstrated in Chapter 6, the implementation of hydropower plants to combat the effects of climate change are likely to increase incidences of saprolegniasis. Further work is need to substantiate the hypothesised effects of climate change on this oomycete and develop effective management procedures to protect important fish species.

Rapid assessment of *Saprolegnia parasitica* diversity

While Rocchi et al. (2017) developed a real-time quantitative PCR (qPCR) method to quantify *S. parasitica* levels, there is no current method to rapidly assess intra-specific diversity without performing whole-genome resequencing on every collected isolate.

Further work in our lab will utilise the whole-genome data of Chapter 3 to identify short genomic regions with enough genetic variability to discriminate between isolates; PCR primers could then be designed to amplify these regions for DNA sequencing. Furthermore, these primers could be used in combination with Whatman® FTA® DNA binding cards that can easily capture/store DNA and moreover be used directly in PCR assays (see Smith and Burgoyne 2004; Zou et al. 2018; Appendix 4). This may provide a simple and non-invasive method for members of the aquaculture/ornamental industries or the Environment Agency (EA) to easily identify the *S. parasitica* isolates infecting their facilities/rivers. Moreover, this data may uncover potential pathways of infection and interactions between wild fish stocks and aquaculture.

Cryopreservation of *Saprolegnia* spp.

For this thesis, maintenance of *Saprolegnia* spp. cultures relied upon serial sub-culturing on artificial media; a method that is both expensive and labour-intensive. Furthermore, previous work has revealed continuous transfer can cause physiological and genetic modifications (Kirsop and Doyle 1991; Ko 2003; Marx and Daniel 1976); indeed attenuation of virulence is known to occur in fungal (Ansari and Butt 2011), bacterial (Almaguer-Chávez et al. 2011) and viral (Druelle et al. 2008) cultures and may account for the drastic differences in virulence observed during the experimental challenge infections of Chapter 3. Developing a method to cryopreserve *Saprolegnia* would enable genome preservation directly after collection in the field and provide a reliable method for archiving isolates. Cryopreservation of *S. parasitica* mycelial cultures was trialled during this PhD (see Appendix 3), but these initial attempts were unsuccessful. Methodologies are currently being optimised in other laboratories and are likely to become available in the near future.

Summary

This thesis has provided an invaluable insight into the epidemiology of *S. parasitica* using a unique combination of genomic and field data alongside *in vitro*/*in vivo* experiments. The influence of host and environmental factors on *S. parasitica* adaptation has been uncovered here for the first time and our understanding of risk factors associated with saprolegniasis outbreaks has been expanded. Ultimately, the data provided here will aid the development of improved genetic tools and rapid

screening approaches to successfully track and mitigate *S. parasitica* infections in aquaculture and inform current and future monitoring, management and protection of wild fish stocks.

References

Abebe, T.D., Naz, A.A. and Léon, J. 2015. Landscape genomics reveal signatures of local adaptation in barley (*Hordeum vulgare* L.). *Frontiers in Plant Science* 6, 813. doi: 10.3389/fpls.2015.00813.

Alderman, D.J. 1996. Geographical spread of bacterial and fungal diseases of crustaceans. *Revue scientifique et technique* 15, pp. 603–632.

Alexa, A. and Rahnenfuhrer, J. 2019. *topGO: Enrichment Analysis for Gene Ontology*. R package version 2.36.0.

Alexander, D.H., Novembre, J. and Lange, K. 2009. Fast model-based estimation of ancestry in unrelated individuals. *Genome Research* 19, pp. 1655–1664.

Ali, E.H. 2009. Antifungal activity of sodium chloride on *Saprolegnia diclina* and *Aphanomyces* sp. *Acta Mycologica* 44, pp. 125–138.

Ali, S.E., Thoen, E., Evensen, Ø. and Skaar, I. 2014. Boric Acid Inhibits Germination and Colonization of *Saprolegnia* Spores *In Vitro* and *In Vivo*. *PLOS ONE* 9, e91878. doi: 10.1371/journal.pone.0091878.

Almaguer-Chávez, J.A., Welsh, O., Lozano-Garza, H.G., Said-Fernández, S., Romero-Díaz, V.J., Ocampo-Candiani, J. and Vera-Cabrera, L. 2011. Decrease of virulence for BALB/c mice produced by continuous subculturing of *Nocardia brasiliensis*. *BMC Infectious Diseases* 11, 290. doi: 10.1186/1471-2334-11-290.

Anderson, G. 1990. *Atlantic salmon: Fact & fantasy.* Montreal: Salar Publishing, pp. 176.

Ansari, M.A. and Butt, T.M. 2011. Effects of successive subculturing on stability, virulence, conidial yield, germination and shelf-life of entomopathogenic fungi. *Journal of Applied Microbiology* 110, pp. 1460–1469.

Atlantic Salmon Trust. 2019. *Putting Wild Salmon First.* Available at: https://atlanticsalmontrust.org/ [Accessed: 23 September 2019].

Bakke, T. and Harris, P. 1998. Diseases and parasites in wild Atlantic salmon (*Salmo salar*) populations. *Canadian Journal of Fisheries and Aquatic Sciences* 55, pp. 247–266.

Barnes, M.E., Ewing, D.E., Cordes, R.J. and Young, G.L. 1998. Observations on Hydrogen Peroxide Control of *Saprolegnia* spp. during Rainbow Trout Egg Incubation. *The Progressive Fish-Culturist* 60, pp. 67–70.

Bartnicki-Garcia, S. 1968. Cell wall chemistry, morphogenesis, and taxonomy of fungi. *Annual Review of Microbiology* 22, pp. 87–108.

Bates, D., Mächler, M., Bolker, B. and Walker, S. 2014. Package Lme4: Linear Mixed-Effects Models Using Eigen and S4. *Journal of Statistical Software* 67, pp. 1-103.

BBC News 2012. *Salmon disease found in Spey fish*. Available at: https://www.bbc.com/news/uk-scotland-north-east-orkney-shetland-18288859 [Accessed: 23 September 2019].

Beakes, G.W. 1983. A comparative account of cyst coat ontogeny in saprophytic and fish-lesion (pathogenic) isolates of the *Saprolegnia diclina-parasitica* complex. *Canadian Journal of Botany* 61, pp. 603–625.

Beakes, G.W. and Bartinicki-Garcia, S. 1989. Ultrastructure of mature oogonium-oospore wall complexes in *Phytophthora megasperma*: a comparison of *in vivo* and *in vitro* dissolution of the oospore wall. *Mycological Research* 93, pp. 321–334.

Beakes, G.W. and Ford, H. 1983. Esterase isoenzyme variation in the genus *Saprolegnia*, with particular reference to the fish-pathogenic *S. diclina-parasitica* complex. *Journal of General Microbiology*. 129, pp. 2605–2619.

Belmonte, R., Wang, T., Duncan, G.J., Skaar, I., Mélida, H., Bulone, V., van West, P. and Secombes, C.J. 2014. Role of pathogen-derived cell wall carbohydrates and prostaglandin E2 in immune response and suppression of fish immunity by the oomycete *Saprolegnia parasitica*. *Infection and Immunity* 82, pp. 4518–4529.

Ben-Ami, R., Lewis, R.E., Leventakos, K. and Kontoyiannis, D.P. 2009. *Aspergillus fumigatus* inhibits angiogenesis through the production of gliotoxin and other secondary metabolites. *Blood* 114, pp. 5393–5399.

Berg, P.R., Jentoft, S., Star, B., Ring, K.H., Knutsen, H., Lien, S., Jakobsen, K.S. and André, C. 2015. Adaptation to Low Salinity Promotes Genomic Divergence in Atlantic Cod (*Gadus morhua* L.). *Genome Biology and Evolution* 7, pp. 1644–1663.

Blaxter, M., Mann, J., Chapman, T., Thomas, F., Whitton, C., Floyd, R. and Abebe, E. 2005. Defining operational taxonomic units using DNA barcode data. *Philosophical Transactions of the Royal Society of London, Series B, Biological Sciences* 360, pp. 1935–1943.

Blazer, V.S., Vogelbein, W.K., Densmore, C.L., May, E.B., Lilley, J.H. and Zwerner, D.E. 1999. *Aphanomyces* as a Cause of Ulcerative Skin Lesions of Menhaden from Chesapeake Bay Tributaries. *Journal of Aquatic Animal Health* 11, pp. 340–349.

Bly, J.E. and Clem, L.W. 1991. Temperature-mediated processes in teleost immunity: *In vitro* immunosuppression induced by *in vivo* low temperature in channel catfish. *Veterinary Immunology and Immunopathology* 28, pp. 365–377.

Bly, J.E., Lawson, L.A., Dale, D.J., Szalai, A.J., Durborow, R.M. and Clem, L.W. 1992. Winter saprolegniosis in channel catfish. *Diseases of Aquatic Organisms* 13, pp. 155–164.

Bolger, A.M., Lohse, M. and Usadel, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, pp. 2114–2120.

Brännäs, E. 1995. First access to territorial space and exposure to strong predation pressure: A conflict in early emerging Atlantic salmon (*Salmo salar* L.) fry. *Evolutionary Ecology* 9, pp. 411–420.

Breau, C., Cunjak, R.A. and Peake, S.J. 2011. Behaviour during elevated water temperatures: can physiology explain movement of juvenile Atlantic salmon to cool water? *The Journal of Animal Ecology* 80, pp. 844–853.

Brooks, M.E., Kristensen, K., van Benthem, K.J., Magnusson, A., Berg, C.W., Nielsen, A., Skaug, H.J., Maechler, M. and Bolker, B.M. 2017. glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. *The R Journal* 9, pp. 378–400.

Bruno, D.W., Noguera, P.A. and Poppe, T.T. 2013. *A Colour Atlas of Salmonid Diseases*. *2nd ed*. Heidelberg: Springer, pp. 220.

Bruno, D.W., van West, P. and Beakes, G.W. 2011. *Saprolegnia* and other oomycetes. In: Bruno, D.W. and Woo, P.T.K. eds. *Fish Diseases and Disorders: Viral, Bacterial and Fungal Infections. 2nd ed.* Wallingford, England: CABI International, pp. 669–720.

Bruno, D.W. and Wood, B.P. 1999. *Saprolegnia* and other Oomycetes. In: Bruno, D.W. and Woo, P.T.K. eds. *Fish Diseases and Disorders. Viral, Bacterial and Fungal Infections.* 2nd ed. Wallingford, England: CABI International, pp. 599–659.

Bruno, M.C., Cashman, M.J., Maiolini, B., Biffi, S. and Zolezzi, G. 2015. Responses of benthic invertebrates to repeated hydropeaking in semi-natural flume simulations. *Ecohydrology* 9, pp. 68–82.

Buckland, F.S., Walpole, S. and Young, A. 1880. *Report on the disease which has recently prevailed among the Salmon in the Tweed, Eden and other rivers in England and Scotland*. London: Her Majesty's Stationery Office (HMSO).

Burr, A.W. and Beakes, G.W. 1994. Characterization of zoospore and cyst surface structure in saprophytic and fish pathogenic *Saprolegnia* species (oomycete fungal protists). *Protoplasma* 181, pp. 142–163.

Carbery, J.T. 1968. Ulcerative Dermal Necrosis of salmonids in Ireland. *Symposia of the Zoological Society of London* 24, pp. 39–49.

Carbery, J.T. and Strickland, K.T. 1968. Ulcerative Dermal Necrosis (UDN). *Irish Veterinary Journal* 22, pp. 171–175.

Casas-Mulet, R., Alfredsen, K., Hamududu, B. and Timalsina, N.P. 2015. The effects of hydropeaking on hyporheic interactions based on field experiments. *Hydrological Processes* 29, pp. 1370–1384.

Casas-Mulet, R., Saltveit, S.J. and Alfredsen, K.T. 2016. Hydrological and thermal effects of hydropeaking on early life stages of salmonids: A modelling approach for implementing mitigation strategies. *Science of the Total Environment* 573, pp. 1660–1672.

Chang, C.C., Chow, C.C., Tellier, L.C., Vattikuti, S., Purcell, S.M. and Lee, J.J. 2015. Second-generation PLINK: rising to the challenge of larger and richer datasets. *GigaScience* 4, 7. doi: 10.1186/s13742-015-0047-8.

Chitty, J.L. and Fraser, J.A. 2017. Purine Acquisition and Synthesis by Human Fungal Pathogens. *Microorganisms* 5, 33. doi: 10.3390/microorganisms5020033.

Clarke, A. and Fraser, K.P. 2004. Why does metabolism scale with temperature? *Functional Ecology* 18, pp. 243–251.

Coker, W.C. 1923. *The saprolegniaceae, with notes on other Water Molds*. North Carolina: University of North Caroline Press, Chapel Hill, pp. 201.

Cooke, D.E., Drenth, A., Duncan, J.M., Wagels, G. and Brasier, C.M. 2000. A molecular phylogeny of *Phytophthora* and related oomycetes. *Fungal Genetics and Biology* 30, pp. 17–32.

Crawley, M.J. 2007. Statistical Modelling. In: *The R book.* England, U.K.: Wiley, pp. 323–386.

Davis, D.J., Klug, J., Hankins, M., Doerr, H.M., Monticelli, S.R., Song, A., Gillespie, C.H. and Bryda, E.C. 2015. Effects of Clove Oil as a Euthanasia Agent on Blood Collection Efficiency and Serum Cortisol Levels in *Danio rerio*. *Journal of the American Association for Laboratory Animal Science* (JAALAS) 54, pp. 564–567.

de Bari, A. 1852. Beitrag zur Kenntnis der *Achlya prolifera* Nees. *Botanische Zeitung* 10, pp. 473–479, 489 - 496, 505 – 511.

de Bruijn, I., Belmonte, R., Anderson, V.L., Saraiva, M., Wang, T., van West, P. and Secombes, C.J. 2012. Immune gene expression in trout cell lines infected with the fish pathogenic oomycete *Saprolegnia parasitica*. *Developmental and Comparative Immunology* 38, pp. 44–54.

de la Bastide, P.Y., Leung, W.L. and Hintz, W.E. 2015. Species composition of the genus *Saprolegnia* in fin fish aquaculture environments, as determined by nucleotide sequence analysis of the nuclear rDNA ITS regions. *Fungal Biology* 119, pp. 27–43.

Diéguez-Uribeondo, J., Cerenius, L. and Söderhäll, K. 1994. Repeated zoospore emergence in *Saprolegnia parasitica*. *Mycological Research* 98, pp. 810–815.

Diéguez-Uribeondo, J., Fregeneda-Grande, J.M., Cerenius, L., Pérez-Iniesta, E., Aller-Gancedo, J.M., Tellería, M.T., Söderhäll, K. and Martín, M.P. 2007. Re-evaluation of the enigmatic species complex *Saprolegnia diclina-Saprolegnia parasitica* based on morphological, physiological and molecular data. *Fungal Genetics and Biology* 44, pp. 585–601.

Dolan, B.P., Fisher, K.M., Colvin, M.E., Benda, S.E., Peterson, J.T., Kent, M.L. and Schreck, C.B. 2016. Innate and adaptive immune responses in migrating spring-run adult chinook salmon, *Oncorhynchus tshawytscha*. *Fish & Shellfish Immunology* 48, pp. 136–144.

Dong, C.-H., Agarwal, M., Zhang, Y., Xie, Q. and Zhu, J.-K. 2006. The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. *Proceedings of the National Academy of Sciences of the United States of America* 103, pp. 8281–8286.

Druelle, J., Sellin, C.I., Waku-Kouomou, D., Horvat, B. and Wild, F.T. 2008. Wild type measles virus attenuation independent of type I IFN. *Virology Journal* 5, 22. doi: 10.1186/1743-422X-5-22

Dudgeon, D., Arthington, A.H., Gessner, M.O., Kawabata, Z.-I., Knowler, D.J., Lévêque, C., Naiman, R.J., Prieur-Richard, A.-H., Soto, D., Stiassny, M.L.J. and Sullivan, C.A. 2006. Freshwater biodiversity: importance, threats, status and conservation challenges. *Biological Reviews* 81, pp. 163–182.

Dunning, H. 2019. *Scientists trial drones to protect coffee plants from devastating fungal disease*. Available at: https://www.imperial.ac.uk/news/190712/scientists-trial-drones-protect-coffee-plants/ [Accessed: 9 August 2019].

Durance, I. and Ormerod, S.J. 2007. Climate change effects on upland stream macroinvertebrates over a 25-year period. *Global Change Biology* 13, pp. 942–957.

El-Feki, M., Hatai, K. and Hussein, M.M.A. 2003. Chemotactic and chemokinetic activities of *Saprolegnia parasitica* toward different metabolites and fish tissue extracts. *Mycoscience* 44, pp. 159–162.

Elliott, J.M. 1991. Tolerance and resistance to thermal stress in juvenile Atlantic salmon, *Salmo salar. Freshwater Biology* 25, pp. 61–70.

Elliott, J.M. and Elliott, J.A. 1995. The effect of the rate of temperature increase on the critical thermal maximum for parr of Atlantic salmon and brown trout. *Journal of Fish Biology* 47, pp. 917–919.

Elliott, J.M. and Elliott, J.A. 2010. Temperature requirements of Atlantic salmon *Salmo salar*, brown trout *Salmo trutta* and Arctic charr *Salvelinus alpinus*: predicting the effects of climate change. *Journal of Fish Biology* 77, pp. 1793–1817.

Environment Agency (EA) 2017. *Impact of catch and release angling practices on survival of salmon: report.* Available at: https://www.wildtrout.org/assets/img/general/Impact_of_catch_and_release_angling_practices_on_survival_of_salmon_-_report.pdf [Accessed: 22 November 2019].

Environment Agency (EA) 2018. *Salmon Stocks and Fisheries in England and Wales in 2018*. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/810720/SalmonReport-2018-assessment_final.pdf [Accessed: 22 November 2019].

Environment Agency (EA) 2019. *Water quality data archive.* Available at: https://environment.data.gov.uk/water-quality/view/landing [Accessed: 31 January 2019].

Feng, M., Zolezzi, G. and Pusch, M. 2018. Effects of thermopeaking on the thermal response of alpine river systems to heatwaves. *Science of the Total Environment* 612, pp. 1266–1275.

Finch, C., Pine, W.E. and Limburg, K.E. 2015. Do Hydropeaking Flows Alter Juvenile Fish Growth Rates? A Test with Juvenile Humpback Chub in the Colorado River. *River Research and Applications* 31, pp. 156–164.

Fisher, M.C., Henk, D.A., Briggs, C.J., Brownstein, J.S., Madoff, L.C., McCraw, S.L. and Gurr, S.J. 2012. Emerging fungal threats to animal, plant and ecosystem health. *Nature* 484, pp. 186–194.

Fleming, I.A. 1996. Reproductive strategies of Atlantic salmon: ecology and evolution. *Reviews in Fish Biology and Fisheries* 6, pp. 379–416.

Foll, M. and Gaggiotti, O. 2008. A Genome-Scan Method to Identify Selected Loci Appropriate for Both Dominant and Codominant Markers: A Bayesian Perspective. *Genetics* 180, pp. 977–993.

Food and Agriculture Organization of the United Nations. 2013. *Fish to 2030: Prospects for Fisheries and Aquaculture*. Available at: http://www.fao.org/3/i3640e/i3640e.pdf [Accessed: 22 November 2019].

Food and Agriculture Organization of the United Nations. 2019. *Aquaculture*. Available at: http://www.fao.org/aquaculture/en/ [Accessed: 10 October 2019].

Fornace, K.M., Drakeley, C.J., William, T., Espino, F. and Cox, J. 2014. Mapping infectious disease landscapes: unmanned aerial vehicles and epidemiology. *Trends in Parasitology* 30, pp. 514-519.

Forneris, G., Bellardi, S., Palmegiano, G.B., Saroglia, M., Sicuro, B., Gasco, L. and Ivo, Z. 2003. The use of ozone in trout hatchery to reduce saprolegniasis incidence. *Aquaculture* 221, pp. 157–166.

Fregeneda-Grandes, J., Rodríguez-Cadenas, F. and Aller-Gancedo, J. 2007. Fungi isolated from cultured eggs, alevins and broodfish of brown trout in a hatchery affected by Saprolegniosis. *Journal of Fish Biology* 71, pp. 510–518.

Futuyma, D.J. and Moreno, G. 1988. The evolution of ecological specialization. *Annual Review of Ecology and Systematics* 19, pp. 207–233.

Gavin, C. 2014. *Energy Trends: March 2014, special feature articles*. Available at: https://www.gov.uk/government/statistics/energy-trends-march-2014-special-feature-articles [Accessed: 7 November 2018].

Geist, J. and Hawkins, S. 2016. Habitat recovery and restoration in aquatic ecosystems: current progress and future challenges. *Aquatic Conservation: Marine and Freshwater Ecosystems* 26, pp. 942–962.

Gieseker, C., Serfling, S. and Reimschuessel, R. 2006. Formalin treatment to reduce mortality associated with *Saprolegnia parasitica* in rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* 253, pp. 120–129.

Gozlan, R.E., Marshall, W.L., Lilje, O., Jessop, C.N., Gleason, F.H. and Andreou, D. 2014. Current ecological understanding of fungal-like pathogens of fish: What lies beneath? *Frontiers in Microbiology* 5, pp. 1–16.

Graham, C.T. and Harrod, C. 2009. Implications of climate change for the fishes of the British Isles. *Journal of Fish Biology* 74, pp. 1143–1205.

Guerriero, G., Avino, M., Zhou, Q., Fugelstad, J., Clergeot, P.H. and Bulone, V. 2010. Chitin Synthases from *Saprolegnia* are involved in tip growth and represent a potential target for anti-oomycete drugs. *PLOS Pathogens* 6, e1001070. doi: 10.1371/journal.ppat.1001070.

Hallett, I.C. and Dick, M.W. 1986. Fine structure of zoospore cyst ornamentation in the Saprolegniaceae and Pythiaceae. *Transactions of the British Mycological Society*. 86, pp. 457–463.

Halsall, D.M. 1976. Zoospore chemotaxis in Australian isolates of *Phytophthora* species. *Canadian Journal of Microbiology* 22, pp. 409–422.

Hannah, D.M. and Garner, G. 2015. River water temperature in the United Kingdom: Changes over the 20th century and possible changes over the 21st century. *Progress in Physical Geography: Earth and Environment* 39, pp. 68–92.

Hansen, J., Ruedy, R., Sato, M. and Lo, K. 2010. Global surface temperature change. *Reviews of Geophysics* 48, RG4004. doi: 10.1029/2010RG000345.

Hari, R.E., Livingstone, D.M., Siber, R., Burkhardt-Holm, P. and Güttinger, H. 2006. Consequences of climatic change for water temperature and brown trout populations in Alpine rivers and streams. *Global Change Biology* 12, pp. 10–26.

Häsler, B., Howe, K.S. and Stärk, K.D. 2011. Conceptualising the technical relationship of animal disease surveillance to intervention and mitigation as a basis for economic analysis. *BMC Health Services Research* 11, p. 225.

Hatai, K. and Hoshiai, G. 1992. Mass mortality in cultured coho salmon (*Oncorhynchus kisutch*) due to *Saprolegnia parasitica* Coker. *Journal of Wildlife Diseases* 28, pp. 532–536.

Hatai, K. and Hoshiai, G.I. 1994. Pathogenicity of *Saprolegnia parasitica* Coker. In: Mueller G.J. ed. *Salmon Saprolegniasis*. Portland, Oregon, U.S.: Department of Energy, Bonneville Power Administration, pp. 87–98.

Helfman, G.S. 2007. *Fish Conservation: A Guide to Understanding and Restoring Global Aquatic Biodiversity and Fishery Resources*. Washington, D.C.: Island Press, pp. 600.

Hockley, F.A., Wilson, C.A.M.E., Graham, N. and Cable, J. 2014. Combined effects of flow condition and parasitism on shoaling behaviour of female guppies *Poecilia reticulata. Behavioral Ecology and Sociobiology* 68, pp. 1513–1520.

Hodgson, A., Kelly, N. and Peel, D. 2013. Unmanned Aerial Vehicles (UAVs) for Surveying Marine Fauna: A Dugong Case Study. *PLOS ONE* 8, e79556. doi: 10.1371/journal.pone.0079556.

Holdich, D.M. 2003. Crayfish in Europe – an overview of the taxonomy, legislation, distribution, and crayfish plague outbreaks. In: Holdich, D.M. and Sibley, P.J. eds. *Management and Conservation of Crayfish*. Proceedings of a conference held on 7th November 2002 at Nottingham Forest Football Club, Nottingham, UK. Bristol: Environment Agency. 15–34.

Hughes, G.C. 1994. Saprolegniasis: then and now: a retrospective. In: Mueller, G.J. ed. *Salmon saprolegniasis*. Portland, Oregon: Bonneville Power Administration, Division of Fish and Wildlife, pp. 3–32.

Huson, D.H. and Bryant, D. 2006. Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution* 23, pp. 254–267.

Hussein, M.M.A. and Hatai, K. 1999. *Saprolegnia salmonis* sp nov. isolate from sockeye salmon, *Onchorhyncus nerka. Mycoscience* 40, pp. 387–391.

Hussein, M.M.A. and Hatai, K. 2002. Pathogenicity of *Saprolegnia* species associated with outbreaks of salmonid saprolegniosis in Japan. *Fisheries Science* 68, pp. 1067–1072.

International Council for the Exploration of the Sea (ICES) 2018. *Report of the Workshop on MSFD biodiversity of species D1 aggregation (WKDIVAGG)*. Copenhagen, Denmark: ICES HQ.

Isasa, M., Suñer, C., Díaz, M., Puig-Sàrries, P., Zuin, A., Bichman, A., Gygi, S.P., Rebollo, E. and Crosas, B. 2016. Cold Temperature Induces the Reprogramming of Proteolytic Pathways in Yeast. *Journal of Biological Chemistry* 291, pp. 1664–1675.

Jensen, A.J., Johnsen, B.O. and Heggberget, T.G. 1991. Initial feeding time of Atlantic salmon, *Salmo salar*, alevins compared to river flow and water temperature in Norwegian streams. *Environmental Biology of Fishes* 30, pp. 379–385.

Jensen, L.F., Hansen, M.M., Pertoldi, C., Holdensgaard, G., Mensberg, K.-L.D. and Loeschcke, V. 2008. Local adaptation in brown trout early life-history traits: implications for climate change adaptability. *Proceedings of the Royal Society of London B: Biological Sciences* 275, pp. 2859–2868.

Jensen, M.H. 1965. *Disease and salmon in Irish rivers, 1964-1965. Report to the Ministry of Agriculture and Fisheries*, *Dublin*. (Cited by Carbery, J.T. and Strickland, K.T. 1968).

Jiang, R.H.Y., de Bruijn, I., Haas, B.J., Belmonte, R., Löbach, L., Christie, J., van den Ackerveken, G., Bottin, A., Bulone, V., Díaz-Moreno, S.M., Dumas, B., Fan, L., Gaulin, E., Govers, F., Grenville-Briggs, L.J., Horner, N.R., Levin, J.Z., Mammella, M., Meijer, H.J.G., Morris, P., Nusbaum, C., Oome, S., Phillips, A.J., van Rooyen, D., Rzeszutek, E., Saraiva, M., Secombes, C.J., Seidl, M.F., Snel, B., Stassen, J.H.M., Sykes, S., Tripathy, S., van den Berg, H., Vega-Arreguin, J.C., Wawra, S., Young, S.K., Zeng, Q., Dieguez-Uribeondo, J., Russ, C., Tyler, B.M. and van West, P. 2013. Distinctive Expansion of Potential Virulence Genes in the Genome of the Oomycete Fish Pathogen *Saprolegnia parasitica*. *PLOS Genetics* 9, e1003272. doi: 10.1371/journal.pgen.1003272.

Johnson, T., Seymour, R. and Padgett, D. 2002. *Biology and the Systematics of the Saprolegniaceae.* Wilmington, N.C.: University of North Carolina at Wilmington, Department of Biological Sciences, pp. 804.

Jonsson, B. and Jonsson, N. 2009. A review of the likely effects of climate change on anadromous Atlantic salmon *Salmo salar* and brown trout *Salmo trutta*, with particular reference to water temperature and flow. *Journal of Fish Biology* 75, pp. 2381–2447.

Joost, S., Kalbermatten, M. and Bonin, A. 2008. Spatial analysis method (SAM): a software tool combining molecular and environmental data to identify candidate loci for selection. *Molecular Ecology Resources* 8, pp. 957–960.

Kales, S.C., DeWitte-Orr, S.J., Bols, N.C. and Dixon, B. 2007. Response of the rainbow trout monocyte/macrophage cell line, RTS11 to the water molds *Achlya* and *Saprolegnia*. *Molecular Immunology* 44, pp. 2303–2314.

Kamoun, S. 2003. Molecular genetics of pathogenic oomycetes. *Eukaryotic Cell* 2, pp. 191–199.

Kawecki, T.J. and Ebert, D. 2004. Conceptual issues in local adaptation. *Ecology Letters* 7, pp. 1225–1241.

Kiesecker, J.M., Blaustein, A.R. and Miller, C.L. 2001. Transfer of a Pathogen from Fish to Amphibians. *Conservation Biology* 15, pp. 1064–1070.

Kirsop, B.E. and Doyle, A. 1991. *Maintenance of microorganisms and cultured cells: A manual of laboratory methods, 2nd edition*. London: Academic Press, pp. 288.

Kitancharoen, N., Yamamoto, A. and Hatai, K. 1997. Fungicidal effect of hydrogen peroxide on fungal infection of rainbow trout eggs. *Mycoscience* 38, pp. 375–378.

Kitancharoen, N., Yuasa, K. and Hatai, K. 1996. Effects of pH and temperature on growth of *Saprolegnia diclina* and *S. parasitica* isolated from various sources. *Mycoscience* 37, pp. 385–390.

Ko, W.-H. 2003. Long-term storage and survival structure of three species of *Phytophthora* in water. *Journal of General Plant Pathology* 69, pp. 186–188.

Koeypudsa, W., Phadee, P., Tangtrongpiros, J., Hatai, K., Koeypudsa, W., Phadee, P., Tangtrongpiros, J. and Hatai, K. 2005. Influence of pH, Temperature and Sodium Chloride Concentration on Growth Rate of *Saprolegnia* sp. *Journal of Scientific Research, Chulalongkorn University*, pp. 123–130.

Koh, L. and Wich, S. 2012. Dawn of drone ecology: low-cost autonomous aerial vehicles for conservation. *Tropical Conservation Science* 5, pp. 121–132.

Lahnsteiner, F. 2012. Thermotolerance of brown trout, *Salmo trutta*, gametes and embryos to increased water temperatures. *Journal of Applied Ichthyology* 28, pp. 745–751.

Le Morvan, C., Troutaud, D. and Deschaux, P. 1998. Differential effects of temperature on specific and nonspecific immune defences in fish. *Journal of Experimental Biology*. 201, pp. 165–168.

Leggett, H.C., Buckling, A., Long, G.H. and Boots, M. 2013. Generalism and the evolution of parasite virulence. *Trends in Ecology & Evolution* 28, pp. 592–596.

Lenth, R.V. 2016. Least-Squares Means: The R Package lsmeans. *Journal of Statistical Software* 69, pp. 1–33.

Li, H. 2013. *Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM*. Available at: http://arxiv.org/abs/1303.3997 [Accessed: 30 July 2019].

Li, H. 2014. Toward better understanding of artifacts in variant calling from high-coverage samples. *Bioinformatics* 30, pp. 2843–2851.

Li, Y., Ziang, X., Mao, R., Yang, J., Miao, C., Li, Z. and Qiu, Y. 2017. Ten Years of Landscape Genomics: Challenges and Opportunities. *Frontiers in Plant Science* 8, pp. 2136.

Liew, N., Mazon Moya, M.J., Wierzbicki, C.J., Hollinshead, M., Dillon, M.J., Thornton, C.R., Ellison, A., Cable, J., Fisher, M.C. and Mostowy, S. 2017. Chytrid fungus infection in zebrafish demonstrates that the pathogen can parasitize non-amphibian vertebrate hosts. *Nature Communications* 8, 15048. doi: 10.1038/ncomms15048.

Maddock, B.G. 1974. A Technique to Prolong the Incubation Period of Brown Trout Ova. *The Progressive Fish-Culturist* 36, pp. 219–222.

Marx, D.H. and Daniel, W.J. 1976. Maintaining cultures of ectomycorrhizal and plant pathogenic fungi in sterile water cold storage. *Canadian Journal of Microbiology* 22, pp. 338–341.

Mawle, G.W. and Peirson, G. 2009. *Economic evaluation of inland fisheries: managers report from science project*. Bristol: Environment Agency. Available at: https://trove.nla.gov.au/version/31540247 [Accessed: 17 October 2018].

May, R.M. and Anderson, R.M. 1990. Parasite-host coevolution. *Parasitology* 100, pp. S89-101.

Met Office 2018. *UK Climate Projections.* Available at: https://www.metoffice.gov.uk/research/approach/collaboration/ukcp/download-data [Accessed 02 December 2019].

Met Office 2019. *Climate summaries*. Available at: https://www.metoffice.gov.uk/climate/uk/summaries [Accessed: 21 January 2019].

Miller, K.M., Teffer, A., Tucker, S., Li, S., Schulze, A.D., Trudel, M., Juanes, F., Tabata, A., Kaukinen, K.H., Ginther, N.G., Ming, T.J., Cooke, S.J., Hipfner, J.M., Patterson, D.A. and Hinch, S.G. 2014. Infectious disease, shifting climates, and opportunistic predators: cumulative factors potentially impacting wild salmon declines. *Evolutionary Applications* 7, pp. 812–855.

Minor, K.L., Anderson, V.L., Davis, K.S., van den Berg, A.H., Christie, J.S., Löbach, L., Faruk, A.R., Wawra, S., Secombes, C.J. and van West, P. 2014. A putative serine protease, SpSsp1, from *Saprolegnia parasitica* is recognised by sera of rainbow trout, *Oncorhynchus mykiss. Fungal Biology* 118, pp. 630–639.

Mjelde, M., Hellsten, S. and Ecke, F. 2013. A water level drawdown index for aquatic macrophytes in Nordic lakes. *Hydrobiologia* 704, pp. 141–151.

Moran, D. and Dann, S. 2008. The economic value of water use: implications for implementing the Water Framework Directive in Scotland. *Journal of Environmental Management* 87, pp. 484–496.

Mörner, T., Obendorf, D.L., Artois, M. and Woodford, M.H. 2002. Surveillance and monitoring of wildlife diseases. *Revue scientifique et technique (International Office of Epizootics)* 21, pp. 67–76.

Munro, A.L.S. 1970. Ulcerative dermal necrosis, a disease of salmonid fishes in the rivers of the British Isles. *Biological Conservation* 2, pp. 129–132.

Natural Resources Wales 2019. *UK Water Quality Sampling Harmonised Monitoring Scheme Detailed Data*. Available at: http://lle.gov.wales/catalogue/item/UKWaterQualitySamplingHarmonisedMonitoringSchemeDetailedData/?lang=en [Accessed: 3 April 2019].

Neish, G.A. 1977. Observations on saprolegniasis of adult sockeye salmon, *Oncorhynchus nerka* (Walbaum). *Journal of Fish Biology* 10, pp. 513–522.

Nelson, F.A. 1986. Effect of Flow Fluctuations on Brown Trout in the Beaverhead River, Montana. *North American Journal of Fisheries Management* 6, pp. 551–559.

O'Flynn, F.M., McGeachy, S.A., Friars, G.W., Benfey, T.J. and Bailey, J.K. 1997. Comparisons of cultured triploid and diploid Atlantic salmon (*Salmo salar* L.). *ICES Journal of Marine Science* 54, pp. 1160–1165.

Oidtmann, B., Peeler, E., Lyngstad, T., Brun, E., Bang Jensen, B. and Stärk, K.D.C. 2013. Risk-based methods for fish and terrestrial animal disease surveillance. *Preventive Veterinary Medicine* 112, pp. 13–26.

Ojanguren, A.F. and Braña, F. 2003. Thermal dependence of embryonic growth and development in brown trout. *Journal of Fish Biology* 62, pp. 580–590.

Ojanguren, A.F., Reyes-Gavilán, F.G. and Braña, F. 2001. Thermal sensitivity of growth, food intake and activity of juvenile brown trout. *Journal of Thermal Biology* 26, pp. 165–170.

Oksanen, J., Blanchet, J.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H. and Wagner, H. 2012. *vegan: Community Ecology Package*. R package version 1.15-1.

Olden, J.D. and Naiman, R.J. 2010. Incorporating thermal regimes into environmental flows assessments: modifying dam operations to restore freshwater ecosystem integrity. *Freshwater Biology* 55, pp. 86–107.

Olson, D.H., Aanensen, D.M., Ronnenberg, K.L., Powell, C.I., Walker, S.F., Bielby, J., Garner, T.W.J., Weaver, G. and Fisher, M.C. 2013. Mapping the Global Emergence of *Batrachochytrium dendrobatidis*, the Amphibian Chytrid Fungus. *PLoS ONE* 8, e56802. doi: 10.1371/journal.pone.0056802.

Oono, H. and Hatai, K. 2007. Antifungal activities of bronopol and 2-methyl-4-isothiazolin-3-one (MT) against *Saprolegnia*. *Biocontrol Science* 12, pp. 145–148.

Orr, H.G., Simpson, G.L., Clers, S., Watts, G., Hughes, M., Hannaford, J., Dunbar, M.J., Laizé, C.L.R., Wilby, R.L., Battarbee, R.W. and Evans, R. 2015. Detecting changing river temperatures in England and Wales. *Hydrological Processes* 29, pp. 752–766.

Papalexiou, S.M., AghaKouchak, A., Trenberth, K.E. and Foufoula-Georgiou, E. 2018. Global, Regional, and Megacity Trends in the Highest Temperature of the Year: Diagnostics and Evidence for Accelerating Trends. *Earth's Future* 6, pp. 71-79.

Parrish, D., Behnke, J.R., Gephard, S.R., McCormick, S.D. and Reeves, G.H. 2011. Why aren't there more Atlantic salmon (*Salmo salar*)? *Canadian Journal of Fisheries and Aquatic Sciences* 55, pp. 281–287.

Paterson, P.J., Seaton, S., Prentice, H.G. and Kibbler, C.C. 2003. Treatment failure in invasive aspergillosis: susceptibility of deep tissue isolates following treatment with amphotericin B. *Journal of Antimicrobial Chemotherapy* 52, pp. 873–876.

Paul, B. and Steciow, M.M. 2004. *Saprolegnia multispora*, a new oomycete isolates from water samples taken in a river in the Burgundian region of France. *FEMS Microbiology Letters* 237, pp. 393–398.

Phillips, A.J., Anderson, V.L., Robertson, E.J., Secombes, C.J. and van West, P. 2008. New insights into animal pathogenic oomycetes. *Trends in Microbiology* 16, pp. 13–19.

Pickering, A.D. and Duston, J. 1983. Administration of cortisol to brown trout, *Salmo trutta* L., and its effects on the susceptibility to *Saprolegnia* infection and furunculosis. *Journal of Fish Biology* 23, pp. 163–175.

Pickering, A.D. and Willoughby, L.G. 1982. *Saprolegnia* infections of salmonid fish. In: *Fiftieth annual report for the year ended 31st March 1982*. Ambleside, UK: Freshwater Biological Association. Available at: http://aquaticcommons.org/5186/ [Accessed: 6 September 2019].

Pickrell, J.K. and Pritchard, J.K. 2012. Inference of Population Splits and Mixtures from Genome-Wide Allele Frequency Data. *PLOS Genetics* 8, e1002967. doi: 10.1371/journal.pgen.1002967.

Pinheiro, J.C. and Bates, D.M. 2000. *Mixed-Effects Models in S and S-PLUS*. New York: Springer-Verlag, pp. 548.

Plummer, M., Best, N., Cowles, K. and Vines, K. 2006. CODA: convergence diagnosis and output analysis for MCMC. *R News* 6, pp. 7–11.

Poesch, M.S., Chavarie, L., Chu, C., Pandit, S.N. and Tonn, W. 2016. Climate Change Impacts on Freshwater Fishes: A Canadian Perspective. *Fisheries* 41, pp. 385–391.

Poulet, N., Beaulaton, L. and Dembski, S. 2011. Time trends in fish populations in metropolitan France: insights from national monitoring data. *Journal of Fish Biology* 79, pp. 1436–1452.

Poulin, R. and Mouillot, D. 2003. Parasite specialization from a phylogenetic perspective: a new index of host specificity. *Parasitology* 126, pp. 473–480.

Pounds, J.A. 2001. Climate and amphibian declines. *Nature* 410, pp. 639–640.

Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M.A.R., Bender, D., Maller, J., Sklar, P., de Bakker, P.I.W., Daly, M.J. and Sham, P.C. 2007. PLINK: a tool set for whole-genome association and population-based linkage analyses. *American Journal of Human Genetics* 81, pp. 559–575.

R Core Team. 2018. *R: A language and environment for statistical computing.* Vienna, Austria: R Foundation for Statistical Computing. Available at: http://www.R-project.org/.

Rach, J.J., Marks, J.A. and Dawson, V.K. 1995. Effect of Water Flow Rates in Hatching Jars to Control Fungal Infections of Rainbow Trout Eggs. *The Progressive Fish-Culturist* 57, pp. 226–230.

Ramaiah, N.S. 2006. A review on fungal diseases of algae, marine fishes, shrimps and corals. *Indian Journal of Marine Sciences* 35, pp. 380–387.

Ravasi, D., de Respinis, S. and Wahli, T. 2018. Multilocus sequence typing reveals clonality in *Saprolegnia parasitica* outbreaks. *Journal of Fish Diseases* 41, pp. 1653–1665.

Réalis-Doyelle, E., Pasquet, A., Charleroy, D.D., Fontaine, P. and Teletchea, F. 2016. Strong Effects of Temperature on the Early Life Stages of a Cold Stenothermal Fish Species, Brown Trout (*Salmo trutta* L.). *PLOS ONE* 11, e0155487. doi: 10.1371/journal.pone.0155487.

Rees, H.C., Maddison, B.C., Middleditch, D.J., Patmore, J.R.M. and Gough, K.C. 2014. The detection of aquatic animal species using environmental DNA - a review of eDNA as a survey tool in ecology. *Journal of Applied Ecology* 51, pp. 1450–1459.

Reynolds, M., Hockley, F.A., Wilson, C.A.M.E. and Cable, J. 2019. Assessing the effects of water flow rate on parasite transmission amongst a social host. *Hydrobiologia* 830, pp. 201–212.

Richards, R.H. and Pickering, A.D. 1978. Frequency and distribution patterns of *Saprolegnia* infection in wild and hatchery-reared brown trout *Salmo trutta* L. and char *Salvelinus alpinus* (L.). *Journal of Fish Diseases* 1, pp. 69–82.

Richards, R.H. and Pickering, A.D. 1979. Changes in serum parameters of *Saprolegnia*-infected brown trout, *Salmo trutta* L. *Journal of Fish Diseases* 2, pp. 197–206.

Roberge, C., Páez, D.J., Rossignol, O., Guderley, H., Dodson, J. and Bernatchez, L. 2007. Genome-wide survey of the gene expression response to saprolegniasis in Atlantic salmon. *Molecular Immunology* 44, pp. 1374–1383.

Roberts, R.J. 1993. Ulcerative dermal necrosis (UDN) in wild salmonids. *Fisheries Research* 17, pp. 3–14.

Roberts, R.J. 2012. *Fish Pathology*. Hoboken, New Jersey: Wiley-Blackwell, pp. 590.

Robertson, E.J., Anderson, V.L., Phillips, A.J., Secombes, C.J., Dieguez-Uribeondo, J. and van West, P. 2009. *Saprolegnia*—fish interactions. In: Lamour, K. and Kamoun, S. eds. *Oomycete Genetics and Genomics: Diversity, Interactions and Research Tools*. New Jersey: John Wiley & Sons, Inc., pp. 407–424.

Robideau, G.P., de Cock, A.W., Coffey, M.D., Voglmayr, H., Brouwer, H., Bala, K., Chitty, D.W., Désaulniers, N., Eggertson, Q.A., Gachon, C.M., Hu, C., Küpper, F.C., Rintoul, T.L., Sarhan, E., Verstappen, E.C., Zhang, Y., Bonants, P.J. and Lévesque, C.A. 2011. DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. *Molecular Ecology Resources* 11, pp. 1002–1011.

Robinson, C.V., Webster, T.M.U., Cable, J., James, J. and Consuegra, S. 2018. Simultaneous detection of invasive signal crayfish, endangered white-clawed crayfish and the crayfish plague pathogen using environmental DNA. *Biological Conservation* 222, pp. 241–252.

Rocchi, S., Tisserant, M., Valot, B., Laboissière, A., Frossard, V. and Reboux, G. 2017. Quantification of *Saprolegnia parasitica* in river water using real-time quantitative PCR: from massive fish mortality to tap drinking water. *International Journal of Environmental Health Research* 27, pp. 1–10.

Rooke, A.C., Palm-Flawd, B. and Purchase, C.F. 2019. The impact of a changing winter climate on the hatch phenology of one of North America's largest Atlantic salmon populations. *Conservation Physiology* 7. doi: 10.1093/conphys/coz015.

Rowlands, D.J., Frame, D.J., Ackerley, D., Aina, T., Booth, B.B.B., Christensen, C., Collins, M., Faull, N., Forest, C.E., Grandey, B.S., Gryspeerdt, E., Highwood, E.J., Ingram, W.J., Knight, S., Lopez, A., Massey, N., McNamara, F., Meinshausen, N., Piani, C., Rosier, S.M., Sanderson, B.M., Smith, L.A., Stone, D.A., Thurston, M., Yamazaki, K., Hiro Yamazaki, Y. and Allen, M.R. 2012. Broad range of 2050 warming from an observationally constrained large climate model ensemble. *Nature Geoscience* 5, pp. 256–260.

Sample, J.E., Duncan, N., Ferguson, M. and Cooksley, S. 2015. Scotland′s hydropower: Current capacity, future potential and the possible impacts of climate change. *Renewable and Sustainable Energy Reviews* 52, pp. 111–122.

Sandoval-Sierra, J.V., Martín, M.P. and Diéguez-Uribeondo, J. 2014. Species identification in the genus *Saprolegnia* (Oomycetes): Defining DNA-based molecular operational taxonomic units. *Fungal Biology* 118, pp. 559–578.

Santiago, J., Garcia de Jalon, D., Alonso, C., Solana-Gutierrez, J., Ribalaygua, J., Pórtoles, J. and Monjo, R. 2016. Brown trout thermal niche and climate change: Expected changes in the distribution of cold-water fish in central Spain. *Ecohydrology* 9, pp. 514–528.

Sarowar, M.N., Cusack, R. and Duston, J. 2019. *Saprolegnia* molecular phylogeny among farmed teleosts in Nova Scotia, Canada. *Journal of Fish Diseases* 42, pp. 1745–1760.

Savolainen, O., Lascoux, M. and Merilä, J. 2013. Ecological genomics of local adaptation. *Nature Reviews Genetics* 14, pp. 807–820.

Seymour, R. 1970. The genus *Saprolegnia*. *Nova Hedwigia* 19, pp. 1–124.

Shah, S.L. 2010. Impairment in the haematological parameters of tench (*Tinca tinca*) infected by *Saprolegnia* spp. *Turkish Journal of Veterinary and Animal Sciences* 34, pp. 313–318.

Smith, L.M. and Burgoyne, L.A. 2004. Collecting, archiving and processing DNA from wildlife samples using FTA® databasing paper. *BMC Ecology* 4, 4. doi: 10.1186/1472-6785-4-4.

Songe, M.M., Thoen, E., Evensen, Ø. and Skaar, I. 2014. *In vitro* passages impact on virulence of *Saprolegnia parasitica* to Atlantic salmon, *Salmo salar* L. parr. *Journal of Fish Diseases* 37, pp. 825–834.

Stewart, A., Hablützel, P.I., Brown, M., Watson, H.V., Parker-Norman, S., Tober, A.V., Thomason, A.G., Friberg, I.M., Cable, J. and Jackson, J.A. 2018. Half the story: Thermal effects on within-host infectious disease progression in a warming climate. *Global Change Biology* 24, pp. 371–386.

Stewart, A., Jackson, J., Barber, I., Eizaguirre, C., Paterson, R., van West, P., Williams, C. and Cable, J. 2017. Hook, Line and Infection: A Guide to Culturing Parasites, Establishing Infections and Assessing Immune Responses in the Three-Spined Stickleback. *Advances in Parasitology* 98, pp. 39–109.

Strand, D.A., Jussila, J., Johnsen, S.I., Viljamaa-Dirks, S., Edsman, L., Wiik-Nielsen, J., Viljugrein, H., Engdahl, F. and Vrålstad, T. 2014. Detection of crayfish plague spores in large freshwater systems. *Journal of Applied Ecology* 51, pp. 544–553.

Strayer, D.L. and Dudgeon, D. 2010. Freshwater biodiversity conservation: recent progress and future challenges. *Journal of the North American Benthological Society* 29, pp. 344–358.

Stuart, M.R. and Fuller, H.T. 1968. Mycological aspects of diseases of Atlantic salmon. *Nature* 217, pp. 90–92.

Stucki, S., Orozco-terWengel, P., Forester, B.R., Duruz, S., Colli, L., Masembe, C., Negrini, R., Landguth, E., Jones, M.R., The NEXTGEN Consortium, Bruford, M.W., Taberlet, P. and Joost, S. 2017. High performance computation of landscape genomic models including local indicators of spatial association. *Molecular Ecology Resources* 17, pp. 1072–1089.

Taylor, S.G. 2008. Climate warming causes phenological shift in Pink Salmon, *Oncorhynchus gorbuscha*, behavior at Auke Creek, Alaska. *Global Change Biology* 14, pp. 229–235.

Therneau, T. 2015. *A Package for Survival Analysis in S version 2.38*. Available at: https://cran.r-project.org/web/packages/survival/index.html [Accessed 21 September 2017].

Thoen, E., Evensen, Ø. and Skaar, I. 2016. Factors influencing *Saprolegnia* spp. spore numbers in Norwegian salmon hatcheries. *Journal of Fish Diseases* 39, pp. 657–665.

Thorstad, E., Næsje, T., Fiske, P. and Finstad, B. 2003. Effects of hook and release on Atlantic salmon in the River Alta, northern Norway. *Fisheries Research* 60, pp. 293–307.

Toffolon, M., Siviglia, A. and Zolezzi, G. 2010. Thermal wave dynamics in rivers affected by hydropeaking. *Water Resources Research* 46, W08536. doi: 10.1029/2009WR008234.

Torto-Alalibo, T., Tian, M., Gajendran, K., Waugh, M.E., van West, P. and Kamoun, S. 2005. Expressed sequence tags from the oomycete fish pathogen *Saprolegnia parasitica* reveal putative virulence factors. *BMC Microbiology* 5, 46. doi: 10.1186/1471-2180-5-46.

UniProt. 2019. Available at: https://www.uniprot.org/ [Accessed 09 August 2019].

Vähä, J.-P., Erkinaro, J., Niemelä, E. and Primmer, C.R. 2007. Life-history and habitat features influence the within-river genetic structure of Atlantic salmon. *Molecular Ecology* 16, pp. 2638–2654.

van den Berg, A.H., McLaggan, D., Diéguez-Uribeondo, J. and van West, P. 2013. The impact of the water moulds *Saprolegnia diclina* and *Saprolegnia parasitica* on natural ecosystems and the aquaculture industry. *Fungal Biology Reviews* 27, pp. 33–42.

van Vliet, M.T.H., Franssen, W.H.P., Yearsley, J.R., Ludwig, F., Haddeland, I., Lettenmaier, D.P. and Kabat, P. 2013. Global river discharge and water temperature under climate change. *Global Environmental Change* 23, pp. 450–464.

van Vliet, M.T.H., Ludwig, F., Zwolsman, J.J.G., Weedon, G.P. and Kabat, P. 2011. Global river temperatures and sensitivity to atmospheric warming and changes in river flow. *Water Resources Research* 47, W02544. doi: 10.1029/2010WR009198.

van West, P. 2006. *Saprolegnia parasitica*, an oomycete pathogen with a fishy appetite: new challenges for an old problem. *Mycologist* 20, pp. 99–104.

van West, P., de Bruijn, I., Minor, K.L., Phillips, A.J., Robertson, E.J., Wawra, S., Bain, J., Anderson, V.L. and Secombes, C.J. 2010. The putative RxLR effector protein SpHtp1 from the fish pathogenic oomycete *Saprolegnia parasitica* is translocated into fish cells. *FEMS Microbiology Letters* 310, pp. 127–137.

van West, P. and Vleehouwers, V.G.A.A. 2004. The *Phytophthora infestans* - potato interaction. In: Talbot, N.J. ed. *Plant Pathogen Interactions Chapter 9*. Blackwell Scientific Publishers, pp. 219–242.

Vangestel, C., Vázquez-Lobo, A., Martínez-García, P.J., Calic, I., Wegrzyn, J.L. and Neale, D.B. 2016. Patterns of neutral and adaptive genetic diversity across the natural range of sugar pine (*Pinus lambertiana* Dougl.). *Tree Genetics & Genomes* 12. doi: 10.1007/s11295-016-0998-7.

Vanzo, D., Siviglia, A., Carolli, M. and Zolezzi, G. 2016. Characterization of sub-daily thermal regime in alpine rivers: quantification of alterations induced by hydropeaking. *Hydrological Processes* 30, pp. 1052–1070.

Vellanki, S., Huh, E.Y., Saville, S.P. and Lee, S.C. 2019. *Candida albicans* Morphology-Dependent Host FGF-2 Response as a Potential Therapeutic Target. *Journal of Fungi* 5, 22. doi: 10.3390/jof5010022.

Venables, W.N. and Ripley, B.D. 2002. *Modern Applied Statistics with S*. New York: Springer, pp. 495.

Vilgalys, R. and Hester, M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* 172, pp. 4238–4246.

Vishwanath, T.S., Mohan, C.V. and Shankar, K.M. 1998. Epizootic Ulcerative Syndrome (EUS), associated with a fungal pathogen, in Indian fishes: histopathology—`a cause for invasiveness'. *Aquaculture* 165, pp. 1–9.

Vladiĉ, T. and Jätrvi, T. 1997. Sperm motility and fertilization time span in Atlantic salmon and brown trout—the effect of water temperature. *Journal of Fish Biology* 50, pp. 1088–1093.

Vollset, K.W., Skoglund, H., Wiers, T. and Barlaup, B.T. 2016. Effects of hydropeaking on the spawning behaviour of Atlantic salmon *Salmo salar* and brown trout *Salmo trutta*. *Journal of Fish Biology* 88, pp. 2236–2250.

Warren, D.R., Robinson, J.M., Josephson, D.C., Sheldon, D.R. and Kraft, C.E. 2012. Elevated summer temperatures delay spawning and reduce redd construction for resident brook trout (*Salvelinus fontinalis*). *Global Change Biology* 18, pp. 1804–1811.

Warrilow, A.G.S., Hull, C.M., Rolley, N.J., Parker, J.E., Nes, W.D., Smith, S.N., Kelly, D.E. and Kelly, S.L. 2014. Clotrimazole as a potent agent for treating the oomycete fish pathogen *Saprolegnia parasitica* through inhibition of sterol 14α-demethylase (CYP51). *Applied and Environmental Microbiology* 80, pp. 6154–6166.

Wawra, S., Bain, J., Durward, E., de Bruijn, I., Minor, K.L., Matena, A., Löbach, L., Whisson, S.C., Bayer, P., Porter, A.J., Birch, P.R.J., Secombes, C.J. and van West, P. 2012. Host-targeting protein 1 (SpHtp1) from the oomycete *Saprolegnia parasitica* translocates specifically into fish cells in a tyrosine-O-sulphate–dependent manner. *Proceedings of the National Academy of Sciences of the United States of America* 109, pp. 2096–2101.

Webb, B.W. and Walling, D.E. 1992. Long term water temperature behaviour and trends in a Devon, UK, river system. *Hydrological Sciences Journal* 37, pp. 567–580.

Webb, B.W. and Walsh, A.J. 2004. Changing UK river temperatures and their impact on fish populations. In: Webb, B., Acreman, M. and Maksimovic, C. eds. *Hydrology: Science and Practice for the 21st Century, Volume II*. Wallingford: British Hydrological Society, pp. 177–191.

Wessels, J.G.H. and Sietsma, J.H. 1981. Fungal cell walls: a survey. In: *Encyclopedia of plant physiology. Plant carbohydrates.* Berlin: Springer Verlag, pp. 352–394.

Whisler, H.C. 1996. *Identification of* Saprolegnia *Spp. Pathogenic in Chinook Salmon : Final Report*. Washington, D.C.: US Department of Energy.

White, D. 1965. (Cited by Carbery, J.T. and Strickland, K.T. 1968).

White, T.J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. eds. *PCR - Protocols and Applications - A Laboratory Manual*. Academic Press, pp. 315–322.

Williams, G.C. 1966. *Adaptation and Natural Selection*. Princeton, New Jersey: Princeton University Press, pp. 328.

Willoughby, L.G. 1969. Salmon disease in Windermere and the River Leven: The fungal aspect. *Salmon and Trout Magazine* 186, pp. 124–129.

Willoughby, L.G. 1978. Saprolegniasis of salmonid fish in Windermere: a critical analysis. *Journal of Fish Diseases* 1, pp. 51–67.

Willoughby, L.G. 1994. *Fungi and Fish Diseases*. Stirling, Scotland: Pisces Press, pp. 536.

Willoughby, L.G., McGrory, C.B. and Pickering, A.D. 1983. Zoospore germination of *Saprolegnia* pathogenic to fish. *Transactions of the British Mycological Society* 80, pp. 421–435.

Willoughby, L.G. and Pickering, A.D. 1977. Viable saprolegniaceae spores on the epidermis of the Salmonid fish *Salmo trutta* and *Salvelinus alpinus. Transactions of the British Mycological Society* 68, pp. 91–95.

Wittwer, C., Stoll, S., Strand, D., Vrålstad, T., Nowak, C. and Thines, M. 2018. eDNA-based crayfish plague monitoring is superior to conventional trap-based assessments in year-round detection probability. *Hydrobiologia* 807, pp. 87–97.

Wittwer, C., Stoll, S., Thines, M. and Nowak, C. 2019. eDNA-based crayfish plague detection as practical tool for biomonitoring and risk assessment of *Aphanomyces astaci*-positive crayfish populations. *Biological Invasions* 21, pp. 1075–1088.

Wohl, E. 2017. Connectivity in rivers. *Progress in Physical Geography: Earth and Environment* 41, pp. 345–362.

Yuasa, K. and Hatai, K. 1995. Relationship between Pathogenicity of *Saprolegnia* spp. Isolates to Rainbow Trout and their Biological Characteristics. *Fish Pathology* 30, pp. 101–106.

Zolezzi, G., Siviglia, A., Toffolon, M. and Maiolini, B. 2011. Thermopeaking in Alpine streams: event characterization and time scales. *Ecohydrology* 4, pp. 564–576.

Zou, Y., Mason, M.G., Wang, Y., Wee, E., Turni, C., Blackall, P.J., Trau, M. and Botella, J.R. 2018. Nucleic acid purification from plants, animals and microbes in under 30 seconds. *PLOS Biology* 16, e1002630. doi: 10.1371/journal.pbio.1002630.

Appendix 1 – *Saprolegnia* isolates

The table below details the 133 *Saprolegnia* spp. isolates collected by the Environment Agency between 2015 and 2017. N/A indicates the information was not available. Isolates marked with the same superscript number were obtained from the same fish host.

Isolate ID	Sampling location	Date isolated	Host species	Location on host	*Saprolegnia* species (Confirmed by ITS sequencing)
EA001	River Esk, Yorkshire, England. (54°26'59.1"N, 0°48'12.42"W)	10/01/2015	Atlantic salmon (*Salmo salar)*	N/A	*S. parasitica*
EA002	River North Tyne, England. (55°01'52.4"N, 2°07'10.5"W)	17/11/2015	Atlantic salmon	N/A	*S. parasitica*
EA003	River South Tyne, England. (54°59'29.6"N, 2°12'31.3"W)	17/11/2015	Atlantic salmon	N/A	*S. parasitica*
EA004	River North Tyne, England.	24/11/2015	Atlantic salmon	Fin	N/A
EA007	River Ouse, Yorkshire, England. (53°50'08.2"N, 1°04'33.4"W)	25/11/2015	European eel (*Anguilla anguilla)*	Skin	*S. parasitica*
EA008	River Ouse Yorkshire, England.	25/11/2015	European eel	Skin	*S. australis*
EA009	River North Tyne, England.	24/11/2015	Sea trout (*Salmo trutta*)	Fin	*S. parasitica*
EA010	River North Tyne, England.	17/11/2015	Atlantic salmon	N/A	*S. ferax*
EA011	River North Tyne, England.	17/11/2015	Atlantic salmon	N/A	*S. parasitica*
EA012	Lake near Romsey, Hampshire, England. (50°59'40.6"N, 1°34'46.7"W)	22/03/2016	Common carp (*Cyprinus carpio*)	Gill	*S. parasitica*
EA013	Lake near Amwell, Essex, England. (51°46'47.6"N, 0°00'11.2"E)	14/04/2016	Common carp	Flank skin	*S. parasitica*
EA014	River Exe, Exeter, England. (50°51'56.1"N, 3°30'01.3"W)	10/05/2016	Atlantic salmon	Dorsal skin	*S. parasitica*

EA015	River Dart, Devon, England. (50°27'36.432"N, 3°41'42.144"W)	07/06/2016	Sea trout	Dorsal fin	N/A
EA016[1]	River Dart, Devon, England.	03/06/2016	Sea trout	Head	*S. parasitica*
EA017[1]	River Dart, Devon, England.	03/06/2016	Sea trout	Ventral Skin	*S. parasitica*
EA018[2]	River Elwy, Wales. (53°14'08.7"N, 3°34'04.4"W)	22/08/2016	Sea trout	Caudal fin	N/A
EA019[2]	River Elwy, Wales.	22/08/2016	Sea trout	Dorsal fin	N/A
EA020	Bells Mill Fishery, Stourbridge, England. (52°28'17.1"N, 2°10'56.9"W)	31/08/2016	Mirror carp (*Cyprinus carpio*)	Flank skin	*S. parasitica*
EA021	Bells Mill Fishery, Stourbridge, England.	31/08/2016	Mirror carp	Flank skin	*S. australis*
EA022	River South Tyne, England.	01/11/2016	Atlantic salmon	Head	*S. declina*
EA023	River South Tyne, England.	01/11/2016	Sea trout	Flank skin	N/A
EA024	River South Tyne, England.	01/11/2016	Atlantic salmon	Adipose fin	*S. parasitica*
EA025	River South Tyne, England.	01/11/2016	Atlantic salmon	Pectoral fin	*S. parasitica*
EA026	River South Tyne, England.	01/11/2016	Atlantic salmon	Head	*S. parasitica*
EA027	River South Tyne, England.	01/11/2016	Atlantic salmon	Caudal fin	N/A
EA028	River South Tyne, England.	01/11/2016	Atlantic salmon	Pelvic fin	N/A
EA029	River South Tyne, England.	01/11/2016	Atlantic salmon	Head	N/A
EA030	River South Tyne, England.	01/11/2016	Atlantic salmon	Head	*S. parasitica*
EA031	River South Tyne, England.	01/11/2016	Atlantic salmon	Head	N/A
EA032	River South Tyne, England.	01/11/2016	Atlantic salmon	Head	N/A
EA033	River South Tyne, England.	01/11/2016	Sea trout	Caudal fin	*S. parasitica*
EA034	River South Tyne, England.	01/11/2016	Atlantic salmon	Caudal fin	N/A
EA035	River South Tyne, England.	01/11/2016	Atlantic salmon	Head	*S. parasitica*
EA036	River South Tyne, England.	01/11/2016	Sea trout	Head	*S. parasitica*
EA037	River Meon, Hampshire, England. (50°55'36.9"N, 1°09'05.1"W)	02/11/2016	Chub *(Squalius cephalus)*	Flank skin	*S. australis*
EA038	River Meon, Hampshire, England.	02/11/2016	Sea trout	Caudal fin	*S. ferax*
EA039	River North Tyne, England.	02/11/2016	Sea trout	Adipose fin	N/A

EA040	River North Tyne, England.	02/11/2016	Atlantic salmon	Adipose fin	N/A
EA041[3]	River Usk, Wales. (51°43'47.5"N, 2°56'55.9"W)	25/11/2016	Atlantic salmon	Pectoral fin	*S. declina*
EA042[3]	River Usk, Wales	25/11/2016	Atlantic salmon	Caudal fin	N/A
EA043	River Usk, Wales.	25/11/2016	Atlantic salmon	Caudal fin	*S. parasitica*
EA044[4]	River Torridge, Devon, England. (50°57'25.1"N, 4°10'00.4"W)	24/11/2016	Atlantic salmon	Pectoral fin	N/A
EA045	River Torridge, Devon, England.	24/11/2016	Atlantic salmon	Skin of caudal peduncle	N/A
EA046	River Torridge, Devon, England.	03/12/2016	Atlantic salmon	Caudal fin	*S. parasitica*
EA047[4]	River Torridge, Devon, England.	03/12/2016	Atlantic salmon	Pectoral fin	N/A
EA048	River Torridge, Devon, England.	09/12/2016	Atlantic salmon	Pectoral fin	N/A
EA049	River Torridge, Devon, England.	09/12/2016	Atlantic salmon	Pectoral fin	N/A
EA050	River Exe, Exeter, England.	15/12/2016	Atlantic salmon	Head	N/A
EA051	River Axe, England. (50°43'21.6"N, 3°03'23.9"W)	27/11/2016	Atlantic salmon	Pectoral fin	N/A
EA052	River Exe, Exeter, England.	14/12/2016	Atlantic salmon	Caudal fin	N/A
EA053	River Exe, Exeter, England.	14/12/2016	Atlantic salmon	Pectoral fin	N/A
EA054	River Gaunless, County Durham, England. (54°37'32.3"N, 1°46'13.6"W)	12/09/2016	Sea trout	Skin from shoulder region	*S. parasitica*
EA055[5]	River Coquet, Northumberland, England. (55°18'25.8"N, 1°55'20.5"W)	18/12/2016	Atlantic salmon	Head	*S. parasitica*
EA056[5]	River Coquet, Northumberland, England.	18/12/2016	Atlantic salmon	Dorsal fin	*S. parasitica*
EA057[5]	River Coquet, Northumberland, England.	18/12/2016	Atlantic salmon	Caudal fin	*S. parasitica*
EA058[6]	River North Tyne, England.	19/12/2016	Atlantic salmon	Head	*S. parasitica*
EA059[6]	River North Tyne, England.	19/12/2016	Atlantic salmon	Pectoral fin	*S. parasitica*
EA060[7]	River North Tyne, England.	19/12/2016	Atlantic salmon	Head	*S. parasitica*

EA061[7]	River North Tyne, England.	19/12/2016	Atlantic salmon	Adipose fin	*S. parasitica*
EA062[7]	River North Tyne, England.	19/12/2016	Atlantic salmon	Caudal fin	*S. parasitica*
EA063[8]	River North Tyne, England.	19/12/2016	Atlantic salmon	Pectoral fin	*S. parasitica*
EA064[8]	River North Tyne, England.	19/12/2016	Atlantic salmon	Dorsal fin	*S. parasitica*
EA065[8]	River North Tyne, England.	19/12/2016	Atlantic salmon	Caudal fin	*S. parasitica*
EA066[9]	River North Tyne, England.	19/12/2016	Atlantic salmon	Head	N/A
EA067[9]	River North Tyne, England.	19/12/2016	Atlantic salmon	Dorsal fin	N/A
EA068[10]	River South Tyne, England.	19/12/2016	Atlantic salmon	Head	*S. parasitica*
EA069[10]	River South Tyne, England.	19/12/2016	Atlantic salmon	Pectoral fin	*S. parasitica*
EA070[10]	River South Tyne, England.	19/12/2016	Atlantic salmon	Dorsal Fin	*S. parasitica*
EA071[10]	River South Tyne, England.	19/12/2016	Atlantic salmon	Caudal Fin	*S. parasitica*
EA072[11]	River South Tyne, England.	19/12/2016	Atlantic salmon	Head	*S. parasitica*
EA073[11]	River South Tyne, England.	19/12/2016	Atlantic salmon	Pectoral fin	N/A
EA074[11]	River South Tyne, England.	19/12/2016	Atlantic salmon	Pelvic fin	N/A
EA075[11]	River South Tyne, England.	19/12/2016	Atlantic salmon	Pelvic fin	N/A
EA076[11]	River South Tyne, England.	19/12/2016	Atlantic salmon	Anal fin	N/A
EA077[11]	River South Tyne, England.	19/12/2016	Atlantic salmon	Caudal fin	*S. parasitica*
EA078	River East Lyn, Somerset, England. (51°13'19.5"N, 3°47'12.5"W)	30/12/2016	Atlantic salmon	Adipose fin	N/A
EA079[12]	River Usk, Wales.	16/01/2017	Atlantic salmon	Pectoral fin	N/A
EA080[12]	River Usk, Wales.	16/01/2017	Atlantic salmon	Caudal fin	N/A
EA081[13]	River Usk, Wales.	16/01/2017	Atlantic salmon	Pectoral fin	*S. parasitica*
EA082[13]	River Usk, Wales.	16/01/2017	Atlantic salmon	Caudal fin	N/A
EA083[14]	River Usk, Wales.	16/01/2017	Atlantic salmon	Caudal fin	N/A
EA084[14]	River Usk, Wales.	16/01/2017	Atlantic salmon	Pelvic fin	N/A
EA085[14]	River Usk, Wales.	16/01/2017	Atlantic salmon	Pectoral fin	N/A
EA086[15]	River Usk, Wales.	16/01/2017	Atlantic salmon	Caudal fin	N/A
EA087[15]	River Usk, Wales.	16/01/2017	Atlantic salmon	Dorsal fin	N/A
EA088[16]	River Usk, Wales.	16/01/2017	Atlantic salmon	Pectoral fin	N/A
EA089[16]	River Usk, Wales.	16/01/2017	Atlantic salmon	Adipose fin	N/A
EA090[16]	River Usk, Wales.	16/01/2017	Atlantic salmon	Caudal fin	N/A

EA091[17]	River Great Ouse, England. (52°18'51.5"N, 0°12'40.2"W)	10/02/2017	Armoured catfish *(Callichthys callichthys)*	Head	*S. ferax*
EA092[17]	River Great Ouse, England.	10/02/2017	Armoured catfish	Head	*S. ferax*
EA093	Belmont Pool, Hereford, England. (52°02'18.4"N, 2°44'55.3"W)	11/04/2017	Mirror carp	Flank skin	*S. parasitica*
EA094	Belmont Pool, Hereford, England.	11/04/2017	Mirror carp	Dorsal fin	*S. parasitica*
EA095	Belmont Pool, Hereford, England.	11/04/2017	Mirror carp	Head	*S. parasitica*
EA096[18]	River Coquet, Northumberland, England.	19/04/2017	Atlantic salmon	Flank skin	*S. parasitica*
EA097[18]	River Coquet, Northumberland, England.	19/04/2017	Atlantic salmon	Adipose fin	*S. parasitica*
EA098[18]	River Coquet, Northumberland, England.	19/04/2017	Atlantic salmon	Flank skin	*S. parasitica*
EA099[18]	River Coquet, Northumberland, England.	19/04/2017	Atlantic salmon	Flank skin	*S. parasitica*
EA100	Dagnam Lake, Dagnam Park, Romford, England. (51°36'56.3"N, 0°14'33.6"E)	26/04/2017	Mirror carp	Flank skin	N/A
EA101	River Dart, Devon, England.	23/06/2017	Atlantic salmon	Skin	*S. parasitica*
EA102	River Dart, Devon, England.	16/06/2017	Atlantic salmon	Anal and pelvic fins	*S. ferax*
EA103	River North Tyne, England.	01/11/2017	Atlantic salmon	Head	N/A
EA104	River North Tyne, England.	01/11/2017	Sea trout	Head	N/A
EA105[19]	River North Tyne, England.	01/11/2017	Atlantic salmon	Head	*S. parasitica*
EA106[19]	River North Tyne, England.	01/11/2017	Atlantic salmon	Flank skin	*S. parasitica*
EA107	River North Tyne, England.	01/11/2017	Sea trout	Flank skin	N/A
EA108	River North Tyne, England.	01/11/2017	Atlantic salmon	Head	N/A
EA109[20]	River North Tyne, England.	01/11/2017	Atlantic salmon	Dorsal fin	*S. parasitica*
EA110[20]	River North Tyne, England.	01/11/2017	Atlantic salmon	Caudal fin	*S. parasitica*
EA111	River North Tyne, England.	01/11/2017	Atlantic salmon	Anal fin	N/A
EA112	River North Tyne, England.	01/11/2017	Atlantic salmon	Head	*S. parasitica*
EA113	River North Tyne, England.	01/11/2017	Sea trout	Pectoral fin	N/A

EA114[21]	River North Tyne, England.	01/11/2017	Sea trout	Caudal fin	*S. parasitica*
EA115[21]	River North Tyne, England.	01/11/2017	Sea trout	Caudal fin	N/A
EA116	River North Tyne, England.	01/11/2017	Atlantic salmon	Head	N/A
EA117	River North Tyne, England.	01/11/2017	Atlantic salmon	Pectoral fin	N/A
EA118	River North Tyne, England.	01/11/2017	Sea trout	Caudal fin	*S. parasitica*
EA119[22]	River North Tyne, England.	01/11/2017	Atlantic salmon	Flank skin	N/A
EA120[22]	River North Tyne, England.	01/11/2017	Atlantic salmon	Dorsal ridge	*S. parasitica*
EA121	River North Tyne, England.	01/11/2017	Sea trout	Dorsal fin	N/A
EA122	River North Tyne, England.	01/11/2017	Atlantic salmon	Skin of caudal peduncle	N/A
EA123[23]	River South Tyne, England.	16/11/2017	Atlantic salmon	Pectoral fin	*S. parasitica*
EA124[23]	River South Tyne, England.	16/11/2017	Atlantic salmon	Head	*S. parasitica*
EA125[23]	River South Tyne, England.	16/11/2017	Atlantic salmon	Anal fin	*S. parasitica*
EA126[24]	River South Tyne, England.	16/11/2017	Atlantic salmon	Caudal fin	*S. parasitica*
EA127[24]	River South Tyne, England.	16/11/2017	Atlantic salmon	Anal fin	*S. parasitica*
EA128[24]	River South Tyne, England.	16/11/2017	Atlantic salmon	Pelvic fin	*S. parasitica*
EA129	River South Tyne, England.	16/11/2017	Atlantic salmon	Caudal fin	*S. parasitica*
CF005	Roath Brook, Cardiff, Wales. (51°29'54.1572"N, 3°9'54.2484"W)	07/08/2016	Three-spined stickleback (*Gasterosteus aculeatus*)	Dorsal fin	N/A
CF006	Roath Brook, Cardiff, Wales.	15/07/2016	Three-spined stickleback	Gill	*S. parasitica*
CF007	Roath Brook, Cardiff, Wales.	15/07/2016	Three-spined stickleback	Caudal fin	*S. parasitica*
CF008	Roath Brook, Cardiff, Wales.	27/07/2016	Three-spined stickleback	Pectoral fin	N/A
CF009	Roath Brook, Cardiff, Wales.	09/08/2016	Three-spined stickleback	Dorsal fin	*S. parasitica*
CF010	Roath Brook, Cardiff, Wales.	15/08/2016	Three-spined stickleback	Dorsal fin	*S. parasitica*

Appendix 2 – Optimisation of *Saprolegnia* zoospore production

Optimisation of zoospore production was attempted; mycelial mats were placed in the conditions outlined in the table below and zoospore production was monitored every 24 h for 7 days. Consistent zoospore production was not achieved using any of the trialled conditions.

Temperatures (°C)	Water conditions
5, 10, 15, 20	Autoclaved dechlorinated water
	Autoclaved aquaria water
	Dechlorinated water
	Aquaria water
	90/10 mix of dechlorinated and aquaria water
	80/20 mix of dechlorinated and aquaria water
	70/30 mix of dechlorinated and aquaria water
	60/40 mix of dechlorinated and aquaria water
	50/50 mix of dechlorinated and aquaria water
	40/60 mix of dechlorinated and aquaria water
	30/70 mix of dechlorinated and aquaria water
	20/80 mix of dechlorinated and aquaria water
	10/90 mix of dechlorinated and aquaria water

Appendix 3 – *Saprolegnia* cryopreservation

Cryopreservation of *Saprolegnia* spp. stock cultures was trialled during this PhD. Mycelial plugs, approximately 5 mm^2 in size, were placed into 2 ml cryogenic vials (Corning External Thread Cryogenic Vials) containing different concentrations of the cryoprotectants Glycerol and Dimethyl sulfoxide (DMSO) alongside glucose-yeast broth (Glucose 10g L^{-1}, Yeast Extract 2.5g L^{-1}) (see table below). The cryogenic vials were subsequently placed into a Mr. Frosty™ Freezing Container (Thermo Scientific) within a -80°C freezer; achieving a cooling rate of approximately -1°C min^{-1}. After 24 h, the samples were thawed rapidly for 5 mins at room temperature and the mycelial plugs were placed onto fresh potato dextrose agar (PDA) (39g L^{-1}) plates immediately. The agar plates were then placed at room temperature and monitored daily for signs of mycelial growth. Revival of the mycelial cultures was not achieved using this method.

Trial	Cryoprotectant		Glucose-yeast broth (%)
	Glycerol (%)	Dimethyl sulfoxide (DMSO) (%)	
1	5	0	95
2	10	0	90
3	15	0	85
4	0	5	95
5	0	10	90
6	0	15	85
7	5	5	90
8	10	10	80

Appendix 4 – *Saprolegnia* DNA capture and storage

Whatman® FTA® DNA binding cards retain nucleic acids and enable their long-term storage at room temperature; this greatly aids sample collection within the field and also reduces the need for laboratory freezers. Furthermore, these cards can be used directly in PCR reactions, allowing easy DNA amplification. *Saprolegnia* spp. DNA capture and amplification directly from Whatman® Indicating FTA® Micro Cards was achieved using the conditions and Whatman® protocols outlined below alongside the PCR protocol implemented in Chapters 3 and 4 of this thesis. However, protocol optimisation for *Saprolegnia* spp. was not achieved, i.e. minimum concentration of zoospores or size of mycelial scrape required for successful DNA amplification and detection.

Zoospore suspensions	
Zoospore concentration (L^{-1})	Method of DNA capture
$3x10^5$	Poured zoospore water onto card
$1.5x10^5$	
$7.5x10^4$	
$3.75x10^4$	
$3x10^5$	Submerged card in zoospore water for 2 mins
$1.5x10^5$	
$7.5x10^4$	
$3.75x10^4$	
$3x10^5$	Submerged card in zoospore water for 1 min
$1.5x10^5$	
$7.5x10^4$	
$3.75x10^4$	
$3x10^5$	Submerged card in zoospore water for 30 secs
$1.5x10^5$	
$7.5x10^4$	
$3.75x10^4$	
Mycelial scrapes	
Size of mycelial scrape (mm^2)	Method of DNA capture
~ 5	Extracted mycelia from live fish host and placed directly onto card

Whatman® protocols:

(1) FTA Protocol BD08: Preparing an FTA® Disc for DNA Analysis.

(2) Nucleic Acid Sample Preparation for Downstream Analyses: Principles and Methods.