Reeling in the Truth: Drivers of Aquatic Infection & Control

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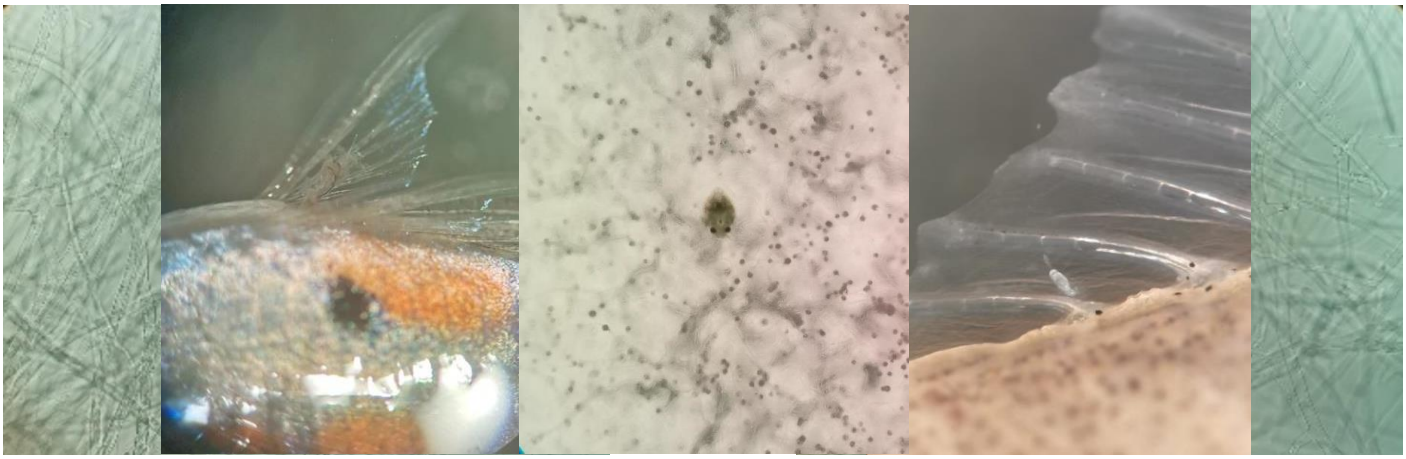

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Contents

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"The circumstances of one's birth are irrelevant; it is what you do with the gift of life that determines who you are." – Mewtwo, Pokemon

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Thesis Abstract

Freshwater habitats are facing high extinction rates, due to anthropogenic influences from habitat loss and over-exploitation. Combine this with the threat posed by infectious disease, and all freshwaters, be they wild, aquaculture or ornamental, require aid to mitigate biodiversity loss. This PhD sought to assess the drivers of aquatic infection and control, with the goal to create a resource to aid management of freshwater systems. Assessing aquatic disease control was accomplished through a literature review of aquatic diagnostics, where the past, present and future methods of aquatic diagnostics were collated and assessed under four pillars of: high sensitivity, specificity, rapid diagnosis, and cost-effectiveness**.** Once the scope of aquatic diagnostics was assessed, the next step was to ensure and optimise a method for adequate supplies of aquatic parasites, to be available for experimental studies. This led to the testing of two storage methods on the aquatic parasite *Saprolegnia parasitica*, mineral oil immersion and cryopreservation. Both methods were effective for short- and mid-term (1 – 50 days) preservation, particularly cryopreservation, which led to experimental studies conducted on the drivers of infection. Drivers were divided into two broad categories, natural and non-natural. For natural drivers, temperature and light were assessed. First, in how they impact the *in vitro* growth of *S. parasitica*, where it was found that growth rate (regardless of strain) was highest at 25°C. Light was found to impact different strains differently, with the common impact being reduced growth when exposed to constant light. This led to assessment of light impacts on *Gyrodactylus turnbulli*. Here, the impacts of light exposure on both natural and experimentally infected guppies (*Poecilia reticulata*) were assessed, revealing greater maximum parasite burdens for fish exposed to constant light. Moving to non-natural drivers, pollutants specifically microfibres were assessed. Three fibre types, polyester, cotton and bamboo, were evaluated on their impacts on guppy-*G. turnbulli* infections and off-host survival of *G. turnbulli*. Polyester was found to increase maximum parasite burdens and hasten parasite mortality, whilst bamboo had significantly lower parasite burdens and no impact on parasite survival. This prompted further investigation into bamboo fibres and their associated dye, where no negative impacts were found in regard to parasite burdens, but respirometry was impacted. The data provided throughout this thesis furthers our understanding of the drivers and control of aquatic infectious disease.

Chapter 1 – General Introduction

Freshwater fish are a core component to life, intrinsic to ecosystems (Postel & Carpenter, 1997; Radinger et al., 2019) and human life, as a protein source and as pets (Modesto et al., 2018; Sinclair et al., 2021; Hossain et al., 2022). Currently estimated at 35,300 extant species of fish (Froese & Pauly, 2023), more than 15,000 of those inhabit freshwater systems (McDermott, 2021) despite freshwater accounting for only 1% of the earth's surface (Lévêque et al., 2008). These species rich systems are at risk of habitat destruction, overexploitation, infectious disease, invasion by non-native species and pollution, all of which have led to higher extinction rates compared to any terrestrial vertebrate group (Dudgeon et al., 2006; Lévêque et al., 2008; Adams et al., 2014; Pimm et al., 2014). The role fish play within ecosystems ranges from food chain interplay to system stability, so the losses these groups face may have detrimental impacts on whole ecosystems.

Aquaculture, the controlled rearing and cultivation of aquatic organisms, emerged from Asia to become a globally important industry in addressing international food security, with the added benefit of economic growth (Subasinghe et al., 2009). The majority of fish cultivated this way are for human consumption, the capability for which has been enhanced due to the intensification of aquaculture over recent years (Ahmad et al., 2021). The scale of food production by aquaculture is only outpaced by that of poultry, with aquaculture producing 157 million tonnes of product for consumption in 2018 alone (FAO, 2020). The greatest proportion of consumable aquaculture production (~77%) is provided by freshwater, including more than 332 aquatic species ranging from molluscs and crustaceans to fish (Metian et al., 2020; Zhang et al., 2022). In parallel, the ornamental fish trade gained substantial popularity as a market contributing to the aquaculture sector's economic significance, with the sector now worth billions of pounds involving trade between more than 50 countries (Biondo & Burki, 2020). As the global population continues to increase, the demand for seafood and ornamental fish has surged, prompting aquaculture to expand rapidly (5.8% annual growth since 2001), e.g. live-weight production was 34 Mt in 1997, increasing to 112 Mt in 2017 (Pouli et al., 2019; FAO, 2020; Schar et al., 2020; Naylor et al., 2021). The combined influence of food and ornamental aquaculture has not only augmented the aquaculture industry's scale on the whole but also brought attention to the challenges faced regarding the health and well-being of aquatic organisms. In this context, understanding and mitigating the impact of stressors on aquatic environments and their inhabitants has become paramount. Despite facing many difficulties

(land use, transport, diet; Ashley, 2007), arguably the greatest difficulty facing aquaculture (including the ornamental trade) is infectious disease.

Aquatic Diseases

Losses attributed to infectious diseases within aquaculture have been estimated to cost upward of £10 billion per annum (Shinn et al., 2015), and given that this was estimated over 8 years ago costs have likely increased, particularly given the rise in antimicrobial usage (Schar et al., 2020). This cost comprises the loss of animal life, cost of treatment and prophylaxis and labour (Oidtmann et al., 2011; Stentiford et al., 2017), but difficulties also arise in regard to targeting the causative agents of these losses. Aquatic organisms must combat various pathogens including bacteria, viruses and fungi, all of which may be categorised as parasites (an organism which is dependent on a host, in turn causing some level of harm to said host) (Méthot & Alizon, 2014). In addition, to the wide scope of pathogens which pose a threat to aquatic organisms, the majority of parasites are cryptic so identification and diagnosis can be difficult. To counter this, effective and efficient methods of diagnosis and treatment are required.

Aquatic Diagnostics

One of the most vital tools against infectious diseases are accurate and effective diagnostics. The adage holds true, where prevention is better than treatment, but diagnosis is required for both aspects. Effective mitigation methods could save aquaculture upwards of £4 billion per year (The World Bank, 2014; Stentiford et al., 2017). Diagnosis of aquatic diseases come with a host of difficulties, due to the inherit properties of water being so dynamic (Davies-Colley & Smith, 2001; Pérez et al., 2013). The field of aquatic diagnostics is multidisciplinary, encompassing historic techniques to novel high-tech methods, providing a comprehensive assessment of aquatic ecosystems and the health of aquatic organisms (Hutson et al., 2023). Recently, advanced molecular and genetic techniques have expanded the scope of aquatic diagnostics, allowing for the identification of specific pathogens, genetic diversity, and earlier detection of emerging threats to aquatic ecosystems (Purcell et al., 2011). The integration of these diagnostic approaches plays a pivotal role in safeguarding the health of aquatic systems, supporting sustainable aquaculture practices, and addressing environmental challenges, ultimately contributing to the conservation and responsible management of our water resources (Adams & Thompson, 2011). Additionally, current diagnostic techniques for assessing aquatic stressors often lack integration and specificity, hindering our ability to comprehensively address these challenges (Adams & Thompson, 2011; Paladini et al., 2017).

Stressors – temperature, light and plastics

Fish, and other organisms, will rarely experience pathogenic infections without external perturbations, be they environmental or physical. It is common for external stressors to facilitate or exacerbate infections (e.g. Avtalion & Clem, 1981; Hedrick, 1998; van Muiswinkel et al., 1999). This is particularly true for aquatic organisms, which live in a turbulent and fluctuating environment. With the transition from the Holocene to Anthropocene, water temperatures are consistently rising (Dokuli et al., 2021). With this global warming, entire ecosystems will be subject to water temperatures outside their optima. Fortunately, with regard to abiotic stressors, temperature-based impacts are relatively well studied (e.g. Bly & Clem, 1991; Brierly & Kingsford, 2009; Buckley & Huey, 2016). We know temperature can impact organism, growth, reproduction, migrations etc. (Buckley & Huey, 2016), but impacts on parasites are understudied compared to their host counterparts. Key to our understanding is the correlation between water temperature and host immune status, where increasing water temperatures can either improve or impair host immune function (species dependent) (Fletcher, 1986; Bly et al., 1997; Bowden, 2008).

Conversely to temperature, light as a stressor remains relatively understudied. Light is another key abiotic factor involved in organism functions from growth and reproduction to immunity (Ángeles Esteban et al., 2006; Taylor et al., 2006; Brown et al., 2014; Ellison et al., 2021). Light pollution, caused by the excessive and improper use of artificial lighting, has been identified as a disruptive factor for various ecosystems. Of note is artificial light at night (ALAN), where artificial light (of anthropogenic origin) disrupts the natural cycle of lighting by illuminating organisms during dark periods (Gaston et al., 2015). In aquatic environments, light pollution can affect the circadian rhythms, behaviour, and physiology of aquatic organisms, ultimately leading to decreased fitness and survival rates (e.g. Bruning et al. 2018; Schligler et al., 2021). As with temperature, what remains understudied is the impact light may have on the parasites of hosts, and the impact it may have on their relationships.

Whilst temperature and light show natural fluctuations and can be manipulated by humans for a benefit, other stressors such as pollutions are much more sporadic and out-with our control (beyond pollution mitigation efforts). Perhaps the most prominent group of pollutants currently in regard to aquatic systems are microplastics (Shim et al., 2018). Deemed as a plastic (any synthetic polymer not of natural origin) particle smaller than 5 mm (GESAMP, 2015), microplastics have gained a reputation for their sheer abundance globally (Kanhai et al., 2017). Whilst most studies and work have focused on granular or spherical microplastics, a large proportion of microplastics are fibrous

in nature (Boucher & Friot, 2017; Horton et al., 2017; Vince & Stoett, 2018; Suaria et al., 2020). Fibre pollution is currently understudied but forms a potentially significant area involved in the disruption of aquatic ecosystems, stemming from the release of microplastics and synthetic fibres into aquatic systems. These microplastics can be ingested by fish, potentially causing physical harm and introducing toxic substances into their bodies (Spanjer et al., 2010; Lusher et al., 2017; Huang et al., 2020; Hu et al., 2020). As these fibres can break down to the nano-scale, even pollution mitigation efforts cannot guarantee absence of fibre pollution.

Aquatic Parasites – *Saprolegnia*

One of the most prominent aquatic parasitic groups, particularly when taken from the view of salmonid aquaculture, are the oomycetes (fungal-like, but more closely related to algae) of the genus *Saprolegnia* (van West, 2006). Present globally in freshwaters, *Saprolegnia* spp. are (as the name suggests) saprotrophic, but also hemibiotrophic and nectrotrophic, feeding on dead fish and other organic substrata (Bruno & Wood, 1994). Of the 23-24 described *Saprolegnia* species the most pertinent with regard to fish health is *Saprolegnia parasitica*, the causative agent of saprolegniosis or saprolegniasis (Sandoval-Sierra & Diéguez-Uribeondo, 2015). Saprolegniasis presents as the growth of “fluffy” white patches on the dermis of fish and is often clearly visible from above the surface of the water (Earle & Hintz, 2014) (Figure 1.1). Typically, clinical signs present primarily on the skin, gills and fins where the mycelia invade the dermal layers causing osmoregulatory disruption and potential death due to haemodilution (Richards and Pickering, 1979; Shah, 2010). Due to their opportunistic nature, *Saprolegnia* spp. were originally only considered secondary pathogens, infecting through lesions or abrasions, however *S. parasitica* is now regarded as a primary pathogen and itself can be highly virulent (Willoughby & Pickering, 1977; Shin et al., 2017; Trusch et al., 2018). For freshwater systems globally this parasite poses an unpredictable problem, with outbreaks occurring seasonally but sporadically (van West, 2006).

Figure 1.1 Young rainbow trout displaying the characteristic signs of saprolegniasis.

The greatest issue with this parasite currently is the lack of effective treatment and prevention methods. Previously, the compound malachite green was utilised as a treatment for saprolegniasis (Earle & Hintz, 2014), as a general fungicide within aquaculture (Tedesco et al., 2018). However, after numerous studies displaying the toxic and carcinogenic properties of malachite green in organisms (Culp et al., 2006; Tedesco et al., 2018), it was swiftly banned globally as a treatment of animals destined for human consumption in 2000 (European Commission, 2010). Despite assessment of numerous alternatives (e.g. salt (NaCl) (Ali, 2005; van West, 2006), boric acid (Ali et al., 2014; Ali et al., 2019), ozone (Forneris et al., 2003), copper sulphate (Marking et al., 1994; Straus et al., 2012) and formalin (Bly et al., 1996; Walser & Phelps, 1994)) there is no current effective and efficient treatment for saprolegniasis which does not potentially harm hosts or those consuming the treated fish (Barde et al., 2020). Given that saprolegniasis is responsible for 10% of all losses in salmonid aquaculture (Hussein & Hatai, 2002; van West, 2006; Phillips et al., 2008; Robertson et al., 2009; van den Berg et al., 2013), knowledge and understanding of *Saprolegnia* spp. remains a requirement for ensuring food security and animal welfare moving into the future.

Aquatic Parasites – *Gyrodactylus*

Another aquatic parasitic group which pose problems to fish globally (including ornamental and farmed fish) are the gyrodactylids, a large group of ubiquitous ectoparasitic monogeneans. Estimated to comprised over 400 named species (Bakke et al., 2007), with the total potentially estimated to be around 20,000 species yet to be revealed (Bakke et al., 2007). Likely the most unique, and characteristic, aspect of these parasites is their hyper viviparity, the capability for the "mother" parasite to contain not only a "daughter" but also a developing "granddaughter" within the "daughter" (Cable & Harris, 2002; Bakke et al., 2007) (Figure 1.2). This adaptation can allow for exponential growth of populations on a host, as a single parasite can populate a host within days. Combine this rapid generation with direct transmission to hosts, and the gyrodactylids form a highly functional and effective parasitic group. To establish themselves on hosts, the gyrodactylids will slightly penetrate the dermis of the fish using hooks, leaving small abrasions and penetrations along the host (Shinn et al., 2005). Established infection is then deemed gyrodactylosis (Malmberg, 1993). The greater the infection burden, the greater the number of worms, and thus the greater the abrasions/lesions created. These abrasions/lesions provide a route of entry for other pathogens, leading to co-infection and deterioration of the host and as such the greater the likelihood of

secondary infections (Cusack & Cone, 1985; Cusack & Cone, 1986; Abdel-Latif et al., 2020). Two species are of particular note, *Gyrodactylus salaris* and *Gyrodactylus turnbulli*. *G. salaris* is notable due to its decimation of Atlantic salmon (*Salmo salar*) populations in Norway in the 1970's (Heggberget & Johnsen, 1982; Johnsen & Jensen, 1992), resulting in the pathogen being deemed "notifiable" by the World Organisation for Animal Health in 1983 (Harris et al., 2011). The outbreaks in Norway were treated seriously due to the severe economic and environmental impacts, where the chemical rotenone was applied as an anti-helminthic to affected rivers. Eradication of the parasites was successful; however, the rotenone also decimated the natural fish populations of treated rivers (Johnsen & Jensen, 1982). This presents a case study on the importance of efficient and effective pathogen control. *G. turnbulli* is a warm-water relative of *G. salaris* with a lesser economic impact. However, the short generation time coupled with a direct mode of transmission between hosts allowed for *G. turnbulli* to become a model pathogen (King & Cable, 2007). Although less pathogenic than *G. salaris*, *G. turnbulli* infections can result in morbidity and mortality of their hosts, generally due to the exponential growth of the parasite population on a single host.

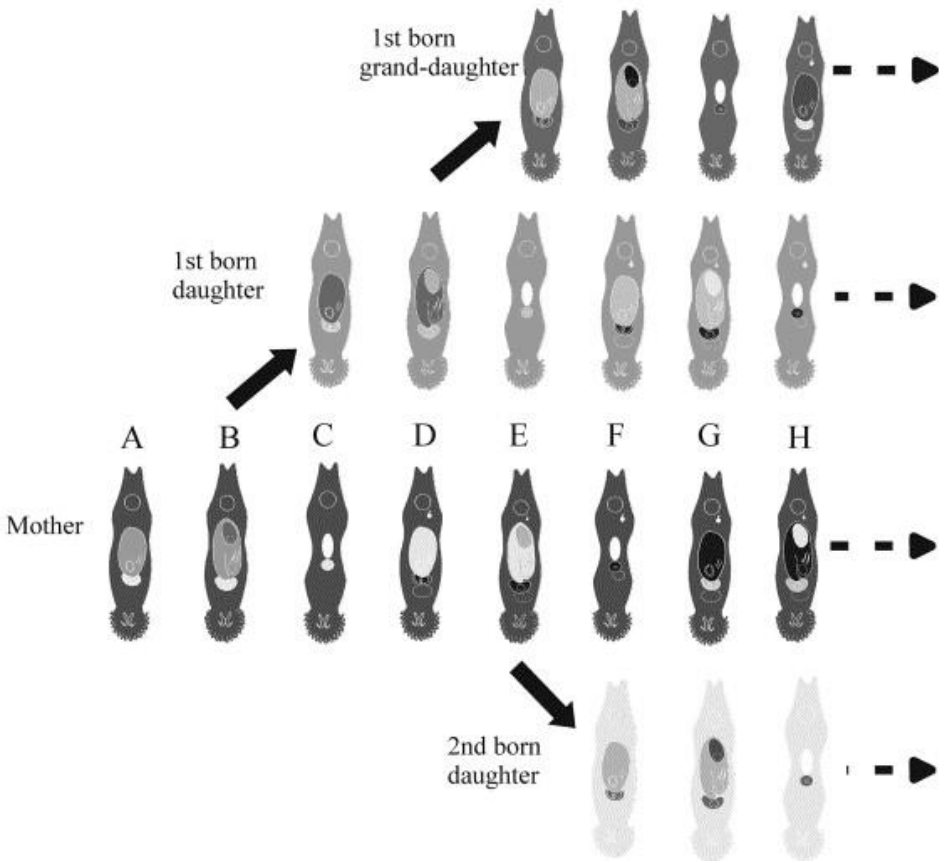

Figure 1.2 Reproduction of *Gyrodactylus turnbulli*. The "mother" parasite will first produce a daughter asexually, with every subsequent daughter after that through either parthenogenesis or sexual reproduction, resulting in morphologically indistinguishable offspring. This can happen every 24 hours under optimal conditions, resulting in exponential population growth (from Cable & Harris, 2002).

Thesis Aims and Objectives

This thesis aims to bridge knowledge gaps in: the scope and potential of aquatic diagnostics, the impacts of natural drivers on host-parasite dynamics, and how pollutants (non-natural drivers) impact fish health. In Chapter 2 the scope of methods available to aquatic diagnostics is directly addressed, with a literature review covering the past, present and future techniques utilised, framed under the pillars of high sensitivity, specificity, speed, and cost-effectiveness. Then, the key data chapters utilise two different host-parasite systems. Firstly, the *in vitro* model of *Saprolegnia parasitica* on potato dextrose agar (PDA) and then *in vivo* the guppy-*G. turnbulli* system. Chapter 3 presents two methods of parasite preservation, specifically for *Saprolegnia parasitica*, as a means for ensuring stocks for research alongside the potential for developing an archival database of parasites. The parasite *Saprolegnia parasitica* is then utilised in Chapter 4, where the impacts of two abiotic factors, temperature and light, on *in vitro* growth are tested. Chapter 5 continues to assess light impacts, this time on the host-parasite system guppy-*Gyrodactylus*. The thesis then shifts to a different stressor, this time fibre pollution, where Chapter 6 assesses the impact of three different fibre types on host-parasite dynamics. This is followed in Chapter 7 by a more in depth look at a specific fibre type, bamboo, and how it and its associated dye can impact fish health. To round off the thesis, Chapter 8 presents a general discussion of the thesis as a whole, beginning with an overview on aquatic diagnostics and where developments are likely to lead, following from Chapter 1. This is followed by discussion on parasite culturing, maintenance and long-term storage, focusing on cryopreservation as in Chapter 2. Then, a discussion on abiotic factors as stressors for host-parasite systems, focusing on those trialled in Chapters 3-6 temperature, light and fibre pollution. Each data chapter is written to be self-contained, with Chapters 2 and 7 published, and Chapters 4, 5 and 6 submitted for publication, so there is some overlap in methodologies.

Animal Ethics

For all experiments in the thesis, procedures and protocols followed ARRIVE guidelines (Kilkenny et al., 2014), were conducted according to the UK Home Office Regulations under licences PPL 303424 (2019-2022) and PP8167141 (2022-2023) and approved by Cardiff University's Animal Ethics Committee.

Chapter 2 – Aquatic diagnosis: moving towards improved surveillance and earlier diagnosis of aquatic pathogens

This chapter is affiliated with the publication MacAulay et al.*, 2022 in the journal Reviews in Aquaculture*

Abstract

Early and accurate diagnosis is key to mitigating the impact of infectious diseases, this however is particularly challenging in aquatic environments due to hidden biodiversity and physical constraints. Traditional methods, such as visual diagnosis and histopathology, are still widely used, but increasingly technological advances such as portable Next Generation Sequencing (NGS) and Artificial Intelligence (AI) are being tested for early diagnosis. The most straightforward methodologies, based on visual diagnosis, rely on specialist knowledge and experience. Future computational remote sensing methods, such as AI image diagnosis and drone surveillance, will ultimately reduce labour costs whilst not compromising on sensitivity, but they require capital and infrastructural investment. Molecular techniques have advanced rapidly in the last 30 years, from standard PCR through loop-mediated isothermal amplification (LAMP) to NGS approaches, providing a range of technologies that support the currently popular eDNA diagnosis. There is now vast potential for transformative change driven by developments in human diagnostics. Here we compare current diagnostic technologies with those that could be used or developed for use in the aquatic environment, against four gold standard ideals of high sensitivity, specificity, rapid diagnosis, and cost-effectiveness.

Introduction

The increased demand for protein to sustain the growing human population could be largely fulfilled by aquaculture (FAO, 2009). In 2017, aquaculture protein production reached 80.1 million tons, but further growth is required to sustain a population predicted to reach over 9 billion by 2050 (FAO, 2012) and replace other less sustainable protein sources. Therefore, facilitating the growth and health of managed fish is a priority, with arguably the greatest challenge to this being infectious disease. Prevention and early detection of pathogens are essential to reduce the estimated £4.2 billion annual losses to aquaculture worldwide (World Bank, 2014; Stentiford, 2017), with parasites accounting for losses of £47-134 million annually to the UK industry alone (Shinn et al., 2015). All animals are subject to disease, with infectious disease outbreaks exacerbated by environmental

disturbance (habitat loss or destruction, pollution, urbanization, ocean acidification, climate shift; reviewed by Cable et al., 2017), population density, diet and intrinsic host factors (immune status, genetics, life-stage, reproductive status; Casadevall & Pirofski, 2001; Jiménez & Sommer, 2017). The old adage '*prevention is better than cure'* still applies with regards to control of infectious disease, but the wider impacts need to be considered if prevention, for example, contributes to antimicrobial resistance or other environmental impacts. Non-chemical interventions, good husbandry, stress reduction, environmental enrichment, dietary supplements, water quality maintenance, stock movement restrictions, quarantine measures, genetically resistant stocks, and regular surveillance all contribute to prevention (Assefa & Abunna, 2018), but complete harmony is difficult to achieve (Rottmann et al., 1992). Even the best management strategies cannot guarantee protection from disease outbreaks and effective mitigation requires early detection diagnostics: identifying the pathogens, and if possible, quantifying them.

Typically, fish health is first assessed visually through general indicators such as behaviour and appearance. Routine monitoring of fish health is more challenging than for terrestrial livestock due to variable and fluctuating water conditions. Turbidity, sediment type, turbulence and the weather can all affect visibility and obscure detection of clinical signs (Evans et al., 2011; Raja & Jithendran, 2015). Like any infectious disease, early diagnosis of aquatic pathogens is vital to minimise morbidity and mortality; once a pathogen or group of pathogens is identified, early intervention can reduce the chances of mass mortalities. For parasites such as *Saprolegnia parasitica* which cause rapid host death (24-48 hours) with no effective cure, early diagnosis is key to reduce population-level losses (Van Den Berg et al., 2013). The goals for early diagnosis can be categorised under three pillars: sensitivity, speed and cost (infrastructure, consumables, and labour). This review assesses the range of early diagnostic techniques currently used in aquaculture, the ornamental trade, wild fisheries and aquatic research, and considers future developments. As novel diagnostic techniques are brought to the forefront for human health, greatly accelerated by the SARS-CoV-19 pandemic, this provides potential for translation to animal health methods. Early detection and identification of problem pathogens will allow for effective implementation of control strategies minimising losses and the spread of infection.

Aquatic diagnosis – difficulties and considerations

As Emerging (and re-emerging) Infectious Diseases become more common, we must consider technologies utilised in other fields or currently in development for use in aquatic systems, bearing in mind the Technology Readiness Level (TRL; scaled 1-7). This metric defines the maturity of a technology in relation to development, with 1 reporting the research backing the technology and 7 representing the operational testing stage (Héder, 2017). Diagnostic techniques showing promise with a TRL 1-3 are in their infancy and will require further development before implementation. Although the TRL is primarily applied to terrestrial technologies, it does flag technologies that could be transferred to aquatic systems, but doing so is not simple as there are significant challenges regarding the variable and dynamic aquatic environment.

The natural aquatic environment is constantly in flux and resident fish are subject to variations in water quality, oxygen concentrations, light levels, enrichment, competitors, and predators, all potentially influencing disease susceptibility. Many fish, especially those in the ornamental trade, are transferred long distances to reach the end user and this movement also increases susceptibility and disease risk through mechanical disturbances (Masud et al., 2019) and reduced water quality from increased CO_2 and build-up of other toxic compounds (Livengood & Chapman, 2007). Within intensive aquaculture systems, water quality including dissolved oxygen levels are controlled, but stocking density is often pushed to its limit, which can also affect disease susceptibility (Ashley, 2007; Ellison et al., 2020). For many species, high densities increase stress, as is the case with Atlantic salmon (*Salmo salar*) resulting in increased disease susceptibility (Ellison et al., 2020). For territorial species, such as Nile tilapia (*Oreochromis niloticus*), high densities can lower stress, as social aggression is reduced (Champneys et al., 2018) and consequently so too is disease susceptibility (Ellison et al., 2018). So, disease mitigation is critically dependent on the system and species. The number of aquatic species cultured greatly outnumbers those in terrestrial environments, with around 600 aquatic species farmed commercially (Mathiesen, 2015). This means there is no “one-size-fits-all” solution for aquatic diagnostics and each method must be tailored towards the culturing system and species.

Resources for aquatic disease diagnosis arise from academic, governmental, and independent organisations. They vary greatly across sectors and geographic regions, and all rely heavily on local specialist knowledge. Within intensive aquaculture, commercial diagnosis routinely utilises off-site or company veterinarians and scientific laboratories, particularly when the pathogens are cryptic (Jones, 2016). For aquafarmers with limited or no technology including internet access, alternative

diagnostic technologies such as tele-diagnosis systems can be employed (Li et al., 2006; Zhang & Li, 2007). With growing consciousness of the effects of overfishing on global aquatic ecosystems, funding is being put in place to aid transitions to sustainable fishing and the development of aquatic and coastal jobs. Ensuring sustainability is a concern and efforts vary globally. The European Union put in place the European Maritime and Fisheries Fund (EMFF) to support sustainability (European Commission, 2015), with funding split between fisheries and aquaculture, monitoring and enforcement of rules, data collection to improve future knowledge, and to the blue economy through creation and growth of marine jobs. In Asia, the fisheries *refugia* approach was implemented with the goal of bringing together the fisheries and environmental sectors of the South China Sea, aiming to reduce fishing pressures and aid in habitat management (Paterson et al., 2013). With the outcome of the fisheries *refugia* concept resulting in local sustainability of target species, such as lobsters (*Panulirus* spp. and *Thenus orientalis*) and tiger prawns (*Penaeus monodon*) by implementing seasonal closing so that the populations can recover (Siow et al., 2020).

Projects such as the fisheries *refugia* allocate areas, however one key issue with aquaculture is site occupation, with farms requiring large areas for enclosures and associated infrastructure. Open water systems pose additional problems for disease, with spillover/spillback effects between natural and farmed populations (Bouwmeester et al., 2021). One approach to combat this is the development of inland 'mega-farms', self-contained units, which prevent disease transmission between wild and farmed fish, allowing treatments to be more targeted thereby reducing pollution (Yogev et al., 2017). For recreational angling, city centre fisheries provide those with limited countryside access an 'authentic' fishing experience from within the city limits. Indoor angling prevents fish from being impacted by weather conditions, inflowing pathogens, invasive non-native species and predators, but requires large setup and maintenance costs. Similar small inner-city venues for small scale locally produced food are appearing with tilapia, for example grown alongside salad crops in aquaponic systems (Rakocy et al., 2013). All these onshore/inland facilities face optimisation challenges, with husbandry and housing conditions (e.g., lighting, enrichment, flow rate) varying between species and facility, in addition to very strict biosecurity, which is why diseases in these facilities have not been eliminated (Assefa & Abunna, 2013). As productivity of these indoor aquatic industries is still limited by infectious disease, the development of novel diagnostic techniques is vital for continued growth.

The health of farmed fish and responsible usage of aquatic resources is managed across different scales; from local/regional to trans-national and global efforts. On a regional or national level, fish

health may be managed by governmental agencies, such as the UK Centre for Environment Fisheries and Aquaculture Science (CEFAS, 2020) and the National Oceanic and Atmospheric Administration (NOAA). At an international or transnational level, the Asia-Pacific Fishery Commission (APFIC) (FAO, 2020b) and the Ornamental Fish International (OFI) organisations, amongst others, contribute to fish health management (Stevens et al., 2017). Wild fish stocks may be managed by different governmental organisations: in England and Wales this is the Environment Agency (also responsible for stocked fish), and for Scotland the Marine Scotland Directorate Fish Health Inspectorate. Intergovernmental organisations, such as INFOFISH and GLOBEFISH, provide information to fisheries worldwide. Aquaculture and the ornamental trade may also benefit from the advice of nutrition companies. Food additives are increasingly included in fish diets to boost the immune system to reduce disease susceptibility (Velasco-Santamaría & Corredor-Santamaría, 2011; Amoah et al., 2017). If farmers are experiencing problems with specific pathogens, then specialist vets can provide targeted advice to combat the infection. However, there is an increasing number of emerging diseases, such as puffy skin disease or red-mark syndrome, for which the causal agents are unknown so relying on treatments/interventions by vets is problematic (Schmidt et al., 2018).

All fish stocks need to be regularly surveyed for pathogens, but progressive budget cuts over recent decades have reduced routine surveillance, such that now surveys only tend to be conducted for research or in response to a disease outbreak (Oidtmann et al., 2013). This is a global problem, especially in Europe, Asia, Africa and South America, with survey results suffering bias through false or inaccurate reporting, which further complicates risk assessments (de Graaf et al., 2011). Without regular surveys of fish health, prevention (and indeed early warning of wider ecosystem problems) becomes increasingly difficult, but early diagnostics can at least help maintain fish health of current stocks.

Visual Diagnosis

Visual diagnosis can range from traditional methods of noting changes in behaviour and condition to remote sensing through drones and AI diagnosis (Figure 2.1).

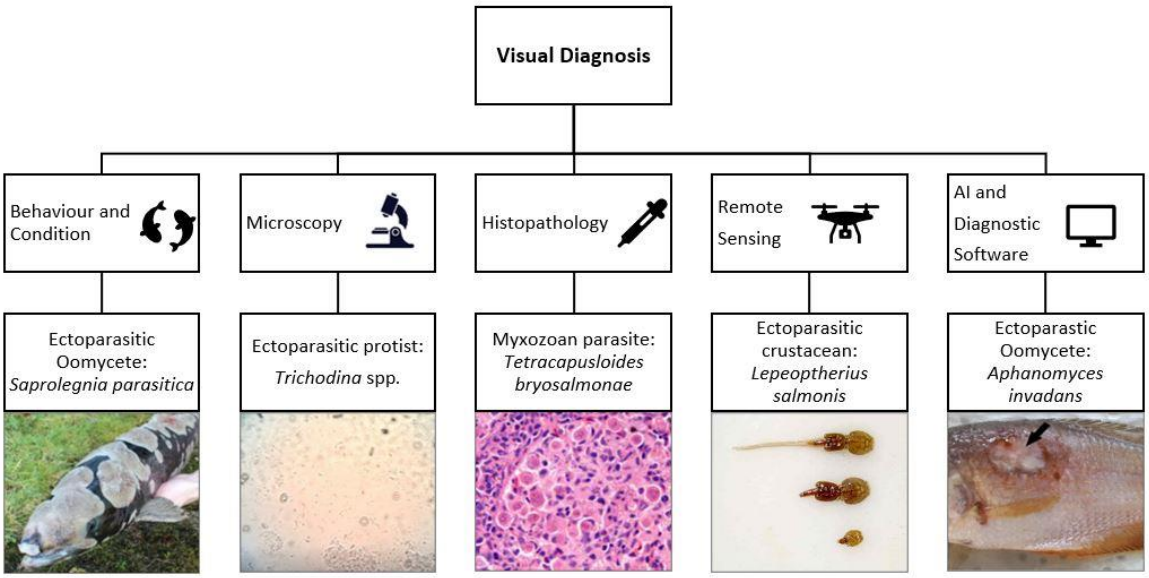

Figure 2.1 Visual diagnostic techniques and example of their application to specific aquatic pathogens. Images sourced as follows: *Saprolegnia parasitica* - Environment Agency, *Trichodina* spp. – KoiQuestion (https://www.flickr.com/photos/koiquest10/26357384027), *T. bryosalmonae* – AquaTT (https://commons.wikimedia.org/wiki/File:T._bryosalmonae_parasites_in_rainbow_trout_kidney._Tissue_section_stained_with_haematoxylin_and_eosin.jpg), *L. salmonis* - Thomas Bjørkan (https://commons.wikimedia.org/wiki/File:Salmonlouse.jpg), *A. invadans* – adapted from Majeed et al. (https://commons.wikimedia.org/wiki/File:Dwarf_gourami_infected_by_Aphanomyces_invadans.png),).

Visual observation for clinical signs and diagnosis

In situ, aberrant behaviour of fish, often followed or accompanied by altered physiology or morphology, are typically early indicators of ill health. Common clinical signs include increased opercular rate, gasping at the surface, loss of equilibrium, lesions or abrasions, and string-like faeces (Noga, 2010). Identification of such characteristics may lead to a more detailed examination for pathogen presence or a full post-mortem, the sensitivity of which relies on the experience and expertise of the observer. Large ectoparasites and or pathogens that cause visible clinical signs can be detected by sight alone. For example, *Saprolegnia parasitica* presents as “fluffy” white patches on the body, head and fins of fish, distinguishable from the water’s surface whilst the fish is submerged (Earle & Hintz, 2014). Adult crustacean parasites, such as freshwater (*Argulus* spp.) (Figure 2.2a) (see Taylor *et al.*, 2005) and marine lice (*Caligus* or *Lepeophtheirus* spp.) can aggregate in large numbers on the body or gills of a fish, visible by eye. Visual diagnosis can be time-consuming depending on the number of fish and the species of both host and pathogen. Diagnostic features may also change during disease progression and secondary pathogens might obscure clinical signs of the primary pathogen (Zeldis and Prescott, 2000). Certain diseases present distinct clinical signs,

such as ulcerations, lesions or exophthalmia, but the causal agents remain unknown; such as in red-mark syndrome or puffy skin disease (Figure 2.2b).

Microscopy is often the next step in visual diagnosis, accuracy of which is again dependent on the expertise of the observer. For microscopic diagnostics, mucus scrapes or tissue sections of the fish are commonly utilised. For example, *Chilodonella hexasticha*, a ciliated protozoan fish parasite, can be visualised from skin/mucous scrapes without the need for staining (McGuigan & Sommerville, 1985), likewise for larger pathogens such as *Diplostomum* or *Trichodina* species. Microscopic diagnosis relies on the pathogen being morphologically distinct, which within the cacophony of aquatic pathogens, is a rarity. For gyrodactylids, with >400 *Gyrodactylus* species described, the majority are morphologically cryptic, requiring sequencing, or electron microscopy, to differentiate species (Harris et al., 2008). For the many thousands of *Gyrodactylus* species, and other fish pathogens, as yet undescribed, sequencing alone is problematic without a morphological reference description, so a combined approach is required (Harris et al., 2008). Other than equipment and labour costs, light microscopy is relatively cheap, but the main caveat is user error and the potential to overlook low level infections. Diagnosis of fish disease through these traditional methods is highly skill dependent, with variation occurring between the individual carrying out the diagnosis (Shinn et al., 2007). Microscopy can generate quantified data, but again is dependent on the accuracy of the diagnostician and the representative samples. Many aquatic pathogens, including viruses, are undetectable through light microscopy and require electron microscopy, which is costly (Nkili-Meyong et al., 2017), and increasingly difficult to find suitable facilities.

Certain external clinical signs can be difficult to diagnose and may require additional measures to improve accuracy. Ulceration, erosion of the skin from mechanical or chemical means, is a common sign of disease in fish, particularly for ectoparasites feeding on the dermis. Ulcers lead to haemodilution and osmotic imbalance in the fish, and often secondary infection. Mortality inducing ulcers are detectable by eye, whereas early-stage ulcers were difficult to detect visually until Noga (2010) suggested a fluorescein test commonly used in terrestrial diagnosis for corneal ulceration. The fish is immersed in fluorescein that enters the damaged epithelial layer and allows skin damage to be visualised under UV (Noga & Udomkusonsri, 2002). Compared to histology (see below), fluorescein is more sensitive at targeting ulcers, lower cost and faster with complete coverage of the fish. Due to high sensitivity but low accuracy however, the method will pick up on minor ulcerations that may have been caused by handling or regular activity and are not attributable to pathogens (Colotello & Cooke, 2011). High concentrations of fluorescein may be toxic to fish, but

short exposure (approx. 6 minutes) at doses (0.1-0.2 mg per ml) used experimentally did not negatively affect fish (Davis & Ottmar, 2006; Noga, 2010; Colotello & Cooke, 2011). Fish anaesthetised with tricaine methanesulphonate, however, may present false negatives as tricaine subdues the fluorescent reaction, or false positives as unbuffered tricaine causes epithelial damage (Davis et al., 2008). Fluorescein is a useful non-lethal methodology for ulcer visualisation but not for pathogen diagnosis.

Figure 2.2 Diseases of fish which can be diagnosed through visual observation. a) Juvenile Argulus foliaceus on the caudal fin of a three-spined stickleback (*Gastrosteus aculeatus*). [Photograph by R. Hunt]. b) Puffy skin disease in a rainbow trout (*Oncorhynchus mykiss*) [Photograph by Environment Agency] c) Red vent syndrome in an Atlantic salmon (*Salmo salar*). [Photograph by Environment Agency]. d) An Atlantic salmon suffering from Saprolegniasis caused by *Saprolegnia parasitica* [Photograph by Environment Agency].

Histopathology

Histology can be a valuable diagnostic tool if host and or pathogen tissue is available. It can be useful for routine monitoring or once infection has been established, but internal examination requires sacrifice of the target species. Sample processing involves the use of chemical preservatives such as 10% formalin (or even Bouin's fluid, potentially explosive when dry) for tissue fixation, embedding (in paraffin or resin), sectioning, affixing onto a slide and staining (Noga, 2010) using generic (such as Haematoxylin and Eosin) or more specific (e.g., Periodic Acid-Schiff) stains (Alturkistani et al., 2016; Smith et al., 2018). Slides are then examined for tissue abnormalities or direct pathogen

identification (Figure 2.3). Histology is a valuable diagnostic method for many diseases, such as furunculosis and syncytial hepatitis of tilapia, and the cryptic salmonid disease ulcerative dermal necrosis (UDN) is currently only detectable through histology (Ferguson et al., 2014; Matthews, 2019). It can be cost-intensive compared to other visual diagnostics (~£35 per slide) but may be cheaper than most molecular techniques (see 5 below). Histological diagnoses can require several days but provides high specificity for target pathogens and semi-quantitative results depending on the replicates analysed.

Immunohistochemistry (IHC) targets specific pathogens with antibodies (Thoresen et al., 1994; Zerihun et al., 2011). *Tetracapsuloides bryosalmonae*, the causative agent of proliferative kidney disease, for example, can be detected through kidney tissue staining with a monoclonal antibody and counter stain (Figure 2.3) (Morris et al., 2000), and the bacterial agent of rainbow trout fry syndrome (*Flavobacterium psychrophilum*) is detectable in fish tissue through IHC (Ekman & Norrgren, 2003). Potential non-specific binding, cross-reactivity of antibodies (Johnson, 1999), ischemia of antigens (Kim et al., 2016) and a lack of standardised methods (Battifora, 1999; Taylor & Levenson, 2006) mean IHC is not deployed as an initial diagnostic method, but as confirmation if a particular pathogen or pathologies are suspected and as with histology only provides semi-quantitative results.

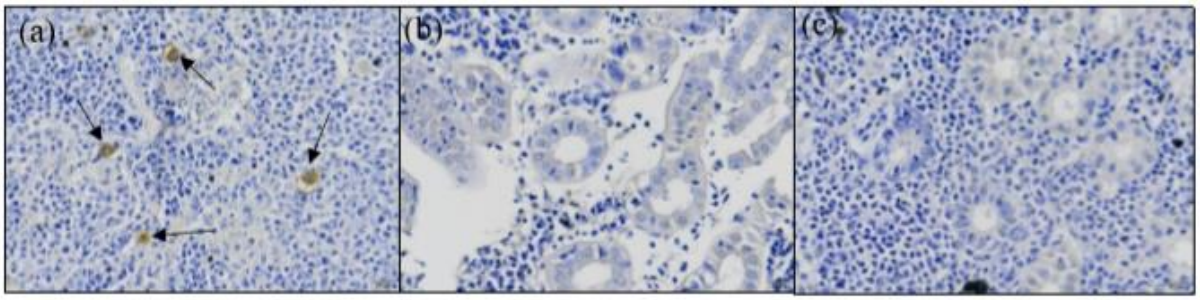

Figure 2.3 IHC staining for *Tetracapsuloides bryosalmonae* in kidney tissue of farmed rainbow trout (*Onchorhynchus mykiss*). a) positive control, *T. bryosalmonae* indicated by arrows. b) and c) negative kidney tissue.

Remote Sensing

Fish suffering infection will often remain at the surface, in a moribund state and can be picked up by farmers, workers or environmental officers patrolling the water body, but surveying of wild stocks is challenging. This is time-consuming and limited to accessible sites. Drones can be implemented to refine this process, by applying an appropriate resolution to the camera, being able to survey the entire water body from the air, and potentially providing images for immediate diagnosis (Oh et al., 2017). Advances in remote sensing techniques have allowed developments in

visual diagnosis, especially for terrestrial organisms, and are expanding to the aquatic environment. Remote sensing, which utilises remote-controlled technologies to transmit or record images or video directly (Zhang et al., 2013), is increasingly used for wildlife monitoring, where unmanned aerial vehicles (UAV or drones) gather real-time data (Mangewa et al., 2019). UAVs have been used to conduct aquatic aerial surveys of macrofauna, such as sharks and crocodiles, with current developments paving the way for underwater surveys (Ezat et al., 2018; Colefax et al., 2019). The benefits to UAV diagnosis include increased survey coverage, less risk to personnel, repeatability and reduced operational costs (Verfuss et al., 2019). Applications of UAVs for disease diagnosis are still developing but have been successfully applied in agriculture (Albetis et al., 2017; Heim et al., 2019). UAVs could be useful for detecting large aquatic ectoparasites, such as sea lice, or those which cause visible external signs, like the white patches of *S. parasitica*.

Not all infected fish rise to the surface, so underwater surveys may be required. Autonomous underwater vehicles (AUVs), fully functional below the water's surface, possess a 360° camera or "eye", allowing for high throughput detection in challenging environments. AUVs have been successful at marine macrofauna (Meyer et al., 2019) and invertebrate (James et al., 2017) identification, highlighting their potential for aquatic disease diagnosis. The "Stingray" drone designed by Norwegian engineer Esben Beck utilised stereo-cameras to detect lice on a fish, and then deployed lasers to kill the lice (Dumiak, 2017). Although no current data is available on the efficacy of "Stingray", field tests and feedback from industry are positive, with drone deployment throughout Norwegian and Scottish salmon farms (Dumiak, 2017). Although remote sensing for pathogen detection and diagnosis is still in its infancy it presents significant potential for remote detection and quantification of pathogens in an elusive and difficult environment.

Artificial-Intelligence (AI) and Diagnostic Software

Gaining sufficient experience to accurately assess and diagnose fish diseases takes years, hence interest in Artificial Intelligence (AI) to automate diagnosis through digital image processing (Park et al., 2007). AI programs are capable of learning and developing through experience (Chrispin et al., 2020). But for each taxon, comprehensive training and test image databases are needed for AI disease detection development (Khirade & Patil, 2015; Mohanty et al., 2016). Images for training AI must be good resolution with no replicated images and must include the pathogen on different backgrounds from different angles. Once training is complete, a new set of images is required for validation. AI detection can also be applied to video footage; similar issues occur, but with the

additional need to account for sudden light changes and multiple objects in the field of view (Matzner et al., 2017). A key problem for AI diagnosis of fish pathogens is the lack of suitable image databases, but citizen science projects could provide such images. Successful image detection has been achieved for epizootic ulcerative syndrome, caused by the oomycete parasite *Aphanomyces invadans*, using different image processing techniques, where the most successful technique successfully identified *A. invadans* 86% of the time (Malik et al., 2017), but such methods have yet to be tested on large databases.

The Fish-Vet diagnostic tool, originally developed by Zeldis and Prescott (2000) as a desktop application for PC, was an early attempt at a diagnostic program for aquatic diseases. The software evolved into a free aquatic diagnostic app (FishVetApp), which provides information and images of 95 fish diseases, covering ornamental, food and wild fish. The FishVetApp is currently in development for mobile devices, allowing it to be more widely used in the field. Others have created web-based aquatic disease diagnosis systems, such as the Fish-Expert implemented in Northern Chinese cities to fish farmers, fishery experts and fish vets with reported positive feedback (Li et al., 2002). This program at inception held information for 126 fish diseases from 9 fish species (Li et al., 2002), but does not appear to have been updated. At the farming level, the program was quite complex and inaccessible to many, and some farms lacked the necessary resources (e.g., microscopes, water quality equipment) to gather the required information (Li et al., 2002).

Clearly, we are in the early stages of remote diagnosis but automating the process through the application of AI and machine learning approaches has the potential to establish a robust high-throughput process with the potential for quantification. They do, however, rely heavily on reference databases and further technology development. Misdiagnosis still may occur due to the generic nature of clinical symptoms of many fish diseases and difficulty controlling for secondary infection.

Microbiology, Biochemistry & Serology

Microbiology

Fish microbial diseases are highly prevalent, as both primary and secondary infections, driven by stress (water quality, poor nutrition, temperature) or other infections (Guzman & Shotts, 1988). Diagnosis has historically involved isolation and culturing of the causative agent. Direct placement or swabbing of diseased tissue or mucus onto agar is a common method for aquatic bacterial diagnosis, and for some aquatic fungal-like pathogens, followed by analysis of biochemical and

morphological traits (Frans et al., 2011). Such methods are selective and susceptible to contamination, requiring serial subculturing to obtain a pure strain of the causative agent. The causative agent of bacterial kidney disease (*Renibacterium salmoninarum*) is particularly fastidious and grows slowly on regular agar, requiring a specialized agar for rapid growth with a 'nurse' microbe (Evelyn et al., 1989). In contrast, the oomycete pathogen *S. parasitica* is regularly cultured on potato dextrose agar (PDA) by obtaining small tufts of mycelia from infected fish and embedding them within the agar, producing growth within 2-4 days (Stewart et al., 2017). Culture dependent methods are limited to pathogens with known nutrient requirements, subject to contamination even with antibiotics in the media, and, for long-term culturing, can be labour intensive. Culturing as a means of diagnosis is unreliable when trying to verify causal agents of polymicrobial infections (Nie et al., 2017). In addition, genetic alteration of microbes may occur over time resulting in strains unrepresentative of natural communities. Culture-independent methods have been instrumental in not only identifying pathogenic microbes but revealing the key role of microbiomes (all microbes within an organism) for fitness, immunity and life span of fish (Xiong et al., 2019). Following successful culturing, routine PCR is often carried out for pathogen confirmation, and sequencing if species-level identification is required.

Though the rise of molecular techniques in recent years has reduced the need for culture-dependent techniques, diagnosis of some pathogens still necessitates these methods. Every organism naturally hosts a range of microbes. This microbiome varies between individuals, species and populations, so understanding what constitutes a 'natural' or core microbiome is important for identifying any dysbiosis, disrupted microbiota. As a diagnostic tool, the microbiome can indicate health status (Meron et al., 2020) as microbiota diversity will alter upon host infection (Nie et al., 2017), treatment (Rosado et al., 2019) and environmental stressors. Fish microbiomes naturally contain both virulent and avirulent pathogens, residing at non-lethal thresholds, which typically do not require intervention and are the baseline against which dysbiosis should be compared. Many fish farms (over)use antibiotics as a proactive treatment, which in turn can promote antimicrobial resistance. In extreme examples, where fish are bred and maintained in sterile environments this could even lead to gnotobiotic fish (which harbour no or reduced microbes). Like any animal with limited prior infection exposure, gnotobiotic fish are at greater risk from common diseases (Perez-Pascual et al., 2021), which can lead to increased mortality (Situmorang et al., 2014), so in this case extreme prevention is not better than a cure. We can monitor for dysbiosis through non-invasive faecal samples (Casen et al., 2015) or skin swabs (Ellison et al., 2021), as well as sampling of tissues.

Typically, this identifies microbes to species level, but does not confirm whether strains are virulent or not (Smith et al., 2012) so interpretation of microbiome data is an important area to focus on now that the molecular methodologies are well developed. Also, more studies need to consider the entire assemblage of microbiota and host - the holobiont (Margulis & Fester, 1991) - rather than just target bacterial species.

Biochemistry

There is increasing interest in the "smell of infection" - targeting chemical signals (volatile organic compounds, or VOCs) released during infection for rapid diagnoses (e.g. Pawluk et al., 2018 who identified chemical cues from infected and uninfected fish). Recently developed rapid medical tests, such as Gene Xpert, can detect *Mycobacterium tuberculosis* (TB) in patient's sputum in 3 hours (Zeka et al., 2011; Javed et al., 2014). In addition to TB, VOCs are in development for lung cancer and other infectious diseases (Philips et al., 2006). The application of these techniques to aquatic animals could utilise water samples to detect a specific target or VOC which is diagnostic for infection (Pawluk et al., 2018). At present there are no commercially available products utilising this methodology for application to fish or aquatic systems.

Biosensors that use biochemical reactions to detect (optical, volatile, electrochemical or mass-sensitive) chemical compounds have gained popularity in plant-fungal diagnosis in recent years (Ray et al., 2017). Optical biosensors function by transduction causing the phase, amplitude, polarisation or frequency of the light input to change. Volatile biosensors are suited for gaseous detection of volatile organic compounds, primarily those released from plants, similar in concept to the "smell of infection". Electrochemical biosensors convert a binding biomolecule into an electrical signal, used for assessing the concentration of a sample or target of interest. Mass-sensitive biosensors detect changes in mass through electrical currents (Ray et al., 2017). Nanoparticles have also been used with biosensors, as a means of increasing surface area available for recognition and thus increasing sensitivity of tests. Patch vital sign monitoring are sensors able to continuously monitor health parameters such as electrocardiography, heart rate, respiratory rate, activity, and or posture (Hofmann & Welch, 2017). The range and variety of biosensors are their main strength allowing for wide applicability but there is difficulty in translating these to the aquatic environment. When considering their application to aquatic diagnostics, the information gained from these health parameters is too general for diagnostics and the benefits would not outweigh the costs.

Serology

While commonly used in terrestrial veterinary practices, serology is used less in aquatic diagnostics due to insufficient development of methodologies (Jaramillo et al., 2017). Until 2012, The World Organisation for Animal Health (OIE)'s *Manual of Diagnostic Tests for Aquatic Animals* stated that serological detection was not an accepted method of diagnosis for fish pathogens (OIE, 2009), although this has since been removed (OIE, 2019). Serology can directly identify pathogens, such as *Trypanosoma carassii* a parasite of cyprinids (Overath et al., 1998), or indicate signs of irregular immune function, such as haemoglobin levels or differential leukocyte counts, caused by a pathogen (Blaxhall & Daisley, 1973). The enzyme-linked immunosorbent assay (ELISA) is a rapid serological test through which antigens in fish sera are detected via a visual colour change, caused by an enzyme-chromogen complex (Adams & Thompson, 1990; Jaramillo et al., 2017). ELISA is available for a range of aquatic disease diagnoses including *Renibacterium salmoninarum* (see Dixon, 1987), *Mycobacterium* spp. (see Adams et al., 1995) and *Aeromonas salmonicida* (see Adams & Thompson, 1990), and is often used in conjunction with molecular techniques.

Serology in terrestrial medicine has a wide range of applications within testing and diagnostics, with significant advances into the early detection of cancers. One such novel technique is utilising immunosignatures where serum from an individual is challenged with an array (tens of thousands to millions) of random-sequence peptides to determine the binding of patient's antibodies (Hofmann & Welch, 2017). The most informative peptides are then identified, based on their ability to differentiate between diseases. Similar diagnoses have been applied to diabetes, Alzheimer's and infectious diseases (Hofmann & Welch, 2017). The wide applicability of this technique in human medicine indicates potential application to the diagnostics and monitoring of infectious aquatic diseases. Terrestrial infectious disease outbreaks often spur diagnostic development, providing potential for translation to the aquatic environment. For example, diagnosis of the Ebola virus requires serological samples, but methods have changed from traditional viral culturing from these samples to molecular diagnosis (Broadhurst et al., 2016). There are serology-based rapid diagnostic tests (RDTs) available for malaria, which can have high sensitivities and limits of detection (Ley & Thriemer, 2020), and utilises small (15 µl) samples of blood, producing results within one minute (Kumar et al., 2020). RDTs could be transferred to aquaculture for aquatic disease diagnosis, but the issue remains of choosing an appropriate target for diagnosis.

Molecular Techniques

The rapid development of our ability to amplify and sequence genetic material has revolutionised every aspect of biological sciences, from behavioural and evolutionary fields to medical and veterinary sciences. Molecular diagnosis ranges from standard PCR to next-generation sequencing and environmental DNA techniques (Figure 2.4). Whilst molecular techniques have advanced rapidly, what now limits their application is the logistics of sampling, storage and transport costs. Storage and transport of samples for molecular analyses can significantly impact the quality of results, with tissue degrading over time, if not fixed sufficiently or kept at low temperatures. Standard agents for transporting tissue include formalin (mostly used for histological samples) or a high percentage molecular grade ethanol (>90%), and samples are usually cooled for long-term storage (Yue & Orban, 2001). Storage by desiccation with silica has been effectively used for tissues (Terra et al., 2004; Rider et al., 2012) and faecal samples (Wasser et al., 1997) from terrestrial animals, and potentially could be utilised more for fish (Lupica & Turner Jr, 2009). Desiccation is short-term and requires samples to be transferred to ethanol for long-term storage but is extremely useful for air transport (Vernesi & Bruford, 2009). When testing for infectious diseases, care must be taken when transporting potentially infective samples. For example, with Ebola samples there is the need to integrate with regional labs for regular testing requiring transport logistics to be addressed for collection of blood samples which are a biohazard. Developments are arising into new stabilising methods that allow for easier/safer transport of genetic material, such as Whatman® FTA® Cards. For small samples, the Whatman® FTA® Cards remove many of these issues (Livia et al., 2006). The target organism (size dependent) or DNA is swabbed onto a sterile FTA card® without the need for fluids. The cards can be kept at room temperature, eliminating the need for freezers, excessive storage space and transport of flammable liquids. FTA cards® have been successfully used for the preservation of fish buccal cells and mucus, as a cheap alternative to freezing or commercial extraction kits (Livia et al., 2006; Navaneeth Krishnan et al., 2016). Brown trout (*Salmo trutta*) and northern pike (*Esox Lucius*) DNA was successfully extracted non-invasively with no cross-contamination from FTA cards® (Livia et al., 2006). Storage of parasite DNA on FTA cards® has been successful, such as with samples containing parasites and parasite eggs (Ahmed et al., 2011; Webster et al., 2019). DNA can be maintained on cards for years at room temperature and amplified following standard protocols (Merck, 2020), but experimentally detectable viral RNA (Genus *Betanodavirus*) decreased after four weeks even when cards were stored at 4°C (Krishnan et al., 2016). A review of 47 studies indicated the maximum storage time for viral RNA on FTA cards® ranged from one to eight months at temperatures from -20°C to 37°C (Cardona-Ospina *et al.* 2019).

Therefore, if using FTA cards as preservation tools, it is recommended to process the samples within a year whilst maintaining them at a maximum of 22°C. Not all diagnostics will target DNA, some require RNA. However, difficulties arise with storage and transport of RNA as it rapidly degrades in tissue and water samples, therefore requires immediate storage at -80°C or use of protective reagents such RNAlater.

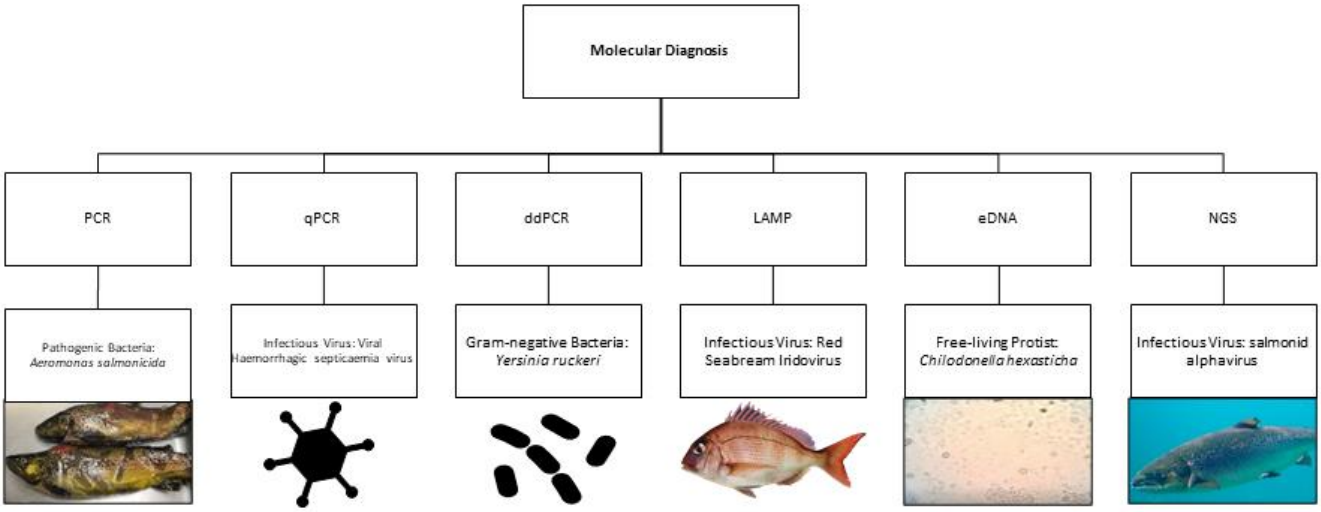

Figure 2.4 Molecular diagnostic techniques, and examples of their application to specific aquatic pathogens. Images sourced as follows: *A. salmonicida* - Robert Durborow (https://commons.wikimedia.org/wiki/File:Furunculosis_on_Brown_Trout_F12-50.JPG), *Chilodonella hexasticha* protist – Picturepest (https://pxhere.com/en/photo/363624), salmonid alphavirus Salmo salar - Hans-Petter Fjeld (https://commons.wikimedia.org/wiki/File:Salmo_salar-Atlantic_Salmon-Atlanterhavsparken_Norway_(cropped).JPG).

PCR and its Successors

PCR revolutionised disease diagnosis, reducing reliance on culturing and histological methods. PCR amplifies target regions of DNA from tissue or environmental sources, providing presence/absence data. Standard PCR methods involve multiple thermoregulated cycles of denaturation, annealing, and extension to facilitate the amplification of a target fragment of DNA. Amplification is targeted by specifically designing primers complementary to regions flanking the region to be amplified, as the PCR reaction cools after denaturation the primers anneal to these regions acting as initiation points for the thermal stable polymerase to generate new daughter strands during the extension phase of the reaction (review by Innis et al., 2012). Each PCR cycle provides a doubling of the targeted fragment resulting in over a billion copies ($1.07x10^9$) from 30 amplification cycles. DNA generating products can be visualized through gel electrophoresis where the size (in bp) can be confirmed against known size markers; a visualization process that historically used the carcinogen ethidium bromide, but there are now alternatives, such as SYBR Safe (Canela et al., 2017). Key to

the success of PCR are the primers, which can either be designed specifically for a group or species of pathogens or non-specific/degenerate when looking for more general groups of pathogens. Sequencing of PCR products is particularly beneficial for disease diagnostics to identify pathogens to species and even strain level, mainly if general primers have been used (Cunningham, 2002).

Quantitative PCR (qPCR, otherwise known as real-time or RT-PCR) is increasingly used for pathogen detection. This method utilises fluorescent primers to quantify the amplified product in real-time by comparing samples to known quantities represented by standard curves (Boulter et al., 2016). The cycling procedures for qPCR are the same as those for standard PCR, but the products are typically shorter (<200 bp). After each cycle, the intensity of fluorescence is measured, which indicates the quantity of DNA amplicons in the sample at the given time (Kralik & Ricchi, 2017). qPCR can potentially be utilised to diagnose any pathogen of interest, dependent on the assay design with the ability to detect specific genes and alleles. qPCR is widely used as it is high throughput, highly sensitive, reproducible, and rapid (Sepúlveda et al., 2013) with reduced potential for cross-contamination (Kralik & Ricchi, 2017). Wide success has been achieved using qPCR for aquatic pathogen detection, including *Anisakis* (see Paoletti et al., 2018), *Ichtyobodo* (see Isaksen et al., 2012), viruses (viral haemorrhagic septicaemia) (Matejusova et al., 2010) and bacteria (*Flavobacterium psychrophilum*; see Orieux et al., 2011; Sepúlveda et al., 2013). Like all DNA methods, a limitation of qPCR is the inability to distinguish live and dead cells (Kralik & Ricchi, 2017), and it can take a long time to optimise the method. If targeting RNA, then this does measure active transcription, however there are issues in handling samples and the instability of RNA.

Building upon qPCR, digital PCR (dPCR or ddPCR) amplifies the target and provides identification and quantification of nucleic acids, without the need for a standard curve. ddPCR partitions the sample into thousands of subset PCR reactions contained within nanodroplets, some containing the target (positive) and others not (negative) (Baker, 2012; Taylor et al., 2017). Fluorescent readings of these droplets identify the target using dye-labelled probes. The negative samples are then used to generate an absolute count, eliminating the need for standards or endogenous controls. Successful aquaculture application of ddPCR has led to the detection of *Flavobacterium pschrophilum* and *Yersinia ruckeria* from recirculating aquaculture systems (Lewin et al., 2020). When compared to qPCR, ddPCR has lower error rates, is more reproducible and remains cost-efficient compared to other molecular techniques (Taylor et al., 2017). In contrast, ddPCR has a limited dynamic range for detection compared to qPCR but provides a similar level of quantification. Nucleic acid amplification

tests (NAATs), other than PCR, are often more complex but offer applicability or sensitivity (Fakruddin et al., 2013; Chui & Li, 2015).

Loop-Mediated Isothermal Amplification (LAMP)

Notomi *et al.* (2000) developed loop-mediated isothermal amplification (LAMP) as an alternative to traditional PCR. In contrast to the multiple, fluctuating temperature-dependent steps (40-98°C) of PCR, DNA is amplified by LAMP within isothermal conditions. LAMP merely requires a water bath to maintain ~65°C, with the addition of *Bst* (*Bacillus staerothermophilus*) polymerase to initiate the reaction. It utilises four specifically designed primers that recognize six distinct regions within the target genome. RT-LAMP (reverse transcriptase) is highly specific; ten times more sensitive than reverse-transcriptase PCR when detecting nodavirus in *Macrobrachium rosenbergii* (see Puthawibool et al., 2010). LAMP is also efficient and rapid, taking only 60 minutes including DNA/RNA extraction, compared to the 90-180-minute for regular PCR without DNA preparation (Notomi et al., 2000). Combining RT-LAMP with chromatographic, lateral flow dipstick (LFD) is highly effective at confirming the products of the RT-LAMP by hybridisation, allowing for rapid visualisation (Biswas & Sakai, 2014). This combination of methods facilitated amplification of Taura syndrome virus in shrimp along with removing the need to use a DNA staining agent (Kiatpathomchai et al., 2008). Detection of red seabream iridovirus (RSIV) was ten times more sensitive by LAMP than standard PCR (Caipang et al., 2004). There is the potential for contamination of target DNA in the final stages due to the high amplification, sensitivity is highly dependent on the designed primers, and the limit of detection may differ for LAMP compared to PCR (Dittrich et al., 2014). By removing the need for expensive (and typically non-portable) thermocyclers and thermally-sensitive reagents, LAMP-based detection methods hold great promise for rapid aquatic pathogen diagnosis in the field and low-income regions

eDNA

Environmental DNA (eDNA) methods have the potential to greatly improve our ability to detect and monitor pathogens in aquatic environments, be that as whole cells or free-floating DNA. eDNA can follow a targeted or passive method; targeted following standard PCR, qPCR or LAMP methodologies to determine presence/absence or abundance of a target species, whilst the passive approach uses primers sharing conserved binding sites to sequence communities of organisms (Harper et al., 2018). During water sample collection, differing filter sizes affect sample sensitivity; larger pores let more material into the sample, clouding the purity of the target DNA, whilst smaller pores aid in targeting DNA but are prone to clogging and limit the volume of water that can be

filtered. Optimal sample volume is dependent on the target species and habitat, but minimal volumes suggested are 1 L of sample water and 14 µl of extracted eDNA (Mächler et al., 2016). Where Huver *et al.* (2015) filtered samples of 500 ml and Wittwer *et al.* (2018) filtered varying volumes of 1.6 L to 10 L, both found successful detection of their target. Novel water collection methods have arisen for both low (up to 5 litres) and high (up to 50 litres) volume sampling, with programmable samplers collecting water over variable tidal flows and cycles (MBARI, 2017). These programmable sample collectors are one solution to the larger logistical issue regarding eDNA, sample collection, transportation, and storage. Factoring in the costs of sample collection and analysis are often at the forefront of our mind, the costs and logistics of transporting samples to and/or from sample sites and laboratories is a less discussed but equally important issue and one of the main challenges going forward before this can be an effective tool. Deciding on optimal sample volume and replicates are also key variables that need to be evidenced with further research, likely being dependant on target DNA and ecological knowledge of the field site and target organism. Just as water bodies show stratification, so does the associated DNA. eDNA samples should match the known location of the target species or, if the sample site is deep, be sampled throughout the water column to represent accurate species distribution and presence. eDNA technologies are consistently evolving, with new technologies applicable within laboratory settings and in field, but perhaps one of the most significant recent advances reducing the problem of transporting water samplers is the eDNA Sampler Backpack (Smith-Root). This kit pumps the water directly on to filters impregnated with preservatives so that the eDNA is stored in this easily transportable form for up to two months, without any need to transport water itself. Similar filters can be used for smaller laboratory experiments with hand-held pumps. Successful preservation enables sampling across more remote, larger areas for longer periods of time. Whilst many studies have focussed on spatial use of eDNA, the method has also been successfully applied temporally, providing insight into seasonal biodiversity of water bodies (Bista et al., 2017). For both spatial and temporal studies though, there are many variables that must be considered when applying DNA methods, such as turbidity, UV exposure and flow rate.

eDNA is most effective in shallow waters where the benefits of eDNA outweigh regular trapping methods (Tréguier et al., 2014). Most experimental studies utilise water samples when targeting DNA, but sediment is a viable alternative (Holman et al., 2019). Asian carp (*Hypophthalmichthys* spp.) DNA was more concentrated (8-1,800 times) in sediment compared to water (Turner et al., 2015), but sedimentary eDNA is more likely to present past-species occupancy

due to resuspension and transport (Roussel et al., 2015). The relative benefit of sediments compared to water for eDNA sampling is debatable and will depend on the target and the habitat. Drones may be deployed to collect water samples once the desired volume or sampling period has been achieved, or drones could collect smaller water samples *ad hoc* (Doi et al., 2017; Bershadsky et al., 2016). Methods such as these can be adjusted depending on the target, with buoys collecting water column samples or coring for benthic demersal layer sampling. False positives may arise due to the introduction or transportation of DNA into the water body, whilst certain species release DNA at a sub-detection threshold, leading to false negatives (Roussel et al., 2015). Water quality also impacts eDNA success, with acidity of water increasing degradation of environmental DNA (Seymour et al., 2018). As eDNA methods become widely implemented, protocols continue to be optimised to overcome issues with sample purity, accurate species detection and choice of target genomic material but as new pathogens emerge, at the moment, each requires method optimisation.

Current eDNA techniques target DNA, which may be present in tissue, living, dead or dormant (e.g., cysts, spores, or eggs). DNA within water or sediment samples may not be indicative of active infectious stages of a pathogen, but if environmental RNA (eRNA) is targeted this does indicate active gene transcription. Detection of fish pathogens through eRNA has not been utilized thus far but there is potential (Trujillo-González et al., 2019). Targeting eRNA can direct users towards the infective stage of a pathogen. Utilising eRNA poses additional challenges as RNA is less stable than DNA, degrading rapidly, and current costs are high (Cristescu, 2019). The greatest benefit of RNA is targeting specific genes only expressed at certain life stages, providing high specificity, but the origins of environmental RNA are poorly understood (Cristescu, 2019). The choice of targeting RNA or DNA is highly dependent on the target pathogen. To date, eDNA has been successfully applied to a range of pathogens from ranavirus' in amphibians (Vilaça et al., 2019) to chytrid fungus in bullfrogs (Kamoroff & Goldberg, 2017). The aquatic host range for eDNA applicability ranges from fish and amphibians (Thomsen et al., 2011) to crustaceans (Wittwer et al., 2017). eDNA has great potential to predict disease outbreaks. One study assessed *Batrachochytrium dendrobatidis* presence before amphibian die-off events, where detection was successful before the mass mortality events (Kamoroff & Goldberg, 2017). eDNA has also been used to predict *Chilodonella hexasticha* prevalence in relation to water quality, although no association was identified (Gomes et al., 2017). eDNA can potentially be a more reliable method of pathogen detection than traditional approaches. For example, eDNA and qPCR detection of signal crayfish (*Pacifastacus leniusculus*) is more reliable than physical trapping (Wittwer et al., 2018). Such molecular methods can also be conducted year-

round, they are not seasonally dependent, and can monitor prevalence; eDNA detection of the trematode *Ribeiroia ondatrae* from water samples matched 90% of those detected through necropsy of amphibians (Huver et al., 2015). DNA in water remained traceable after 21 days in the laboratory at 25°C, so sample identification can occur up to three weeks post-sampling. Logistically, eDNA can be twice to ten times more cost-efficient than traditional sampling (see review by Smart et al., 2016).

Next-Generation Sequencing and Bioinformatics

Next generation sequencing (NGS) technologies provide massive parallel sequencing capability generating millions of high-quality reads, far exceeding the targeted Sanger sequencing approaches (reviewed by Behjati and Tarpey, 2013). NGS falls into two broad categories: 1) sequencing covering entire (or representation of) genomes/transcriptomes ("shotgun sequencing") or 2) massively parallel sequencing of specific sequence fragments (ampliconseq).

For shotgun approaches, bioinformatics is used to map sequence reads to available reference sequences, or they can be used for *de-novo* assembly of genomes or transcriptomes. Sequences can be derived from a single or a mixture of organisms, allowing characterisation of individuals or communities (meta-omics). Infections are rarely monopathogenic, and often are either caused by or lead to multiple pathogens within a host. Metagenomic/transcriptomic applications derive sequence data from all nucleic acids present in a sample/tissue, but demands significant sequencing depth, which can be costly both in direct NGS costs but also in computational time for analysis. Metagenomics allows characterisation of all genomes within a given sample whilst metabarcoding describes the species present on a taxonomic level (Zepeda Mendoza et al., 2015). Successful application of metagenomics, such as detection of parasites within swine faeces including first time discovery of *Blastocystis* within swine faeces (Wylezich et al., 2019), and metabarcoding, such as describing ape parasite assemblages from faecal samples (Gogarten et al., 2019), have been applied terrestrially but less so for aquatic environments.

Targeting NGS towards specific genetic sequences, or 'barcodes', with high taxonomic resolution and where significant database resources exist allows the technology to efficiently provide community species composition, an approach referred to as metabarcoding. Interpretation of NGS data is improving rapidly with development of databases, such as GenBank and the Barcode of Life Data System (BOLDSYSTEMS, 2020) which in Jan 2021 held >9,154k barcodes yielding 713k unique sequences representing 320 species (A'Hara & Cottrell, 2020) whilst Genbank has over 226 million sequences as of February 2021 (NCBI, 2021). Metabarcoding of eDNA is a potential path for aquatic

development of these techniques as it allows the characterization of the species and communities contributing to their ecosystems from a simple water sample (Harper et al., 2018).

Classical NGS platforms, such as Illumina sequencers, have technical limitations associated with the length of individual sequences generated (<300 bp from a single read) and also require substantive capital infrastructure investments. Recent innovations in microfluidics and pore-based sequencing, such as those supplied by Oxford Nanopore, provide mobile/desktop sequencers that can generate significantly longer sequence reads, routinely >100 kb in length. Platforms using this technology include the PromethION for ultra-high throughput centralized infrastructure, as well as the MinION platform, a portable sequencer able to generate long reads in real-time with field capability. NGS has successfully identified aquatic viruses (Nkili-Meyong et al., 2016), with nanopore technology leading the way through detection of salmonid alphavirus (Gallagher et al., 2018) and infectious salmon anaemia virus, and sequencing the full 16S rRNA gene of the sea louse *Caligus rogercresseyi* (see Gonçalves et al., 2020). NGS issues primarily arise around substantial costs and the quality of data produced, but error rates are still improving.

The need for real-time disease diagnostics has been highlighted by the SARS-CoV-19 pandemic, resulting in tests that can provide quantifiable results in 90 minutes. Methods such as the LamPORE (able to analyse 96 samples in one hour) and laboratory free DnaNudge (Oxford Nanopore) for example, could be repurposed for animal diseases, in the aquatic environment substituting a cheek swab for a mucus or water sample and alternative primers. Concerns immediately arise over costs, as to scale these tests for national COVID testing would cost around £100 bn, current tests number 350,000 per day aiming to upscale to 10 million per day (Iacobucci & Coombes, 2020). Applying these tests to aquaculture and fisheries would never match this scale but would require significant monetary input (Iacobucci & Coombes, 2020). But as with all novel technologies, costs rapidly decrease with time. Also, quality of data and portability will improve with the potential to revolutionise diagnostics of emerging diseases and cryptic pathogens.

Comparison to Gold Standard Pillars

The methods covered in this review are summarised in Table 1 and Table 2 with respect to their sensitivity, speed and cost. Visual diagnosis of pathogens is monetarily efficient, but not highly specific and is reliant on observer experience. Microscopy and histology are generally low budget, but time taken to identify the pathogen and sensitivity are both observer dependent. In its current state, remote sensing diagnostic detection is high cost, most of this cost consumed by the machinery

and operational costs, relatively sensitive but not time-efficient, with future work improving upon sensitivity and time efficiency. AI diagnosis can be highly sensitive, cost-efficient compared to UAVs, and rapid, but development of specific methodologies and technologies is ongoing. LAMP is rapid, inexpensive, and highly sensitive, making it an ideal methodology for pathogen diagnosis. eDNA is cost and labour efficient but specificity depends on suitable primers, taking a moderate time to produce results. NGS is highly sensitive and fast to sequence but very expensive to purchase the machinery, thus most expedite the process to companies, and run, and takes significant time to analyse.

Table 2.1 Visual diagnostic methods reviewed in relation to the four pillars of a gold standard technique: sensitivity, specificity, speed and cost (instrumentation, labour and running costs).

Trait of Test	**Visual Diagnosis**						
	Behaviour & Condition	*Fluorescein*	*Histology*	*Microscopy*	*AI*	*Remote Sensing*	***Serology***
Sensitivity	Low	High	High	Observer dependent	Low-High	Observer/Technology dependent	**High**
Specificity	Low	Low	High	Generally Low but species dependent	Low	Low	**Moderate**
Speed	Slow	Fast (15-30 mins)	Slow (1-2 days)	Observer depended (generally fast)	Long to train, fast once established	Moderate	**Moderate**
Cost	Low	Low	Low	Low	Low	High	**Medium**
Labour	Medium	Low	Medium	High	High	High	**Medium**
Lethality of host	**Never**	**Never**	**Almost always**	**Sometimes**	**Never**	**Never**	**Not Often**

Table 2.2 Molecular diagnostic methods reviewed in relation to the four pillars of a gold standard technique: sensitivity, specificity, speed and cost (instrumentation, labour and running costs).

Trait of Test	**Molecular Diagnosis**					
	PCR	*qPCR*	*ddPCR*	*LAMP*	*eDNA*	*NGS*
Sensitivity	High	High	High	High	High	High
Specificity	Med	High	High	High		
Speed	Slow	Slow but real time output	Slow but real-time output	Fast	Slow	Med
Cost	Med	High	High	Low	Low	High
Labour	Low	Medium	Medium	Low	High	High
Lethality of host	Dependent on tissue sequenced	Dependent on tissue sequenced	Dependent on tissue sequenced	Dependent on tissue sequenced	Never	Dependent on tissue sequenced

Recommendations and Conclusions

The lack of transference of terrestrial techniques to the aquatic environments is due to issues of translation, changing something suited for terrestrial applications to the aquatic environment is not easily done, and requires significant interest and/or funding. The recent thrust in diagnostic development will result in progress not only for human medicine, but diagnostics across disciplines Advances in early pathogen diagnosis have typically been driven by infections of terrestrial hosts, highlighted by the current COVID-19 crisis. One benefit of this pandemic has been the rapid increase in efficient and rapid diagnostic techniques, such as lateral flow immunochromatographic assays providing results within 90 minutes or adapted LAMP technology. Such advances will hopefully boost the entire diagnostic field, including aquatic pathogens but as previously stated, will require a significant driver to bring in financial support. Lateral flow tests have always had potential for disease diagnosis but were relegated primarily to pregnancy tests due to the lack of sufficient drivers to develop the technology for other users (O'Farell, 2009). The COVID-19 crisis demanded utilisation of every tool available, and thus the potential of lateral flow tests was harnessed for rapid diagnostics of the virus and informs how we can turn the retrospective into a reactive approach (Yu et al., 2020). The diagnostic potential of many terrestrial diagnostic methods will not be translated for aquaculture without sufficient ecological or monetary drivers. Indeed, even human neglected diseases are facing the same hurdles (Ung et al., 2021). Nevertheless, here we evaluated a variety of diagnostic methods in light of the three pillars for a gold standard diagnostic technique: high sensitivity, low cost, and speed. Going forward, emphasis should be put on two main techniques to advance aquatic diagnostics: AI for visual diagnosis and eDNA for molecular diagnostics. AI has the

potential to drastically reduce the time required to survey fish for disease whilst simultaneously allowing for higher throughput but requires significant input in “teaching” the AI to detect specific diseases. eDNA enables detection and quantification both on-site and in the laboratory, making it one of the most versatile diagnostic techniques once sampling methods have been optimised. As our knowledge of these pathogens increases so do our technological advances, where preventing pathogen outbreaks from occurring is the end-goal and these techniques aid this. Human medicine receives more monetary support for research on novel diagnostic methods, but there is always potential for these methods to be transferred to the aquatic environment should the industry or researchers take the time to adapt them.

Chapter 3 - The shelf life of *Saprolgenia*: short- and mid-term storage of the freshwater oomycete parasite *Saprolegnia parasitica*

Abstract

Parasites pose a complex problem to aquaculture due to their abundance, cryptic nature, adaptability and unpredictability. A parasite of particular economic interest is *Saprolegnia parasitica*, renowned for causing losses in salmonid aquaculture, estimated to kill 10% of all farmed salmon. As such, methods to study and understand this pathogen are essential. Establishing viable stocks of these parasites for present and future work requires efficient storage methods, two of which are reviewed here. We assessed the efficacy of mineral oil and cryopreservation on the short- and mid-term (1 – 50 days) storage and resuscitation of *Saprolegnia parasitica* strains. Mineral oil storage was assessed using agar slopes containing healthy colonies of *S. parasitica* immersed with sterile mineral oil for either one week or two months. Cryopreservation was assessed using two methods: utilising the VIA Freeze ™ cell freezer and a method developed by Eszterbauer et al. (2020). We found 100% survival of all *S. parasitica* strains preserved under mineral oil for both one week and two months. However, *S. parasitica* stored using the VIA Freeze ™ had variable success, with a majority of strains not surviving storage and those that did having survival rates of 25 and 57%. Conversely, all strains frozen using the method developed by Eszterbauer et al. (2020) had a 100% survival rate, irrespective of duration frozen. Our work here adds to knowledge on preservation of *Saprolegnia parasitica* strains though only short- and mid-term storage durations were assessed, aiding in the creation of a global *Saprolegnia* archive.

Introduction

Aquaculture is the fastest growing food industry, of key importance in meeting the food demands of an ever-growing world population. The biggest challenge facing the industry is the threat and impact of infectious disease, with disease-associated global economic losses estimated to be around £7.9 billion per annum (Shinn et al., 2015). Parasites pose a particularly complex problem due to their abundance, cryptic nature, adaptability and, particularly in the light of climate change, unpredictability. They alone account for losses of £47-134 million annually to the UK aquaculture (Shinn et al., 2015). Of particular concern are *Saprolegnia* spp. (Heterokontophyta: Oomycota), oomycetes or water moulds, found globally in freshwater systems (van West, 2006). They target fish at various life stages (eggs, smolts, juveniles and adults), leading to, at best, impaired meat quality and at worst mass mortality events (Eissa et al., 2013; Van Den Berg et al*.,* 2013. Two of the most

economically relevant fish species, Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*)(FAO, 2022), are both highly susceptible to *Saprolegnia* infections. An (under)estimated one in every ten farmed salmon will succumb to saprolegniasis, making *Saprolegnia* spp. of significant economic and welfare importance to aquaculture. *Saprolegnia* spp. cause significant economic losses to freshwater aquaculture, with stock losses, for example, up to 50% in Japanese Coho salmon (*Oncorhynchus kisutch*) and with cost to catfish farming in the USA at $40 million annually (CABI, 2019).

Control and treatment of *Saprolegnia* remains a major problem, and research in this field is hampered by obtaining and maintaining live parasite cultures. Issues with *S. parasitica* escalated when malachite green treatment was banned due its carcinogenic properties, with no effective alternative in place (van West, 2006; Earle & Hintz, 2014). Chemical and physical alternatives have been tested, such as formaldehyde and formalin (Gieseker et al., 2006) or ozone (Forneris et al., 2003), but sufficient control of the pathogen has not been achieved. Current control and surveillance rely on reporting from fisheries and anglers to know where and when the disease is apparent to obtain samples (see Chapter 2). Further, in aquaculture and the ornamental trade once a breakout occurs it is the farmers priority to treat the fish and minimise losses; acquiring samples for research is, understandably, secondary. Thus, viable *S. parasitica* samples are valuable and essential for research. For research purposes, *Saprolegnia* can be maintained *in vitro,* with standard methodologies involving sub-culturing from one agar plate to another (Stewart et al., 2017).

Parasite culturing itself can vary greatly (Ahmed, 2014) from *in vivo* culturing of ectoparasites such as *Gyrodactylus turnbulli* on tropical freshwater fish (see King & Cable, 2007) and *Argulus* spp. on temperate freshwater fish (see Hunt et al., 2021) to *in vitro* culturing of *Chilodonella* spp. using pond water as media and rice grains to stimulate bacterial growth as a nutrient source (Bellec et al., 2014; Li et al., 2022). To facilitate animal welfare and compliance with the 3Rs alternative, *in vitro* culturing is always preferred but not always possible. For example, complete life cycle *in vitro* culturing of *Ichthyopthirius multifiliis* or *Cryptocaryon irritans* has still not been achieved (Li et al., 2023). Fortunately, *in vitro* culturing of *Saprolegnia* spp. is possible, but is heavily skewed towards the asexual portion of the life cycle. Culturing *Saprolegnia* spp. can require specialist media, such as Sabouraud dextrose agar (SDA) or yeast extract agar (Eissa et al., 2013; Magray et al., 2019), although potato-dextrose agar (PDA) is commonly used (Stewart et al., 2017). In the UK, if a saprolegniasis outbreak is reported, (where possible) fresh samples of ‘fluffy’ lesions are swapped directly from fish onto agar plates in the field site. This sampling in-field risks contamination,

primarily combatted through incorporating antibiotics such as chloramphenicol into the agar (Meneses et al., 2022), although this is only possible on a small scale where it is certain that no antibiotic allergies exist amongst those handling the plates. If this sampling is successful, *Saprolegnia* strains can be maintained *in vitro* via repeated plating (weekly to monthly), but may lose virility, pathogenicity and the ability to sporulate (Dahmen et al., 1983; Songe et al., 2014). Stewart et al. (2017) recommended passaging the cultures through fish cell lines to reduce the potential loss of virulence, but this is cost and labour intensive. The burden of re-culturing can be lessened through long term storage in mineral oil, by which *Saprolegnia* spp. have survived for up to two years before being re-cultured (Ravimannan et al., 2012). Historically mineral oil preservation has been commonly used (see Buell & Weston, 1947; Reischer, 1949; Stebbins & Robbins, 1949) and remains in use currently (e.g. Gieseker et al., 2012; Ravimannan et al., 2012; Kannojia et al., 2020), however to induce sporulation, *Saprolegnia* must be transferred to a liquid media and temperature shocked (Stewart et al., 2017), further increasing the time and labour input. Thus, for long-term storage and maintenance of this parasite cryopreservation is the recommended 'gold standard' for which success has been achieved, but only on at a small scale for species such as *S. parasitica, S. australis and S. ferax* (see Eszterbauer et al., 2020) (Table 3.1).

Table 3.1 Cryopreserving oomycete species from previous studies. DMSO = dimethylsulphoxide. LN = liquid nitrogen. PEG = polyethylene glycol, RT = room temperature. * 66% survival after 6 months. ** 100% survival after 3 and 9 months.

Oomycete Pathogen	**Cryoprotectant**	**Temperature (°C)**		**% survival (no. of replicates)**	**References**
		Cooling Rate	**Thawing**		
Phytophthora infestans	DMSO	-1°C min^{-1} to -40°C then LN immersed	40°C	22% (n=3)	Dahmen *et al.* (1983)
Phytophthora infestans	Skim-Milk Glycerol	-1°C min^{-1} to -40°C then LN immersed	40°C	42% (n=3)	Dahmen *et al.* (1983)
Phytophthora & *Pythium*	10% Glycerol	-1°C min^{-1} to -40°C then -2°C min^{-1} to -80°C	30°C	100% (n=18)	Nishii & Nakagiri (1991)
Phytophthora & *Pythium*	10% DMSO	-1°C min^{-1} to -40°C then -2°C min^{-1} to -80°C	30°C	100% (n=18)	Nishii & Nakagiri (1991)
Phytophthora & *Pythium*	10% PEG	-1°C min^{-1} to -40°C then -2°C min^{-1} to -80°C	30°C	40% (n=18)	Nishii & Nakagiri (1991)
Saprolegnia parasitica	10% Glycerol	-10°C min^{-1}	35°C	32.5% (n=4)	Morris *et al.* (1988)
S. parasitica, S. australis & S. ferax	10% Glycerol	-60°C to -80°C min^{-1}	RT	66%* (n=3) 100%** (n=3)	Eszterbauer *et al.* (2020)

For cryopreservation, the parasite is preserved at either -80°C or, with liquid nitrogen, -196°C (Morris et al., 1988), which theoretically would enable indefinite storage of viable *Saprolegnia* samples. Initial attempts at cryopreservation of oomycetes, including *Saprolegnia*, were variable (Dahmen et al., 1983; Morris et al*.,* 1988; López-Lastra et al., 2002) (see Table 1). For *S. parasitica* Morris *et al.* (1988) achieved a 32.5% survival rate, low compared to the 97% survival of some other cryopreserved oomycetes (Nishii & Nakagiri, 1991). Eszterbauer et al. (2020) achieved 100% survival of *S. parasitica* after 3 and 9 months of cryopreservation, and 66% survival after 6 months, but samples were just 3 per treatment. With the looming threat of these pathogens to aquaculture and the requirement to have readily available samples at hand, verifying the efficacy and optimising the cryopreservation methodology is key for maintaining a regular stock of *Saprolegnia*.

Here we assessed the success of two storage methods, mineral oil submersion and cryopreservation, on the survival of different *S. parasitica* strains in the short and mid-term, here defined as 1 to 50 days in duration. We hypothesised both methods would show success with short- and mid- term storage.

Methods

Sample Collection and Identification

Saprolegnia strains were obtained from various sources described in Table 3.2 and maintained as per Stewart et al. (2017). Briefly, this involved inoculating potato dextrose agar (PDA; Potato Glucose Agar (Sigma-Aldrich); 39g L^{-1}) petri dishes (90 mm) (Sigma-Aldrich) with a 5 mm plug of *Saprolegnia* mycelia) incubated at 18±1°C and monitored daily for growth.

Table 3.2 *Saprolegnia* species and origin utilised in current study. *denotes the reference strain (C65).

S. parasitica	***Host***	***Date Isolated***
CBS223.65*	Pike**	1965 (originally)
N12	Atlantic Salmon	Unknown
HO	Atlantic Salmon	16/08/2017
VWB	Atlantic Salmon	Unknown
AB15	Fish (unknown) Tissue from pectoral fin	10/09/2013

** Wuensch *et al.* 2018

To ensure accurate identification of samples to species level, samples were extracted and then sequenced to ensure all were *Saprolegnia parasitica*. DNA extraction utilised the Qiagen DNeasy

Plant Kit (Qiagen) and followed the manufacturer's protocol (Qiagen, 2020). Approximately 0.3 g of *Saprolegnia* mycelia were used per extraction. PCR amplification of the rDNA ITS regions 1 and 2 from purified DNA 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 (10 µM), 2 µl genomic DNA and 5 µl nuclease-free water to give a total reaction volume of 25 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. Confirmation of correct PCR products (target 710 bp) was carried out through running on a 1% agarose gel and visualising under UV-transillumination. Following visual confirmation of PCR amplicon, samples were sent to Eurofins Genomics for sequencing (10 µl nuclease-free water, 5 µl PCR product, 2 µl primer at 10 µM concentration, forward or reverse). All chromatograms generated were assessed to ensure good sequence quality (defined as clear single peaks for each base), with forward and reverse sequences paired and ends trimmed using BioEdit version 7.2.5 (Hall, 1999). The resulting contigs for each sample were then put through an NCBI BLAST search for related sequences, with confirmation of species taken upon a match of >98% sequence identity.

All species were identified as *Saprolegnia parasitica* but were isolated at different time points (e.g. C65, VWB, AB15 and HO strains; Table 2).

Mineral Oil Immersion

Sterile glass tubes (250 ml) were filled with 125 ml PDA, angled at a 45° slope until solidified. PDA was then inoculated with a 5 mm plug of fresh *S. parasitica* mycelia and incubated at 18±1°C until approx. 0.5 cm vertical mycelial growth was observed. Following this, sterile mineral oil (25 ml) was added to n = 20 tubes until mycelia were completely immersed, n = 10 slopes remained uncovered by mineral oil to act as controls. Immersed slopes were then incubated at 18±1°C under a 12:12 h light regime. After one week and 2 months of immersion, the mineral oil was removed from the glass tubes of immersed samples and sub-cultures of the mycelia taken and plated onto fresh agar to assess survival from both immersed and non-immersed control samples.

Cryopreservation

Cultures were maintained in sterile Petri-dishes contained PDA prior to the experiment, with 5 mm plugs transferred into 1.5ml cryovials for freezing. Multiple freezing and thawing protocols were carried out, as described in Table 3; variables tested were: cooling rate, cryoprotectant (glycerol or DMSO), anti-desiccant (hemp seeds or perlite), pre-freezing conditions and strain of *S. parasitica*. After freezing at -80°C, samples were thawed at the designated temperature (see Table 3) until the aqueous media had defrosted sufficiently to allow removal of a plug and/or anti-desiccant media. This was then embedded within a fresh Petri dish containing PDA and incubated at 14±1°C. Successful revival was deemed once hyphal growth exceeded 10 mm diameter from the embedded plug. All controls were exposed to the same mixture of nutrient broth, cryoprotectant and anti-desiccant, and handled in the same manner as the treatment protocols but without freezing.

Statistical Analysis

All statistical analysis was conducted using R statistical software version 4.2.3 (http://www.R-project.org/). Models were refined through removal of non-significant terms based on Analysis of Variance (Crawley 2007) and the robustness of models was assessed using residual plots (Pinheiro and Bates 2000). The level of significance for all tests was taken as $p < 0.05$. A General Linear Model (GLM) using the gaussian family and identity link functions was run to investigate how survival rates per method compared to controls. A GLM using the gaussian family and identity link functions was run to assess the differences in survival rates for duration.

Results

Mineral Oil Immersion

All mineral oil immersed samples successfully resuscitated post-immersion after one week, achieving full plate coverage. There was no significant difference in the survival of *Saprolegnia* immersed in mineral-oil compared to those which were sub-cultured on agar absent of mineral oil. Duration was significant (GLM: $p < 0.05$), with 100% survival of immersed cultures after 1 week and 100% survival of immersed cultures after 2 months compared to 100% survival of control cultures after 1 week but 20% survival of control cultures after 2 months.

Cryopreservation

The survival rates of *Saprolegnia* varied depending on the method of cryopreservation method (see Table 3.3), with 100% success rate for all strains tested subject to the Eszterbauer et al. (2020) method. Survival using the VIA Freeze™ varied from 0% to 57% compared to 100% survival in the

unfrozen controls. The cryoprotectant nor anti-dessicant had any impact on survival, nor was there any difference between *Saprolegnia* strain survival.

Table 3.3 Summary of the trials/protocols used for the cryopreservation of *Saprolegnia parasitica* strains including survival post-thawing. * denotes survival of mycelia post-thawing. Sample size refers to the number of cryovials containing one plug of mycelia which were frozen.

***Saprolegnia* Strain**	**Freezing Apparatus**	**Cryoprotectant**	**Anti-Desiccant**	**Sample Size**	**Freeze Duration (Days)**	**Survival (%)**
C65	VIA Freeze™	10% Glycerol	N/A	20	1	0
C65	VIA Freeze™	10% Glycerol	N/A	20	4	0
C65	VIA Freeze™	10% DMSO	N/A	20	4	0
VWB	VIA Freeze™	10% Glycerol	N/A	12	6	25*
C65	VIA Freeze™	10% Glycerol	Perlite	15	9	0
C65	VIA Freeze™	10% DMSO	Perlite	15	9	0
01	VIA Freeze™	10% Glycerol	N/A	7	2	57*
C65	VIA Freeze™	10% Glycerol	Hemp Seeds	16	2	0
C65	VIA Freeze™	10% DMSO	N/A	15	14	0
C65	VIA Freeze™	Water	N/A	15	50	0
C65	VIA Freeze™	10% Glycerol	N/A	12	42	0
C65	-80°C	10% Glycerol	Hemp Seeds	15	7	100*
01	-80°C	10% Glycerol	N/A	10	7	100*
AB15	-80°C	10% Glycerol	N/A	10	7	100*
HO	-80°C	10% Glycerol	N/A	10	7	100*
AB15	-80°C	10% Glycerol	N/A	10	24	100*

Discussion

Here, we primarily assessed short and mid-term (1 – 50 days) storage of *Saprolegnia parasitica*. Mineral oil storage was universally successful for storing and reviving all Saprolegnia samples, regardless of strain or duration. Cryopreservation exhibited varying success rates across methods, but the approach based on Eszterbauer et al. (2020) consistently achieved 100% survival rates for all strains.

Historically, mineral oil has been used as long-term storage for cells and colonies (e.g. *Fusarium* spp., Actinomycetes and Phycomycetes) including *Saprolegnia* (Buell & Weston, 1947; Reischer, 1949), which has been stored successfully for up to two years (Ravimannan et al., 2012). The efficacy of which was shown in the current study, with 100% survival of all strains regardless of storage

duration. The main drawbacks of this method, when compared to cryopreservation, are an increased likelihood of contamination. Further work would look to increase the duration of immersion from mid-term (2 months) to longer-term (6 months) and assess the viability of immersed colonies post-immersion.

As theorised two decades ago (Engelmann, 2004), cryopreservation has become frequently utilised and available as a method of long-term preservation and conservation of precious samples. Where common usage has been seen in reproductive (Vajta & Kuwayama, 2006) and plant biology (Engelmann, 2004), it has always held the potential for widespread application. As such, many sought to use cryopreservation on *Saprolegnia* (Morris et al., 1988; Smith & Thomas, 1997) with the hope that success would follow that of fungi and other oomycetes (see Table 1). However, success with *Saprolegnia* spp. was not as common as other oomycete pathogens, and methods required optimisation. Methods of cryopreservation generally retain similar procedures for preparation of the sample; a cryoprotectant, then freezing at a set rate and thawing at a set temperature. It within these parameters that optimisation of methods occurs e.g. different cryoprotectants, different freezing rates and different thawing temperatures.

Cryoprotectants tested on oomycetes include dimethyl sulphoxide (DMSO), glycerol and various mixtures including skim milk or L-proline (Dahmen et al., 1982; Nishii & Nakagiri, 1991) (see Table 1). As such, we found it appropriate to choose DMSO and glycerol as the cryoprotectants for this work. Other studies altered the substrate in which the oomycete was frozen or grown, for example: growth medium (glucose-yeast broth), vitrification of the cryoprotectant solution, entrapment within calcium alginate beads or the use of a media such as hemp seeds or perlite (Morris et al., 1988; Homolka, 2013; Wolkers & Oldenhof, 2015). It has been suggested that the optimal freezing temperature for *S. parasitica* is 10°C min^{-1} (Morris et al.,1988), which our results neither support nor refute. We found some success utilising freezing rates of -1 to -2°C min^{-1}, although the survival was lower than those frozen at faster rates, but nevertheless were still successful and viable. Controlled freezing was carried out using the VIA Freeze ™ (Cytiva), targeted toward medical cryopreservation of cells (e.g. Morris et al., 2017; Mitchell et al., 2017) but with the potential for controlled and effective preservation of *Saprolegnia*. The unique selling point of this tech is the mode of freezing, where conventional freezing methods often freeze with convection cooling, the VIA Freeze ™ utilised conduction cooling, where samples are cooled evenly (Cytiva, personal communication). We found limited success here using this technology, potentially due to the limited freezing rate maximum of -2°C min^{-1}. Alternatively, the method developed by Ezsterbauer et al. (2020) does not

specify a controlled freezing rate, instead opting for a more rapid but less controlled freezing rate determined by the freezer used. Surprisingly, the cooling rate used by this method resulted in total survival of all strains frozen, regardless of duration. Further work, following the results obtained here, would be to increase the duration of freezing from mid- to long-term (ideally greater than 6 months) to assess efficacy of long-term storage. Following successful long-term cryopreservation, the next steps would be to ensure pathogenicity of the strains frozen (Hardy et al., 2023). Whilst we did observe successful growth on agar (matching that of non-frozen controls), we were unable to test the infectivity of these frozen strains. Recent work using the same cryopreservation method as Eszterbauer et al. (2020) suggests that freezing will not impair pathogenicity (Hardy et al., 2023), regardless of strain, even after more than 2 years of cryopreservation.

Ensuring an adequate viable supply of pathogens are available facilitates research, potentially allowing for the creation of an archive. Creation of an effective standardised protocol for preservation of fickle parasites, such as *Saprolegnia parasitica*, also supports work in this field. Our work here adds to knowledge on preservation of *Saprolegnia parasitica* strains using short- and mid-term storage results through the use of mineral-oil immersion and cryopreservation, both of which were found to be successful.

Chapter 4 – Growing strong: vegetative growth of freshwater parasitic oomycete *Saprolegnia parasitica* in response to temperature and light.

Abstract

For aquatic systems, infectious diseases caused by fungal or fungal-like pathogens pose severe threats in terms of biodiversity, welfare and food security. Of particular importance to wild, cultured and farmed fish is *Saprolegnia parasitica,* an oomycete water mould, which can kill fish within 24 h. The rate at which *Saprolegnia* infections spread within and between hosts is dependent on a range of biotic and abiotic factors, with temperature and light playing pivotal roles for many pathogens. Within aquaculture, temperature and light are manipulated to varying degrees, with potential implications for *Saprolegnia* depending on the system. Indoor systems offer precise control over all abiotic conditions, while outdoor systems generally contend with natural environmental variation and rely on supplemental artificial light. For *Saprolegnia* species*,* temperature particularly affects infection success. We know little of the impact of light on *Saprolegnia* spp., although in terrestrial oomycete species, light can suppress sporulation. Additionally, artificially lengthened photoperiod can increase host stress and susceptibility to infection. Here, vegetative growth of wild (obtained from riverine fish in England and Wales) and laboratory (cultured off-host, in one case for more than 50 years) strains of *Saprolegnia parasitica* was investigated under different temperature and light regimes. Both temperature and light had strain specific effects on growth, with temperature being the most predictable. Growth generally increased with rising temperatures, peaking at 25°C before declining at 30°C. For light, *S. parasitica* strain CBS223.65 exposed to 0 h: 24 h, 6 h: 18 h or 12 h: 12 h light: dark grew consistently, with growth supressed by 10-20% at 18 h: 6 h and 24 h: 0 h light:dark. For *S. parasitica* strain A01, growth was suppressed at 0 h: 24 h and 24 h: 0 h light:dark but amplified at 18 h: 6 h light:dark. This work provides a clearer understanding of how vegetative growth of the pathogen *in vitro* responds to environmental conditions; growth is affected by temperature predictably and light variably, with both responses being strain specific.

Introduction

Saprolegnia spp. (Heterokontophyta: Oomycota), commonly known as water moulds or oomycetes, are ubiquitous in freshwater ecosystems and play a significant role in both natural and farmed aquatic populations (van West, 2006; Masigol et al., 2021; Masigol et al., 2023). Being opportunistic pathogens, *Saprolegnia* spp. can infect a wide range of organisms, often leading to substantial

mortality in species such as fish, frogs, and crustaceans (Pickering & Willoughby, 1982; Wolinska et al., 2008; Ruthig, 2009; van den Berg et al., 2013). In the wild, *Saprolegnia* outbreaks can be unpredictable, often associated with declines in wild salmonid populations, posing a threat to ecosystems and communities dependent on these fish (van West, 2006; Phillips et al., 2008). Furthermore, *Saprolegnia* spp. pose a severe economic threat to the aquaculture industry. Atlantic salmon (*Salmo salar*), the second most economically valuable aquaculture species globally, and rainbow trout (*Oncorhynchus mykiss*), the ninth most produced species by quantity, are highly susceptible to *Saprolegnia* infections (Cai et al., 2017; FAO, 2022). In fact, saprolegniosis is responsible for an estimated 10% of annual global economic losses in salmonid aquaculture (Hussein & Hatai, 2002; van West, 2006; Phillips et al., 2008; Robertson et al., 2009; van den Berg et al., 2013), though actual losses may be as high as 50% (Bly et al., 1992; Hatai & Hoshiai, 1992; van West, 2006; Bruno et al., 2011).

To combat these outbreaks in both wild and farmed populations, it is important to have a comprehensive understanding of environmental predictors and effective treatments against this oomycete. Since the ban of malachite green, there is no guaranteed treatment or cure for *Saprolegnia* infections (Earle & Hintz, 2014). Once an infection has established, the only recourse is culling the affected fish. Therefore, current mitigation efforts primarily focus on minimizing the impact of saprolegniasis (Torto-Alalibo et al., 2005). These measures include routine monitoring for clinical signs of the pathogen, conducting light salt baths upon detecting potential lesions, and quarantining fish (Holan et al., 2020). These methods, however, are limited in their effectiveness in preventing *Saprolegnia* outbreaks (van West, 2006; Barde et al., 2020; see Chapter 2). To address saprolegniasis, it is essential to conduct a comprehensive assessment of the environmental factors contributing to proliferation, in conjunction with understanding the life history of the parasite. *Saprolegnia* spp. exhibit both sexual and asexual life stages (Figure 4.1), which may react differently to environmental changes. During the asexual stage, free-living zoospores are responsible for locating and attaching to potential hosts (Pickering & Willoughby, 1982; Hatai et al., 1990; van den Berg et al., 2013). Sporulation can be triggered by sudden decreases in ambient water temperatures (Bly et al., 1992), indicating a clear relationship between temperature and infection. Once cysts successfully attach to a host, the vegetative stage is responsible for host colonization, with hyphal branches penetrating the host's epidermis, muscle layers, and blood vessels (Boddy, 2016). Mycelial growth results in visible clinical signs of *Saprolegnia* infection, which can also develop on other

organic substrates such as fish eggs and hemp seeds (Ali et al., 2013; Songe et al., 2016), and even on agar plates for *in vitro* culturing (Stewart et al., 2017).

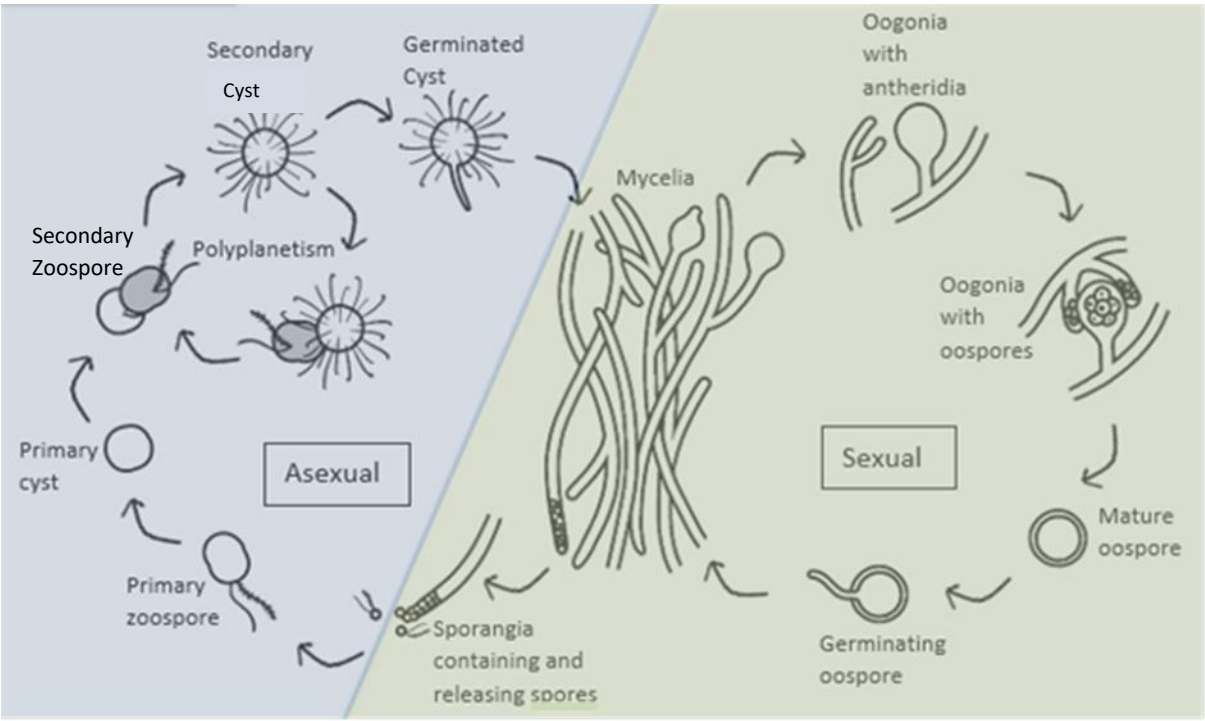

Figure 4.1 The life cycle of *Saprolegnia parasitica*, presenting the asexual cycle on the left and the sexual cycle on the right. Phases in blue represent free living stages, while phases in green represent stages occurring on host (*in* situ) or agar (*in vitro*). The current study focussed on the asexual vegetative growth on agar (blue phases), however all stages also develop on the host (green and blue). Adapted from van West, 2006.

Key environmental factors influencing *Saprolegnia* spp. include temperature, pH, oxygen levels, nutrients, and water flow (Pickering & Willoughby, 1982; Smith et al., 1984). Some anecdotal links suggest that incidences of saprolegniasis are associated with low flow and increased water temperatures in temperate systems (van West, 2006). Temperature is the abiotic factor most frequently linked to *Saprolegnia* infections. In indoor aquaculture, temperature is typically maintained at an optimal range for the specific fish stock (Summerfelt, 2000). In contrast, outdoor aquaculture and wild populations must balance temperature between climatic conditions and supplementation (Lamoureux, 2003). Temperature also affects the host's immune status, with increased water temperatures either improving or decreasing immune function depending on the fish species (Fletcher, 1986; Bly et al., 1997; Bowden, 2008). Given global warming, understanding the relationship between temperature and host immunity is vital. Sudden temperature changes in spring and winter can have immunomodulatory effects, rendering fish more susceptible to infection (Bly & Clem, 1991; Le Morvan et al., 1998). Notably, mass *Saprolegnia*-induced mortalities during

winter months on catfish farms in the United States led to the coining of the term “winter kill syndrome” (Bly et al., 1992). Laboratory studies have shown that the temperature at the time of *Saprolegnia* exposure significantly influences infection success, highlighting the need for temperature data alongside immunological status to predict disease risk (Stewart et al., 2018).

While the impacts of temperature on organisms are well-documented, light exposure is a relatively neglected stressor, despite its direct influence on growth, appetite, reproduction, sex determination, and immunity (Ángeles Esteban et al., 2006; Taylor et al., 2006; Brown et al., 2014; Ellison et al., 2021). While wild fish experience natural daily and seasonal variations in the light cycle, photoperiod is often manipulated in aquaculture to enhance productivity (Bowden, 2008; Volkoff et al., 2010). Extended photoperiods can stimulate increased feeding, resulting in faster fish growth and higher protein yields. Conversely, altering the light regime can also increase stress and susceptibility to infection (Valenzuela et al., 206; Ellison et al., 2021; Valenzuela et al., 2022). Similarly, early maturation of Atlantic salmon (*Salmo salar*) as a result of disrupted photoperiods, reduces food conversion ratios and yields resulting in economic loss (McClure et al., 2007).

It is evident that multiple abiotic and biotic factors influence *Saprolegnia* infectivity and host immunity, underscoring the importance of establishing a strong baseline understanding. One practical way to do this is by assessing *in vitro* mycelial growth of *Saprolegnia* under varying temperature and light conditions. Here, a series of laboratory-based experiments examined the impact of these abiotic factors on wild and laboratory strains of *Saprolegnia parasitica*, through examination of vegetative mycelial growth on agar. We hypothesised that growth rate would increase with both temperature and light, with no significant differences between strains.

Methods

Origin and maintenance of parasite strains

All 13 strains of *Saprolegnia* sp. were either donated from laboratory cultures or collected from fish present in water bodies in England and Wales (see Table 4.1 for details). Two laboratory strains of *Saprolegnia parasitica* were used: reference strain CBS223.65, originating from the Netherlands supplied by the Aberdeen Oomycete Laboratory, and strain A01 originating from Aberdeen Oomycete Laboratory. These strains allowed us to examine the effect of serial sub-culturing on agar for many years (CBS223.65 for 50+ years and A01 for 10 years), and CBS223.65 has been utilised as a reference strain for many previous studies (e.g. van West et al., 2010; Jiang et al., 2013; Belmonte

et al., 2014; Srivastava et al., 2018; Kumar et al., 2020). The 11 unique wild strains of *Saprolegnia* were obtained from UK rivers (Table 4.1).

Following collection, strains were cultured *in vitro* under laboratory conditions according to Stewart et al. (2017). In brief, *Saprolegnia* was inoculated into potato dextrose agar (PDA) Petri dishes (145 mm x 20 mm) (Potato Glucose Agar (Sigma-Aldrich); 39g L^{-1}) incubated at 18±1°C, and signs of growth were checked daily. Once growth was 40 mm from the original tissue, 2-3 agar plugs (approximately 5 mm^3) were taken from the plate using the base of a sterile 200 µL pipette tip and embedded into a fresh PDA plate. These new plates were grown at 18±1°C for 4 days, again with daily monitoring (Stewart et al., 2017). Strains were maintained through repeated sub-culturing every 2-4 weeks onto PDA to ensure healthy fresh stocks.

Table 4.1 *Saprolegnia parasitica* isolates used in the current study.

Isolate ID	Location (Grid Reference)	Fish species	Date isolated
CF006	Roath Brook, Cardiff, Wales.	Three-spined stickleback (*Gasterosteus aculeatus*)	15/07/2016
CF009	Roath Brook, Cardiff, Wales.	Three-spined stickleback	09/08/2016
CF010	Roath Brook, Cardiff, Wales.	Three-spined stickleback	15/08/2016
EA001	River Esk, Yorkshire, England.	Atlantic salmon (*Salmo salar*)	10/01/2015
EA002	River North Tyne, England.	Atlantic salmon	17/11/2015
EA007	River Ouse, Yorkshire, England.	European eel (*Anguilla anguilla*)	25/11/2015
EA009	River North Tyne, England.	Sea trout (*Salmo trutta*)	24/11/2015
EA012	Lake near Romsey, Hampshire, England,	Common carp (*Cyprinus carpio*)	22/03/2016
EA016	River Dart, Devon, England.	Sea trout	03/06/2016
EA020	Bells Mill Fishery, Stourbridge, England.	Common carp	31/08/2016
EA097	River Coquet, Northumberland, England.	Atlantic salmon	19/04/2017
CBS223.65	Centraal Bureau voor Schimmelcultures (CBS), the Netherlands	Pike (*Esox Lucius*); thereafter lab culture	1965
A01	Aberdeen Oomycete Lab, Scotland.	Mealworm Bait; lab culture	02/10/2013

Species identification and confirmation

To ensure all strains were the same species, and thus comparable, samples were extracted and sequenced for identification. DNA extraction was carried out using Qiagen dNeasy Plant Kit (Qiagen) and following the manufacturer's protocol (Qiagen, 2020). Subsequently, PCR amplification of the rDNA ITS regions 1 and 2 from purified DNA 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 (10 µM), 2 µl genomic DNA and 5 µl nuclease-free water to give a total reaction volume of 25 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 of the expected fragment size (approx. 700 bp), they were run on a 1% agarose gel and visualised under UV-transillumination. Following visual confirmation of PCR amplicon, samples were sent to Eurofins Genomics for DNA sequencing (10 µl nuclease-free water, 5 µl PCR product, 2 µl primer at 10 µM concentration, forward or reverse). All chromatograms generated were assessed to ensure good sequence quality (defined as clear single peaks for each base), with forward and reverse sequences paired and ends trimmed using BioEdit version 7.2.5 (Hall, 1999). The resulting contigs for each sample were then put through an NCBI BLAST search for related sequences, with confirmation of species taken upon a match of >98% sequence identity.

Measurement of Saprolegnia parasitica *vegetative growth*

Freshly inoculated agar plates were utilised for all experiments. All *Saprolegnia* strains for each experiment were photographed every 12 h until mycelial growth reached the edge of the Petri dish, the duration of which varied from 96 h to 132 h. For temperature experiments, this involved using a Samsung Galaxy S7 camera against a black background with overhead strip lights (240 mm above the plate) to provide standard lighting conditions and minimise reflections. For light exposure experiments, photographs were taken using ZOSI Technology D/N Digital security cameras (1AC-2617A) positioned 190 mm above the Petri dish. During the light period, photographs were taken without any additional light, during the dark period photographs were taken using infrared lights built into the cameras. All photographs were analysed by measuring the area of culture growth using the imaging software ImageJ v1.51j8 (Rasband, 2018).

Growth of Saprolegnia parasitica *isolates at different temperatures*

The growth rates of the 11 wild *S. parasitica* isolates were initially assessed over a broad temperature range (5-30°C). This first experiment was performed in two separate batches, under consistent 12 h L: 12 h D lighting (500 lux, where lux is a standard unit of illumination). During batch 1, growth rates were obtained at 15, 25 and 30°C. During batch 2, the growth rates were recorded at 5, 10 and again 15°C. Petri dishes (145 mm dia., Greiner Bio-One) with ~50 mL of PDA (39g L^{-1}) were inoculated in the centre with an agar plug (5 mm dia.) of healthy white mycelia taken from the stock *Saprolegnia* culture. A total of 10 replicates were produced for each isolate, at each of five temperatures (5, 10, 15, 25 and 30°C) for strain analysis.

To further examine lower temperature impacts on *Saprolegnia parasitica* growth in a second experiment, isolates from the rivers Coquet, Dart and Esk further *in vitro* (145 mm dia. Petri dish) growth rate analyses at smaller intervals of 4, 6, 8, 10, 12 and 15°C. These reflect the typical range of UK river temperatures throughout the year. Methods were identical to the prior temperature experiment 1 outlined above. A total of 10 replicates were produced for each isolate, at each of the six temperatures (4, 6, 8, 10 12 and 15°C) for strain analysis.

Growth of Saprolegnia parasitica *under different light regimes*

The mycelial growth rates of *S. parasitica*, reference strain CBS223.65 and laboratory strain A01, were assessed under five different light regimes (hours of light:dark respectively), 0:24, 6:18, 12:12, 18:6 and 24:0 to provide a broad scope of photoperiods under which organisms may be exposed. This was initially carried out using standard 90 mm diameter Petri dishes, but to minimise surface area as a limiting factor, larger 145 mm diameter petri-dishes were subsequently used (identical to those used for both temperature experiments above). For all regimes, *S. parasitica* were exposed to ~210 lux during the light period. *S. parasitica* grown on PDA plates (n = 11 per light regime) were acclimated to each light regime for 2 weeks at 19±1°C. Following 2 weeks of acclimation, a plug of white mycelia (5 mm dia., taken using same method as for culturing) was removed from each plate and used to inoculate fresh PDA plates under laboratory artificial lights (~210 lux), except for the 0:24 treatment where red-light was used (1-2 lux), at 19±1°C.

Data Analysis

For temperature, statistical analyses were conducted using R statistical software version 4.0.4 (R Core Team, 2021) utilising packages: “ggplot2” for visualisation of the data (Wickham, 2011), “lme4” to run General linear mixed models (GLMM) (Bates et al., 2014), “effects” to visualise general trends (Fox, 2003) and “*emmeans*” to allow analysis of interactions (Lenth et al., 2018). Models were refined through removal of non-significant terms based on Analysis of Variance (Crawley, 2012) and the robustness of models was assessed using residual plots (Pinheiro & Bates, 2000). The level of significance for all tests was taken as $p < 0.05$.

The growth rates of the wild 11 *S. parasitica* isolates at 15°C were first assessed for each batch (batch 1 = 15, 25 and 30°C; batch 2 = 5, 10 and 15°C) to confirm no significant difference between the strains grown at 15°C. 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 vs 2) and the interaction between these terms, with time and replicate ID as random terms. Replicate ID identified

the isolate, batch and replicate number. This analysis revealed no batch effect (p >0.05). 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 replicate ID were included as random terms. *Post-hoc* analysis ('*emmeans'*) 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, followed by the same *post-hoc* analysis ('*emmeans'*).

General Linear Mixed Models (GLMM) fitted with gaussian family and identity link function were used to assess whether the growth, rate of increase and growth rate (dependent term) of *Saprolegnia* strains varied with light regime, time and the interaction between regime and time (fixed effects), with plate ID included as a random factor to account for pseudo-replication. To assess whether the rate of change in growth varied with light regime, time and the interaction between the two, a GLM fit with the gaussian family and identity link function was used. To assess whether the growth rate varied with light regime, time and the interaction between the two, a GLM fit with the gaussian family and identity link function was used. Direct comparisons between growth at individual time points was then examined using *post hoc* analyses with '*emmeans*'.

Results

For all isolates across all experiments, no vegetative growth was observed before 24 h as mycelia were establishing within the agar.

For all *Saprolegnia* isolates, temperature had a significant impact on vegetative growth rates, peaking at 25°C before declining at 30°C (Figure 4.2). Responses were isolate specific (GLMM; df=52, p <0.0001), with EA012 displaying the highest growth rate across all strains. Comparisons between isolates revealed no specific trends according to the disease status (normal vs. elevated levels of saprolegniasis) of the respective water body, or *Saprolegnia* species. Furthermore, the growth rates exhibited by the reference isolate CBS223.65 (lab strain) were similar to the 13 isolates (wild strains) obtained between 2015-2017, despite an extended time spent in culture (for CBS223.65, 50 years in culture prior to this experiment).

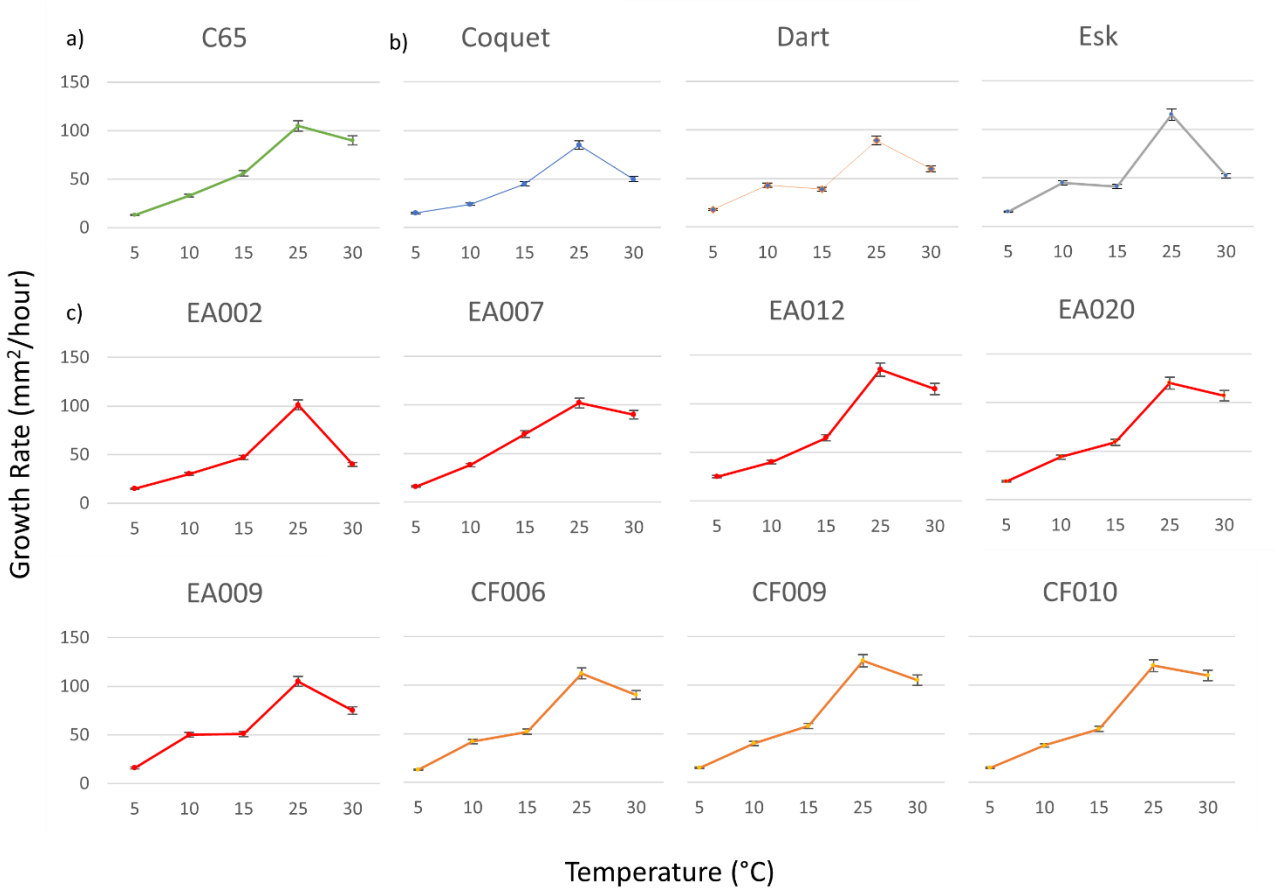

Figure 4.2 Growth rates of *Saprolegnia parasitica* isolates (±SE) at 5, 10, 15, 25 and 30°C. a) The reference isolate CBS223.65. b) Three isolates from different rivers selected to assess any lower temperature preferences (Rivers Coquet, Dart and Esk; see Table 2, Figure 3). c) Eight isolates from sites that have experienced normal levels of saprolegniasis (River North Tyne (EA002, EA009); 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.

Effect of temperature on Saprolegnia parasitica growth rate

More detailed examination of growth rate across a narrower temperature range typical of UK rivers again revealed a general increase in growth rate with temperature, with isolate dependent variance (GLMM; *df*=10, *p* <0.0001). The Coquet isolate grew faster than the other two isolates at 6 and 8°C, the Dart isolate grew faster at 15°C and slower at 8°C, while the Esk isolate grew slower at 10 and 12°C (Figure 4.3).

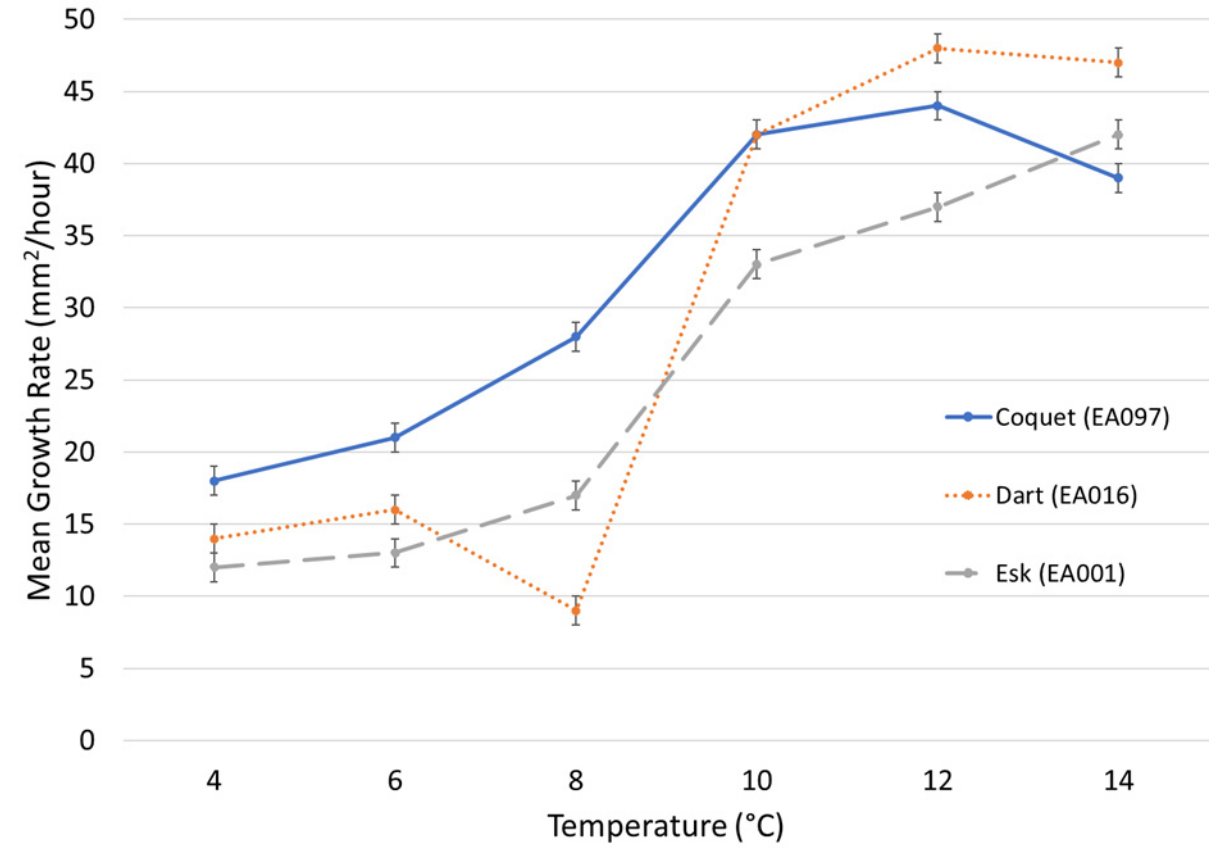

Figure 4.3 Growth rates of *Saprolegnia parasitica* isolates (±SE) from Rivers Coquet (EA097), Dart (EA016) and Esk (EA001) at 4, 6, 8, 10, 12 and 15°C. Potato dextrose agar plates were inoculated with these isolates, a total of 10 replicates were obtained for each isolate at each temperature.

Effect of photoperiod on Saprolegnia parasitica *growth rate*

S. parasitica strain CBS223.65 exposed to 0 L: 24 D, 6 L: 18 D or 12 L: 12 D grew consistently, with growth dampened by 10-20% at 18 L: 6 D and 24 L: 0 D. For strain A01, growth was suppressed at 0 L:24 D and 24 L:0 D but amplified at 18 L:6 D. Photoperiod significantly affected the vegetative growth, growth rate and rate of increase for mycelial growth for *Saprolegnia* laboratory strains CBS223.65 (GLMM; df=28, p <0.0001; Figure 4.4a) and A01 (GLMM; df=28, p <0.0001; Figure 4.4b). The only commonality in response to a treatment for the two strains was significantly impaired growth under 24:0 light:dark (GLMM; CBS223.65, df=28, p <0.0001. A01, df=28, p <0.0001).

The growth rate of A01 peaked earlier than CBS223.65, however overall, the growth rate of A01 was lower than CBS223.65 (Figure 4.5). Considering the increase seen in the 0 L: 24 D, 6 L: 18 D and 12 L: 12 D plates from around 75% plate coverage to >90% from 72 to 84 h post inoculation, there was ample room for the 18 L: 6 Dand 24 L: 0 D plates to grow, suggesting the decrease in growth rate was not due to limited space. Available area for growth is accounted for with growth rate, as peak

growth rates for all strains under all regimes occur within 48 h and severely decrease after this point, long before the hyphae reached the edge of the petri dish. Further data of all significant interactions between light regime and growth can be found in Appendix Tables S4.1, S4.2 and S4.3.

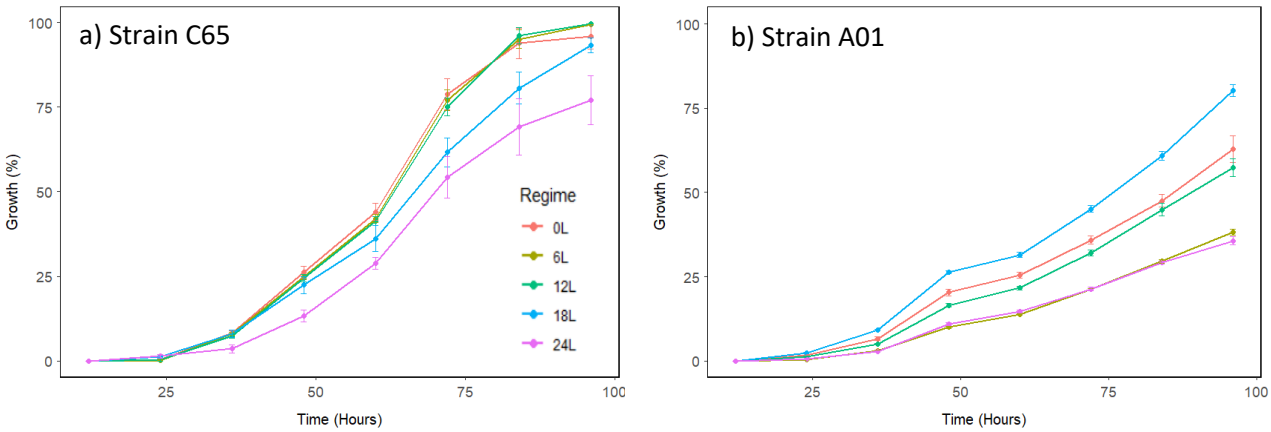

Figure 4.4 a) The vegetative growth of *Saprolegnia parasitica* strain CBS 223.65 (C65), expressed as percentage of petri-dish cover minus the inoculate area, across 96 hours under differing light regimes (0, 6, 12, 18 & 24 hours of light). b) The vegetative growth of *Saprolegnia parasitica* strain A01, expressed as percentage of petri-dish cover minus the inoculate area, across 96 hours under differing light regimes (0, 6, 12, 18 & 24 hours of light).

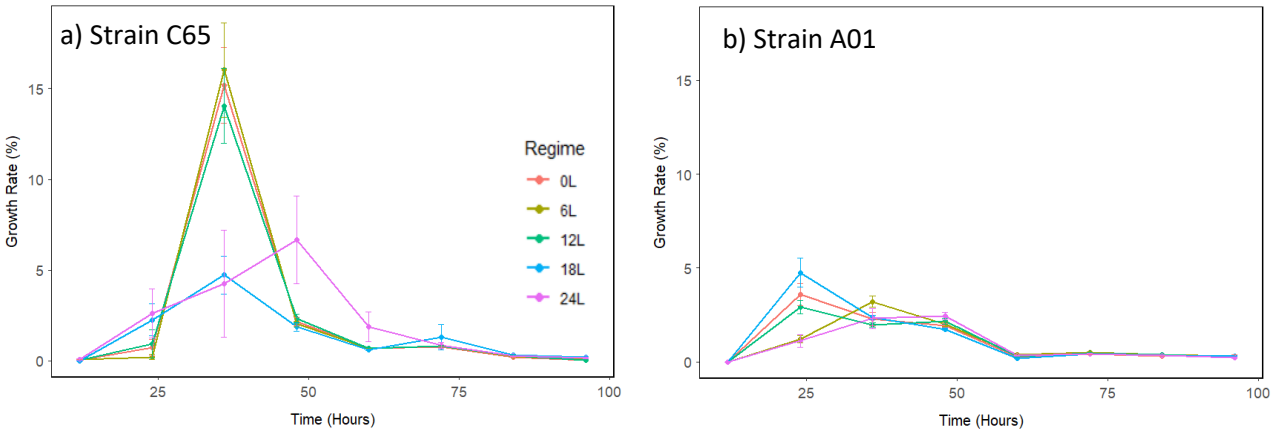

Figure 4.5 a) The vegetative growth rate of *Saprolegnia parasitica* strain CBS 223.65 (C65), expressed as percentage growth from one time point to the next, where the y-axis represents the percentage change as a decimal value. b) The vegetative growth rate of *Saprolegnia parasitica* strain A01, expressed as percentage growth from one time point to the next, where the y-axis represents the percentage change as a decimal value.

Discussion

Saprolegnia parasitica is arguably one of the most important pathogens in salmonid aquaculture (van den Berg et al., 2013), and without a cure we must look to predict and prevent outbreaks where

possible. Temperature and *Saprolegnia* have an established relationship (Bly & Clem, 1991; Kitancharoen et al., 1996; Morvan et al., 1998; Stewart et al., 2017) potentially making it a useful abiotic factor to predict outbreaks. Light on the other hand is much less studied with regard to *Saprolegnia* spp. (with the exception of Szaniszlo, 1965). The interaction between *Saprolegnia* and environmental factors is highly strain-specific (current study; Bangyeekhun et al., 2001; Matthews, 2019) likely due to the high diversity among *Saprolegnia* strains (Matthews et al., 2021). Temperature increased *Saprolegnia* growth rate until 25°C, followed by a decrease in growth at 30°C for all strains and both species. Photoperiod also influenced growth, where 24 hours constant light exposure diminished growth irrespective of strain, whilst all other light conditions resulted in variable growth responses. Such variation in growth between strains, regardless of temperature and light exposure, indicates diagnosis of saprolegniasis via the characteristic white mycelial patches (Earle & Hintz, 2014) is not time reliable, assuming this *in vitro* growth is reflective of *in vivo* growth.

For both wild sourced and laboratory strains, 25°C was the optimal temperature for growth with strain specific variance (for instance the Esk and Dart strains exhibited higher growth rates at 10°C vs 15°C, while all other strains showed the opposite). Our findings marry with the literature showing increased growth of *S. parasitica* from 5 to 25°C (Koeypudsa et al., 2005; Liu et al., 2017; Tandel et al., 2021) but differ for 30^{o}C where we found a decrease in growth for all strains, compared to the increase seen for some other strains (Liu et al., 2017). Within a strain, the growth rates reported appeared repeatable; at least for the experiment performed twice here at 15°C. The associations observed here between vegetative *Saprolegnia* growth and temperature supports work on the relationship between fish health and temperature, including the well-established effects of temperature on fish immunity (Bly & Clem 1991; Morvan et al., 1998) and *Saprolegnia* sporulation (Bly et al., 1992; Kitancharoen et al., 1996).

With regard to photoperiod exposure, the only commonality observed between the two laboratory strains of *S. parasitica* tested (CBS223.65 and A01) here was a suppression of growth under constant light (24 L: 0 D). In contrast, previous findings found growth of one *S. parasitica* strain was inhibited by constant artificial light whilst others grew best under constant artificial light (Lee, 1962; Lee, 1965). Under constant dark conditions, the vegetative growth of strain CBS223.65 was unaffected, whilst the growth of strain A01 was suppressed. Similar variation was found with *S. diclina* which showed both suppressed and heightened (compared to controls) growth under total darkness (Szaniszlo, 1965). Preference for shorter light exposures could be an adaptation for northern hemisphere waters where photoperiods can be relatively short and high turbidity waters reduce

light penetration and sporulation may have different time requirements. For the terrestrial oomycete *Phytophthora infestans*, sporulation only occurs in the dark, possibly related to a lack of UV protection in the sporangia (Mizubuti et al., 2000; Xiang & Judelson, 2014). The response to 18 L: 6 D was significantly different between strains, with CBS223.65 showing suppressed growth compared to A01 which grew optimally. These differences mirror that of early work where some strains thrived and others suffered in excess light (Lee, 1962; Lee 1965). Here, they may be due to the differences in culture duration and or genetic variation. For fungi, light may both be a stimulus and inhibitor of sporulation (Hawker, 1957), however oomycetes sporulate in darkness, and as such, the artificial lighting used in aquaculture may inadvertently inhibit oomycete sporulation, growth and/or propagation, as seen here with both strains showing suppressed growth under constant light conditions (Cohen et al., 1975; Judelson et al., 2009; Cohen et al., 2013). Light-induced oomycete suppression has also been observed in terrestrial species, such as *Peronospora belbahrii*, a parasite of sweet basil, where red light inhibited sporulation and reduced epidemics, leading to decreased fungicide application (Cohen et al., 2013). Although constant light has the potential to suppress saprolegniasis, the negative impact on fish hosts (increased stress, reduced immunity (Owen et al., 2010; Vitt et al., 2017)) would outweigh the benefits.

While there are commonalities in *Saprolegnia* outbreaks, including stress, water quality, and season, predicting and preventing outbreaks remains a challenge. Laboratory studies typically focus on a single environmental factor, however organisms *in situ* experience multiple environmental stimuli in concert, becoming multi-stressors if they deviate from optimal ranges (DeLorenzo et al., 2021). Here, when investigating temperature or light impacts on growth we maintained either a consistent light source (500 lux) or consistent temperature (19±1°C), respectively. Whilst beyond the scope of the current project, next steps could involve combining both of these stimuli concurrently and monitoring growth (started by Szaniszlo, 1965) but also marrying *in vitro* data with on-host infections, as we currently lack evidence to know whether the two match. Species and strain responses to environmental stimuli vary, and as such so will the optima for growth under temperature and light (Lee, 1962; Liu et al., 2017; Tandel et al., 2021). Interestingly, it has been suggested that environmental stimuli may fall into a "ranking" of sorts in regards to importance of impacts (Bowden, 2008), for example where it has been found that low temperatures limited photoperiod impacts on Atlantic salmon (McCormick et al., 2000). For parasites, we must consider the indirect impact of abiotic stimuli through host responses. Host circadian rhythms can in turn entrain parasite circadian rhythms, and host stress/immune responses will change across light

regime/temperature, influencing infection outcome (Ellison et al., 2021; Hunter et al., 2022; Arapi et al., in Press).

Our results further strengthen the connection between *Saprolegnia* and temperature, with predictable growth of *Saprolegnia* at specific temperatures. Temperature remains a key predictor of disease outbreaks. The connection between *Saprolegnia* and light is much less clear but does serve to highlight strain and species levels of variation.

Chapter 5 – *Gyrodactylus* in the spotlight: how exposure to light impacts disease and feeding behaviour of the freshwater tropical guppy (*Poecilia reticulata*)

Abstract

Artificial light at night (ALAN) negatively impacts organisms in many ways, from their feeding behaviours to their response and ability to deal with disease. Our knowledge of ALAN is focused on hosts, but we must also consider their parasites, which constitute half of all described animal species. Here, we assessed the impact of light exposure on a model host-parasite system (fish host guppy *Poecilia reticulata* and the ectoparasitic monogenean *Gyrodactylus turnbulli*). First, parasite-free fish were exposed to 12 h: 12 h light: dark (control) or 24 h: 0 h (ALAN) for 21 days followed by experimental infection. Second, naturally acquired *G. turnbulli* infections were monitored for 28 days during exposure of their hosts to a specified light regime (either 6 h: 18 h, 12 h: 12 h or 24 h: 0 h L:D). Experimentally infected fish exposed to constant light had, on average, a greater maximum parasite burden than controls. Host feeding behaviour was also significantly affected, fish under ALAN fed faster and took more bites than controls, whilst fish exposed to reduced light fed slower. Thus, ALAN can impact parasite burdens, even in the short term, and altering light conditions will impact fish feeding behaviour, but not mortality. Such responses could initiate disease outbreaks or perturb food-webs with wider ecological impacts.

Introduction

Urbanisation has led to prolonged exposure of organisms to artificial light, particularly artificial light at night (ALAN). This has the potential to disrupt or alter various biological functions, including flight patterns, mating and orientation, maturation, all of which are dictated by light cues (Sweeney et al., 2003; Muheim et al., 2006; Dechaine et al., 2009). Despite being a known issue, the drivers behind changes in ALAN often revolve around socio-economic issues, such as energy costs and greenhouse gases, rather than addressing the necessity to reduce environmental stressors on ecosystems (Stone et al., 2012; Holker et al., 2021; Hooker et al., 2022). Light intensity, quality, colour and duration all impact organisms and their interactions with the environment (Boeuf & Le Bail, 1999). This complexity is amplified in variable aquatic environments, where organisms constantly move within 3-dimensions and light is reflected, refracted, and attenuated as depth and turbidity increase (Sumpter, 1992; Evans, 2004; Stoner, 2004). Photoreception by fish occurs both via the eyes and pineal gland, and is species and life stage dependent (Tabata, 1992). Most species require minimal light exposure per day to develop and grow, linked to activities like movement, hunting and visual

foraging (e.g. Richardson & McCleave, 1974; Iigo & Tabata, 1997). In aquaculture, light exposure is intentionally increased to boost production (Wang et al., 2023). Overexposure to light, however, can increase stress in fish (Tian et al., 2015; Ellison et al., 2021) and even result in mortalities (Schligler et al., 2021). ALAN has been shown to reduce sex steroids and mRNA expression of gonadotropins in European roach (*Rutilus rutilus* L.) and perch (*Perca fluviatilis* L.) (see Bruning et al. 2018). Behaviourally, ALAN can increase fish predation on invertebrate prey and alter emergence of fish (Kurvers et al., 2018; Czarnecka et al., 2019).

All fish are infected by a range of naturally occurring parasites, which often only become detrimental to the host following environmental perturbation (Wood & Johnson, 2015). We know ALAN can influence interactions between species, such as foraging and predation, less is known about the impacts of ALAN on parasites (Rydell, 1992; Dwyer et al., 2013; Rodriguez et al., 2017). While exposure to increased photoperiods can indirectly exacerbate the problem of infectious diseases (e.g. via reduced immunity in fish; Tian et al., 2015; Ellison et al., 2021; Bakke et al., 2021, or increased parasite reproduction; Gannicott & Tinsley, 1997), parasites are also directly impacted by light and exhibit daily rhythmicity in gene expression (de Bekker et al., 2017; Rijo-Ferreira et al., 2017; Smith et al., 2020; Hunt et al., 2022). For macroparasites, such as *Argulus* spp. (aquatic lice), we know that they display diurnal behavioural patterns off-host, with males showing increased activity at the change of light, while females exhibit a delayed pattern (Hunt et al., 2021). For the microparasite *Gyrodactylus turnbulli* (a monogenean), this ectoparasite is more active in the dark and it even induces restlessness in its host, the guppy *Poecilia reticulata* (Peters 1859) (hereafter 'guppy'), particularly at night (Arapi et al., in press). It is with this non-invasive freshwater model that we continue to investigate how light can impact the relationship between parasites and their hosts.

Here, we posed the following two questions: do altered light regimes impact infection dynamics between the guppy and *G. turnbulli*? Is the feeding behaviour of the host (both infected and uninfected) impacted by different light regime exposure? We hypothesised that increased light exposure would result in an increase in parasite burdens, and that increased light would influence the feeding behaviour of hosts. These questions were investigated under laboratory conditions: firstly, experimental infection with *G. turnbulli* was assessed under control light (12 h: 12 h light:dark) and total light regime and secondly, naturally acquired infections of *G. turnbulli,* along with host feeding behaviour, were assessed under three different light regimes (6: 18, 12: 12 and

24 h: 0 h light:dark). In both instances, exposure to light alterations was relatively short-term (3 to 5.5 weeks).

Methods

Host-Parasite System

We utilised the established guppy-*Gyrodactylus* system for this study. The guppy (*Poecilia reticulata*) is a well-studied model fish host and the genus *Gyrodactylus* is a species-rich group of fish pathogens that are both ecologically and economically important and globally pervasive (Bakke et al., 2007). *G. turnbulli* is the primary monogenean ectoparasite of guppies and a major pest in the ornamental fish trade, known as a "Russian-Doll parasite" due to its hyperviviparous reproductive capabilities (Bakke et al., 2007). One key benefit of this parasite-host system as an experimental system is the ability to non-invasively track individual host parasite burdens over time.

Size-matched (254-294 mm) mixed ornamental adult female guppies were imported to Cardiff University (n=300) and acclimated in shoals of 10-15 fish for 24 hours, maintained within 40 L aquaria at 24±0.5^{o}C on a 12 h:12 h light:dark photoperiod (500 lux, lights abruptly on 7am and off at 7pm). This stock was used for both experiments. All fish were screened to assess ectoparasite communities. All fish originated from commercial pet stocks and were confirmed to host *G. turnbulli* through both screening and sequencing identification (position of worms was an indicator of species, then samples were sequenced via PCR for confirmation following the protocol outlined in Schelkle et al., 2012). Briefly this involved extraction of DNA using Qiagen dNeasy blood & tissue kit (Qiagen) following manufacturer's instructions, using primers from Harris et al. (1999) which amplified the 5.8s gene and partial ITS-1 and ITS-2 regions (R1 = ACTCCATG- TGGTGGATC and F3 = TTGCTGCACTCTTCATC). PCR of extracted DNA was performed as described by Faria et al. (2011), and extracted DNA was sent to Eurofins Genomics for sequencing (10 µl nuclease-free water, 5 µl PCR product and 2 µl of each primer at 10 µM concentration). The resulting contigs for each sample were then subject to an NCBI BLAST search for related sequences, with confirmation of species taken upon a match of 100% sequence identity to *Gyrodactylus turnbulli*.

Prior to the investigation, n=210 fish were treated with Levamisole (Norbrook ®, UK) according to Schelkle et al. (2009) and n=90 retained with their parasite load for the infected treatments in Experiment 2. The treated fish were then screened and confirmed free of ectoparasites if no parasites were detected through microscopic examination on three consecutive times (see Schelkle et al., 2009). This involved mildly anaesthetising individual fish using 0.02% tricaine

methanosulphate (MS-222) and observing the surface of each fish for visible signs of parasitaemia (e.g., raised fins, white spots, abnormal growths), where any infected fish were excluded. The remaining fish (n=90) were monitored daily for signs of abnormalities or mortalities. All fish, prior to experimental infections, were measured for standard length by mildly anesthetising individuals as above. All fish were then individually isolated into 1 L aquaria (for the duration of both experiments) and maintained under the same environmental conditions as before (24±1^{o}C on a 12 h:12 h light:dark photoperiod) for 3 days until the beginning of the experiment. Fish were fed twice daily on flake food (Aquarian®) one day and freshly hatched *Artemia* nauplii on alternate days. Feeding regimes were maintained throughout, and any/all mortalities of either infected or uninfected fish were recorded. As the infection trajectory for individual fish was followed, pseudo-replication was accounted for during analysis (see Statistical Analysis section below).

Experiment 1 – Impact of Artificial Light at Night (ALAN) on experimental G. turnbulli *infections*

Female ornamental guppies (n = 120, all confirmed parasite free) were separated into two groups (n = 60) and exposed to either 12 h: 12 h light: dark (control light of 500 lux) or 24 h light (artificial light at night - ALAN) for 21 days. Then, half of the fish (n=30) from each regime were infected with *G. turnbulli* according to the method described by Schelkle et al. (2009). Briefly, this involved anaesthetising recipient fish lightly with 0.02% MS-222 and bringing this recipient gently towards a deceased donor until two individual *G. turnbulli* attached onto the recipient's caudal fin. Every two sequential days following initial exposure, all infected fish had their parasite numbers assessed through mildly anesthetising infected fish (using 0.02% MS-222) and counting the number of worms present under a dissecting microscope with fibre optic illumination until day 19 when most fish had cleared their infection or host mortality had occurred. The uninfected fish were handled in exactly the same manner as the infected fish throughout the experiment, starting with sham infections (anesthetised and handled but without the introduction of parasites).

Experiment 2a – Impact of differing light regimes on established G. turnbulli *infections*

Fish (n=60 per regime, n=180 total) were exposed to one of three light regimes: 6 h: 18 h (short day), 12 h : 12 h (control, 500 lux) or 24 h: 0 h (long day/artificial light at night). Within each regime, n=30 fish were host to variable naturally acquired *G. turnbulli* infections (all were pre-quantified, range 8-153 worms per host) and n=30 were confirmed ectoparasite clear to act as uninfected controls to account for exposure mortality. Every two sequential days following initial light

exposure, all infected fish had their parasite numbers assessed as in Experiment 1. The last parasite screen occurred on day 28, when almost all fish had cleared their infections or died.

Experiment 2b – Impact of differing light regimes on host feeding behaviour

To understand the effect of light exposure on the feeding behaviour of fish, feed trials were conducted once a week during the experiment for both infected and uninfected fish. Fish were fed as in Experiment 1. Feeding trials involved introducing 1 mL of *A. salina* nauplii into the individual fish aquaria, illuminated under artificial light (500 lux, identical to pre-exposure lighting) equally to minimise shadowing, commencing 3 hours after lights on. Upon immediate introduction, feeding latency (time from the introduction of food until the first bite) was noted, and after which feeding rate (number of feed lunges occurring within 30 s after the first bite) was recorded. Unsuccessful lunges towards individuals of *A. salina* were still recorded as bites, and occurrences where the *A. salina* were eaten, expelled, and re-ingested were counted as separate bites. Following the feeding trials, all fish were returned to their experimental light conditions.

Statistical Analysis

All statistical analyses were carried out using Rstudio version 4.2.3. (http://www.R-project.org/) and the following packages: '*MASS*' for general and generalised linear models and data transformation, '*ggplot2*' for visualisation of data, '*lme4*' for general and generalised linear mixed models and '*effects*' for graphical overviews of data.

For analysis of infection data (for both Experiment 1 and 2) the following variables were measured in regard to parasite metrics: Area Under Curve (AUC), duration of infection, host death day, maximum parasite burden, peak day, death day, mean parasite intensity. To calculate AUC, a common parasite metric which quantifies overall pathogen burdens across a known trajectory, we utilised the trapezoid rule (White, 2011). Duration of infection was classified as the time until a fish either cleared its parasite burden or died (host death day). The maximum parasite burden was defined as the highest number of *G. turnbulli* a single host achieved on a single day during the duration of the experiment, the day of which was defined as the peak day. Mean parasite intensity was taken as the average number of *G. turnbulli* worms remaining within the system across the duration of the experiment. Standard length was initially included in all statistical models, but as it did not explain significant variation it was removed from subsequent models, as part of model refinement (Thomas et al., 2013). For analysis of AUC sum, we transformed the data using the Box-

Cox transformation method within the *MASS* package in R, as no combination of family or link functions could satisfy the assumptions of the GLM prior to transformation. Following transformation, a General Linear Model (GLM) using the gaussian family and identity link functions was run to investigate how AUC varied with regime. To analyse how maximum parasite count varied between light regimes, we used a Generalised Linear Model (GisedLM) with a negative binomial family and identity link function. For the analysis of variation between peak day and light regime, a Box-Cox transformation was again used, and a GisedLM using the inverse gaussian family and identity link function run. Infection duration variation between regimes was analysed with a GLM using a gaussian family and identity link function. For the analysis of mean parasite intensity, we must account for pseudo replication in regard to individual fish, a Generalised Linear Mixed Model (GisedLMM) in the '*lme4*' package was used with a negative binomial family and identity link function. Within this model, parasite number was our response variable, regime and the interaction between regime and time our dependent variables and individual fish ID was our random factor.

For the analysis of the host feeding ecology data (Experiment 2), latency to feed was analysed against regime and the interaction between week and regime using a GisedLMM. For this, latency was our response variable, with light regime, week and the interaction between the two as our dependant variables and fish ID as our random variable, with the gaussian family and sqrt link function. For analysis of number of bites over time, we used a GisedLMM with a poisson family and a log link function link. The analysis of both maximum bite count and mean bite count per fish were carried out using a GisedLM with the negative binomial family and identity link function.

Results

In both experiments for all light regimes, parasite burden on the individually isolated hosts varied significantly with time (GisedLMM: df = 3, ChiSq =1618.49, $p < 0.0001$). Time was also a significant factor for all treatments with regard to feeding latency (GLMM: SE = 0.02, z = -34.75, $p < 0.0001$).

Experiment 1 – Impact of Artificial Light at Night (ALAN) on experimental G. turnbulli *infections*

Fish exposed to both ALAN (24 h : 0 h light:dark) and controls (12 h: 12 h light:dark) exhibited similar parasite burden trends, increasing until a peak and then decreasing (Figure 5.1a), but the maximum parasite count varied significantly, with fish exposed to 24 h light (ALAN) having a greater maximum parasite burden than those under 12 h of light (GisedLM: SE = 16.87, t = 2.20, $p = 0.02$). For mean parasite intensity across the experiment, fish exposed to 24 h light did not significantly vary

compared to controls (GisedLMM: SE = 0.93, z = 0.018, *p* = 0.98). Similarly, the AUC.sum, duration of infection, peak day nor death day did not significantly vary between treatments (see Table 5.1 for statistical outputs).

Table 5.1 Statistical outputs for parasite parameters (AUC, maximum parasite count, peak day, infection duration, parasite abundance, death day and parasite count) for Experiment 1, using control as a baseline. Included are the standard errors, the t or z value (test dependent) and p value for all parameters tested (* denotes significance).

Treatment (Hours of Light)	Standard Error	t/z Value	*p* Value
AUC.Sum – GLM (family = gaussian, link = identity)			
24	0.0065	0.17	0.86
Max Parasite Count – GisedLM (family = negative binomial, link = identity)			
24	16.87	2.20	0.02*
Peak Day – GLM (family = gaussian, link = identity)			
24	1.35	1.36	0.18
Infection Duration – GisedLM (family = gaussian, link = log)			
24	0.04	-0.59	0.5
Abundance – GisedLM (family = negative binomial, link = identity)			
24	3.90	1.30	0.19
Death Day – GLM (family = gaussian, link = identity)			
24	4.86	1.61	0.15
Parasite Count – GisedLMM (family = negative binomial, link = identity)			
24	0.93	0.01	0.98

Experiment 2 – Impact of differing light regimes on established G. turnbulli *infections*

At the start experiment, the mean number of gyrodactylids per treatment group was equal (n = 65) with individual host burdens ranging 8-153 worms (Figure 5.1b, day 0). As expected from naturally acquired infections, when the hosts were isolated the parasite burden generally declined with time (there were exceptions where certain individuals were particularly susceptible (n = 15 total, n = 5 per treatment) and their burden increased until mortality occurred). When compared to the control treatment (12 h of light), none of the measured parasite metrics (AUC, maximum parasite count, average parasite burden, peak day and duration of infection) varied significantly between treatments (Table 5.2). When looking at parasite count over time (accounting for pseudoreplication), there was no significant difference between treatments (GLMM: 6 h; SE = 0.25, z = 1.08, *p* = 0.28, 24 h; SE = 0.25, z = 1.63, *p* = 0.10).

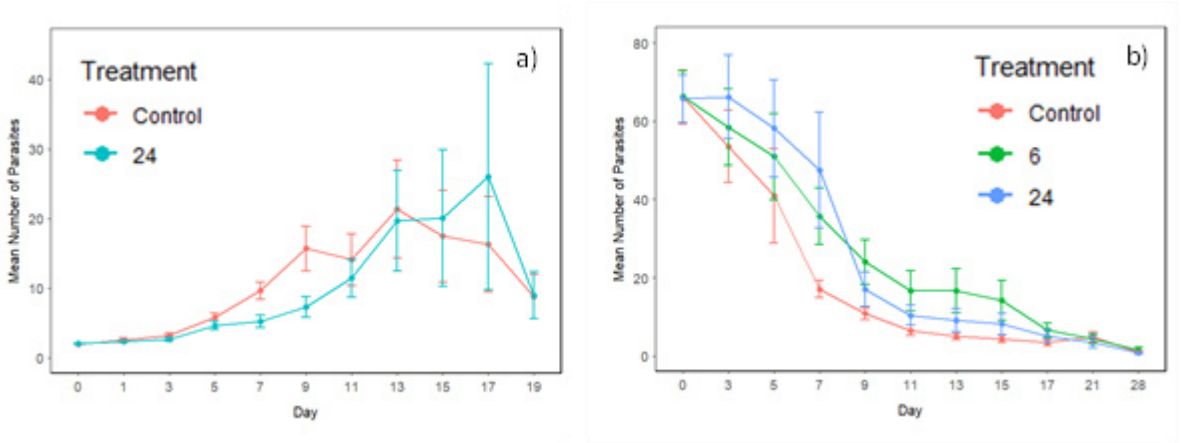

Figure 5.1 Mean parasite intensities of *Gyrodactylus turnbulli* per light regime (distinguished by colour) per day (including standard error) on their host *Poecilia reticulata* a) Experiment 1, where fish were exposed to either control (12 L: 12 D) or ALAN (24 L: 0 D) light regimes over 19 days. All naïve fish in the two treatments were experimentally infected with two individual parasites on Day 0 after 21 days pre-exposure to the different light conditions. B) Experiment 2, where fish were exposed to either control (12 L: 12 D), low light (6 L: 18 D) or ALAN (24 L: 0 D) light regimes over 28 days starting with naturally acquired infections with a mean starting infection level in the 3 different treatments of 65 worms per treatment. In this experiment, fish were all exposed to the same control light conditions until Day 0.

Table 5.2 Statistical outputs for parasite parameters (AUC, maximum parasite count, peak day, infection duration, average worm count and parasite count) for Experiment 2, using control as a baseline. Included are the standard errors, the t or z value (test dependent) and p value for all parameters tested (* denotes significance).

Treatment	Standard Error	t/z Value	*p* Value
AUC.Sum – GLM (family = gaussian, link = identity)			
6	0.07098	1.087	0.280
24	0.07098	1.424	0.158
Max Parasite Count – GisedLM (family = negative binomial, link = identity)			
6	13.954	0.148	0.882
24	14.865	0.868	0.385
Peak Day – GisedLM (family = inverse gaussian, link = identity)			
6	0.7944	1.762	0.0815
24	0.6635	0.955	0.3424
Infection Duration – GLM (family = gaussian, link = identity)			
6	0.14433	0.418	0.677
24	0.14433	0.742	0.460
Average Worm Count – GisedLM (family = negative binomial, link = identity)			
6	5.806	0.810	0.418
24	6.031	1.117	0.264
Parasite Count – GisedLMM (family = negative binomial, link = identity)			
Treatment	Df 2	ChiSq – 2.75	0.25
Treatment:Day	Df 3	ChiSq – 1618.49	<0.0001*
6	0.25	1.08	0.28
24	0.25	1.63	0.10

Experiment 2 – Fish Feeding Ecology

Latency to feed was impacted by altered light exposure (Table 5.3), with fish exposed to 24 h light responding significantly faster (shorter latency) than the controls (GLMM: SE = 0.14, z = -10.79, *p* <0.0001), whilst those exposed to 6 h light fed significantly slower (longer latency) compared to controls (12 h) (GLMM: SE = 0.14, z = 2.98, *p* = 0.002) (Figure 5.2a). Fish exposed to 24 h light took significantly more bites than controls (GLMM: SE = 0.07, z = 8.30, *p* <0.0001) (Figure 5.2b). Fish exposed to 24 h decreased their bite count over time (GLMM: SE = 0.017, z = -13.36, *p* <0.0001), whereas the bite count of fish exposed to 6 h light remained constant (GLMM: SE = 0.019, z = -0.39, *p* = 0.70) (Figure 5.2). Neither the maximum bite count nor the mean bite count per fish significantly varied between treatments (GisedLM Maximum Bite Count: 6; SE = 2.96, t = -1.58, *p* = 0.12, 24; SE = 3.13, t = 1.18, *p* = 0.24, GisedLM Mean Bite Count: 6; SE = 2.44, t = -1.26, *p* = 0.21, 24; SE = 2.57, t = 0.90, *p* = 0.37). Infection with *G. turnbulli* did not impact feeding behaviours (GisedLMM Latency to feed: SE = 0.11, z = -0.76, *p* =0.44, GisedLMM Number of Bites: SE = 0.05, z= 0.73, *p* = 0.48).

Table 5.3 Statistical outputs for feeding behaviours (latency to feed, number of bites in 30 s, maximum bite count and mean bite count) for Experiment 2, using control as a baseline. Included are the standard errors, the t or z value (test dependent) and p value for all parameters tested (* denotes significance).

Treatment	Standard Error	t/z Value	*p* Value
Latency to Feed – GisedLMM (family = gaussian, link = log)			
6	0.14	2.98	0.002*
24	0.14	-10.79	<0.0001*
6:Week	0.03	-4.09	<0.0001*
24:Week	0.02	28.07	<0.0001*
Infection	0.11	-0.76	0.44
Week	0.02	-34.75	<0.0001*
Number of Bites over time – GisedLMM (family = poisson, link = log)			
6	0.08	-1.18	0.23
24	0.07	8.30	<0.0001*
6:Week	0.019	-0.39	0.70
24:Week	0.017	-13.68	<0.0001*
Infection	0.05	0.73	0.48
Week	0.01	14.52	<0.0001*
Maximum Bite Count – GisedLM (family = neg binomial, link = identity)			
6	2.96	-1.58	0.12
24	3.13	1.18	0.24
Mean Bite Count – GisedLM (family = neg binomial, link = identity)			
6	2.44	-1.26	0.21
24	2.57	0.90	0.37

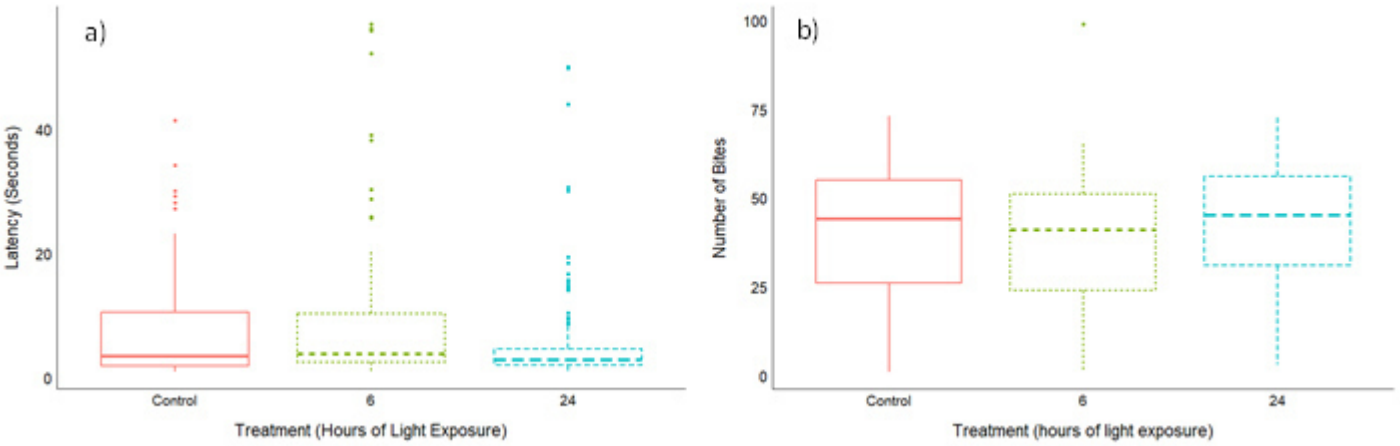

Figure 5.2 Feeding behaviour of *Poecilia reticulata* in Experiment 2. a)) Latency (in seconds) to first bite per treatment across the duration of the experiment. b) Bite count (recorded as number of bites taken within 30 seconds) per treatment across the duration of the experiment. Box plots show the median (line), interquartile range (box) and the 1.5x interquartile range (whiskers). Any filled circles represent values outside the 1.5x interquartile range.

Discussion

Here we sought to assess the relationship between fish exposure to artificial light at night (ALAN, 24 h:0 h light dark) and infection with the ectoparasitic monogenean *Gyrodactylus turnbulli*. Exposure to ALAN following experimental infection resulted in increased maximum parasite burdens. Fish feeding behaviour was also influenced, with fish exposed to ALAN showing a shorter latency to feed and increased bite count, whilst those exposed to reduce light (6 h:18 h light:dark) had a longer latency to feed.

Chronic light exposure has been shown previously to significantly impact gyrodactylid dynamics in controlled laboratory settings. Subjecting laboratory reared 3-spined sticklebacks (*Gasterosteus aculeatus* L.) to altered light conditions (specifically, 6 months under a 16 h: 8 h light:dark cycle) resulted in increased susceptibility to *Gyrodactylus gasterostei* (see Whiting et al., 2020). More acutely, we found experimentally infected guppies exposed to ALAN for 21 days prior to infection experienced a significantly higher maximum parasite burden. All other parasite metrics analysed here showed no significant changes in response to altered photoperiod. Manipulating photoperiod though, even in the short term, can influence fish immunity and induce stress (Bowden, 2008; Ellison et al., 2021), factors that likely contribute to the observed increase in parasite burden. Direct comparisons with other host-parasite systems, however, are problematic, especially when comparing temperate (*G. gasterostei*) and tropical (*G. turnbulli*) species. The temperature-

dependent nature of parasite reproduction (and host immunity) affects the timing of their response to any environmental perturbation. In our Experiment 2, we observed a steady decrease in parasite numbers over time, reflecting the parasites' inability to sustain infrapopulations on immunologically active fish. This decline was not affected by increased light presumably due to the overriding impact of host immunity. Such parasite trajectories are representative of infections that have past their peak, in contrast to newly established experimental laboratory infections (e.g., King & Cable, 2007, Smallbone et al., 2016, Stewart et al., 2017, Masud et al., 2020). We also know that the activity of gyrodactylids increases in the dark, as demonstrated by *G. gastorostei* off the host (Brooker et al., 2011) and by *G. turnbulli* on the host (Arapi et al., in press). In the latter case, this heightened activity results in restlessness of their diurnal hosts (Arapi et al., in press); another factor which might have been at play in the current experiments.

Light is vital to fish that are visual hunters (López-Olmeda & Sánchez-Vázquez, 2010). Diurnal feeders rely on light to illuminate prey, which are then more vulnerable to predation. The impact of ALAN could worsen this, and heightened light can also lead to increased invertebrate emergence, potentially disturbing food webs (Kurvers et al., 2018; Czarnecka et al., 2019). We found both fish latency to feed and bite count were impacted by altering light regimes despite all our feeding behaviour recordings being conducted under well-lit conditions, consistent between regimes. As there was also no enrichment in the tanks to obstruct the prey, vision (Diehl, 1988; Aksnes & Giske, 1993; Brooker et al., 2011) nor olfaction of the fish is unlikely to have been impacted (Stoner, 2004). Our ALAN-exposed fish showed the shortest feeding latency, consistent with prior research on light impacting fish activity (Scherer & Harrison, 1988; Marchand et al., 2003; Trippel & Neil, 2003; Stoner, 2004). Increased light exposure also correlates with heightened fish activity and feeding (Czarnecka et al., 2019) as more energy is required to maintain this level of activity (Adegboye et al., 2017; Adegboye et al., 2020). Increased activity may reduce overall fitness and increase susceptibility to parasites, if the increased nutrient intake does not sufficiently compensate for the expended energy. The observed latency in fish exposed to 6 hours of light and 18 hours of darkness could be due to lethargy, resulting in reduced activity (also shown in Jones et al., 2017; Jones & Hale, 2020). During the current study, we found no impact of short-term light exposure on host mortality. In contrast, long term exposure (>18 months) to ALAN (4.3 lux) decreased average survival in anemonefish (*Amphirion chrysopterus* Cuvier 1816) by 36%, and decreased their growth (Schligler et al., 2021).

In aquaculture, light is manipulated for economic benefit (Frenzl et al., 2014; Hou et al., 2019), however, hobbyists and pet owners may be encouraged to illuminate their aquarium tanks 24 hours a day. Informed adjustment of light regimes could potentially reduce parasite infection by optimising photoperiods to enhance fish growth while minimising or negatively impacting parasite infections. The potential for light as a disease management tool (chronotherapy) would heavily depend on the host-parasite system. In terrestrial environments, increased red light exposure for plants increased their salicylic acid levels, a defence mechanism against infection (Gallé et al., 2021). Oomycete pathogens sporulate in darkness, and as such, the artificial lighting used in aquaculture may inadvertently inhibit their sporulation, growth and/or propagation (Cohen et al., 1975; Xiang & Judelson, 2014; see Chapter 2). Chronotherapy could also enhance treatment efficacy, for instance, by guiding the timing of treatments for *Argulus foliaceous* based on when they are most vulnerable. This approach is supported by the discovery that the genes targeted by treatments display a daily rhythmicity (Hunt et al., 2022). Manipulation of photoperiod offers a non-invasive mitigation strategy for aquaculture to improve pathogen mitigation and provides a counterargument to the drawbacks of extended photoperiods on fish health; unfortunately, this is likely very host-pathogen specific.

Our work adds to the data on ALAN impacting fish, in particular increased host feeding activity prior to and during infection with *G. turnbulli*. Here, we observed mild effects of acute ALAN exposure on parasite burdens of a freshwater fish host, which highlights the implications for chronic exposure, especially with regard to multiple stressors.

Chapter 6 – Killer threads: the impacts of synthetic and cellulose based fibres and their associated dyes on fish hosts and parasite health

This chapter is affiliated with the publication MacAulay et al., *2023 in the journal Environmental Science and Pollution Research.*

Abstract

Plastic pollution is now a ubiquitous feature of freshwater systems and the majority of this is fibrous. Here, we test the effects of plastic and cellulose-based fibres (polyester, cotton, and bamboo from commercial clothing) on fish host-parasite interactions using a freshwater fish host-parasite model system (guppy *Poecilia reticulata*-*Gyrodactylus turnbulli*). For uninfected fish, polyester exposure was associated with significantly higher mortality rates compared with the other two fibre types. For infected fish, while polyester and cotton exposure were not associated with any significant changes to parasite burdens, fish exposed to bamboo fibres had significantly reduced maximum parasite burdens compared with fish not exposed to any fibres, indicating that the bamboo fibres and/or associated dyes conferred some degree of resistance or tolerance. While unable to determine the exact nature of the chemical dyes, when testing off-host parasite survival on exposure to the fibre dyes, cotton and particularly polyester dyes were associated with higher parasite mortality compared to bamboo. Overall, we add to the growing body of evidence which shows that polyester microplastic fibres and their associated dyes can be detrimental for both fish and parasite survival, and we highlight the need for increased transparency from textile industries on the chemical identity of fabric dyes.

Introduction

Textile industries are key players contributing to the ever-increasing burden of particulate pollution, especially microplastics, an ecological issue that has recently taken centre stage. Over 80% of environmental microplastics (plastics <5 mm) are fibrous (freshwater: Horton et al., 2017; marine: Vince & Stoett, 2018; terrestrial: Rillig & Lehmann, 2020) and 35% of oceanic microplastic pollution is attributed to the fashion industry that produce garments from non-degradable synthetic polymers (United Nations Climate Change, 2018). Fast fashion produces cheap, low-quality clothing per year (Young, 2021) providing affordable fashion to those on a budget, but such garments are only worn on average ten times before being discarded and deposited in landfills (Barnardos, 2015; TRAID, 2018). Fibres from discarded garments typically persist in the environment, many ending up in water

bodies through run-off, and are incorporated throughout the food web (De Falco et al., 2019). The sheer scale of fibre waste generation is concerning, with a single household wash cycle releasing 100-300 mg of fibres per kg of fabric (De Falco et al., 2019). Scaling this up leads to exorbitant quantities; estimates in Finland, for example, reveal annual household production of 154,000 kg for synthetic polyester fibres and 411,000 kg for cotton fibres (Sillanpaa & Sainio, 2017). Whereas natural polymers are degraded by microbes (Pekhtasheva et al., 2012; Arshad et al., 2014), synthetic fibres are resistant to such breakdown (Resnick, 2019) so greater use of natural polymers is one mitigation strategy in the global initiative to reduce non-degradable plastic pollution. It is essential, however, to assess whether these products marketed as 'ecologically friendly' are less harmful for organisms when released into multiple habitats. Indeed, one of the key issues raised in response to the EU Directive 2019/904 is giving a green light to non-plastic polymers, such as bamboo, hemp, or fruit fibres, before their biological impact is assessed (Eunomia Research & Consulting, 2020).

To date, biological assessments of fibre exposure and/or consumption are mostly limited to invertebrates and reveal no clear trend and typically focus on plastic fibres, ignoring cellulose-based fibres such as cotton, rayon, and bamboo (Suran, 2018). Consumption of polyester fibres by earthworms (*Lumbricus terrestris*) revealed no effect on mortality or avoidance behaviour (Prendergast-Miller et al., 2019), whereas land snails (*Achatina fulica*) exposed to polyethylene terephthalate (PET) fibres showed reduced feeding ability and increased oxidative stress (Song et al., 2019). For vertebrates, fibre pollution has been detected in the guts of many animal groups including fish (Lusher et al., 2017), birds (Lourenço et al., 2017) and mammals (Lusher et al., 2015), but only two studies to the best of our knowledge have assessed the direct biological effects of fibres on fish health. Adult Japanese medaka (*Oryzias latipes*) exposed to polyester and polypropylene fibres for 21 days were not impacted in terms of reproductive changes and mortality, but did show aneurysm in lamellae, opercular swelling and abnormal mucosal cell proliferation (Hu et al., 2020). Juvenile chinook salmon (*Oncorhynchus tshawytscha*) that were exposed shorter term (10-days) to polyester fibres showed no changes in gut mass (Spanjer et al., 2010). Fibre pollution could also impact fish health by reducing resistance to parasitic infections, however this has yet to be tested. Indeed, it is well known that pollutants can directly impact fish resistance to parasitic disease by influencing underlying immunity (reviewed in Tort, 2011). Microplastics, which are a key component of fibre pollution within aquatic environments, can impact fish immunity by impacting the regulation of gene expression and immune cells (Limonta et al., 2019; Zwollo et al., 2021), and

have been shown to reduce resistance to parasitic infections in freshwater sticklebacks (*Gasterosteus aculeatus*; see Masud et al., 2022).

Here, we investigate the impact of plastic and cellulose-based fibres (polyester, cotton, and bamboo) on fish host-parasite interactions. Synthetic polyester and cotton were chosen as they are two of the most used fibre types for clothing material (Carr, 2017). The durability of synthetic polyesters makes them resistance to natural degradation (Carr, 2017), whereas cotton is a natural fibre crop, but with a large water footprint (Chapagain et al., 2006). Bamboo, on the other hand, requires substantially less water than cotton and is also a natural polymer. Therefore, a key question here is whether cellulose based fibres are 'better' in relation to their potential impact on fish welfare compared with polyester fibres. Furthermore, we assessed whether dyes associated with these fibres had any impacts on the independent (i.e., off host) survival of parasites. We hypothesised that polyester fibres would be associated with increased disease susceptibility, whereas the cellulose-based fibres (cotton and bamboo) would have more attenuated impacts on fish welfare, and dyes associated with the tested fibres, would impact off-host parasite survival. To test these hypotheses, we used an established host-parasite system, the guppy (*Poecilia reticulata*)-*Gyrodactylus turnbulli* model.

Methods

Host-Parasite System

The guppy is a tropical fish species, native to the Caribbean Islands and Venezuela, and an invasive non-native species on every continent except Antarctica (Magurran, 2005). The genus, *Gyrodactylus,* is a species rich group of fish parasites that are ecologically and economically important (Bakke et al., 2007). The primary monogenean ectoparasite *G. turnbulli* is a major pest in the ornamental fish trade (Cable, 2011). For this investigation, we used size-matched, mature, mixed ornamental male guppies (*Poecilia reticulata* from Guppy Farm UK- 6-8 months old). Upon arrival at Cardiff University, all fish (n=240) were acclimated for 24 hours in groups (~10-15 individual fish) within 40 L aquaria at 24^{o}±0.5^{o}C on a 12:12 light/dark photoperiod. All fish were then confirmed as ectoparasite free, via three daily consecutive microscopic screens (see Schelke et al., 2009). Briefly, this involved mildly anaesthetising individual fish using 0.02% MS-222 and observing the surface of each fish for visible signs of parasitaemia (e.g., raised fins, white spots, abnormal growths) and any infected fish were excluded. To rule out bacterial infection, all fish were treated

with the antibacterial treatment (Myxazin) as per manufacturer instructions (Waterlife) 2 weeks prior to the start of the experiment. We acknowledge that chemical derivatives from the Myxazin treatment may have persisted in the tissue of the experimental fish, but as our model parasite is an epidermal feeder, any tissue-based toxicity is unlikely to have impacted the parasites. Furthermore, all fish (controls and fibre treatments) were exposed to Myxazin. For experimental infections, we used the *Gt3* strain of *Gyrodactylus turnbulli*, isolated from a Nottingham aquarium pet store (King & Cable, 2007; see Figure 6.1A). This parasite has been cultured under laboratory conditions since establishment in November 1997. All fish, prior to experimental infections, were weighed on an electronic scale by mildly anesthetising individuals with 0.02% MS-222; and fish were then weighed again at day 52 (i.e., end of experiment) to determine if fibre exposure and consumption impacted wet mass.

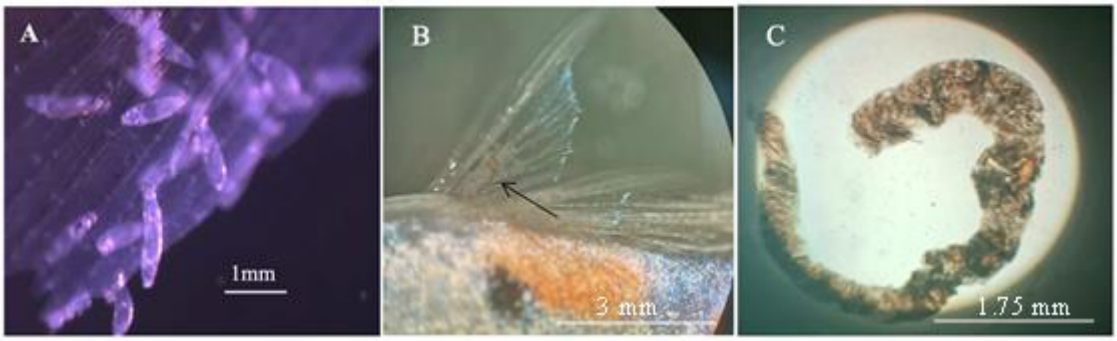

Figure 6.1 The host-parasite system. A) *Gyrodactylus turnbulli* individuals on the caudal fin of guppy (*Poecilia reticulata*) under x10 fibre optic illumination. B) Fibre (arrow) being egested by male guppy at 2x fibre optic illumination. C) Faecal pellet of a guppy with a fibre encased within, indicating the passage of fibres through the guppy gastrointestinal tract at 4x magnification.

Fibre Preparation, Exposure and Chemical Analysis

Fish were separated into four treatment groups: 1) control (n=60), 2) polyester fibre (n=60), 3) cotton fibre (n=60), and 4) bamboo fibre (n=60). For the polyester and cotton fibres, we sourced 100% polyester and 100% cotton shirts respectively. The bamboo fabric consisted of 95% Bamboo viscose and 5% elastane. Due to the structural property of bamboo (i.e., short inflexible fibres), 100% bamboo is typically not utilised for commercial bamboo clothing and elastane is added to ensure flexibility of finalised fabrics (Muthu, 2017). All materials were black (to avoid colour as a confounding variable) and obtained from commercial retailers. While we were unable to ascertain the exact nature of the dye (due to commercial sensitivity), the most commonly used black dye is

Reactive black 5 (industrial name Setazol Black) and therefore the most likely to be found in the fabrics tested in this experiment (Bilińska et al., 2016; Al-Tohamy et al., 2020).

The fabrics were first cut into 7.5 cm^2 squares, then shred into 1.5 cm^2 pieces using sterile scissors and immersed in 1 L of aquarium water (i.e., the same water used for fish tanks mentioned above) and agitated to promote fibre shedding to simulate a washing cycle. After 24 hours, the fibre water was aliquoted into 50 ml bottles and autoclaved. A drop of fibre water (50 µL) was then viewed under a compound microscope and the number of fibres counted. This was repeated 10 times per fibre treatment (fibre number ranged for bamboo 22-52; cotton 22-50; and polyester 21-65) to calculate the mean number per 1 mL, which was circa. 700 fibres/L. The size distribution of fibres used in this study was determined using a Titachi TM3030 Plus benchtop microscope in Back Scattering Electron (BSE) mode at 15 kV. To isolate the fibres from the solution, a centrifuge was used to spin 50 mL of soaked fibre solution at 4000 xg. The resulting pellets were washed with deionised water three times and resuspended in 5 mL. To provide optimal contrast for fibre identification, three drops of this highly concentrated solution from each treatment were sequentially drop cast onto a steel disc. We counted a total of 100 fibres from each treatment and plotted a particle size distribution with a Gaussian kernel-smooth curve fit (see Figure 6.2).

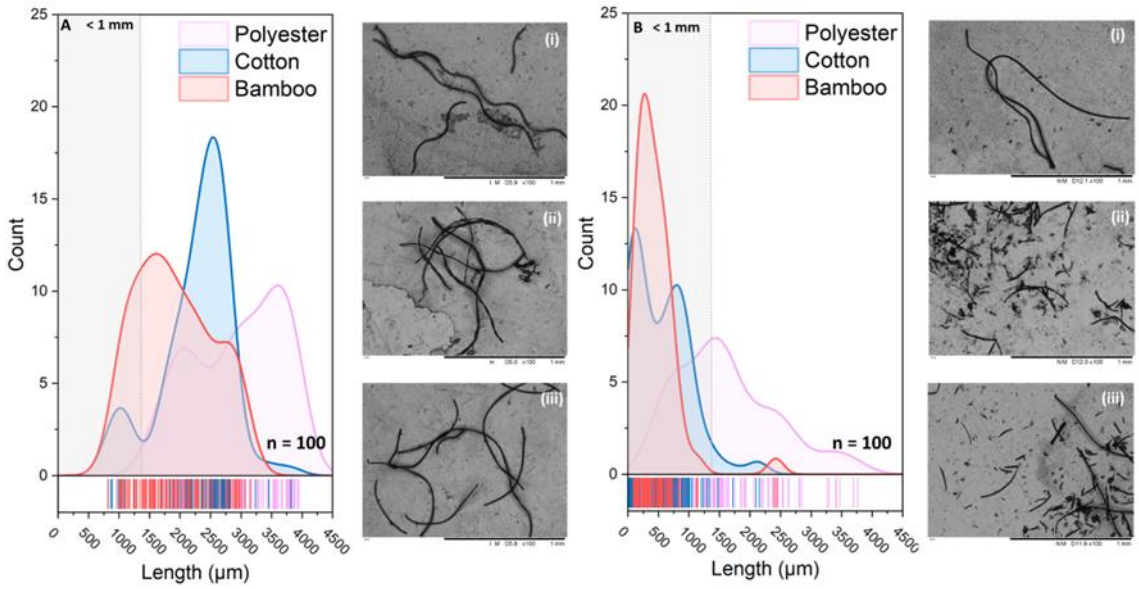

Figure 6.2 Particle size distribution of cotton, polyester, and bamboo fibres shed over 24 hours (A) and after 2 months soaking (B). In (A), the mean particle sizes with standard deviation are reported for each fibre type (2933.7 µm for polyester, 2273.7 µm for cotton, and 1867.7 µm for bamboo), along with SEM micrographs of a polyester fibre (A(i)), cotton fibre (A(ii)), and bamboo fibre (A(iii)). In (B), the mean particle sizes with standard deviation are reported after 2 months soaking **(**615.5 µm for polyester, 539.1 µm for cotton, and 446.7 µm for bamboo), along with SEM micrographs of a polyester fibre (B(i)), cotton fibre (B(ii)), and bamboo fibre (B(iii)). Also noted is the physical degradation of fibres, more greatly seen for cotton and bamboo and less for polyester

Our analysis showed that the mean fibre length of all three fibre types decreased with prolonged soaking time, with a greater decrease observed in cotton and bamboo fibres than polyester fibres. After a 24-hour soak, the average fibre length was 2933.7 µm for polyester, 2273.7 µm for cotton, and 1867.7 µm for bamboo. This decreased further after 2 months of soaking to 1615.4 µm for polyester, 539.1 µm for cotton, and 446.8 µm for bamboo (Figure 6.2). The change in fibre size over time was 1318.3 µm for polyester, 1734.6 µm for cotton, and 1420.9 µm for bamboo. Additionally, we observed evidence of particle degradation in the micrographs of cotton and bamboo fibres. In contrast, although some bowing and roughness could be seen on the surface of polyester fibres, there were no clear areas of degradation evident. As guppies are gape limited predators (Magurran, 2005) and fibres are inherently pliable, the male guppies in this study with a gape of diameter range 1.5-2.5 mm, would have been able to consume all the fibres in this study.

A preliminary trial was conducted on n=5 fish per fibre treatment where individual fish were maintained in 1 L containers. Fish were exposed for 7 days to ~700 fibres/L, equivalent to fibre loads found in some natural environments (Carr, 2017). This involved adding 1 mL of the fibre mixture at the same time as adding flake food (Aquarian®) to each 1 L container. Control fish (n=5) were maintained under the same conditions but without fibre exposure. Each day, faecal matter from the water was transferred using a glass pipette into a pre-cleaned glass petri dish and then dissected under a dissecting microscope to count the number of fibres encapsulated within the faeces. Fibres were clearly observed within all faeces of fish exposed to fibres even within the first 24 h of exposure (fibre range = 12-23 per faecal pellet; see Figure 6.1B & C). Faecal analysis of control fish (not exposed to fibres) revealed no fibres in the faeces.

For the main experiment, all fish were isolated into 1 L containers (i.e., 1 fish per 1 L container) and fish were exposed to fibres (i.e., ~700 fibres/L) for 21 days. This time frame of exposure was chosen as it corresponds to significant effects seen in changes to host-parasite responses to granular microplastic exposure on the guppy-*G. turnbulli* system (Masud & Cable, 2023). Control fish were fed the same quantity of flake food (2% of body weight) without fibre addition, to ensure that nutrition was not a confounding variable. Due to this immersion mode of fibre exposure, it is likely that consumption of fibres occurred passively. A full water change (for the preliminary trial and the main experiment fish) occurred every alternate day coinciding with feeding, which involved removing all water from the 1 L containers in which fish were housed and replacing with fresh temperature controlled dechlorinated water. It is acknowledged that water changes may cause some degree of stress to fish, however as this cleaning method was applied across all treatments

using the same technique, any potential confounding effects of handling stress would average out. During feeding, all precaution was taken to ensure that the experimenters clothing did not contribute to fibre contamination by ensuring short sleeved clothing was worn during all feeding regimes. However, we acknowledge that even under tightly controlled laboratory conditions atmospheric fibre contamination is possible (Gwinnett & Miller, 2021), but would have been consistent across treatments.

Experimental Infection

After 21 days of fibre exposure, half of the fish in each treatment group were infected (n=30) and half remained uninfected (n=30). Fish to be infected with *G. turnbulli* were lightly anaesthetised with 0.02% MS-222 and then held in water alongside a donor fish. Using a dissecting microscope with fibre optic lighting, transfer of two gyrodactylid worms to the caudal fin of the recipient fish was observed following the method of King & Cable (2007). Uninfected fish were anaesthetised and handled in the same manner without the introduction of parasites to control for any handling stress (sham infections). All infected and sham infected fish were maintained within 1 L containers throughout the experiment to ensure transmission was not a confounding variable for this experiment. Parasite numbers were assessed every 48 hours for 31 days and this involved mildly anesthetising infected fish (using 0.02 % MS-222) and counting the number of worms present under a dissecting microscope with fibre optic illumination (see King & Cable, 2007 for detailed description). Infection monitoring terminated at 31 days as this was the time point at which all fish had either cleared their infections and/or mortality of hosts occurred. As we were able to follow infection trajectories on the same fish over time, pseudo-replication was taken into consideration when statistically analysing parasite data from each fish/replicate (see Statistical Analysis section below). Fish were categorised as either resistant (parasite numbers on a host fail to increase and individual hosts often manged to clear their infections), susceptible (parasite numbers consistently increase) or responder (parasite numbers increase but then plateau or decrease) (see Bakke et al., 2002 for more in-depth explanation of these categories). The same feeding regimes continued during the infection phase of the experiment, i.e., both fibres and flakes. Any host mortalities were recorded throughout the study.

Gyrodactylus turnbulli *off-host survival*

As all fibres within our stock solution released dyes into the water (see Supplementary Material for chromatographic analysis of dyes), we wanted to determine the effect of any chemical dye exposure on off host parasite survival. To investigate this, 40 wells of sterile 96-well plates were inoculated with 100 µl of the same liquid that the fish were exposed to during each fibre treatment (i.e., stock solution) as these contained any leached-out dyes. Individuals of *G. turnbulli* were removed from a recently sacrificed infected fish by gentle agitation with a needle and transferred to the prepared wells via pipette. Worms exposed to fibre dye treatments (n=120, 40 worms per treatment) and control worms exposed to dechlorinated water (n=40) were observed and survival monitored every hour under a dissecting microscope. Any worms that died (mortality confirmed via worm immobility and non-responsive to pin touch) within the first hour were excluded from the experiment. Survival was then monitored until the last *G. turnbulli* worm died at 33 hours; hour of death was noted for every expired worm.

Statistical analysis

All statistical analysis were conducted in R Studio Version 1.3.1073. When analysing host infections, the following response variables were considered: maximum parasite burden, peak parasite day, Area Under Curve (AUC), rate of parasite increase and host disease status. Here, maximum parasite burden is defined as the maximum number of *G. turnbulli* worms at a particular time point (day), defined as peak parasite day. To calculate AUC, a common parasite metric quantifying total parasite burdens over the course of an entire infection trajectory, we used the trapezoid rule (White, 2011). Rates of parasite increase, indicative of parasite reproduction was calculated as the slope of the curve of individual infection trajectories.

To analyse host infection responses, we used Generalised Linear Models and Generalised Linear Mixed Models (GLMs and GLMMs). For analysing maximum parasite burdens, we used a GLM with a negative binomial error family and a square root link function, within the *MASS* package in R Studio. When analysing peak parasite day and AUC, a GLM with a Gamma error family and log link was used. For rates of parasite increase we used a GLMM within the *lme4* R package to prevent pseudo-replication as rates of parasite increase were calculated on each fish at multiple time points. For the GLMM we used a Gamma error family and a square root link function. When analysing host disease categories (i.e., fish that were categorised as either resistant, susceptible or responders, see methodology above for more details), a GLM with a Poisson error family and log link function was used. A GLM with a gaussian error family and log link function was used to determine if there was a

significant association between fish wet mass change before and after fibre exposure and the type of fibre used, where the difference in mass was the response variable and fibre treatment was the independent variable. After all model assumptions were met (i.e., normality of residuals and homogeneity of variance), all final models were chosen based on the lowest AIC values (Thomas et al., 2013).

Kaplan-Meier survival analyses were also conducted for *in vitro* effects of fibres and/or chemical dye exposure on parasite and host survival (infected and uninfected groups). For analysing parasite survival, we used a parametric model and a cox-proportional hazards model for host survival and in this regard, we used the R *survival* package. To visualise survival data, we plotted a probability distribution using the *survminer* and *ggfortify* work package. All final survival models were chosen based on the lowest AIC values.

Results

Host and parasite survival

For infected fish, there was no significant difference in mortality between any of the infected treatments related to fibre exposure and/or consumption (p >0.05, see Supplementary Material for all test statistics outputs). When analysing mortality of uninfected fish, those exposed to polyester suffered significantly earlier mortality compared with control uninfected fish (coef=2.06, exp(coef)= 7.85, p = 0.004; see Figure 6.3A for prediction plot), while there was no significant difference for cotton or bamboo exposed fish. No significant association was found between the change of fish body mass over 52 days of fibre exposure and the type of fibre that the fish were exposed to (p >0.05). No mortalities were observed in control uninfected fish (i.e., not exposed to any fibres/dyes or parasites).

Gyrodactylid parasites removed from their hosts and exposed to polyester and cotton fibre dyes *in vitro* died significantly earlier compared to control parasites in just dechlorinated water (i.e., with no dye addition, Cotton: z=-2.58, SE=0.11, p = 0.01; Polyester: z=-3.35, SE=1.55, p = 0.0008). In contrast, exposure to bamboo dye solutions *in vitro* did not impact parasite survival (Figure 6.3B).

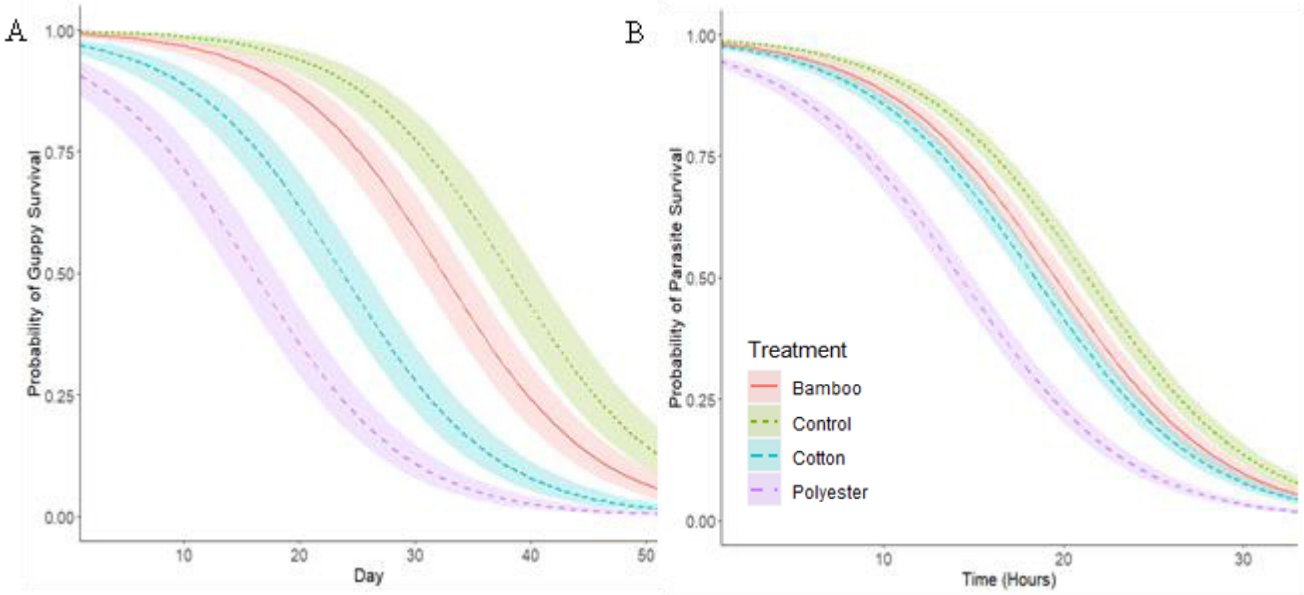

Figure 6.3 Host (guppy, *Poecilia reticulata*) and off-host parasite (*Gyrodactylus turnbulli*) survival. A) Prediction curve shows the probability of uninfected *P. reticulata* survival over 52 days of fibre exposure. All mortalities were monitored daily for 52 days with *P. reticulata* isolated in individual 1 L pots. B). Prediction curve shows the probability of *G. turnbulli in vitro* survival over 33 hours of fibre exposure in 96-well microtiter plates.

Host disease response

Fish exposed to bamboo fibres had the lowest maximum parasite burdens compared with control fish not exposed to any fibre (GLM: Est.=-3.15, SE=0.97, p = 0.001; Figure 6.4A). All other fish within the remaining fibre treatments (cotton and polyester) did not significantly vary in their maximum parasite burdens when compared with control fish (see Table 1 for all test statistic outputs). Polyester exposed fish achieved the highest mean parasite intensity compared with bamboo exposed which achieved the lowest (Figure 6.4B), but these were not statistically significant. Peak parasite burden occurred significantly later in bamboo and polyester exposed fish compared with control infected fish (GLM: Bamboo, Est=0.17, SE=0.03, p <0.001; Polyester, Est=0.08, SE=0.03, p=0.01). Supporting this, rates of parasite increase were significantly lower for fish in the bamboo treatment compared with the controls (GLMM: Bamboo, Est.=-1.95, SE=0.85, p = 0.02). When analysing total parasite burdens over time using AUC metrics though, there was no significant difference between any of the treatments (p >0.05, Figure 6.4B; see Table 6.1). Furthermore, in terms of host disease categories (i.e., resistant, susceptible and responder), there was no significant difference in the proportion of fish in each category between the different treatments.

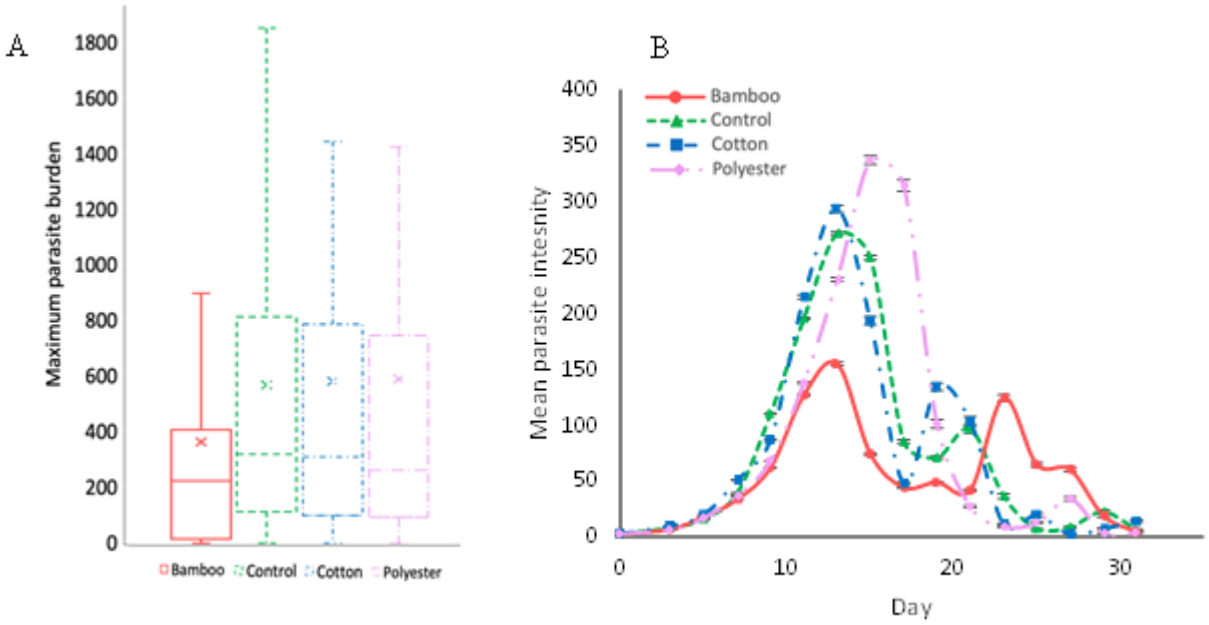

Figure 6.4 Infection dynamics of guppies exposed to fibres and subsequently infected with *Gyrodactylus turnbulli*. A) Box plot distribution with mean marker (x), median line and inter-quartile range of maximum parasite burdens of guppies (*Poecilia reticulata*) exposed to fibres for 21 days and then infected with *G. turnbulli* and B) the mean parasite intensity over the entire 31-day infection trajectory for each fibre exposure treatment. The shape of the smoothed function for each treatment is influenced by mortality of hosts and clearance of parasites. Standard error of the mean also shown for each graph.

Table 6.1 All independent variables for host-parasite metrics analysed via Generalised Linear Models and Linear Mixed Models (GLM and GLMM). Shown here are the models estimates, standard error and respective *p* values. Control fish not exposed to any fibres (not shown here) and subsequently infected are the baseline against which all other treatments (i.e., Bamboo, Cotton, and Polyester) are compared within the statistical models. * denotes significant *p* values.

Treatment	Est.	Std. error	*p* value
Maximum parasite burden (GLM):			
Bamboo	-3.15	0.97	0.001*
Cotton	1.09	1.01	0.28
Polyester	-1.41	1	0.15
Peak parasite day of (GLM):			
Bamboo	0.17	0.03	<0.001*
Cotton	0.02	0.03	0.52
Polyester	0.08	1	0.01*
Area under curve for (GLM):			
Bamboo	-0.48	0.26	0.07
Cotton	-0.42	0.25	0.09
Polyester	-0.09	0.25	0.71
Rates of parasite increase (GLMM):			
Bamboo	-1.95	0.85	0.02*
Cotton	-1.16	0.81	0.51
Polyester	-1.28	0.82	0.11

Discussion

Fibre contamination is now recognised as the major constituent of particulate pollution in marine, freshwater, and terrestrial ecosystems (Horton et al., 2017; Vince & Stoett, 2018). In freshwater systems, fibre contaminants are present in multiple fish species (e.g., Silva-Cavalcanti et al., 2017; Su et al., 2019). Despite the prevalence of fibres in freshwater systems, this study is the first to assess the functional impacts of plastic and cellulose-based fibres and associated dye exposure and consumption (polyester, cotton, and bamboo) on fish host-parasite dynamics, specifically host mortality, disease resistance, and off-host parasite survival. For infected fish, while polyester and cotton had no major effects on disease dynamics, bamboo was associated with significantly lower maximum parasite burdens. Polyester exposure and/or consumption were also associated with significantly increased mortality of uninfected hosts. Analysis of fibre-based dyes on off-host

parasite survival revealed that cotton and polyester associated dyes were linked to significantly reduced parasite survival, with the polyester dyes associated with the highest parasite mortalities.

Polyester, specifically polyethylene terephthalate, is the most common thermoplastic polymer used for clothing (Ji, 2013) and unsurprisingly most prevalent in multiple surveys of fibre types in fish gastrointestinal tracts (Su et al., 2019). The only studies we are aware off that have tested polyester health effects in fish revealed no significant detrimental effects (Spanjer et al., 2020; Hu et al., 2020). Juvenile chinook salmon successfully cleared 94% of their gut polyester fibres over 10 days (following in-feed exposure of 20 fibres per food pellet with guts sampled at days 0,3,5,7 and 10) and polyester exposure had no effect on their mean gastrointestinal mass when compared with fish not exposed to any fibres (Spanjer et al., 2020). This chinook salmon study though was based on short term exposure and the authors commented that longer term studies may reveal detrimental health effects. In contrast, exposure of adult medaka for 21 days to polyester at levels x14 greater than those tested in the current study (10,000 fibres/L versus 700 fibres/L) reported denuded epithelium on gill arches, fusion of primary lamellae, and increased mucus production, but no changes in adult growth, weight or mortality, nor embryonic mortality (Hu et al., 2020). In contrast, the current study which exposed fish to polyester fibres for 52 days demonstrated significantly reduced survival for uninfected hosts, and we predict that longer exposure times may have revealed even more pronounced effects. A plausible reason why polyester was associated with increased mortality of fish and also a limitation of the current study is that polyester fibres were, on average, larger particles than bamboo and cotton fibres. This is certainly linked to the greater physical degradation over time that we observed with cotton and bamboo compared with polyester. Therefore, the larger polyester fibres may have been linked to increased gut blockages and/or morbidities associated with the intestinal lining (e.g., necrosis and lesions), certainly something noted for other microplastic types within fish in previous studies (e.g., polystyrene in Ahrendt et al., 2020). However, in the current study we were unable to assess gut morphology or control for the size of the fibres being released from the commercial fabrics.

When assessing the impacts of fibre associated dyes on parasite mortality, we observed that cotton and polyester dyes were linked to significantly higher off host parasite mortalities compared with bamboo dyes and controls. Off-host survival for this *G. turnbulli* strain typically ranges from 2 to 31 hours (mean 13.7 hours at 25^{o}C; Schelkle et al., 2013), which was reflected in our control worms (i.e., not exposed to any dyes). Ecological parasitology is increasing our understanding of how pollutants, such as the fabric chemical dyes in this study, impact the health of parasites, making

parasites potential indicators of environmental health (reviewed by Vidal-Martínez, 2010; Sures et al., 2017). Typically, studies on parasite responses to pollutants have tended to focus on their ability to bioaccumulate toxins, especially heavy metals, particularly for endoparasites (e.g., cestodes and acanthocephalans; Palm & Rückert, 2009) and fewer studies have assessed survival of free-living stages of parasites such as trematode cercariae (e.g., Hock & Poulin, 2012). For this studies parasite species, *G. turnbulli*, which has a direct life cycle (i.e., no intermediate free-living stage), while we did reveal impacts of the dyes on the parasite's direct survival (underlying mechanism unknown), we did not attempt to assess the ability of these monogenean worms to bioaccumulate dyes. Of the three fibre dye types, polyester had the highest number of parasite mortalities and while we were unable to tackle the mechanism underlying this increased parasite or host mortality, polyester was leaching a specific dye component (as identified via LCMS analysis; see Appendix 1 & 2) more than bamboo and cotton, and this might have been a contributing factor. The chromatographic analysis also revealed that comparable amounts of dye were released from the bamboo and cotton fibres tested, but for some reason the cotton associated dye/s led to higher parasite mortalities. Current industry practices mean that the chemical identity of fabric dyes tends to remain confidential (Chen & Burns, 2006), and unfortunately, we were unable to determine the exact nature of the dye. While it has been shown that wastewater effluent containing textile dyes can be toxic to fish (Zhang et al., 2013; Kaur & Due 2015), targeting specific fabric dyes and their impacts on fish welfare is much harder without actually knowing the specific dyes in question. However, plausible reasons why fibres and their dyes were associated with increased host and off-host parasite mortality include 1) development of biofilms harbouring infectious agents (Di Pippo et al., 2020; Tang et al., 2021); 2) gastrointestinal related pathologies (i.e., gut blockage and lesions; Jovanovic, 2017); 3) toxic effects related to a breakdown product from either the fibres and/or the associated dyes (e.g. RB5 bis-vinyl (FIOSH, 2021), and/or 4) immune priming associated with microfibres being treated as antigens leading to reduced host survival (Tort, 2011). Little is currently known of the products resulting from the breakdown of these fibres and their additives. Given the synthetic nature of polyester, it does not degrade readily (see Figure 6.2), whilst the breakdown products of bamboo and cotton are likely to be some form of cellulose. There may be synergistic or antagonistic relationships between fibre type and additional treatments which influence the impact of products resulting from fibre breakdown; however, there is little empirical evidence of these.

Unlike the thermoplastic plastic polymer polyester, cotton and bamboo are cellulose based fibres and with the push to utilise natural alternatives, such as bamboo and hemp (Raj, 2021), the

biological impacts of these alternatives must be established. Tropical fish, such as guppies used in this study, would naturally consume cellulose-based foliage (Zandona et al., 2011), and the current study revealed that, compared with controls not exposed to any fibres, cotton consumption did not impact disease burden or mortality. Though bamboo is also composed of cellulose, the bamboo utilised in this study was 95% viscose based and therefore technically synthetic in nature (i.e., chemically treated) using regenerated cellulose (structurally the same as natural cellulose; Kauffman, 1993) and 5% elastane, which is a synthetic polymer. Elastane is known for its extreme flexibility and hence an essential addition to such clothing, but it is also recognised as non-recyclable and not easily degraded *in natura* (see Yin et al., 2014). For this study bamboo exposure and consumption was associated with significantly lower maximum parasite burdens compared with fish not exposed to any fibres, while also reaching these peaks much later than the control treatments. It is unclear why bamboo consumption was associated with such low maximum parasite burdens, but this could be related to the host immune system being primed to a chronic pollutant leading to a heightened disease response (i.e., increased resistance- see Tort, 2011) and/or reduced parasite survival and/or reproduction rate in response to the bamboo fibre and associated dye exposure. However, off-host parasite survival was not affected by direct bamboo dye exposure; leaving potential reproductive changes to the parasite, host immune related effects or increased host tolerance to the parasites themselves as the likely explanation behind why bamboo may be conferring some degree of protection to the fish.

Concluding remarks

This study investigated the effects of three fibre types, polyester, cotton, and bamboo and their associated dyes on host-parasite dynamics utilising a freshwater fish model, specifically testing disease resistance, host mortality and off-host parasite survival. Our results have revealed two key findings. Firstly, polyester fibres were associated with increased host mortality, and polyester and cotton leachate were also linked to increased off-host parasite mortality. Secondly, bamboo fibre exposure and consumption were associated with significantly reduced maximum parasite burdens. With fibre pollution being a dominant form of anthropogenic waste within freshwater environments, studies such as the current one provides important biological assays on potential detrimental impacts on organism welfare.

Chapter 7 – Beneficial bamboo? Analysis of bamboo fibres and their associated dye in the parasite-host dynamics of freshwater fish

Abstract

With the growth of the fashion and textile industries into the 21st Century, associated pollution has become pervasive. Of these pollutants, fibre-based microplastics are the most common types of plastics recovered from aquatic ecosystems encouraging the increased trend in organic fibre usage. Often marketed as biodegradable and 'environmentally friendly', organic textile fibres are seen as less harmful. Here, we assess the health effects of reconstituted bamboo viscose fibres, processed bamboo-elastane fibres (both at 700 fibres/L) and their associated dye (Reactive Black-5, at 1 mg/L) on fish, with an emphasis on disease resistance utilising an established host-parasite system: the freshwater guppy host (*Poecilia reticulata*) and *Gyrodactylus turnbulli* (monogenean ectoparasite). Following three weeks exposure to the bamboo fibres and associate dye, half the experimental fish were infected with *G. turnbulli*, after which individual parasite trajectories were monitored for a further 17 days. Overall, exposure to reconstituted bamboo-viscose fibres, processed bamboo-elastane fibres or dye were not associated with any change in host mortality nor any significant changes in parasite infection burdens. When analysing the routine metabolic rate (RMR) of fish, we noted that uninfected fish had, on average, significantly impacted RMR when exposed to processed bamboo elastane (increased RMR) and reconstituted bamboo viscose (decreased RMR). Hosts exposed to reconstituted bamboo viscose and the associated dye treatment showed significant changes in RMR pre- and post-infection. This study bolsters the growing and needed assessment of the potential environmental impacts of alternative non-plastic fibres; nevertheless, more research is needed in this field to prevent potential greenwashing.

Introduction

The fashion and textile industries contribute significantly to environmental pollution via wastewater containing additives and other associated chemicals, in addition to fibres shed from clothing. Around 35% of oceanic microplastic pollution is attributed to the fashion industry, mostly non-degradable, synthetic, petroleum-based polymers such as nylon, spandex and polyester (Boucher & Friot, 2017; Suaria et al., 2020). Global textile production is dominated by petroleum-based synthetic fibres (~60% of total production) compared to naturally derived (~30% of total production) and other fibre types (~10% of total production) (Carr, 2017). Petroleum-based fibre usage has risen with the advent

of ‘fast fashion’ that produces billions of clothing items per year (Niinimäki et al., 2020). Fast fashion garments are only worn on average ten times before thrown away, where they are sent to landfill more often than are recycled (TRAID, 2018; Barnardos, 2021). The affordability of these garments comes at an environmental cost (Niinimäki et al., 2020). Non-degradable polymers commonly used in these garments constantly release fibres, which end up in water bodies through run-off and wastewater. Regular household fibre waste generation alone can reach worrying scales, with fibre effluent counts reaching in the millions per wash, not accounting for industrial scale generators (Xu et al., 2018), such as netting from fishing equipment and masks from medical waste (Sillanpaa & Sainio, 2017; De Falco et al., 2019). One mitigation strategy to reduce this waste and its harmful effects is the drive toward more plant derived (i.e., nature based) products, which in theory are degraded *in-natura* by microbes compared to petroleum-based fibres which resist breakdown (Pekhtasheva et al*.,* 2011; Arshad et al*.,* 2014; Resnick, 2019). It is essential, however, to ensure that products marketed as ‘ecologically friendly’ are less damaging to the environment by empirical testing under controlled conditions. Indeed, the EU Directive 2019/904 highlighted the potential problem of transitioning to non-plastic polymers, such as bamboo or hemp, without sufficient knowledge of their environmental and biological impact (Hann et al., 2020).

The negative impacts of granular microplastics on organisms are increasingly well documented (Wright et al., 2013a; Wright et al., 2013b; de Sá et al., 2018; Ockenden et al., 2021), but data on fibre exposure is limited. Granular petroleum-based microplastic consumption in fish not only increased their parasite burden but also increased host mortality (Masud & Cable, 2023), and similar effects were seen following exposure to petroleum-based microplastic polyester fibres (see Chapter 6). In contrast, exposure to bamboo fibres (for 52 days) from a commercially available t-shirt, interestingly, significantly reduced parasite burdens in adult fish compared with fish not exposed to any fibres (see Chapter 6). Such work supports the drive to utilise plastic alternatives, with bamboo being a prime contender as a bio-based polymer, leaving a lower carbon footprint and requiring less water during culturing than other biofibres such as cotton (Afrin et al., 2009; Waite, 2010; Ogunwusi, 2013). A further consideration regarding transitioning to alternative fibres is that fibres shed from commercial textiles (no matter the origin) are very different to raw non-processed fibres (Yaseen & Scholtz, 2018). Although, bamboo is entirely cellulose based, the rigidity of the plant means it requires considerable processing before it is suitable for textile use, hence bamboo cellulose is chemically regenerated to increase malleability (Kauffman, 1993). The resulting bamboo viscose is then combined with a

petroleum-based polymer, e.g., elastane, to increase flexibility and allow it to function as a comfortable textile garment.

The assumption that biobased fibres are inherently 'better' might be an example of '*Greenwashing*' (see de Freitas Netto et al., 2020), with natural fibres, for instance, sharing sorbing capabilities with petroleum-based fibres (Ladewig et al., 2015; Stanton et al., 2019) and finished products containing similar additives. Fibres, particularly those from textiles, have been altered and treated to suit the end product, which involve chemical alteration such as bleaching or dying (Holkar et al., 2016; Yaseen and Scholtz, 2018). Reactive dyes are commonly used in textiles which easily (and strongly) bind to common textile fibre types, such as cotton and wool (Chavan, 2011; Shang, 2013). These dyes, however, hydrolyse with water even without an auxiliary compound, such as salt (other dyes may require additional compounds to ensure fastness), to produce dyed fabrics (Gopalakrishnan et al., 2019). When fabrics enter the aquatic environment, these additives have a greater likelihood of leaching from fibres into the water column. This suggests that dyes may interact with aquatic organisms in two ways: through direct consumption of the dyed fibre or passive consumption of the dye tainted water. Dyes, including reactive dyes such as the widely used Reactive Black-5, have known negative impacts on aquatic organisms including developmental defects and cell death observed in zebrafish embryos (Manimaran et al., 2018; Joshi and Pancharatna, 2019). This highlights how each aspect of textile pollution, from whole fibres to additives, must be considered in any ecological assessment.

With wastewater containing a cacophony of pollutants, including fibres and their additives, fish (especially those in freshwaters) are often some of the first organisms exposed to these pollutants and this can be detrimental to their welfare. Previous findings revealed that adult male guppies (*Poecilia reticulata*) exposed to fibres released directly from a commercial bamboo-viscose (with elastane) garment experienced reduced parasite burdens whilst the leachate from these fibres had no impact on the parasite itself (*Gyrodactylus turnbulli* see Chapter 6). Building on this, here we assess the impacts of both the whole and individual components of bamboo textile fibres. We tested reconstituted bamboo-viscose fibres, and processed bamboo-viscose with elastane fibres (from a commercially available black t-shirt) alongside a reactive black dye (commercially used in the textile industry) on juvenile fish metabolism, disease resistance and mortality. We hypothesised that both the reconstituted bamboo-viscose and processed bamboo-viscose with elastane would reduce infection burdens (as in Chapter 6) but that the reactive black dye would increase burdens. Further,

we hypothesise that both bamboo fibre types would have no impact on metabolism, but the reactive black dye would negatively impact metabolism.

Methods

Host-Parasite System

We utilised the established guppy-*Gyrodactylus* model for this study, which allows us to non-destructively monitor parasite burdens over time for individual hosts. Size-matched mixed ornamental juvenile guppies (n = 240 laboratory strain, established in November 1997) were maintained within 70 L aquaria at 24±0.5^{o}C on a 12 h:12 h light/dark photoperiod (lights on 7am and off at 7pm) prior to the investigation. For experimental infection, we utilised the *Gt3* strain of *G. turnbulli*, isolated from a Nottingham aquarium pet store and cultured under laboratory conditions since establishment in November 1997 (King & Cable, 2007). All fish, prior to experimental infections, were measured (standard length) and weighed on an electronic scale by mildly anesthetising individuals with 0.02% MS-222.

Fibre and Dye Preparation

The black bamboo fabric (from BAM Bamboo) was of the same origin as used in Chapter 6 and consisted of 95% bamboo viscose and 5% elastane. Bamboo fabric was cut into 7.5 cm^2 squares, then shred into 0.5-1.5 cm^2 pieces using sterile scissors and immersed in 1 L of dechlorinated water and agitated to promote fibre shedding to simulate a washing cycle. The same volume of raw reconstituted bamboo-viscose fibres (regenerated cellulose from bamboo plants) was agitated in 1 L dechlorinated water. A drop of each fibre water was then viewed under a compound microscope at 40x magnification, and the number of fibres counted on days 1, 3, 5 and 7 of soaking. This was repeated 10 times per fibre treatment to calculate the average number per 1 mL, which were then all diluted to 700 fibres/L, equivalent to levels found in some natural systems (Carr, 2017; Velasco et al., 2022) . The reactive black dye, obtained from Sigma-Aldrich (Merck), is analogous to the setazol black SDN dye previously confirmed by BAM Bamboo to be used during manufacture of bamboo clothing products. Wastewater has been found to contain concentration of dye upwards of 10 mg/L (Munagapati et al., 2018; Jalali Sarvestani & Doroudi, 2020), due to ethical considerations, a concentration of 1 mg/L was utilised here.

Experimental Design

The experiment was conducted in two batches and batch effect was accounted for during statistical analysis. Fish were separated into four treatment groups: 1) control (n = 60), 2) processed bamboo-viscose t-shirt with 5% elastane (n = 60), 3) raw reconstituted bamboo-viscose fibres (n = 60), and 4) reactive black dye (n = 60).

A preliminary trial was conducted on n=5 fish per fibre treatment where individual fish were isolated and maintained in 500 mL containers. Fish were exposed for 7 days to ~700 fibres/L for either reconstituted bamboo-viscose or processed bamboo-elastane fibres, equivalent to fibre loads found in some natural environments (Carr, 2017). This involved adding 1 mL of the fibre mixture at the same time as adding flake food (Aquarian®) to each 500 mL container. Control fish (n=5) were maintained under the same conditions but without fibre exposure. Each day, faecal matter from the water was transferred using a glass pipette onto a pre-cleaned glass slide, crushed under a cover slip, and observed under a dissecting microscope to confirm the presence of fibres encapsulated within the faeces (Figure 7.1).

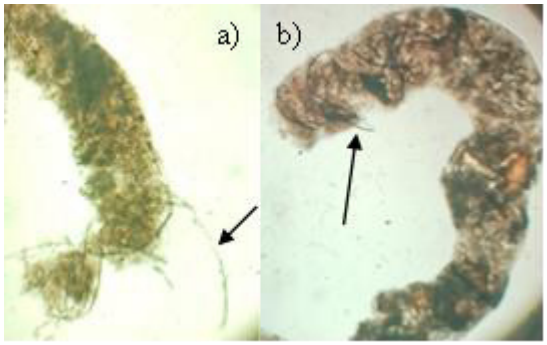

Figure 7.1 Faecal casings from fish (*Poecilia reticulata*) exposed to: a) raw bamboo-viscose fibres, where fibres are contained within and without the casing and b) processed bamboo-elastane, with arrow indicating presence of individual fibres expulsed from the faecal casing after being compressed under a cover slip.

For the main experiment, all fish were isolated into 500 mL containers (i.e., 1 fish per 500 mL container) and exposed to fibres (i.e., ~700 fibres/L) or dye (concentration 1 mg/L) for 21 days. Both fibre mixtures were agitated prior to exposure to ensure thorough mixing of the fibres within the water column for equal dispersion when introduced into the containers. Control fish were fed the same quantity of flake food (10% of body weight; Frederickson et al., 2021) without fibre or dye addition, to ensure that nutrition was not a confounding variable. Due to the exposure method (immersion), it is likely that consumption of fibres and dye occurred primarily passively, with active consumption probable but not verifiable (see Figure 7.1). A full water change (for both the

preliminary trial and main experiment) occurred every alternate day prior to feeding but after respirometry, which involved removing all water from the 500 mL containers in which fish were housed and replacing with fresh temperature controlled dechlorinated water. Upon refilling, the fish were then exposed to their respective treatment and fed the flake food (Aquarian®). During feeding, precaution was taken to ensure that the experimenters clothing did not contribute to fibre contamination by always wearing short sleeved clothing, but total elimination was not guaranteed (Gwinnett & Miller, 2021).

Experimental Infection

After 21 days of fibre exposure, half of the fish in each treatment group were infected (n = 30 total) and half remained uninfected (n = 30 total). Fish to be infected with *G. turnbulli* were lightly anaesthetised with 0.02% MS-222 and then held in water alongside a donor fish. Using a dissecting microscope, with fibre optic lighting, two gyrodactylid worms were transposed to the caudal fin of the recipient fish following the standard methods of King & Cable (2007). Uninfected fish were anaesthetised and handled in the same manner without the introduction of parasites to control for any handling stress (sham infections). All infected and sham infected fish were maintained within 500 mL containers throughout the experiment to ensure transmission was not a confounding variable for this experiment. Parasite numbers were assessed every 48 h for 17 days and this involved mildly anesthetising infected fish (using 0.02 % MS-222) and counting the number of worms present under a dissecting microscope with fibre optic illumination (see King & Cable, 2007 for detailed description). Fish were categorised as either Resistant (parasite numbers on a host fail to increase above 8 worms and most individual hosts cleared their infections), Responder (parasite numbers increased but then plateaued or decreased) or Susceptible (parasite numbers consistently increased) (see Bakke et al., 2002 for more in-depth explanation of these categories). The same feeding regimes continued during the infection phase of the experiment, i.e., both exposure treatments and foods. Any host mortalities were recorded throughout the study. The experimental design is summarised in Figure 7.2.

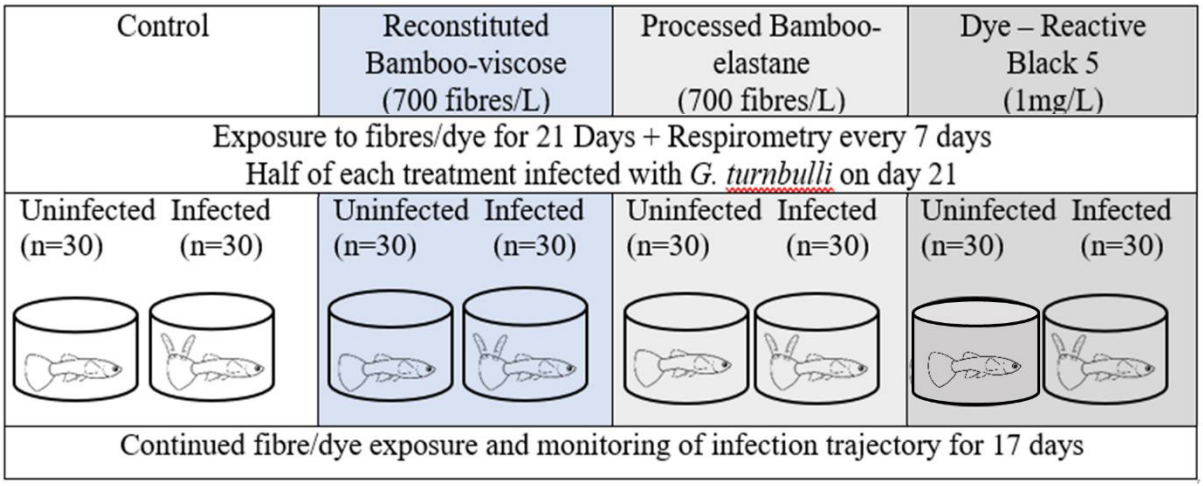

Figure 7.2 Schematic representation of experimental design. Four treatments; controls (exposed to only dechlorinated water), reconstituted bamboo-viscose (exposed to 700 fibres/L of reconstituted bamboo-viscose fibres), processed bamboo-elastane fibres (exposed to 700 fibres/L of bamboo-elastane fibres) and dye (reactive black 5 dye at 1 mg/L), where exposure was conducted for 21 days. On day 21, half the fish from each treatment were infected with two *Gyrodactylus turnbulli* and the infection trajectory monitored for a further 17 days whilst continuing previous treatment exposure.

Respirometry

To investigate whether exposure to either the fibres, dye or both impacted the routine metabolic rate (RMR) (Chabot et al., 2016), infected guppies (prior and during infection with *G. turnbulli*) (n = 24) were transferred to respirometer chambers on days 0, 7, 14, 21, 28 and 35 of exposure, with each treatment tested on the same exposure days but in batches of 4 fish. For day 21, when infections occur, respirometry was measured prior to infection. All measurements were conducted in a respirometry set-up that permitted monitoring of fish alongside a control simultaneously and temperature for the duration of measurements was maintained at 24±0.5°C. All water used for experimental purposes was autoclaved prior to use and then brought to the desired temperature. The static respirometry set-up consisted of individual glass chambers (130 mL, sealed DuranTM square glass bottles with polypropylene screw caps, Fisher), which were briefly washed with ethanol (Sigma-Aldrich) prior to commencing measurements to minimize background noise before the start of each respirometry trial. Chambers were fitted with individual contactless oxygen sensor spots attached to probes that were connected to a FireSting O_2 meter (PyroScience, Aachen, Germany). The O_2 concentration within respirometry chambers was measured every 1 s for 30 minutes total (10 min acclimation time and 20 min for recordings) using the following equation: $RMR = \frac{\Delta O2}{M} \times Vc$, where M is fish mass in grams, V_c is the volume of the respirometer chamber in mL and ΔO_2 is the

rate of oxygen decline (Bonneaud et al., 2016) calculated as the slope of a linear regression. During respirometry, the O_2 levels never dropped below 7 mg L^{-1} and were maintained within the recommended levels for freshwater tropical fish (OATA, 2008). Each individual fish was weighed immediately following respirometry, but only prior to infection as weighing hosts with ectoparasites could influence parasite burdens. Following infections, the average weight increase (0.03 g for all treatments) was calculated and added onto the weights for measurements. All respirometry measurements were taken prior to any handling or water changing stress.

Statistical Analyses

All statistical analyses were carried out under RStudio version 4.2.3 (http://www.R-project.org/). For this study, the following response variables were measured in relation to parasite metrics: parasite count over time, maximum parasite burden, peak infection day, Area Under Curve (AUC), duration of infection, and rate of parasite increase. Here, maximum parasite burden is defined as the maximum number of *G. turnbulli* worms at a particular time point, defined as peak infection day. To calculate AUC, a common pathogen metric utilised to quantify total pathogen burdens over the course of an entire infection trajectory, we utilised the trapezoid rule (White, 2011). Rates of parasite increase, indicative of parasite reproduction, were calculated as the slope of the curve of individual infection trajectories. To analyse mean parasite intensity, maximum parasite burden, peak parasite day, AUC, average RMR and duration of infection, we utilised Generalised Linear Models (GLMs). Standard length was initially included in the models, but as it did not explain significant variation it was removed from subsequent models, as part of model refinement (Thomas et al., 2013). For both mean parasite intensity and maximum parasite count, we used a GLM with a negative binomial error family and the log link function, within the *MASS* package in R Studio. For analysing AUC sum, we had to transform the data using the Box-Cox transformation method also within the *MASS* package in R, as no family structure and link function could satisfy the assumptions of GLMs with the raw data, i.e., normality of standardised residuals and heterogeneity of variance. Subsequently, a GLM with a gaussian error family and the identity link function was used, which did satisfy all model assumptions. A GLM with a gaussian error family and the inverse root link function was used to for analysing peak infection day. For the analysis of parasite count over time, where we needed to account for pseudo replication as the same fish was observed for parasite numbers over multiple time points, we utilised a generalised linear mixed model (GLMM) from the 'lme4' package. This was carried out as a negative

binomial GLMM where treatment, day and the interaction day and treatment were our fixed factors and fish ID was included as the random factor. For analysing host metabolism, we assessed how mean routine metabolic rate (RMR) of fish varied between experimental treatments using a GLM with an inverse gaussian family and the identity root link function. We analysed individual RMR trends using a GLMM with gaussian family and identity link functions, where the treatment, day and the interaction between treatment and day were fixed factors and fish ID was included as a random factor. This GLMM was used to create a prediction plot using the *ggpredict* function within the "*ggeffects"* package in R (Johnson & O'hara, 2014). In addition, emmeans post-hoc analysis was applied to assess significance of day and treatment using the '*emmeans*' package.

For all statistical models described above, model assumptions were tested, specifically normality of standardised residuals and homogeneity of variance and all final models were chosen based on the lowest Akaike Information Criterion (http://CRAN.R-project.org/package=lme4).

Results

Host survival and disease burdens

Neither reconstituted bamboo-viscose fibres, processed bamboo-elastane fibres nor RB5 dye had any significant impact on juvenile fish mortality, infected nor uninfected ($p > 0.05$), as number of deaths across treatments did not vary significantly. After 17 days of infection, there was no significant difference between the AUC sum, maximum parasite burden nor peak day between any of the treatments ($p > 0.05$) (Figure 7.3). Treatment had no impact on the day in which the parasites reached their peak, however Batch 2 reached peak day significantly earlier (approximately 3 days) than Batch 1 (GLM: Batch2, Est=-0.017, SE= 0.049, p = 0.0007). Parasite count over time was not significant between treatments (GLMM: reconstituted bamboo-viscose; SE=10.858885, t=0.2703594, p=0.786883, dye; SE=10.836791, t=-0.8526725, p=0.3938409, processed bamboo-elastane; SE= 10.869252, t=-0.5640176, p=0.5727422) nor was the interaction between day and treatment (GLMM: $p > 0.05$) whilst day was significant (GLMM: $F_{9, 240} = 1573.9$, $p < 0.001$). Infection status (Resistant, Responder or Susceptible) did not vary significantly between treatments ($X^2 = 7.1238$, df=6, p=0.3095). In all treatments, the dominant status was that of Susceptible (control n=17, raw bamboo-viscose n=20, dye n=19, and processed bamboo-elastane n=22), followed by Responders (control n=11, raw bamboo-viscose n=10, dye n=7, and processed bamboo-elastane n=7, with the fewest (or

none) being Resistant (control n=2, raw bamboo-viscose n=0, dye n=4, and processed bamboo-elastane n=1).

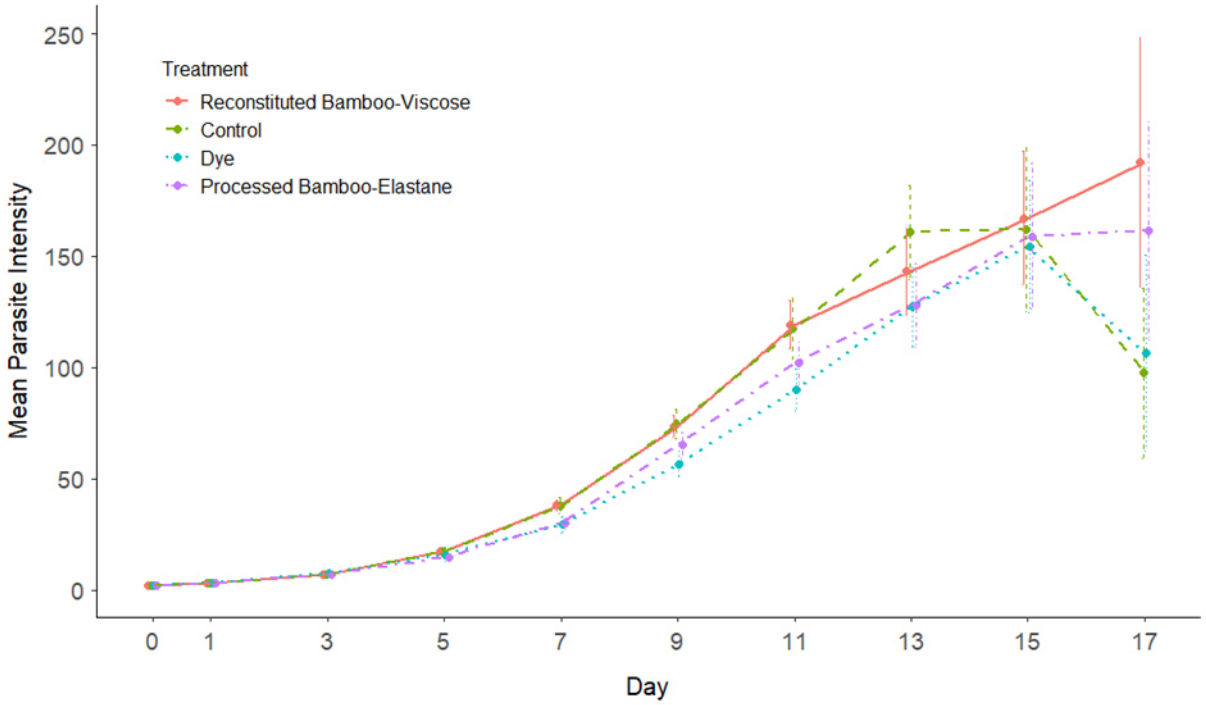

Figure 7.3 Mean parasite intensities of *Gyrodactylus turnbulli* per treatment (distinguished by colour and line type) per day (including standard error) on their host *Poecilia reticulata*.

Respirometry

The average RMR for control fish (across the duration of the experiment) was 1.136 mg O_2 g^{-1} h^{-1} $fish^{-1}$, while fish exposed to reconstituted bamboo-viscose fibres had an average RMR of 1.052 mg O_2 g^{-1} h^{-1} $fish^{-1}$, processed bamboo-elastane fibres of 1.350 mg O_2 g^{-1} h^{-1} $fish^{-1}$ and dye of 1.182 mg O_2 g^{-1} h^{-1} $fish^{-1}$. This translated to no significant difference in the average RMR between control and dye exposed fish (GLM: Est = 0.025263, SE = 0.054156, p = 0.64617), however the average RMR of processed bamboo-elastane exposed fish was significantly higher than control fish (GLM: Est = 0.207028, SE = 0.061548, p = 0.00326) while reconstituted bamboo-viscose exposed fish had significantly lower average RMR than control fish (GLM: Est = -0.111341, SE = 0.049450, p = 0.03638) (Figure 7.4). For RMR, day 21 represents the final day of exposure without infection, and measurements for days 28 and 35 represent RMR during infection with *G. turnbulli*. The interaction between treatment and day was significant for RMR for all fish (GLMM; $p < 0.05$). Looking at within treatment differences when fish had been infected after 21 days of bamboo and dye exposure, there

was no significant difference between control and processed bamboo-elastane RMRs across the experiment. However, for reconstituted bamboo-viscose, there were significant differences in their RMR between days 7 and 28 (emmeans: Est = 0.2943, SE = 0.106, p = 0.0443), and days 7 and 35 (emmeans: Est = 0.4040, SE = 0.111, p = 0.0030), and for the dye between days 7 and 14 (emmeans: Est = 0.3771, SE = 0.107, p = 0.0043), days 7 and 28 (emmeans: Est = 0.5471, SE = 0.107, p < 0.0001), and days 7 and 35 (emmeans: Est = 0.4577, SE = 0.120, p = 0.0015). These results indicate a treatment specific influence of infection on RMR for the reconstituted bamboo-viscose and dye treatments, but not for controls or processed bamboo-elastane (Figure 7.5).

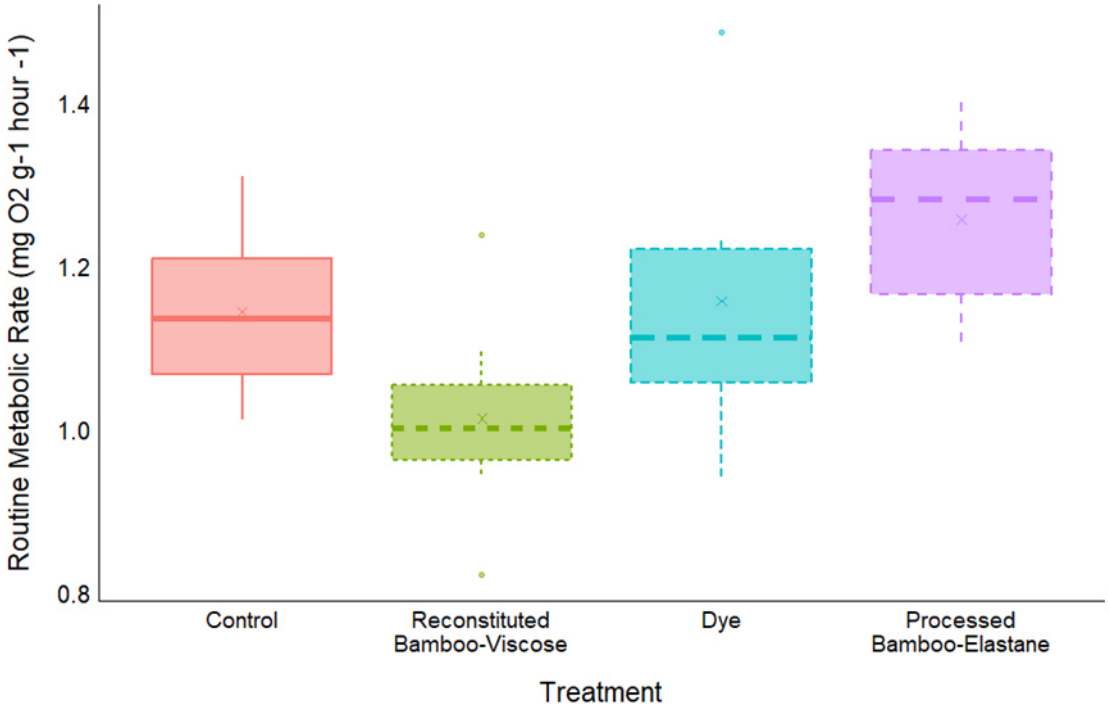

Figure 7.4 The average routine metabolic rate (RMR) of fish per treatment, accounting for all measurements taken for each treatment across the duration of the experiment. Box plot shows the median (line), mean (cross) interquartile range (box) and the 1.5x interquartile range (whiskers). The filled circles represent values outwith the 1.5x interquartile range. Each box is outlined with different line-types to represent each treatment.

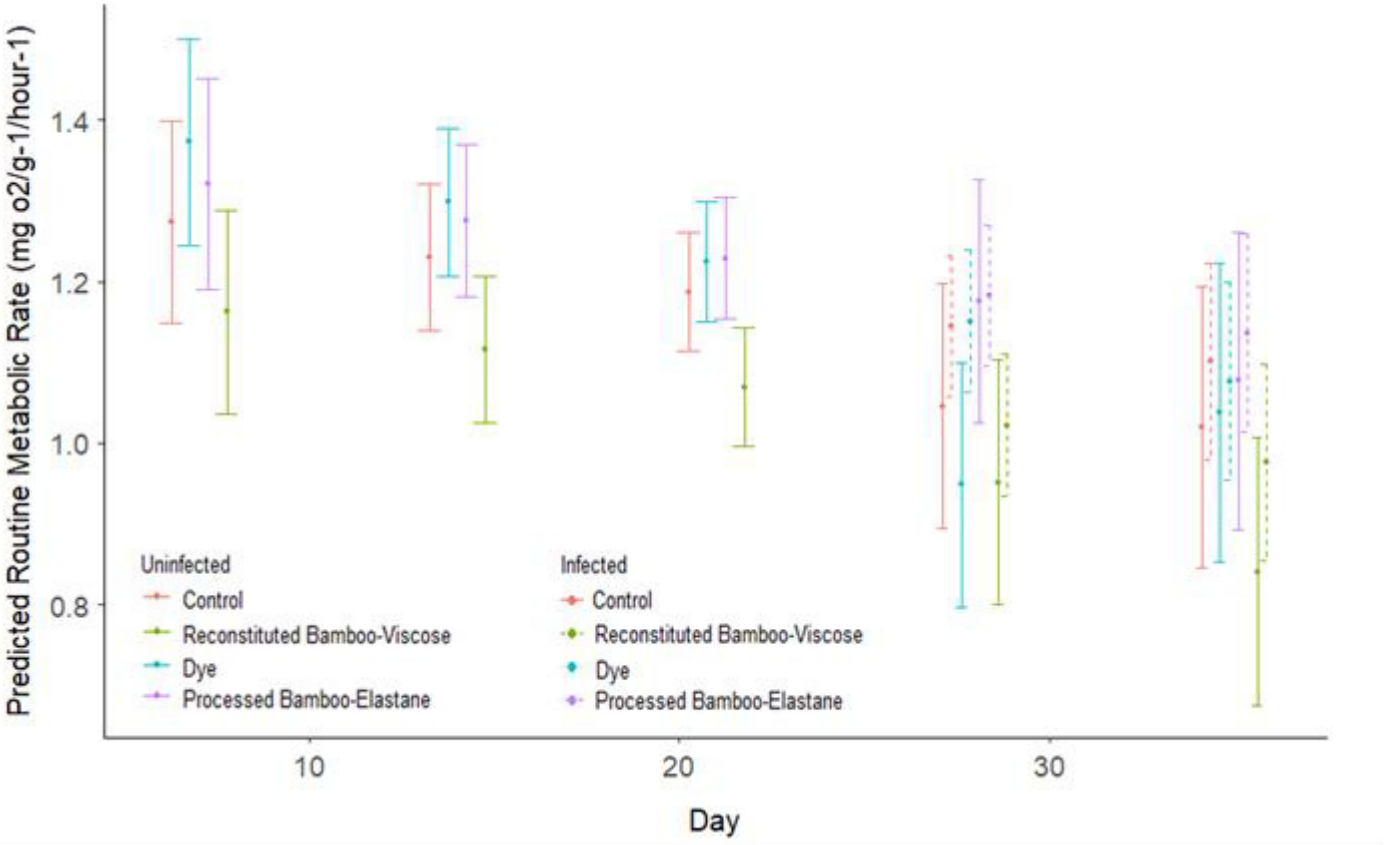

Figure 7.5 Predicted range of routine metabolic rates (RMRs) (mg O2/g-1/h-1) for fish, per treatment per day, across 38 days. Fish were exposed to their respective treatments across the entire experiment, but days 28 and 35 represent RMRs where some fish were actively infected (post day 21) with *Gyrodactylus turnbulli* (dashed error bars) and some remained uninfected (solid error bars).

Discussion

Textile pollutants, which includes microfibres and their associated dyes, are pervasive within freshwaters, and understanding their biological impacts on freshwater organisms is important for any welfare assessment. Despite the prevalence of studies on non-degradable fibres, the dominant proportion of aquatic microfibres are cellulose based and likely of anthropogenic origin, where it has been suggested that coloured cellulose-based textile fibres have been misidentified as microplastics for many years (Wesch et al., 2016; Cesa et al., 2017; Stanton et al., 2019; Suaria et al., 2020). This highlights the need to understand the potential impacts of cellulose-based textile fibres on aquatic environments. The current study suggests that reconstituted bamboo fibres, processed bamboo fibres and the raw dye associated with these fibres do not negatively impact disease susceptibility or host survival, at least following 38 days exposure. However, physiological impacts of fibre and dye exposure revealed that processed bamboo-elastane did impact metabolism by significantly increasing routine metabolic rate (RMR) compared to baseline control fish. Conversely, fish exposed

to reconstituted bamboo-viscose showed a significantly lowered RMR, indicating that fibres and their associated dyes can impose metabolic stress on fish. We also reveal that infections had treatment specific impacts on RMR, specifically for fish exposed to reconstituted bamboo-viscose and the Reactive Black-5 dye.

As bamboo is entirely comprised of cellulose it is biodegradable, but it is extremely rigid in its base structure. As such, for textile usage, the base structure of bamboo has to be chemically regenerated, and this reconstituted bamboo-viscose is considered semi-natural or semi-synthetic (Bien, 2021). Elastane is then added to provide flexibility to the fabric (Kauffman, 1993). Previously, we demonstrated that exposure of adult male guppies for 52 days (21 days prior to infection plus 31 days exposure during infection) to processed bamboo-elastane fibres resulted in lower *G. turnbulli* burdens, along with no adverse impact on mortality of the guppy host nor the parasite (Chapter 6). The current study time focussed on juvenile fish exposed for a shorter (38 day) period (21 days prior to infection plus 17 days during infection). From our current results, we reveal no impact of either reconstituted bamboo-viscose nor the processed bamboo fibres on disease dynamics using the same host-parasite system, however we add to this knowledge by revealing that changes in metabolism are detectable when fish are exposed to processed bamboo-elastane and reconstituted bamboo-viscose in the same host-parasite system. Juvenile guppies exposed to microplastics for 28 days retain particles within the gut (Huang et al., 2020), which likely also occurs for fibres (in future, this could be confirmed through microscopy of the intestine), indicating the importance of assessing long-term impacts. Exposure to microplastics can stimulate juvenile fish immunity, potentially priming their immune system for infection (Huang et al., 2020). If this was the case in the current study, this might explain why these juveniles tolerated *G. turnbulli* infection more effectively than adult fish. The key difference between this and Chapter 6 is the life stage of the fish, which we know can influence immune response, where juvenile fish have a generally underdeveloped immune system versus the established immune system of mature fish (Zapata et al., 2006; Uribe et al., 2011). Despite no observable impacts being found in the current study, micro- and nano-level effects were not directly assessed: techniques such as histopathology, ELISA and transcriptomics may reveal impacts at the organ, cellular and DNA level (Petitjean et al., 2019; Huang et al., 2020).

The dye utilised here was Reactive Black-5 (RB5; also known as Remazol Black B), a readily available black dye commonly used in textile colouring. The wet fastness of reactive dyes is touted as a benefit, but when fibres dyed with these reactive dyes enter the water column (be that during washing or as

waste products), it is possible to visually observe dye leaching out of the fibres and into the water. The dyes, and any other associated chemicals contained within the textile, will leach out of the fibres and enter the water column as leachate. In zebrafish, textile leachate and RB5 specifically can induce cytotoxicity within cells lines, cause malformations during larval development and increase mortality of embryonic fish (de Oliveira et al., 2016; Manimaran et al., 2018). These dyes can also impact behaviour, reducing activity in rainbow trout and competitive behaviours in fathead minnows following exposure to wastewater effluent *in-natura* (Garcia-Reyero et al., 2011; Almroth et al., 2021). Dye concentrations have been found to cause observable effects, under laboratory conditions, at concentrations greater than 1 mg/L, although 1 mg/L did increase mortality of zebrafish embryos (Manimarin et al., 2018). Within our study, fish parasite burdens were not impacted following exposure to 1 mg/L dye and no other observable detrimental effects were observed, however this may be limited by the experimental duration and further investigations over a longer exposure period may be necessary. Malformations observed in previous studies focussed on larval zebrafish arguably at greater risk of developmental difficulties (Kato et al., 2004; Rojo-Cebreros et al., 2018) than the juvenile fish used within our study. RB5 is degradable by bacteria, which break down and decolour the dye within water (El Bouraie & Din, 2016). The aquarium water used for the current study was not sterile but as it was completely changed and re-dosed every two days so it is unlikely that bacterial breakdown would have deactivated the dye during the course of our experiment, but we acknowledge some breakdown products may have been generated.

For average routine metabolic rate (RMR), we observed no significant differences between dye exposed fish and controls, but we did observe differences for reconstituted bamboo-viscose and processed bamboo-elastane exposed fish. We recorded a lower average RMR for the reconstituted bamboo-viscose exposed fish and a higher average RMR for processed bamboo-elastane exposed fish, compared to control fish. This suggests that reconstituted bamboo-viscose is associated with metabolic depression, although the reason for this is unclear. We know that freshwater fish often have cellulose-based detritus in their diets and that they can digest and utilise the nutrients from bamboo (Magurran, 2005; Saha et al., 2006), which may explain why fish exposed to processed bamboo-elastane had significantly increased metabolism. It is plausible that the processed bamboo-elastane fibres, which are associated with multiple additives, including RB5 (which we tested in this study), was causing metabolic stress, seen here as a significant increase in RMR. Processed fibres, such as our processed bamboo-elastane, often contain chemicals such as sodium hydroxide,

formaldehyde and hydrogen peroxide (Yaseen & Scholtz, 2018), all of which may influence RMR (Tavares-Dias, 2021; Wood et al., 2021) but we are unable to say for certain. Here we show that parasitic infections have a treatment specific influence on RMR, where the RMR is depressed, matching the trend seen for other fish with parasitic infections (Hvas et al., 2017; Guitard et al., 2022; Schaal et al., 2022). In terms of temporal RMR variation across the experiment, we did not see a difference in RMR for fish between pre- (days 0-21) and post-infection (days 28 and 35) that were not exposed to fibres nor dye, whereas previous results indicate an increase in RMR with infection of *G. turnbulli* (see Masud et al., 2020; Robison-Smith et al., submitted). This could be due to two factors: fish strain variation and life stage. The RMR of wild-origin fish under similar lab conditions was lower on average than the ornamental strain used here, and the wild-origin fish showed increased oxygen consumption post-infection (Masud et al., 2022; Robison-Smith et al., 2023). We used juvenile fish, which may have been under increased energy demands needed for maturation and sexual development, and as such infection did not significantly influence the RMR (Joblin, 1994; Pichavant et al., 2001). Smaller fish will display a higher metabolic rate than larger fish, when weight is considered (Urbina & Glover, 2013; Guitard et al., 2022). Overall, the decreased trend observed in RMR across the experiment (Figure 7.5) matches what we would expect and is due to a combination of infection and growth, which directly correlates to lower RMR, as the fish were growing, gaining ~0.003 g per week (Urbina, 2013).

Our current knowledge of fibre pollution is lacking. The majority of studies pertaining to fibre pollution focus on assessing the type and scale of fibre pollution, typically within a 'plastic focus' framework (e.g., Collard et al., 2017; Halstead et al., 2018; Henry et al., 2019; Ross et al., 2021). This work does, however, highlight the sheer pervasiveness of fibres (Collard et al., 2017; Pazos et al., 2017; Ragusa et al., 2021; Ross et al., 2021), supporting the need for continued and improved assessment of their functional impact. Whilst 60% of fibres produced are synthetic (Carr, 2017), and as such can be classified under the microplastic umbrella, the notoriety of microplastics has driven an upsurge in 'alternative' or biobased fibre types to reduce plastic pollution and their negative impacts. This work assesses the potential for bamboo fibres as a 'green alternative' to plastic fibres, by testing their impacts on freshwater fish metabolism and disease resistance. The results of this study do not give an unadulterated green light for these fibres as they highlight that even nature-based fibres may be detrimental for fish welfare over extended periods of exposure.

Chapter 8 - General Discussion

With freshwaters facing worrying levels of extinction, primarily due to anthropogenic influences (Sala et al., 2000, Ormerod et al., 2010; Stendra et al., 2012) combined with infectious disease, we must work to mitigate these losses across all three freshwater systems: wild, aquaculture and ornamental. This thesis sought to provide a resource collating freshwater diagnostics and empirical data on the impacts of stressors on freshwater host-parasite dynamics. This was tackled around four subject areas: (1) assessment of aquatic diagnostics (past, present and future) available alongside methods with potential for translation to aquatic diagnostics from other fields, addressed in Chapter 2 with references throughout, (2) the establishment and insurance of pathogen stocks available for research in Chapter 3, (3) how do abiotic stressors (temperature and light), that are actively manipulated in aquaculture, impact freshwater fish and their parasites, addressed in Chapters 4 and 5, and (4) how do pollutants, stressors we do not actively control, impact freshwater fish and their parasites in Chapters 6 and 7.

Predominantly this thesis is framed around aquaculture, due to their economic importance and innate value to humanity. Chapter 2 aims to be broad in the assessment of diagnostic methods, being that those described may be used in any of the systems, be they wild, aquaculture or ornamental, however due to the controlled nature of the latter two systems, some methods may be more applicable. The data chapters (Chapters 3 to 7) utilise parasites which pose threats to all systems, and as such the results of those chapters should apply to all systems. Chapters 3 and 4, which use *Saprolegnia parasitica*, do have a focus on aquaculture due to the economic issue this parasite presents in that system, but as it is also a problem in the wild and ornamental systems, we can apply this knowledge to those systems also. Chapters 5, 6 and 7 use *Gyrodactylus turnbulli* as the model pathogen with the intent that the results can be transplanted to similar pathogen systems.

Diagnostics

Within the scope of aquatic diagnostics, methods vary in their complexity, from human visual observation through to artificial intelligent (AI) remote diagnosis (**Chapter 2**). These were broadly categorised as visual, microbiological and molecular diagnostics, with overlap between all three categories. Visual diagnostics are considered the “historical” diagnostic methods, where the presence of clinical signs is noted and investigated further (Noga, 2010). However, not all diseases present with distinct clinical signs (Bernoth & Crane, 1995; Beran et al., 2006; Noga, 2010), combine

this with visual observation of aquatic organisms often being difficult or obstructed (Evans et al., 2011; Raja & Jithendran, 2015), and visual diagnosis holds many areas for improvement. Further, if clinical signs are detectable, these are often conserved in aquatic diseases (Noga, 2010) compared to terrestrial diseases which tend to have more distinct characteristics (e.g. Deem et al., 2000; Galluzi et al., 2005; Iqbal et al., 2013). This often results in the need for closer inspection, however the handling of fish itself could exacerbate infection or create routes for secondary infection (Robertson et al., 1987; Ward & Beyens, 2015; Masud et al., 2019). Diagnosis without direct involvement is a more recent approach being undertaken using AI and remote sensing. Remote sensing allows visual diagnosis using remote controlled technologies (Zhang et al., 2013; Oh et al., 2017; Manegawa et al., 2019) whilst AI can automate diagnosis (Park et al., 2007). Despite increasing in complexity when compared to standard personal visual diagnosis, these methods share similar issues, with a lack of diagnostic specificity and requiring users with experience. Added onto this is the time and cost these technologies require to achieve efficiency (Khirade & Patil, 2015; Mohanty et al., 2016). These methods are effective for those with experience or small systems, but the issues drove development, progression and application of more complex techniques for diagnosis. PCR may arguably be the most significant development made for diagnostics (Bustin, 2010; Perkel, 2014). With the capability to diagnose aetiological agents which may be invisible to the naked eye, it can even allow for diagnosis of pathogens with no distinct clinical signs, in essence answering the issues with visual diagnosis (Zhu et al., 2020). From its inception in 1985 (Mullis et al., 1986; Zhu et al., 2020), PCR has undergone further development and optimisation to compensate for its shortcomings. Those being a lack of quantification, addressed by qPCR (Bustin & Vandesompele, 2009), and the potential for errors.

Aside from these diagnostics methods, the adaptation of terrestrial diagnostics is a route of interest. This has long been stymied by challenges, including issues of translation and a dearth of interest and funding (O'Farell, 2009). The recent surge in diagnostic development, primarily propelled by the urgency of terrestrial infections such as the COVID-19 crisis, has catalysed breakthroughs in diagnostics. Lateral flow immunochromatographic assays and adapted LAMP technology, initially constrained, have emerged as potent tools, delivering results within an unprecedented 90-minute window. These advancements, although transformative for human medicine, underscore a critical need for analogous progress in aquatic diagnostics. However, the biggest roadblock for this transition is lack of financial support and drive (Ung et al., 2021). Despite the current emphasis on rapid diagnostics, the translation of terrestrial diagnostic methods to aquaculture demands a significant

impetus. This challenge is not unique to aquatic diagnostics, as even human neglected diseases encounter similar hurdles. However, amidst these challenges, a comprehensive evaluation of diagnostic methods unveils promising pathways to advance aquatic diagnostics. Chapter 2 provides a resource which was lacking, a summary of diagnostic methods available for aquatic systems, where a further review solely focusing on terrestrial diagnostics with translation potential to aquatic systems may be of benefit. A weakness of Chapter 2 is that it will be a victim of time, where diagnostic methods will continue to develop and evolve meaning this piece of work will require updating.

Pathogens Stocks for Research

Diagnosis of pathogens is key, however research into pathogen impacts requires empirical testing, which in-turn requires pathogen stocks. Long-term storage of economically and ecologically relevant parasite is a focal point of research, particularly for research on elusive pathogens which rely on sourcing from wild outbreaks (**Chapter 3**). Ensuring an adequate viable supply of pathogens facilitates research and potentially allows for the creation of an archive (Boyle et al., 2003; Patwardhan & Lawson, 2016). Methods such as mineral oil immersion and cryopreservation have been successful for a plethora of organisms (e.g. Buell & Weston, 1947; Reischer, 1949; Boyle et al., 2003; Ravimannan et al., 2012), however this is not the case for all pathogens. The creation of an effective standardised protocol for preservation of fickle parasites is exemplified by *Saprolegnia parasitica*, for which effective long-term storage has been a challenge (Morris et al., 1988; Smith & Thomas, 1997). Recent works (Eszterbauer et al., 2020; Hardy et al., 2023) have highlighted successful methods for cryopreservation of *S. parasitica*, which Chapter 3 sought to verify on different isolates whilst comparing the less complex mineral oil immersion method. Within expectations, the method first trialled in Eszterbauer et al. (2020) was successful (100% resuscitation) regardless of strain and duration, though this was only up to a duration of 24 days frozen. The study contributes to our understanding of short- and mid-term preservation of *Saprolegnia parasitica* strains, connecting the diagnostic realm to the importance of preserving these pathogens for sustained research efforts. Further work would involve assessing preservation in the long-term (upwards of 6 months), utilising the resuscitated samples for infection trials, however cost-benefit analysis would be performed to assess if the potential loss of fish would be worth the outcome. Potentially alternative cryoprotectants and anti-dessicants could be assessed, however with the success observed with glycerol and hemp seeds respectively (Eszterbauer et al., 2020; Hardy et al., 2023) this is unnecessary.

These methods allowed for successful short- to mid-term storage of aquatic pathogens. Future work, in addition to the increased duration of storage (> 6 months), may assess the viability of these methods in creating an archive of *Saprolegnia* species and strains, to characterise genetic diversity among species, and allow for samples to be readily available worldwide for experimental use to tackle this problematic parasite group.

Natural Drivers – Temperature & Light

Transitioning from theoretical to practical applications, storage of pathogens provides pathogen stocks, in turn facilitating laboratory studies. When considering drivers of aquatic infection, they can be generally categorised into: natural drivers with a scope for anthropogenic manipulation, and those which generally lie out-with anthropogenic manipulation (e.g. pollution). In **Chapter 4,** two prominent environmental drivers, temperature and light, which are actively manipulated or controlled in different systems, are assessed on their impact on the *in vitro* growth of multiple *Saprolegnia parasitica* strains. Temperature is often manipulated to improve productivity within aquaculture (Pankhurst & King, 2010). The impact assessments of these drivers have primarily focused on hosts (Fletcher, 1986; Bly et al., 1997; Bowden, 2008; Valenzuela et al., 206; Ellison et al., 2021; Valenzuela et al., 2022), however their impacts on pathogens may be just as insightful. Temperature is known to influence sex, growth, reproduction and immunity (Islam et al., 2022; Lindmark et al., 2022), with some overlap for light (Ángeles Esteban et al., 2006; Taylor et al., 2006; Brown et al., 2014; Ellison et al., 2021). Marrying with previous literature (Koeypudsa et al., 2005; Liu et al., 2017; Tandel et al., 2021), it was found that *S. parasitica* grew optimally at 25°C regardless of strain which may suggest an increase in *S. parasitica* outbreaks following the rise in water temperatures due to global warming (Stewart et al., 2018; Sformo et al., 2021; Li et al., 2022). Interestingly, we found conflicting results between strains for the impact of light exposure, particularly when exposed to 18 L : 6 D. As only 2 strains were tested here, further work using multiple strains should be conducted to increase result robustness. The commonality observed was suppression/reduced growth of *S. parasitica* strains under 24 hours of light exposure, highlighting a potential mitigation method (chronotherapy). However, the impacts this light exposure would have on the fish hosts would likely outweigh the disease mitigation of this single pathogen (Owen et al., 2010; Vitt et al., 2017).

These results prompted further work into the impact of light exposure on pathogens, and how that impacts host-parasite dynamics (**Chapter 5**). Utilising the guppy-*G. turnbulli* host-parasite model system, the impacts of low (6 L : 18 D) and constant (24 L : 0 D) light exposure was assessed in regard to parasite burdens and feeding behaviours. The constant light was analogous to artificial light at night (ALAN), an increasing issue globally due to urbanisation (Gaston et al., 2015; Gaston et al., 2017). The maximum parasite burdens of fish exposed to ALAN was significantly higher than controls but this was the only observed change for parasite metrics. Light did not impact parasite burdens of pre-infected hosts, suggesting a low level of influence on infections once established. Feeding was significantly impacted by light exposure, with both latency to feed and bite count significantly differing between ALAN exposed fish and controls. Specifically, ALAN exposed fish fed faster and took more bites compared to control fish. This suggests the impacts ALAN may have on systems, potentially disturbing natural food webs through increased predation (built upon as ALAN is known to increase invertebrate emergence (Kurvers et al., 2018; Czarnecka et al., 2019) or causing ornamental fish to over-exert themselves whilst not having the resources to re-coup these losses. Whilst aquaculture often manipulates light, particularly salmonid aquaculture, to improve yields (Frenzl et al., 2014; Hou et al., 2019) whilst the ornamental industry may disregard light impacts by having store lights or tank light illuminating 24 hours a day. From the results in Chapters 4 & 5, chronotherapy as a treatment method may not show many positive qualities, however success has been found for other pathogens such as with *Argulus foliaceous*, where time-based targeting of treatments can improve their efficacy (Hunt et al., 2022). Bringing both aspects from Chapters 4 & 5 together would be the logical step forward, a multi-stressor analysis on how temperature and light together can impact host and their associated parasites, assessing similar parameters as in these studies.

Non-Natural Driver - Fibre Pollution

Having elucidated the effects of temperature and light on two parasite systems, the thesis then sought to establish the impacts of non-natural drivers, particularly pollutants. One of the most prevalent groups of pollutants for freshwaters currently are the microplastics. Typically defined as synthetic polymers smaller than 5 mm (Wang et al., 2020), the dominant proportion of these microplastics in aquatic systems are fibrous (Horton et al., 2017; Alak et al., 2022). Fibrous microplastics, and even non-plastic fibres, have been relatively understudied compared to their

granular counterparts. **Chapter 6** sought to address this by providing empirical data as to the impacts of textile fibres on fish and their associated parasites. Three different fibre types were assessed (all from commercial retailers) in their impacts on parasite burdens and parasite survival off-host: polyester a synthetic microplastic, cotton a common bio-based fibre, and bamboo a novel bio-based fibre. It was observed that fish exposed to polyester fibres had significantly greater maximum parasite burdens than controls, in contrast to other work which found no detrimental effects of polyester on fish health (Spanjer et al., 2020; Hu et al., 2020). This further adds empirical evidence towards the detrimental impacts of microplastics. Interestingly, fish exposed to bamboo fibres has significantly lower maximum parasite burdens compared to controls. Of note however is that the bamboo fibres are not 100% bamboo, but a mixture of 95% bamboo and 5% elastane, a synthetic polymer. Presently, the mechanism behind why these beneficial effects occurred is unknown, but combined with the results of the off-host survival, it indicates the effect is related to host immunity or tolerance (Tort et al., 2011). For the off-host survival, it was found that *G. turnbulli* exposed to either polyester or cotton fibres died significantly earlier than control worms. It was theorised this may have been due to the leached dyes from the fibres, however chromatographic analysis revealed it was likely (but not confirmed) that all fibre types were leaching the same dye compound. This exploration into fibre pollution, a dominant form of anthropogenic waste in freshwater environments, sheds light on potential detrimental impacts on organism welfare.

These results provided a clear route for further examination, specifically of bamboo fibres and the compounds with which they are treated/contain. **Chapter 7** sought to focus on bamboo fibres, based on the positive results obtained from Chapter 6. One key notion to address was that of '*greenwashing'* (Majláth, 2017), by which nature-based products are assumed to be beneficial or non-harmful inherently without substantiated support (de Freitas Netto et al., 2020). Whilst we seek to reduce the usage of plastics, there must be a suitable and effective alternative in place, verified by efficient testing (Yaseen & Scholtz, 2018; Hann et al., 2020). This chapter tested bamboo fibres obtained from clothing, raw bamboo fibres not yet formed for textile use and a common textile black dye (Reactive Black-5) on fish-parasite dynamics and host metabolism. In contrast to Chapter 6, there was no impact observed of either processed or un-processed bamboo fibres on parasite metrics, nor did the dye. This was potentially due to difference in maturation of the fish hosts, with fish in Chapter 6 being fully mature males but juveniles in this chapter, and for a shorter (14 days) period. The juvenile immune system may have been readily primed to tolerate infection compared to those in

Chapter 6 (Huang et al., 2020). There may have been impacts that were unobservable e.g. cellular changes or gene expression, warranting further study (Petitjean et al., 2019; Huang et al., 2020). The metabolism of host fish was impacted however, where fish exposed to the processed bamboo has, on average, a higher average routine metabolic rate (RMR) compared to controls. Whilst fish exposed to un-processed raw bamboo fibres has a lower average RMR compared to controls. Although we cannot say for certain, there is the potential for the additives in the processed bamboo fibres to be causing the observed increase in RMR (Yaseen & Scholtz, 2018; Tavares-Dias, 2021; Wood et al., 2021) but one might then expect this to also be seen in the RMR of dye exposed fish, which it was not. This chapter does provide support towards to move to alternative fibres such as bamboo but also highlights the avenues needed for further study, such as cellular level impacts and multi-stressor analysis on parasite dynamics and metabolism. In a world increasingly seeking sustainable alternatives, a nuanced understanding of their ecological impacts becomes imperative for informed choices. The results gained from both chapters 6 & 7 suggest positive potential for alternative fibres in regular textile usage, however further examination into genetic impacts or populations impacts would also be beneficial. Further, chapters 4 & 5 could be brought together with chapters 6 & 7 to investigate a large multi-stressor level experiment on the impacts of abiotic factors and pollution of freshwater hosts and their associated parasites.

In Summary

The overarching theme for this thesis was understanding the drivers for aquatic control and infections. The scope of aquatic diagnostics is summarised in Chapter 2, with notes on the potential for the transfer of techniques such as lateral flow from terrestrial to aquatic diagnostics given sufficient drivers. Ensuring adequate supplies of pathogen for research was successful using both mineral oil immersion and cryopreservation. When assessing the impact of temperature on *S. parasitica* growth, it was found that optimal growth occurred at 25°C regardless of strain. Light exhibited inhibitory effects on the growth of *S. parasitica* when exposure was constant, whilst increasing the maximum burden of *G. turnbulli* on guppy hosts during constant exposure. Light also influenced feeding behaviour, with constant exposure increasing bite count and reducing latency, whilst reduced light exposure increased latency and reduced bite count. Exposure to different fibre types resulted in greater maximum parasite burdens for fish exposed to polyester, whilst those exposed to bamboo had lower maximum parasite burdens. Polyester also reduced the survival duration of *G. turnbulli* off-host. Further investigation into bamboo and its associated dye revealed

no impacts on the parasite burdens of juvenile fish, but did impact respirometry of the fish host. Using literature and experimental work on two prevalent aquatic parasites, *Saprolegnia parasitica* and *Gyrodactylus turnbulli*, this body of work will act as a reference point for the scope of aquatic diagnostics available and how various stressors can impact host-parasite dynamics. As the global population surpasses 8 billion, fish remain a prominent source of protein around the world, and as such we must ensure we are using this resource responsibly and mitigating issues such as disease and pollution where we can.

Appendix 1

Figures S1 & S2 - Supplementary data for Chapter 3 showing the rate of increase for *Saprolegnia parasitica* strains under different light regimes. a) strain C65. b) strain A01.

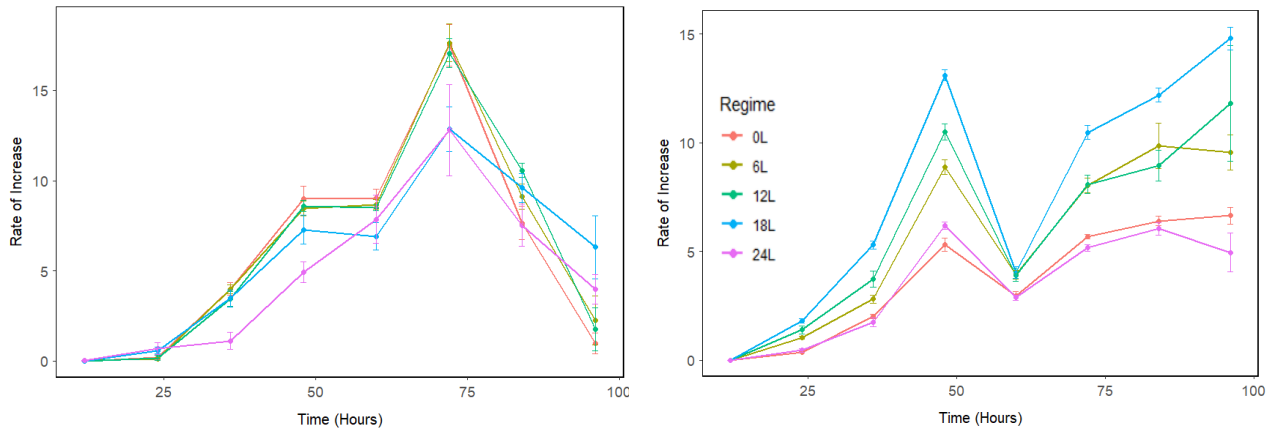

Figure S1. The rate of increase in growth between time points across 96 hours under differing light regimes (0, 6, 12, 18 & 24 hours of light). a) *Saprolegnia parasitica* strain CBS 223.65 (CBS223.65). b) *Saprolegnia parasitica* strain A01. The decrease shown in a) from 72 h onwards indicates the mycelial mats reaching the edge of the petri dish, with no room left to grow. The decrease observed at 60 h for b) is not area related, but a response for something not measured..

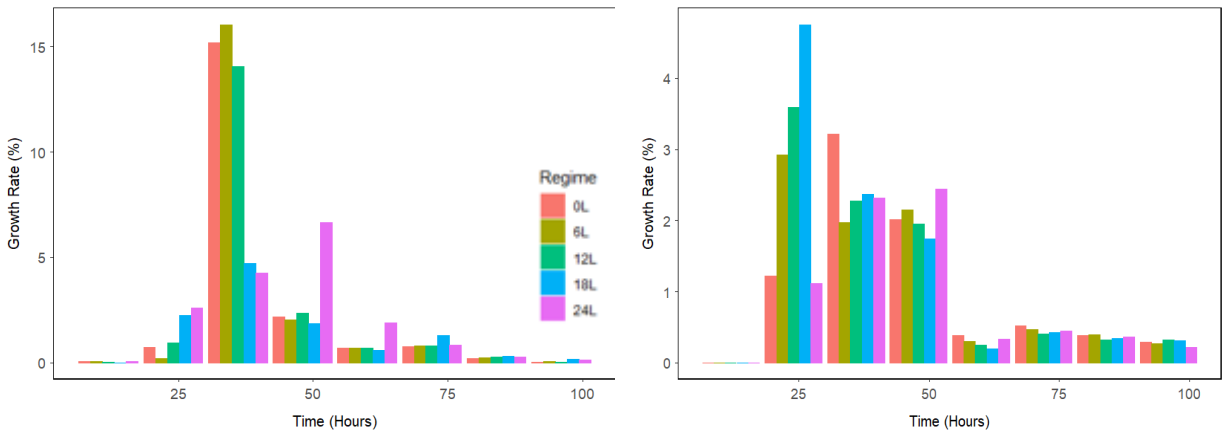

Figure S2. The vegetative growth rate of two *Saprolegnia parasitica* strains expressed as percentage growth from one time point to the next, where the y-axis represents the percentage change as a decimal value. a) strain CBS223.65. b) strain A01. Note the growth rate axis difference between a) and b).

Appendix 2

Tables S1, S2 & S3 – Supplementary data for Chapter 3 detailing the significant results obtained from post-hoc analysis comparing between light regimes.

Table S1. '*emmeans'* post-hoc analysis of the GLMM model for the impact of photoperiod and time on growth of *Saprolegnia parasitica* isolates CBS 223.65 and A01. Reported values are for all time points between which there were significant differences.

Time Point	*Saprolegnia parasitica* Strain	Light Regime Contrast	Estimate	Standard Error	t.ratio	*p* value
36	A01	0L:18L	-6.15	1.45	-4.24	0.0003
	A01	6L:18L	-4.24	1.45	-2.92	0.03
	A01	18L:24L	-6.39	1.45	-4.40	0.0002
48	A01	0L:6L	-6.54	1.45	-4.51	0.0001
	A01	0L:12L	-10.31	1.49	-6.94	<0.0001
	A01	0L:18L	-16.28	1.45	-11.22	<0.0001
	CBS223.65	0L:24L	12.86	3.96	3.25	0.01
	A01	6L:18L	-9.74	1.45	-6.72	<0.0001
	CBS223.65	6L:24L	11.58	4.04	2.87	0.04
	A01		5.63	1.45	3.88	0.001
	A01	12L:18L	-5.97	1.49	-4.01	0.008
	CBS223.65	12L:24L	11.13	4.04	2.76	0.05
	A01		9.41	1.49	6.33	<0.0001
	A01	18L:24L	-15.37	1.45	-10.60	<0.0001
60	A01	0L:6L	-7.81	1.45	-5.38	<0.0001
	A01	0L:12L	-11.56	1.49	-7.78	<0.0001
	A01	0L:18L	-17.68	1.45	-12.19	<0.0001
	CBS223.65	0L:24L	15.20	3.96	3.84	0.001
	A01	6L:18L	-9.87	1.45	-6.81	<0.0001
	CBS223.65	6L:24L	13.23	4.04	3.279	0.01
	A01		7.005	1.45	4.829	<0.0001
	A01	12L:18L	-6.13	1.49	-4.12	0.0005
	CBS223.65	12L:24L	12.45	4.04	3.09	0.02
	A01		10.75	1.49	7.23	<0.0001
	A01	18L:24L	-16.88	1.45	-11.64	<0.0001
	A01	0L:6L	-10.87	1.45	-7.49	<0.0001
	A01	0L:12L	-14.65	1.49	-9.86	<0.0001
	CBS223.65	0L:18L	17.03	3.96	4.302	0.0002
	A01		-23.89	1.45	-7.49	<0.0001
	CBS223.65	0L:24L	24.52	3.96	6.192	<0.0001

72	CBS223.65	6L:18L	15.30	4.10	3.728	0.002
	A01		-13.02	1.45	-8.98	<0.0001
	CBS223.65	6L:24L	22.87	4.10	5.552	<0.0001
	A01		10.758	1.45	7.416	<0.0001
	CBS223.65	12L:18L	13.41	4.04	3.322	0.01
	A01		-9.24	1.49	-6.21	<0.0001
	CBS223.65	12L:24L	20.90	4.04	5.176	<0.0001
	A01		14.54	1.49	9.78	<0.0001
	A01	18L:24L	23.78	1.45	16.39	<0.001
84	A01	0L:6L	-15.35	1.45	-10.58	<0.0001
	A01	0L:12L	-17.965	1.49	-12.08	<0.0001
	CBS223.65	0L:18L	12.34	3.96	3.116	0.02
	A01		-31.42	1.45	-21.66	<0.0001
	CBS223.65	0L:24L	24.78	3.96	6.259	<0.0001
	CBS223.65	6L:18L	13.50	4.04	3.337	0.01
	A01		-16.06	1.45	-11.07	<0.0001
	CBS223.65	6L:24L	25.91	4.04	6.421	<0.0001
	A01		15.71	1.45	10.83	<0.0001
	CBS223.65	12L:18L	14.50	4.04	3.592	0.004
	A01		-13.45	1.49	-9.05	<0.0001
	CBS223.65	12L:24L	26.94	4.04	6.676	<0.0001
	A01		18.32	1.49	12.32	<0.0001
	CBS223.65	18L:24L	12.44	4.23	2.940	0.03
	A01		31.77	1.45	21.91	<0.0001
96	A01	0L:6L	-19.12	1.45	-13.18	<0.0001
	A01	0L:12L	-24.66	1.49	-16.59	<0.0001
	A01	0L:18L	-41.99	1.45	-28.95	<0.0001
	CBS223.65	0L:24L	18.84	3.96	4.760	<0.0001
	A01	6L:12L	-5.54	1.49	-3.72	0.002
	A01	6L:18K	-22.87	1.45	-15.77	<0.0001
	CBS223.65	6L:24L	22.46	4.04	5.568	<0.0001
	A01		21.67	1.45	14.94	<0.0001
	A01	12L:18L	-17.33	1.49	-11.66	<0.0001
	CBS223.65	12L:24L	22.54	4.04	5.586	<0.0001
	A01		27.21	1.49	18.31	<0.0001
	CBS223.65	18L:24L	16.96	4.23	4.008	0.0008
	A01		44.55	1.45	30.71	<0.0001

Table S2. '*emmeans'* post-hoc analysis of the GLMM model for the impact of photoperiod and time on growth rate of *Saprolegnia parasitica* isolates CBS 223.65 and A01. Reported values are for all time points between which there were significant differences. * Denotes values which border on significance.

Time Point	*Saprolegnia parasitica* Strain	Light Regime Contrast	Estimate	Standard Error	t.ratio	*p* value
24	A01	0L:6L	-169.72	30	-5.65	<0.0001
	A01	0L:12L	-236.75	30.7	-7.70	<0.0001
	A01	0L:18L	-352.69	30	-11.761	<0.0001
	A01	6L:18L	-182.96	30	-6.10	<0.0001
	A01	6L:24L	180.25	30	6.01	<0.0001
	A01	12L:18L	-115.93	30.7	-3.77	0.001
	A01	12L:24L	247.28	30.7	8.04	<0.0001
	A01	18L:24L	363.22	30	12.11	<0.0001
36	A01	0L:6L	123.62	30	4.12	0.0004
	A01	0L:12L	93.50	30.7	3.04	0.02
	A01	0L:18L	84.74	30	2.82	0.04
	CBS223.65	0L:18L	10.12	1.30	7.80	<0.0001
	A01		-6.15	1.45	-4.21	0.0003
	CBS223.65	0L:24L	10.92	1.30	8.41	<0.0001
	A01		89.67	30	2.99	0.02
	CBS223.65	6L:18L	10.97	1.32	8.28	<0.0001
	A01		-4.23	1.45	-2.92	0.03
	CBS223.65	6L:24L	11.77	1.32	8.89	<0.0001
	CBS223.65	12L:18L	8.99	1.35	6.64	<0.0001
	CBS223.65	12L:24L	9.79	1.35	7.23	<0.0001
	A01	18L:24L	6.38	1.45	4.40	0.0002
48	CBS223.65	0L:24L	-4.48	1.30	-3.451	0.0056*
	CBS223.65	6L:24L	-4.64	1.32	-3.50	0.005
	CBS223.65	12L:24L	-4.31	1.32	-3.26	0.01
	CBS223.65	18L:24L	-4.77-	1.39	-3.44	0.0059*

Table S3. '*emmeans'* post-hoc analysis of the GLMM model for the impact of photoperiod and time on rate of increase of *Saprolegnia parasitica* isolates CBS 223.65 and A01. Reported values are for all time points between which there were significant differences.

Time Point	*Saprolegnia parasitica* Strain	Light Regime Contrast	Estimate	Standard Error	t.ratio	*p* value
36	A01	0L:18L	-3.27	0.74	-4.34	0.0001
	A01	6L:18L	-2.48	0.74	-3.36	0.007
	A01	18L:24L	3.55	0.74	4.80	<0.0001
48	A01	0L:6L	-3.55	0.74	-4.80	<0.0001
	A01	0L:12L	-5.18	0.76	-6.84	<0.0001
	A01	0L:18L	-7.78	0.74	-10.52	<0.0001
	CBS223.65	0L:24L	4.11	1.14	3.62	0.003
	A01	6L:18L	-4.23	0.74	-5.72	<0.0001
	CBS223.65	6L:24L	3.60	1.16	3.08	0.02
	A01		2.67	0.74	3.62	0.003
	A01	12L:18L	-2.60	0.76	-3.43	0.006
	CBS223.65	12L:24L	3.20	1.16	3.20	0.01
	A01		4.31	0.76	5.69	<0.0001
	A01	18L:24L	6.91	0.74	9.34	<0.0001
72	A01	0L:6L	-2.35	0.74	-3.18	0.013
	A01	0L:12L	-2.38	0.76	-3.14	0.015
	A01	0L:18L	-4.77	0.74	-6.45	<0.0001
	CBS223.65	0L:18L	4.44	1.14	3.91	0.001
	CBS223.65	0L:24L	4.70	1.14	4.14	0.0004
	CBS223.65	6L:18L	4.57	1.18	3.86	0.001
	A01		-2.41	0.74	-3.27	0.01
	CBS223.65	6L:24L	4.83	1.18	4.10	0.0005
	A01		2.88	0.74	3.90	0.001
	CBS223.65	12L:18L	4.25	1.16	3.45	0.006
	A01		-2.39	0.76	-3.15	0.01
	CBS223.65	12L:24L	4.26	1.16	3.68	0.002
	A01		2.91	0.76	3.84	0.001

	A01	18L:24L	5.30	0.74	7.17	<0.0001
84	A01	0L:6L	-3.44	0.74	-4.66	<0.0001
	A01	0L:12L	-2.54	0.76	-3.36	0.007
	A01	0L:18L	-5.79	0.74	-7.82	<0.0001
	A01	6L:18L	-2.34	0.74	-3.16	0.01
	A01	6L:24L	3.80	0.74	5.15	<0.0001
	A01	12L:18L	-3.24	0.76	-4.27	0.0002
	A01	12L:24L	2.98	0.76	3.83	0.001
	A01	18L:24L	6.15	0.74	8.31	<0.0001
96	A01	0L:6L	-2.89	0.74	-3.91	0.001
	A01	0L:12L	-5.14	0.76	-6.97	<0.0001
	CBS223.65	0L:18L	-5.28	1.14	-4.64	<0.0001
	A01		-8.13	0.74	-11.0	<0.0001
	A01	6L:12L	-2.25	0.76	-2.97	0.02
	CBS223.65	6L:18L	-4.02	1.16	-3.47	0.005
	A01		-5.24	0.74	-7.08	<0.0001
	A01	6L:24L	4.59	0.74	6.20	<0.0001
	CBS223.65	12L:18L	-4.50	1.16	-3.89	0.001
	A01		-2.98	0.76	-3.94	0.0009
	A01	12L:24L	6.84	0.76	9.02	<0.0001
	A01	18L:24L	9.83	0.74	13.29	<0.0001

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