A fishy tale: the impact of multiple stressors on host behaviour, physiology, and susceptibility to infectious disease

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Table of Contents

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Collaborations

"*Science is a collaborative effort. The combined results of several people working together is often much more effective than could be that of an individual scientist working alone*."

John Bardeen, from his second Nobel Prize Banquet speech

John Bardeen, the only Physicist to be awarded a Nobel Prize twice, summed upped scientific collaborations perfectly. This PhD is the product of several scientific collaborations, whom I acknowledge here.

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Abstract

Aquatic habitats are facing increased anthropogenic stressors that are associated with multiple species demise. The loss of species is, sadly, part of a wider global crisis with current extinction levels estimated to be a thousand-fold higher than the background extinction rate. Freshwater habitats in particular are facing higher rates of degradation than any other habitat and within these, fish species are being lost faster than their terrestrial counterparts. Beyond the importance of fish as keystone species, they are an invaluable source of protein for humanity and stocks are facing a state of collapse. A key threat facing the fish industry, including wild stocks, is increased infectious disease burdens. A major reason we are witnessing a continued increase in losses to disease is because fish species are experiencing increased stressors that are compromising host welfare that in turn impacts disease susceptibility.

This PhD project focussed on how fish welfare is impacted by different biotic and abiotic stressors, with an emphasis on host immunity and disease resistance. To accomplish this project, freshwater fish host-parasite models were utilised permitting long-term monitoring of infections in real time. Most stressors investigated negatively impacted fish disease resistance, with the first stressor investigated being mechanical disturbance associated with routine transportation practices. Beyond the increased susceptibility to disease seen in fish hosts exposed to stressors, this PhD also revealed that noise pollution significantly increases host mortality rates. However, in response to the ecological stressor, flow, no changes to fish immune gene expressions or pathogen burdens were seen. With regards to implementing simple measures for effective disease control, this project has revealed how the addition of structural enrichment to fish tanks significantly improved disease resistance while also reducing agnostic behaviour. The universal physiological cost of infection by significantly increasing host metabolic rates was also revealed. The final experimental study assessed how one of the most prevalent contaminants, microplastic, impacted host disease resistance, growth and mortality. Microplastic at variable concentrations significantly increased disease susceptibility and host mortality. Ultimately, this PhD project has furthered our understanding of how multiple emerging and widespread stressors are impacting fish host welfare through the lens of host-pathogen dynamics.

Chapter 1

General introduction

1.1.Why fish welfare?

Fish are a paraphyletic group and comprise the most abundant vertebrates on the planet, with over 34,000 known species (FishBase, 2020). Beyond their taxonomic significance, fish are found in nearly all aquatic habitats, with the possible exception of the deepest 25% of the oceans (Yancey et al., 2015) and therefore, unsurprisingly, fish also constitute keystone species in many aquatic habitats (Collen et al., 2014; Dudgeon et al., 2006). Alas, the rate of extinction of fish seriously threatens their diversity (Pimm et al., 2014). For freshwater fish, in particular, the available data on species extinction suggests a higher rate than any other terrestrial vertebrate group (Dudgeon et al., 2006; Adams et al., 2014). Furthermore, freshwater fish as a group remain incredibly data deficient; with only 5685 of the 15000 species being assessed for their conservation status (IUCN Red List, 2020). Despite many local initiatives existing that are aimed at preventing fish species extinction (see Maceda-Veiga et al., 2016; King, 2019), emphasis still tends to be placed on charismatic 'poster' species. With fish playing a key role in food chains and ecosystem stability, the 'silent extinction' of fish may have far reaching ecological implications.

For humanity, fish occupy a key role. We consume more fish than all other animals combined (except poultry) and aquaculture continues to be the fastest growing food sector globally (FAO, 2018). Even within the food sector, the 'silent extinction' of fish is gaining attention, with 63% of fish stocks facing collapse (Worm et al., 2009, 2016). This is particularly concerning for native island populations whose livelihoods depend on reliable fish stocks and with trawling decimating wild fish populations, the stability of fish populations must be secured to prevent island communities from facing ruin (Herbert et al., 2019). Beyond their role as a vital food source, fish are also companion animals, indeed being the most populous pets in western households (American Pet Products Association, 2012). Despite their economic and cultural importance, fish welfare has been secondary to that of terrestrial vertebrates, possibly as the knowledge that fish can neurologically respond to pain emerged only in 2003 (Sneddon et al., 2003).

The internationally recognised framework for animal welfare was proposed by the UK Farm Animal Welfare Council in 1979 - the Five Freedoms (The National Archives, 2020). Since its inception, the Five Freedoms have been adopted as 'the golden standard' by many animal welfare organisations, the most notable being the World Organisation for Animal Health (OIE Aquatic Animal Health Code, 2019). The five freedoms are applicable for fish welfare in a broad context, with modifications made as and when necessary (see Braithwaite et al., 2013). The third freedom as currently listed - freedom from pain, injury and disease – is arguably, the greatest welfare concern with transmissible diseases caused by parasites plaguing the fish industry.

1.2.Host-parasite dynamics: The Red Queen's game

Parasites that cause infectious disease are a global issue for the animal trade (Oidtmann et al., 2013). A parasite is any organism that is metabolically dependant on a host and causes some degree of harm. In terms of economic loss, the burden imposed by disease related mortality in the fish trade is a major concern (FAO, 2018; Stentiford et al., 2017). The cost imposed by disease for aquaculture goes beyond host mortality and includes the cost of treatment and prevention (Oidtmann et al., 2011; Stentiford et al., 2017). Beyond their impacts in the fish trade, parasites are now recognised as the 'dark matter' of ecosystems, dominating the biomass of surveyed habitats (Kuris et al., 2008) and because they impact all aspects of host life history traits, their functional importance in ecosystem food chains is now clear (Poulin, 1999; Lefèvre et al., 2009).

The relationship between hosts and their parasites is modulated by the host's immunity. Fish, like mammalian vertebrates, possess elements of both innate and adaptive immunity. The innate immune system provides a non-specific first line of immune defence with phagocytic activity and secretion of antimicrobial molecules (Tort, 2011). The adaptive immune response leads to the generation of random and highly diverse repertoires of T and B-lymphocyte receptors encoded by recombinant activation genes (RAGs) and this contributes to a more specific and efficient response against infections (reviewed by Alvarez-Pellitero, 2008). The fish host's immune response and the parasites' ability to evade or resist cellular and chemical responses leads to the host-parasite arms race typified by Red Queen selection dynamics, omnipresent in both natural and captive environments (Salathe et al., 2008). Parasites are not the only factor modulating teleost immunity. Environmental stressors have a significant impact on fish immunity (reviewed in Tort, 2011). A stressor can be defined broadly as any stimulus that instigates a stress response, which causes biological changes within an organism (Barton, 2002; Tort, 2011). One of the tertiary level effects of long-term stress responses is changes to disease resistance due to suppressed immunity (reviewed in Tort, 2011). And now we are becoming increasingly aware that many stressors that fish are exposed to under captivity can impact immunity and resistance to infectious disease, such as poor water quality (Ackerman et al., 2006; Smallbone et al., 2016) and nutritional deficiency (Xu et al., 2016).

Natural stressors are often a result of oscillatory patterns in ecosystems such as seasonality that leads to variations in temperature and access to resources that will always be a constraint on host immunity and as a result, disease resistance (Brown et al., 2016; Jackson et al., 2020). However, anthropogenic stressors are now so consistently pervasive within aquatic habitats, that there is no doubt they are implicated in global species decline (Pimm et al., 2014; Adams et al., 2014). Mounting evidence suggests that disturbed habitats can drastically change the diversity of parasites on fish hosts, for example, by reducing overall species diversity but increasing specific parasite species abundance (Dušek et al., 1998; Dunne et al., 2013). Fundamentally, however, hosts that are stressed are more likely to succumb to parasitic infections, which even if mortality does not ensue, may lead to changes in foraging, mate choice and reproductive output and therefore the structure of populations (reviewed by Lefèvre et al., 2009; Hatcher et al., 2012). However, much remains to be understood about these most fundamental organism interactions and how they are responding to increasing environmental change in the Anthropocene.

1.3. Utilising models: ideal study systems

A model organism is a non-human species that is extensively studied to understand biological processes with the expectation that the discoveries made in those model organisms will provide generalisable insight into what occurs in other organisms (Fields & Johnston, 2005). To this extent, this PhD project utilises two ecological and parasitological model organisms, the Trinidadian guppy (*Poecilia reticulata*) and the three-spined stickleback (*Gasterosteus aculeatus*) (Figure 1.1).

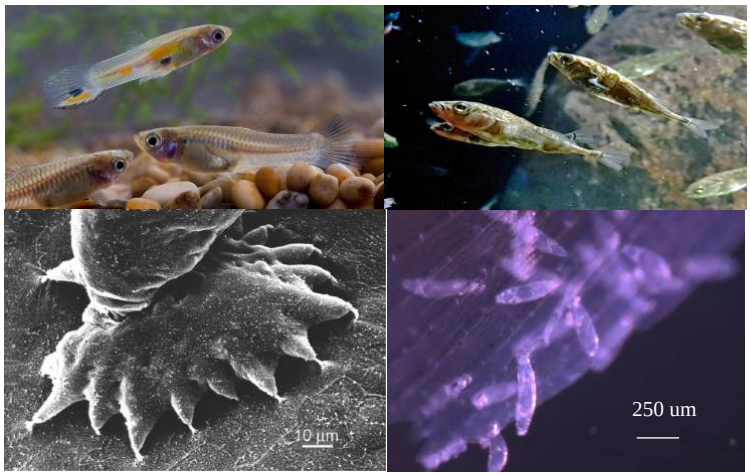

Figure 1.1. The model fish hosts, and parasites used for this PhD. Shown here; the guppy (*Poecilia reticulata*, top left), the three-spined stickleback (*Gasterosteus aculeatus*, top right); the ectoparasite *Gyrodactylus turnbulli* on the tail of a guppy (bottom picture) and scanning electron microscopy of *Gyrodactylus salaris* opisthaptor attached to Atlantic salmon (Bakke et al., 2007).

The guppy is a tropical freshwater fish native to Antigua and Barbuda, Barbados, Brazil, Guyana, Jamaica, the Netherlands Antilles, Trinidad and Tobago, the U.S. Virgin Islands, and Venezuela and one of the most globally distributed aquatic pet species (FishBase, 2020; Global Invasive Species Database, 2020). Their popularity is in large part due to their adaptability to different environmental conditions and live bearing capacity, which makes them easy to breed under captive conditions (Magurran, 2005). Guppies have now been introduced to every continent except the Antarctic and unsurprisingly have been studied extensively as ecological models (Magurran et al., 2001a). Being sexually dimorphic, the males have evolutionary selected phenotypes which also makes them ideal for behaviour and reproductive studies (Khoo et al., 1999). The three spined stickleback is common and easily found in most inland coastal water bodies north of 30^0 and combined with their hardiness is a major reason for their suitability as a laboratory species. Ecologically, this species shows great morphological variation over multiple habitats, which has made it an excellent subject for evolutionary and population genetic studies (Behm et al., 2011). Furthermore, their elaborate breeding behaviour, with males showing extreme territoriality, nest building, and egg and fry caring behaviour combined with their sociality has made them popular for ethological studies (e.g., Milinski, 1987; Braithwaite & Barber, 2000). Another key reason the guppy and stickleback are model organisms is because both have annotated genomes (Jones et al., 2012; Künstner et al., 2016). Perhaps most importantly, for this PhD, both species have had their

parasite fauna extensively studied and catalogued and the stickleback in particular is incredibly rich with parasite fauna (Cable, 2011; Barber, 2013).

The parasite species utilised for this PhD project belong to the genus *Gyrodactylus*. This highly species rich genus contains fish pathogens that have impacts on both ecosystems and the fish trade (Bakke et al., 2007). This genus is constantly updating with the addition of new or novel introduced species. These fish ectoparasites are well known for their hyperviviparous and precocious reproduction (gaining them the name 'Russian dolls'), where a parasite is born with a developing F1 generation *in utero*, which also contains the next generation (F2 offspring) (Cable & Harris, 2002). Therefore, it is not uncommon to witness parasite populations exploding exponentially on hosts and spreading rapidly through populations (Johnson et al., 2011). These notorious fish pathogens are microparasites and are directly transmitted from host-to-host without intermediates and typically for transmission to occur some form of social interaction between fish is required (Richards et al., 2012). To complicate matters, gyrodactylids can survive for brief periods in the water column or on benthic surfaces and in the case of *G. salaris*, dead hosts (Olstad et al., 2006) and infections can result via these routes. As gyrodactylids are socially transmitted, these parasites often find themselves under ideal transmission scenarios within captive stocking conditions where host densities can be extremely high (see Johnson et al., 2011). The most notorious species, *G. salaris*, is a notifiable disease of global importance, which is unsurprising given its historical precedence of nearly wiping out populations of Norwegian salmon (Johnson, 1978). Even within the ornamental trade, this genus is replete with species that plague the aquarium trade (Maceda-Veiga & Cable, 2019).

While this PhD thesis does not majorly focus on host immunity to gyrodactylosis, elucidating underlying immunity towards these hyper-prevalent pathogens has remained a challenge and our understanding is far from complete (reviewed in Bakke et al., 2007). However, host innate and acquired immunity have both been shown to play a role in disease resistance. Innate immunity certainly plays a key role in fighting off these infections, with off host investigations demonstrating that gyrodactylids are effectively killed with complement factors extracted from fish serum (see Harris et al., 1998, Buchmann, 1998). Even with immunological gene expression studies, increased expression of genes associated with pro-inflammatory cytokines is significantly associated with effective gyrodactylid clearance (Lindenstrom et al., 2003, 2004 but also see Buchmann & Bresciani, 1998 for microenvironment analysis of mucosal cells). Regarding acquired immunity, epidemiological studies reveal that fish with prior history of infections mount a more effective response to secondary infections (see van Oosterhout & Cable, 2007). Interestingly, studies have yet to detect specific antibodies in response to gyrodactylid infections (see Lindenstrom et al., 2003 and Zhou et al., 2018 for example). However, very recently MHC supertype depletion within guppy strains have been implicated in *G. turnbulli* resistance in guppies (Smallbone et al., 2021). Conversely, *G. kobayashii* infections in goldfish (*Carassius auratus*) did not affect MHC supertypes expression (see Zhou et al., 2018) which may be unsurprising considering the sheer diversity of gyrodactylid species, which would lead to species level differences in immunity (see Bakke et al., 2007).

Despite our growing understanding behind gyrodactylid immunity, effective *en masse* treatments do not exist for any species (Schelkle et al., 2009). The implications of this are significant because even if a treatment reduces infection load in most individuals within a population, a single surviving parasite is enough to initiate an epidemic. Ecologically, gyrodactylid species are known to impact major aspect of life history behaviours such as mate

choice and reproduction (Kennedy et al., 1987), shoaling (Reynolds et al., 2018) and food acquisition (Kolluru et al., 2008). For this PhD project, the gyrodactylid species utilised are *G. turnbulli*; the primary monogenean ectoparasite for guppies and *G. gasterostei*; one of the primary monogenean parasites for three-spined sticklebacks.

1.4.PhD outline

This PhD project investigates how multiple stressors (anthropogenic and ecological) impact fish welfare utilising freshwater host-parasite models. While the major welfare parameter considered is susceptibility to disease with emphasis on the infection phenotype, fish behaviour, metabolic rates, growth and mortality are also recorded.

The bulk of the thesis consists of five data chapters followed by a review with two experimental Appendices. In Chapter 2 the impacts of transportation practices on disease susceptibility and host mortality are studied. Chapter 3 investigates the impact of acute and chronic noise pollution on fish health. The biological costs of depriving fish of environmental enrichment are shown in Chapter 4 by measuring changes to host behaviour, metabolic rate and disease resistance. Then, Chapter 5 focusses on one of the most recalcitrant environmental pollutants, microplastic, and how its consumption by fish impacts their growth, mortality and disease resistance. The final experimental chapter, Chapter 6, demonstrates how an ecological stressor, flow, constrains immunity and impacts disease resistance. Chapter 7 provides a wider context for the preceding data chapters by reviewing the current biosecurity policies in relation to fish disease prevention and control and concludes with a recommendation for integrated fish welfare strategies with biosecurity. The thesis closes in Chapter 8 with a general discussion on the implications of all the experimental results gathered throughout the entire PhD project.

1.5. Ethics statement

All animal work undertaken during this thesis was approved by Cardiff Universities Animal Ethics Committee and conducted under UK Home Office Licence (PPL 303424) following ARRIVE guidelines.

Chapter 2

A neglected fish stressor: mechanical disturbance during transportation impacts susceptibility to disease in a globally important ornamental fish

This chapter is affiliated with the publication Masud et al., 2019a in the journal Diseases of Aquatic Organisms

2.1. Abstract

The transport of fish in aquaculture and the ornamental trade exposes fish to multiple stressors that can cause mass mortalities and economic loss. Previous research on fish transport has largely focused on chemical stress related to deterioration in water quality. Mechanical disturbance during routine fish transport, however, is unpredictable and is a neglected potential stressor when studying fish welfare. Stress induced immunosuppression caused by mechanical disturbance can increase the chances of contracting infections and significantly increase infection burden. Here, using the model guppy-*Gyrodactylus turnbulli* host-parasite system and a new method of bagging fish (Breathing Bags ™), which reduces mechanical disturbance during fish transport, investigations were conducted on how parasite infections contracted after simulated transport impact infection trajectories on a globally important ornamental species. Guppies exposed to mechanical transport disturbance suffered significantly higher parasite burden compared to fish that did not experience transport disturbance. Unfortunately, there was no significant reduction in parasite burden of fish transported in the Breathing Bags ™ compared to standard polythene carrier bags. Thus, transport induced mechanical disturbance, hitherto neglected as a stressor, can be detrimental to disease resistance and highlights the need for specific management procedures to reduce the impact of infectious diseases during routine fish transport.

2.2. Introduction

For the animal industry, transportation can lead to maladaptive traits, including reduced feeding, altered immune response and mortality (Cattle: Stockman et al., 2013, Swine: Zou et al., 2017, Poultry: Matur et al., 2016, Fish: Momoda et al., 2007, Castro et al., 2016). Although the impact of transport stress is a general animal welfare issue, priority of research has been placed on terrestrial livestock (Schwartzkopf-Genswein et al., 2012) over aquatic species. Furthermore, current research on transport stress in fish focusses on food fish and neglects the ornamental trade (Ashley 2007; Stevens et al., 2017) despite fish being the most abundant pet in western households (American Pet Products Association, 2012). Indeed, with over 4500 freshwater fish and 1450 marine fish species traded globally as pets, the ornamental trade is a lucrative business valued at US $800 million to $30 billion annually (Stevens et al., 2017) and this demand for ornamentals is increasing with expansion of the global pet trade (Saxby et al., 2010). Increased fish transport is an inevitable consequence of rising demands for exotic

species and emphasis on meeting these demands includes minimising transport costs which may lead to fish being transported in sub-optimal conditions.

Stressors experienced by fish during transport can lead to immunosuppression, with the proposed mechanism linked to the release of catecholamines and glucocorticoid hormones as a stress response (Barton, 2002; Ackerman et al., 2006), which may increase disease susceptibility (Caruso et al., 2002; Ramsay et al., 2009). Experimental investigations into transport related stressors and disease susceptibility are limited, and the link between a stress event and suppressed immunity is far from clear in fish studies (see Tort, 2011 for review). Whether a stressor is chronic or acute will often determine how the immune system responds with certain stress events enhancing or suppressing immune pathways (Dhabbar, 2000). For example, chronic stress in Atlantic salmon (absence of enrichment) was found to suppress transcriptional immune responses to pathogenic challenge, whereas acute stress (cold-shock treatment) enhanced it (Webster et al., 2018). Thus, with transport stressors that remain under the radar, we remain in the dark on how the immune system responds. Further complications arise when variations in susceptibility to disease are linked to both host and pathogen species making the outcome of transportation on fish welfare uncertain. Chinook salmon (*Oncorhynchus tshawytscha*) and ayu (*Plecoglossus altivelis*), for example, exposed to transport conditions showed increased susceptibility to bacterial infections (Iguchi et al., 2003; Ackerman et al., 2006). Channel catfish (*Ictalurus punctatus)* that experienced low water crowding stress as part of simulated transport conditions, only showed increased susceptibility when exposed to *Ichthyophthirius multifiliis*, but not to inoculation with the channel catfish virus (Davis et al., 2002).

Typically, fingerlings, juveniles and small fish are transported in plastic bags, filled with 25-30% water and 70-75% air or pure oxygen (Carneiro & Urbinati 2001; Conte, 2004). Presence of air pockets in polythene bags for fish increases the chances of mechanical stress due to water movement. In mechanical terms, stress is defined as a force applied across a surface per unit area for all orientations of that surface (Chen & Han 2007). However, from a biological perspective and for the purposes of this study mechanical stress will be defined as any physical disturbance that induces a stress response and has a measurable impact on welfare. In addition to potential mechanical stress, accumulation of carbon dioxide from respiring fish can lead to displaced available oxygen, especially if stocking densities are high (Conte, 2004). Thus, traditional transport carriers can expose fish to multiple stressors, including capture, handling, overcrowding, abrupt changes in temperature and physical trauma (Robertson et al., 1988; Stevens et al., 2017). A decline in water quality caused by the accumulation of ammonia, fluctuations in dissolved oxygen and pH, which are known fish stressors, is another consequence of transportation (Patterson et al., 2003; Dhanasiri et al., 2011, Ackerman et al., 2006). Micro-porous transport bags (Breathing Bags ™, Kordon ®) unlike traditional polythene bags allow exchange of respired carbon dioxide with atmospheric oxygen. Being porous the bags can be completely filled with water (without the need to add air or oxygen) and since water is incompressible relative to air, this should provide natural cushioning for fish being transported (Thiagarajan et al., 2011). The impact of many of the stressors associated with traditional fish transport (highlighted above) on fish welfare and techniques to alleviate them have been investigated (water quality: Ackerman et al., 2006; Dhanasiri et al., 2011, capture and handling: Caruso et al., 2002; Thompson et al., 2016, stocking densities: Ramsay et al., 2009) whereas mechanical stress has thus far remained entirely neglected.

The transport procedure for fish varies globally depending on local animal trading laws and whether fish are transported locally or internationally. The latter routinely involves fish quarantine procedures before transport and border inspections post-arrival (Portz et al., 2006). In addition, such fish will experience extended transport disturbance including multiple handlings due to inspections. Fish transportation procedures typically lack routine screening procedures for parasites and is therefore a wide-scale welfare issue (Ashley, 2007; Stevens et al., 2017). Ornamentals transported from the wild or local pet shops may be reservoirs of undiagnosed infections that become more pernicious due to stress-imposed immunosuppression following transport (Bonga, 1997). Due to mixing of species from different geographic regions, disease dynamics in wholesalers, retailers and hobbyist aquaria may result in parasite host switching and increased virulence (Kelly et al., 2009). Accidental or intentional introduction of exotic species into local fish populations can cause transmission of highly virulent parasites to which native fish species may be especially susceptible (Smit et al., 2017).

Ornamental fish trade practices routinely involve the addition of antiparasitic chemicals into water and removal of weak or diseased fish which reduces disease outbreaks and keep parasite numbers to a minimum (Stevens et al., 2017). Diseases with distinctive symptoms, such as those caused by *Ichthyophthirius multifiliius* or *Saprolegnia parasitica*, are relatively easy to detect through visual inspection of fish, leading to either quarantine or euthanizing infected individuals to halt spread of infections (FAO, 2012; Stentiford et al., 2017). Such standard practice for fish farmers and hobbyists does reduce maintenance cost and for legal reasons many countries only sell or display fish that appear healthy (Washington & Ababouch, 2011). However, many parasites at low levels of infection do not affect fish phenotype, making them undetectable to non-specialists. Ectoparasites, such as *Gyrodactylus* species, typically require thorough microscopic examination to determine parasite burden (Maceda-Veiga & Cable, 2018), which is not a routine procedure for fish at any point in the aquaculture or ornamental trade. For gyrodactylosis, there is no 100% effective treatment and parasites can remain at low frequencies in fish populations that are being transported and then in favourable conditions they can increase exponentially until stock survival is severely affected (Cable, 2011). Thus, even if species harbour low-level infections due to the presence of anti-parasitic chemicals, stressful transport conditions can sufficiently weaken the immune system allowing large infection sources to be established in healthy stocks.

Amongst the most popular tropical fish species is the guppy (*Poecilia reticulata*, see Maceda-Veiga, 2016), which has been transported worldwide as an ornamental and biological control agent, with 41 recorded introductions outside its native habitat (Magurran, 2005). The most common parasites of wild and ornamental guppies are viviparous monogenean *Gyrodactylus* spp. known for their ‘Russian doll’ reproduction and direct transmission (Cable, 2011). This makes them capable of rapidly colonising a fish population, affecting their behaviour, including courtship, feeding and shoaling (Kennedy et al., 1987; Kolluru et al., 2009; Hockley et al., 2014) and survival (Cable & van Oosterhout, 2007; Yamin et al., 2017).

Here investigations were conducted to determine the impact of simulated transportation on fish infection dynamics. Specifically, assessments were made on how mechanical disturbance associated with traditional polythene carrier material impacts susceptibility to disease in fish exposed to parasites after simulated transport. In addition, the efficacy of Breathing Bags ™ in helping alleviate mechanical disturbance-induced elevated disease susceptibility and mortality were tested.

2.3. Materials and methods

2.3.1. Host and parasite species maintenance

Male guppies (standard length: 12.1-17.4 mm) bred from a stock originating in the Lower Aripo River in Trinidad, were initially housed at Exeter University before being transferred to Cardiff University in October 2014. Guppies were kept in 70 L breeding tanks, containing artificial plants and refugia. They were maintained under a 12 h light: 12 h dark photoperiod (lights on 07:00-19:00) at 24 ± 1°C and fed daily on dry food flake (Aquarium ®) and every alternate day on live freshly hatched *Artemia* nauplii. Experimental infections utilized the Gt3 strain of *Gyrodactylus turnbulli*, isolated from a Nottingham aquarium shop in October 1997 and subsequently maintained at Cardiff University since 1999 on inbred guppies prior to this study.

2.3.2. Experimental design

To test the impact of traditional polythene bags versus Breathing Bags ™ on fish susceptibility to disease, guppies (20 per experimental treatment) were experimentally infected after experiencing simulated transport. All guppies were netted carefully from breeding tanks to minimise handling stress and transferred to separate tanks for a 24 h holding period. Fish were not fed for the holding period to ensure a post-absorptive stage and to minimise build-up of nitrogenous waste, as per standard aquacultural practice (Berka, 1986). To simulate transport stress, fish were randomly allocated into either 48 x 21 cm polythene bag treatments (provided by Aquatic World, Cardiff) or 36 x 19 cm Breathing Bags ™ treatments. The polythene bags were filled with one-third dechlorinated water to two-thirds air which is the most common method of transporting small fish in aquaculture (Conte, 2004). Air was not added to the Breathing Bags ™ as per supplier instructions to reduce mechanical disturbance due to sloshing (Thiagarajan et al., 2011). Fish stocking density was 4 fish/l for both bag treatments, which falls within guidelines for tropical freshwater species stocking densities (OATA Water Quality Criteria) and each bag used water volumes of up to 1.5L. To prevent handling fish with nets, they were placed into bags while fully submerged. Bagged fish were then contained in an insulated sealed thermal box (dimensions: 30 x 24 x 19 cm, 24±1°C) and placed onto an orbital shaker (Stuart®) for 24 h at 50 rpm to simulate transport motion. The rotator allowed for orbital movement on a horizontal platform, similar to any flat surface fish would be placed on in a transport vehicle or aircraft (Portz et al., 2006). Control fish (n=20) were kept in bags without turning on the orbital rotator, adjacent to an operating rotator to ensure fish were exposed to the same noise levels.

2.3.3. Experimental infections

To perform controlled infections, guppies were lightly anaesthetised with 0.02% MS222 and each fish was infected with two gyrodactylid worms. Parasite transfer was conducted using a dissection microscope with fibre optic illumination following standard methods of King & Cable (2007). Briefly, two worms from heavily infected donor fish were transferred to the caudal fin of recipient hosts by placing the anaesthetised donor fish in close proximity to an anaesthetised naïve host with the transfer monitored continuously using the dissecting microscope. To avoid an outcome where the two parasites transferred were too old to reproduce, all fish were re-examined the day after infection and those which had lost both parasites were re-infected with two new worms, and for these two fish the time was re-set to Day 0. Parasite infections were then monitored every 48 h by anaesthetising fish and the total number of gyrodactylids counted over the first 17 days of infection. At Day 17, all fish that survived were treated with Levamisole (Norbrook ®, UK) according to Schelkle et al., (2009) and their post-treatment recovery and any further mortalities monitored for 3 weeks.

2.3.4. Water quality

As water quality can impact disease susceptibility (Ackerman et al., 2006), water ammonia (freshwater master test kit, API ®), pH (battery powered checker HANNA ®) and oxygen saturation (dissolved oxygen meter, Lutron Electric Enterprise CO., LTD.) were measured to ensure this did not vary between the treatment and control groups (n=5 per experiment). All water quality levels within the polythene bags and Breathing Bags ™ post-transport was within normal ranges (ammonia levels undetectable for both bag treatments), pH (pH 7.1-7.8) and oxygen saturation (20.4-21.4 %) and consistent between treatments (Fisher's Exact test: oxygen, p= 0.958, pH, p=0.909).

2.3.5. Statistical analysis

All statistical analyses were conducted using RStudio version 1.0.143 (R Development Core Team, 2015). *G. turnbulli* mean intensity for all experiments, was defined as the average number of worms on infected hosts (Bush et al., 1997). A generalized linear mixed model (GLMM) with a negative binomial error family in the MASS R package was used to analyse the relationship between transport treatments (polythene bags and Breathing Bags ™) and mean parasite intensity. Host standard length, bag type (polythene bags and Breathing Bags ™) and treatment (transport and no-transport) were treated as fixed factors. As parasite intensity was recorded for each individual fish at different days, 'Fish ID' and 'days since initial infection' were included as a random effect in the GLMM to avoid pseudoreplication by incorporating repeated measures. Fish length was included in the initial model but was removed because the size range did not explain significant variation (Thomas et al., 2013). Area under the curve (AUC) is a statistical parameter that provides a measure for analyzing infection trajectories over time using the trapezoid rule (White, 2011). Area under the curve was also analysed using a GLMM with a negative binomial error family. Finally, a Generalised Linear Model (GLM) was used to analyse how peak parasite day and maximum parasite count varied with treatment. For analysing maximum parasite count a negative binomial error family with a log link function was used and a gaussian error family with an identity link function for peak parasite day. All error families were determined based on the lowest Akaike Information Criterion (AIC) value.

A logistic regression was used to analyse mortality between transported and control fish and between bag types.

2.4. Results

Parasite dynamics were influenced by fish simulated transport, with guppies being transported suffering significantly higher parasite burden than untransported control fish (GLMM: $Z_{79,691}$= 2.51, p=0.009; Figure. 2.1). The carrier type used to simulate transport (polythene bags or Breathing Bags ™), however, did not affect the parasite count between transported and untransported fish (GLMM: $Z_{79,691}$= 2.51, p=0.19). Total infection trajectory over 17-days, as measured through Area Under Curve (AUC) was significantly greater in fish that experienced simulated transport versus controls (GLMM: $Z_{79,612}$=2.42, p=0.01). Similarly, peak parasite day ($t_{7679.2,698}$=2.24, p=0.02) and the associated maximum parasite count ($Z_{781.6,698}$=6.73, p<0.001) were significantly different between transported and control fish: with maximum parasite count on peak days having approximately 51% greater parasite load compared to controls in both carrier types (Breathing bags™= 50.9% greater, polythene bags= 51.2% greater). There was no significant difference in mortality between simulated transport and control fish within the same bags or between Breathing Bags™ and polythene bags (between bags, GLM: $Z_{1.1,3}$ =0.18, p= 0.85; within same bags, GLM: $Z_{0.26,1}$= 0.89, p= 0.371).

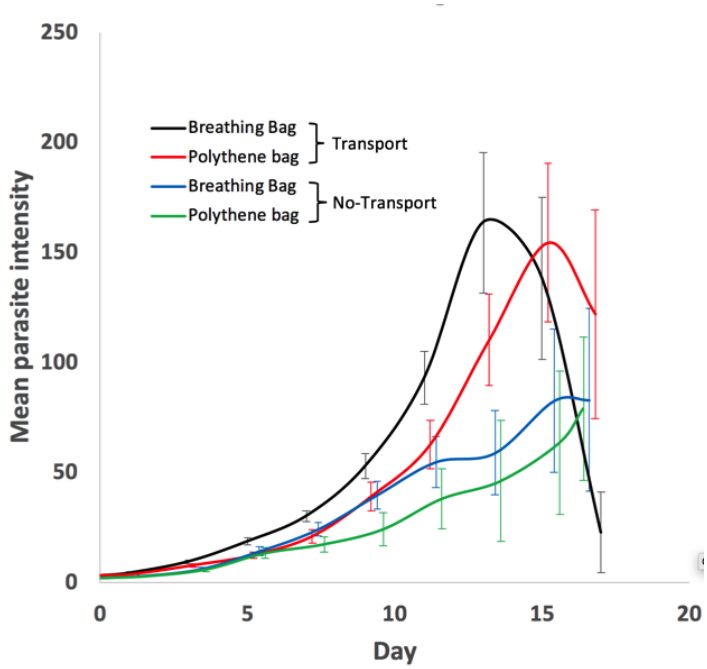

Figure. 2.1. *Poecilia reticulata* exposed to *Gyrodactylus turnbulli* infections after being exposed to mechanical disturbance during simulated transport in polythene bags and Breathing Bags ™. All guppies exposed to mechanical disturbance in both bag types suffered significantly higher parasite burden than control fish. Standard Error bars slightly transposed to one side to prevent overlap.

2.5. Discussion

Simulated transport significantly affected guppy susceptibility to infections with *Gyrodactylus turnbulli* showing for the first time the impact of mechanical disturbance on disease dynamics. Unfortunately, increased parasite burdens were not ameliorated following use of specialized Breathing Bags ™ even though these bags did reduce the level of water sloshing during mechanical disturbance. While it was visually apparent that the Breathing Bags did indeed reduce water sloshing during simulated transport, the possibility of mechanical stress during the transport simulation as a physical quantity was impractical to measure and therefore this could not be ruled out as a possible influencing factor. Thus, fish transported in Breathing Bags may indeed have experienced a form of mechanical stress despite reduced water sloshing leading to increased susceptibility to disease. Designing transport bags which are more impervious to mechanical stress as opposed to simply reducing water sloshing may be the only way to tackle the issue of mechanical disturbance as a neglected welfare issue.

Susceptibility to disease in fish is influenced by stressors linked to the production of cortisol, which is an immunosuppressant (Tort et al., 2003; Tort, 2011). While the relationship between a stress event and immunosuppression is far from clear (reviewed by Tort, 2011), the transport process for fish is associated with multiple stressors including handling and netting, with water quality deterioration considered the major stressor linked to high stocking density (see Braun & Nuñer, 2014), which has been implicated in elevated cortisol levels, increased disease susceptibility and significant mortality levels (Caruso et al., 2002; Iguchi et al., 2003; Cho et al., 2009; Robertson et al., 2017). However, for the current study fluctuating water quality, temperature, lighting, noise, netting, and stocking densities were controlled, leaving mechanical disturbance as the major stressor. While the length of time a stress response would last in guppies post-transport is unknown, as there is likely a species level difference in cortisol production (Honryo et al., 2018), mechanical disturbance in these transported guppies could have caused elevated cortisol production during a stress response leading to immunosuppression. Surprisingly, guppies exposed to gyrodactylid infection immediately prior to experiencing mechanical transport disturbance did not show a significant effect of transportation on subsequent parasite levels compared to untransported fish (see Appendix 1), which indicates immune status at the time of initial infection is the most important factor determining disease outcome.

Undiagnosed infections on imported fish are a major biosecurity risk in the ornamental trade (Maceda-Veiga & Cable, 2018), particularly as they may introduce novel parasite species to which local hosts have no immunity (Paterson et al., 2012). The current study emphasises the need for stricter screening procedures after transport, as diseases such as gyrodactylosis are difficult to diagnose without thorough microscopic screening and can cause an explosion in parasite burden due to transport stress. Application of anesthetic agents, like clove oil and MS-222, into water prior to transport has shown limited efficacy in reducing stress and mortality in transported fish (Rubec et al., 2000) and is associated with the risk of respiratory failure (Wagner et al., 2003; Pramod et al., 2010). In contrast, addition of compounds, such as salt, prior to fish transportation, can reduce transport-related mortality (Oyoo-Okoth et al., 2011);

however, they have variable efficacy on diseases such as gyrodactylosis, as treatment is often time, concentration, and species dependent (Schelkle et al., 2011). Studies of parasite diversity in the ornamental trade (pet shops, retailers, and home aquaria) highlight *Gyrodactylus* spp. as one of the most common group of parasites detected during screening procedures (Trujillo-González et al., 2018; Maceda-Veiga & Cable, 2018). Thus, the impact of this monogenean infection remains a serious welfare issue for global ornamental trade. For the first time this investigation highlights that even when water quality, stocking density and temperature are stable, mechanical disturbance during transport, hitherto neglected as a potential stressor, significantly impacts susceptibility to infections in fish. With disease remaining the major factor limiting the expansion of global fish trade (FAO, 2016), investigating stressors that have remained under the radar thus far may prove crucial in a growing trend emphasizing the need for improved fish welfare.

Chapter 3

Noise pollution: acute noise exposure increases susceptibility to disease and chronic exposure reduces host survival

This chapter is affiliated with the publication Masud et al., 2020a in the journal Royal Society Open Science,

3.1. Abstract

Anthropogenic noise is a pervasive global pollutant that has been detected in every major habitat on the planet. Detrimental impacts of noise pollution on physiology, immunology and behaviour have been shown in terrestrial vertebrates and invertebrates. Equivalent research on aquatic organisms has until recently been stunted by the misnomer of a silent underwater world. In fish, however, noise pollution can lead to stress, hearing loss, behavioural changes and impacted immunity. But the functional effects of this impacted immunity on disease resistance due to noise exposure have remained neglected. Parasites that cause transmissible disease are key drivers of ecosystem biodiversity and a significant factor limiting the sustainable expansion of the animal trade. Therefore, understanding how a pervasive stressor is impacting host-parasite interactions will have far reaching implications for global animal health.

Here, the impact of acute and chronic noise on vertebrate susceptibility to parasitic infections was investigated, using a model host-parasite system (guppy-*Gyrodactylus turnbulli*). Hosts experiencing acute noise suffered significantly increased parasite burden compared to those in no noise treatments. In contrast, fish experiencing chronic noise had the lowest parasite burden. However, these hosts died significantly earlier compared to those exposed to acute and no noise treatments, demonstrating a potential functional trade-off between improved parasite-resistance and shorter life span. By revealing the detrimental impacts of acute and chronic noise on host-parasite interactions, this study adds to the growing body of evidence demonstrating a link between noise pollution and reduced animal health.

3.2. Introduction

With species loss occurring 1000 times above the background rate of extinction, there is an urgent need to understand how anthropogenic activity influences ecosystem biodiversity and animal welfare (Pimm et al., 2014). Anthropogenic noise is a global pollutant. It has marked impacts on human health, from reduced cardiovascular function (Babisch et al., 1999, 2005; Babisch, 2003; Sobotova et al., 2010) to elevated cortisol levels and disrupted sleep patterns (Ising & Kruppa, 2004; Shepherd et al., 2011). Indeed, from long term cross-sectional surveys, people report a significant reduction in their quality of life when subject to chronic noise (Shepherd et al., 2011). Stress responses to sound pollution have also been shown in non-human vertebrates (reviewed in Ising & Kruppa, 2004). Bird communities, such as the greater

sage-grouse (*Centrocercus urophasianus*), have elevated faecal corticosteroid metabolites and show a decline in male lek attendance when exposed to chronic and intermittent noise (Blickley et al., 2012 a, b). Reproductive behaviour, including anuran mate calling, is affected by chronic roadside noise, with frogs for example having to increase song pitch leading to greater energy expenditure (Parris et al., 2009). More than any other vertebrate system, mouse models have demonstrated that noise can impact behaviour, reproduction, metabolism, the cardiovascular system and immunology (reviewed in Kight & Swaddle, 2011). Even invertebrates are not exempt from the detrimental impacts of noise pollution (Gurule-Small & Tinghitella, 2019).

For aquatic organisms, including fish, the potential impact of noise pollution has only recently gained attention linked to the significant rise in underwater sonar, pile driving, seismic activities and motorised vehicle activity (Hildebrand, 2009). Freshwater fish in particular are a global welfare concern, recognised as the most endangered group of animals on the planet (Dudgeon et al., 2006; Collen et al., 2014), in addition to being a major source of animal protein for human consumption (FAO, 2018). Multiple fish species have displayed primary (e.g., cortisol production; Wei et al., 2018), secondary (e.g., cellular immune response; Filiciotto et al., 2017) and tertiary level impacts (e.g., potential disease resistance; Anderson et al., 2011) of noise exposure. However, while a range of tertiary level impacts of noise exposure on fish have been shown (e.g., Anderson et al., 2011; Filiciotto et al., 2017; Wei et al., 2018), limited work exists on disease resistance.

Typically, animal species respond in one of three ways to noise exposure: i) no apparent response to the sound stimulus (e.g., Wysocki et al., 2007); ii) an initial stress response followed by acclimation (e.g., Nedelec et al., 2017); or iii) consistent long-term detrimental health effects (e.g., Gurule-Small & Tinghitella, 2019). Reduced resistance to transmissible disease is arguably the most significant long-term welfare concern of noise exposure. This is because if left untreated and/or immune suppression occurs, transmissible disease will impact primary and secondary stress responses and ultimately cause mortality. To date, only two animal studies have assessed the impact of noise on susceptibility to infections (Wysocki et al., 2007; Anderson et al., 2011). Of these, only Wysocki et al. (2007) demonstrated an unambiguous effect; rainbow trout (*Oncorhynchus mykiss*) appeared unaffected by chronic 8-month noise exposure followed by subsequent *Yersinia ruckeri* inoculation. Parasites causing transmissible disease are recognised as one of the most significant causes of economic loss, due to host mortality in global animal trade (see Shinn et al., 2015; FAO, 2018). For industries such as aquaculture, infectious disease has reached crisis status exacerbated by neglected stressors that compromise host immunity (Stentiford et al., 2017). Therefore, the functional importance of stressors such as noise and its relation to disease resistance extends to impacts on valuable human resources.

The guppy-*Gyrodactylus turnbulli* host-parasite system has been utilised to understand how anthropogenic stressors impact disease resistance (e.g., nitrate enrichment: Smallbone et al., 2016, animal transport: Masud et al., 2019). This model allows us to monitor individual infection trajectories in real-time, which is done here to assess how acute and chronic noise exposure impacts resistance to transmissible disease. The host is a globally important freshwater fish, the Trinidadian guppy that is an established eco-evolutionary model (e.g., Magurran, 2005). The genus *Gyrodactylus* is a group of hyperprevalent monogenean ectoparasite species of ecological and aquaculture importance (Bakke et al., 2007; Kolluru et al., 2008; Reynolds et al., 2018). These so called ‘Russian-doll killers’ employ progenesis and hyperviviparity allowing parasite numbers to exponentially rise threatening host survival

(reviewed in Bakke et al., 2007). *G. turnbulli* is a primary parasite of guppies and of major concern in the ornamental trade (Cable, 2011). As the conservation status of freshwater fish is critical (Sadovy et al., 2013), understanding how anthropogenic noise impacts their resistance to transmissible disease is extremely timely.

3.3. Materials and methods

3.3.1. Host and parasite origins and maintenance

Mixed strain ornamental guppies (*Poecilia reticulata*, n=200) were purchased and transported from GuppyFarm UK to Cardiff University in September 2018. All fish were ectoparasite free on arrival, confirmed through three consecutive screens using a dissecting-microscope with fibre optic illumination (Stewart et al., 2017). For experimental infections, the Gt3 strain of *Gyrodactylus turnbulli* was used which originated from a single worm isolated from an ornamental guppy in 1997. This parasite population has since been maintained in culture pots containing at least four naïve fish collectively infected with a minimum of 30 worms. Naïve guppies are added to the culture when worm numbers decrease, and heavily infected fish removed (treated) and replaced to prevent parasite extinction (Reynolds et al., 2018). All fish were maintained at 24 ± 1°C under a 12 h light/12 h dark lighting regime and fed a daily diet of tropical flakes (Aquarian®) along with freshly hatched *Artemia* nauplii every alternate day. Experimental fish were size matched adult females (SL range 14-27 mm).

3.3.2. Experimental design: acute and chronic noise exposure

To investigate how noise exposure impacts fish resistance to parasitic infections, guppies were allocated to either acute noise (24 h, n=24, SL range 16-25 mm) or chronic noise (7 d, n=28, SL range 14-21 mm) treatments prior to parasite exposure. For each treatment, control fish (acute, n=24; chronic, n=28, SL range 14-27 mm) were placed in identical conditions but with no noise exposure. The experimental set-up for both acute and chronic treatments (Figure 3.1A) involved placing individual guppies in 1L containers within a glass tank (60 x 30 x 30 cm^3) equidistant from an omnidirectional underwater speaker (UW-30, Illuminate Design Ltd., Witham). Each tank consisted of four 1L containers per speaker (acute noise = 6 replicates; chronic noise = 7 replicates). The water level in the tanks was just below the rim of the 1L containers and sufficient to ensure full submergence of the speakers. This host isolation was necessary to monitor individual infection trajectories as *G. turnbulli* can directly transfer between conspecific fish upon contact (King & Cable, 2007). To maintain water quality, all experimental fish in 1L containers underwent complete water changes every alternate day. Underwater speakers were connected to an amplifier and subsequently a laptop to deliver the same sound file into each experimental tank. The speaker played random, intermittent white noise covering the 100-10,000 Hz range (Smith, 2004). These noise files were generated using VCV Rack, an open-source additive synthesis software (https://vcvrack.com/), and then randomly enveloped (Figure 3.1B) to generate individual "bursts" of sound between 0.1 - 10s, interleaved with silence of the same random duration range. In the control (n=13) tanks, the speakers were turned off and disconnected from the main power source. No noise was transmitted between the noise exposure and control tanks and the same noise levels were recorded within each 1L container in each experimental tank, confirmed through hydrophone

(Reson TC 4013) recordings and data acquisition system (Picoscope 5443B). The white noise emitted from the speaker was altered by reflections due to tank geometry, and the mechanical characteristics of the medium and of the tank wall and contents. Figure 3.2 shows the resulting power spectrum, measured at mid depth of the 1L fish containers and averaged over ~10s. While fish can respond to particle motion (Nedelec et al., 2016), this could not be measured as a suitable accelerometer could not be obtained. However, these sound pressures are in line with mild sound levels recorded in concrete raceways, earthen ponds, and indoor aquaculture systems (Davidson et al., 2007).

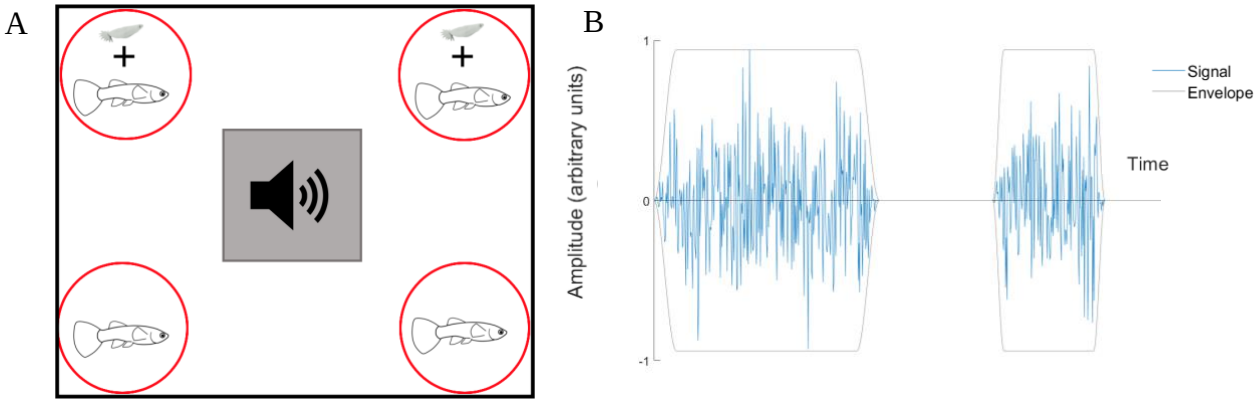

Figure 3.1 . (A) Schematic of general experimental design. Guppies were exposed to one of three treatments: acute noise (n=24), chronic noise (n=28) or no noise, controls (n=52). This was followed by experimental infections of half the fish with *Gyrodactylus turnbulli* parasites (shown here as grey worms, not to scale). Sound treatment design shown here, the black rectangle represents a glass tank (60 x 30 x 30 cm^3) with an underwater speaker (grey filled square; turned off in the no noise controls). Each red circle with a female guppy represents 1 L containers in which hosts were isolated for the duration of acute and chronic noise exposure as well as control treatment. (B) White noise enveloped (i.e., turning a continuous sound into bursts of shorter sounds of random length, followed by silence of random length) to generate 'bursts' of noise that was used for both the acute and chronic treatments.

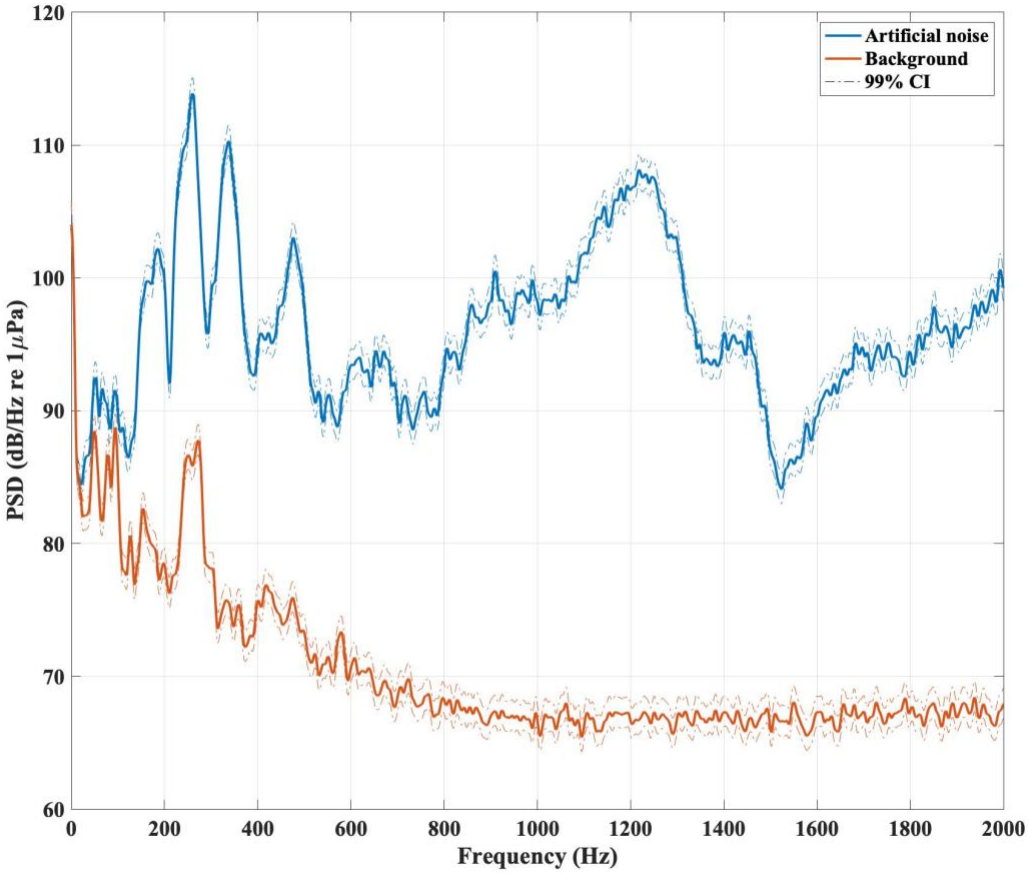

Figure 3.2. Power spectral density of the noise hosts were exposed to compared with the background noise inside a tank. Average of 10 distributions over 1s intervals (frequency resolution = 1Hz) and 99% CI.

3.3.3. Experimental infections

Guppies were experimentally infected after acute (24 h) or during chronic noise exposure (day 7) (Figure 3.3). For the chronic noise treatment, hosts that were infected with parasites continued to experience noise during infection trajectories. Thus, chronic exposure fish, experienced noise for a total of 24 days. Experimental infections involved lightly anaesthetizing individual guppies with 0.02% MS-222, and each fish was infected with two gyrodactylid worms. Parasite transfer was conducted following standard methods of King & Cable (2007). Briefly, two worms from heavily infected donor fish were transferred to the caudal fin of recipient hosts by placing the anaesthetized donor fish near an anaesthetized naïve host, monitored continuously using a dissecting microscope with fibre optic illumination. Parasite infections were then monitored every 48 h by anaesthetizing fish and counting the total number of gyrodactylids over the first 17 d of infections.

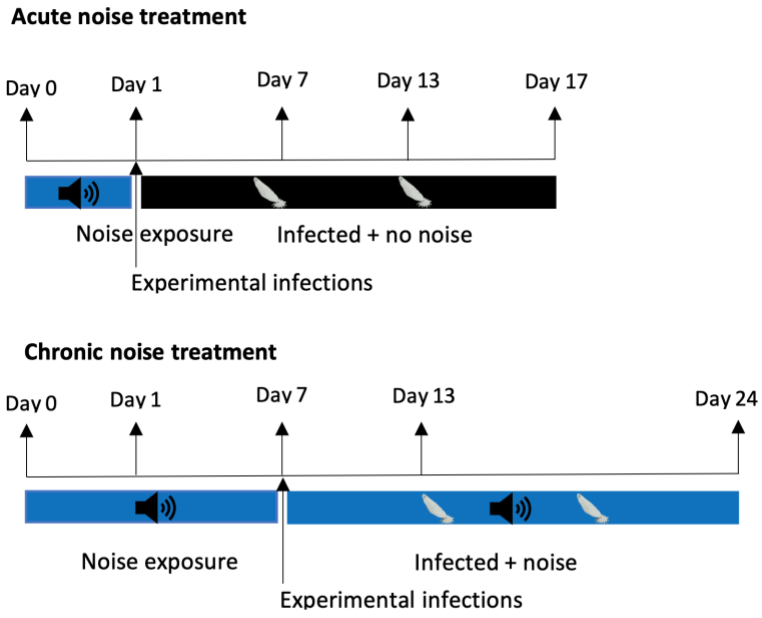

Figure 3.3 Timeline of when hosts were exposed to noise and controlled infections for both acute and chronic noise treatments.

To determine whether there was any immediate impact of noise exposure on *G. turnbulli* reproduction on the host, n=10 size matched female guppies from the same mixed ornamental stock were infected with 15 parasites each and exposed to 24 h of noise as detailed above. Control fish (n=10) were also infected but not exposed to noise. Over the 24 h time period, fish were removed and screened at two different time points (2 h and 24h) to record whether or not parasite infrapopulations had increased.

Mortality was recorded for all treatments and any fish that survived parasite infection studies were treated with Levamisole (Norbrook®) according to Schelkle et al. (2009). Post-treatment fish were monitored for 3 weeks and no mortalities occurred during this time. Fish mortality only occurred during infections.

3.3.4. Statistical analysis

All statistical analyses were conducted using RStudio v2.1 and final models were all selected based on the lowest Akaike's information criterion (AIC) value. Peak parasite burden is the maximum number of parasites at a given time point, defined here as peak day (Bush et al., 1997). To quantify total infection trajectory over the 17 days, Area Under the Curve (AUC) was calculated using the trapezoid rule (White, 2011). To analyse peak parasite burden, peak day and AUC a Generalised Linear Model (GLM) with a negative binomial error family and a log link function in the R MASS package was used. Explanatory variables for the GLM's were treatment (no noise, acute noise, chronic noise) standard length and mortality day. All GLM error families were chosen based on the lowest dispersion parameter, theta (Thomas et al., 2015).

A Generalised Linear Mixed Model (GLMM) with a negative binomial error family and log link function was used to analyze intrinsic rate of parasite increase. A GLMM was utilised as parasite data was recorded for each fish at different time points and therefore to prevent pseudoreplication, Fish ID was treated as a random factor. Standard length, and treatment (no noise, acute noise, chronic noise) were treated as explanatory variables. As experimental fish were placed in n=6 (acute treatment) and n=7 (chronic treatment) tanks, tank number was also treated as a fixed factor to rule out batch effect. For all models used in analysis, no batch effect was found for either noise exposure treatments ($P > 0.05$ for all models). Model refinement was conducted by removing standard length in the GLM and GLMM used to analyse AUC and intrinsic rates of parasite increase as it was a non-significant explanatory variable (AUC: $Z=0.72$, $SE=0.01$, $P=0.47$; intrinsic rate of parasite increase: $Z=0.719$, $SE=0.04$, $P=0.47$).

For analysing the *in vivo* impact of 24 h noise exposure on parasite infrapopulations, a GLMM with a Poisson error family was utilised to analyse parasite count over time to prevent pseudo-replication as fish were screened at two time points. The explanatory variable for this model being 'treatment' (i.e., noise exposure versus no-sound) and fish ID being a fixed factor. A GLM with poisson error family and log link function was utilized for analyzing death day, where the explanatory variable was treatment as fish mortality only occurred if they were infected. Furthermore, a Kaplan-Meier survival analysis using Cox's proportional hazards model was also utilized to analyze the proportion of deaths per noise treatment and host survival was visualized graphically using a probability distribution of survival within the *ggplot* package in R.

3.4. Results

Guppies exposed to acute noise and subsequently infected had significantly greater parasite burdens over time as measured through AUC compared to no noise controls (GLM: $Z=0.08$, $SE=-4.14$, $P<0.001$; Figure 3.4A). Fish exposed to acute noise also had significantly higher peak parasite burdens compared to controls (GLM, $Z=-6.44$, $SE=0.09$, $P<0.001$; Figure 3.4B). In contrast, guppies exposed to chronic noise had significantly reduced peak parasite burden and infection trajectories compared to controls (GLM, peak parasite burden: $Z=-8.4$, $SE=0.07$, $P<0.001$; AUC: $Z=-9.9$, $SE=0.06$, $P<0.001$). Fish exposed to chronic noise also showed a reduced intrinsic rate of parasite increase compared to control guppies (GLMM: $Z=-3.554$, $SE=0.10219$, $P<0.01$).

Parasite *in vivo* noise exposure revealed that *G. turnbulli* numbers consistently increased and there was no significant difference in total parasite count over the 24 h period (GLMM: $Z=-0.43$, $SE=0.06$, $P=0.66$) indicating no direct impact of sound on the parasites.

Day on which host mortality occurred was significantly associated with peak parasite count and AUC for both acute and chronic noise treatments (AUC: $Z=52.23$, $SE= 0.007$, $P<0.001$, peak count: $Z=39.8$, $SE=0.008$, $P<0.001$). Moreover, guppies that experienced chronic noise treatment died significantly earlier than fish experiencing acute or no noise (chronic treatment average death day= 12, compared to average death day= 14 for acute and control fish, GLM: $Z= 3.08$, $SE=0.03723$, $P=0.002$). Interestingly, though fish within the chronic noise treatment were dying earlier than fish in the no noise treatment, there was no significant difference in the proportion of hosts dying between chronic exposure and no exposure (Coxph: $Coef=0.46$, $Z= 1.34$, $p=0.17$). However, a significantly greater proportion of hosts within the acute noise

treatment were dying compared with fish not experiencing any noise (Coxph: Coef=0.91, Z=2.77, p=0.005)

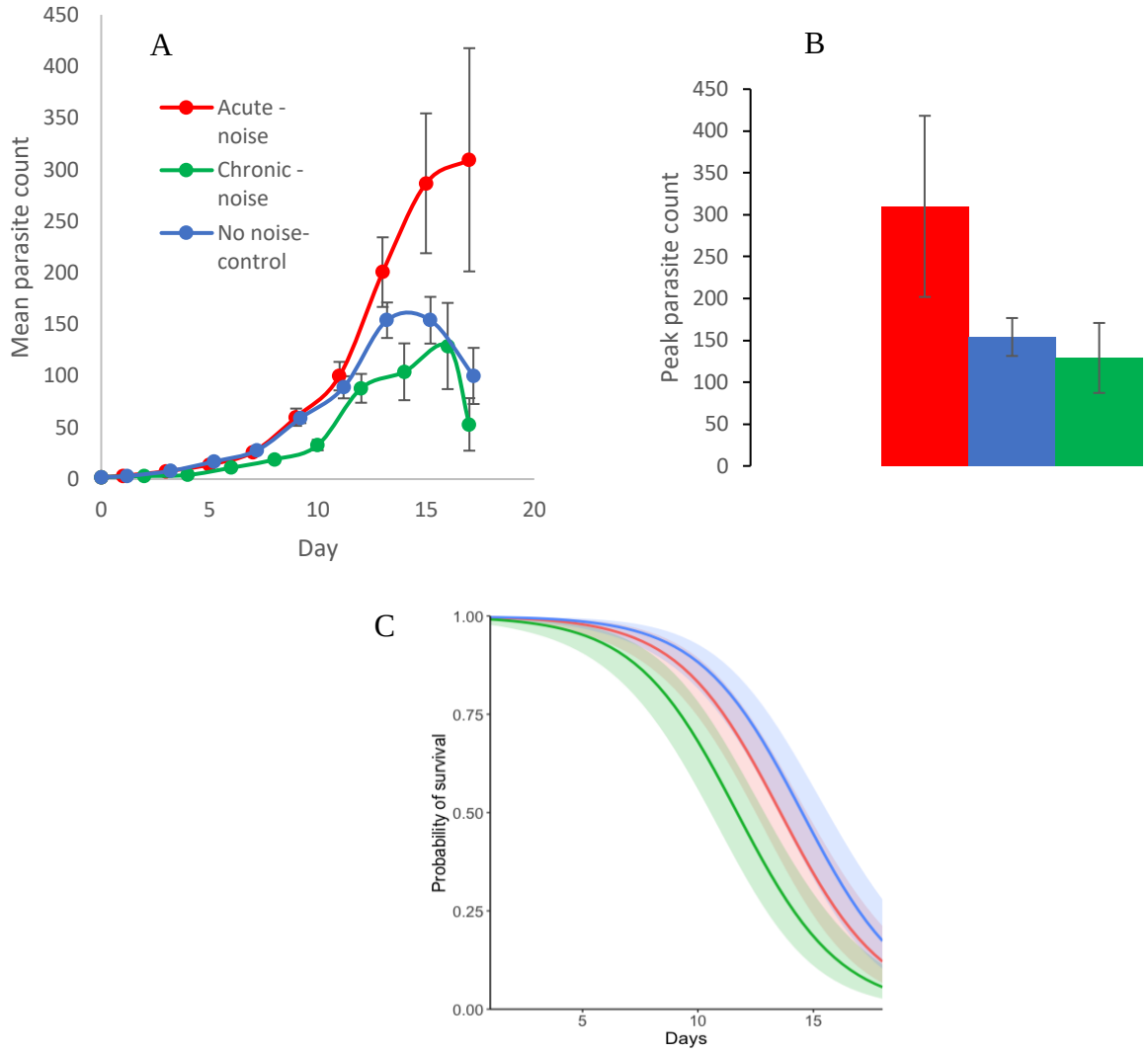

Figure 3.4. Mean count (A) and peak parasite burden (B) of *Gyrodactylus turnbulli* infections in guppies (*Poecilia reticulata*) exposed to either acute, chronic or no noise (controls) and the (C) probability distribution of host survival within each treatment. All error margins shown are standard error.

3.5. Discussion

Anthropogenic noise pollution is now a recognised welfare concern, with international regulations (e.g., Smith, 2015) aiming to restrict potential detrimental health impacts. Regulations in the European Parliament, for example, have imposed restrictions on noise levels for motorised vehicles as well as introducing silencing systems, in recognition that noise can have wide ranging health impacts. This study shows fish experiencing acute noise suffered increased disease susceptibility. In contrast, chronic noise exposure significantly reduced parasite burden, but fish were prone to earlier mortality. It is likely that acute noise caused a stress response without providing sufficient time for the immune system to respond before pathogenic challenge. Acute stress has been linked to increased lymphocyte trafficking and expressions of protein cytokines from leukocytes, whereas chronic stress is associated with reduced leukocyte function (Dhabhar, 2006; Fast et al., 2008). Chronic stress is generally accepted as detrimental to immunity and acute stress as potentially adaptive (Dhabhar et al., 2006). However, knowledge of how stressors impact the immune system is largely based on *in vitro* investigations (reviewed in Tort, 2011), although there is an increase in studies on how stressors influence disease resistance. This is unsurprising considering the economic cost of disease for animal husbandry (Shinn et al., 2015; FAO, 2018) and certainly for farmed aquatic species classic stressors, such as stocking density and water quality, are now known to significantly impact immune responses and disease resistance (Ackerman et al., 2006; Ellison et al., 2018). In addition to stressors associated with husbandry practices, those linked to environmental change are also being investigated in relation to disease resistance (Rohr et al., 2011). There is particular concern regarding such stressors that cross critical thresholds, termed 'planetary boundaries' (Rockström et al., 2009), that induce physiological stress leading to system dysfunctions that includes increased disease susceptibility (Cable et al., 2017). Noise pollution, however, that may be contributing to the breach of planetary boundaries has previously been neglected in terms of disease resistance. Therefore, *in vivo* experiments, combined with immunological expression studies, are needed to determine how noise has functional impacts on disease resistance.

Chronic noise exposure can activate the immune system, with gilthead sea bream (*Sparus aurata*), for example, showing significantly higher total oxidant status, lysozyme activity and antiprotease activity in response to 40 days of chronic aquaculture noise compared to no noise controls (Filiciotto et al., 2017). Chronic noise exposure in mice can cause immune alterations but this is dependent on strain type, with T-cell dependant antibody production and *ex vivo* T-cell proliferation significantly reduced in C57Bl/6 but not BALB/c mice (Pascuan et al., 2014). In comparison to a classic stressor (physical restraint) also applied to the C57Bl/6 mice, chronic noise had more impact on antibody production and immune cell proliferation (see Pascuan et al., 2014). This study revealed chronic noise exposure significantly reduced parasite burdens suggesting active priming of host immunity. Alternatively, direct exposure to sound could cause parasites to actively move off the host, die or disrupt their reproduction. However, the *in vivo* investigations suggest that, at least over 24 h of noise exposure, there are no immediate impacts on parasite infrapopulations as their numbers consistently increased. The only research showing that sound exposure can directly impact ectoparasites utilised ultrasonic waves that are of frequencies several orders of magnitude higher (e.g., Skjelvareid et al., 2018) than those used in the current study. Furthermore, at ultrasonic frequencies, sound only impacted ectoparasitic lice when at close range to the emitted sound (see Skjelvareid et al., 2018). The

apparent increased resistance to parasites demonstrated in this study for the fish exposed to chronic noise was linked to earlier host mortality, indicative of a possible functional trade-off. Such a trade-off is unsurprising considering the biological cost of an active immune response (McKean et al., 2008), and similar trade-offs have been shown among invertebrates (Libert et al., 2006). While exposure to noise has been shown to impact fish mortality (e.g., Nedelec et al., 2017) this is the first study to demonstrate how increased disease resistance linked to chronic noise exposure reduces survival.

Animal food industries, including aquaculture, are projected to see a further rise in disease burden linked to increased stressors (FAO, 2018; Stevens et al., 2017). Here, for the first time the detrimental impact of noise exposure on disease resistance and mortality are revealed. With animal husbandry focussed on increasing output to meet human food chain demands, increased automation and machinery use is exposing animals to further noise (Kight & Swaddle, 2011; Hildebrand, 2009). While this study has isolated noise as an individual stressor under laboratory conditions animals typically face multiple stressors during routine husbandry. Future work must consider how noise pollution in conjunction with other common anthropogenic stressors, for instance enrichment use (Näslund & Johnsson, 2016), transport (Masud et al., 2019) and manual handling (Falahatkar et al., 2009), impact on animal health. Currently, there are no effective treatments for many of the diseases that plague animal industries and the renewed emphasis on ‘prevention rather than cure’ means that now more than ever identifying key stressors associated with increased disease burden is an important goal towards developing sustainable preventive measures.

Chapter 4

Cost of a deprived environment – increased intraspecific aggression and susceptibility to pathogen infections

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4.1. Abstract

A lack of environmental enrichment can be severely detrimental to animal welfare. For terrestrial species, including humans, barren environments are associated with reduced cognitive function and increased stress responses and pathology. Despite a clear link between increased stress and reduced immune function, uncertainty remains on how enrichment might influence susceptibility to disease. For aquatic vertebrates, we are only now beginning to assess enrichment needs. Enrichment deprivation in fish has been linked to increased stress responses, agonistic behaviour, physiological changes, and reduced survival. Limited data exist, however, on the impact of enrichment on disease resistance in fish, despite infectious diseases being a major challenge for global aquaculture. Here, using a model vertebrate host-parasite system investigations were conducted on the impact of enrichment deprivation on susceptibility to disease, behaviour and physiology. Fish in barren tanks showed significantly higher infection burdens compared to those in enriched enclosures and they also displayed increased intraspecific aggression behaviour. Infections caused hosts to have significantly increased Standard Metabolic Rates compared to uninfected conspecifics, but this did not differ between enriched and barren tanks. This study highlights the universal physiological cost of parasite infection and the biological cost (increased susceptibility to infection and increased aggression) of depriving captive animals of environmental enrichment.

4.2. Introduction

Lack of environmental enrichment for captive terrestrial species is an established global welfare concern (Erwin et al., 1976; Appleby & Wood-Gush, 1988; Carughi et al., 1989). Even for humans, environments lacking enrichment such as colour and structural variation cause reduced cognitive stimulation and are implicated in early onset neurodegenerative diseases (reviewed by Kramer et al., 2004; Milgram et al., 2006). For non-human vertebrates, commercial farming, in particular, represents a major welfare challenge with its focus on maximizing outputs often at the cost of depriving species of enrichment (Ashley, 2007; Wells, 2009; Stevens et al., 2017). But addition of structural enrichment, in the poultry industry, for example, can reduce intra-specific aggression, mortality levels and stress responses to human contact (Jones & Waddington, 1992; Gvaryahu et al., 1994). Reducing stress is particularly important in captive animals as it has knock-on positive effects for immunity. Much of our understanding of this connection between stress and immunity is based on research conducted in fish (see Tort, 2011), where enrichment has been shown to reduce stress that is linked to decreased cortisol production (e.g., Pounder et al., 2016; Giacomini et al., 2016). However, it remains to be seen if using structural enrichment will translate to improved disease resistance.

Managing disease burden in fish is a global priority; fish are the most consumed source of animal protein and aquaculture is the fastest growing food industry globally (Shinn et al., 2015; FAO, 2018). Parasitic diseases pose the most significant biosecurity and economic risk for aquaculture (Shinn et al., 2015) and stock management strategies are now emphasizing husbandry practices that minimize stressors to prevent stress-related immunosuppression (Conte, 2004; Ashley, 2007). The monogenean gyrodactylids are a group of hyperviviparous ectoparasites that historically have been a challenge to manage in aquaculture and the ornamental trade, with no effective cures that can be applied to fish stocks *en masse* (Schelkle et al., 2009). Norwegian salmon were decimated by *Gyrodactylus salaris* in the 1970s (Johnsen, 1978; Appleby & Mo, 1997) and despite the use of rotenone in rivers to kill all potential fish hosts, the parasite persisted in adjacent water bodies (Erikson et al., 2009). Even for parasite species that may not cause mortality, the metabolic cost of infection will have life history consequences, such as reduced growth and fecundity, for hosts (Sheldon & Verhulst, 1996; Bonneaud et al., 2016).

Here the hypothesis that inclusion of environmental enrichment for captive animals can increase disease resistance is tested using a model host-parasite system (guppy-*Gyrodactylus turnbulli*). The guppy host, *Poecilia reticulata*, is an established ecological and parasitological model (Magurran, 2005). *P. reticulata* has been introduced as a pet and biological agent to every major continent, except Antarctica (Deacon et al., 2011), and is a key economic species in the ornamental trade (Maceda-Veiga et al., 2016). The hyperviviparous ectoparasite *G. turnbulli* is a primary monogenean parasite of the guppy and a major concern in the ornamental trade (reviewed by Cable, 2011). This is the first study of its kind to investigate the impact of enrichment deprivation simultaneously on fish disease resistance, behaviour and physiology (Standard Metabolic Rate; SMR).

4.3. Materials and methods

4.3.1. Study system

For this study, size matched male guppies, measured using callipers under 0.02% MS-222 induced mild anaesthesia (*Poecilia reticulata*, size range: 14-19 mm) were used. Fish were bred from a stock caught in the Lower Aripo River in Trinidad in 2012 and initially housed at Exeter University before being transferred to Cardiff University in 2014. All guppies were maintained in 70 L breeding tanks (closed systems- 60 cm x 40cm x 30 cm) utilising dechlorinated water from a main source at 24 ± 0.5^{o}C under a 12 h light: 12 h dark photoperiod (lights on 07:00-19:00) and fed dry food flakes (Aquarian ®) *ad libitum* and freshly hatched *Artemia* nauplii every alternate day. Water quality levels are tested on a weekly basis and prior to removing fish for experimental investigations the water quality level was Ammonia non detectable, pH: 7.8, Nitrite levels: >0<0.21 mg/L, Nitrate levels: <20 mg/l (API® Freshwater Master Test Kit). All fish stock tanks are consistently aerated with air stones connected to a main air supply. Each stock tank was provided with the same environmental enrichment consisting of 2 cm pea gravel substrate, plastic flowerpots, plastic reeds, and tubing. Sufficient refugia were available to ensure all individual fish were able to use them when required. For investigating susceptibility to disease, experimental infections used the Gt3 strain of *Gyrodactylus turnbulli*, isolated from a Nottingham aquarium shop in October 1997 and subsequently maintained at Cardiff University on inbred guppies prior to this study (see King & Cable, 2007).

4.3.2. Experimental design

All fish used for this study were size matched with callipers under mild anaesthesia (0.02% MS-222- see above). Experimental fish were assigned to one of two treatments: enriched or barren tanks (16 L - 36 cm x 21 cm x 21 cm). Each enriched tank contained gravel (2 cm pea gravel substrate), plastic tube, flowerpot, and plastic reeds (purchased from Aquatic World, Cardiff) and these enrichments were consistent between each batch. Barren tanks contained no enrichment and were visually isolated from enriched tanks. Guppies were removed from stock tanks and a batch of fish (5 fish per batch x 12 replicates per treatment) randomly assigned to an enriched or barren treatment tank. To ensure the effect of displacement and a novel environment did not confound results, fish prescribed to enriched and barren treatments were maintained in their respective experimental tanks for 2 weeks to allow acclimatisation prior to starting experiments; this is sufficient time for the formation of shoals based on familiarity (Griffiths & Magurran, 1997).

4.3.3. Behavioural observations

To investigate the effect of enrichment deprivation on guppy behaviour, focal observations were conducted pre-infection (days 13 and 14 of acclimatisation) as *G. turnbulli* is known to influence guppy inter-specific interactions (Reynold et al., 2018). Focal observations involved an observer choosing a single male, identifiable from distinct colouration (out of 5 fish per tank) and recording all interactions between the focal male and conspecifics. For the enriched tanks, the time spent interacting with the structural enrichment was also recorded as preliminary observations revealed that guppies will interact with enrichment by either pecking at structures (gravel, flowerpot, plastic tube, and reeds) or seeking refuge (in flowerpots, plastic tubes and reeds). To ensure that observer bias did not influence recording behavioural metrics, two observers (one who was unaware of the expected outcomes of this study) recorded agonistic behaviours for a subsample of tank treatments (5 enriched and barren tanks). A Kendall's Tau correlation analysis (chosen because several 'tied' observations were reported between observers) revealed no significant difference between observer data (i.e., a significant association was detected; $z= 11.729$, $p< 0.001$).

All observations were conducted between 10 am and 2 pm, and prior to each behavioural recording, the experimenter allowed 10 min for the fish to acclimatise to their presence. Aggression between male guppies is characterised by chasing and nipping behaviour (Houde, 1997). Two behavioural metrics are reported for this study: 1) aggression index= number of nips + chases 2) time spent associating with enrichment = nibbling enrichment + swimming into plastic pot or tubing + swimming between plastic reeds.

4.3.4. Experimental infection

To investigate the effect of enrichment deprivation on susceptibility to disease, guppies from tank treatments (barren = 42 fish, enriched= 41 fish) were lightly anaesthetised with 0.02 % MS222 and all fish infected with two gyrodactylids each. Parasite transfer was conducted using a dissection microscope with fibre optic illumination (following standard methods of King & Cable, 2007). Briefly, two parasites from donor fish were transferred to the caudal fin of each recipient hosts by placing the tail of a heavily infected donor fish close to that of a naïve host. Control fish (barren= 20 fish, enriched= 20 fish) were treated the same way infected

fish were (anaesthetizing without pathogen inoculation) to ensure that handling was not a confounding variable.

After experimental infections, fish were returned to their respective experimental tanks where they were housed for a further 17 days. As gyrodactylids naturally transfer between fish upon contact, every 48 h guppies were removed from their tanks and mean parasite intensity was calculated for each fish. Parasite infections were monitored by anaesthetising fish and counting the total number of gyrodactylids. Individual male guppies could be recognized by distinct colouration based on photographs taken on an iPhone (Apple Inc).

4.3.5. Respirometry

For investigating how environmental enrichment and infection impacted SMR, individual infected (n=29) or uninfected (n=28) guppies from both barren (n=14) and enriched (n=15) tanks were placed in respirometer chambers on days 3 and 13 of the 17-day infection trajectory to determine the impact of low and high parasite burden on Standard Metabolic Rate (SMR). All measurements were conducted in a respirometry set-up that permitted monitoring of 3 fish and 1 blank control simultaneously and temperature for the duration of measurements maintained at 24 ± 0.5°C. All water used for experimental purposes was autoclaved. The static respirometry set-up consisted of individual glass chambers (130 ml, sealed Duran™ square glass bottle with Polypropylene screw cap, Fisher). Glass chambers were autoclaved and rinsed with ethanol prior to commencing measurements to minimise background noise before the start of each respirometry trial and each chamber contained a false bottom with a magnetic stirrer to ensure a homogenous distribution of oxygen within it. Chambers were fitted with individual contactless oxygen sensor spots attached to probes that were connected to a FireSting O_2 meter (PyroScience, Aachen, Germany). Food was withdrawn for 24 h before each fish was tested to ensure they were in a post-absorptive state so SMR measurements were not influenced by thermal effects of food in the digestive tract. The decline in O_2 concentration within respirometry chambers was measured using the below formula in repeated 1s measurement cycles over ca. 1 h 20 min, with 1 h acclimation time and 20 min for recordings:

$$\text{SMR} = \frac{\Delta O2}{fishmass} \times Vc$$

Where V_c is the volume of the respirometer chamber and $\Delta O2$ is the rate of oxygen decline (Bonneaud et al., 2016) calculated as the slope of a linear regression. During measurements dissolved oxygen levels never fell below 7 mg/L, which is within recommended levels for freshwater tropical fish (OATA, 2008). The mean background oxygen consumption (typically *ca.* 20% of fish SMR) was subtracted from fish SMR for analysis.

4.3.6. Statistical analysis

All statistical analyses were conducted using RStudio version 1.0.143 (R Development Core Team, 2015). Here, three host disease categories are defined: hosts on which parasite numbers consistently increased (susceptible); those on which parasite numbers increased followed by a consistent decline indicative of an immune response (responders) or hosts which cleared their parasites (resistant) (Bakke et al., 2002). Total infection trajectory over 17 days was calculated by Area Under Curve (AUC), using the trapezoid rule. A generalized linear mixed model

(GLMM) with a negative binomial error family in the MASS R package was used to analyse both AUC and mean parasite intensity. Host standard length and treatment were treated as fixed factors. Parasite count was recorded on each fish at multiple time points over a 17-day infection trajectory so 'Fish ID' was included as a random effect in the GLMM to avoid pseudoreplication by incorporating repeated-measures. Fish length was included in the initial model but was removed because the size range did not explain significant variation. A Generalised Linear Model (GLM) was used to analyse how peak parasite day, maximum parasite count and mortality varied with treatment. For analysing maximum parasite count, a negative binomial error family with a log link function was used; a quasiPoisson error family with a log link function for peak parasite day and a Poisson error family with log link function for mortality count. A Fisher's exact test was used to investigate the difference between fish disease categories.

For analysing behaviour data, a GLMM with a negative binomial error structure was used to analyse agonistic behaviour between treatments, to prevent pseudoreplication as each experimental tank was observed at two time points and over two days. Agonistic behaviours (number of nips and chases) were combined into a single aggression index for analysis. Any aggression observed in enriched tanks was hypothesized to be associated with the time spent interacting with enrichment. Thus, a GLMM with a Restricted Maximum Likelihood (REML) function was also used to analyse the association between the time spent interacting with enrichment and the number of agonistic interactions within enriched tanks. Data in the REML model had to be rescaled due to very large eigenvalues and overdispersion (Thomas et al., 2013). Rescaling maintained data structure and minimized dispersion, generating a robust model structure.

For analyzing the effect of tank treatments (barren versus enriched) and infection on SMR, a GLM with an inverse gaussian error family and log link function was used. Additionally, a linear regression analysis was utilized to assess the relationship between parasite count and SMR. All models used for analyses were chosen and refined based on the lowest Akaike Information Criterion (Bates et al., 2014).

4.4. Results

Mortality did not significantly differ between fish in enriched tanks and barren ones (GLM: $Z=-0.11$, $SE=0.21$, $p=0.91$) but fish from barren tanks were significantly more susceptible to infection (Fig. 4.1B; barren: 26/42; 62%; enriched: 12/40; 30%) and showed significantly higher mean parasite intensity compared to fish housed in enriched tanks (Fig. 4.1A; GLMM: $Z=-8.16$, $SE=0.08$, $p<0.001$). Fish from barren tanks also had significantly higher peak pathogen burdens (Fig. 4.2A; GLM: $Z=-16.03$, $SE=0.07$, $p<0.001$) and this peak was achieved significantly later in fish from barren tanks compared with enriched ones (Fig. 4.2B; GLM: $t=-7.893$, $SE=0.02$, $p<0.001$). In addition, significantly more fish (Fisher's exact text: 95% C.I. = 3.29, $p<0.001$) cleared infections (resistant) in enriched tanks (13/40; 33%) compared to barren tanks (1/42; 2%). Enrichment did not significantly affect SMR (Fig. 4.3A; GLM: $t=-1.66$, $SE=0.11$, $p=0.09$) but fish with high parasite burdens (parasite range: 30-330; parasite mean: 120) had significantly greater SMR compared to uninfected ones regardless of enrichment (Fig. 4.3B; GLM: $t=3.38$, $SE=0.25$, $p<0.001$). Moreover, a linear regression analysis revealed that a significant proportion of the SMR of infected fish could be explain by parasite count (Fig. 4.3C; LM: $R^2=0.31$, $t=5.16$, $p<0.001$).

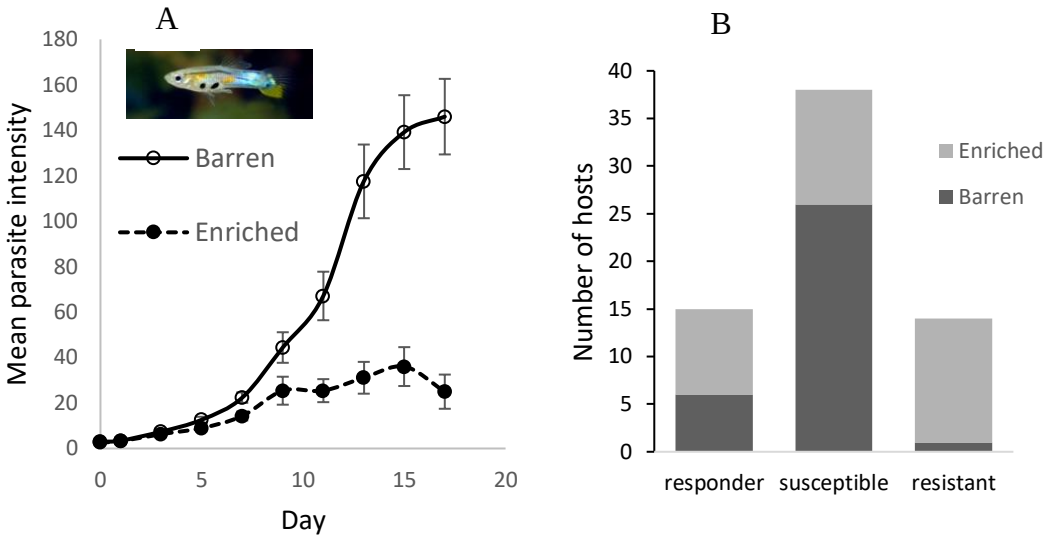

Figure 4.1. (A) Mean (±1 SEM) parasite intensity in guppies (*Poecilia reticulata*) exposed to *Gyrodactylus turnbulli* infection was significantly higher in fish in barren tanks (n=42) than enriched ones (n=41). (B) The number of hosts raised in either enriched or barren tanks classed as susceptible (hosts on which parasite numbers consistently increased), responders (hosts on which parasite numbers increased followed by a consistent decline indicative of an immune response), or resistant (hosts which cleared their parasites). Hosts from barren tanks were significantly more susceptible to disease (n=26) compared to those from enrichment treatments (n=12).

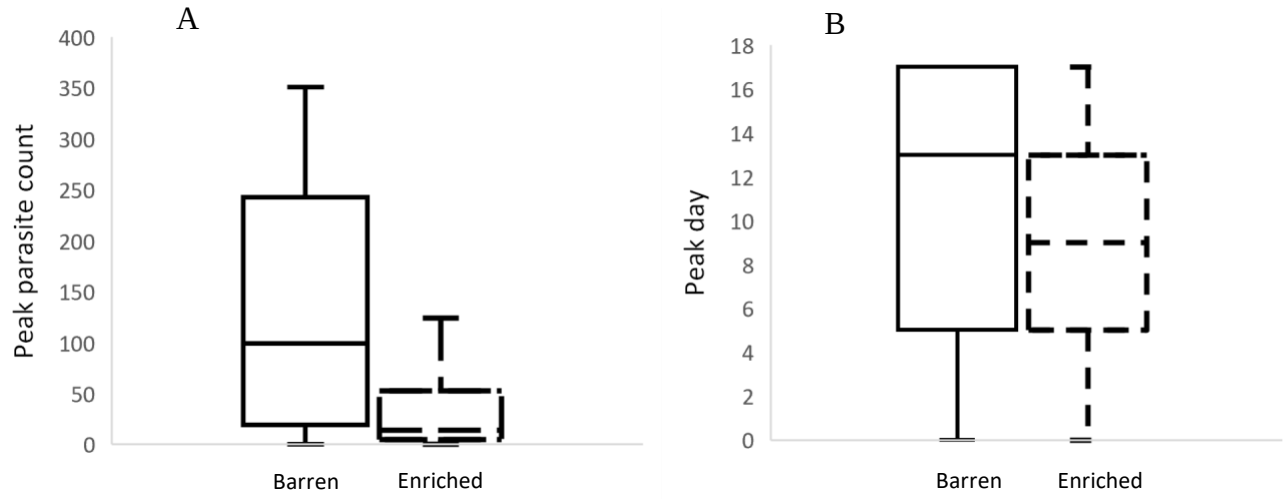

Figure 4.2. (A) Hosts from barren tanks (n=42) had significantly higher peak parasite counts than their enriched counterparts (n=41) and (B) peak parasite burdens occurred significantly later (peak day) for hosts in barren tanks compared to those in enriched tanks. Box plots show median (line), interquartile range (box) and 1.5x interquartile range (whiskers).

Fish in barren tanks displayed significantly more aggressive behaviour (nipping and chasing) towards conspecifics compared to those in enriched tanks (GLMM: Z=-11.21, SE= 0.15, P<0.001). In addition, aggression observed in enriched tanks was significantly associated with time spent interacting with enrichment and fish that spent more time using enrichment showed significantly less agonistic behaviour compared to those that used less enrichment (GLMM: t= -5.34, SE= 0.0008, P<0.001).

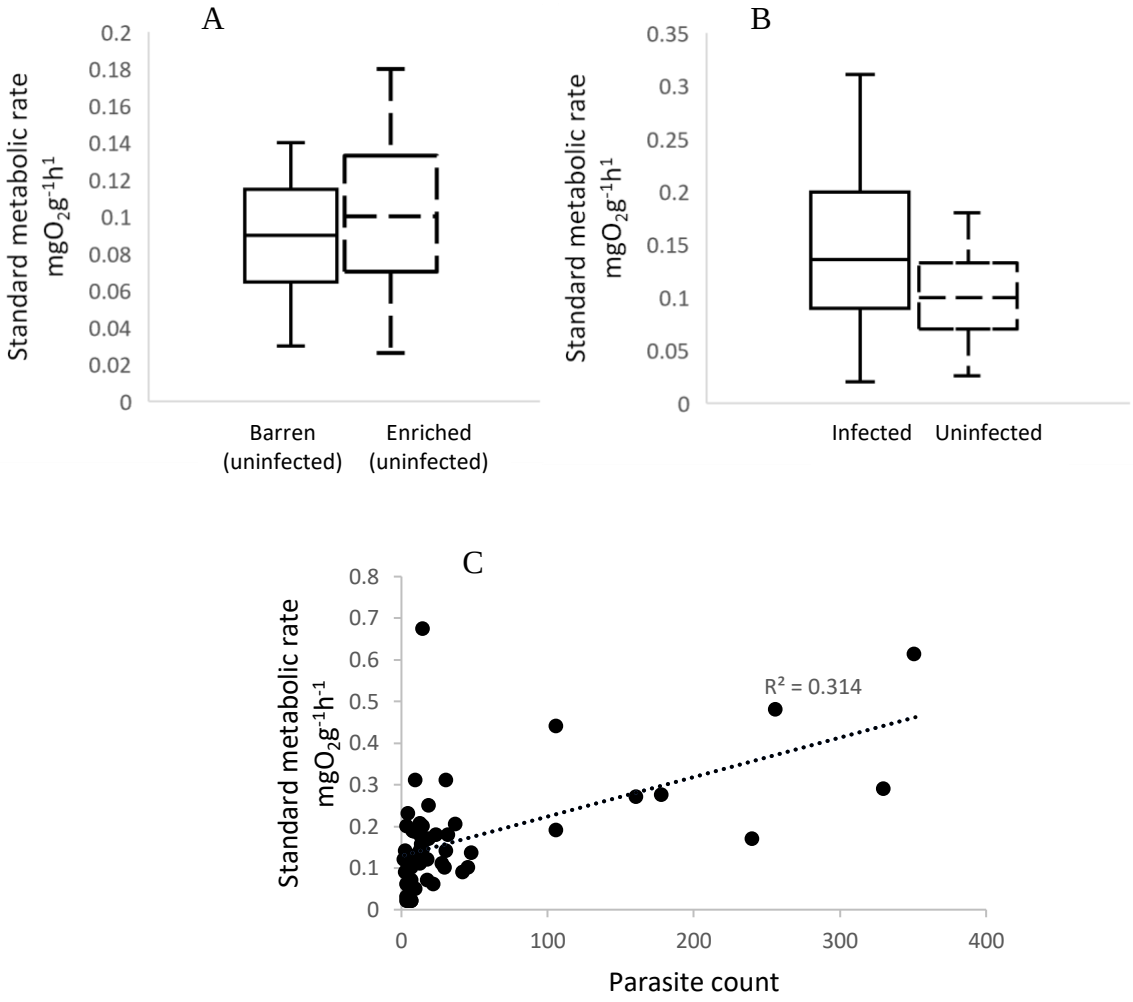

Figure 4.3. Relationship between fish SMR ($mgO_2g^{-1}h^{-1}$), tank treatment (barren versus enriched) and infectious status. (A) No significant association was found between SMR and tank treatment (n= 29 barren and n=28 enrichment- no infections) but (B) fish that were infected (n=29) had significantly higher SMR compared to uninfected conspecifics (n=28). Moreover, (C) a significant proportion of SMR of infected hosts could be explained by parasite count.

4.5. Discussion

Transmissible disease is one of the most significant factors limiting the expansion of aquaculture globally (Stentiford et al., 2017) and there is now a renewed emphasis on developing sustainable methods for disease management. Here, this study reveals that inclusion of environmental enrichment significantly reduces disease burden of ornamental fish. It is also revealed that behavioural modification (i.e., increased aggression) occurs, caused by depriving hosts of enrichment that could facilitate disease transmission and increased disease burden significantly elevates SMR of hosts. Taken together, these results show how relatively simple measures could sustainably improve welfare of captive animals by reducing disease burden and maladaptive behaviours.

Previous studies on the impact of environmental enrichment on host-pathogen dynamics are so limited, and use different methodologies, that this precludes direct comparisons. This study, however, does directly support the observation that farmed piglets reared with environmental enrichment and subsequently inoculated with both Porcine Reproductive and Respiratory Virus (PRRSV) and *Actinobacillus pleuropneumoniae*, showed greater disease resistance compared to piglets in barren enclosures (van Dixhoorn et al., 2016). It was clear in this study that fish from barren enclosures were less resistant to pathogen infections compared to hosts from enriched tanks and peak pathogen burdens were also significantly higher in barren enclosures (Fig. 4.1B). Moreover, hosts from enriched tanks cleared pathogen infections more effectively, suggesting application of environmental enrichment can improve immune responses to infectious disease. This finding is particularly compelling as pathogen exposure is likely to occur in most captive environments because sterile enclosures are not sustainable, especially in large scale facilities. Therefore, ensuring maintenance conditions maximise hosts' immune responses should be a priority.

Variations in the amount and type of enrichment can also impact host-pathogen interactions. Certain enrichment substrates may act as a medium for pathogen growth and increase the chances of infection. However, enrichment substrates are unlikely to facilitate reproduction in directly transmitted microparasites such as *Gyrodactylus* spp. used in this study which cannot survive for long off a host (reviewed in Bakke et al., 2007). Under certain enriched conditions, conversely, bacterial pathogens such as *Flavobacterium columnare*, can actually increase propagation due to the formation of biofilms, increasing host susceptibility to disease (see Karvonen et al., 2016; Räihä et al., 2019). Moreover, the source of enrichment might not only influence biofilm growth but also present an additional hazard as a source of macrofauna contamination; for instance, intermediate hosts, such as snails, vectoring other infectious pathogens. Ultimately, the importance of managing disease burden with interventions such as environmental enrichment is linked to the trade-off between the labour costs of enrichment maintenance and risk of contamination versus the potential to reduce the economic and welfare costs imposed by pathogens.

Most infections lead to the reallocation of metabolic resources to the immune system from general physiological functions (Sheldon & Verhulst, 1996). This study is the first to show that gyrodactylosis increases the SMR of hosts. *Gyrodactylus* spp. are of major welfare concern in both the ornamental and aquaculture trade (Bakke et al., 2007; Maceda-Veiga & Cable, 2019), particularly because there are no effective *en masse* treatments. This increased metabolic demand, even if hosts survive, will impact health reducing physical condition and potentially fecundity. Increased metabolic rates linked to parasitism has been demonstrated in both

invertebrate and vertebrate hosts (e.g., crabs: Haye & Ojeda, 1998; brown trout: Filipsson et al., 2017), and this study further highlights the universal physiological impact of parasitism. Enrichment deprivation on its own, however, did not affect fish SMR, suggesting that the increased aggression seen in fish in barren tanks was not driven by increased basal metabolism.

Increased aggression, as seen in this study for hosts in barren tanks, may have increased disease burden. Chronic aggression can elevate stress levels (see Giacomini et al., 2016) and chronic stress does suppress immunity and increase disease susceptibility (Khansari et al., 1990; Dhabhar, 2009). Furthermore, higher aggression levels will lead to increased contact rates, which can increase the probability of direct transmission for pathogens such as *Gyrodactylus* (e.g., Reynolds et al., 2018). While this study did provide two weeks for fish to acclimate in experimental tanks, which is sufficient for this species to form familiar shoals (Griffiths & Magurran, 1997), it is possible that removing fish from enriched stock tanks might have impacted stress levels. However, as fish hosts in this study demonstrated significantly higher aggression levels in only barren tanks, this does suggest that enrichment deprivation has an overriding influence on stress related behaviour. Through aggression associated nips and chases, contact rates would have increased, and it is plausible that this facilitated pathogen transmission.

To conclude, this study highlights the biological costs of enrichment deprivation: increased susceptibility to disease and interspecific aggression levels. It is also revealed how elevated disease burden linked to enrichment deprivation has a significant metabolic impact. Aquaculture industries have displayed reluctance in using environmental enrichment due to additional time spent cleaning structures and catching fish. However, if we are to prioritise animal welfare, this study recommends industries to investigate which enrichment conditions are most effective at managing aggressive behaviour and disease outbreaks while minimising cleaning and capture time. This study shows that at least on a small-scale enrichment can be a useful tool in health management.

Chapter 5

Microplastic exposure and consumption significantly impacts host susceptibility to disease and mortality

5.1. Abstract

Investigations into the impacts of microplastics on ecosystems have dominated the ecological research landscape. This is unsurprising as microplastics have been found in all surveyed ecosystems and in multiple organisms' diets. Detrimental health impacts of this consumption include abnormal growth rates, reproductive changes, metabolic stress and immune alterations for both invertebrates and vertebrates. Yet despite investigations showing that microplastics can impact immunity for invertebrates and vertebrates, no information exists on how disease resistance may be affected. This knowledge gap must be addressed as parasites are a key factor shaping the structure of food chains and pose significant threats to the human food industry. Here, investigations are conducted on how different microplastic concentrations (0.01 mg/L and 0.05 mg/L) impact host susceptibility to disease and mortality, utilising the freshwater fish guppy-*Gyrodactylus turnbulli* system. This studies results revealed that fish consuming microplastic at both concentrations demonstrated a significantly higher pathogen burden over time compared with fish fed a plastic free diet. While microplastic on its own did not impact mortality, for fish that were infected, microplastic consumption (at both tested concentrations) was associated with significantly reduced life spans. With parasite infections dominating food chains, this study reveals that microplastic consumption can significantly impact host-parasite dynamics.

5.2. Introduction

Microplastics are a recognised global pollutant of high ecological concern. These micropollutants are classified as any type of plastic less than 5 mm in diameter and were first termed 'microplastic' in 2004 after a survey of the British shoreline debris revealed the majority to be plastic smaller than 5mm (see Thompson et al., 2004). Since then, all ecosystems that have been surveyed revealed the presence of microplastics (Wagner et al., 2014; Vince & Stoett, 2018; Rios Mendoza and Balcer, 2020; Rillig and Lehmann, 2020). There are two main modes of microplastic generation: 1) primary production of microplastic in the form of microbeads or microspheres used extensively in household cosmetic products, for example, and 2) degradation of larger macroplastic pieces due to weathering into smaller pieces. The omnipresence of microplastic raised concerns about their consumption by organisms and bioaccumulation in food chains, which has subsequently been confirmed (Lim, 2021). While there are extensive investigations regarding the types and frequency of microplastic in multiple habitats, much less is known about the health effects that these pollutants have in organisms (reviewed in Horton et al., 2017 and Lim, 2021). Thus far, studies have shown there are four typical health effects of microplastic consumption: 1) no discernible effects (Malinich et al., 2018) 2) primary level effects (e.g., endocrine disruption; Pannetier et al., 2020) 3) secondary effects (e.g., impacted cellular immunity and tissue damage) and 4) tertiary level emergent effects (e.g., stunted growth and reproduction, Naido & Glassom, 2019). Of course, these effects can occur in conjunction, especially over extended periods of exposure (e.g., Pannetier et al., 2020).

Arguably, the greatest emphasis in the microplastic crisis has been paid to oceans, largely in response to popular nature documentaries (reviewed by Vince & Stoett, 2018 and Barboza et al., 2018). With freshwaters, however, being the most disturbed habitats globally (Dudgeon et al., 2006; Pimm et al., 2014; Tickner et al., 2020), we are now seeing an increased emphasis on microplastic contamination here (reviewed by Triebskorn et al., 2019). Furthermore, freshwater fish, in particular, are facing higher extinction rates than any other vertebrate group and increasing anthropogenic contaminants are implicated in species loss (Collen et al., 2014; Adams et al., 2014; Pimm et al., 2014). Microplastics are found in freshwater fish diets (Horton et al., 2017; Triebskorn et al., 2019), but our understanding of their health impacts is still in its infancy, to date ranging from no observable effects (Rochman et al., 2017) to metabolic stress (Alomar et al., 2017), reproductive changes (Wang et al., 2019), impacted growth and increased mortality rates (Naidoo & Glassom, 2019). The effects of microplastic consumption are further complicated by their ability to adsorb chemical contaminants, which often amplifies their toxicity (reviewed by Wang et al., 2018).

A particular aspect of fish welfare that remains poorly understood is how microplastic consumption may impact immunity. Changes to immunity is arguably one of the most concerning effects that could occur in response to microplastic consumption, as parasites that cause transmissible diseases are not only recognised as the predominant biomass within ecosystems (Kuris et al., 2008), but they also impact all aspects of organism's life history traits (Poulin, 1999; Lefèvre et al., 2009). In the absence of human interventions and anthropogenic stressors, Red Queen selection dynamics tend to hold hosts and their parasites in a state of equilibrium *in natura* (Hatcher et al., 2012). If a pollutant as ubiquitous as microplastics can suppress fish immunity, this could tip the scales in favour of parasite propagation. Thus far, transcriptional changes in immune genes, and altered cellular immune responses have been observed in fish exposed to microplastic (Greven et al., 2016; Limonta et al., 2019), which has the potential to impact disease susceptibility. However, to the best of this researchers' knowledge, no prior studies have revealed how underlying changes in immunity (both genetic and cellular) caused by microplastic exposure impact disease resistance. Investigations not associated with this PhD have shown that wild freshwater sticklebacks (*Gasterosteus aculeatus*) with gyrodactylosis (monogenean infection) maintain their infections for significantly longer when exposed to microplastics (Masud et al., in review). While the stickleback study did reveal that phenotypically disease responses can be impacted in a close to natural scenario, it was based on pre-existing infections and it is acknowledged that immunological memory could be a confounding factor. Ideally, to determine if microplastic is the major factor that influences disease resistance, pathogen specific immunologically naïve fish would be needed. To this extent, the current study utilises fish that had no prior exposure to gyrodactylosis.

Here, investigations are conducted to determine the impact of variable microplastic (polypropylene) concentrations on host-parasite interactions and host mortality. In this study the focus is on polypropylene microplastic as it is the second most widely used plastic commodity (PlasticEurope, 2019). Furthermore, it is known that different types of microplastics can have interactive effects (see Rochman et al., 2017), making it much more challenging to disentangle the impacts of consumption on disease dynamics, without needing unsustainably large live animal samples. The host is the ecological and parasitological model, the Trinidadian guppy (*Poecilia reticulata* – see Magurran, 2005) and the parasite is a common monogenean ectoparasite for this host, *Gyrodactylus turnbulli*. This parasite has a direct life cycle and transfers between hosts upon direct contact (King & Cable, 2007). The genus

Gyrodactylus contain species that are both ecologically and aquaculturally important and are known for their unique hyperviviparous mode of reproduction, linked to progenesis (reviewed by Bakke et al., 2007). This is the first study of its kind that investigates the functional impact of microplastic exposure and consumption on disease resistance.

5.3. Materials and methods

5.3.1. Host-parasite system

Size matched female guppies were used for this study (*Poecilia reticulata* - size range: 19-22 mm standard length) and bred from a stock originally caught in the Lower Aripo River in Trinidad in 2012. All guppies were maintained in 70 L breeding tanks at 24º±0.5ºC under a 12-h light: 12-h dark photoperiod (lights on 07:00-19:00) and fed dry food flakes (Aquarian®) daily and freshly hatched *Artemia* nauplii every alternate day. To assess their susceptibility to disease, experimental infections utilized the *Gt3* strain of *Gyrodactylus turnbulli*, isolated from a Nottingham aquarium shop in October 1997 (King & Cable, 2007). This parasite population has since been maintained using established methods in our laboratory (Reynolds et al., 2018). To measure wet mass, all hosts were weighed on an electronic scale (0.01 mg accuracy, OHAUS ®) prior to commencing treatment feeds and at days 7 and 25 of the infection trajectory.

5.3.2. Microplastic preparation and dietary exposure

As microplastics readily absorb toxins, which are detrimental to fish health (Rochman et al., 2013; Wang et al., 2018), pristine polypropylene pellets (Sigma-Aldrich <0.5 mm, further ground into microplastics using pestle and mortar) were used for microplastic dietary preparation for this study. To avoid procedural contamination, all equipment was rinsed with acetone followed by ethanol and preparation of diets conducted under a fume hood. Immediately before grinding, pellets were placed in cryogenic vials (STAR LAB Ltd) and dipped in liquid nitrogen (Rochman et al., 2013). After grinding, plastics were sieved in pre-cleaned stainless-steel metal sieves with 0.3 mm aperture to collect fragments. Therefore, all microplastics used for this investigation were <0.3 mm. It has been previously confirmed that our aquarium water supply is not contaminated with microplastics (Masud et al., in review).

In a preliminary trial, guppies were clearly observed consuming microplastic when sprinkled on the water surface and 1-week later microplastics were detected in faecal matter of these fish using a dissecting microscope. For dietary exposure, fish were fed 2% bodyweight per day. Experimental fish were divided into three treatments, corresponding to three levels of microplastic: 1) 0.05 mg/L, n=74; 2) 0.01 mg/L, n=74; and 3) 0 mg/L controls, n=74. These concentrations were chosen based on previous dose-response studies on fish microplastic consumption (Barboza et al., 2018) and correspond to levels of microplastics detected in freshwater systems (reviewed in Fisher et al., 2016). Control fish (n=74, not exposed to microplastic) were fed the same quantity of food as fish exposed to microplastic, to ensure all hosts were given the same nutritional input. All experimental fish were isolated in 1L containers for the duration of the experiment. Fish were fed once a day by sprinkling their diet on the water surface of each container. A 100% water change was conducted daily at 4pm to standardize timing of feeding. Fish were experimentally infected after 3 weeks of dietary exposure and the same feeding regimes continued throughout infection.

5.3.3. Experimental infections

From each of the three diet treatments, half the fish were experimentally infected. Briefly, this involved lightly anaesthetizing individual guppies with 0.02% MS-222, and each fish infected with two gyrodactylid worms. Parasite transfer was conducted following standard methods of King & Cable (2007). Parasite infections were then monitored every 48 h for a maximum of 45 days, by anaesthetizing fish and counting the total number of gyrodactylids over the entire infection trajectory.

5.3.4. Statistical analysis

All statistical analyses were conducted using RStudio v2.1. To analyze the relationship between treatments (microplastic exposure and controls) and disease resistance the following parasite metrics were used: mean intensity; maximum parasite count and Area Under Curve (AUC). Maximum parasite count is the highest parasite number achieved at a given time point (defined here as peak day), over the infection trajectory. To quantify total infection trajectory, the Area Under the Curve (AUC) was utilized and calculated with the trapezoid rule (White, 2011). To analyse maximum parasite count and AUC a Generalized Linear Model (GLM) with a negative binomial error family and a log link function was used in the R MASS package.

To analyse the relationship between microplastic exposure and time of mortality (days) for fish that were exposed to infections, a GLM with an inverse gaussian error family and log link function was utilized. Whereas a GLM with a poisson error family was utilized to compare mortalities between all treatments. All GLM error families were chosen based on the lowest dispersion parameter, theta and Akaike Information Criterion (AIC) value. No deaths occurred for control fish that were not exposed to infections and therefore not included in the mortality analysis. For all GLM's, standard length of hosts and treatment were treated as fixed factors. Further to the GLM analysis of time to mortality, a Kaplan-Meier survival analysis was conducted using a Cox's proportional hazards model within the *survival* package in R. A Generalised Linear Mixed Model (GLMM) was fitted to the live wet mass and treatment fish were exposed to, to assess the impact of microplastic exposure on body condition. As the same fish wet mass were measured at different time points, to prevent pseudoreplication a GLMM was needed. The GLMM analysis was conducted using the lme4 package in R with a gaussian error family and log link function. The error family for the GLMM was chosen based on which model achieved the lowest AIC value (Thomas et al., 2017).

5.4. Results

Hosts consuming microplastic (0.01 mg/L and 0.05 mg/L) had significantly higher maximum parasite burdens compared to controls fed on standard flake food (GLM: 0.05 mg/L exposure $z=4.46$, S.E.=0.11, $p<0.001$; 0.01 mg/L exposure $z=8.23$, S.E.=0.11, $p<0.001$; Fig. 5.1). However, a *post-hoc* analysis revealed no significant difference in maximum parasite counts between the two microplastic treatments ($X_2=2.881$, df = 1, $p = 0.08963$).

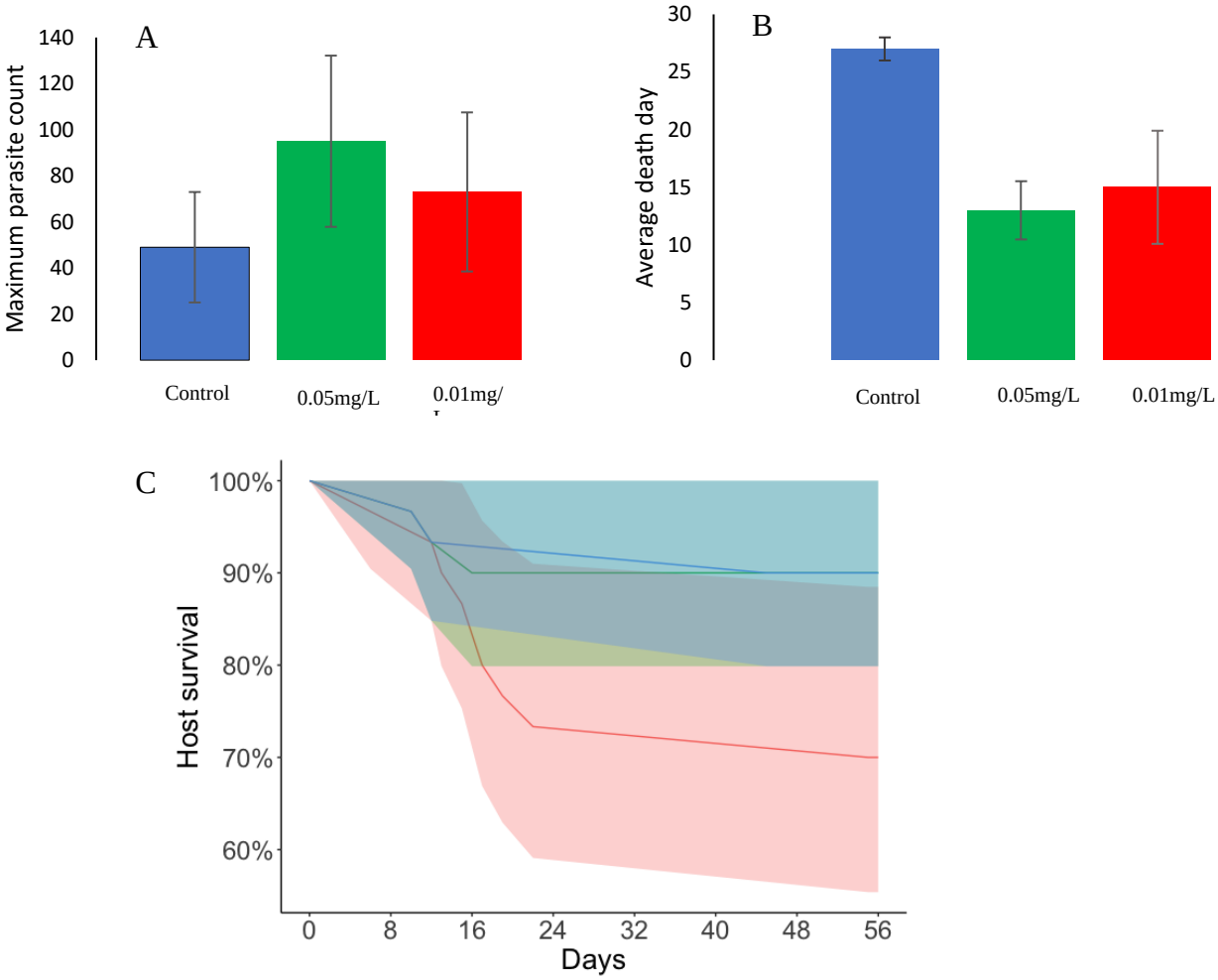

Fig. 5.1 Hosts (*Poecilia reticulata*) exposed to 0.01 mg/L or 0.05 mg/L microplastic and subsequently infected with *Gyrodactylus turnbulli* suffered A) significantly higher maximum parasite count and B) died significantly earlier compared to control fish fed on standard flake food and C) there was a marginally significant increase in the proportion of hosts dying in fish exposed to 0.01 mg/l. Standard error bars shown for maximum parasite count and average death day with 95% confidence intervals for percentage host survival.

Total infection trajectories as measured through AUC were significantly greater for hosts that were feeding on microplastics compared to controls (0.05 mg/L exposure: GLM Z=-16.87, S.E.=0.13, $p<0.001$, 0.01 mg/L exposure: GLM Z= -14.26, S.E.=0.13, $p<0.001$; Fig. 5.2).

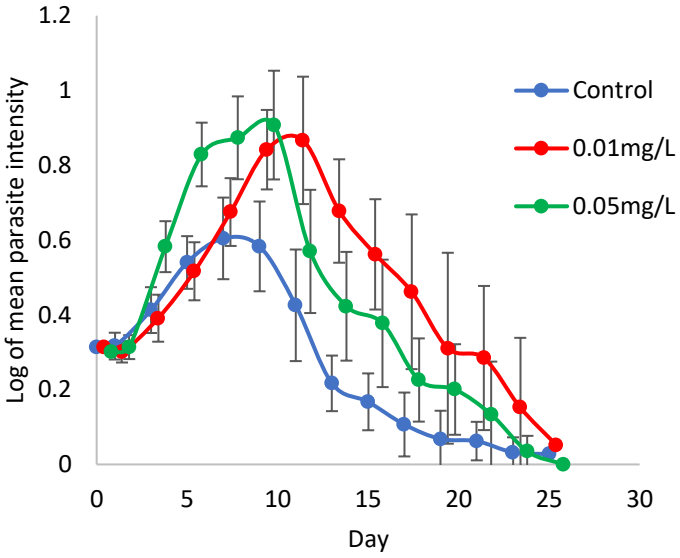

Fig 5.2. Mean parasite count of hosts (*Poecilia reticulata*) exposed to microplastic concentrations (0.01 mg/L and 0.05 mg/L) were significantly higher than those of controls that were fed standard flake food. Shown here, Log of parasite intensity with standard error bars for microplastic exposure and controls.

When comparing mortalities between all treatments, fish exposed to the higher dose of microplastic (0.05 mg/L) and subsequently infected died significantly earlier compared to fish in all other treatments (GLM: z= -2.165, S.E.=0.33, P=0.03-Table 5.1). Interestingly, when only comparing mortalities between fish that were infected, both concentrations of microplastic exposure led to significantly earlier mortalities compared to controls (0.05 mg/L + infections: GLM: t=-9, S.E.=0.08, p<0.001; 0.01 mg/L + infections: GLM: t=-7, S.E.=0.07, p<0.001). Survival analysis revealed that for fish fed microplastic at both concentrations and subsequently infected, only hosts consuming microplastic at 0.01mg/l had marginally significant increases in the proportion of deaths (Coxph: Coef= 1.17, z=1.76, p=0.07- Figure 5.1C- number of deaths in 0.01mgl treatment =9 out of 30 fish). However, no difference was seen in the proportion of deaths between control infected fish not fed any microplastic and those exposed to 0.05mg/l and subsequently infected (Coxph: Coef: 0.01, z=0.01, p=0.98- Figure 1C- number of death in control and 0.05mg/l identical = 3 out of 30 fish).

Table 5.1. Average death day and corresponding p -values from Generalised Linear Model analysis for all fish that were either exposed to microplastics or control treatments and subsequently infected with parasites.

Treatments	**Average death day**	**Z- value**	**Standard error**	**P- value**
Control + infections	27	-0.26	0.26	0.79
0.01 mg/L exposure + infections	15	-1.82	0.32	0.06
0.05 mg/L exposure + infections	13	-2.16	0.33	0.03
0.01 mg/L exposure	29	0.26	0.26	0.78
0.05 mg/L exposure	20	-1	0.29	0.30

No impact of any treatment was detected to significantly impact the wet mass of fish and majority of fish increased in mass during the experiment ($p > 0.05$ for all treatments).

5.5. Discussion

Plastic pollution is considered one of the greatest environmental concerns of this century (Moore et al., 2001; Galloway et al., 2017; Rios Mendoza & Balcer, 2020). This legacy contaminant in the form of microplastics is found in multiple organism diets and we are only now beginning to understand the impacts of this consumption for animal welfare (Horton et al., 2017; Triebskorn et al., 2019). This study investigated the impact of variable microplastic consumption on fish susceptibility to disease and host mortality. Fish consuming microplastic (at 0.01 mg/L and 0.05 mg/L) demonstrated significantly higher parasite burdens compared with control fish fed only flake food indicating that microplastic at these concentrations is detrimental for disease resistance. How the changes in disease resistance relate to underlying cellular and genetic immune responses is currently unknown and studies on microplastics impact on fish immunity are very limited. Zebrafish that consume microplastic show a downregulation in key genes involved in innate immunity, specifically epithelium integrity (Limonta et al., 2019). This might suggest that for zebrafish, there is reduced pathogen control at epithelial barriers and rising chances of infections at mucosal sites. The zebrafish study did not, however, investigate disease resistance (Limonta et al., 2019). In this study, the pathogen investigated is an ectoparasite that forages on the mucous and epithelial cells of fish (see Bakke et al., 2007). Therefore, if as suggested by the zebrafish study, downregulation of immune genes at epithelial barriers is occurring in guppies, this would explain the increased severity of gyrodactylosis in fish consuming microplastic.

In previous investigations not associated with this PhD, three-spined stickleback (*Gasterosteus aculeatus*), that are a cold-water species, maintained their gyrodactylid infections for much longer when consuming microplastic (polypropylene at 0.05 mg/L) and showed a marginally elevated pathogen burden (Masud et al., in review). However, it is worth noting that the hosts in the current study were maintained at a much higher temperature (24ºC versus 14ºC) and infections tend to progress much faster as temperatures increase; certainly, seen for gyrodactylid species (see Stewart et al., 2017a). Furthermore, the fish in this investigation were effectively immunologically naïve to gyrodactylosis unlike the sticklebacks which may well have had prior exposure to these ectoparasites, and it is known that prior exposure impacts subsequent disease responses (Cable & Van Oosterhout, 2007). Regardless of temperature related dynamics and immunological history effects on infections, this study and the related investigation on sticklebacks reveal that microplastic consumption can negatively impacts disease resistance in fish.

Another possibility beyond the scope of this study is to investigate the impact of nanoplastics on disease resistance. While this study did use microplastics that were <0.3mm, the method of preparation could have fractured the polypropylene pellets into nanoplastics and therefore we cannot rule out the possibility that nanoplastics were interacting with fish cellular immunity. *In vitro* investigations have revealed that nanoplastics cultured with fathead minnow (*Pimephales promelas*) neutrophils, that are a key element of fish innate immunity, demonstrated increased extra cellular trap release and degranulation (see Greven et al., 2016)

suggesting nanoplastics could affect disease resistance. However, identifying, and characterising nanoplastics is a major challenge and considered ‘the needle in the haystack, but the needle looks like the hay’, because even the most current imaging technologies struggle to differentiate between plastic and biological samples at a nanoscale (see Lim, 2021). However, experimentally it has been shown that cell lines will readily absorb nanoplastics and this is considered a biological ‘ticking time bomb’ for many researchers specialising in micropollutants (see Caldwell et al., 2021).

Host mortality rates were also impacted by microplastic consumption. Infected fish consuming microplastic at 0.05 mg/L died significantly earlier compared with fish from all other treatments. Interestingly, when excluding uninfected fish from the mortality analysis, both microplastic concentrations were associated with significantly earlier mortality, which suggests that microplastic on its own was not enough to impact mortality, at least over the time scale of the experiment. Furthermore, the survival analysis also revealed that, paradoxically, at the lower concentration of microplastic exposure (0.01mg/l), a greater proportion of deaths occurred compared with the higher concentration (0.05mg/l), though it is acknowledged this was only marginally significant. It is unclear from this study what the underlying reason behind the increased total number of host deaths in the lower microplastic dose treatment was. Nonetheless, it seems that in terms of time to mortality, both concentrations of microplastic were associated with earlier deaths in infected fish indicating that microplastic was reducing the tolerance of fish to infections. Though non-significant, it is worth noting that, for uninfected fish, at 0.05 mg/L there was a negative association between microplastic consumption and time of death, implying that at this concentration, microplastic was marginally reducing the life expectancy of adult fish. While many studies have shown that microplastic consumption does impact mortality levels of invertebrates (e.g., Jemec et al., 2016; Gray & Weinstein, 2017; Horn et al., 2019), studies in fish species are much rarer. Those studies that have investigated microplastic associated mortality in fish have found variable results with comparisons between studies confounded by use of different fish species, life stages (juvenile or adult) and microplastic type and concentration. Most studies that have shown a significant effect on mortality tend to be in juveniles, linked to stunted growth, cellular toxicity, and DNA damage (Naido & Glassom, 2019; Pannetier et al., 2020), whereas the link between microplastic consumption and mortality is less apparent in adult fish (Rochman et al., 2013; Guven et al., 2018).

To conclude, this study has shown that microplastic consumption at relatively high concentrations can significantly impact host-parasite interactions by increasing pathogen burdens for host. Furthermore, for infected hosts, microplastic consumption is associated with significantly high mortality. Future research will need to pair phenotypic infection data presented here with underlying immune transcriptomics, to understand what the underlying reasons are behind changes to host-parasite dynamics. Furthermore, investigations will also have to consider as a matter of priority whether nanoplastics, which have been shown to enter cultured cell lines, are part of this story, which would require further development of imaging technologies that currently struggle to distinguish between biological material and plastic at nanoscales. With plastic pollution being a ubiquitous feature of all food chains, such research is of high priority.

Chapter 6

Not going with the flow: locomotor activity does not constrain immunity in a wild fish

This chapter is affiliated with the publication Masud et al., 2019b in the journal Ecology & Evolution

6.1. Abstract

Immunity is a central component of fitness in wild animals, but its determinants are poorly understood. In particular, the importance of locomotory activity as a constraint on immunity is unresolved. Using a piscine model (*Gasterosteus aculeatus*) this study combines a 25-month observational time series for a wild lotic habitat with an open flume experiment to determine the influence of locomotor activity (counter-current swimming) on natural variation in immune function. To maximize the detectability of effects in this flume experiment flow velocity and duration (10 cm s^{-1} for 48 h) are set just below the point at which exhaustion would ensue. Following this treatment, measurements of expression in a set of immune-associated genes and infectious disease resistance were made through a standard challenge with an ecologically relevant monogenean infection (*Gyrodactylus gasterostei*). In the wild, there was a strong association of water flow with the expression of immune-associated genes, but this association became modest and more complex when adjusted for thermal effects. In the flume experiment, although statistically well-powered and based on a scenario near the limits of swimming performance in stickleback, detected no counter-current swimming effect on immune-associated gene expression or infection resistance. The field association between flow rate and immune expression could thus be due to an indirect effect and this study tentatively advances hypotheses to explain this. This study clarifies the drivers of immune investment in wild vertebrates; although locomotor activity, within the normal natural range, may not directly influence immunocompetence, it may still correlate with other variables that do.

6.2. Introduction

This study examines the consequences of locomotor activity for immunity in a model wild vertebrate, the three-spined stickleback, *Gasterosteus aculeatus*. Like most animals, sticklebacks need to undertake locomotor activity to survive. In particular, individuals living in flowing water must maintain station within suitable habitat through counter-current swimming (rheotaxis) (Arnold, 1974). This expends energy and may functionally interfere with other physiological processes (Kieffer, 2000), perhaps altering immune allocation and function (van Dijk & Matson 2016). Alteration in immunity, in turn, is likely to affect health and fitness at the individual and population level - influencing the development of disease within individuals (Parkin, 2001) and constraining the transmission of infectious disease between individuals (Hellriegel, 2001). Thus, being able to understand and predict sources of variation in immune function will often be necessary to understand the dynamics of disease. Moreover, despite increasing recognition that immune variation is generated largely by environmental effects, perhaps including locomotory responses to the environment, the sources of this

variation are poorly understood, even in humans and laboratory mice (Brodin et al., 2015; Beura et al., 2016).

Physical activity is widely thought to influence the immune system (Pedersen & Hoffman-Goetz, 2000; Walsh et al., 2011), and, furthermore, has often been considered to exert suppressive, generally transient, effects that increase disproportionately at more extreme levels of activity (Nieman, 2000; van Dijk & Matson, 2016). Nonetheless, the latter paradigm has also been challenged and immunological changes following intense exercise interpreted differently – as a beneficial heightening of immune surveillance and regulation (Campbell & Turner, 2018). The evidence for these contrasting paradigms in naturally occurring vertebrates is even less clear-cut and largely derived from a limited number of studies in birds, with fewer studies in other vertebrate classes (Brown & Shine, 2014; Husak, Ferguson & Lovern, 2016). In birds, flight experiments (Matson et al., 2012; Nebel et al., 2012; Nebel et al., 2013) mostly suggest immunosuppressive effects of sustained flight, but contrary observations of no effect (Hasselquist et al., 2007) have also been reported.

Through integrating field observation with matched experimental manipulation of sticklebacks, the present study aims to place the effects of water flow, and associated counter-current swimming, within the context of overall environmental effects on immunity in the wild. To achieve this, this study considers both field records of water flow and immune gene expression in a lotic habitat and effects estimated in an experiment in which acclimated fish were made to swim in an open flume under controlled conditions. This study design is intended, as far as possible, to avoid the artificiality of laboratory models and the very weak inference typically possible in purely observational studies (due to collinearity and confounding of variables).

For the field component of this study, an analysis of a 25-month time series was made, containing monthly expression data for immune-associated genes and fine scale thermal and flow data (all of which showed strong sinusoid-like circannual oscillation). For the flume experiment, effectively wild fish (from a naturalistic, parasite-exposed habitat) were used that had first been treated to remove parasites and acclimatized. As immune system expression is likely to depend on past individual experience of the environment, this ensured that experimental subjects had a history of natural environmental exposures and a relatively natural immunophenotype. (In contrast, laboratory-bred subjects would have had past environmental exposures very different to those in the wild, and very different immunophenotypes, unrepresentative of those in nature).

In both the field and experiment a standard set of gene expression measurements were made that have been previously demonstrated to precisely report a dominant genome-wide seasonal oscillation in immune-associated gene expression in wild sticklebacks (Brown et al., 2016; Stewart et al., 2018b). This oscillation corresponds to experimentally determined infection resistance (Stewart et al., 2018a; Stewart et al., 2018b) and is partly driven directly by environmental temperature and partly by other, as yet unidentified, seasonal environmental variation that might include variation in flow effects (Stewart et al., 2018a; Stewart et al., 2018b). Seasonal progression explains more genome-wide variation in immune-associated gene expression than other relevant factors (including geographic site, sex, and ontogeny) and is characterized by outlying expression values in the late winter and late summer (Brown et al., 2016). For simplicity, as previously described (Stewart et al., 2018b), this study combines the set of gene expression measurements into a single representative index (seasonal reporter

index, SRI). Moreover, to cross-reference gene expression variation and the experimental treatments to a functional phenotype direct measurements of infection resistance are also made in the flow experiment. This was based on challenge infections with the ecologically relevant (pathogenic and naturally occurring) monogenean ectoparasite, *Gyrodactylus gasterostei* (Stewart et al., 2017), a directly transmitted viviparous species that proliferates *in situ* on the host skin.

This study was thus designed to allow partitioning of the effects of locomotory activity from other sources of variation in immune allocation in nature and to quantify them. In the event, no effect of sustained and intense counter-current swimming was found, suggesting that variation in locomotory activity has a negligible direct influence on immune allocation and function in wild fish. Nonetheless, seasonal flow rates were correlated with immune allocation in the wild and so this study further considers possible indirect effects of water flow on seasonal immune variation.

6.3. Materials and methods

6.3.1. Field site and field observations

The study site (RHD, 52.4052, -4.0372) was a small area in an oligotrophic, fully freshwater, side-channel of the River Rheidol. As the Rheidol traverses an unusually steep gradient and is subject to anthropogenic water releases from the Cwm Rheidol dam (upstream of the study site), it experiences a very variable flow regimen, with maximum flows above 30 m s^{-1} (Whoriskey & Wootton, 1987) possible, even in side channels. The site held a large population of sticklebacks with an approximately annual life history (young of the year largely replacing the previous year cohort by early autumn) (Wootton, 2007). A calibrated TinyTag Aquatic 2 data logger was placed at a representative depth within the habitat, recording temperature readings every 5 min throughout the study. To provide information on water flow in the river, water level readings, taken every 15 min, were obtained from a gauge at Cwm Rheidol (data kindly supplied by National Resources Wales). Ten fish were sampled per month from RHD for 25 months between October 2013 and October 2015. Monthly data were missing for October and December 2014 due to high water levels. Gene expression data for the RHD fish has previously been reported (Stewart et al., 2018b). As previously described, fish were individually captured with dipnets and immediately killed by concussion and decerebration, then conserved in RNA stabilization solution and transferred to -80°C for long term storage (Brown et al., 2016). Standard length (tip of snout to tail fork, mm), weight (mg) and sex were recorded for all fish.

6.3.2. Open flume experiment

Sticklebacks, from a self-propagating population in semi-natural (lentic) outdoor fishponds (Surrey, UK), were obtained in January 2017 and transported to Cardiff University. As the source population was exposed to a natural community of parasites, the fish were subject to anti-parasitic treatment. Fish were first submerged in 0.004% formaldehyde solution for 1 h, with a half hour rest period in between, and then maintained in 1% aquarium salt and 0.002g/L methylene blue for 48 h to prevent secondary infections. Subsequently, each individual fish was visually screened for ectoparasites three times by anesthetising them in 0.02% MS-222 and observing them using a dissecting microscope with fibre optic illumination. This screen

involved removing any remaining ectoparasites with a watchmaker's forceps following the methods of Schelkle et al.(2009). After treatment, sticklebacks were maintained in 70 L tanks with dechlorinated water (temperature: 14 ± 1°C; photoperiod: 12 h light/12 h dark; approximating late summer conditions). Fish were fed daily, to satiation, on a diet of frozen bloodworms and acclimatized to laboratory conditions over a period of 8 weeks. The above temperature, lighting and diet conditions were maintained throughout the experimental trials.

In employing fish from a lentic habitat for the experiment it was expected that the fish might be less pre-conditioned to sustained swimming, and thus allow more sensitive detection of any locomotion effect on immune gene expression. It is also noted that natural variation in the gene expression readouts that are employed below is largely due to consistent environmental responses, with fish of different genetic identity in different habitats responding similarly to seasonal change (Brown et al., 2016; Stewart et al., 2018b). Thus, differences in the genetic background of the study fish are expected to be unimportant.

The flow experiment was conducted in a perspex open channel recirculating flume (channel length: 150 cm, depth: 16 cm and height: 20 cm), filled to 15 cm depth, to expose fish to water flow. To create flow, an impeller with a diameter of 10 cm was attached to a 1 horsepower three phase 4-pole motor with a maximum shaft speed of 1500 rpm (Machine Mart) and wired to a 1.1 kW inverter (RS Components) which controlled motor speed. Aluminium honeycomb flow straighteners (width: 20 mm, cell diameter: 6.4 mm) were inserted at both ends of the flume to provide laminar flow, restricting the fish to a 100 cm length section. Based on preliminary trials, a flow speed of 10 cm s^{-1} (25Hz) was chosen for this experiment as it evoked counter-current swimming (rheotaxis) without leading to exhaustion, defined as an inability to maintain station (Whoriskey & Wootton 1987). An identical flume with static conditions was used as a control. Flow speed was measured by recording the time taken for a neutrally buoyant ball to flow 1 m downstream, averaged over 10 replicates. Fish were placed in the flume at a stocking density of 5 fish per flow trial and the flume was run for 48 h (equivalent to travelling 17.3 km).

To determine immune expression at the end of the 48 h flow treatments (see Figure 5.1 for a summary of the experimental design), fish exposed to flow (n = 15) and control fish in static conditions (n = 15) were removed from the flume and immediately killed by an overdose of anaesthetic (MS222) followed by decerebration ("baseline" fish). Further fish were removed from the flume and experimentally infected with two *Gyrodactylus gasterostei* worms as previously described (see King & Cable, 2007). Briefly, a heavily infected donor stickleback was placed near a recipient fish and monitored under a dissecting microscope with fibre optic illumination until two worms were observed transferring to the caudal fins of the recipient (procedure lasting < 5 min). Control fish, experiencing no-flow conditions, were infected at the same time as flow-treated fish. Infected fish were then maintained in 1 L containers, where a subset (n = 18 flow treatment; n = 16 control) were screened for parasites (full count of individuals) every 4 days (King & Cable 2007) over a 24-day infection trajectory (Figure 6.1). The remaining infected fish were sampled to determine immune expression (as above) at days 10 or 20 of the infection trajectory (n = 15 fish per treatment × time combination) (Figure 6.1). The viviparous *in situ* infrapopulation growth of *G. gasterostei*, on isolated fish at the temperatures used here, typically features an initial post-infection increase ("up phase"), followed by a peak and decrease ("down phase") to zero or very low numbers (Harris, 1983). The 10- and 20-day time points used above respectively correspond to the "up-phase" and "down-phase" of population growth in the present set of infections.

All fish killed at sampling points were immediately conserved in RNA stabilization solution, as previously described (see Hablutzel et al., 2016), and transferred to -80°C for long term storage. Standard length, weight and sex were recorded for all fish processed for gene expression and standard length for challenge infection fish. The range of standard length in experiment fish sampled for gene expression was 45-60 mm (compared to 15-61 mm in wild fish from RHD). The experiment fish were, on average, in poorer body condition than wild fish at RHD, suggesting that any relevant energy or nutrition-mediated effect of locomotory activity on immune activity would not have been masked by an excessively high level of nutrition in the feeding regimen that was employed.

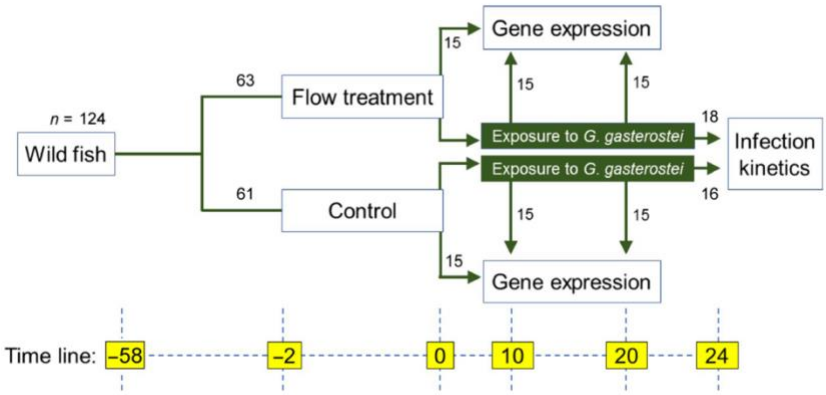

Figure 6.1. Schematic summarising experimental design. Time (lower boxes) is expressed in days relative to the time of exposure with *Gyrodactylus gasterostei* (i.e., negative before parasite exposure).

6.3.3. Gene expression measurements

Gene expression was quantified in RNA extracted from homogenized whole fish by reverse transcription quantitative real-time PCR (QPCR) following previously described methods (Brown et al., 2016; Hablützel et al., 2016; Stewart et al., 2018b). The advantages of using whole fish samples are considered in Hablützel et al. (2016). A set of 5 genes (seasonal reporter, SR, genes) were used whose expression have been shown to reflect a major immune-system-wide seasonal expression signature (Brown et al., 2016; Stewart et al., 2018b). These include three genes expressed highly in summer: *cd8a* (Ensembl gene identifier: ENSGACG00000008945), *foxp3b* (ENSGACG00000012777) and *ighm* (ENSGACG00000012799); and two genes expressed highly in winter: *orai1* (ENSGACG00000011865) and *tbk1* (ENSGACG00000000607). As noted above, gene expression data for the field study at RHD have previously been considered, and detailed methods reported, in Stewart et al. (2018b). Equivalent methods were used to generate the gene expression dataset for the present flume experiment. Briefly, RNA was extracted from whole fish samples preserved in RNA stabilization solution using the Isolate II RNA mini kit (Bioline). Whole individual fishes were homogenized in kit lysis buffer using a 5 mm stainless steel bead (Qiagen, 69989) in a Qiagen TissueLyser LT system and a standard aliquot of the homogenate passed through the manufacturer-recommended protocol. RNA extracts were DNAse treated and converted to cDNA using the High-Capacity RNA-to-cDNA™ Kit (ThermoFisher), according to manufacturer's instructions, including reverse transcription

negative (RT-) controls for a subsample. Assays were pipetted onto 384 well plates by a robot (Pipetmax, Gilson) using a custom programme and run on a QuantStudio 6-flex Real-Time PCR System (ThermoFisher) at the machine manufacturers default real-time PCR cycling conditions. Reaction size was 10 µl, incorporating 1 µl of template and Applied Biosystems™ Fast SYBR™ Green Master Mix (ThermoFisher) and primers at the machine manufacturer's recommended concentrations. Samples from different experimental treatment groups were dispersed across 3 plates. Each plate contained all target gene expression assays and two endogenous control gene assays, for samples (in duplicate) and a calibrator sample (in triplicate). Endogenous control genes (*yipf4, acvlr1*) were previously validated (Brown et al., 2016), as a pairing, for stability under seasonal variation. Primers used were reported in Brown et al., (2016) and Hablutzel et al., (2016). In addition, no template controls for each gene were included on each plate. Template cDNA (see above) was diluted 1/20 prior to assay. The calibrator sample (identical on each plate) was created by pooling cDNA derived from whole fish RNA extracts from wild sticklebacks captured in summer. Relative gene expression values used in analyses are RQ values calculated by the QuantStudio 6-flex machine software according to the ΔΔCt method, indexed to the calibrator sample. Melting curves and amplification plots were individually inspected for each well replicate to confirm specific amplification.

6.3.4. Parasite material

Parasites originated from a laboratory culture of *Gyrodactylus gasterostei* recently derived from wild sticklebacks at Roath Park, Cardiff (51.506, -3.175) and passaged on uninfected sticklebacks.

6.3.5. Statistical analysis

The field and experiment gene expression datasets respectively contained 219 and 54 individuals with no missing values. In the experiment, only a random subsample of the fish sampled at day 10 and day 20 (6 per treatment group) were processed for gene expression (see Figure 5.1). The *G. gasterostei* challenge infection dataset from the experiment contained 34 individuals, 3 of which had missing standard lengths but no other missing values.

All analyses were carried out using *R* version 3.4.4 (R Core Team 2018). For the field dataset, gene expression measurements were combined into an additive index (seasonal reporter index, SRI) that have been previously shown to reflect a major pattern of seasonality in the expression of stickleback immune-associated genes. For this, each raw relative gene expression variable was first $\log_{10}$ transformed and standardized. The values for each gene variable were then summed, assigning negative or positive values to genes according to whether they were most expressed in winter (negative) or in summer (positive) in the transcriptomic study of Brown et al. (2016) (i.e., *cd8a + foxp3b + ighm - tbk1 - orai1*). High values of the SRI reflect high expression of genes linked to the adaptive arm of the immune system. For the field dataset, this study does not present the variation in individual gene expression variables contributing to SRI here as this has been previously considered in detail elsewhere (Stewart et al., 2018b) and the individual variables all correlate very strongly with SRI.

For the field dataset, initially variation in flow and temperature in generalized additive models (GAMs) were analysed separately and variation in SRI in generalized additive mixed models

(GAMMs) (Wood, 2006). The non-parametric smoother term in these models was used to flexibly represent temporal trends, without presupposing a particular relationship. The GAMs for flow and temperature contained a thin plate spline smoother for time as an explanatory term. The GAMMs for gene expression variables additionally contained fixed effects for length and sex (male/female), and random intercepts for assay plate. GAMs and GAMMs (with normal errors) were implemented using the *gam* command in the *mgcv* package, with the random component in the GAMMs represented as penalized regression terms. To test the association of flow with SRI, whilst accounting for the effects of temperature variation (of known causal importance (Stewart *et al.* 2018b)), a further GAMM was constructed with SRI as the response. Initially these were of the same form as the SRI model above, except that the non-parametric smoother for time was replaced by a linear term for temperature. An additional term (either linear or a smoother) for flow was then added to the model.

To obtain an overall test of the effects of flow treatment on gene expression in the experimental (flume) dataset, initially a permutational multivariate analysis of variance was applied based on a distance matrix (PERMANOVA-DM) to the 5 (untransformed) gene expression variables (*adonis* command; package *vegan*). The full model included main effect factor terms for flow treatment (flow or no flow), *Gyrodactylus* infection stage (baseline, up-phase and down-phase), sex and assay plate. It also included main effect continuous terms for standard length (mm) and body condition (residual from a quadratic regression of weight on length) and an interaction term for flow treatment and *Gyrodactylus* infection stage. Terms were assessed for significance through a backward selection procedure whereby the *P* value for individual terms was determined by addition (last) to the full model. The least significant term was omitted and then the process repeated to obtain a minimal model with only significant terms. Results reported below are based on addition to a minimal model containing only significant terms. Secondarily analyses on variation in SRI and on each of the individual gene expression variables were also conducted separately, employing linear mixed models (LMMs) (*lmer* command; package *lme4*). For these analyses the individual gene expression variables were first power transformed (determined through a Box-Cox procedure) and then standardized (zero mean, unit standard deviation). A separate LMM was constructed for each variable containing the same fixed terms as in the PERMANOVA-DM (above). Additionally, each LMM contained random intercepts for flow trial (experiment batch) and RNA extraction batch. Fixed effects were tested via a backward selection procedure and results below for flow treatment and experimental stage are presented when these terms were respectively added to a base model (containing all other significant fixed terms and terms for plate, flow trial and RNA extraction batch, regardless of significance). Other model selection strategies gave identical conclusions about the effect of flow and infection stage.

To quantify the individual 25-day *Gyrodactylus* infection trajectories (see above), area under the curve for worm counts ($AUC^{worm\ count}$) was utilised, peak worm count and the maximum intrinsic rate of increase (Birch, 1948), *r*, observed over any of the 4-day observation periods. *r* was calculated from the relationship:

$$N_t = N_0\, e^{rt}$$

where N is the worm count, t is time in days and e is the base of the natural logarithm). These were analysed as the response in general linear models (GLMs; *lm* command) with flow treatment (flow or no flow) as a factor and fish standard length as a continuous explanatory term. Prior to analysis, $AUC^{worm\ count}$ and peak worm count were optimally transformed by a

Box-Cox procedure. Standard diagnostic plots were inspected for all additive (*gam.check*) and linear (*plot.lm*, *plot.merMod*) models to check their suitability.

6.4. Results

6.4.1. Flow rate and immune allocation were seasonal and correlated in a wild lotic habitat

In the 25-month time series from a lotic habitat (RHD), there was a strong crude correlation between water flow, water temperature and SRI (which reflects high adaptive immune activity at high values) (Figure 6.2). All three of these variables showed sinusoid-like circannual variation: water flow (winter peak) tending to vary in antiphase to SRI and temperature (summer peaks) (Figure 6.3). It has been previously demonstrated experimentally that temperature drives SRI variation, with an effect size sufficient to account for a substantial part of the circannual oscillation in SRI at RHD (Stewart et al., 2018b). Thus, the first questions asked is whether flow could explain any SRI variation over and above that explained by temperature in a conventional linear statistical model. Whilst bearing in mind that collinearity would likely wholly or partly mask any separate effects of temperature and flow, it is also noted that flow was often irregular (due to irregular rainfall and anthropogenic dam release; see Figure 6.3) and that this might generate sufficient orthogonality with temperature variation to establish an independent association of flow and SRI (if not an interpretable effect size). Therefore, a GAMM was constructed to explain SRI in terms of confounder variables (sex and length) and temperature, and then flow was added as a further explanatory term to this base model. Flow was non-significant as a linear term, but significant when added to the model as a non-linear non-parametric smoother, explaining a modest additional amount (up to 3.7 percentage points) of model deviance (Table 6.1, Figure 6.4).

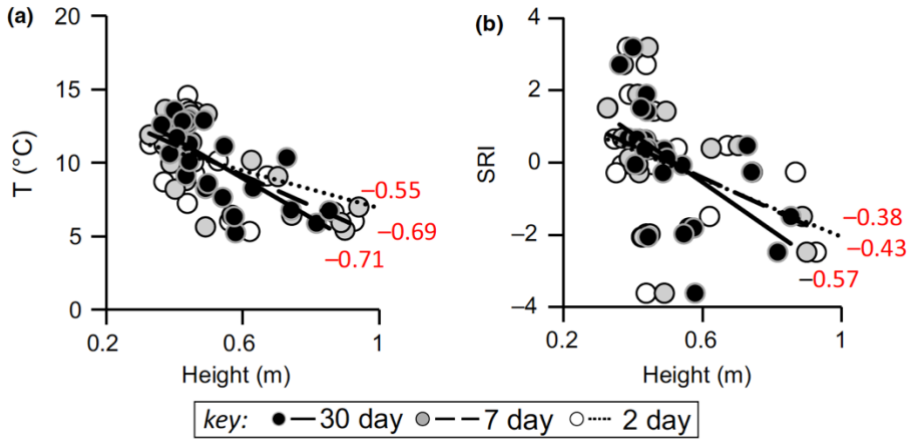

Figure 6.2. Crude correlation between water flow (Height, m), water temperature (T, °C) and immune-associated gene expression (SRI) at the field site (RHD). Plots show mean water temperature (a) and monthly mean SRI (b) against mean water flow. The plotted data for temperature and flow are means for 2, 7 and 30 days prior to the monthly sampling point for SRI (see key). Figures on the plots are Pearson correlation coefficients. SRI (seasonal reporter index, SRI) is an additive index based on the expression of 5 separate genes (seasonal reporter genes, SR) known to reflect a consistent major seasonal oscillation in immune-associated gene expression in wild stickleback (Brown *et al.* 2016; Stewart *et al.* 2018b).

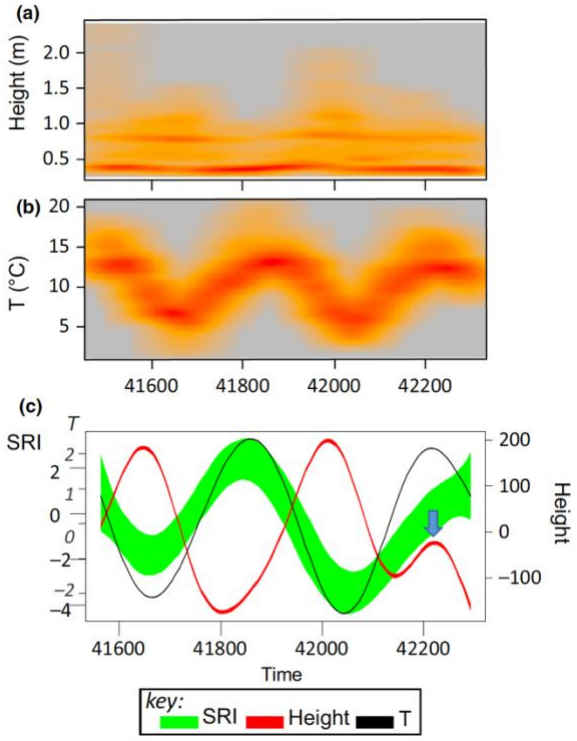

Figure 6.3. Temporal variation in water flow (Height, m), temperature (T, °C) and immune-associated gene expression (SRI) at the field site (RHD). (a) Water height at a gauge upstream of the study locality plotted against time and shown as a smoothed colour density representation obtained through a (2D) kernel density estimate; based on recordings taken every 15 min. (b) Water temperature (T) plotted against time and shown as a colour density representation (see above); based on recordings taken every 5 min. (a-b) Increasing density of observed points is indicated by yellow to red colours. (c) 95% confidence intervals for (centred) non-parametric smoothers against time for water flow (red), water temperature (black) and immune-associated gene expression, SRI (green). SRI (seasonal reporter index) is an additive gene expression index based on 5 genes (seasonal reporter genes, SR) known to report a dominant immunome-wide seasonal oscillation; high values reflect high expression of genes involved in adaptive immunity (Brown et al. 2016; Stewart et al. 2018b). On all plots time is shown as days since Jan 1st, 1900 (a standard format used by many computer programs); 41600 = 22nd November, 2013; 42200 = 15th July 2015. Note: later in the time series smoothed flow, unlike smoothed temperature, does not fully conform to a simple circannual sinusoid (with a late additional peak in the second year; arrow (c)) and throughout flow is more intermittent in character than temperature (a, b), meaning these environmental variables sometimes vary orthogonally.

Table 6. 1. Results of a generalised additive mixed model (GAMM) explaining variation in immune associated gene expression in a 25-month time series from a wild lotic habitat (RHD). The gene expression response variable is an additive index (seasonal reporter index, SRI) based on the expression of 5 separate genes (seasonal reporter genes, SR) known to reflect a consistent major seasonal oscillation in immune-associated gene expression in wild stickleback (Brown et al. 2016; Stewart et al. 2018b). Different terms representing flow (height, m) were added separately to a base model for SRI already containing fixed terms for sex, standard length (L, mm) and temperature (°C) and random intercepts for assay plate. Flow data were based on averages over 2, 7 and 30 days prior to the gene expression sample point and terms for these added to models were either linear or non-parametric smoothers. The percentage of total deviance explained (%Dev) is shown for the different models, with the best smoother model explaining only an additional 3.7 percentage points of the total deviance above the 53.6% explained by the base model.

Base model	% Dev	Flow term	*P*	% Dev
Sex + *L* + tempera-ture + *assay plate*	53.6	2 day (linear)	ns	53.6
		7 day (linear)	ns	53.5
		30 day (linear)	ns	53.4
		2 day (smoother)	ns	55.2
		7 day (smoother)	7.9×10^{-4}	57.5
		30 day (smoother)	0.001	57.3

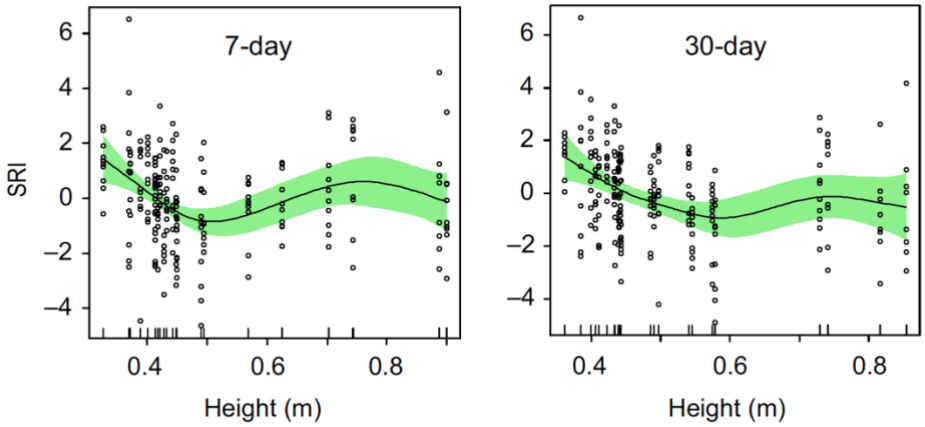

Figure 6.4. Confounder-adjusted association of immune-associated gene expression (SRI) with water flow (Height, m). Plotted lines are nonparametric smoothers (centred) from generalized additive mixed models (GAMMs) on the scale of the model linear predictor, with 95% confidence intervals shaded; scatter of points shows partial residuals. Analyses based on mean flow in the 7 days and 30 days prior to the monthly sampling points for SRI. SRI (seasonal reporter index, SRI) is an additive index based on the expression of 5 separate genes (seasonal reporter genes, SR) known to reflect a consistent major seasonal oscillation in immune-associated gene expression in wild stickleback (Brown et al. 2016; Stewart et al. 2018b).

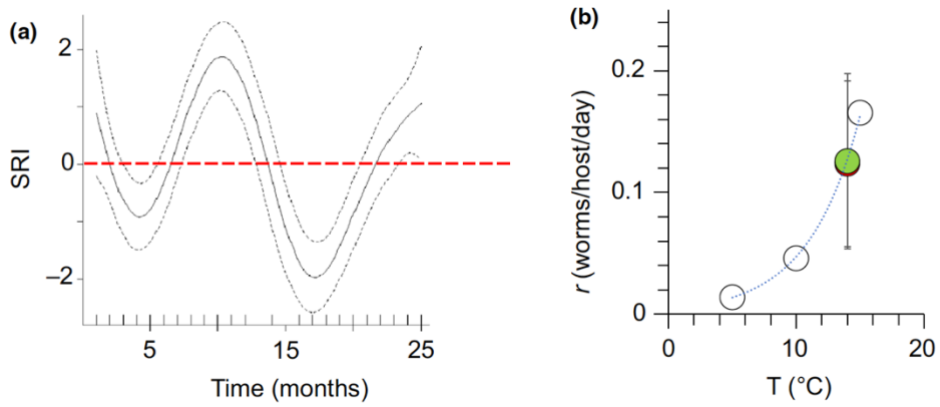

Figure 6.5. Power and effect size for the flow experiment, in the context of the known effects of other environmental variables. (a) Flow parameters estimate for immune-associated gene expression (SRI) and 95% confidence interval (right) shown alongside smoothed SRI temporal (seasonal) variation (black line) in the field at RHD with 95% confidence interval (black dashed lines). The experiment and field SRI values are measured on the same scale, indicated for both by the y – axis on the main plot. SRI (seasonal reporter index, SRI) is an additive index based on the expression of 5 separate genes (seasonal reporter genes, SR) known to reflect a consistent major seasonal oscillation in immune-associated gene expression in wild stickleback (Brown et al. 2016; Stewart et al. 2018b). (b) Least squares means for *Gyrodactylus gasterostei* intrinsic rate of increase (*r*) in the flow (red) and no flow (green) treatments of the challenge infection experiment (conducted at 14°C); bars indicating 95% confidence intervals. Open circles show *r* for *G. gasterostei* reported by Harris (1983) at different temperatures (*r* estimated by a regression method); a best fit exponential line is shown for reference.

6.4.2. Sustained intense swimming in an open flume had no effect on immune allocation or infection susceptibility

In the flume experiment, there was no overall effect of flow treatment on immune-associated gene expression (PERMANOVA-DM, $F_{1,50} = 0.116$, $P = 0.967$) or of infection stage ($F_{2,49} = 1.682$, $P = 0.151$) and no interaction between these ($F_{2,46} = 0.581$, $P = 0.752$). Nonetheless, a substantial association with sex was detectable ($F_{1,51} = 8.930$, $P = 0.001$, $R^2 = 13.9\%$) and a modest association with condition ($F_{1,51} = 4.509$, $P = 0.012$, $R^2 = 7.0\%$). Consistent with this, when analysed individually, neither SRI nor any of the single gene expression variables were significantly associated with flow treatment, infection stage or their interaction, but some associations were observed with length, condition and especially sex. To put possible effect size into perspective, the estimated flow parameter for SRI (negative for the zero-flow treatment) is in the opposite direction to that required to drive the observed field circannual SRI fluctuation (where low flows coincide with high SRI). Furthermore, the upper 95% confidence limit for this parameter represents only ~18% of the smoothed temporal SRI range observed in the field (see also Figure 6.5). Thus, any undetected effect of counter-current swimming on immune-associated gene expression is likely to be at most small or negligible compared to other natural environmental variation.

For the *G. gasterostei* challenge infection, there was no significant effect of flow treatment on $AUC^{\text{worm count}}$ (GLM, $F_{1,32} = 0.84$, ns), peak intensity (GLM, $F_{1,32} = 1.29$, ns) or r (GLM, $F_{1,32} = 0.002$, ns; parameter = 0.002 ± 0.0486). To put possible effect size into perspective, the upper 95% confidence limit for the r flow parameter magnitude was equivalent to the rise in r due to a ~2.3°C rise in temperature from 14°C (the temperature of the current experiment), based on data reported by Harris (1983) (see also Figure 6.5). Thus, any undetected effect of counter-current swimming is likely to be at most small or negligible compared to thermal effects.

6.5. Discussion

This study aimed to quantify the role of locomotor activity as a constraint on immune activity in wild fish. To achieve this, this investigation matched long-term records for water flow and immune gene expression in the wild with experimental estimates of the effect of sustained counter-current swimming on immune gene expression and infection resistance in acclimated fish derived from a naturalistic outdoor habitat.

It was initially observed that, in a natural lotic habitat across a 25-month period, water flow was strongly correlated with temperature and stickleback immune-associated gene expression (SRI). It has been previously shown that temperature is a powerful causal driver of immune-associated gene expression in sticklebacks (Stewart et al., 2018b) and so it was asked if a direct effect of counter-current swimming in response to flow could explain any variation over and above that explained by temperature variation. In this analysis the assumption was that any direct causal effect of flow on immune-associated gene expression would be linear, i.e., that increasing flow would result in proportional increases in counter-current swimming, which in turn would drive a directional change in gene expression. In fact, no linear effect of flow on (thermally adjusted) immune-associated gene expression (represented by SRI) was found. Nonetheless, it is recognized that there is a possibility that a (real) flow effect might have been obscured in observational data (for example, by confounding with temperature or an unmeasured environmental variable). Thus an experiment was designed, using an open channel

flume, to directly assess the effect of counter-current swimming on immune-associated gene expression.

In the flume experiment, acclimated wild fish were exposed to a water flow that stimulated rheotropism (counter-current swimming). Sticklebacks are relatively weak, primarily labriform (Walker & Westneat, 2002), swimmers that readily seek refugia under conditions of high flow. Previous studies on sticklebacks from the RHD study locality suggest most individuals exhaust (cease to maintain station) within 8 h at 20-30 cm s^{-1} (Whoriskey & Wootton, 1987). In this study (following initial trials) the current was set at 10 cm s^{-1}, allowing continuous swimming for an extended period (48 h) which approached the limit at which some individuals would succumb to exhaustion. The experiment was thus intended to reflect relatively extreme conditions, maximizing the detectability of any locomotion effects. In the event, it was found that there was no effect of sustained intense swimming on immune-associated gene expression or infection resistance. Taken together with the lack of detectable linear flow effects in the field, this is taken as strong evidence that locomotor activity negligibly constrains immune activity under natural conditions. It seems likely that in the wild, in practice, sticklebacks would exhaust (and likely die or be lost downstream), or seek flow refugia, before the effects of extreme physical activity on immunity were manifested.

Having ruled out an important direct effect of locomotory activity, it might further be considered whether flow itself has indirect effects in the wild. As this study could not detect a (thermally-adjusted) linear relationship between flow and immune-associated gene expression (SRI) in wild fish, as would have been consistent with a direct flow effect via counter-current swimming, the possibility of a (thermally-adjusted) non-linear association was also addressed. It was found that this non-linear association was highly significant but only explained a modest additional increment of the variation seen in the wild fish (beyond that explained by temperature). The form of the association was such that higher levels of adaptive immunity occurred at low and high flows, adjusting for thermal effects.

One notional environmental driver of immunity that might follow a complex non-linear trend with increasing flow would be water quality. In principle, for example, at low flows, chemicals and eutrophicating agents might inherently be concentrated by small water volume. Conversely, at high flows increased terrestrial run-off or more powerful currents in river channels might mobilize certain substances more than normally. Other potential explanations could also be considered. Amongst these is an indirect influence of flow via effects on foraging, as has been recently shown with strong diet effects on immunity (Friberg, Taylor & Jackson, 2019). In this scenario, arthropod prey (which promote elevated adaptive immune activity) might be concentrated at low flows simply by the smaller volume of the river channel. In high flows such organisms, having some counter-current locomotory capacity (Richardson, 1992; Lancaster, 1999; Sidler, Michalec & Holzner, 2018), might accumulate in flow refugia (areas of slack water) alongside their predator. Another possibility is altered pathogen exposure according to flow regimen. For example, there might be increased transmission when fish are at high density due to low water volumes or when occupying confined refugia during high flows. These explanations (foraging or transmission effects) have the advantage of predicting gene expression in the observed direction (high adaptive gene expression, due to enhanced foraging for arthropods or increased pathogen transmission, at times of low and high flow).

An important caveat to the interpretation of the thermally-adjusted statistical association between flow and immune gene expression is that the thermal adjustment does not exclude the

possibility that some real (indirect) flow effects may be obscured by collinearity with temperature. If this were the case then flow may, overall, be a negative indirect driver of SRI (as the crude correlation is strongly negative), possibly with a substantial real effect size. In this eventuality, each of the feeding, pathogen exposure or hydrochemical explanations above might still be relevant – but with adverse hydrochemistry, pathogen pressure or poor feeding efficiency predominantly driving low adaptive immune activity at high flows. Although further testing of these hypotheses is beyond the scope of the present study, nonetheless inferences can be made that the effect size of any hydrochemical influence is unlikely to be large. Thus, water chemistry would be expected to vary seasonally and, if important, to drive different seasonal patterns in sites subject to very different hydrological conditions. In fact, it has been recently recently reported (Stewart et al., 2018b) similar seasonal patterns occur in SRI variation (tending to track temperature) in a lowland river (RHD), an upland lake and even in mesocosms filled from mains water supply. Moreover, this study did not detect any effect of experimental *G. gasterostei* infection on SRI (or individual gene expression variables), suggesting "force of infection" in the wild is unlikely to drive seasonal SRI variation, although the effects of other common pathogens remain to be studied.

In summary, the key finding of this study is that locomotory activity *per se* is unlikely to be an important constraint on immunocompetence in healthy sticklebacks under normal natural circumstances. The potential generality of this result is supported by the fact that sticklebacks are relatively poorly adapted to sustained rapid swimming and thus may be an especially sensitive system in which to detect locomotory effects on immunocompetence. In addition, this investigation found that, despite the lack of a direct locomotory effect on immune expression, water flow was still statistically associated with the latter in wild sticklebacks: and this study concludes that this association must be driven by an unknown indirect mechanism.

Chapter 7

Prevention and control of infectious disease: current and future trends in global fish trade

7.1. Abstract

A state of crisis has been described within the aquaculture fish trade, with over half of global stocks facing collapse. A major culprit in this crisis is infectious disease caused by transmissible pathogens which significantly hamper sustainable expansion of the trade to meet rising demands for fish. With fish playing a key role in feeding humanity, implementing effective biosecurity policies remains a top priority. This review critically assesses current global biosecurity policies applicable to the fish trade, with emphasis on the European Union and the United Kingdom. In doing so, this review highlights the trend hallmarked by compartmentalisation of disease risk assessments from strategies emphasising host welfare. This compartmentalisation was particularly evident in the European Union's Animal Health Law (2016) that comes into effect in April 2021. Yet, high standards of host welfare might provide the missing link between infectious disease prevention and long-term control sustainable control. This review also proposes a novel biosecurity strategy of Integrated Disease Prevention and Control (IPAC), which comprises three key elements: host welfare policies; disease risk assessments and disease responses, that are applicable to global fish trade. The application of IPAC is assessed using examples of infectious disease of international importance and this study highlights instances where this has already shown promise. Ultimately, this review aims to provides a template for effective prevention and control of infectious disease within the fish trade.

7.2. Introduction

The fish trade comprises one of the most important sources of animal protein for humans, exceeding that of the combined consumption from all terrestrial animals, with the exception of poultry (FAO, 2018). Furthermore, non-trawling fishing as a means of food production has the lowest carbon footprint of all animal farming (Tilman & Clark, 2014). With terrestrial farming being the largest source of natural habitat degradation and a major factor implicated in biodiversity loss (Pimm et al., 2014; Poore & Nemecek, 2018), culturing fish can provide a sustainable means of feeding an ever-growing human population (WTO, 2018; FAO, 2018). The key point being, however, that the fish trade needs to be sustainable. As it stands, aquaculture practices are placing unprecedented pressures on fish stocks in the wild and in captivity, with approximately 63% of fisheries requiring rebuilding due to stock collapse (Worm et al., 2009; Worm, 2016). The future of fish stocks is also inherently linked to the state of ecosystem biodiversity as fish constitute keystone species in majority of aquatic habitats (Dudgeon et al., 2006; Adams et al., 2014).

Apart from their value as food, fish are also utilised as pets and/or showcase animals in the fish ornamental trade. Indeed, numerically, fish are the most populous pets in western households (American Pet Products Association, 2012). Unlike aquaculture, in the ornamental trade long survival of fish is paramount as opposed to optimising survival until harvest for consumption

(Stephenson et al., 2017; King, 2019). While there are more species traded within the ornamental industry (>4000) as opposed to aquaculture (~100), these fish account for <1% of marine fish caught globally and consequently the ornamental trade has a significantly smaller ecological footprint (Maceda-Veiga et al., 2016; King, 2019).

A major issue facing the fish trade, specifically aquaculture, and its sustainable expansion, is infectious disease (Shinn et al., 2015; Stentiford et al., 2017). The disease crisis within the fish trade is driven by the increasing abiotic and biotic stressors fish stocks are facing (Oidtmann et al., 2013; Stentiford et al., 2017). A stressor in its broadest context can be any stimulus (physical and/or psychological) which elicits a stress response and causes biological changes within an organism (Barton, 2002; Tort, 2011). Biological stress responses are highly conserved amongst vertebrates, and it is no surprise that the same endocrine responses (e.g., adrenaline and cortisol release) occur in fish and humans (Wendelaar Bonga, 1997; Barton, 2002). Acute stress can be adaptive and increase the chances of an organism's survival by, for example, initiating a rapid escape response from a harmful stimulus. Chronic stress, caused by persistent stressors from which an organism cannot escape, decrease the chances of survival. Many anthropogenic stressors are persistent and prevalent, which seriously impacts fish welfare (Dias et al., 2017).

Stress compromises animal welfare through impacting immunity (reviewed by Tort, 2011). Common stressors in aquatic environments have a detrimental impact on immunity and susceptibility to infectious disease (e.g., Ackerman et al., 2007; Smallbone et al., 2017; Masud et al., 2019a, 2020). While the link between increased disease susceptibility and stressors is well established (Martin et al., 2010; Tort, 2011), biosecurity policies for the fish trade that are aimed at prevention and control of transmissible disease tend not to integrate this knowledge. The need for this review emerged from an assessment of the European Union's Animal Health Law (2016) that will come into effect in April 2021. A renewed and more strategic focus on disease prevention and control was clearly highlighted in the EU Law, and yet prioritisation of animal welfare (including fish) was not included. Effectively, animal welfare constitutes separate legislation for the EU (EU Strategy for the Protection and Welfare of Animals 2012-2015), which is concerning because this is essential to ensure effective and sustainable disease prevention and control. The EU constitutes a major economic powerhouse within the global fish trade (FAO, 2018) and the standards they apply for biosecurity have ripple effects globally. Unfortunately, Lower Middle-Income Countries (LMICs) have more limited legislation and transparency with regards to biosecurity (Woolhouse et al., 2001; Schurer et al., 2016), which presents a major challenge with regard to assessing their disease prevention strategies.

This review attempts to offer a novel approach towards biosecurity policies by proposing integration with improved fish welfare strategies. While this review offers a global perspective wherever possible, the emphasis will be on the EU and UK, which will be used as a reference point for assessments. The review commences by assessing current strategies used in the prevention and control of transmissible pathogens within the fish trade and, in particular, highlight the disparity between policies on fish welfare and disease control. Then this study proposes improvements to biosecurity measures in relation to disease surveillance and control, with examples of globally important fish pathogens. Ultimately, this review aims to develop a potential global template for sustainable disease prevention and control within the fish trade by unifying best welfare practices with disease risk assessment and surveillance.

7.3. Biosecurity protocols, disease assessments and risks - a global perspective

The highest level of international laws and protocols on infectious disease are covered within the treaty of the World Trade Organisation (WTO) called the Agreement on the Application of Sanitary and Phytosanitary Measures, also known as the SPS Agreement. This Agreement effectively came into force with the establishment of the WTO in 1995 (Miano, 2006), aiming to protect human, animal and plant life from disease risks (Bossche & Zdouc, 2013). The standards set by the SPS Agreement are based on the methodologies and principles devised by three key organisations: The Codex Alimentarius Commission (Codex), World Organization for Animal Health (OIE) and the Secretariat of the International Plant Protection Convention (IPPC) (see Figure 7.1). From the perspective of fish welfare, including disease prevention and control, the OIE established the Aquatic Animal Health Code (2019), also referred to as the Aquatic Health Code. Broadly, this Code covers standards and practices for the improvement of aquatic animals (molluscs, crustaceans, fish, and amphibians) and this includes preventing and controlling transmissible disease and improving welfare of farmed fish (Chapters 4 and 7 respectively of the Aquatic Health Code, 2019).

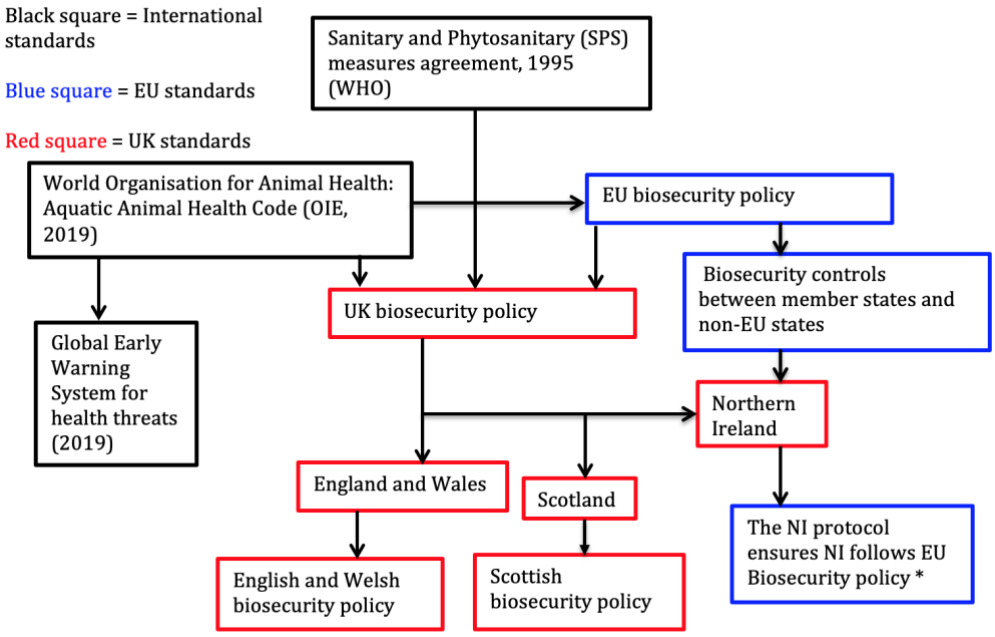

Figure 7.1. An overview of biosecurity policy and how international, EU and UK biosecurity policies are currently interlinked. International standards (Black) are often the base line for EU (Blue) and national (UK, Red) biosecurity standards and recommendations. *Northern Ireland (NI) is uniquely positioned, subjected to both EU and UK law on a situation dependant basis.

While organisations, such as the OIE, have made major progress in providing a global template for aquatic animal health, including fish, it is evident from international biosecurity policies that a hierarchy of taxa exists. Terrestrial animals are consistently placed higher in importance than aquatic organisms with regards to welfare standards and disease assessments (Oidtmann et al., 2013; Schurer et al., 2017). This is understandable from an anthropocentric perspective as zoonoses are more likely derived from terrestrial than aquatic animals, in addition disease surveillance is more challenging in aquatic environments (Oidtmann et al., 2013; Brugere et al., 2017). From an animal welfare perspective, however, there should be no distinction

between aquatic and terrestrial vertebrates. The OIE (in conjunction with the SPSS Agreement) implemented the establishment of disease-free zones in OIE member countries. Nation states can apply to be recognised as free of a particular disease and refuse fish imported from countries not recognised as disease free (OIE, 2019, Chapter 1.4.4). Implementation of these disease-free zones in aquaculture is especially important where there is an urgent need for increased regulation on infectious disease control to prevent severe economic losses (Moss et al., 2012). In communities dependent on subsistence aquaculture, disease outbreaks could decimate local production and potentially lead to starvation when other sources of food (especially protein) are less readily available (Herbert et al., 2019).

In the ornamental fish trade, there is even less emphasis on disease assessment (Rio-Rodriguez, 2006; Maceda-Vega & Cable, 2019). Many fish destined for the ornamental trade from across the world spend extended periods of time in hub countries (Whittington & Chong, 2007; King, 2019). Here, different species from distinct geographical locations may end up sharing the same water, potentially facilitating pathogen transfer (Whittington & Chong, 2007). At the EU level, for any bulk movement of fish monitoring occurs at both the supranational and national level. Article 194 of the EU Animal Health Act requires that animal health certificates be provided for each bulk movement of fish between different EU countries. Furthermore, once fish arrive at their destination, they become the nation state's responsibility and local regulations apply. In the UK, an approved veterinarian conducts a fish health check whether the fish are of EU origin or not (The Aquatic Animal Health (England and Wales) Regulations 2009; The Aquatic Animal Health (Scotland) Regulations 2009).

Within the UK, there are differences with regards to fish health assessments between the devolved nations of Scotland, Wales, England and Northern Ireland, as well as differences for aquaculture (native species) versus exotic fish species (see Aquatic Animal Health Regulations, 2009 for details). With regards to species provenance, Northern Ireland follows the EU's rules and directives regardless of fish provenance or purpose of use (The Wildlife (Northern Ireland) Order 1985; The EU Directive 2006/88/EC). Within England, for example, the core regulatory framework states that a minimum of 30 fish or 10 individuals of each species be provided for inspection and there is also a requirement to provide a selection of different sizes where applicable (Environment Agency, 2020). Exotic tropical aquaculture species and/or ornamental fish however are monitored through the annual import-sampling programme, which is conducted by the Fish Health Inspectorate (FHI) in England and Wales and the Scottish Fish Health Inspectorate in Scotland (SFHI). Within the devolved nations of the UK, there are different regulations: for example, in England and Wales, it is the operator (e.g., fish stocker, wholesaler) who decides which fish to submit for inspection, whereas in Scotland, members of the Scottish Fish Health Inspectorate conduct fish health checks directly. A potential biohazard exists between the UK's devolved nations as different screening process in England and Wales may miss diseases that were not overlooked at the Scottish border and vice versa. Furthermore, there are no health checks conducted for the movement of fish destined for either aquaculture or the ornamental trade between the devolved nations of the UK so that fish which have passed England and Wales health checks, for example, can be transported to Scotland from local and international destination via private hobbyists or operators.

For ornamental fish, wholesalers can potentially facilitate pathogen transmission if fish are not properly quarantined and subject to appropriate health checks or if staff are not trained to identify and/or report signs of disease. Another biosecurity concern is the release of fish by pet owners into local waters. While specific policies prohibit this in the UK (the Wildlife and

Countryside Act 1981), people do fall foul of such policies without awareness of associated risks (King, 2019). The release of exotic species into local waters may be introducing novel pathogens that leads to host switching, the consequences of which can be devastating for local species (Pinder et al., 2005; Hossain et al., 2014; Reyda et al., 2019). The improper disposal of dead fish is another potential disease risk. International standards for disposal of moribund fish are available and do provide a good framework for safe disposal (Article 194, EU Animal Health Act 2019, OIE Aquatic Health Code, Chapter 4.7). However, it is local operators and not veterinarians who decide how to dispose of moribund fish. The disposal of fish is a potential biosecurity hazard as, unfortunately, many pathogens remain infectious post-mortem and will spread in the water and contaminate other fish even after the infected host has been removed (Joseph & Carnahan 1994; Rakus et al., 2013; Faisal et al., 2019).

Despite there being international policy-based disease mitigation strategies for the fish trade, there remains a distinct compartmentalisation between disease control and the welfare of fish hosts. Strategies for disease control largely consist of isolation, treatment and/or culling, which can be effective and reduce pathogen transmission (Kent et al., 2009; Beest et al., 2011; Lieke et al., 2020). However, mass stock culling as a strategy is both costly and potentially ineffective (Erikson et al., 2009; Morters et al., 2013; Bolzoni et al., 2014). The monogenean ectoparasite *Gyrodactylus salaris*, for example, can move from a dead host to a live one (Olstad et al., 2006) and strategies to eliminate this pathogen via mass host culling using rotenone proved ineffective with the parasite surviving in adjacent water bodies and on dead hosts in addition to major ecological fallout (Eriksen et al., 2009). Therefore, sustainable disease prevention and control requires an emphasis on fish welfare as this reduces strain on the immune system and thereby improving disease resistance (Conte, 2004; Segner et al., 2012; Tapia-Paniagua et al., 2014). Failure to emphasise the welfare of hosts will always result in disease prevention and control defaulting to a scorched earth strategy.

7.4. Emphasising fish welfare: the missing link needed to improve effective disease prevention and control

The OIE provides a robust definition of animal welfare, which also forms the basis of the EU legislation on fish welfare, documented within their Strategy for the Protection and Welfare of Animals 2012-2015, which states "*An animal is in a good state of welfare if (as indicated by scientific evidence) it is healthy, comfortable, well nourished, safe, able to express innate behaviour, and if it is not suffering from unpleasant states such as pain, fear and distress*". Fish welfare is compromised if it experiences a state of heightened stress (Barton, 2002; Conte, 2004). A stress response can be either acute, which in certain instances is adaptive and has evolved to increase survival, or chronic, which is generally maladaptive and decreases chance of survival (Dhabhar 2002, 2006, 2009). Our understanding of fish stress responses and their relationship to welfare has increased tremendously due to improvements in non-invasive measurements of stress (e.g., glucocorticoid measurements in water and faecal matter, reviewed by Sadoul & Geffroy, 2019). Both acute and chronic stress responses can have a measurable impact on fish immunity and disease resistance (reviewed by Tort, 2011). Therefore, emphasis on fish host welfare is central to prevention and control of transmissible pathogens. Despite this, the welfare of terrestrial animals has been prioritised over that of fish (EU Strategy for the Protection and Welfare of Animals 2012-2015). This is linked to EU communications stating that the welfare of farmed fish within aquaculture will be evaluated based on scientific evidence and that they would take "*appropriate action on the basis of the outcome of that evaluation*". Effectively, this communication implies fish do not need the same

level of welfare consideration as terrestrial counterparts. Furthermore, the analysis specifically focuses on fish for aquaculture and entirely omits the welfare of ornamentals. The ability of fish to neurologically respond to noxious stimuli (i.e., nociception) was first demonstrated by Sneddon et al. (2003). Subsequent reviews on fish welfare still treated the topic of whether fish are capable of suffering as debatable (Conte, 2004; Huntingford et al., 2006). However, much new information now exists relating to fish cognition with enough neurological and behavioural complexity demonstrated in multiple species to conclude that fish welfare deserves equal footing as terrestrial counterparts (see Braithwaite et al., 2013).

This study will review why emphasising fish welfare is the crucial bridge that connects policies on the use of commercial and non-commercial fish and those on the prevention and control of transmissible disease. This review will highlight stressors that fish may encounter under captivity that can impact disease resistance. While it is impractical to list all possible stressors that a fish may encounter under captivity, there are welfare practices utilised globally that warrant analysis about their impact on disease resistance, and these are considered below. Additionally, this review outlines and assess best welfare practices that are applicable to aquaculture and the ornamental trade that minimise disease risks, which is especially important in light of the EU Animal Health Law (2016), which focusses on disease prevention, and the compartmentalised legislations on animal welfare practices (EU Strategy for the Protection and Welfare of Animals 2012-2015). This review will also highlight welfare areas that may be unique to each trade and how to address these biosecurity issues.

7.4.1. Handling, transport and stocking

With global aquaculture production at approximately 120 million tonnes (FAO, 2018) and between 350 million and 1.5 billion live ornamental fish traded annually (Miller & Morgan, 2009; Steven et al., 2017), handling, transport and stocking of fish is an unavoidable process that presents opportunities for increased stress and pathogen transmission. Handling of fish is often used for key management tasks including restraint for vaccine delivery and movement of fish between tanks, for instance when re-sizing stocks (Plant & LaPatra, 2011). For such handling routines the cost of an acute short-term stress response (e.g., increased cortisol production and lymphocyte trafficking - Cho et al., 2009; Tort, 2011) may be unavoidable.

To minimise the cost of transporting large quantities of fish and maximise farmed fish outputs, high stocking densities are typical for both transport and rearing (Ashley, 2007; Steven et al., 2017). High stocking densities can be associated with a suite of stressors including deterioration in water quality, abrasion and aggression induced wounds (Ashley, 2007; Portz et al., 2006). Densities at which aquaculturally important fish are maintained can impact fish immunity and disease resistance, but this is further complicated by a species level difference in immune responses to stocking density (Ellison et al., 2018, 2019). Most well-known stressors associated with transport and handling have been shown to impact disease susceptibility (e.g., netting: Caruso et al., 2002; manual handling: Davis et al., 2002; ammonia accumulation: Ackerman et al., 2006). These stressors, under competent handlers, are usually minimal. Certain aspects of transport such as mechanical disturbance due to vehicular motion have only recently been analysed and shown to impact disease resistance (Masud et al., 2019). Even within the international Aquatic Health Code (OIE 2019, Article 7.2.7), negating ‘uncontrolled movement’ is mentioned as a single point and it is recommended that more focus is given to the development of transport containers that are mechanically stable.

Interestingly, while the welfare of farmed and ornamental fish is poorly covered in the EU legislation for animal welfare, at least for transporting fish, there is a global standard (OIE Aquatic Health Code - Article 7.2), which covers essential elements of what constitutes good health practice (e.g., checking water quality, minimal handling, reduced crowding and noticing signs of disease). Within the EU member states, however, the European Commission's final report on what constitutes good transport practices for animal welfare, does not mention fish at all, nor does it specify why they have been excluded (European Union, 2019). Within the UK, there are strict biosecurity measures in relation to transporting fish, which follow the OIE guidelines of good international practice but with the key addition of ensuring disinfection protocols for transport vehicles (CEFAS, 2019 - Finfish Biosecurity Measures). However, it must be appreciated that the sheer number and species of fish (both aquaculture and ornamental) that are handled and stocked for transport makes it impractical to cater to species specific needs, and it does raise the concern that as a result, disease responses may remain under the radar.

7.4.2. Water quality

The OIE guidelines (OIE Aquatic Health Code 2019, Article 7.2.4) state that during transport, water quality should be monitored and kept within ranges appropriate for the relevant fish species. Deterioration of water quality (encompassing any physio-chemical property of water, including temperature, salinity, pH, dissolved oxygen (DO_2), CO_2 and NH_3 levels) commonly occurs during transport or as consequence of poor welfare practices. High stocking densities and prolonged transport in closed systems exert stress on fish which, consequently, leads to lower DO_2 levels and an increase in CO_2 and NH_3 concentrations (Vijayan, 1990; Ellis et al., 2002; Singh et al., 2004; Portz et al., 2006; Hong et al., 2019). To purge the gut and reduce metabolic waste, fish are commonly starved for at least one day prior to transport (Lim et al., 2003). Altered water quality can increase disease susceptibility by increasing fish stress in response to the environmental changes (Barton & Iwama, 1991; Ellis et al., 2002; Magnadottir, 2010). For example, fish mortality caused by two economically important pathogens, *Aeromonas salmonicida* and *Edwardsiella tarda*, is highest during temperature and organic pollution peaks as well as decreased DO_2 (Kingsbury, 1961; Meyer & Bullock, 1973; Wedemeyer, 1996, Uhland et al., 2000; Plumb, 2001). Similarly, Channel catfish (*Ictalurus punctatus*) subjected to low DO_2 and high NH_3 and CO_2 show increased susceptibility to *A. hydrophila* and *E. tarda* compared to catfish that were not environmentally stressed (Walters & Plumb, 2000).

A neglected water quality variable, CO_2, is gaining traction as projected atmospheric CO_2 levels approach 1000 µatm by 2100 (Pörtner et al., 2014). This is expected to affect growth, development, behaviour, and sensory systems of marine and freshwater fish (reviewed in Munday et al., 2019). Under farming conditions, far higher CO_2 concentrations are recorded, with intensive aquaculture protocols listing safe CO_2 levels at 15-40 mg/L (>7,000-28,000 µatm at 35 ppt salinity and 12^{o}C; Fivelstad et al., 1999; Blancheton, 2000; Petochi et al., 2011). Data from 2009 shows that over 40% of salmon smolt hatcheries in Norway reported CO_2 levels over 12.7 mg/L (>5,400 µatm) (Noble et al., 2012). Furthermore, Orellana and Wecker (2014) recorded a CO_2 concentration of up to 130 mg/L (>70,000 µatm) following a 10 h transportation of Atlantic salmon by well boat. Indeed, throughout lengthy transportation, water pH decreases as CO_2 levels increase and this correlates with increased mortality in both aquaculture and ornamental fish (e.g., Fivelstad et al., 1998; Lim et al., 2003; Paterson et al., 2003; Sampaio et al., 2019). Ornamental fish may be particularly susceptible to water quality

deterioration as they may be transported for long periods for international consignments (>24 h), during which mortality rates often increase (Olivier, 2011; Stevens et al., 2017). Despite the concerningly high CO_2 levels reported in the fish trade, the effect of increased or fluctuating CO_2 levels on disease resistance is poorly documented. The limited data available suggests that increased CO_2 concentrations may increase disease susceptibility (Boleza et al., 1999; Walters & Plumb, 2000; Kaya et al., 2013).

Water quality should always be kept within species-appropriate ranges. As the norm is to keep multiple species of ornamental fish in the same aquarium (Stevens et al., 2017), it is important to select optimal water quality for all displayed species. While the sheer number of species makes it challenging to cater to species specific need; keeping species that have similar water quality requirements together would significantly reduce the burden associated with water quality variation. To maintain water quality during transport, various tactics may be employed such as gradually reducing water temperature, and using salt, anaesthetics, and pH buffers (Lim et al., 2003; Pramod et al., 2010, etc). Competent fish handlers that follow water quality guides such as those set out by the OIE and OATA (see OATA Water Quality Guide, 2008; OIE Aquatic Health Code, 2019), should be able to maintain water quality within healthy limits for both aquaculture and ornamental fish species.

7.4.3. Nutrition

The link between nutrition and healthy immunity is well established (reviewed in Trichet, 2010). For fish to develop an effective immune response to pathogens (i.e., immunocompetence), a healthy diet must contain the minimum requirements of macronutrients (amino acids and essential fatty acids) and micronutrients (vitamins, minerals, and carotenoids) appropriate for the species of fish grown and cultured (NRC, 1993; Sales, 2003). Though there are data on the minimum dietary requirements of most aquaculture fishes, although rare, nutritional deficiencies still occur. The most common dietary deficiencies seen in cultured fish are due to insufficient vitamins (most commonly vitamins C and E) and lipids, and this can impact disease resistance (Hardy, 2001; Oliva-Teles, 2012). Higher mortality was recorded among groupers (*Epinephelus malabaricus*) fed vitamin C deficient diets following *Vibrio carchariae* infection compared to groupers given excess levels of the vitamin (Lin & Shiau, 2005). Similarly, in studies on grass carp (*Ctenopharyngodon idella*), vitamin C deficiency led to higher gill rot-related mortality following *Flavobacterium columnare* infection, while an absence of vitamin E led to increased susceptibility to *Aeromonas hydrophila* infection compared to carp given optimal vitamin E levels (Xu et al., 2016; Pan et al., 2017). Though rare, amino acid deficiencies may also impair immunity. Mortality following *Photobacterium damselae piscicida* infection was significantly higher in European seabass (*Dicentrarchus labrax*) lacking dietary tryptophan than in seabass fed diets with adequate tryptophan levels (Machado et al., 2019).

In addition to ensuring optimal nutrition, supplementary nutrients (over minimum required amounts), as well as supplying compounds that are non-essential for fish health (i.e., additives), can enhance immune system function (i.e., immunonutrition, reviewed in Pohlenz & Gatlin, 2014). Examples of additives include non-digestible polysaccharides (glucans and alginates), whole or components of microorganisms (probiotics) and non-digestible carbohydrates (prebiotics) (see Kiron, 2012). Arginine-enriched diets, for example, significantly improved the survival of channel catfish (*Ictalarus punctatus*) when infected with *Edwardsiella ictalurid* (see Buentello & Gatlin, 2001). Similarly, lower mortality following exposure to infectious

salmon anaemia virus or *Piscirickettsia salmonis*, as well as lower sea lice (*Lepeophtheirus salmonis*) burden was observed in Altantic salmon (*Salmo salar*) after supplementation of standard commercial diets with dietary nucleotides (Burrells et al., 2001). This supplementation also improved survival of rainbow trout following exposure to infectious pancreatic necrosis virus (Leonardi et al., 2003).

Improving welfare through optimum nutrition is a growing area of aquaculture investment, with the estimated value of the collective aquafeed market being $100 Billion (FAO, 2018). It is not clear how much of this collective aquafeed investment is aimed at immunonutrition, but in the UK BioMar (2019), for example, invested >£700K per year on Research & Development, producing 'functional feeds' supplemented with glucans, probiotics, nucleotides, vitamins and/or minerals. Nonetheless, immunonutrition is still in its infancy as our current understanding of the teleost immune system remains fragmented (Klimpel, 2019). In relation to ornamental fish, the available data on nutrient requirements is very limited (Yanong, 1999; Sales, 2003). Several factors may contribute to this; perhaps the most important being that many more species are cultured within the ornamental trade compared with aquaculture (x40 more, Garibaldi, 1996; Sales, 2003; Sneddon et al., 2016; Stevens et al., 2017). This makes determining the dietary requirements of the fewer aquaculture species more feasible. Furthermore, in 2014 alone, <4% of all fish used within the fish trade were ornamental, compared to 87% used for human consumption (FAO, 2018). Also, information on the dietary requirements of ornamental fishes has been extrapolated from those of food fishes which may not be applicable (Sales, 2003). There is a distinct lack of information regarding optimal nutrition of ornamental fishes compared to food fishes; considering the remarkably diverse number of species being kept as pets, this welfare gap needs to be addressed.

7.4.4. Genetics and selective breeding

Aquaculture has limited selective breeding programmes, with <10% of global aquaculture production in 2010 coming from selectively bred fish (Gjedrem et al., 2012). In the ornamental trade, the majority of marine fish are wild caught, whereas the reverse is true for freshwater species with most fish bred in captivity (Stevens et al., 2017; King, 2019). With regards to food fish, the major emphasis of selective breeding programmes has historically been on improving aquaculture yields to meet rising demands (Gjedrem & Rye, 2018). The average improvement in growth per generation across several farmed fish species such as Atlantic salmon, Rainbow trout and Channel catfish, was estimated at 14.3%, considerably higher than the genetic gains measured in livestock (Gjedrem & Rye, 2018). One of the first family-based aquaculture selective breeding programmes was started for Atlantic salmon in Norway in 1975, initially selecting only for improved growth rate (Gjedrem, 2000). Selective breeding of Atlantic salmon has continued and, currently, nearly 100% of global Atlantic salmon production is based on genetically modified stocks (reviewed in Gjedrem et al., 2012).

Attempts at improving disease resistance by selective breeding programmes show that significant results can be achieved in as little as one generation. For example, the F1 generation of Rohu selected for resistance against *Aeromonas hydrophila* had 56.6% higher survival following experimental infection with the pathogen compared to the parental generation (Sahoo et al., 2011). Similarly, the average survival over 8 years of a rainbow trout strain selected for resistance against the Infectious Pancreatic Necrosis (IPN) virus was 96.1% following infection with the virus, compared to 4.3% for an unselected strain (Okamoto et al.,

1993). Other experiments had similar results, successfully increasing resistance to IPN virus and *A. salmonicida* in Atlantic salmon (Gjedrem & Gjøen, 1995; Storset et al., 2007), *Streptococcus* spp. in Nile tilapia (LaFrentz et al., 2016; Suebsong et al., 2019) and to *Flavobacterium psychrophilum* in rainbow trout (Leeds et al., 2010).

In contrast to food fish, limited work has been done on selective breeding in ornamental fish for improved disease resistance, as traditionally breeding has selected for aesthetic and domestication traits (Nakajima & Taniguchi, 2001; Stevens et al., 2017; Chen et al., 2020). Selection for disease resistance can be achieved by individual mass selection (i.e., selecting specific individuals by observation) during infection challenge experiments. However, mass selection may result in inbreeding (Moav & Wohlfarth, 1976; Teichert-Coddington & Smitherman, 1988; Huang & Liao, 1990), high levels of which can reduce growth and reproductive success and even increase mortality (Mrakovčič & Haley, 1979; Kincaid, 1983). To add to this, prior mass selection of desirable traits (for colour, size etc) may have occurred at the cost of increased susceptibility to parasites (Arkush et al., 2002; Consuegra & de Leaniz, 2008; Eszterbauer et al., 2015; Smallbone et al., 2016).

Establishing selective breeding programmes to improve fish disease resistance would require novel data on quantitative trait loci that govern resistance for many different species (e.g., Palaiokostas et al., 2018). Furthermore, while providing selective pressure for existing pathogens, this might facilitate the emergence of novel, more infectious strains. Though selective breeding has shown promise for specific pathogens, with the emergence of novel diseases, combined with evolution of resistant strains, the jury is still out on the sustainable application of selective breeding programs. However, selective breeding can be applied as part of a more holistic fish welfare-based biosecurity strategy that also includes controlling stressors and optimising water quality and diet.

7.5. Lessons learnt from key diseases: can we create a global template for disease prevention and control?

Typically, when biosecurity protocols are established and/or updated, they tend to be based on disease risk assessments and any subsequent actions (e.g., isolation and treatment) that may emerge from those assessments (see Oidtmann et al., 2011, 2013). Thus, most biosecurity policies are reactionary rather than proactive and pre-emptive. A proactive approach must cater to promote fish welfare to ensure that stress levels are kept to a minimum thereby ensuring immunocompetence if and when infections do occur. The stressors we expose fish to under captivity impact disease resistance and therefore separating fish welfare from biosecurity is unwise. The EU Animal Health Law (2016), which highlights the need for renewed emphasis on disease prevention and control, fails to relate back to animal welfare, even though this is covered by separate EU legislation. This review tentatively proposes a global biosecurity template for the fish trade, Integrated Disease Prevention and Control (IPAC), which contains three key components, and hopefully will appear in any new legislation:

1) Host welfare policies: aimed at assessment and implementation of species-specific needs;
2) Disease risk assessments: aimed at early detection and must cover both known and emergent diseases;
3) Disease response: actions taken after confirmed detection of disease.

While implementation of these three components will vary based on multiple factors related to host and pathogen biology such as host susceptibility and pathogen virulence, good host welfare practice and disease risk assessments should be a routine. Here, the review analyses biosecurity protocols for three key fish diseases of global importance (*Aeromonas* spp., including *A. hydrophila*, *A. veronii*, *A. salmonicida* and *A. sobria*), *Cyprinid herpesvirus 3* (CyHV-3) and *Gyrodactylus salaris*. These diseases are selected because of their known impacts on both aquaculture and ornamental trade, and thus their classification by the OIE as diseases of global concern (Johnsen & Jensen 1986; Ilouze et al., 2011; Tavares-Dias & Martines 2017; OIE, 2019). The review then assesses whether the three key components of what is considered effective biosecurity measures are applied to these diseases and how effective they have been in prevention and control. Where and why targeted biosecurity measures fail to work will be considered as well as what could be done to further combat the spread of these pathogens.

7.5.1. Aeromonas

Aeromonas spp. are one of the most globally distributed aquatic bacterial pathogens, inhabiting marine, fresh, brackish and even chlorinated water systems (Tomás, 2012). Early diagnosis of *Aeromonas* infections is difficult visually as symptoms often occur in the gills, internally or in tiny patches at the base of the fins (Bartkova et al., 2016). When outbreaks of *Aeromonas* spp. occur, the infection manifests externally, in the form of dermal and ocular ulcerations, as well as internally, causing fatal internal ulcerations in organs such as the kidney and liver (Huizinga et al., 1979; Citarasu et al., 2011; Sudheese et al., 2012). Unfortunately, *Aeromonas* spp. quickly adapted to treatment and several species have developed antibiotic resistance (Ansari et al., 1992; Vivekanandhana et al., 2002; Figueira et al., 2011; Scarano et al., 2018). Generally, *Aeromonas* outbreaks correlate with poor environmental conditions and improvements in basic welfare practice is known to reduce outbreaks (Walters & Plumb, 1980; Hrubec et al., 1996; Dror et al., 2006; Räihä et al., 2019; Das et al., 2020; Zdanowicz et al., 2020; Zhang et al., 2020). Due to the ubiquitous nature of *Aeromonas* spp., pathogen screening is recommended as part of a routine risk assessment (Tomás, 2012). A PCR assay (such as that developed by Griffin et al., 2013) is highly recommended for the aquaculture industry, and can be used to distinguish between different strains, for example, *Aeromonas hydrophila* stain vary in virulence and host pathogenicity (Griffin et al., 2013). This also allows for a higher degree of flexibility, targeted treatments and the possibility to intervene before an *Aeromonas* spp. outbreak occurs. However, due to antibiotic resistance, culling may be the only option for uncontrollable outbreaks (see Qvillier et al., 2020).

7.5.2. Cyprinid herpes virus 3

Cyprinid herpes virus 3 (CyHV-3 from here on) is an alloherpes virus infecting cyprinids, most commonly *Cyprinus carpio* (see Negenborn et al., 2015; Boutier et al., 2015). The pathophysiological symptoms of CyHV-3 manifest as lesions in the skin, gills and kidneys, which if left untreated interfere with ion uptake and osmoregulation leading to mass mortality (Negenborn et al., 2015). Other cyprinids such as Tench (*Tinca tinca*), Rudd (*Rutilus rutilus*) and Crucian carp (*Carrassius carrasius*) are asymptomatic carriers and transmitters of CyHV-3 (Gaede et al., 2017), suggesting that (cyprinid) mixed-species assemblages are particularly vulnerable to CyHV-3 outbreaks (Gaede et al., 2017). CyHV-3 spreads through skin contact (primary infection route, Costes et al., 2009; Rakus et al., 2013; Miwa et al., 2014; Adamek et al., 2014) and orally, via water containing contaminated skin mucus, faeces, and urine (Gilad

et al., 2004; Dishon et al., 2005; Negenborn et al., 2015). CyHV-3 is infectious once shed in the water for up to 4 hours (Rakus et al., 2013), allowing it to spread rapidly in environments where centralised filtration systems are employed or where wastewater is recycled.

Current eDNA methods are not effective in detecting CyHV-3, making early outbreak prevention and detection difficult (Trujillio-Gonzales et al., 2019). But CyHV-3 outbreaks can be limited through the application of higher welfare standards (reducing stress) in already infected animals (Bergmann & Kempter, 2011; Lin et al., 2017). This is due to the way in which CyHV-3 behaves as a virus. After initial infection, surviving animals retain the dormant virus and are immune to reinfection (Bergmann & Kempter, 2011; Lin et al., 2017). However, when conditions become unfavourable and the host is placed under prolonged stress, the immune system weakens and the CyHV-3 virus can increase its rate of replication within host cells (Lin et al., 2017). Reducing physiological stress can only be achieved by increasing welfare standards such as reduced stocking densities, increased oxygenation, and reduced handling (Ruane et al., 2002; Bergmann & Kempter, 2011; Lin et al., 2017). Due to a lack of effective treatments and only partially effective vaccines against CyHV-3 infection (Gao et al., 2018; Boutier et al., 2019), the best way to avoid initial CyHV-3 outbreaks is to avoid adding already infected fish to non-infected stock, this would require each batch of fish to be tested possibly via PCR as suggested by Loose et al. (2020). Avoiding mixed (cyprinid) species assemblage will also prevent cross infection from carrier species to common carp (Gaede et al., 2017). Unfortunately, if a stock outbreak of CyHV-3 does occur, despite the economic fallout, wide scale culling may be the only solution (Ziarati & Hassantabar, 2020).

7.5.3. Gyrodactylus salaris

Unlike the previous two pathogens, *Gyrodactylus salaris* is a monogenean ectoparasite, which primarily infects salmonids (Jensen & Johnsen 1992; Bakke et al., 2002, 2007; Ozerov et al., 2010). *G. salaris* is currently limited in distribution and infection severity varies across fish populations. High Atlantic salmon *(S. salar*) mortality rates in response to *G. salaris* infections, are observed in the western Atlantic, for example in Norway (Johnsen & Jensen 1991; Pike et al., 1994). Conversely, limited mortality is seen in Baltic salmon populations (Cable et al., 2000) and no mortality on some Finnish Atlantic salmon populations (Rintamäki-Kinnunen & Valotonen, 1996). Central and southern European and Canadian *S. salar* populations show varying degrees of genetic resistance to *G. salaris* (see Bakke & Mackenzie 1993; Dalgaard et al., 2003, 2004; Paladini et al., 2014; Denholm et al., 2016). Apart from strict measures to prevent introducing *G. salaris* into stocks with low resistance to the parasite, improving welfare through stressor reduction should be an important tool to reduce mortality rates caused by *G. salaris*. Measures such as reduced stocking densities and improved water quality in populations with intermediate resistance to the disease is effective by benefiting fish immunity (Räihä et al., 2019; Liu et al., 2019). Improving fish welfare is certainly known to reduce the severity of other gyrodactylid infections by for example application of environmental enrichment (e.g., Masud et al., 2020). Alongside measures to boost the immune system, *G. salaris* can be detected in water via the use of eDNA technology (Rusch et al., 2018; OIE Aquatic Health Code, 2019) and early detection of this pathogen is an important step to prevent mass spreading and subsequent mortality of hosts.

The pathogens highlighted above, *Aeromonas* spp., CyHV-3 and *G. salaris*, like many other infectious diseases within the fish trade do not have any effective *en masse* treatments (Oidtmann et al., 2011; OIE Aquatic Health Manual, 2019). Therefore, it is especially

important to ensure that high welfare standards are maintained for hosts to be immunocompetent (Ramírez et al., 2015). Ultimately, by integrating host welfare policies into biosecurity strategies, it is possible to not only reduce infection rates, but also massive economic losses associated with host mortality, treatment and potential wide scale culling (Hrubec et al., 1996; Conte, 2004; Bergmann & Kempter, 2011; Senger et al., 2012; Tapia-Paniagua et al., 2014; Lin et al., 2017; Liu et al., 2019).

7.6. Future directions and conclusion

Perhaps one of the biggest unknowns in the struggle to tackle disease burden in fish is identifying which specific stressors have dominance effects in relation to impacting disease resistance. Multi-stressor investigations in relation to disease resistance are limited (see Crain et al., 2008; Martin et al., 2010) but are crucial as most environments present fish hosts with multiple and often interacting stressors, the impacts of which remain largely unknown. By identifying which stressors have dominance effects on host disease resistance, targeted approaches can be utilised in mitigation strategies.

Disease mitigation strategies are also influenced by the global political climate. In response to the COVID-19 pandemic, there has been a concerning rise in the number of calls by individuals and environmental NGO's for a blanket ban in the trade of exotic species. Such a ban could also cease trade of ornamental fish. Such a blanket ban on the wildlife trade would encourage underground unregulated trade practices with even less chance of promoting welfare practices. For the ornamental fish trade, for example, the synergy of local communities and hobbyists with trade organisations (e.g., OATA, OFI) provides critical feedback that ensures that welfare standards for fish are consistently high (Maceda-Veiga et al., 2016; King, 2019). Even for aquaculture, the current political climate is making regulating welfare standards for fish more challenging. At the time of writing, a trade deal between the UK and Europe was achieved for Brexit. One of the major negotiating hurdles was access to fishing rights in shared UK- EU waters. The following five and a half years will remain a transition period with regards to how fishing resources are shared out between the UK and the EU and uncertainty remains about how this might impact fish stocks and their welfare.

Despite some of the challenges the global fish trade will face, with regards to disease prevention and control at least, this review does tentatively propose a step in the right direction in the form of an Integrated Disease Prevention and Control biosecurity strategy (IPAC), with emphasis on improved fish host welfare. The EU Animal Health Law (2016), which comes into effect in April 2021, has unfortunately maintained the division between fish welfare and disease prevention. If the fish trade is to achieve sustainability by limiting the burden of infectious disease, this review strongly recommends the application of IPAC to global fish stocks.

Chapter 8

Discussion

8.1. Key lessons

This PhD project has revealed that stressors can have unpredictable and often detrimental consequences on host-parasite dynamics, host physiology, behaviour and mortality. Experimental investigations showed that most stressors studied in this project had negative impacts on disease resistance. From mechanical disturbance associated with routine transport of fish, to enrichment deprivation and acute noise exposure and the recalcitrant global pollutant, microplastic, exposure to all these stressors significantly increased fish disease susceptibility. This was evident in heightened pathogen burdens over the course of an individual host's infection trajectory. Increased metabolic rates and agonistic behaviour were also recorded in fish that were deprived of environmental enrichment. Furthermore, significantly increased mortality levels were recorded in fish exposed to chronic noise and high concentrations of microplastic. In contrast, for the ecological stressor, flow, there was no evidence of constrained immunity nor changes to disease resistance.

8.2. Implications and applications

Perhaps the most direct application of this project is information that can be utilised by the ornamental trade, including trade associations (e.g., OATA UK) and hobbyists' groups, providing recommendations for improved practices (e.g., transport and building tank environments) that have a positive impact on fish welfare. Chapter 3 revealed the effects of mechanical disturbance during transportation - information requested by trade organisations, notably OATA. Furthermore, by testing novel Breathing Bags ™, advertised as preventing mechanical disturbance, the study also shows that these bags do not, in fact, make any difference to disease resistance compared with standard transport bags, at least over simulated 24h transportation. This information is extremely useful for organisations that may consider using these Breathing Bags under the believe that they improve fish welfare. However, future work will need to determine whether utilising different types of transport bags over longer periods of international transport that can take days will impact disease resistance. While there is no set time limit to how long fish can be transported for, IATA which sets international standards for live animal transport recommends that fish are prepared for at least 48 hrs of transport (twice the transportation time that Chapter 2 exposed fish to).

For stressors such as noise pollution, studied in Chapter 4, that are now so widespread trying to regulate them remains a challenge (reviewed in Slabbekoorn et al., 2010). However, Chapter 4 provides empirical support for international policies that are trying to regulate noise levels for human and animal welfare (e.g., Smith, 2015). The noise pollution study also provides novel information showing that human welfare intervention in the fish trade, such as pumps and air filters, required for improving welfare, generates noise levels that may be having unintended negative consequences (see Wysocki et al., 2007).

Though anthropogenic stressors are now a common feature in aquatic environments, with 43% of European rivers, for example, reporting two or more types of stressor (see Birk et al., 2020)

ecological stressors are almost unavoidable in natural habitats. Often, ecological stressors are the result of natural fluxes such as seasonality leading to differential access to resources (Brown et al., 2016; Jackson et al., 2020). Increasing incidences of natural flooding means that flow is becoming a common variable in aquatic habitats, however this did not impact immune gene expression or disease resistance (Chapter 5), despite the hosts (three spined sticklebacks) being pushed to the limits of counter current swimming. The results from Chapter 5 certainly suggest that sticklebacks are adapted or able to cope with flow as a stressor and more broadly other investigations suggest that fish species can adapt to stressors over time (see Schulte, 2007; McBryan et al., 2008). The three-spined stickleback, a species that underwent postglacial colonization of freshwater habitats from a marine environment are uniquely suited for a changing world (Rogers et al., 2013). Indeed, considering this species inhabits a thermal spectrum ranging from 4-22^{0}C (Barret et al., 2011), changes such as global warming may not hand this species the short end of the stick.

Preventative measures in the struggle to curb disease outbreaks in captivity means ensuring the host's environment is one that is conducive to their health with minimal stressors. Environmental enrichment is a simple measure that may improve not only the psychological disposition of animals (Benaroya-Milshtein et al., 2004) but lower stress levels through cognitive stimulation which has knock on consequences by improving immunity (Giacomini et al., 2016; van Dixhoorn et al., 2016). This PhD has shown in Chapter 6 that depriving fish of simple structural enrichment significantly increases their levels of aggression. Therefore, it is not unreasonable to conjecture that depriving fish of enrichment will cause a state of suffering. Conversations with aquaculture organisations as a direct result of the enrichment study revealed that there is reluctance in adopting an enrichment approach because of the associated increase in workload of more cleaning and capture time. However, if small-scale studies are reporting improved welfare parameters, such as reduced aggression and disease burdens, when enrichment is applied, choosing not to adopt such strategies at a large scale is a questionable ethical decision, and particular types of enrichment that could easily be removed from tanks could solve this problem. Even for large scale fisheries, minimising stressors through curbing widespread activities such as 'catch and release' can reduce the outbreak of diseases which typically may not be virulent under low stress conditions (Dias et al., 2017, also see Appendix 2).

Arguably, one of the most sobering implications of the wider research conducted around this PhD project is how anthropogenic pollutants have scarred ecosystems. A major recalcitrant pollutant is microplastic and the extent of their spread is truly alarming, with recent studies even finding them in human placentas (Ragusa et al., 2021). Beyond the concern for microplastic having potential human transgenerational effects, the bioaccumulation of microplastic within food chains is raising alarm bells (Horton et al., 2018; Vince & Stoett, 2018). Chapter 7 revealed that fish host-parasite dynamics are significantly impacted by variable microplastic concentrations and while the levels of microplastic used were at the high end of the concentration spectrum, they are still lower than levels found in aquatic habitats where the combined mass of plastic is greater than the biomass of organisms (see Moore et al., 2001; Fischer et al., 2016). This legacy contaminant, which can seriously impact immunity and disease resistance, is also an ideal habitat for the development of antibiotic resistant pathogens (termed the plastisphere- see Zettler et al., 2013), which may be implicated in the emergence of novel diseases at sites of high industrial waste (Arias-Andres et al., 2018; Zhang et al., 2020). How the plastisphere and its associated pathogens impact host disease resistance, however, is a question that remains to be answered.

8.3. Hope for the future: questions to answer

The experimental investigations for this project focussed on how stressors impacted fish welfare, with an emphasis on host-pathogen dynamics. Of course, most environments are subject to multi-stressors; this is a term used to refer to stressors that may have interactive effects on organisms. There are four typical responses to combining stressors: dominance, additive, synergism and antagonism (reviewed in Crain et al., 2008; Birk et al., 2020). Briefly, dominance effects occur when one stressor from a pair or group has the dominating influence on a biological response (e.g., 1 +2 =1); additive effects occur when combining stressors equals the sum of the two stressors (e.g., 1+1=2); synergism occurs when the combined stressors have a greater effect than each of the individual stressors (e.g., 1+1= 3) and antagonism occurs when adding stressors has a dampening effect on the biological response (e.g., 1+1= 1.5). In this era where we are witnessing the effects of multi-stressors in ecosystems, experimental designs must be adapted such that we can account for biological responses to multiple stressors. This is particularly important for identifying key stressors that may have overarching influences when interactions occur *in natura*, which would be especially pertinent if stressor dominance occurs (see Birk et al., 2020). In this regard, we remain largely in the dark about how host-parasite interactions are responding to multi-stressors with very few studies having been conducted (reviewed in Martin et al., 2010). However, this is slowly changing, with, for example, investigations revealing what happens when naturally infected wild fish (three spined stickleback) are exposed to microplastic together with a globally prevalent herbicide, glyphosate from Roundup (Masud et al., in review). The results of the multi-stressor study revealed some unpredictable and worrying effects, with the interacting stressors (microplastic and glyphosate) causing an additive effect on pathogen burdens and a highly interactive effect by causing mass mortality of fish (Masud et al., in review).

A major unknown that remains to be tackled with regards to how host-parasite interactions are being disrupted in a changing world is scaling up what we see happening at an individual host level to a food-chain level (Lefèvre et al., 2009; Cable et al., 2017). One strategy to tackle this is to design mesocosms that go some way in replicating mini ecosystems, while allowing us to manipulate variables within an established food chain (see Stewart et al., 2017 for example). At the time of submitting this PhD thesis, experimental designs are underway specifically to investigate how an established food chain (within mesocosms) contaminated by anthropogenic pollutants would affect host-parasite interactions.

An alternative approach to understand the effect of perturbations on infection dynamics is by developing *in silico* models (either deterministic or stochastic); which are parameterised or calibrated based on experimental data. Simulations with mathematical models can help explore the effects of underlying model parameters (e.g., birth rates, death rate, parasite reproduction rate) and other covariates (e.g., pollutant levels) on the overall infection dynamics (Grassly & Fraser, 2008). Indeed, such models have been used to accurately simulate the infection dynamics for the guppy-*G. turnbulli* host-parasite system, extensively utilised in this PhD project (see van Oosterhout et al., 2008; Tadiri et al., 2019). This unison of experimental investigation with mathematical models, would allow us to simulate how infections dynamics may respond when specific variables (e.g., pollutant level) are changed. The broader applications of such simulations being, of course, that a robust model should be applicable to any host-parasite system where direct transmission occurs.

8.4. Conclusion

This PhD project has made progress in understanding how emerging and widespread stressors impact fish welfare. The fate of the most abundant vertebrate group on the planet is a double-edged sword. One blade points at the alarmingly high extinction rates of fish and the other edge points at what has been described as a disease crisis within the fish trade, implicated in global stocks facing a state of collapse. This project has demonstrated that neglected but all too common anthropogenic stressors, like mechanical disturbance, noise pollution and enrichment deprivation, can be severely detrimental to disease resistance and negatively impact host physiology and behaviour. The investigation on microplastic, one of the most common anthropogenic pollutants of this era, has for the first time shown a functional impact of consumption on disease resistance and added to the growing body of information showing how plastic pollution is impacting species welfare. In the last experimental chapter, one of the most common variables in aquatic habitats, flow, unlike the other anthropogenic stressors, did not constrain immunity or impact disease resistance, and this hints at wild fish being able to adapt to naturally occurring stressors even at the limits of their physical endurance. Beyond the empirical information this project provides on how stressors are impacting fish welfare, this thesis also critically assesses why integrating fish welfare policies within biosecurity will be invaluable if the fish trade is to remain sustainable for an ever-growing human population.

The overarching message of this thesis is: the welfare of fish is being threatened by anthropogenic activities and research is revealing which activities must either be regulated more strictly, or in which instances we must develop alternative strategies. This PhD project provides empirical information for policy change that is needed not only for the fish trade but also for protecting aquatic ecosystems in which fish play a key role.

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Appendix 1

Transport-induced mechanical stress impact on infection trajectories of guppies with pre-existing infections

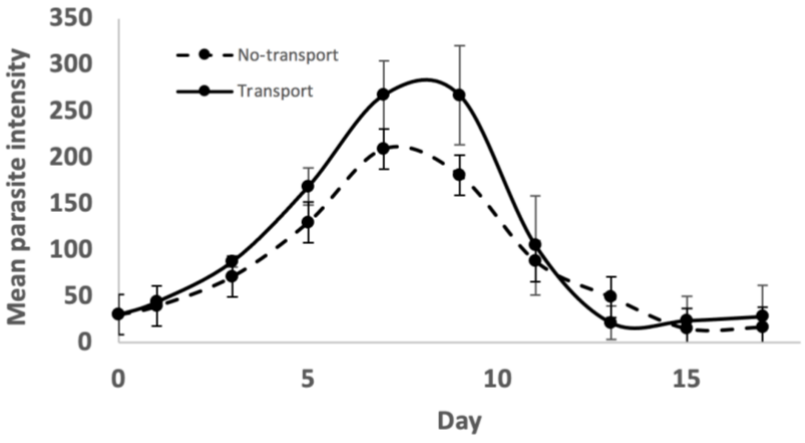
This study investigated how mechanical disturbance during simulated transport impacted pre-existing high burden infections in guppies, *Poecilia reticulata*. This Appendix complements Chapter 2 which investigates how mechanical disturbance impacts disease resistance when hosts are infected post-transport.

A.1.1. Materials and Methods

Each guppy (n = 20 per treatment) was experimentally infected with 30 *Gyrodactylus turnbulli* worms, packaged in standard polythene bags and then exposed to simulated transport (as described in Materials and methods section above) or left as un-transported controls. After 24 h of simulated transport, all fish (transport and control) were individually isolated in 1 l pots and screened every 48 h over 17 d to monitor their infection trajectories. Data were analysed as described in the statistical analysis section of the main text.

A.1.2. Results

Mean parasite intensity and total infection trajectories over 17 d as measured by the area under the curve (AUC) were not significantly different between transported guppies and controls (mean parasite intensity: GLMM: Z = 1.64, SE = 0.1, p = 0.1; AUC: GLM: Z = 0.6, SE = 0.38, p = 0.54; Fig. A1). Peak parasite burden, however, was significantly higher in guppies that experienced simulated transport (GLM: Z = 2.72, SE = 0.05, p = 0.006) and timing of peak parasite burden was also earlier in these guppies compared to controls (GLM: t = −3.83, SE = 0.03, p = 0.0001).



A.1. *Poecilia reticulata* exposed to a starting point *Gyrodactylus turnbulli* infection of 30 worms prior to transport did not suffer significantly elevated parasite burden compared to controls.

Appendix 2

Temperature dynamics and infectivity of an important fish pathogen: *Gyrodactylus sprostonae* infections in carp

A2.1. Introduction

Emergence of novel disease-causing pathogens is a major concern for farming as it threatens global food security (Murray & Peeler, 2005; Bruijn et al., 2018). Aquaculture is the world's fastest growing food sector and production intensification means that fish stocks are under unprecedented stress to meet rising demands (Worm et al., 2009; Worm & Branch, 2012; FAO, 2018). Infectious diseases remain a recalcitrant problem for aquaculture and industry wide losses exceed US $6 billion per annum, which rivals the projected proportional losses experienced by terrestrial livestock (Shinn et al., 2015; Stentiford et al., 2017). Emergent diseases pose a unique challenge due to cryptic aetiologies and lack of knowledge on specific disease dynamics. Emergence of new pathogens from pre-existing pathogenic strains is especially challenging as there is already an *a priori* disease threat and pre-existing disease assessments and risk mitigation strategies might not be applicable for newly introduced or novel species (Murray & Peeler, 2005).

Within aquaculture and the ornamental trade, the pathogenic genus *Gyrodactylus* has historically proven to be a major pest with significant reported economic losses (see Bakke et al., 2007; Maceida-Veiga & Cable, 2019). This genus includes an OIE listed species, *Gyrodactylus salaris*, within the top ten list of globally important transmissible pathogens (OIE, 2019). With hyperviviparous reproduction, direct transmission and currently no effective *en masse* treatments, this genus will remain a major challenge for the foreseeable future. While effective treatment can be achieved for gyrodactylosis in cultured environments, for wild fish stocks in lakes and ponds, it is almost impossible to treat (Schelkle et al., 2009). Furthermore, new species are described regularly within this incredibly diverse genus (Bakke et al., 2007). In 1962, a new species of gyrodactylid was identified in the gills of common carp (*Cyprinus carpio*) in China (Mo-en, 1962), *Gyrodactylus sprostonae*. Since then, this species of gill parasite has spread and been identified in other carp species (e.g., silver carp and bighead carp) in multiple countries including, Syria, Iran, Hungary, Germany (Barzegar et al., 2017) and recently the UK. Within the UK, this disease has been implicated in significant losses within UK fisheries with reported symptoms and mortality reported only in large, prized, carp. Symptoms of this infection include gill hyperplasia and respiratory distress and mortality (CW personal obs). However, transmission and infection dynamics of this pathogen are unknown.

This study investigates two key aspects of *G. sprostonae* infection dynamics: 1) infection dynamics (i.e., pathogen number changes) under two temperature scenarios (cold: 14^{o}C and warm: 24^{o}C), and 2) transmission potential between adult and juvenile fish. Understanding these two fundamental aspects of infection dynamics will lead to a clearer picture of the nature of this pathogen, the potential for spread and overall pathogen burden.

A2.2. Material and Methods

A.2.2.1. Carp and parasite source and maintenance

Juvenile carp (0+ age, standard length 70-90cm, *Cyprinus carpio*, n=222) supplied by the Environment Agency (EA) from a carp farm in southern England free from parasites and historic infections, arrived at Cardiff University in September 2018. Upon arrival, carp were acclimatised to laboratory conditions which consisted of dividing half the juveniles into two separate temperature-controlled rooms to investigate how *G. sprostonae* infection dynamics responded to temperature differences. Here, juvenile carp were maintained at the two different temperatures for a week prior to infections (14°C and 24°C). In each temperature treatment, carp were separated into infection tanks (n=86) or control tanks (n=25) at a density of 1 fish per 2.5L. Carp were maintained under 12 h dark-12 h light cycles and fed twice daily on commercial trout pellets for 1 week prior to experimental infections.

Infected adult donor carp were obtained from a recreational fishery in England with a history of *G. sprostonae* infections (65-75 cm, n=4). Each adult fish was maintained individually in 400l tanks constantly aerated by air stones connected to air pumps. All experiments in this study utilised the adult carp known to be infected with *G. sprostonae*, as donor hosts for the experimental juveniles. Infections in adult donors were confirmed via gill examination utilising microscopic analysis of opisthaptor morphology (Paladini et al., 2009). All adult carp were co-infected with *Dactylogyrus* spp. Adult carp were humanely sacrificed, via anaesthetic (clove oil) overdose and cranial destruction to obtain gill samples for infections (Home Office Schedule 1 method).

A.2.2.2. Experimental infections

Three methods were used to optimise chances of establishing infections on juvenile carp: individual, group and cohabitation infections.

1) Individual infections: Juvenile carp (n=128) were experimentally infected using adult carp gill filaments with established *G. sprostonae* infections. Infections were conducted under a dissecting microscope with fibre optic illumination according to King and Cable (2007). Each juvenile carp, under mild anaesthesia (0.02% MS-222), was exposed to two gyrodactylids obtained from sacrificed infected adult carp (see above) close to the operculum. This involved bringing a gill filament containing worms near a host and observing parasite transfer onto the operculum or on the skin near the gill. Fish were then allowed to recover fully from anaesthesia and returned to experimental tanks. Control fish (n=47) underwent anaesthesia without exposure to infection. All Infected and control fish were housed separately, but with infected fish being held together at 14°C or 24°C at a density of one fish per 4L (64 fish per temperature treatment). Experimental fish were screened at day 7, 14 and 38 after initial infections (n=10 per time point), which involved humanely euthanising carp and placing dissected gills in fresh dechlorinated water and observing individual gill filaments under a dissection microscope with fibre optic illumination, with watchmaker forceps used to tease apart individual gill filaments to determine worm burden.

2) Group infections: Then groups of juvenile carp were infected at 24°C with *G. sprostonae*. This temperature was chosen as it was the only temperature at which gyrodactylids were still

found after day 7 using our first infection protocol (see below). To this extent, 5 naïve juvenile carp with individual gill filaments from infected adult carp containing *G. sprostonae* worms (mean worm range per 1L pot = 45-65), obtained from infected adults, were placed in 1 L pots under dark conditions to facilitate parasite transmission (Brooker et al., 2011) for 24 h. Fish were then examined after 24 h for parasites by humanely euthanising fish and extracting gills and viewing them under a dissecting microscope.

3) Cohabitation infections: To investigate the infectivity of *G. sprostonae* between adult and juvenile carp at the two stated temperatures, infections via cohabitation were conducted, where juvenile carp were placed in 120 L tanks with an adult known to be infected with *G. sprostonae* at a density of 5 juveniles per 1 infected adult. After 24 h cohabitation, juveniles were removed from tanks and placed individually in 5 L containers at 14 or 24ºC. To test the effectiveness of cohabitation, the first batch of fish (n=5) was humanely euthanised via anaesthetic overdose and worm number was counted on the body surface and gill arches. This confirmed that cohabitation was successful at transferring worms to the body surface and gills. Three subsequent batches were screened non-destructively after 24 h cohabitation to quantify the number of *G. sprostonae* on the body of juvenile carp. Screening of the body surface was then performed every other day to monitor infection trajectories. The fish body surface was divided into seven regions based on parasite location (head, body, pectoral fins, dorsal fin, ventral fin, anal fin, and caudal fin) and the number of worms in each area counted. On days 11 and 17, when worm numbers on the body had declined, which was inferred as the point at which parasites had either moved into gills or died, a subsample of carp from each temperature treatment (n=5) were euthanised and parasite burden on gills determined.

A.2.2.3. Statistical analysis

All statistical analyses were performed using RStudio version 1.0.143 (R Development Core Team, 2015). A Fisher's Exact Test for count data was performed to analyse the difference in number of fish on which *G. sprostonae* worm numbers increased on the body surface and the persistence of infections between temperature treatments (i.e., length of time infections lasted). A Generalised Linear Mixed Model (GLMM) was used to analyse Area Under Curve (AUC), calculated using the trapezoid rule (White, 2011) based on parasite counts on juvenile body surface between temperature treatments with a negative binomial error family and log link function, with temperature and standard length as a fixed factor. A GLMM was chosen to prevent pseudo-replication in model design as individual fish were screened at multiple time points, and fish ID was therefore used as a random variable in our modelling. A GLM with a Poisson error family and log link function was utilised to analyse the association between number of parasites on external body regions after infections and temperature treatment, with host standard length and temperature being fixed factors.

To analyse the difference in parasite numbers on the gills of juvenile carp, post-cohabitation, between temperature treatments, a GLM with a Poisson error family and log link function was used, with temperature and host standard length being fixed factors. All error families for models were chosen based on normality of residuals and the lowest AIC value indicative of goodness of fit (Thomas et al., 2017).

A.2.3. Results

Here it is shown that for *Gyrodctylus sprostonae* infecting carp: 1) temperature had an overarching effect on the persistence of infections and pathogen numbers, and 2) all the juvenile carp, regardless of the infection protocol utilised, cleared their infections and displayed no symptoms of *G. sprostonae* infections (increased opercular beat rate; surface breathing; gill hyperplasia).

A.2.3.1. Individual infections

At day 7 post-infection, only 2 out of 10 juvenile fish in the warm treatment were infected with a single gyrodactylid each on the gills, whereas no fish (n=10) remained infected from the cold treatment. All juveniles screened on days 14 and 38 were uninfected in both temperature treatments (n=10, per time point).

A.2.3.2. Group infections

With regards to infecting groups of fish with individual gill filaments containing parasites, two weeks after maintaining the carp at 24°C, dissections (n=5 fish) and microscopic analysis of the body surface and gills revealed the *G. sprostonae* population had gone extinct. However, co-infecting dactylogyrids were newly established and present on all fish gill filaments screened.

A.2.3.3. Infections via cohabitation

There was a significant difference in total infection trajectories on the body surface of fish post-cohabitation between temperature treatments, with the cold treatment having higher worm numbers than the warm treatment (GLMM of AUC: temperature $Z=-2.08$, $SE=0.54$, $p=0.03$, Figure A2.1A). However, gill dissections after cohabitation trials revealed that fish from the warm treatment had significantly higher *G. sprostonae* burdens compared with fish from the cold treatments (GLM: $Z=8.08$, $SE=0.22$, $p<0.001$, Figure A2.1B). Host standard length did not significantly correlate with number of worms (Standard length $Z=1.12$, $SE=0.03$, $p=0.26$). At 14ºC, the number of *G. sprostonae* worms increased on 10 out of 15 fish, whereas at 24ºC, parasite numbers only increased on 1 out of 15 carp (Fisher's Exact Test, $p=0.001$). Infections persisted for longer at the lower temperature on the body surface (maximum of 15-17 days at 14ºC compared to 7-9 days at 24ºC). However, overall, there was no significant difference between the persistence of infection between the two temperature treatments (Fisher's Exact Test, $p=0.057$, Figure A2.1A).

Externally, significantly more parasites were found on the head and pectoral fins compared with other regions, representing a combined 56% and 80% of the parasite distribution from cold and warm treatments respectively (GLM: head $Z=6.56$, $SE=0.42$, $p<0.001$; pectoral fin $Z=5.62$, $SE=0.42$, $p<0.001$, Figures A2.C and D).

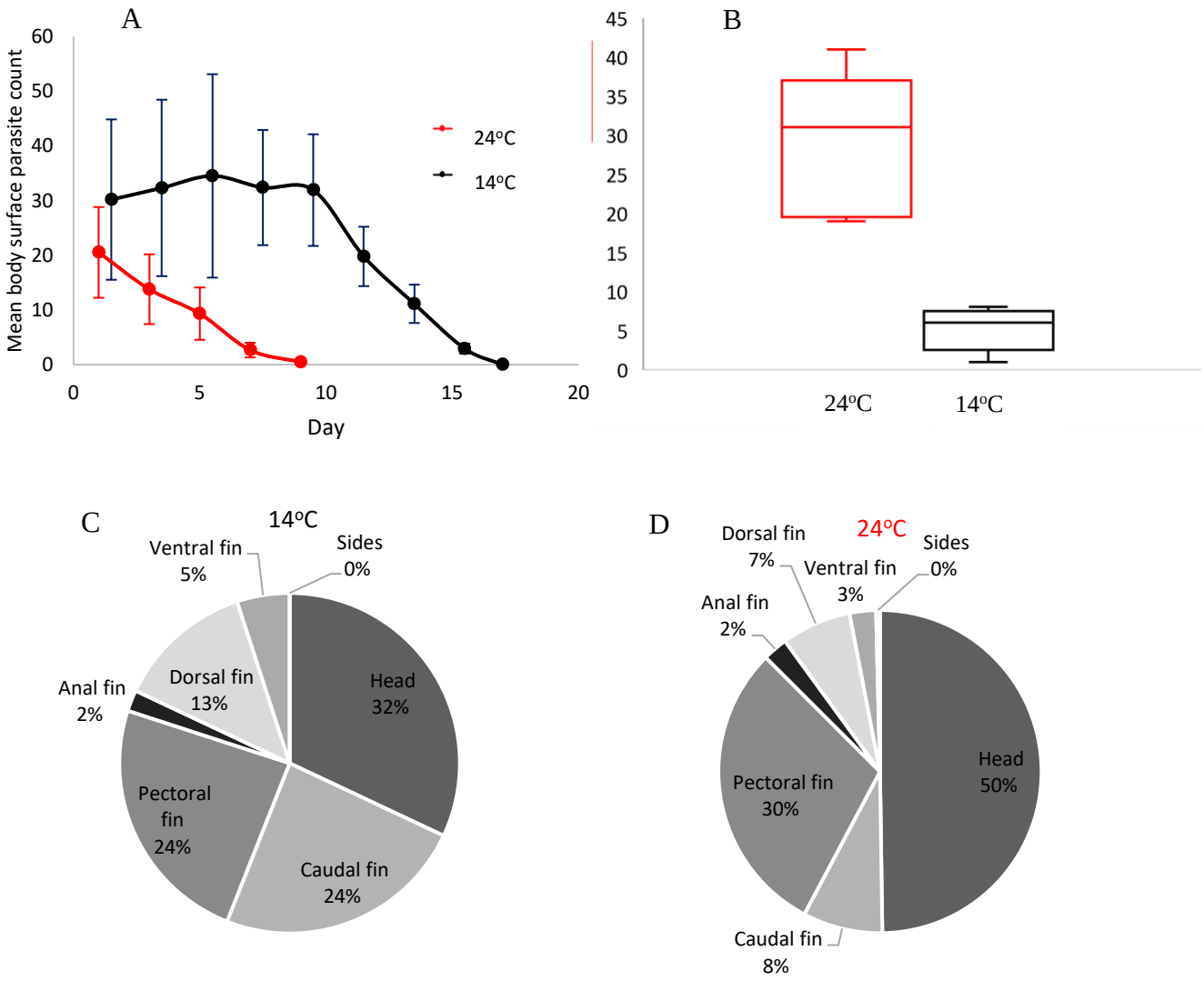


Fig A2.1. (A) Mean parasite count (with standard error bars) of *Gyrodactylus sprostonae* worms on body surface of juvenile carp over 17 days from warm (24°C) and cold (14°C) treatments after cohabitation (24 h) with infected adult carp. (B) Mean *G. sprostonae* count on gills of juvenile carp after cohabitation with infected adults, with box plot showing the median line, inter-quartile range (box) and 1.5x inter-quartile range (whiskers). (C) and (D) Percentage parasite distribution on juvenile carp body surface between temperature treatments after cohabitation.

A.2.4. Discussion

This study investigated the infection dynamics of an important fish pathogen, *Gyrodactylus sprostonae*. Temperature significantly affected the pathogen burden on the gills and body surface of juvenile carp as well as the persistence of infections. Pathogen burdens were significantly higher at the warm temperature treatment, whereas infections persisted for longer at colder temperatures. However, all juvenile carp, regardless of the infection protocols utilised failed to maintain infections, as all hosts lost their infections supporting anectodal observations that this is an infection of large and/or old carp.

For *Gyrodactylus* species, and indeed all pathogens, temperature has a universal impact on infection dynamics (Bakke et al., 2007; Stewart et al., 2017a). Data reported by UK carp fisheries indicate that higher disease burdens for *G. sprostonae* occurs during the warmer summer months in adult fish (EA unpublished data) supporting this studies laboratory observations. Further evidence comes from examination of common carp from Germany reporting that *G. sprostonae* infections were highest in June and August (Lux, 1990). This may be linked to parasites higher innate capacity to increase (r_m) at warmer temperatures, demonstrated experimentally in other gyrodactylid species (see Scott & Nokes, 1984; Jansen & Bakke, 1991). Further support for this comes from this investigations gill dissection data that revealed at warmer temperatures parasite burden was significantly higher than at colder temperatures, indicating that this parasite, like all organisms, have a physiological optimum.

Regardless of the temperature dynamics seen for this infection in common juvenile carp, the transmission potential of *G. sprostonae* between adult and juvenile fish was very poor. None of the infection protocols led to sustained infections nor were there any observable symptoms of *G. sprostonae* infections, which in adults typically consists of surface breathing, increased opercular beat rates, lethargy and gill hyperplasia and mortalities of common carp in the UK are associated with tens of thousands of parasites per host (EA unpublished data). The mechanism behind why juvenile common carp could not sustain gill infections at either temperature is not clear but the answer may lie in common carp not being the only suitable hosts. In Iran, it was found that *G. sprostonae* had the widest host range in warm water freshwater fish of all the gyrodactylid species discovered and hosts included not only common carp but also the silver carp (*Hypophthalmichthys molitrix*) and the big head carp (*Hypophthalmichthys nobilis*) from almost all Iranian farms surveyed (Jalali et al., 2005). Interestingly, another study in the Gilan province of Iran showed that bighead carp had the same mean *G. sprostonae* abundance as common carp (Roohi et al., 2019). If, as evidence suggests, that sustained infections and high virulence of *G. sprostonae* is localised to only adult carps then the disease burden for this infection may have unpredictable consequences at a population level compared with other gyrodactylid species where juveniles are negatively affected (e.g., *G. turnbulli*, see Cable & van Oosterhout, 2007).

Of course, it is possible that the experimental approaches utilised for this study are not ideal for gill monogeneans but more suited to gyrodactyilds that primarily infect the skin surface (Bakke et al., 2007; King & Cable, 2007). Additionally, as the route of transmission for *G. sprostonae* is not yet fully understood, it is difficult to establish an experimental protocol that may successfully establish infections. It is also worth noting that this gill infection may be associated with stressful host conditions. Certainly, the infected adult carp obtained for this study to act as donors were from recreational fisheries associated with catch and release methods that do inflict stress on fish. It may be that the best way to prevent large mortalities associated with *G. sprostonae* infections is to ensure that carp stocks are maintained under low stress conditions.

Infectious disease continues to be one the greatest challenges preventing the sustainable expansion of aquaculture (Shinn et al., 2015; Stentiford et al., 2017). As it stands, certain reports are revealing that over 60% of global fish stocks are facing a state of collapse (Worm et al., 2009) and infectious disease continues to be a major reason why. A key reason we are witnessing what has been described as a disease crisis (Stentiford et al., 2017) is the unprecedented level of stressors fish stocks are under combined with unnaturally high host densities (Ashley, 2007; Worm et al., 2009). Hosts facing such increased levels of stress will

suffer immunosuppression, which makes individuals more susceptible to disease (Tort, 2011) and thereby creating a vicious cycle of sustained infections. While host-pathogen red queen dynamics does mean that novel pathogens emerge *in natura* (Salathé et al., 2008); high host densities combined with altered selective pressures under captivity, may be creating the 'perfect storm' for novel pathogen emergence and spread (Murray & Peeler, 2005). The monogenean gyrodactylids continue to be a major pest for fisheries globally and other than *G. salaris* as a disease being monitored globally, we maintain an 'eyes wide shut' policy for other gyrodactylid species (OIE, 2019). This means that the potential for other *Gyrodactylus* species spread is largely unchecked. For *G. sprostonae* that was first reported in China, its spread across Europe and emergence in UK carp farms in recent years certainly suggests a potential to spread globally. However, we have remained largely in the dark about the infection dynamics of this fish pathogen. This study, at least, sheds light by suggesting that utilising the experimental approaches detailed here, *G. sprostonae* infections are not dangerous to juvenile carps. As farmers continue to report high mortalities for this pathogen in adult prized carp, future research will need to determine if host specificity combined with immune expression can account for the differences seen between the disease dynamics of juvenile and adult carp. There is also a further need to develop and standardise a protocol for future experiments involving gill monogeneans as currently methods to establish and monitor infections remain very challenging.

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