Behavioural Responses of Fish to Parasitism and Environmental Conditions
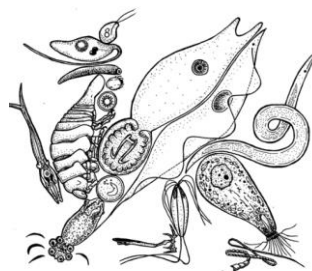
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A thesis submitted to Cardiff University in accordance with the requirements for the degree of Doctor of Philosophy in the School of Biosciences, Cardiff University

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DECLARATION

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Acknowledgements

Abstract

Every aspect of an individual's behaviour is, to some extent, mediated by parasite exposure. Potential hosts can, for example, initiate evasive behaviours towards infected conspecifics to reduce infection risk. If infected, individuals may exhibit adaptive behavioural responses aimed at reducing pathological symptoms. In addition to infection-mediated behavioural modifications, hosts behaviourally adapt to the environment in which they reside. Disentangling the effects of parasitism from environmental variables on host behaviour can be challenging. In a series of self-contained experiments, this thesis investigates three research areas. Firstly, how thermal and hydrological environmental conditions impact freshwater host-parasite interactions; secondly, how parasite infections mediate host behavioural modifications; and finally, how such behavioural changes have population level effects with respect to social structuring. The first experiment in this thesis describes how, when presented with a range of thermal conditions, Trinidadian guppies, *Poecilia reticulata* Peters 1859, infected with a common monogenean ectoparasite, *Gyrodactylus turnbulli* Harris 1986, frequent warmer thermal conditions to self-medicate against infection (Chapter 2). In a second experiment, *G. turnbulli* infected guppies experiencing dissimilar flow conditions showed a significant decrease in shoaling tendencies; but only in the absence of flowing water (Chapter 3). During this experiment, infected fish were observed increasing body contact with conspecifics: a behavioural adaptation presumably aimed at offloading parasite burdens (investigated in Chapter 4). Furthermore, infected hosts exhibited nocturnal restlessness, which may have further repercussions for host health (Chapter 5). Finally, parasite-mediated host behavioural modifications had significant population level effects with respect to social structuring. *G. turnbulli* infected guppies significantly increased their social rank within a population and instigated more contacts than they received, in contrast to their uninfected counterparts (Chapter 6). The infection status of an individual therefore determines its significance in mediating a population's social dynamics, and so driving disease transmission processes.

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Chapter 1

General introduction

1.1   Climate change and freshwater ecosystems

Despite only covering 0.8% of the Earth's surface, freshwater ecosystems encompass approximately 6% of all described species (Gleick 1996; Dudgeon et al. 2006). Significant global freshwater biodiversity declines, exceeding that of terrestrial and marine habitats, have arisen from intensified exploitation of water resources, habitat destruction, pollution, flow modification, and the introduction of non-native species (Strayer and Dudgeon 2010; Vörösmarty et al. 2010). The limited dispersal capabilities of many freshwater organisms within changing environments increase their extinction vulnerability, which subsequently undermines ecological functioning (Bellard et al. 2012; Bush and Hoskins 2016). Furthermore, global climate change exacerbates variations in abiotic factors, including thermal and hydrological regimes and thus fostering biodiversity declines (Webb et al. 2008; Woodward et al. 2010).

By the end of the current century, mean surface water temperatures are predicted to increase by 1.5-5.8 °C (Houghton et al. 2001). The resulting impacts will be most evident in higher altitudinal and latitudinal habitats (Rangwala et al. 2013; Hassan et al. 2005). In Canada, for example, increased mean air and water temperatures have shortened ice coverage on rivers and lakes by approximately 20 days since the 1800's (Schindler 2000; Magnuson et al. 2000). Generally, mean daily temperatures are used as climate change indicators (Qu et al. 2014). However, global warming is more complex than a steady increase in average temperature. It is important to consider diurnal temperature variation; as for many regions minimum daily temperatures are increasing, whilst maximum daily temperatures remain consistent (Easterling et al. 1997). This

narrows the thermal range to which freshwater organisms can adapt, and could prove particularly challenging for ectothermic taxa, including freshwater fishes, which rely on environmental temperature for optimising physiological performance (Huey et al. 2012). Many ectotherms exhibit behavioural modifications to remodel their physiology and buffer the effects of chronic or recurring thermal stress (reviewed by Seebacher et al. 2015). For example, juvenile Atlantic salmon (*Salmo salar*) actively avoid potentially lethal thermal conditions by moving towards and residing in cool water sites (Breau et al. 2011). Thermal elevations therefore drive changes in species distributions, which may facilitate the spread and establishment of non-native organisms into unfamiliar environments (Stachowicz et al. 2002). Consequently, increased predatory pressure, competition for resources and habitat alteration endanger native flora and fauna (e.g. Capinha et al. 2013).

In addition to thermal variation, freshwater bodies have endured significant flow regime modifications as a consequence of anthropogenic activity (Malmqvist et al. 2008), and climate change constitutes another factor in flow alteration (Schneider et al. 2013). A major bi-product of global warming, depending on location and season, is elevated evaporation rate (Alcamo et al. 2007). The magnitude of evaporation is determined not only by temperature, but also soil characteristics, water inputs, run-off, and vegetation cover (Helfer et al. 2012). A significant reduction in water discharge leads to an increased incidence of drought in extreme cases (Easterling et al. 2000). Contrastingly, climate mediated acceleration of the hydrological cycle intensifies precipitation, particularly within the tropics, resulting in frequent spate conditions within river systems (Milly et al. 2005). The duration, frequency, timing and rate of change in flow variability shapes habitat features (Poff et al. 1997; Bunn and Arthington 2002), and thus supports different regional biodiversity (Allan et al. 2005; Schneider et al. 2013). Life history traits of freshwater species including growth rate, fitness, and survival are

linked to the hydrological regimes in which they reside (Mims and Olden 2013). Environmental variability therefore represents a selection pressure towards adaptation or the evolution of phenotypic plasticity (Chevin et al. 2010).

1.2 Climate change and aquatic disease

Aquatic habitats significantly influence disease ecology by providing habitats for larval vectors and, most importantly, facilitating water-borne transmission amongst hosts (Marcogliese 1995; Johnson and Paull 2011; Cable et al. 2017). Conversely, the presence or absence of particular parasites, most notably helminths (see Sures 2001; Vidal-Martínez 2007), provides important information concerning the ecological integrity of an ecosystem (Huspeni and Lafferty 2004; Marcogliese 2005; Blanar et al. 2009). Changes within parasite community composition can be indicative of biological invasions, habitat degradation and climate change (MacKenzie et al. 1995; Valtonen et al. 1997), with the latter predicted to have profound indirect impacts on aquatic animal health with respect to infectious disease (Skuce et al. 2013).

The potential for climate change to impact aquatic host-parasite interactions is well recognised (reviewed in Lafferty 2009; Marcogliese 2008, 2016), with elevated temperatures not only prompting distributional shifts of organisms and their associated pathogens (Nye et al. 2009; Baker-Austin et al. 2012), but also enhancing disease expression (reviewed in Harvell et al. 2002). For sedentary taxa, or those with limited dispersal and/or acclimation capabilities, climate-mediated physiological stress often reduces host immunocompetence, therefore promoting disease emergence (Harvell et al. 1999). In contrast to marine and terrestrial environments, freshwater habitats have received little attention with respect to disease emergence (Dudgeon et al. 2006). For freshwater hosts, particularly fish, physiological and immune functioning is determined by ambient temperature (Bowden et al. 2007; Marcos-López et al. 2010). Similarly,

associated pathogens also exhibit optimal thermal ranges for replication (Marcos-López et al. 2010), with elevated temperatures directly affecting parasite ecology by shortening life cycle completion rates (e.g. Macnab and Barber 2012), and increasing parasite virulence (Harvell et al. 2002; Marcogliese 2008). Conversely, extreme thermal elevations can increase parasite mortality (e.g. Scott and Nokes 1984), to the benefit of the host.

Although temperature is considered the predominant abiotic factor affecting aquatic parasite ecology (Marcogliese 2001), there is growing evidence relating to how hydrological variation impacts host-parasite dynamics. Aquatic pathogens often depend on surface waters for their transmission and persistence (Akullian et al. 2012). Reduced flow rates as a consequence of drought, for example, can increase retention times and concentrate free-living infectious stages (Hofstra 2011). Conversely, elevations in water discharge transport pathogens away from a source to downstream populations. This has implications for the implementation of efficient control programmes, as shown by schistosomiasis: a waterborne parasitic disease infecting 200 million people worldwide (Chitsulo et al. 2000). Free-swimming schistosome larvae are often transported via hydrologic connections from upstream to downstream communities. Thus, conventional targeted treatment of villages with high infection, without regard to interconnections between villages, may not necessarily lead to optimum control (Gurarie and Seto 2008). As well as facilitating the distribution of pathogens between populations, hydrology directly mediates interactions between pathogens and their hosts. For example, infection intensities of *Lepeophtheirus salmonis,* an ectoparasitic copepod infecting Atlantic salmon, were 2.5 times greater in moderate than high flow conditions. Elevated water velocities coupled with the rapid swimming speeds of salmon prevented successful parasite establishment and reduced host-parasite contact times, thus diminishing

infection risk (Samsing et al. 2015). Hydrological variations therefore have diverse outcomes for different aquatic host-parasite interactions, of which an imperative understanding is necessary to predict and prevent disease spread in the face of anthropogenically driven climate change.

1.3   *Environmental change, host behaviour and parasitism*

Environmental heterogeneities directly affect host behaviour (e.g. Lupandin 2005), which can indirectly influence disease transmission dynamics. Furthermore, parasitism itself often drives host behavioural changes, which can be an adaptive response of the host, the parasite, or simply a by-product of infection (Levri 1999). Discerning between these can be particularly challenging (Poulin 1994; Poulin et al. 1994), and in many cases parasitic manipulation is favoured if host phenotypic alterations facilitate parasite transmission necessary for life-cycle completion. The majority of host-parasite interactions are likely reciprocal, with host behaviour feeding back on parasite transmission and vice versa (reviewed in Ezenwa et al. 2016). Host behavioural changes can also be the first line of defence against parasitism (Hart 2011). For example, typically social hosts employ evasive actions towards infected conspecifics to reduce infection risk (Kiesecker et al. 1999; Behringer et al. 2005; Poirotte et al. 2017). Behavioural reduction of infection risk is only beneficial should the costs of infection outweigh the rewards of group membership. Indeed, sociality confers many benefits including enhanced predatory detection (Ward et al. 2010a), foraging efficiency (Dyer et al. 2009) and mating opportunities (Krause and Ruxton 2002). However, the close proximity of individuals can promote disease transmission (Kappeler et al. 2015). Consequently, a continual trade off exists with disease status often determining group formation.

Host sociality results in the formation of highly structured populations, which is evident across multiple taxa (e.g. insects: Gordon 2016; fish: Croft et al. 2004; mammals: Ryan et al. 2013). Using social network analysis to better understand social complexity (Coleing 2009; Silk et al. 2017), specifically contact patterns between conspecifics, can reveal influential group members mediating disease transmission (Salathé and Jones 2010). An individuals' social position ultimately determines its disease acquisition, or if already infected, its probability of propagating disease to conspecifics (White et al. 2015; Silk et al. 2017). Furthermore, environmental changes influence interactions between individuals that underpin group structuring (Webster et al. 2013; Lantz and Karubian 2017; Krause et al. 2017), with resulting consequences for disease spread (reviewed in Craft 2015). For example, as a population becomes dispersed in an increasingly fragmented landscape, social clustering results in the confinement, as oppose to the spread, of infectious disease (Sah et al. 2017). Conversely, populations once distributed over large geographical scales may become confined into smaller patches of suitable habitat, restructuring social complexity and increasing pathogen exposure. Environmental change therefore mediates the evolutionary dynamics of host-parasite interactions at both individual and population levels (Brunner and Eizaguirre 2016).

1.4 Fish behaviour and parasitism

In natural systems it is often difficult to disentangle the effects of environmental variation and parasitism on host behaviour. Synergistic stressors in freshwater habitats, including thermal stress and pollution, can unbalance host-parasite equilibria by causing physiological and mechanical damage to fish hosts and thus increasing disease susceptibility (Lafferty and Kuris 1999; Arkoosh et al. 1998). To mitigate such circumstances, fish can employ 'behavioural resistance' (Karvonen et al. 2004),

whereby habitat choice, prey selection and social decisions are likely to have, in part, evolved to limit exposure to parasites (reviewed in Barber et al. 2000). Acquiring infection can subsequently stimulate a fish to engage in simple or complex self-medication behaviours by, for example, physically dislodging parasites through skin abrasion against substrata (Urawa 1992).

An abundance of evidence concerning host-driven behavioural responses to parasitism exists, yet there are also examples of parasite-mediated host behavioural manipulation. Frequently, a succession of hosts is necessary for parasite life-cycle completion, and fishes often fulfil intermediate host positions within these complex cycles (Auld and Tinsley 2015). It is therefore beneficial for a parasite to 'manipulate' its host to maximise transmission success between trophic levels. In most cases, parasites control their hosts' phenotype through chemical signal exploitation (Lafferty and Shaw 2013). For example, *Euphalorchis californiensis* infected and uninfected killifish significantly differ in their serotonin and dopamine expression (Shaw et al. 2009). As *E. californiensis* resides within the hosts' brain, this strongly suggests crucial neurotransmitters are manipulated by the parasite. Similarly, the development of *Schistocephalus solidus* worms within three-spined stickleback hosts has been associated with immune and neuroendocrine abnormalities (reviewed in Barber and Scharsack 2010). In both cases, neurochemical interference results in distinct differences in anti-predatory behaviour whereby infected fish are more susceptible to avian predation, to the benefit of their parasites (Barber et al. 2004; Lafferty and Morris 1996).

1.5   Model species

When investigating the interplay between parasitism, environmental variability and host behaviour, it is important to utilise well-studied model organisms to differentiate abnormal from normal phenomena, particularly in behavioural experiments. Two established model species are used here to investigate environmental impacts on host behaviour and the resulting disease transmission: the tropical Trinidadian guppy (*Poecilia reticulata*) and the temperate three-spined stickleback (*Gastersteus aculeatus*). These species are arguably the most well established models used in aquatic behavioural experiments and a wealth of knowledge in their evolutionary ecology exists (Magurran 2005; Huntingford and Ruiz-Gomez 2009). Furthermore, an in depth understanding of their parasite assemblages and associative costs has been achieved through extensive experimentation in both natural and artificial settings (e.g. guppies: Stephenson et al. 2015; Pérez-Jvostov et al. 2015; Hockley et al. 2014, and sticklebacks: Schade et al. 2014; Kuhn et al. 2015; Talarico et al. 2017). Finally, these species are easily bred, reared and maintained in the laboratory, without captive conditions constraining natural behaviours.

1.5.1   Trinidadian guppy - Gyrodactylus turnbulli system

Trinidadian guppies (*Poecilia reticulata*) are small, live-bearing tropical poeciliids native to South America and the islands of Trinidad and Tobago (Rosen and Bailey 1963). In their natural environments, guppies occupy a range of habitats from stagnant ponds and lakes to fast flowing mountain streams (Deacon et al. 2011). Waterfalls bisecting streams in which guppies reside represent physical barriers to migration: isolating populations and resulting in parallel but independent evolutionary divergence in a number of guppy traits including morphology (Liley and Seghers 1975), anti-predatory behaviour (Magurran and Seghers 1994) and life expectancy (Reznick et al.

2005). The majority of trait differences emerge as a consequence of dissimilar predatory regimes experienced by up- and downstream populations (Houde 1997; Magurran 2005; Reznick and Endler 1982). For example, greater predatory pressure in lower river courses result in stable differentiated social structures of shoals, important for endorsing cooperative anti-predatory behaviour (Dugatkin 1991; Heathcote et al. 2017). Consequently, lower course guppies, specifically females, exhibit higher parasite prevalence than upstream populations (Stephenson et al. 2015), presumably due to their greater shoaling tendencies facilitating disease transmission (Magurran and Seghers 1994; Richards et al. 2010). In the wild, female guppies typically aggregate into groups of 2-20 individuals (Croft et al. 2004), between which males move in search of mating opportunities (Griffiths and Magurran 1998). Whilst shoal formation confers many benefits (Krause and Ruxton 2002), it can be counterintuitive by promoting disease epidemics, particularly of directly transmitted *Gyrodactylus* ectoparasites (Johnson et al. 2011): a common pathogen of wild guppies (see Appendix A).

Gyrodactylids are monogenean helminths ubiquitous on teleosts (Harris et al. 2004; Bakke et al. 2007). The viviparous reproductive strategy of these small worms (<1 mm) often results in exponential population growth, particularly within warm water that shortens parasite generation times (for *G. turnbulli* 24 h at 25 ℃; Scott and Nokes 1984). Direct transmission through host-host contact promotes rapid epidemic outbreaks that have profound socio-economic impacts. Most notably within aquaculture is the introduction of *Gyrodactylus salaris* into Norwegian Atlantic salmon populations (Peeler et al. 2004), costing the Norwegian salmonid industry US$ 55 million per annum to control (Denholm et al. 2016). Next to *G. salaris, G. turnbulli* is the most well studied gyrodactylid, in conjunction with its guppy host. Intense *G. turnbulli* infections impose severe pathology, including extensive skin damage (Cone and Odense 1984), fin

clamping (Cable et al. 2002) and ultimately host mortality (Scott and Anderson 1984). Infected fish also exhibit differences in foraging, shoaling and courtship behaviours compared to uninfected conspecifics (Kennedy et al. 1987; Houde and Endler 1990; Kolluru et al. 2009). Within the laboratory, *G. turnbulli* population trajectories can be monitored *in situ* at repeated time points without sacrificing a host (Bakke et al. 2007). The ease of quantifying *G. turnbulli* intensity, prevalence and transmission rate therefore provides an appropriate model parasite for the experiments presented within this thesis.

1.5.2   *Three-spined stickleback – Argulus foliaceus system*

The temperate three-spined stickleback (*Gasterosteus aculeatus*) has a widespread geographical distribution across much of the northern hemisphere (Wootton 1976), typically inhabiting slow flowing fresh, brackish or saltwater (DeFaveri and Merilä 2013). Sticklebacks have emerged a supermodel organism for assessing host-parasite associations, mainly due to a thorough understanding of the hosts' natural history, evolutionary biology (reviewed in Barber 2013), and parasitological repertoire (see Chappell 1969). Publication of the stickleback genome enhanced its use in molecular studies investigating evolutionary and developmental biology (e.g. McKinnon et al. 2004; Shapiro et al. 2004; Hohenlohe et al. 2010), whilst ecologically these notoriously hardy fish can act as bioindicators of environmental pollution (Katsiadaki 2007; Muldoon and Hogan 2016).

The use of sticklebacks in parasitological studies has been fundamental to understanding the evolution of parasite life-cycle complexity (Barber and Scharsack 2010) and the behavioural consequences of infection for hosts, e.g. in their competitive ability (Barber and Ruxton 1998), shoaling and foraging behaviours (Barber et al. 1995,

Ranta 1996), sexual selection (Folstad et al. 1994) and reproduction (Heins and Baker 2008). Much of this work has focussed on *Schistocephalus solidus* infected fish (reviewed in Barber and Scharsack 2010), of which the parasite is a specialist infecting only three-spined stickleback hosts (Bråten 1966). However, sticklebacks are also infected with 'generalist' parasites, including *Argulus* or 'fish lice', which are regarded one of the most widespread and problematic ectoparasites in freshwater aquaculture (Walker et al. 2004; Hakalahti-Sirén et al. 2008). Specifically, *A. foliaceus* has been responsible for mass mortalities in rainbow trout and common carp aquaculture (Menezes et al. 1990; Pekmezci et al. 2011; Chris Williams personal communication). This parasite can be readily cultured and maintained on sticklebacks within the laboratory, and the ease of identifying and quantifying individual parasites on a hosts' body surface provides an appropriate model for investigating epidemiological questions (reviewed in Stewart et al. 2017).

1.6   *Thesis overview*

In a series of self-contained experiments, the aims of this thesis is to assess the interplay between environmental variability, social behaviour of freshwater hosts and the resulting disease transmission dynamics. In **Chapter 2**, the Trinidadian guppy-*Gyrodactylus turnbulli* system is utilised to show how, when presented with a range of thermal conditions, infected hosts' have a significant preference for warmer water. By visiting temperatures outside the parasites thermal range, guppies can self-medicate against infection. Although temperature remains the predominant abiotic factor affecting aquatic parasite ecology, it is increasingly recognised how hydrological variation has both direct and indirect effects on disease transmission, with respect to modifying host behaviour. **Chapter 3** therefore assesses the effect of continuous, intermittent and no-flow conditions on the shoaling behaviour of fish, and the resulting

disease transmission. This research revealed that *G. turnbulli* transmission was greater in guppy shoals experiencing intermittent flow conditions: representative of seasonal floods, which are likely to become more frequent with future climate change. Additionally, in the absence of flowing water, parasitism had a greater impact on the social behaviour of hosts. Social preferences of individuals often change depending on the infection status of conspecifics, and infection itself can instigate behavioural modifications of hosts. Previously, *G. turnbulli* infected guppies have been observed increasing body contact with conspecifics. It is hypothesised that such behaviour is presumably aimed at 'offloading' parasite burdens, which could be beneficial considering the severe pathology of *G. turnbulli* infection. The significance of this parasite-driven host behavioural response in determining successful parasite transmission is investigated in **Chapter 4**. Additionally, whether or not infected individuals extend their activity into nocturnal periods, when typically diurnal fish enter a restful state, is addressed in **Chapter 5**. Here, both the guppy-*Gyrodactylus* and three-spined stickleback-*Argulus foliaceus* host-parasite systems were used to show how parasites cause nocturnal restlessness in hosts. Parasite-mediated social behaviour of hosts can have population level effects with respect to social structuring; investigated in **Chapter 6**. Using a social network approach, this study showed that the introduction of parasites stabilised the social structure of guppy populations. Additionally, *G. turnbulli* infected guppies significantly increased their connectivity with other shoalmates. Finally, the wider repercussions of such behavioural responses to infection for host health and the implications for animal behaviour studies of diurnal species are discussed in **Chapter 7**[1].

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[1] All applicable institutional guidelines for the care and use of animals in scientific research were followed. Procedures and protocols were conducted under UK Home Office licence (PPL 303424) by personal licence holder Michael Reynolds (E73FD85A), with approval by the Cardiff University Animal Ethics Committee.

Chapter 2

Getting into hot water: sick guppies frequent warmer thermal conditions[2]

2.1   Abstract

Ectotherms depend on environmental temperature for thermoregulation and exploit thermal regimes that optimise physiological functioning. They may also frequent warmer conditions to up-regulate their immune response against parasite infection and/or impede parasite development. This adaptive response, known as behavioural fever, has been documented in various taxa including insects, reptiles and fish, but only in response to endoparasite infections. In this study, a choice chamber experiment was used to investigate the thermal preferences of a tropical freshwater fish, the Trinidadian guppy (*Poecilia reticulata*), when infected with a common helminth ectoparasite *Gyrodactylus turnbulli*. The temperature tolerance of *G. turnbulli* was also investigated by monitoring parasite population trajectories on guppies maintained at a continuous 18, 24 or 32 °C. Parasites maintained continuously at 32 °C decreased to extinction within three days, whereas mean parasite abundance increased on hosts incubated at 18 and 24 °C. This study shows for the first time that gyrodactylid-infected fish have a preference for warmer waters suggesting that sick fish exploit the upper thermal tolerances of their parasites to self medicate.

2.2   Introduction

Temperature is perhaps the most important environmental determinant of activity and performance of ectothermic vertebrates, and is particularly critical for fishes that, unlike amphibians and reptiles, are inefficient thermoregulators (Atkinson 1994). Fish behaviourally regulate their body temperature by selecting habitats with thermal regimes that optimise physiological performance (Reynolds et al. 1976; Ward et al. 2010b). The metabolism, feeding rate and activity levels of ectotherms generally increase with temperature until conditions become stressful. Thermal stress can have long lasting effects on fish behaviour with respect to migration (Jonsson and Jonsson 2009), reproductive success (Pankhurst and Munday 2011), predatory avoidance (Marine and Cech 2004), and shoaling (Weetman et al. 1998; Weetman et al. 1999). For temperate fish, this results in marked seasonal and diel behaviours, but even tropical species are subjected to distinct temperature heterogeneities (Webb et al. 2008).

In addition to optimising physiological performance, ectotherms exploit thermal regimes to hinder parasite transmission and development. A change in a hosts' thermal preference driven by pathogenic infection, otherwise known as behavioural fever, has been documented in several taxa including bumblebees (Müller and Schmid-Hempel 1993), locusts (Elliot et al. 2002), lizards (Vaughn et al. 1974) and fish. The first evidence of behavioural fever in fish was observed in largemouth bass (*Micropterus salmoides*) and bluegill sunfish (*Lepomis macrochirus*), of which both species displayed a significant increase (+2.7 $^{\circ}$C) in mean temperature preference when inoculated with bacteria (Reynolds et al. 1976). This response was associated with bacterial pyrogens (a fever-inducing chemical) acting directly on the host's hypothalmentic thermoregulatory centre (Reynolds et al. 1976). A subsequent study speculated that an increase in thermal preference by the fish host up-regulates the immune response against parasite infection

(Covert and Reynolds 1977). Using zebrafish (*Danio rerio*) infected with viraemia carp virus it was confirmed that host behavioural fever induces a major up-regulation of the innate immune response, in this case expression of anti-viral genes, which subsequently cleared viral infections within infected fish (Boltaña et al. 2013).

Acute thermal changes can be detrimental to the immune functions of fish (reviewed in Martin et al. 2010). However, some immune responses including elevations in lysozyme and immunoglobulin M levels are positively correlated with temperature until thermal limits are exceeded (Bowden et al. 2007; Marcos-Lopez et al. 2010). Thermal stress can reduce host immunocompetence thereby increasing disease susceptibility in ectotherms (Rohr and Raffel 2010). Interactions between these factors ultimately determine whether infections lead to severe pathology and even mortality, or host recovery. Parasites also respond directly to thermal variation, as elevated temperatures typically reduce development time. For example *Schistocephalus solidus* pleroceroid larvae, infecting three-spined sticklebacks, have faster growth rates and become infectious to their definitive host sooner at 20 ^{o}C compared to 15 ^{o}C (Macnab and Barber 2011).

For directly transmitted ectoparasites, including monogenean gyrodactylids, the rate of reproduction is positively correlated within a temperature range from 17 to 28 ^{o}C in tropical gyrodactylids, and 2.5 to 19.5 ^{o}C in temperate species (Scott and Nokes 1984; Jansen and Bakke 1991). Gyrodactylids are ubiquitous on teleosts, feeding on the skin and fin tissues of a host (Kearn 1996; Harris et al. 2004). Their life history traits, transmission and population dynamics have been extensively studied using the Trinidadian guppy-*Gyrodactylus* system (reviewed by Cable 2011). *Gyrodactylus turnbulli*, a common guppy ectoparasite, exhibits a viviparous reproductive strategy (Cable and Harris 2002), often resulting in explosive population growth, which can

significantly impede host survival (e.g. Cable and van Oosterhout 2007a). As gyrodactylid embryonic development is temperature dependent (reviewed by Bakke et al. 2007), natural variations in water temperature can determine parasite population growth.

Whilst guppies exhibit broad temperature tolerance (Reeve et al. 2014), small changes in water temperature can dramatically modify gyrodactylid life history traits (Bakke et al. 2007), and temperatures exceeding >30 $^{\circ}$C impede *G. turnbulli* survival (Scott and Nokes 1984). The present study investigates the thermal preferences of guppies in female-only and mixed-sex shoals, when uninfected and infected with the ectoparasite *G. turnbulli*. It is hypothesised that guppies infected with *G. turnbulli* will frequent warmer water, in comparison to when they are uninfected, and exposure to extreme thermal conditions has benefits in terms of self-medication against parasites.

2.3   Materials and Methods

2.3.1   *Host and parasite origin*

Guppies from the Lower Aripo (LA) River, Trinidad, were collected in 2010 and stock populations housed in breeding tanks at 24±0.5 °C at Exeter University. Fish were transferred to Cardiff University in April 2012 where they were maintained in 120 L aquaria at 24±0.5 °C and fed daily on Aquarian® tropical fish flakes and occasionally frozen bloodworm.

The *Gt3* strain of the parasite *Gyrodactylus turnbulli* was isolated from ornamental pet shop guppies in 1997, and was maintained on small numbers of fish (4-6 individuals) in laboratory cultures at 24 $^{\circ}$C on a 12 h light: 12 h dark lighting regime. To prevent parasite extinction, each culture pot was subsidised with naïve fish twice weekly. Each

pot contained a minimum of 4 culture fish collectively infected with approximately 40 gyrodactylid worms. To quantify mean parasite abundance (the total number of worms / the number of hosts including zero counts), guppies were anaesthetised with buffered 0.02% tricaine methanesulfonate (MS222) and screened using a dissecting microscope with fibre optic illumination. For experimental infections, a heavily infected donor fish from the parasite culture was sacrificed and 2-6 worms transferred through direct contact onto the caudal fin of an anesthetised recipient fish, as observed using a dissecting microscope. To remove parasites, fish were chemically treated using 0.1% dilution of Levamisole (Norbrook, UK), and subsequently screened weekly over three consecutive weeks to ensure that infection had been eliminated (see Schelkle et al. 2009).

2.3.2   Choice chamber set-up

The experimental arena consisted of three plastic aquaria (30 x 20 x 20 cm) connected by two plastic tunnels (10 cm length x 4 cm diameter; Fig. 2.1). All tanks were filled with dechlorinated water to a depth of 15 cm. The apparatus was surrounded by black paper on 5 sides to reduce disturbance by external stimuli, with one side left open to allow observations. The experiment was conducted in a temperature-controlled room (15 ± 0.5 ^{o}C), under a 12 h light: 12 h dark lighting regime. Across the arena, a temperature gradient was established using heating mats under the central chamber (Chamber B), and an aquarium heater in one side chamber (Chamber C). Chambers A and B also contained small aquarium heaters that were not switched on to ensure uniform conditions within each chamber. Chamber A was maintained at 18 ± 0.5 °C, Chamber B at 24 ± 1 °C and Chamber C at 32 ± 0.5 °C. These temperatures were selected based on the thermal range to which guppies are exposed in the wild (Reeve et al. 2014). Each chamber was uniformly aerated to prevent a thermocline developing within the tank.
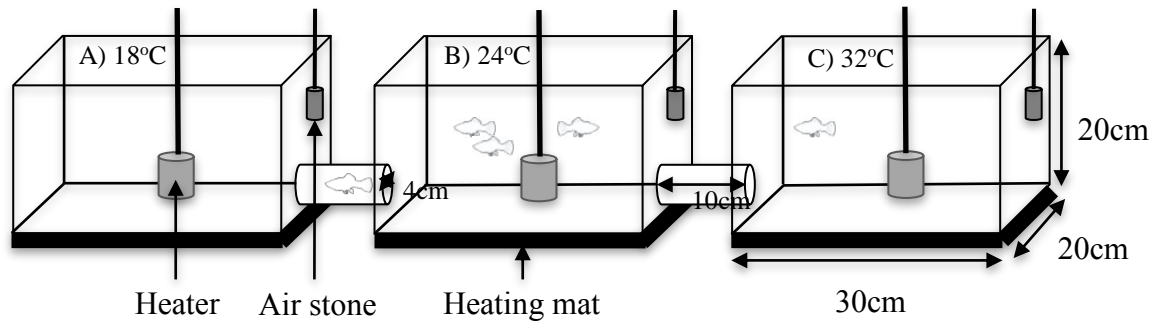
FIGURE 2.1:   The experimental arena consisted of three aquaria interconnected by two plastic tubes (10 cm length x 4 cm dia.). The terminal and back walls of the arena were surrounded by black paper to reduce disturbance to the fish, with one side left open for observations. Tanks were filled with 15 cm depth dechlorinated water. Placing air stones, aquarium heaters and heating mats in and under each chamber established a thermal gradient across the arena, and a consistent temperature within each tank. Chamber A was maintained at 18 °C, Chamber B at 24 °C and Chamber C at 32 °C, each ±0.5 °C. Fish were always introduced and returned to Chamber B during an experimental trial.

2.3.3   Experimental procedure

Female-only (5 females) or mixed-sex (3 females: 2 males) shoals (n = 14 per shoal type) were placed in a 30 x 15 x 15 cm aquaria to familiarise for 7 days prior to an experimental trial (according to Richards et al. 2010). Females typically aggregate in small shoals, between which males frequently move between (Griffiths and Magurran 1998), hence the rationale for using female-only and mixed-sex shoals with natural sex ratios in the current study. Using a crossover experimental design, shoals (6 female-only and 6 mixed-sex) were infected at the start of the trial and monitored for 2 days. On Day 3, these fish were artificially cleared of parasites and observed for a further 2 days. The remaining shoals (8 female-only and 8 mixed-sex) started trials with uninfected individuals and were subsequently infected on Day 3. Thus, each shoal served as its own infected/uninfected control, whilst the crossover design controlled for the potential effect of time by alternating the infection point. Experimental infections were conducted

by housing each shoal with a heavily infected donor fish (>200 *Gt3* worms) for 2 days in an infection tank (20 x 10 x 10 cm). Fish were then anaesthetised, and their gyrodactylid intensities recorded. To achieve a moderate infection (mean gyrodactylid intensity 22.5; range 13-34) per fish, additional worms were manually transferred to the caudal fin of some hosts from an infected donor fish (as previously described). Control fish were sham infected, whereby they endured the same handling and anaesthetic time, but were not exposed to parasites.

During a trial, either a female-only or mixed-sex shoal was introduced to Chamber B of the arena. Profiles were created for each fish within a shoal documenting unique body colouration and markings enabling individuals to be distinguished. Over two consecutive days, each individual was observed five times per day with 2 h intervals in between. During a focal follow, the chamber (A, B or C) of each fish was recorded every 10 s for one minute, accumulating 10 min of observational data per fish. Individuals were then removed from the arena being scooped up in a plastic 10 x 10 cm container to prevent parasite dislodgement, anaesthetised using 0.02% MS222 and screened for *G. turnbulli* using a dissection microscope with fibre optic illumination. Fish were then either experimentally infected, or their parasites chemically removed, thereby reversing their infection status. Following chemical treatment, individuals were screened to ensure no parasites remained on the host. At the end of a trial, these fish were screened twice more to ensure they were not infected, confirming they were parasite-free during their final observation days. Uninfected fish were also exposed to levamisole and anaesthetic to account for their potentially confounding effects on fish behaviour. Fish monitoring was then resumed for a further two consecutive days. For these trials, fish were again introduced into Chamber B of the experimental arena and allowed to habituate for 2 h. This design allowed us to compare fish behaviour when the

host was infected vs. uninfected, whilst also testing whether prior infection status influenced temperature preference.

2.3.4   *Gyrodactylus turnbulli temperature tolerance*

Parasite naïve sexually mature experimental fish (>3 months old) were acclimated and maintained at water temperatures of 18, 24 or 32 $^{\circ}$C (n=20 per treatment). This entailed increasing or decreasing daily water temperature by 1 $^{\circ}$C for 8 or 6 days to reach 32 $^{\circ}$C or 18 $^{\circ}$C respectively (n = 20 for each treatment). These fish were then maintained for a further 14 days under these conditions before being experimentally infected with two *Gt3* worms on their caudal fin (according to the above protocol). Fish were individually housed in 1L containers and screened daily over a 7-day period to record *Gyrodactylus* infection trajectories.

2.3.5   *Statistical analysis*

Analyses were conducted using R statistical software (version 3.1.3, R Development Core Team 2009). Statistical models were refined by deleting non-significant terms from the starting model, based on Analysis of Variance (Crawley 2007). Model robustness was assessed using residual plots (after Pinheiro and Bates 2000). A Generalised Linear Mixed Model (GLMM) was used to investigate whether the mean temperature preference of fish was significantly different before and after infection. Temperature preference was the dependant term in the model and fixed effects included infection status (infected or uninfected), shoal type (female-only or mixed-sex), infection regime (fish infected at the beginning or second half of a trial) and standard length (mm). Fish ID was nested within shoal number and included as a random factor within the model. A negative binomial Generalised Linear Mixed Effects Model (GLMM; "glmmADMB" statistical package) was used to investigate the effects of

temperature, host standard length (mm), the day of infection (i.e. how many days a fish had been infected prior to a particular screen day), on gyrodactylid trajectories over the 7-day infection period. An interaction between temperature and the day of infection was also incorporated into the model, with Fish ID included as a random term.
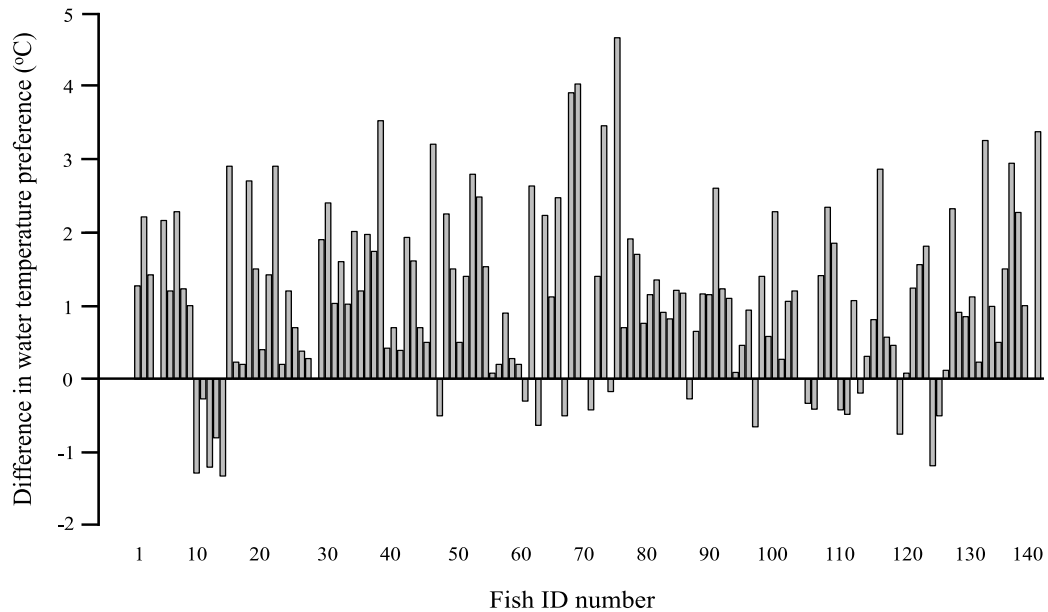
FIGURE 2.2: The mean temperature preference of individual fish when infected with *Gyrodactylus turnbulli*, minus their mean temperature preference when uninfected. All the positive bars indicate individual fish that moved to warmer waters following infection; only the negative bars indicate individuals that moved to cooler waters when infected.

2.4 Results

2.4.1 Fish thermal preference

Infection status had a significant effect on the mean temperature preference of fish (GLMM: $LRT_{1, 137} = 819.97$, $P<0.0001$), which was significantly higher when infected (mean = +0.97 °C) than when uninfected (Estimate = -1.08, SE = 0.10, $P <0.0001$; Fig. 2.2). Shoal type, standard length and infection regime did not influence temperature preference ($P >0.05$ for all variables).

2.4.2   Gyrodactylus turnbulli temperature tolerance

Mean parasite abundance significantly increased over the 7 day infection period (GLMM: LRT = 638.70, *df* = 6, *P* <0.001), however, this was dependant on temperature (LRT= 75.76, *df* = 2, *P* <0.001). Parasite population increase was higher at 24 °C, compared to 18 °C (Estimate = -0.95, SE = 0.22, *P* < 0.0001) and 32 °C (Estimate = -3.01, SE = 0.29, *P* < 0.0001) (Fig. 2.3).
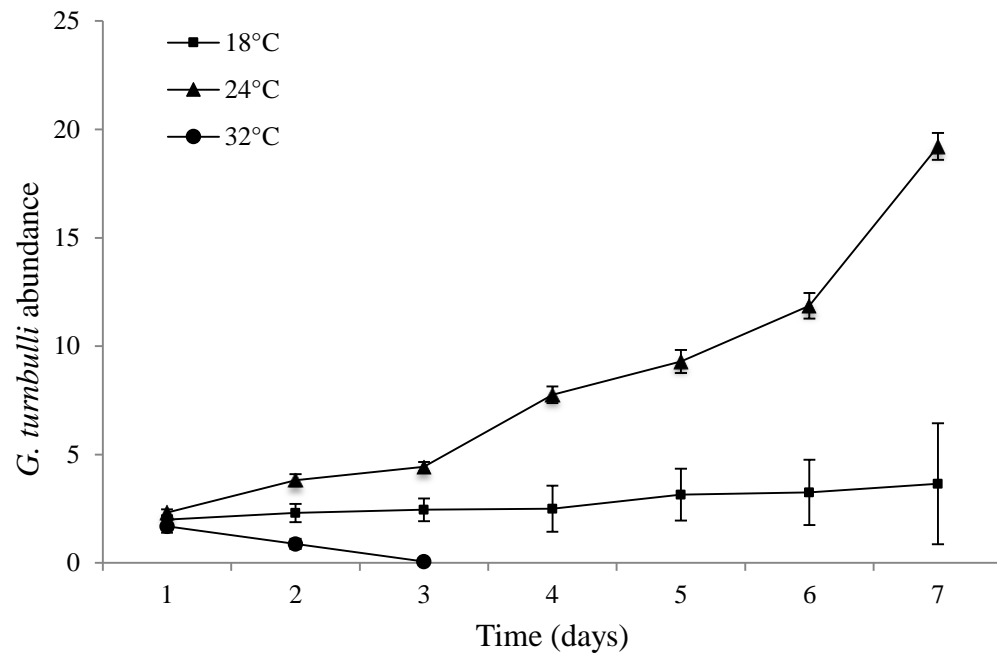
FIGURE 2.3: Mean *Gyrodactylus turnbulli* abundance (±SE) on guppies experimentally infected with two parasites on Day 0, and maintained at three different temperatures (18, 24 and 32 °C) for a 7-day duration.

2.5   Discussion

This study demonstrates, for the first time, that gyrodactylid infected fish have a preference for warmer waters: exploiting the upper thermal tolerances of their parasites to self-medicate against infection. In addition, the guppy immune response may be upregulated by the increase in temperature, which is consistent with the elevation of lysozyme and immunoglobulin M levels observed in other fish (Bowden et al. 2007). This study also confirms the findings of Scott and Nokes (1984), that population growth of *Gyrodactylus turnbulli* is significantly impacted by temperature.

When exposed to a constant temperature, *G. turnbulli* infecting guppies at 32 ℃ declined to extinction within 3 days. Parasite mean abundance increased on fish maintained at 18 and 24 ℃, however population growth was less pronounced at the 18 ℃ treatment. Despite *G. turnbulli* population growth being reduced at cooler temperatures, fish residing within such conditions may compromise their metabolic and immunological benefits of warmth. This may explain why when given a choice of three temperatures (18, 24 and 32 ℃), guppies' frequented the 32 ℃ chamber more often compared to when uninfected; indicated by a significant increase in mean temperature preference. Three-spined sticklebacks infected with *Schistocephalus solidus* also exhibit a preference for warmer water in comparison to uninfected conspecifics. However, unlike our guppy-gyrodactylid system, this observed thermal shift promotes parasite growth, fecundity and ultimately transmission (Macnab and Barber 2011). The mechanisms involved in this seemingly maladaptive behavioural response are complex and in the stickleback system could be affected by both direct and indirect host behavioural manipulation by the parasite (Barber et al. 2004; Scharsack et al. 2007). Although gyrodactylids do cause behavioural changes in their hosts, these are almost certainly by-products of infection rather than parasitic host manipulation (e.g. Kolluru et al. 2009). Therefore, a significant increase in mean temperature preference is likely an adaptive host response. It is speculated that this behavioural change directly imposes thermal stress on the parasite to increase mortality, as observed when parasites were maintained at a constant 32 ℃, and/or up-regulates the host's immune system to counteract gyrodactylid infection.

Guppies exhibit innate and acquired resistance to *Gyrodactylus* species, however, little is known about the precise mechanisms involved in guppy immunocompetence (Cable and van Oosterhout 2007b). Although the innate immune response of guppies is

probably activated at the onset of *G. turnbulli* infection (Scott 1985; van Oosterhout et al. 2008), parasite population declines are most apparent 7-11 days post-infection at 25 °C. This is presumably associated with the induction of acquired immunity. Our results show that *G. turnbulli* infection did not persist for longer than three days on any hosts at 32 °C. The failure of the parasite population at this temperature, particularly in such a short time, indicates that thermal stress, as oppose to the host immune defence, may be the predominant factor compromising parasite survival by impeding physiological function. The parasites used in the current study, however, were not acclimatised to the lower and upper temperature treatments prior to the experiment. Short generation times may facilitate rapid evolution of a wider thermal tolerance within gyrodactylids, although there is no empirical evidence to support this. Due to their small sizes and faster metabolic rates, parasites could acclimate faster than their hosts to thermal shifts, but only if physiological performance is improved at the acclimated temperature (Paull et al. 2015).

Guppies are native to Trinidad and Tobago where they typically reside in warm water ranging between 18-32 °C (Kent and Ojanguren 2015). They have a remarkable capacity for thermal adaptation with populations successfully establishing in environments with very different thermal regimes to their native habitats (Deacon et al. 2011). Water temperatures within freshwater streams can fluctuate ~10 °C daily (Reeve et al. 2014), and exposure to these temperature heterogeneities often result in marked behavioural changes. Juvenile guppies, for example, increase average swimming speed and depth when exposed to elevated temperatures (Kent and Ojanguren 2015). Female guppies preferentially associate with larger, more cohesive shoals at high (26 °C) compared to low (22 °C) water temperatures, particularly in the presence of cichlid predators (Weetman et al. 1998; Weetman et al. 1999). Although associating with larger

shoals may promote gyrodactylid transmission (Richards et al. 2010), by exploiting warmer thermal conditions, fish may self-medicate against parasite infection, particularly monogenean ectoparasites as shown here.

To summarise, the guppy-*G. turnbulli* model is used to highlight how elevated temperatures can significantly impact host-parasite interactions within freshwater environments. *G. turnbulli* mean abundance increased at 18 and 28 °C, whilst thermal extremes of 32 °C caused population extinction. Additionally, it is shown how temperature selection by fish is influenced by parasite infection, with infected individuals frequenting warmer water more often than if uninfected. It is speculated that this adaptive host behavioural response inhibits physiological functioning of gyrodactylid worms. This information helps understand how existing natural variation in water temperature, at a local scale, influences disease outbreaks. In the future, such data could be used to model how climate-driven population responses alter disease epidemics in wild and managed fish stocks, within both tropical and temperate regions. Temperate species in particular face additional challenges associated with elevated temperature, including oxygen depletion within warmer water that subsequently impedes gill respiratory processes. Whether or not temperate species will tolerate thermal conditions outside their own temperature optima in order to self-medicate against parasite infection remains unknown.

2.6 Acknowledgements

Chapter 3

Assessing the effects of flow rate on parasite transmission amongst a social host[3]

3.1 Abstract

Freshwater habitats have endured intensive anthropogenic modifications that have substantially altered natural flow conditions. Consequently, freshwater fauna are increasingly exposed to variable flow conditions, altering their behaviour and subsequently disease transmission amongst social hosts. This study investigates the effects of continual, interrupted or no-flow conditions on the shoaling behaviour of Trinidadian guppies (*Poecilia reticulata*), and the resulting transmission of a directly transmitted ectoparasite, *Gyrodactylus turnbulli*. Shoals exposed to continuous flow exhibited significantly greater *G. turnbulli* peak intensities than those in interrupted or no-flow conditions. Parasite transmission rate was greater in shoals exposed to interrupted flow, resulting in parasites becoming more distributed amongst shoal members and thus reducing mean intensity in comparison to continuous flow shoals. Furthermore, as parasite prevalence increased from an initial infected host, the distances between shoaling conspecifics increased at greater rates in interrupted and no-flow conditions compared to continuous flow; indicating that in the absence of flowing water parasitism may have a greater effect on shoaling behaviour. This data highlights how fish behaviourally respond to variable flow conditions and the implications for disease transmission.

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[3] This chapter has been submitted for publication: ***Reynolds, M**., Hockley, F., Wilson C. A. M. E., and Cable, J. Assessing the effects of flow rate on parasite transmission amongst a social host.*

3.2   Introduction

Anthropogenically-driven climate change is a major threat to ecosystems worldwide (Marcos-López et al. 2010), and freshwater habitats are particularly vulnerable to these changes as water availability is directly mediated by climate (Heathwaite 2010). Additionally, anthropogenic pressure including water abstraction for human consumption, agriculture and energy generation (Ormerod 2009) promotes habitat degradation and fragmentation, imposing dispersal constraints on organisms residing within changing environments (Woodward et al. 2010). The combination of anthropogenic and climatic stressors will increase the incidence of extreme climatic events such as flooding and drought within freshwater habitats (Graham and Harrod 2009). Consequently, freshwater fauna will be exposed to highly variable flow conditions, which will have direct and indirect effects on fish, particularly with respect to behaviour (reviewed in Liao 2007).

Shoaling behaviour in fish primarily enhances predator detection and avoidance (Magurran 1990), but also increases foraging efficiency, mating opportunities and energy conservation during locomotion (Pitcher and Parish 1993; Marras et al. 2015). Shoaling can, however, promote disease transmission between individuals (Barber et al. 2000). The speed of this transmission is determined by the transmission coefficient 'β', which encompasses two fundamental processes: the contact rates between infected and uninfected conspecifics, and whether such contact leads to successful transmission (Antolin 2008; McCallum et al. 2017). Historically, population-based modeling categorised transmission as either density- or frequency dependent, however, it is now recognised that both transmission modes simultaneously promote disease persistence within populations (Ryder et al. 2007; Ferrari et al. 2011; Hu et al. 2013; McCallum et al. 2017). For social species, including shoaling fish, parasite transmission between

individuals may not only be affected by population density, but the frequency of contacts between infected and susceptible hosts, allowing parasite persistence even at low host densities (Johnson et al. 2011).

Considering aquatic habitats significantly influence disease ecology by, for example, facilitating water-borne transmission amongst hosts (Cable et al. 2017), an understanding of how fish pathogen transmission is affected by changing environmental conditions is particularly important in the wild, aquaculture and aquarium trade. Whilst the effects of temperature on disease risk are well documented (reviewed by Graham and Harrod 2009; Karvonen et al. 2010), the effects associated with flow modification remain largely overlooked. Of the few studies that have investigated the relationship between flow condition and parasite transmission, the trend has been towards a higher prevalence and infection intensity within populations inhabiting reduced flows (Barker and Cone 2000; Bodensteiner et al. 2000; Hallett and Bartholomew 2008). However, it is difficult to disentangle whether this is because of poor quality habitat (e.g. high turbidity or hypoxia) leading to elevated host stress and therefore a greater susceptibility to disease (Leniham et al. 1999), or if a host's energy resource is debilitated by infection therefore prompting them to frequent energetically favourable regions including shallower, slow flowing water.

Alternatively, fish can conserve energy by swimming in a conspecifics wake: a substantial benefit associated with shoaling behaviour. Marras et al. (2015), showed how the energy expenditure of grey mullet swimming alone was significantly greater than those in a shoal, with individuals that trailed conspecifics conserving the most energy. Frequenting reduced flow velocity regions may be particularly beneficial when shoals are exposed to variable flow conditions, yet shoaling tendencies have been observed to diminish in higher flow rates (Garner 1997; Sneddon et al. 2006;

Suriyampola et al. 2017). Contrastingly, Hockley et al. (2014) showed that in no-flow conditions the shoaling tendencies of *Gyrodactylus turnbulli* infected Trinidadian guppies (*Poecilia reticulata*) were significantly reduced compared to those experiencing flowing water, indicating that parasitism may have a greater influence than flow in determining shoaling preferences.

Gyrodactylids are ubiquitous teleost ectoparasites, of which over 400 species have been described from both tropical and temperature hosts (Bakke et al. 2007). In particular, the tropical Trinidadian guppy-*Gyrodactylus turnbulli* system has been utilised extensively to investigate social behaviour (e.g. Edenbrow et al. 2011), evolutionary ecology (O'Steen et al. 2002), and epidemiology (Stephenson et al. 2015; Smallbone et al. 2016). The parasite's direct lifecycle, short generation time (*ca*. 24 h at 25 °C; Scott and Nokes 1984) and extreme progenesis often results in exponential population growth, making its reproductive strategy more akin to a micro- rather than a macroparasite (Bakke et al. 2007). In practical terms, parasite intensities and transmission can be accurately monitored *in situ* at repeated time points from the same individual, without sacrificing a host. This host-parasite system is therefore an ideal model for the current study, which aims to assess, over a prolonged period, how *G. turnbulli* transmission dynamics and the shoaling behaviour of Trinidadian guppies are influenced by continuous, interrupted and no-flow conditions.

3.3   Materials and Methods

3.3.1   *Host and parasite origin*

Experimental Trinidadian guppies (*Poecilia reticulata*) were laboratory-reared descendants of a wild fish stock caught from the Lower Aripo River, Trinidad, in 2012. Fish were initially maintained at Exeter University, and transferred to Cardiff University in 2014. Female guppies (>3 months) were size matched (± 3.8 mm) into

groups of five individuals, representative of a natural shoal size. Each group was housed in 6 L aquaria to familiarise and form shoals for a minimum of 14 days (see Griffiths and Magurran 1997a). Shoals were maintained under a 12 h light: 12 h dark photoperiod at 22-24 $^{\circ}$C and fed daily on Aquarian® Tropical fish flakes supplemented weekly with live *Daphnia magna* and *Artemia*. A highly inbred strain of *Gyrodactylus turnbulli* (*Gt3*), isolated from ornamental guppies in 1997, was used for experimental infections.

3.3.2 Flume set-up

Transmission experiments were conducted in two identical open channel re-circulatory flumes, each measuring 150 cm length x 16 cm depth x 20 cm width (Fig. 3.1). Flumes were filled with dechlorinated water to a depth of 15 cm and maintained at 22-24 °C. A 10 cm diameter impeller was attached to a 1hp 3-phase 4-pole motor with a maximum shaft speed of 1500 rpm (Machine Mart), wired to a 1.1 kW inverter (RS Components) to control motor speed. At either end of the flume was a 2 cm thick aluminum honeycomb flow straightener with 6.4 mm cell diameter used to remove the secondary flow currents produced from the bends in the recirculatory pipe system. Flow straighteners also prevented fish from entering the impeller region therefore restricting shoals to a 100 cm length section (Test Arena; Fig. 3.1). The flow depth was fixed and maintained at 15 cm throughout all tests. Three flow conditions were chosen to investigate how parasite transmission and shoaling behaviour are affected by flow. A 'static' condition (no-flow condition) was used as a control with the impeller switched off. Secondly, a continuous 'flow' condition with a depth-averaged velocity of 6 cm s^{-1} (measured using a Nixon miniature propeller flow meter), which evoked rheotaxis without exhausting the fish over a trial duration. This corresponds to a flowrate of 1.9 L s-1 akin to that recorded within guppy habitats in Trinidad (Kodric-Brown and Nicoletto 2005). Finally, a 12 h flow: 12 h static 'interrupted flow' condition (depth-averaged flow velocity 6 cm s^{-1}), was used to represent the time guppies frequent

shallow refuge where flow is minimal to rest, particularly at night, in habitats with high predation regimes (Seghers 1974; Croft et al. 2003). The flow was synchronised with the rooms' photoperiod so that fish were only exposed to flow during daylight hours.
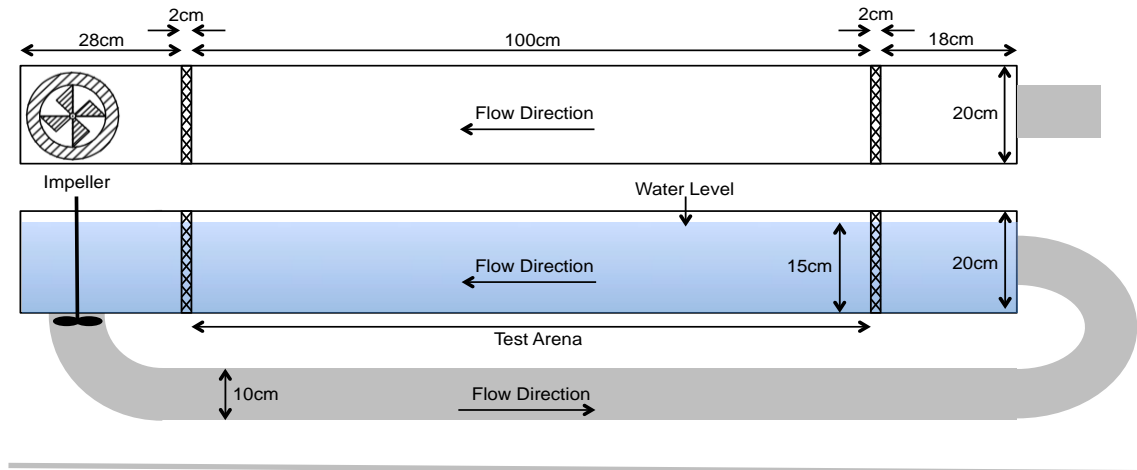
FIGURE 3.1: 2D schematic of the open channel re-circulatory flume used to assess *Gyrodactylus turnbulli* transmission and shoaling behaviour in Trinidadian guppy shoals for three flow conditions. Top: plan view, and bottom: side view. Fish were retained within the 100 cm long test arena using 2 cm thick aluminum honeycomb flow straighteners (6.4 mm cell diameter). Flow depth was maintained at 15 cm.

3.3.3   Preliminary trial of parasite mobility

Preliminary trials assessed whether the recirculartory nature of the flumes facilitated transmission of dislodged *G. turnbulli* worms. Familiarised shoals of five female guppies were placed into either a continual (n=3 shoals), interrupted (n=3 shoals) or no-flow (n=3 shoals) condition for a 24 h acclimation period, prior *G. turnbulli* exposure. Following acclimation, a heavily infected donor fish was sacrificed and placed in a dark cupboard for *ca.* 30 min, whereby such conditions encouraged worms to detach from the deceased host and attach to the water film surface (as in Cable et al. 2002). A sterile pipette was then used to transfer 30 individual worms to a watch glass containing 2 mm depth dechlorinated water, which was subsequently placed in the 18 cm terminal end of the flume (Fig. 3.1). After 1 h, the watchglass was removed and inspected under a

dissection microscope to ensure worms had moved into the water column of the flume. The fin and skins of fish were screened under 0.02% tricaine methanesulfonate (MS222) sedation 24, 48 and 72 h post *G. turnbulli* introduction to confirm if any worms had successfully infected a host. If no worms had attached to a host post 72 h (beyond *in vitro G. turnbulli* survival time; Schelkle et al. 2013), worms were presumed deceased and the trial terminated. No 'dislodged' worms reinfected hosts in any treatment, confirming that any parasite transmission observed during subsequent experimental trials would be as a result of host behaviour, and not the experimental setup.

3.3.4 *Experimental procedure*

Each shoal of five female guppies (n=17 shoals) was placed into a flume for a 24 h habituation period prior to a 7-day trial. A total of 6 shoals were trialled in the no-flow condition, 5 in continuous flow and 6 in the interrupted flow condition. On Day 0, guppies were removed from the flume and one randomly selected fish was infected with *ca.* 30 *G. turnbulli* worms (range 28-34 worms; no significant difference in starting intensity between treatments: ANOVA: $F_{2\ 16} = 1.82$, $P = 0.194$), by anaesthetising the fish with 0.02% MS222 and placing a heavily infected 'donor' fish within close proximity to transfer worms via direct contact. Parasite transfer was observed continuously using a dissecting microscope with fibre optic illumination. The remaining 4 fish were sham infected by anaesthetising and manipulating under the microscope without exposure to parasites. Each fish was measured according to its standard length ('SL'; mm) and profiled by making a detailed diagram of unique body markings enabling identification of each individual at repeated time points to monitor parasite transmission over the 7-day trial. Fish were placed in 1 L dechlorinated water for an hour period, whilst remaining in visual contact to one another, and fed 3 individuals of

Daphnia magna before being returned to the flume. Infection was confirmed the following day (Day 1), and each fish screened every other day thereafter (Days 3, 5 and 7). For each screening, all fish were removed from the flume for a maximum of 30 min, and individually isolated in 1 L dechlorinated water whilst remaining in visual contact. Each fish was anaesthetised and screened to quantify prevalence (the percentage of infected individuals within the shoal) and mean intensity (the mean number of parasites found on infected hosts). Transmission rate was calculated as the number of new host infections per day.

Observations of shoaling behaviour, including the number of fish shoaling, maximum shoal size, shoal size and nearest neighbour distance (a standard measure of shoal cohesiveness), were measured at one-minute increments during a 5 min test period, every day at 9:00 by an observer. This time was selected to coincide with peak guppy activity. Fish were recorded as shoaling if they were within four body lengths of one another (Pitcher et al. 1983), and the number of fish in the largest shoal defined the maximum shoal size. A 2 cm^2 grid adhered to the side and base of the flume was used to visually estimate the distance between neighbouring fish whilst shoaling.

3.3.5   Statistical analysis

Analyses were conducted using R statistical software (version 3.1.3, R Development Core Team 2009). Using the *lme4* library (Bates et al. 2015), Generalised Linear Models (GLMs) were used to investigate the effect of flow condition and fish size on *Gyrodactylus turnbulli* transmission dynamics. Dependent terms in each model included (1) *G. turnbulli* mean transmission rate, (2) *G. turnbulli* peak prevalence, (3) Time to reach peak prevalence, (4) *G. turnbulli* peak intensity, and (5) Time to reach peak

intensity. Fixed effects in these models included flow condition, shoal mean SL, and *G. turnbulli* start intensity. Models were fitted with Gaussian error families with identity or inverse link functions. Model structures were selected based on the lowest residual deviance and AIC values.

Generalised Linear Mixed-Models were constructed to assess the influence of parasitism, fish size and flow condition on the shoaling behaviour of Trinidadian guppies. Dependent terms within these 'global' models included (6) the number of fish shoaling, and (7) the mean nearest neighbour distances between shoaling conspecifics. The independent terms included flow condition (no-flow, interrupted or continual flow), *G. turnbulli* mean intensity and prevalence (as defined by Busch et al. 1997), host mean SL and *G. turnbulli* start intensity. Interaction terms between flow x parasite mean intensity, flow x prevalence and flow x day were also incorporated into the models. To account for repeated measures, shoal number was included as a random term. Models were fitted with Gaussian error structure and log link functions, and model robustness assessed using residual plots.

During model refinement, several equally well-supported models were identified based on comparisons of Akaike's Information Criterion (AIC). Thus, an information theoretic approach to multi-model inference was employed to assess the relative importance of each independent term in influencing dependent variables within the global GLMMs (following methods in Burnham and Anderson 2002). As variables were measured on different scales, model parameters within each global model (containing all independent, dependent and random terms) were standardised to a mean of 0 and standard deviation 0.5 using the *arm* library (Grueber et al. 2011; Gelman and Su 2013). The 'dredge' function within the *MuMIn* package (Bartoń 2014) was then

used to generate a set of 'top' models, which fell within 2.5 AICc of the best model. Averaged parameter estimates from this top set of models were then calculated using the 'model.avg' function, and the relative importance of each parameter generated by summing the Akaike weights across the models in which the parameter occurred (Burnham and Anderson 2002).

3.4 Results

Shoals exposed to interrupted flow exhibited significantly greater *Gyrodactylus turnbulli* mean transmission rates compared to those in continuous and no-flow conditions (Table 3.1, Model 1; Fig. 3.2a). Over time, prevalence increased in all treatments, and a greater mean transmission rate in interrupted conditions resulted in a significantly higher mean peak prevalence of 100% being reached, compared to 92% and 83% in continuous and no-flow conditions, respectively (Table 3.1, Model 2).
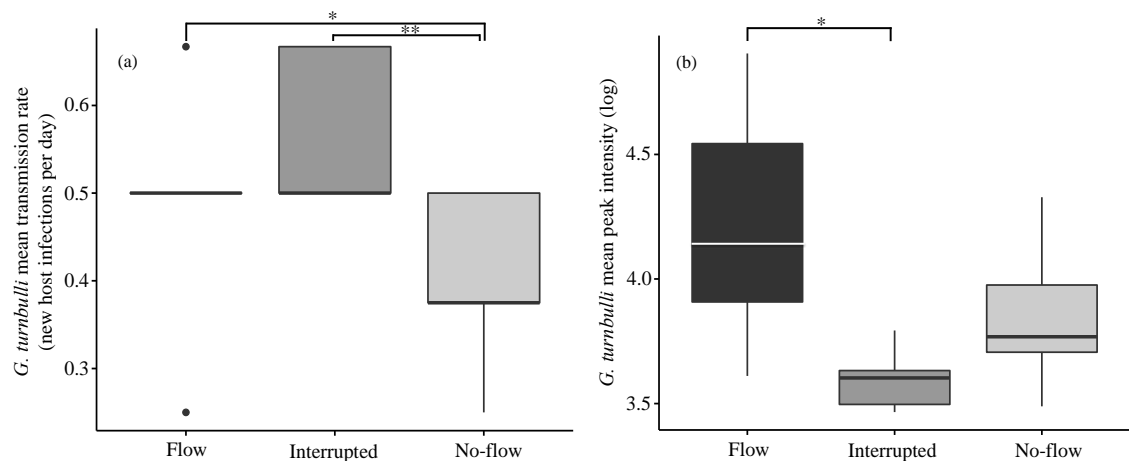
FIGURE 3.2: Mean *Gyrodactylus turnbulli* transmission rate (a), and mean *G. turnbulli* peak intensity (b) within shoals exposed to continuous flow (black), interrupted (dark grey), and no-flow (light grey) conditions. Black dots represent outliers; bars the upper and lower limits; the box the first and third quartile with the median. Pairwise comparisons are denoted by a solid black line between treatments, and the level of significance by an asterisk as follows: * $P < 0.05$; ** $P < 0.005$.

TABLE 3.1: Results output from General Linear Models investigating the effects of flow condition, fish size and *Gyrodactylus turnbulli* starting intensity on *G. turnbulli* transmission dynamics in Trinidadian guppy shoals. The level of significance is denoted by an asterisk as follows: * *P* <0.05; ** *P* <0.005; ***P<0.001.

Model	Dependent Variable	Predictor	Estimate	SE	T value	*P* value
1	Mean Transmission Rate	Intercept Flow condition:	1.053	0.516	2.041	0.064
		Flow	0.176	0.080	2.187	0.049*
		Interrupted	0.241	0.068	3.549	0.004**
		Mean SL	-0.010	0.009	-1.059	0.310
		Start Intensity	-0.018	0.016	-1.105	0.291
2	Peak Prevalence	Intercept Flow condition:	188.531	46.631	4.043	0.002
		Flow	17.945	7.255	2.473	0.004**
		Interrupted	22.159	6.132	3.614	0.029*
		Mean SL	-0.785	0.811	-0.968	0.352
		Start Intensity	-3.186	1.475	-2.160	0.052
3	Time to Peak Prevalence	Intercept Flow condition:	6.861	4.861	1.411	0.184
		Interrupted	-0.630	0.639	-0.985	0.344
		Flow	-0.112	0.756	-0.148	0.885
		Mean SL	0.036	0.085	0.431	0.674
		Start Intensity	-0.027	0.154	-0.177	0.863
4	Peak Intensity	Intercept Flow condition:	0.093	0.012	7.673	<0.001
		Interrupted	0.008	0.004	2.290	0.041*
		Flow	-0.002	0.003	-0.771	0.456
		Mean SL	-0.001	0.0002	-2.429	0.032*
		Start Intensity	-0.002	0.0004	-5.123	<0.001***
5	Time to Peak Intensity	Intercept Flow condition:	14.942	10.695	1.397	0.188
		Interrupted	-2.425	1.406	-1.724	0.110
		Flow	-0.488	1.664	-0.293	0.775
		Mean SL	-0.358	0.186	-1.928	0.078
		Start Intensity	-0.095	0.338	-0.281	0.783

Significantly higher *G. turnbulli* mean peak intensities were reached in continuous flow compared to interrupted, but not no-flow conditions (Table 3.1, Model 4; Fig. 3.2b). Shoals that had a higher *G. turnbulli* start intensity also reached higher peak intensities (Table 3.1, Model 4). Additionally, there was a positive correlation between the mean SL of a shoal and *G. turnbulli* mean peak intensity (Table 3.1, Model 4). There was no effect of flow condition, mean standard length of the shoal or *G. turnbulli* start intensity

on the time taken to reach *G. turnbulli* peak prevalence or intensity (Table 3.1, Models 3 and 5, respectively).
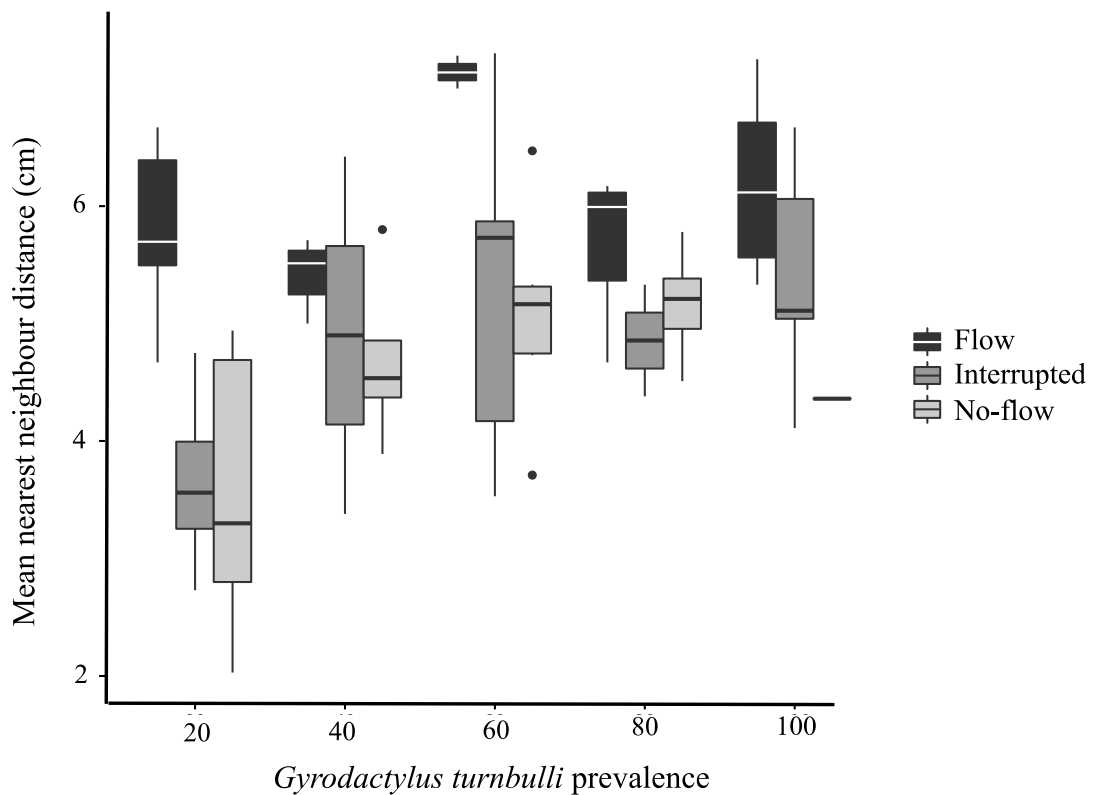
FIGURE 3.3: The mean nearest neighbour distances between shoaling conspecifics in relation to *Gyrodactylus turnbulli* prevalence in continuous (black, n=5), interrupted (dark grey, n=6), and no-flow (light grey, n=6) conditions. Black dots represent outliers; bars the upper and lower limits; the box the first and third quartile with median.

Infection with *Gyrodactylus turnbulli* had a significant effect on the shoaling behaviour of Trinidadian guppies. As parasite prevalence and intensity increased, the number of fish shoaling decreased: significantly more so within flowing, compared to interrupted or no-flow conditions (Table 3.2, Model 6). As parasite prevalence increased, the distances between shoaling conspecifics increased at greater rates in interrupted and no-flow treatments compared to continuous flow conditions (Fig. 3.3); indicating that in the absence of flowing water, parasitism may have a greater effect on shoaling behaviour (Prevalence x Flow interaction: Table 3.2, Model 7).

TABLE 3.2: Standardised averaged model predictors explaining variation in shoaling behaviour of *Gyrodactylus turnbulli* infected Trinidadian guppies within continuous, interrupted and no-flow conditions. Highlighted rows show predictors that confidence intervals did not bound zero and are therefore considered significant. CI=confidence intervals, SE = standard error, SL=Standard length. * Approaching significance.

Model	Dependent Variable	Predictor	Estimate	Adjusted SE	95% CI 2.5%	97.5%	Predictor Importance
6	Number of fish shoaling	Intercept	1.189	0.067	1.058	1.320	
		Day	-0.206	0.101	-0.404	-0.008	0.48
		Flow Condition:					0.39
		Flow	-0.235	0.101	-0.437	-0.033	
		Interrupted	-0.022	0.087	-0.196	0.151	
		Mean Intensity	-0.103	0.051	-0.203	-0.003	0.47
		Mean SL	0.192	0.094	0.009	0.376	0.73
		Prevalence	-0.237	0.089	-0.411	-0.063	0.71
		Start Intensity	0.069	0.088	-0.104	0.241	0.14
7	Nearest Neighbour Distance	Intercept	0.414	0.033	0.348	0.480	
		Day	0.151	0.044	0.065	0.237	1.00
		Day x Flow condition:					0.17
		Day x Flow	-0.104	0.066	-0.234	0.026	
		Day x Interrupted	0.014	0.067	-0.118	0.145	
		Flow Condition:					1.00
		Flow	0.151	0.049	0.056	0.247	
		Interrupted	0.024	0.047	-0.069	0.118	
		Mean Intensity	0.001	0.034	-0.066	0.068	0.09
		Mean SL	0.012	0.040	-0.067	0.091	0.09
		Prevalence	0.020	0.092	-0.161	0.201	0.22
		Prevalence x Flow Condition:					0.12
		Prevalence x Flow	-0.131	0.079	-0.286	0.023*	
		Prevalence x Interrupted	0.012	0.078	-0.139	0.164	
		Start Intensity	-0.037	0.041	-0.118	0.044	0.13

3.5   Discussion

The current study investigated *Gyrodactylus turnbulli* transmission dynamics within a social host, the Trinidadian guppy, under prolonged exposure to continuous, interrupted and no-flow conditions. Mean *G. turnbulli* transmission rate was significantly higher in interrupted compared to continuous and no-flow conditions; resulting in a significantly higher prevalence being reached. Parasite peak intensity was significantly greater in

shoals exposed to continuous flow, and also depended on the starting *G. turnbulli* intensity on the experimentally infected donor fish on Day 1. Additionally, shoals with a larger mean body size reached higher peak parasite intensities.

A significantly greater *G. turnbulli* transmission rate was observed in interrupted flow conditions, resulting in higher parasite prevalence compared to continuous and no-flow conditions. As gyrodactylids reproduce *in situ* (Cable and Harris 2002), the transmission potential amongst hosts' increases as infrapopulation densities also increase (Bakke et al. 2007). Competition for resources and evasion of a host's immune response (see Rubio-Godoy et al. 2012) prompt parasite transmission to new hosts, resulting in the parasite population becoming more evenly distributed throughout a shoal; referred to as the encounter-dilution effect (Mooring and Hart 2002). Within interrupted flow conditions, there may be multiple transmission opportunities; firstly, through direct contacts whilst shoaling in flowing water, and secondly during a nocturnal 'rest' period when the impeller was switched off. Although guppies are presumed a diurnal species with shoaling tendencies ceasing as light intensity diminishes (O'Connor and Krause 2004), nocturnal behaviours, for example prolonged foraging activity, have been observed particularly in the absence of predation (see Fraser et al. 2004). Furthermore, *G. turnbulli* infected individuals exhibit nocturnal restlessness (Chapter 5); frequently encountering and initiating body contact with resting conspecifics during which parasite transmission may occur (Croft et al. 2011). Similarly, for shoals in no-flow conditions, transmission may predominantly occur via nocturnal activity of infected fish moving between uninfected conspecifics attempting to 'offload' their parasite burdens (Chapter 4).

Within continually flowing environments, where *G. turnbulli* mean peak intensity was

significantly greater than both interrupted and no-flow conditions, a greater transmission rate may have been expected. However, parasites infecting these fish were subject to continuous water flow over the hosts' body, and coupled with an increased tail beat frequency may have been more vulnerable to dislodgement. Scott and Anderson (1984) estimated that *ca.* 40% of *G. turnbulli* became dislodged whilst attempting transmission from donor to recipient fish in static water alone, which is likely to be significantly greater within flowing conditions. Following dislodgement, mortality is inevitable should a parasite fail to encounter a new host, as seen in our preliminary trials.

For fish enduring continual flow, compromised immunity within such energetically challenging environments may have resulted in significantly higher *G. turnbulli* peak intensities. Whilst guppies exhibit both innate and acquired immunity towards gyrodactylid infections (Scott 1985; Cable and van Oosterhout 2007b), the efficiency of these responses may become compromised as energy reserves are diverted to alternative fitness-related traits, including locomotion (as in Zamora-Camancho et al. 2014; Husak et al. 2016). Additionally, greater parasite intensities were reached in shoals of larger fish. Within the wild, larger fish generally harbour more parasites with maximum parasite loads increasing exponentially with host body size (see Cable and van Oosterhout 2007a). Larger hosts provide more space and resources for parasites, which in turn exhibit a reduced transmission propensity. Consequently, a smaller proportion of hosts are infected with the majority of parasites, as observed here.

As *G. turnbulli* prevalence and intensity increased, the number of fish shoaling decreased across all treatments; significantly more so within continuous flow conditions. Additionally, we confirm the findings of Richards et al. (2010) that the distances between shoaling guppies increased with parasite prevalence. Although

marginally non-significant, we show that shoals became less cohesive at a greater rate within interrupted and no-flow conditions, indicating that in the absence of flowing water, parasitism may have a greater effect on shoaling behaviour. A reduction in shoaling tendency associated with parasitism has been reported in several previous studies (Dugatkin et al. 1994; Krause and Godin 1996; Hockley et al. 2014), which is not surprising given it is beneficial for individuals to discriminate between infected and uninfected conspecifics, and respond accordingly (see Stephenson and Reynolds 2016). By remaining within a shoal, but increasing nearest neighbour distances, an individual can simultaneously experience the benefits of swimming in the reduced velocity region of a conspecific's wake (Marras et al. 2015), whilst also reducing infection risk. When the costs associated with *G. turnbulli* infection (reviewed in Bakke et al. 2007) outweigh the benefits of remaining with a group, individuals may isolate themselves reducing the overall number of fish shoaling, as observed here. Such isolative sickness behaviour is not uncommon and is demonstrated across multiple taxa (insects; Rueppell et al. 2010, birds; Brown and Brown 1992, mammals; Proudfoot et al. 2014). Furthermore, exclusion of infected individuals by uninfected conspecifics, and/or the failure of an infected fish in sustaining its shoaling position, particularly within flowing environments (see van Oosterhout et al. 2007), may contribute to a reduction in shoaling tendency.

The specific goal of this study was to identify how variable flow conditions impact parasite transmission dynamics and shoaling behaviour in a social host. Our results highlight how flow alteration substantially modifies parasite transmission dynamics, whereby parasitism was more prevalent in shoals exposed to interrupted flow conditions due to greater transmission rates. Additionally, fish experiencing continuous flow developed intense parasite infections: likely having severe implications for host health. Parasitism also appeared to have a greater significance on host shoaling behaviour in the

absence of flowing water. Understanding how disease ecology is affected by anthropogenic and climate mediated changes to river flows is particularly important considering that parasite infections can substantially modify host population dynamics. Furthermore, our data could be used to inform aquaculture of the optimal flow conditions to prevent disease transmission in stocks.

3.6   Acknowledgements

Chapter 4

Parasite-mediated host behavioural modifications: *Gyrodactylus turnbulli* infected Trinidadian guppies increase contact rates with uninfected conspecifics[4]

4.1 Abstract

While group formation provides antipredatory defences, increases foraging efficiency and mating opportunities, it can be counterintuitive by promoting disease transmission amongst social hosts. Upon introduction of a pathogen, uninfected individuals often modify their social preferences to reduce infection risk. Infected hosts also exhibit behavioural changes, for example, removing themselves from a group to prevent an epidemic. Conversely, this study shows how Trinidadian guppies infected with a directly transmitted ectoparasite, *Gyrodactylus turnbulli,* significantly increase their contact rates with uninfected conspecifics. As uninfected fish never perform this behaviour, this is suggestive of an infection-induced behavioural response of infected hosts, presumably to offload their parasites. In the early stages of infection, however, such behavioural modifications are ineffective in alleviating parasite burdens. Additionally, it is shown how fish exposed to *G. turnbulli* infections for a second time spent less time associating than those exposed to parasites for the first time. It is speculated that individuals develop and retain an infection cue repertoire, enabling them to rapidly recognise and avoid infectious conspecifics. This study highlights the importance of considering host behavioural modifications when investigating disease transmission dynamics.

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[4] This chapter has been published: **Reynolds, M.**, *Arapi, E. A., and Cable, J. 2017. Parasite-mediated host behavioural modification: Gyrodactylus turnbulli infected Trinidadian guppies increase contact rates with uninfected conspecifics. Parasitology. 8, 1-7.*

4.2   Introduction

Sociality confers many benefits, particularly with respect to antipredatory defences (Krause and Ruxton 2002; Sansom et al. 2009). Group formation can, however, be counterintuitive by increasing competition for scarce resources, mating opportunities, and the conspicuousness of prey to predators; all of which can directly impede fitness (Alexander 1974; Sherman et al. 1995). Most notably is increased disease susceptibility owing to chronic stress induced by social competition (Glaser and Kiecolt-Glaser 2005; Proudfoot and Habing 2015; Schneider et al. 2016), and the close proximity of conspecifics facilitating disease propagation amongst hosts (Côté and Poulin 1995). This often results in more intense, prevalent infections as group size increases (reviewed in Patterson and Ruckstuhl 2013).

At a population level, a significant determinant of a disease transmission pathway is a group's social structure, which arises from consistent interaction patterns between conspecifics (Whitehead 1997; Wey et al. 2008). The rate of disease transmission depends on the 'socialness' and infection status of group members. Well-connected individuals, for example, are at greater risk of acquiring infections (Bell et al. 1999; Danon et al. 2011), and can subsequently act as superspreaders of disease (Lloyd-Smith et al. 2005; Craft 2015). As infection becomes more prevalent within a population, conspecifics often respond to visual and/or chemical cues indicative of infection by modifying their associative preferences to reduce their infection risk. Such behavioural avoidance has been documented in a number of taxa including amphibians (Kiesecker et al. 1999; Koprivnikar and Penalva 2015), fish (Ward et al. 2005) and mammals (Curtis 2014; Poirotte et al. 2017).

Infected hosts often show remarkable adaptive behavioural traits aimed at combatting disease. Infected fish, for example, frequent warmer thermal conditions outside the thermal tolerance of their associated pathogens to 'self-medicate' against infection (see Chapter 2). Alternatively, infected hosts can isolate themselves to protect the integrity of a group. Extreme forms of altruistic behaviour, such as 'altruistic suicide', have been observed within social insects including ants and honeybees; upon acquiring infection, these hosts remove themselves from a colony to prevent disease epidemics (Heinze and Walter 2010; Rueppell et al. 2010). Conversely, by forming larger groups, parasite 'attacks' can become diluted amongst group members (Duncan and Vigne 1979; Rätti et al. 2006), with such encounter-dilution effects mitigating parasite-mediated costs of sociality (Mooring and Hart 1992).

Whether or not infected individuals increase their contact rates with uninfected conspecifics remains questionable. Indeed, 'offloading' parasites onto conspecifics could be beneficial in terms of alleviating parasite burdens and their associative costs, whilst also serving to 'vaccinate' recipients against subsequent infections (Faria et al. 2010). Here, the well-studied social Trinidadian guppy and its directly transmitted ectoparasite, *Gyrodactylus turnbulli*, are used to investigate how parasitism drives adaptive behavioural responses in hosts, which could aid in alleviating parasite burdens. Specifically, this study investigates (a) if association times and direct contact patterns between fish change depending on infection status, (b) assess the significance of these behavioural responses in determining successful parasite transmission, and (c) assess how the parasite infrapopulation influences transmission dynamics, irrespective of behaviour.

4.3 Materials and Methods

4.3.1 Host and parasite origin

Experimental Trinidadian guppies (*Poecilia reticulata*) were laboratory-reared descendants of a wild stock caught from the Lower Aripo River, 2012. Fish were initially housed at Exeter University, before being transferred to Cardiff University in October 2014. Here, fish were maintained in 70 L dechlorinated water tanks under standard conditions of 24 ± 1 ℃ on a 12 h light: 12 h dark photoperiod (lights on 07:00-19:00), and fed daily with Aquarian tropical fish flakes subsidised with *Daphnia magna* and *Artemia* spp.

Experimental fish were infected with *Gyrodactylus turnbulli* (strain *Gt3*), originating from, and subsequently maintained on, an inbred ornamental guppy stock since 1997. A single parasite was isolated onto a naïve 'culture' fish, and following successful establishment reproduced forming a highly inbred parasite population. This population has since been maintained under standard conditions in culture pots, each containing a minimum of four naïve culture fish collectively infected with *ca.* 30 *G. turnbulli* worms. Naïve fry were added to the culture pots biweekly to prevent parasite extinction.

4.3.2 Experimental design

Only female guppies were used during experimental trials due to their increased propensity to shoal compared to males (Griffiths and Magurran 1998). In the wild, females typically form small shoals (2-20 individuals), between which males move in search of mating opportunities (Croft et al. 2004). Thus by excluding males from this study eliminated the potentially confounding factors associated with mating attempts in influencing parasite transmission. Unfamiliarised female guppies were size-matched according to their standard length (SL; ±4.1 mm) into pairs (n = 50), and individually

housed in 1 L dechlorinated water under standard conditions. As infection histories of these fish differed, we categorised dyads into five treatments summarised in Table 4.1. Treatment 1 utilised parasite naïve guppies and formed a control group. During trials these fish were sham infected to account for handling time but never exposed to parasites. Naïve guppies were also used for treatment 2, which were experimentally infected with a predetermined dose of *Gyrodactylus turnbulli* worms ('Primary infection': see Table 4.1), and tested 24 h post infection. Treatments 3-5 comprised dyads that had experienced *G. turnbulli* infection in a previous experiment (ranging from 20-40 worms), but had been clear of parasites for a minimum of three months prior to this experiment. During a trial, these fish were experimentally infected with 40 *G. turnbulli* worms ('Secondary infection'), and tested 24 (treatment 3), 48 (treatment 4) and 72 h (treatment 5) post-infection.

TABLE 4.1: A summary of the experimental treatments (1-5) including *Gyrodactylus turnbulli* exposure status (primary or secondary infection), dyad sample size (n), *G. turnbulli* dose administered and infection time before a behavioural trial.

Treatment	Primary or secondary *G. turnbulli* infection	Dyad sample size (n)	*G. turnbulli* intensity dose	Infection period (h)
1 (control)	NA	17	NA	NA
2	Primary	15	10 worms	24
		10	20 worms	
		6	40 worms	
3	Secondary	12	All 40 worms	24
4	Secondary	12	Range: 41-72 worms	48
5	Secondary	10	Range: 36-90 worms	72

Behavioural trials took place in a partitioned glass tank (30 x 30 x 45 cm) filled with 7 L dechlorinated water and maintained at 24 ± 1 °C. A removable translucent barrier separated dyads prior to a trial, allowing visual but not physical contact. The tank was placed in an experimental chamber surrounded by white fabric on three sides to prevent

external disturbance, with one side left open for behavioural observations. A 2 cm^2 grid adhered to the sides of the tank enabled visual estimation of the distances between conspecifics. The arena was lit from above using daylight mimicking strip lights (Sylvania T5 F13W/54-765 G5 Luxline Standard Daylight bulb) diffused by white fabric.

4.3.3   Experimental procedure

Each dyad underwent a two-stage trial comprising behavioural and parasite transmission procedures. On day 1, dyads were placed into the partitioned experimental tank for a 24 h acclimation period. On day 2, both guppies were transferred from the tank to individual 1 L dechlorinated water pots using a plastic container. One fish in each dyad was then infected with a predetermined number of *G.turnbulli* worms (see Table 4.1 for *G. turnbulli* dose and sample sizes). Experimental infections entailed sacrificing a heavily infected culture fish, and placing it in close proximity to a temporarily anaesthetised recipient fish (using 0.02% buffered MS222), allowing direct transmission of worms. The infection process was observed continuously under a dissection microscope with fibre optic illumination, and once infected a recipient was revived in 1 L dechlorinated water. Its uninfected conspecific was sham infected to control for handling time and each pair returned to the partitioned tank for a subsequent 24 h, thus ensuring fish were familiar with this environment to minimise exploratory behaviour during a subsequent trial.

On day 3, both fish were removed from the partitioned tank, as above, temporarily anaesthetised and screened under a dissection microscope to quantify the starting *G. turnbulli* intensity of a donor, and confirm the uninfected status of their conspecific. The number of individual worms was counted three times to ensure exact intensities were

recorded. Variability in *G. turnbulli* start intensity, particularly for treatments 4 and 5 (see Table 4.1) were observed given the parasites 24 h reproductive potential (Bakke et al. 2007). Fish were returned to the partitioned tank for 10 min prior to removal of the translucent barrier, which allowed dyads access to one another. A 10 min behavioural trial began when one guppy crossed the central partition line. During a trial, the proportion of time dyads spent associating was quantified (seconds) using a stopwatch based on direct observation. An association was defined as when fish were ≤ four body lengths from one other; a standard measure of shoaling (Pitcher 1983). Additionally, the number of direct contacts initiated by the infected fish was tallied. Infected guppies exhibit clamped fins (Cable et al. 2002), and appear to 'rub-up' against conspecifics, although previously only anecdotal evidence of such behaviour exists (Croft et al. 2011; Stephenson et al. 2017). This unique behavioural response of infected fish coupled with pathological symptoms of infection made the identity of each fish obvious during a trial. A direct contact was therefore defined as when an infected fish instigated skin-skin contact (typically lasting <1 sec) with an uninfected conspecific, which is particularly important for facilitating *G. turnbulli* transmission (Bakke et al. 2007). Following a behavioural trial, both fish were again individually removed from the partitioned tank, temporarily anaesthetised and screened to quantify successful transmission of individual *G. turnbulli* worms.

To assess the significance of *G. turnbulli* intensity on potential *G. turnbulli* transmission dynamics, guppies remained anaesthetised following screening, and were placed in the same Petri dish submerged in 1 cm depth dechlorinated water. Using a pipette tip, fish were manipulated so that they were in direct contact with one another. The number of *G. turnbulli* worms to transmit from a donor to a recipient fish was then monitored for a 5 min period. At the end of a trial, guppies were chemically treated using 0.1%

levamisole and screened clear of infection, on 3 consecutive inspections, before returning to stock tanks.

4.3.4   *Statistical analysis*

Statistical analyses were performed in R (3.0.2; R Core Team 2013). Using the *lme4* library (Bates et al. 2015), two Generalised Linear Mixed Effects Models (GLMMs), fitted with binomial family and 'logit' error structures were used to assess variables influencing the proportion of time dyads spent associating (the dependent term in both models). The first model included donor *Gyrodactylus turnbulli* intensity, dyad mean SL, treatment, and interactions between each variable, as independent terms. Treatment 1 data were excluded from this model, as there was no possible relationship between the uninfected control dyads and parasite intensity. To address differences in dyad association time between treatments (including Treatment 1), a second GLMM was performed whereby dyad mean SL, treatment and an interaction between these terms were included as independent terms, with dyad association time the dependent term. Parasite intensity was excluded from this model. Both models included dyad ID as a random term to account for repeated measures.

Using the *glmmADMB* package (Bolker et al. 2008) a negative binomial GLMM was used to investigate the effects of dyad mean SL, donor *G. turnbulli* intensity, association time and treatment on the total number of direct contacts observed between dyads, instigated by an infected donor. Interactions between association time x *G. turnbulli* intensity and treatment x *G. turnbulli* intensity were incorporated into the model. A final negative binomial GLMM investigated the significance behavioural trials, transmission trials, dyad mean SL, treatment and donor *G. turnbulli* intensity had on determining total *G. turnbulli* transmission. Dyad ID was incorporated into each model

as a random term to account for repeated measures. Models were refined via the sequential removal of non-significant terms to minimise Akaike Information Criterion (Pinheiro and Bates 2000; Bates et al. 2015), and model robustness assessed using residual plots.

4.4   Results

4.4.1   Behavioural trials

Infection with *Gyrodactylus turnbulli* significantly influenced the proportion of time dyads spent associating (GLMM: $z = -7.27$, SE = 0.022, P <0.001), which also differed between experimental treatments (GLMM: $z = -4.33$, SE = 0.189, P <0.001). For fish infected with *G. turnbulli* up to 24 h (Treatments 2 and 3), the time dyads spent associating increased with parasite intensity (Fig. 4.1a). Conversely, for dyads experiencing secondary infections of 48 and 72 h, association time decreased as a donor infection intensified (Treatments 4 and 5, respectively: Fig. 4.1a). Dyad mean SL significantly influenced association time, which also depended on treatment (Dyad mean SL x Treatment interaction, GLMM: $z = -10.48$, SE = 0.004, P <0.001). For control dyads and naïve fish enduring a 24 h primary *G. turnbulli* infection (Treatment 2), larger dyads spent more time associating (Fig. 4.1b). For fish infected with 24, 48 and 72 h secondary infections (Treatments 3, 4 and 5, respectively), the relationship between dyad shoaling time and mean SL was less pronounced (Fig. 4.1b).

Association time and *G. turnbulli* intensity significantly influenced the number of direct contacts initiated by an infected donor towards its uninfected conspecific. Specifically, more direct contacts occurred the longer dyads spent associating (GLMM: $z = 1.98$, SE = 0.767, $P < 0.005$), and as a donor's parasite intensity increased (GLMM: $z = 2.80$, SE

= 0.029, P = 0.0309). However, only on two occasions did successful *G. turnbulli* transmission occur as a consequence of host behaviour.
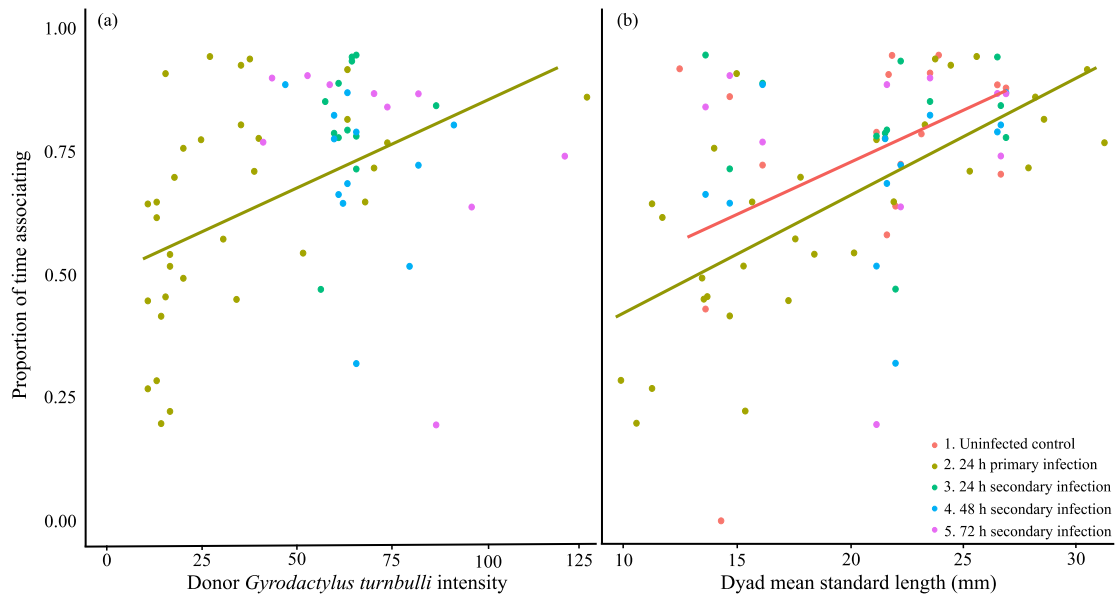
FIGURE 4.1: The relationship between the proportion of time dyads spent shoaling and (a) *Gyrodactylus turnbulli* intensity of the donor fish, and (b) dyad mean standard length (mm). The solid lines represent the regression between the proportion of time dyads spent associating on (a) donor *G. turnbulli* intensity (Treatment 2: r^2 = 0.227, $F_{1, 30}$ = 8.79, P = 0.005), and (b) dyad mean standard length (Treatment 1: r^2 = 0.182, $F_{1, 15}$ = 3.34, P = 0.005; Treatment 2: r^2 = 0.514, $F_{1, 30}$ = 31.77, P < 0.001). Only significant regression lines are presented here.

4.4.2 *Gyrodactylus turnbulli* transmission

When investigating the potential for *Gyrodactylus turnbulli* transmission following host behavioural trials (i.e. placing an anaesthetised donor fish in direct contact with an uninfected recipient for a 5 min period), we found that the total number of *G. turnbulli* to transmit was significantly influenced by a donors' parasite intensity (GLMM: z = 2.09, SE = 0.006, P = 0.037). As parasite intensity increased, the number of parasites transmitting to a recipient also increased (Fig. 4.2). The duration of infection did not affect total *G. turnbulli* transmission (Treatment main effect: GLMM: z = 1.08, SE = 0.10, P = 0.278).
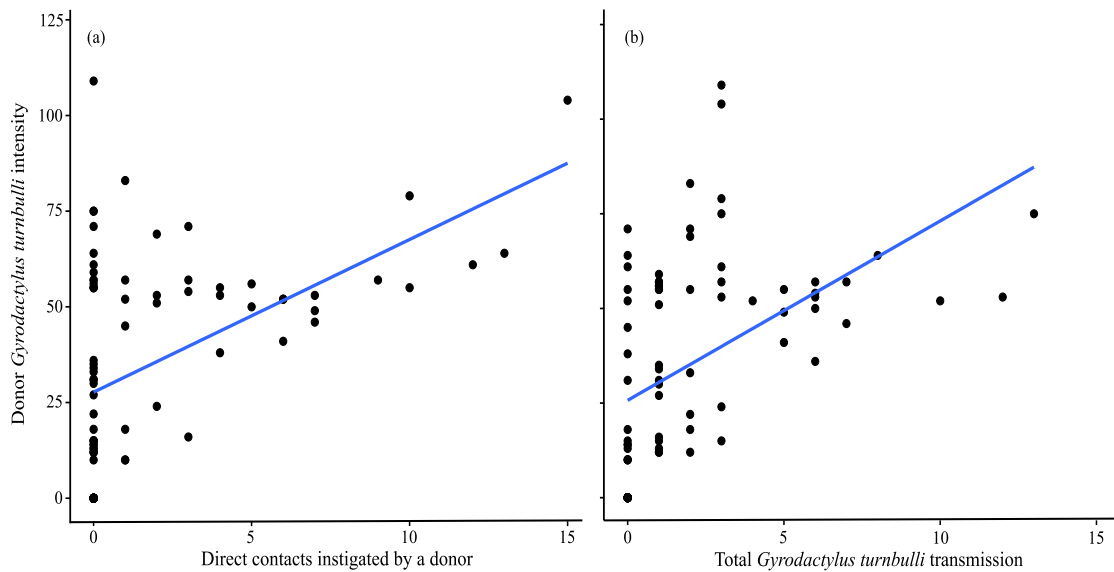
FIGURE 4.2. Association between donor *Gyrodactylus turnbulli* intensity and (a) the number of direct contacts instigated by an infected donor fish, and (b) the number of parasites to transmit to an uninfected recipient over a 5-minute duration when both fish were anaesthetised and placed in direct contact. The solid line represents the regression between a donors *G. turnbulli* intensity on (a) the number of direct contacts instigated by a donor ($r^2 = 0.156$, $F_{1, 81} = 14.96$, $P < 0.001$), and (b) the number of worms transmitted to an anaesthetised recipient host ($r^2 = 0.239$, $F_{1, 81} = 25.38$, $P < 0.001$).

4.5   Discussion

This study demonstrates how infected fish significantly increase direct contact rates with conspecifics. As uninfected fish did not perform such behaviour, it is speculated that this parasite-driven host behavioural response attempts to reduce parasite burdens. Additionally, it is shown how association times between fish exposed to secondary *G. turnbulli* infection was less than those experiencing infection for the first time. The potential for fish to build an infection cue repertoire, enabling them to rapidly identify and subsequently avoid infectious conspecifics, is discussed as a possible mechanism to explain this observation.

The results of this study are consistent with Croft et al. (2011), who provided anecdotal evidence of infected guppies initiating direct body contact with conspecifics. Although gyrodactylid infections do cause host behavioural modifications (see Bakke et al. 2007), these are merely by-products of infection (e.g. Kolluru et al. 2009). Behavioural manipulation by *G. turnbulli* would imply host neurochemical interference (Adamo and Webster 2013), which has previously never been shown by these ectoparasites. Furthermore, *G. turnbulli* worms did not exhibit a strong transmission propensity during behavioural trials, indicating that increased contact rates are likely an adaptive behavioural response of the host as oppose to parasite manipulation.

Alleviating ectoparasite burdens often involves hosts participating in interspecific, intraspecific and/or self-grooming behaviours (reviewed in Hart 2011). Intraspecific grooming is particularly evident in higher animal taxa, most notably mammals such as ungulates. Reciprocal allogrooming in impala (*Aepyceros melampus*), for example, significantly reduced tick infestations by up to 95% that of controls (Mooring et al. 1996). Fish also engage in grooming activity, whereby cleaners including gobies, wrasse or shrimps remove clientele ectoparasites, which provides nutritional and health benefits for both parties, respectively (Grutter 1996; Whiteman and Côté 2002; Titus et al. 2017). Cleaning services are typically concentrated to tropical coral reefs (Côté, 2000), although freshwater intraspecific examples do exist (e.g. Common carp; Soto et al. 1994). For fishes that do not engage in cleaning services, alternative parasite removal strategies are employed. For example, physical dislodgement of parasites through skin abrasion against substrata (Urawa 1992), behavioural fever (Reynolds et al. 1976; Chapter 2), or as observed here increasing contact frequencies with uninfected conspecifics in the early stages of infection. The number of direct contacts instigated by infected guppies towards their uninfected conspecifics significantly increased with *G.*

turnbulli intensity. Direct contact between hosts governs *G. turnbulli* transmission dynamics (see Johnson et al. 2011), and it could be expected that parasite transmission increase linearly with direct contact frequencies. Contrary to predictions, successful *G. turnbulli* transmission only occurred twice during behavioural trials, irrespective of the number of direct contacts between infected and uninfected fish. This indicates that such behavioural responses adopted by a host were ineffective in reducing parasite burdens, at least in the initial stages of infection.

Transmission dynamics are mediated by a complexity of factors from both host and parasite perspectives (see Stephenson et al. 2017). Ectoparasites adopting a direct transmission strategy risk dislodgment and subsequent mortality. Dislodged *G. turnbulli*, for example, have a maximal off host survival of 31 h at 25 ^{o}C (Schelkle et al. 2013). In the initial stages of infection when the parasite infrapopulation is low, worms do not compete for host resources and therefore the transmission risk outweighs the cost of remaining on an initial host. Although not examined here, in the later stages of infection competition for resources due to a greater parasite density, coupled with activation of the host's immune response (Buchmann and Bresciani 1998), may prompt parasite transmission away from unfavourable host microhabitats (Boeger et al. 2005; Pie et al. 2006; Stephenson et al. 2017).

For parasite naïve controls, dyad association time increased with mean SL. Similarly, this trend was observed for dyads experiencing a 24 h primary *G. turnbulli* infection, although a slight decrease in association time was evident. Larger fish generally have greater shoaling tendencies than smaller, younger individuals (Pitcher et al. 1983; Paxton 1996; Rodgers et al. 2011). This may be associated with 'safety in numbers' as large individuals are more conspicuous to predators. Fish experiencing secondary

infections, however, only associated when infection intensities were low. These individuals may have developed a chemical cue repertoire of infection, which overrides their social preferences irrespective of size. During development, individuals imprint on both behavioural and chemical cues of conspecifics to build a 'phenotypic template', which is important for social decisions later on in life (see Mateo 2004). Using these templates, individuals decipher between normal and abnormal cues emitted from conspecifics, to which they can respond accordingly. Such cues important in communicating the infection status of individuals could be associated with a host's immune response.

Fish exhibit both innate and acquired immune responses to gyrodactylid infections (e.g. Scott and Robinson 1984; Scott 1985; Cable and van Oosterhout 2007b), which are directed to the hosts' epidermis (Richards and Chubb 1996). Here, immune by-products including host complement (Buchmann 1998), changes in mucosal secretion composition (Moore et al. 1994), and cortisol release into the surrounding water (Stoltze and Buchmann 2001) translate into chemical cues indicative of infection. Additionally, fish may also perceive excretory compounds of ectoparasites, although to date this remains unknown. Detection of these cues subsequently mediates avoidance behaviours to reduce infection risk, particularly in the late stages of infection (Stephenson and Reynolds 2016). Furthermore, the combination of both innate and acquired immunity during secondary infections could have emitted stronger 'infection cues' resulting in a significant reduction in the association times between infected and uninfected fish exposed to 48 and 72 h secondary *G. turnbulli* infections. It should be noted, however, that acquired resistance diminishes post-recovery (Scott 1985; Cable and van Oosterhout 2007b), and as the experimental fish in this study had been uninfected for over 3 months, it is unlikely that acquired immune responses contributed to the

production of cues indicative of infection. Alternatively, the development and retention of a chemical cue repertoire of infection during primary parasite exposure may have been important in instigating evasive fish behaviours during subsequent infection exposure. Such parasite-mediated avoidance behaviour is particularly beneficial considering the severe pathological costs of gyrodactylid infection (reviewed in Bakke et al. 2007).

To conclude, this study shows that in the early stages of infection, parasite-mediated behavioural modifications of infected hosts is not an effective strategy for reducing parasite burdens. Such behavioural responses may have greater significance in determining parasite transmission during the later stages of infection, when infra-parasite competition and the host's immune response are more pronounced. However, as infection progresses the benefits may be short lived, particularly if the parasite's reproductive rate outweighs a host's offloading ability. Successful transmission as a consequence of increased contact rates would also be costly to conspecifics, and is counterintuitive to the evolutionary theory of sociality. Although this study provides the first quantified example of such behaviour, it may be more widespread within the Animal Kingdom as infected hosts attempt to alleviate their infections. Finally, this study provides evidence that fish exposed to secondary parasite infections may have developed infection repertoires, enabling them to instigate evasive behaviours towards infected conspecifics sooner than fish experiencing infection for the first time.

4.6 Acknowledgements

Chapter 5

Restless nights when sick: Ectoparasite infections increase nocturnal activity of their fish hosts[5]

5.1   Abstract

Disturbances to sleep-wake cycles can increase an individual's disease susceptibility, and infection itself can instigate sleep disturbances by, for example, prolonging nocturnal activity, which could be detrimental to host health. Previous experimental work highlighting the association between nocturnal restlessness and parasite infection almost exclusively focuses on endoparasite infections, which are rarely quantified. Here, we assessed diel activity patterns of two typically diurnal hosts, the Trinidadian guppy and three-spined stickleback, in relation to their common ectoparasite infections. We show that *Gyrodactylus turnbulli* infected guppies and *Argulus foliaceus* infected sticklebacks were generally more active than their uninfected counterparts, significantly more so during nocturnal hours. The causes and consequences of such behavioural responses to infection are discussed.

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[5] This chapter has been submitted for publication in Biology Letters: **Reynolds, M**., *and Cable, J. Ectoparasite infections increase nocturnal activity of their fish hosts.*

5.2 Introduction

Every aspect of an individual's behaviour is, to some degree, mediated by parasite exposure (Ezenwa et al. 2016). Behavioural traits including sociality, foraging and mating strategies are often adversely affected by parasitism (Ezenwa 2004; Raveh et al. 2011; Ashby and Boots 2015). Furthermore, parasites can interfere with an individual's activity pattern to ultimately compromise host fitness. The cestode worm, *Schistocephalus solidus,* for example, increases the activity of its copepod intermediate host: maximising host predation in favour of the parasite (see Hafer and Milinski 2015).

Parasites can also interfere with an individual's sleep-wake cycle (see Ibarra-Coronado et al. 2015). The irritative feeding activity of ectoparasitic tick, lice, flea and mite infestations endorses excessive itching in hosts (e.g. canines: Ugochukwu and Nnadozie 1985, ungulates: Samuel 1989, and birds: Nemejc and Lukesova 2012). Such physical irritation likely impacts the quality and quantity of host sleep, yet nocturnal restlessness as a consequence of ectoparasite infection is rarely empirically quantified. Only one study documents how the presence of a parasite can shift host rest time-budgets: Great tits, *Parus major*, inhabiting mite-infested nests significantly reduced sleeping bouts compared to birds in uninfested nests (Christe et al. 1996). However, this study did not distinguish whether sleep reduction was a direct consequence of mite feeding or simply an overwhelming desire of the host to perform nest sanitation activities.

The current study therefore investigates ectoparasite induced nocturnal host restlessness using two different host-parasite associations: the tropical Trinidadian guppy (*Poecilia reticulata*) - *Gyrodactylus turnbulli,* and the temperate three-spined stickleback (*Gasterosteus aculeatus*) - *Argulus foliaceus* systems. Both host-parasite models have been subject to extensive epidemiological and behavioural investigations (Taylor et al.

2006; Bakke et al. 2007). Although both parasites do cause behavioural modifications in these typically diurnal hosts (e.g. shoaling and swimming performance (Poulin and FitzGerald 1989; Kolluru et al. 2009)), nocturnal host behavioural responses to infection have, until now, been overlooked. We hypothesise that infected individuals are significantly more nocturnally active than their uninfected conspecifics, and discuss the implications this may have for host health.

5.3   Materials and Methods

5.3.1   *Host and parasite origins*

Trinidadian guppies, *Poecilia reticulata,* were laboratory-reared descendants of a wild caught stock from the Lower Aripo River, Trinidad in 2012. Fish were maintained parasite free in 22-24 ^{o}C dechlorinated water on a 12 h light: 12 h dark photoperiod (lights on 07:00-19:00), and fed daily on Aquarian® tropical fish flakes. Aquaria were checked weekly for fry, which were transferred to rearing tanks. Sexually mature female guppies (>4 months) were subsequently size matched according to their standard length (±1.5 mm) into shoals of five individuals (n = 16 groups). For experimental infections, *Gyrodactylus turnbulli* (strain Gt3) were used; isolated in 1997 from, and subsequently maintained on ornamental guppies (as in Chapter 2).

Three-spined sticklebacks, *Gasterosteus aculeatus*, were transferred to Cardiff University from a commercial supplier (DC Freshwater Fish) in December 2016. Fish were treated for parasite infections using 0.1% dilution of Levamisole (Norbook, UK) and Pimafix^{TM}, and placed in 16±1 ^{o}C 70 L dechlorinated water on a 12 h light: 12 h dark photoperiod (lights on 07:00-19:00). Fish were fed daily with frozen bloodworm. Sticklebacks of unknown sex were sized matched according to their standard length (±2.5 mm) into groups of five individuals (n = 18 groups). As sticklebacks only exhibit

sexual dimorphism when in mating condition, it was not possible to determine the sex of each fish without destructive invasive procedures, which would have proven inconclusive here given the small size of the fish. Adult *Argulus foliaceus* (>2 months old), sourced from a North Lincolnshire still water fishery in 2012 (see Stewart et al. 2017), were used for experimental infections. Following size matching of guppies and sticklebacks, individual groups were housed in 6 L familiarisation aquaria to form shoals over a minimum of 14 days (Griffiths and Magurran 1997a), prior to trials.

5.3.2 *Experimental set-up*

The diel activity of infected and uninfected fish was monitored in open channel re-circulatory flumes, each measuring 150 cm length x 16 cm depth x 20 cm width (Fig. 5.2). Flumes were maintained under the same photoperiod and thermal settings as stocking conditions above. Shoals were isolated to a 100 cm 'test arena' using honeycomb flow straighteners, which ensured laminar flow throughout the chamber and restricted fish from entering the impellor region. Two flow conditions were chosen to investigate diel activity patterns of infected and uninfected guppies: a static condition (control), and a continuous flow rate (mean velocity 6.2 ± 0.05 cm s^{-1}), akin to that recorded within natural guppy habitats (Kodric-Brown and Nicoletto 2005). The mean flow rate was calculated by recording velocity every 10 s for a 5 min period using a Nixon miniature propeller flow meter. For stickleback trials, only a static condition was used to minimise *Argulus foliaceus* dislodgement and loss within the re-circulatory system. During nocturnal observations, red light (light intensity 1.2-1.3 lx; Precision Gold Digital Light Meter) enabled visual observations by an observer without disturbing focal shoals.

5.3.3   Experimental procedure

Each familiarised guppy or stickleback shoal was transferred to either a static (n = 9 guppy shoals; n = 18 stickleback shoals) or continual flow condition (n = 7 guppy shoals) for a 24 h acclimation period. At 08:00 the following morning (Day 1), fish were removed from the flume and one guppy or stickleback infected with exactly 30 *G. turnbulli* worms or a sexually mature adult *A. foliaceus,* respectively (following procedures in King and Cable 2007; Stewart et al. 2017). These comparatively low burdens were representative of natural infections (van Oosterhout et al 2003; Walker et al. 2008). The remaining 4 fish in each shoal were sham infected to control for handling time. Fish were placed in individual 1 L pots for 30 min, whilst remaining in visual contact to one another, and fed 3 *Daphnia magna* before being returned to the flume. On Day 2, a stopwatch was used to quantify the proportion of time (sec) an infected and a randomly selected uninfected fish spent actively swimming during a 5 min focal follow over five time points; three diurnal (08:00, 13:00 and 18:00 h) and two nocturnal (22:00 and 01:00 h). The selected time points represented dawn, dusk, midday and midnight, with a second nocturnal observation to assess consistencies in nocturnal behaviour. Fish were deemed actively swimming when propelling themselves forward. Data collected from uninfected individuals were used as a control.

At the end of the trials, infected guppies were treated with 0.1% dilution levamisole and confirmed clear of infection during three sequential inspections separated by 4 days. Any fish still infected were re-treated and screened three times before returning to stock tanks. A pair of fine forceps was used to dislodge *Argulus foliaceus* from infected sticklebacks, which were returned to the parasite culture.

5.3.4   Statistical analysis

Using the *lme4* package (Bates et al. 2015) in R (3.1.3; R Development Core Team 2009) two Generalised Linear Mixed Models (GLMMs), fitted with binomial error family and logit link function, were used to assess diel activity patterns of infected and uninfected guppies (Model 1) and sticklebacks (Model 2). The proportion of time fish remained actively swimming during a 5-min focal follow was the dependant term, and fixed effects included infection status (infected or uninfected), observation time (08:00; 13:00; 18:00; 22:00; 01:00), mean standard length (mm) and flow condition (guppy model only). An interaction term between infection status and time was also incorporated into the model, and shoal ID included as a random term to account for repeated measures. Model robustness was assessed using residual plots (Pinheiro and Bates 2000).

5.4   Results

Infection status had a significant effect on the swimming activity of both Trinidadian guppies and three-spined sticklebacks (infection status main effects; Table 5.1), which also depended on time (Time x Infection status interactions; Table 5.1). Infected fish were generally more active than uninfected conspecifics at each time point, significantly so during nocturnal hours (Fig. 5.1). There was no significant effect of mean standard length for either fish species or flow condition (guppy trials only) on host activity (Table 5.1).

TABLE 5.1: Generalised linear models explaining variation in diel activity patterns of *Gyrodactylus turnbulli* infected Trinidadian guppies and *Argulus foliaceus* infected three-spined sticklebacks.

Host	Parameter	Estimate	Standard Error	*z* value	*P* value
Trinidadian guppies	Intercept	-0.2177	0.0154	-14.126	<0.001
	Mean standard length	-0.1199	0.0887	-1.35	0.1767
	Flow	0.0170	0.0976	1.74	0.0815
	Time	-0.0694	0.0114	-6.094	<0.001
	Infection status	-1.2450	0.0498	-24.999	<0.001
	Time x Infection status	0.0705	0.0148	4.749	<0.001
Three-spined sticklebacks	Intercept	-0.0256	1.0022	0.0026	0.980
	Mean standard length	0.0059	0.0276	0.2140	0.831
	Time	0.0371	0.0089	4.1520	<0.001
	Infection status	-0.2817	0.0419	-6.728	<0.001
	Time x Infection status	-0.0959	0.0126	-7.602	<0.001

5.5   Discussion

We provide the first empirical evidence of ectoparasites directly instigating nocturnal activity within typically diurnal hosts. Using two fish-ectoparasite systems, we show how infected individuals are generally more active than their uninfected counterparts, significantly more so during nocturnal hours. Ectoparasites likely inflict physical discomfort to their hosts during establishment, infection and post-infection stages. Gyrodactylids, for example, attach to their host using hooks, and extrude digestive enzymes onto the hosts' epidermis from which digested epidermal cells and mucus are subsequently ingested (Bakke et al. 2007). In addition to digestive enzymes, argulids utilise a proboscis to pierce the hosts' epidermis to directly access a blood meal (Alas et al. 2010). Consequently, tegument damage and excessive skin scraping performed by aggravated hosts facilitates secondary bacterial infections, often resulting in host mortality (Xu et al. 2007; Alas et al. 2010). Here, gyrodactylids provoked a more pronounced effect on nocturnal guppy activity, compared to *A. foliaceus* infected sticklebacks. The frequent movement of gyrodactylids across the guppies' skin, potentially associated with the avoidance of localised host immune responses (Richards
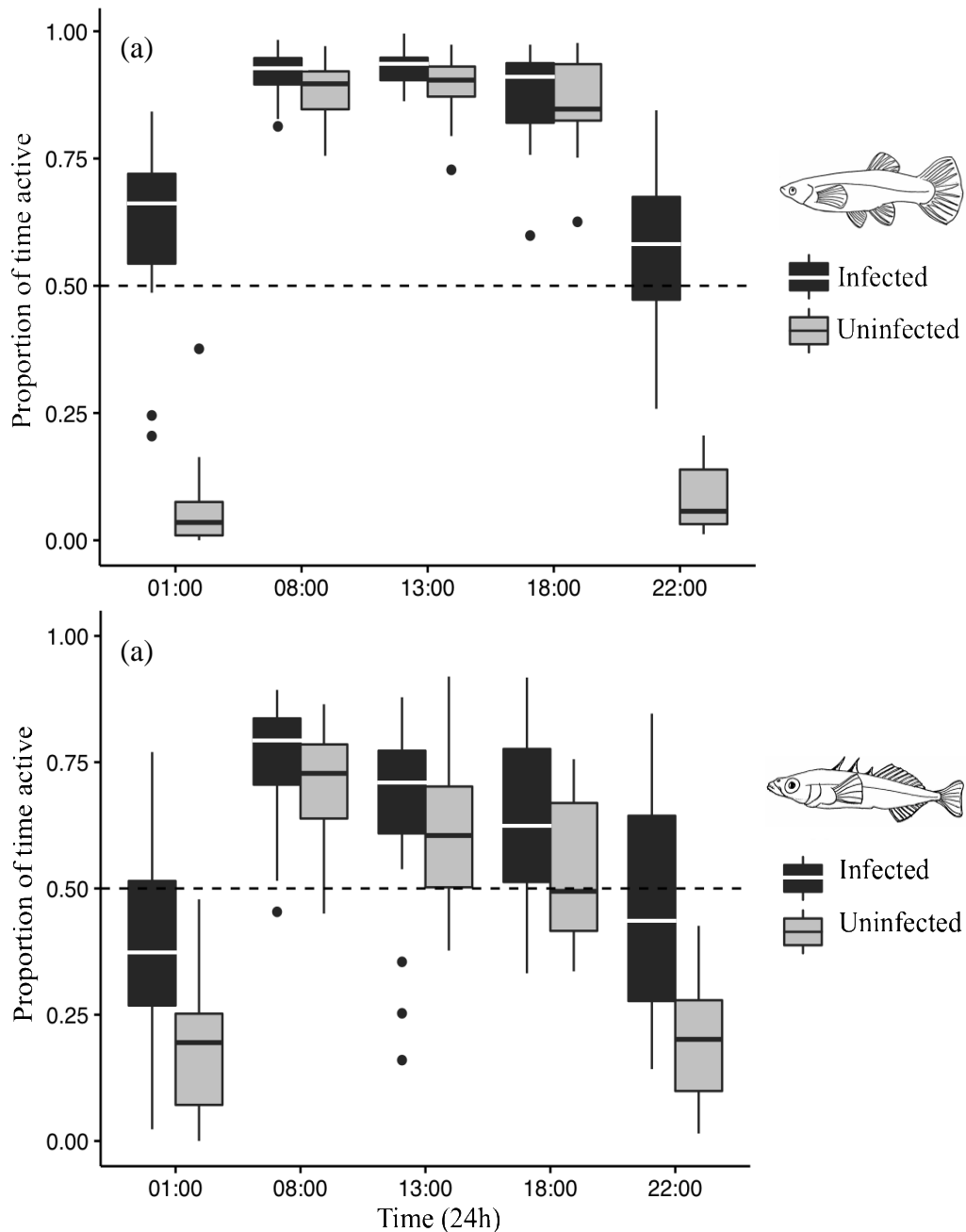
FIGURE 5.1. The proportion of time (a) *Gyrodactylus turnbulli* infected and uninfected Trinidadian guppies, and (b) *Argulus foliaceus* infected and uninfected three-spined sticklebacks remained active during 10 min focal follows over five time points (3 diurnal and 2 nocturnal observations). Black dots represent outliers; bars the upper and lower limits; the box the first and third quartile with median, and the dashed line 50% of the time in which fish remained active during a focal follow.

and Chubb 1998), may have caused greater irritation to a host than argulids, which remained relatively immobile during trial periods.

Increased swimming activity is presumed an adaptive host strategy promoting parasite dislodgement, as shown in argulid-infected sticklebacks (see Walker et al. 2004). However, in natural environments such behavioural changes may be counterintuitive with respect to attracting predatory attacks, which is likely population specific. Guppies residing in up- and downstream populations, for example, experience dissimilar predatory regimes, which have been shown to mediate parasite dynamics (Stephenson et al. 2015). Downstream populations are exposed to greater predation risk and thus prolonged activity of infected hosts could prove fatal. Contrastingly, for upstream populations where predatory threats are relaxed, elevated activity may be beneficial whereby infected fish move between, directly contact and dilute their parasites amongst potential hosts (Chapter 4; Mooring and Hart 1992).

In conclusion, we show that ectoparasites alter nocturnal activity of their hosts, likely as a consequence of the attachment and feeding ecology of these parasites. This has direct implications for animal behaviour studies, which largely overlook nocturnal activity of diurnal species in relation to infection status. Such behaviour could be detrimental to a host, with respect to secondary infection and/or predatory attraction.

5.6 Acknowledgements

Chapter 6

Parasites drive social network dynamics[6]

6.1   Abstract

Epidemiological networks are a popular tool for modelling disease transmission amongst social hosts. Existing modelling approaches typically show how static networks determine disease transmission. This study uses the Trinidadian guppy-*Gyrodactylus turnbulli* host-parasite system to highlight a duality, whereby infection also drives temporal variation in network structure. Specifically, the social network properties of guppy populations, including weighted in- and out-degree, closeness and betweenness, significantly increase post-parasite perturbation, irrespective of whether infection is seeded in the most or least connected host individuals. Infected individuals increased their social rank to occupy, along with their respective shoalmates, consistent network positions post-parasite perturbation. The introduction of parasites therefore stabilised network structure. Additionally, infected and uninfected individuals instigated and received more contacts, respectively: indicating that infected individuals potentially act as superspreaders of disease, whereas uninfected conspecifics were at greater risk of acquiring infection. The short- and long-term behaviour of a system may differ considerably given that infection can substantially mediate host sociality. Further network restructuring may be expected in the later stages of an epidemic as individuals modify their behaviour to reduce infection risk. This study demonstrates the importance of integrating temporal variations in social and transmission elements of social networks.

6.2   Introduction

Network analysis assesses the structural dynamics of interacting units, and has applications in a diverse number of fields ranging from computing (Barabási et al. 2000) and transportation systems (Guimerà et al. 2005), to social behaviour (Makagon et al. 2012). Epidemiological networks have become a popular tool for modelling how social organisation influences disease propagation (reviewed by Danon et al. 2011). Traditionally, mathematical modelling of pathogen transmission assumed homogenous mixing in populations and equal exposure of individuals to disease (Anderson and May 1992). In reality, most populations are highly structured, arising from consistent interactions between conspecifics (see Sah et al. 2017). Social affiliations between individuals, termed 'edges', naturally define a network (Danon et al. 2011). The resulting network, coupled with abiotic factors, host physiology and disease characteristics, determine pathogen transmission.

An underlying mechanism of network heterogeneity is individual behaviour (Wey et al. 2008; Aplin et al. 2013), which may alter in relation to infection (reviewed in Curtis 2014). For example, female house mice, *Mus musculus*, use chemical cues to discriminate between healthy and parasitised males, resulting in behavioural avoidance to reduce infection risk (Penn and Potts 1998; Ehman and Scott 2001). The behaviour of individuals can also lead to unexpected disease dynamics, notably, an increase in bovine tuberculosis (bTB) prevalence after reactive culling of the European badger, *Meles meles* (see Carter et al. 2007; McDonald et al. 2008; Weber et al. 2013). Disruption of stable social structures as a consequence of culling can give rise to a 'perturbation effect' where increased immigration of infectious individuals and mixing between remaining neighbouring groups facilitates bTB propagation (Carter et al. 2007; Weber et al. 2013). A reciprocal host behaviour-parasite feedback mechanism exists (reviewed

in Ezenwa et al. 2016), whereby parasite transmission at a population level is determined by the contact frequency of individuals, which itself may fluctuate in response to the presence of infection.

Typically, studies of infectious diseases examine static networks (see Keeling and Eames 2005; Fefferman and Ng 2007; Danon et al. 2011), and whilst topological descriptions at a specific time point are useful, both behaviour and the infection process are dynamic. A potential duality exists in that infection itself may induce host behavioural changes that ultimately determine network topology, and so drive the dynamics of infectious diseases. Social insects, for example, evolved 'social immunity' as a means of combating an increased disease transmission risk arising from sociality (reviewed in Cotter and Kilner 2010). Bees and ants even instigate extreme forms of behavioural altruism, whereby infected individuals remove themselves from a colony to prevent disease epidemics (Heinze and Walter 2010; Rueppell et al. 2010). Additionally, social exclusion or infection-induced mortality has the potential to rewire networks in a substantial manner. Detailed temporal resolution of data is essential for an in-depth understanding of transmission processes (Lentz et al. 2016).

Controlled experimental approaches to network perturbation have previously focussed on the removal or replacement of key individuals (e.g. Flack et al. 2006; Firth and Sheldon 2016). Networks can, however, exhibit resilience to perturbation events. The general social network properties of Trinidadian guppies, for example, are highly robust to habitat alterations (see Wilson et al. 2015). Specifically, individuals sustain fission-fusion dynamics and occupy consistent network positions before and after habitat manipulation (Krause et al. 2017), indicating a strong selection pressure of individuals to work actively against changes in their social dynamics. Sustaining strong social ties

following biotic perturbation events, specifically the introduction of disease, could, however, be counterintuitive by increasing exposure and infection risk for naïve individuals. Given that, in experimental settings, uninfected individuals actively avoid infection cues (see Stephenson and Reynolds 2016), network restructuring is likely to be an inevitable consequence of parasite perturbation. In the present study, the Trinidadian guppy (*Poecilia reticulata*) and its directly transmitted ectoparasite, *Gyrodactylus turnbulli,* are used to investigate how parasitism drives temporal variation in social network properties by determining (a) if centrality indices of shoal members change post-parasite infection, and (b) if individuals occupy consistent network positions before and after parasite perturbation.

6.3   Materials and Methods

6.3.1   *Host and parasite origin*

Trinidadian guppies (*Poecilia reticulata*) were laboratory-reared descendants of wild caught stock from the Lower Aripo River, Trinidad in 2012. Fish were initially housed at Exeter University, before transfer to Cardiff University in 2014 to be maintained in 70 L dechlorinated water tanks under standard conditions of 24 ºC on a 12 h light: 12 h dark photoperiod (Lights on 07:00 – 19:00). Fish were fed daily on Aquarian® Tropical fish flakes, subsidised with live *Artermia* spp. and *Daphnia magna*. Aquaria were checked weekly for fry, which were transferred to rearing tanks from which female fish were isolated at 8-12 weeks. For experimental infections, *Gyrodactylus turnbulli* (strain *Gt3*) were used; isolated in 1997 from, and subsequently maintained on, ornamental guppies (culture fish).

6.3.2   Experimental design

Experimental trials took place in a 70 L tank of dechlorinated water maintained under standard conditions, as above. A 2 cm layer of fine substrate filled the base of the aquarium, which was lit from above using daylight mimicking strip lights (Sylvania T5 F13W/54-765 G5 Luxline Standard Daylight bulb) diffused by white fabric. The chamber was surrounded on three sides with opaque white fabric to prevent external disturbances, with one side left open to allow observations.

6.3.3   Experimental procedure

A total of 20 replicate shoals, each containing six sized-matched female guppies, were monitored daily for 10 days, with experimental infection being given at Day 5 in 15 randomly selected replicates, and a sham infection in the remaining five controls. Each fish was uniquely marked using visual implant elastomer (VIE), enabling individual fish identification during a trial. To do this, fish were briefly anaesthetised using 0.02% tricaine methansulfonate (MS222), and VIE injected into the ventral or dorsal muscle tissue: a marking procedure extensively used in guppies (Croft et al. 2006, 2009; Wilson et al. 2015; Hasenjager and Dugatkin 2017) that does not appear to influence social behaviour (Croft et al. 2004). Fish standard length (SL; mm) was measured before each group was placed into a separate 5 L aquarium to form shoals over a 2-week familiarisation period (see Griffiths and Magurran 1997a), before transferring to an experimental chamber to acclimate for 24 h.

Focal animal sampling was used to assess node-level (individual) network metrics. Each shoal member was monitored daily for a 10 min period (between 9:00 – 10:00) over a 10-day trial. As *G. turnbulli* exhibits a direct transmission strategy (Bakke et al. 2007), direct contact frequencies between individuals were recorded during focal follows (e.g.

skin-skin contact including a bite or the brushing of fins, typically lasting <1 sec). Additionally, the directionality of potential transmission was also recorded by determining which individual initiated the contact.

Following focal follows on Day 5, all fish were temporarily isolated in individual 1 L pots and either the most or least connected shoal member (determined by assessing accumulated contact frequency data until Day 5) was infected with exactly 30 *G. turnbulli* worms. This formed three experimental treatments: most connected infected (n=7 shoals), least connected infected (n=8 shoals), and uninfected controls (n=5 shoals). Experimental infection entailed anaesthetising a recipient fish and placing a heavily infected culture fish within close proximity so that worms would transfer via direct contact, as observed under a dissection microscope. The remaining five fish in each shoal, as with each fish in the control groups, were sham infected by anaesthetising and manipulating under the microscope, without exposure to parasites. Fish were revived in 1 L dechlorinated water and returned to the experimental chamber. Infection was confirmed after focal follows on Day 6, and each fish screened on consecutive days thereafter (Days 7, 8, 9 and 10) to quantify *G. turnbulli* intensity.

6.3.4   *Social network structure*

For each shoal, adjacency matrices were generated for each day (1-10), from which node-level (i.e. individual-level) metrics were computed using UCINET 6 software (Borgatti et al. 2002). As transmission risk increases as individuals spend more time interacting (Silk et al. 2017), each individual's *weighted in-* and *out-degree*: the number of contacts received and emitted by an individual, respectively, were calculated. An individual's in-degree reflects its potential exposure to infection, whereas its out-degree represents its potential to transmit infection (Gates and Woolhouse 2015). *Betweenness*

centrality measured the number of times an individual connected other nodes in a network, identifying fish acting as 'bridges' between others. Individuals with a high betweenness score may control parasite transmission between others individuals with which it connects (Craft 2015; Silk et al. 2017). Finally, *closeness centrality* scored individuals based on their 'closeness' to others in a network. Those fish with a higher closeness index occupy more central network positions, and thus have higher probabilities of influencing parasite transmission (Silk et al. 2017).

All statistical analyses were performed using R software (core development team v.3.2.2). As social network data are inherently non-independent, therefore violating the assumptions of conventional parametric statistics (Croft et al. 2011; Farine 2016), randomisation tests were incorporated into analyses. Using the *lme4* R package (Bates et al. 2015), Linear Mixed Effects Models (LMEs) were used to assess the association between *G. turnbulli* infection and each network metric. Contact data were pooled from Days 1-5 (pre-) and 6-10 (post-infection), from which mean weighted in- and out-degree, betweenness and closeness was calculated and included in separate LMEs as the dependent terms. Parasite exposure (pre- or post-infection) was the independent term, and shoal ID included as a random factor to account for repeated measures. The coefficient estimate for the magnitude of the slope from the observed data was then compared against a distribution of 10,000 randomised coefficients produced by a null model. A *P* value was calculated based on the position of the observed slope estimate, relative to the distribution of slopes calculated from the randomised data (Farine 2013, 2017).

6.3.5   Temporal network stability

The temporal stability of social structure over Observation Days 1-5 and 6-10 (pre- and post-infection) was examined by ranking guppies (from 1-6) within their respective shoals, based on their weighted in- and out-degree, betweenness and closeness for each day. Individuals with larger scores for each metric occupied higher-ranking positions. The ranks of individual network measures were randomised for 10,000 iterations, from which a 'pre-' and 'post-infection' test statistic was computed by summing the variances of individual ranks between Days 1-5 and after infection at Days 6-10 (Krause et al. 2017). A *P* value < 0.05 indicates that individuals consistently occupy the same ranking positions between days, thus representing a stable social structure. A *P* value $>$ 0.05 reveals variability in social rankings over time and therefore network instability. A final LME was used to assess how *G. turnbulli* intensity, the independent term in the model, affected an individual's rank, the dependent term, within its respective shoal. Fish ID was nested within shoal and incorporated into the model as a random term to account for repeated measures. *P* values were derived from comparing the observed data coefficient to a distribution of 10,000 randomised coefficients generated by a null model, as above. Finally, quantile-quantile plots were used to compare weighted in- and out-degrees of *G. turnbulli* infected and uninfected guppies visually.

6.4   Results

Irrespective of whether the most or least connected individuals were targeted for experimental infection, there was a significant increase in a shoal's mean weighted in-degree (LME, $t = 6.937$, $P < 0.001$), out-degree (LME, $t = 7.625$, $P < 0.001$), betweenness (LME, $t = 3.534$, $P < 0.001$) and closeness (LME, $t = 3.851$, $P < 0.001$) post-infection (Fig. 6.1). In contrast, the network metrics of the control populations remained unchanged post-infection (mean weighted in-degree: LME, $t = 0.230$, $P =$

0.8182; weighted out-degree: LME, $t = 0.235$, $P = 0.814$; betweenness: LME, $t = -0.124$, $P = 0.9009$ and closeness: LME, $t = 0.255$, $P = 0.7986$).

Individuals infected with *Gyrodactylus turnbulli* significantly increased their rank within their respective shoals (LMM, $t = 5.867$, $P < 0.001$). Generally, the most and least connected individuals and their associated shoalmates occupied consistent shoal ranks, with respect to centrality indices, post-infection (Table 6.1); indicating that the introduction of parasites stabilised network structure. Rankings were random in the parasite-free control populations, with the exception of one of five populations, where rankings became stable for three of the four metrics both before and after a sham infection (Table 6.1). Although the addition of parasites stabilised social ranks with respect to out-degree, betweenness and closeness, the in-degree rankings were more inconsistent, and were not stabilised by parasitism. Thus highlighting variability in the number of contacts received by individuals (Table 6.1).
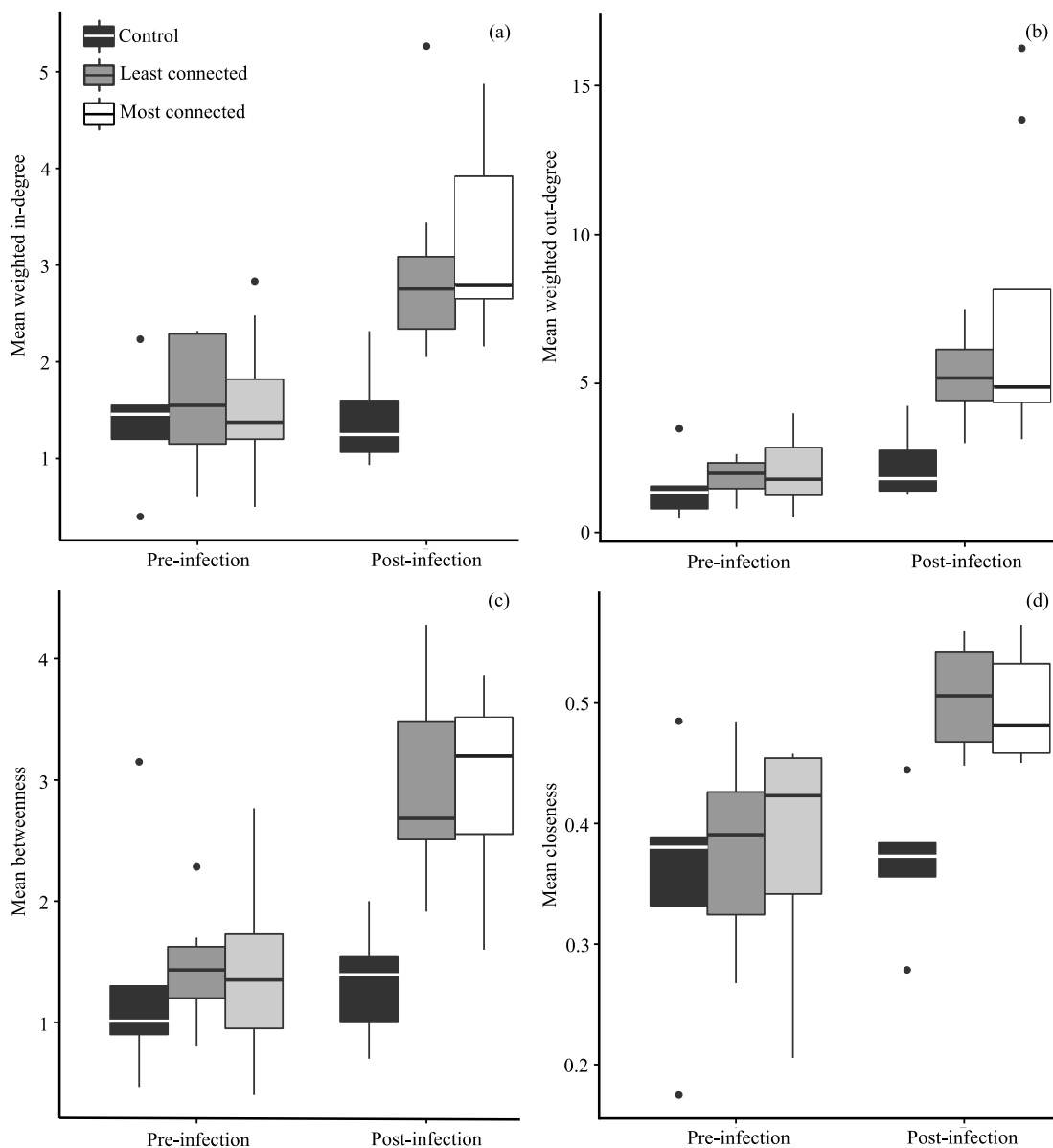
FIGURE 6.1: Patterns in mean (a) weighted in-degree, (b) weighted out-degree, (c) betweenness and (d) closeness centrality indices pre- and post-infection with *Gyrodactylus turnbulli* across treatments. Black shading represents control shoals, not exposed to parasites (n=5 shoals); dark grey, shoals where infection was initiated in the least connected individual (n=8 shoals); white, shoals where infection was initiated in the most connected individual (n=7 shoals). Black dots represent outliers; bars the upper and lower limits; the box the first and third quartile with the median. Note the difference in axis scales.

TABLE 6.1: Summary of the consistency in guppy social rank based on weighted in- and out-degree, betweenness and closeness scores pre- (Days 1-5) and post- (Days 6-10) *Gyrodactylus turnbulli* network perturbation for three experimental treatments: control, most connected infected and least connected infected. Significant *P* values <0.05 (highlighted in grey) indicate consistency in network ranking between days.

Shoal ID	Treatment	Weighted in-degree		Weighted out-degree		Betweenness		Closeness	
		Before (Days 1-5)	After (Days 6-10)	Before (Days 1-5)	After (Days 6-10)	Before (Days 1-5)	After (Days 6-10)	Before (Days 1-5)	After (Days 6-10)
H	Control	0.8482	0.3846	0.0750	0.5498	0.0659	0.8254	0.1716	0.9737
K	Control	0.0710	0.1880	0.2563	0.1540	0.1210	0.2686	0.2951	0.1791
O	Control	0.2728	0.5296	0.0600	0.0289	0.0315	0.0393	0.0325	0.0123
P	Control	0.1137	0.4451	0.1215	0.4512	0.1840	0.2899	0.2537	0.3027
T	Control	0.9985	0.5799	0.5694	0.7457	0.6057	0.4143	0.4488	0.2168
Aa	Least	0.2474	0.0096	0.5890	0.0154	0.0998	<0.0001	0.2216	<0.0001
G	Least	0.4334	0.8467	0.7681	0.2119	0.5846	0.4245	0.7952	0.1614
J	Least	0.8716	0.0020	0.7717	0.0012	0.6440	0.0080	0.4551	0.0027
N	Least	0.8133	0.1961	0.5181	0.0029	0.6794	0.0026	0.5742	<0.0001
Q	Least	0.6125	0.4154	0.1883	<0.0001	0.2382	<0.0001	0.2303	<0.0001
U	Least	0.7254	0.3756	0.3797	0.0364	0.3043	0.0590	0.3288	0.0130
Y	Least	0.0524	0.2204	0.3458	0.0013	0.6416	<0.0001	0.6291	<0.0001
Z	Least	0.2722	0.7155	0.2892	<0.001	0.3964	<0.0001	0.1536	<0.0001
I	Most	0.9830	0.3806	0.1611	<0.0001	0.1185	<0.0001	0.1758	<0.0001
M	Most	0.0168	0.1513	0.4547	<0.0001	0.5566	0.0034	0.5522	0.0034
R	Most	0.0096	0.0902	0.4056	0.0020	0.4674	0.0019	0.4635	0.0026
S	Most	0.6538	0.1954	0.3448	0.0023	0.2963	<0.0001	0.5863	<0.0001
V	Most	<0.0001	0.0457	0.0027	<0.0001	0.0321	<0.0001	0.0055	0.0073
W	Most	0.0353	0.1985	0.7229	0.0026	0.7831	0.0013	0.6905	0.005
X	Most	0.984	0.6978	0.6760	0.0447	0.5129	0.2770	0.9273	0.1083

There was an overall increase in weighted in- and out-degree of uninfected and *G. turnbulli* infected fish, respectively, in comparison to control populations (Fig. 6.2). Specifically, a greater weighted out-degree of infected individuals indicated potential superspreaders of infection. Generally, uninfected individuals avoided making contact with conspecifics post-infection.
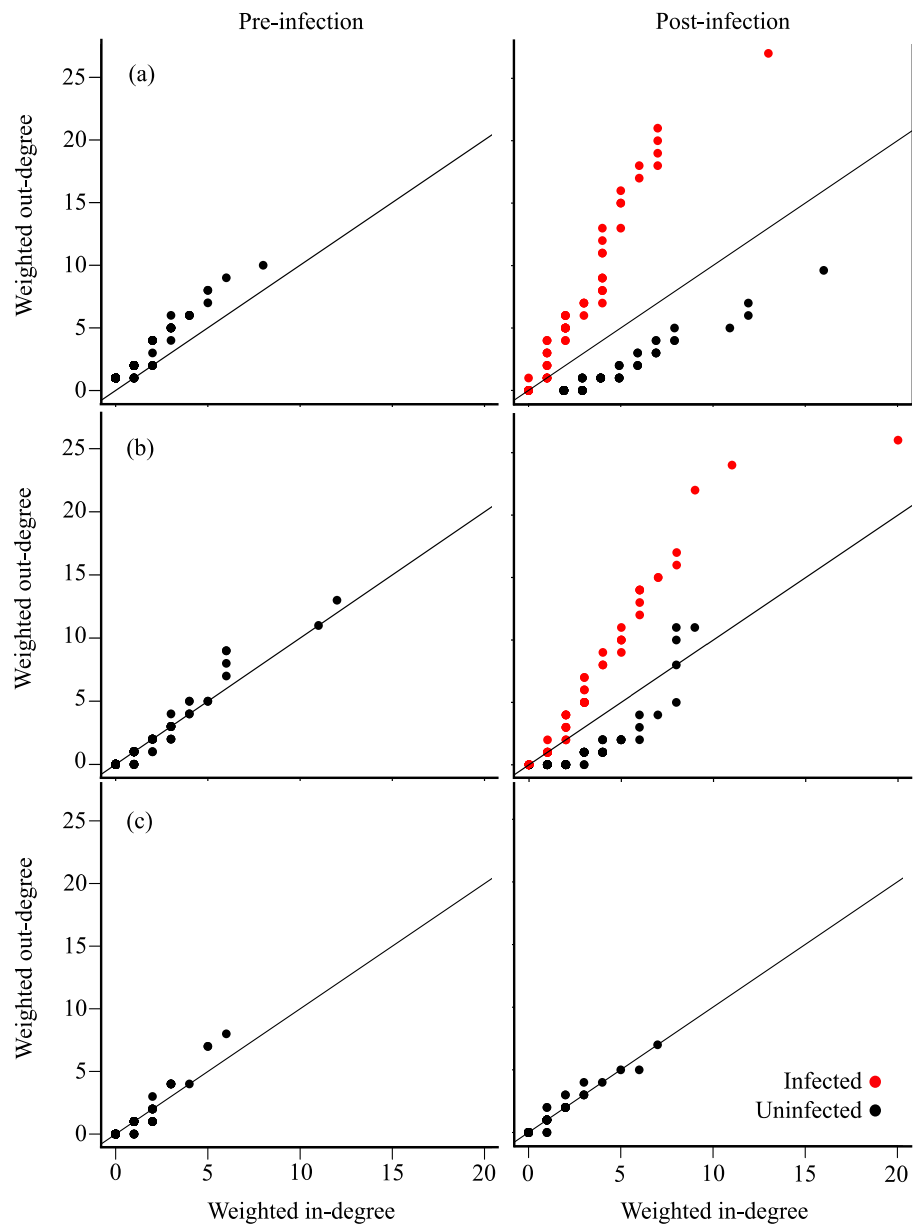
FIGURE 6.2: Quantile-quantile plots comparing in- and out-degree strength of uninfected (black) and *Gyrodactylus turnbulli* infected (red) Trinidadian guppies, pre- and post-parasite perturbation of a network, for three experimental treatments: (a) most connected, (b) least connected and (c) control. Plotted straight line is the 0:1 reference line.

4.5   Discussion

This study assessed social network properties pre- and post-infection, using the Trinidadian guppy-*Gyrodactylus turnbulli* host-parasite system, to determine if parasites drive social network dynamics. Irrespective of whether the most or least connected host individuals were targeted with infection, there was a significant increase in network indices (weighted in- and out-degree, betweenness and closeness) post-infection, compared to control shoals. Upon infection with *G. turnbulli,* hosts significantly increased their social rank to occupy, along with their respective shoal mates, consistent network positions post-infection with respect to centrality indices. The introduction or parasites therefore stabilised a populations' social structure. Additionally, the resulting network dynamics were driven by a parasite-mediated behavioural response of the hosts: infected fish instigated more contacts than they received (i.e. presented higher weighted out- than in-degree), in contrast to uninfected control fish. Thus indicating that these individuals potentially act as superspreaders of infection.

A population's social structure influences disease transmission dynamics (e.g. Ryan et al. 2013; Springer et al. 2017). Social clustering, for example, can delay and/or confine an epidemic (Sah et al. 2017). Yet, studies often overlook the reciprocity of parasitism driving temporal variation in social network properties. Consequently, interactions between network structure and transmission processes are often over-simplified. This study demonstrated how parasite infection altered social network structure by instigating behavioural modifications in hosts. Specifically, infected individuals significantly increased their social rank, owing to increased direct contact frequencies with shoalmates. These individuals, in contrast to their uninfected conspecifics, presented higher weighted out- than in-degree: governed by an adaptive behavioural

response to infection presumably aimed at 'offloading' parasite burdens (Chapter 4). Should the infection process have been monitored over an extended period, greater network 'rewiring' may have been observed, particularly as individuals modify their social preferences by avoiding heavily infected conspecifics (see Kavaliers et al. 2005; Stephenson et al. 2016; Poirotte et al. 2017).

Given that almost every aspect of an individual's behaviour can be associated with parasite exposure (see Ezenwa et al. 2016), it is surprising that reciprocal network structure-parasite feedback mechanisms are so poorly understood. Typically, it is assumed that a higher contact rate between hosts enhances parasite transmission (Moller et al. 1993), with centrality indices mediating an individual's infection risk (e.g. Leu et al. 2010). Transmission processes are, however, complex, whereby the nature of behavioural interactions between individuals is particularly important in determining the directionality of disease transmission. Drewe (2010), for example, demonstrated that in a meerkat society, the most socially interactive animals were not necessarily at the highest risk of infection. Instead, meerkats that regularly groomed others were at greatest risk of orally acquiring tuberculosis infection, irrespective of their overall contact frequency in the network (Drewe 2010). Additionally, it is important to consider how parasite related traits influence transmission dynamics (see Stephenson et al. 2017). The precarious transmission strategy of *Gyrodactylus turnbulli,* for example, can result in parasite dislodgement and mortality (see Scott and Anderson 1984). In the early stages of infection, when the parasite infrapopulation and competition for resources is low, the transmission risk may outweigh the benefits of locating a new host. Thus, highly connected infected individuals are not necessarily superspreaders of infection if their parasites lack a transmission propensity.

The costs of parasitism on sociality can, to a certain degree, be offset through changes in host behaviour (e.g. selecting alternative sleeping sites; Reckardt and Kerth 2007). This itself underpins temporal variability in social structure. In the current study, and contrary to predictions, a strengthening in network metrics was observed post-parasite perturbation. There is growing evidence whereby social resilience mitigates the impacts of network perturbation (see Wilson et al. 2015). For example, the overall connectedness of a great tit, *Parus major*, population significantly increased in response to the loss of conspecifics (Firth et al. 2017). Such compensatory behaviour is predicted to sustain antipredatory defences and information sharing amongst conspecifics regarding food sources (Aplin et al. 2012; Firth et al. 2017). The resulting social structure could, however, also have important implications in facilitating disease spread, as shown following culling events of facial tumour infected Tasmanian devils (Beeton and McCallum 2011), bTB infected badgers (Weber et al. 2013), and rabid vampire bats (Streicker et al. 2012). Within the current fish-parasite system, by increasing contacts with conspecifics, infected individuals may benefit by diluting their parasites amongst potential hosts (see Mooring and Hart 2002).

To conclude, this study demonstrates how parasites significantly influence social network dynamics. A significant increase in centrality parameters was observed post-infection, irrespective of whether infection was seeded in the most or least connected individuals. Infected hosts significantly increased their social rank: a parasite-mediated behavioural modification of hosts that led to them instigating more contacts than they received, presumably to offload parasite burdens. An initial rewiring of social structure in response to parasite perturbation was followed by a period of network stability. The short- and long-term behaviour of a system may differ considerably given that infection can substantially mediate host sociality, and thus further network rewiring may be

expected in the later stages of an epidemic. Whereas most network epidemiology studies demonstrate how the social structure of a group determines disease transmission at a given time point, the current study highlights a duality in this interaction; whereby infection itself also drives temporal variation in network structure. The failure to measure and integrate ecological, social and transmission elements temporally may subsequently lead to inaccurate and misleading predictions of disease transmission processes.

4.6   Acknowledgements

Chapter 7

General Discussion

7.1   Summary

This thesis has focused on three research areas: Firstly, how environmental variability, specifically in thermal and hydrological regimes, impact freshwater host-parasite interactions; secondly, how parasite infections mediate host behavioural modifications; and finally, how such behavioural changes have population level effects with respect to social structuring. The key findings in this thesis demonstrate that (a) fish alter their thermal preferences to combat parasite infection (Chapter 2), (b) variable flow conditions modify host sociality with implications for disease transmission (Chapter 3), (c) infected hosts perform parasite-mediated behavioural responses to infection presumably aimed at offloading parasite burdens (Chapter 4), and (d) parasite infection instigates nocturnal restlessness in hosts, with potential repercussions for host health (Chapter 5). Perhaps the most important finding to emerge from this research is the significance of parasites in driving temporal variation in a populations' social structure (Chapter 6), whereas previous work primarily focused on how static networks influence disease transmission processes.

7.2   Environmental change and freshwater host-parasite interactions

Environmental variations, specifically in thermal and hydrological regimes, are predicted to have profound impacts on freshwater host-parasite interactions (reviewed in Marcogliese 2008). Thermal elevations, for example, are often presumed to favour parasites over their hosts by reducing life-cycle completion rates (Poulin 2000), expanding distributional ranges (Lafferty 2009), and in some cases reducing host immunocompetence (Pounds et al. 2006). However, host behavioural plasticity can mitigate the severity of parasite infections, as shown here in Chapter 2. Using the

Trinidadian guppy-*Gyrodactylus turnbulli* host-parasite system, this study demonstrated how hosts benefit from elevated temperatures, at a cost to their parasites. Specifically, by frequenting warmer thermal conditions outside of the parasites thermal optima, fish could self-medicate against infection. Previous research has demonstrated the immunological benefits of warmth in up regulating host immune responses (Reynolds et al. 1976), whilst extreme thermal conditions can physiologically impair parasites. In an increasingly warming environment, ectotherms including fish may therefore benefit in terms of rapid activation of immune responses to infection, until conditions become stressful.

Mitigating thermal stress through physiological plasticity or evolutionary adaptation is essential for species persistence in a changing environment. Thermal elevations impose a selection pressure for acclimation in both parasites and their hosts. The acclimation capabilities of parasites are predicted to exceed that of their hosts (Raffel et al. 2013) given their small size and faster metabolisms (Gillooly et al. 2001; West et al. 2002; Brown et al. 2004). Advances in molecular technologies have enabled the precise mechanisms underpinning thermal acclimation to be investigated (e.g. Oksala et al. 2014; Morley et al. 2017). In the future, assessing genomic variation of parasites over time will elucidate links between host- and environmental-induced selection pressures on evolutionary adaptations of parasites (preliminary data for *G. turnbulli* presented here in Appendix B).

The effects of climate change on aquatic disease are usually framed primarily in terms of an increase in temperature. Additional abiotic factors, such as precipitation, cause significant hydrological variations with resulting impacts on host-parasite interactions. Understanding how river hydraulics influence fish disease is particularly important

considering that infections substantially modify host population dynamics (Peeler et al. 2006). It is often difficult to disentangle, however, the effects of environmental variability from parasitism on host behaviours. In Chapter 3, for example, parasitism significantly reduced the cohesiveness of fish shoals, but only in the absence of flowing water. The benefits of shoaling in an energetically demanding, flowing environment may therefore outweigh the costs of potentially acquiring parasite infection. Furthermore, despite shoaling in close proximity to infected conspecifics, infection risk may be reduced as parasites become dislodged in a flowing environment. Parasite transmission was greatest in shoals exposed to intermittent flow representative of spate conditions that are likely to increase in magnitude and frequency with proposed climate change. Within the wild, seasonal rains displace infected fish downstream (see van Oosterhout et al. 2007), thus potentially spreading gyrodactylid parasites between populations. In the future, it is essential that fish health professionals, river managers and fish farmers recognise complex reciprocities between environmental change, host behaviour and their associated pathogens, to mitigate the severe socioeconomic costs of disease epidemics. Whilst some of these questions can be addressed using model systems under controlled laboratory conditions, it is essential future studies corroborate laboratory experiments with field observations. For example, whilst the effects of flow and parasitism on host behaviour could be disentangled in this study, in natural environments additional abiotic factors including water turbidity, pollution and predation pressure add further complexity to host-parasite interaction outcomes (Marcogliese and Pietrock 2011; Gheorgui et al. 2012; Stephenson et al. 2015). It is only following an integrative experimental approach that the significance of biotic and abiotic variables in mediating host-parasite interactions can be achieved, and effective disease control strategies subsequently implemented. For example, adjusting stocking conditions (Amend 1976), small-scale habitat manipulation (Thompson 2011),

transitioning from flow-through to water reuse systems (Summerfelt et al. 2004), introducing effective bio-control (Verschuere et al. 2000), and the strategic timing of fish stocking to reduce pathogen exposure (Chiaramonte et al. 2016).

7.3   *Host behavioural modifications to infection*

Chapters 2 and 3 demonstrated how hosts modified their behaviour in response to infection. Firstly, by adapting thermal preferences to combat parasite infection and secondly increasing shoaling distances between conspecifics to reduce infection risk in static environments. During these behavioural trials it became apparent that *G. turnbulli* infected guppies increased their body contact with conspecifics (anecdotal evidence in Croft et al. 2011; Stephenson et al. 2017): a unique behavior performed only by infected fish presumably to offload parasite burdens. However, adopting this behaviour proved ineffective in reducing infection intensities during the early stages of infection (Chapter 4), highlighting that transmission processes are mediated by a complexity of factors from both host and parasite perspectives (Ezenwa et al. 2016; Stephenson et al. 2017). Indeed, offloading parasites onto conspecifics could be beneficial considering the severe pathology of gyrodactylid infection (Bakke et al. 2007), and could also serve to vaccinate recipient hosts (Faria et al. 2010). Extension of the study period until the point of host recovery, in conjunction with quantification of host behaviour and immune parameters, would elucidate the significance of host and parasite-mediated traits in determining transmission amongst hosts.

Parasite infections were also shown to mediate the diurnal activity of hosts (Chapter 5). The guppy-*Gyrodactylus turnbulli* and three-spined stickleback-*Argulus foliaceus* host-parasite systems were used to show how infected fish were significantly more active during nocturnal hours than their uninfected conspecifics. The mechanisms underlying

such observations are likely associated with the irritative feeding ecology of these ectoparasites, particularly in the early stages of infection. During prolonged infections, complex interactions between host hormonal and immunological parameters may also contribute to nocturnal host restlessness (Bryant et al. 2004; Scheff et al. 2010), with significant repercussions for host health. This forms the premise for future research into the effects of infection on host circadian rhythms. Specifically, how the interplay between circadian gene expression, which drives immune modulation (Besedovsky et al. 2012; Scheiermann et al. 2013), and host pathogens can impact future disease susceptibility. An understanding of these interactions would be particularly important in acquaculture, where fish stocks often endure prolonged light periods to maximise growth (Boeuf and Bail 1999). Such conditions could have profound impacts on fish circadian rhythmicity, and thus immune modulation.

Finally, this thesis showed how parasite-mediated behavioural changes had substantial population level impacts with respect to social structuring (Chapter 6). Infected individuals significantly increased their social rank to subsequently sustain, along with their respective shoalmates, consistent network positions. Contrary to predictions, the introduction of parasites stabilised a population's social structure. Similarly, social ties between individuals strengthened following physical manipulation of guppy habitats, indicating that guppies work actively against changes in their social dynamics (Krause et al. 2017). Given that *G. turnbulli* infection intensities are typically low in wild populations (see Appendix A), the benefits of social stability (e.g. enhanced information transfer of predators and food resources; Swaney et al. 2001) may outweigh the costs of potentially acquiring and enduring a low intensity infection. Such network analysis offers an invaluable tool for understanding and managing disease transmission in both domesticated and wild animals. A thorough understanding of a systems parasite

ecology, host behaviour and social dynamics is necessary for developing disease control strategies. In wildlife studies, for example, reactive culling of individuals, with disregard to a population's social structure and an incomplete understanding of the system of interest, proved ineffective in restricting disease spread (e.g. Beeton and McCallum 2011; Streicker et al. 2012; Weber et al. 2013). It is important to note that both behaviour and the infection process are dynamic. Infection itself often induces host behavioural changes (Chapter 2, 3, 4 and 5) that ultimately determine network topology (Chapter 6), and so driving dynamics of infectious disease. Thus, the collection of high-resolution network data from before, during and after a management intervention is necessary to quantify its effectiveness in preventing disease spread.

7.4 Conclusion

This thesis has used an integrative approach to assess how environmental variation, host sociality and behavioural modifications influence disease transmission dynamics. Whilst the Trinidadian guppy-*Gyrodactylus turnbulli* host-parasite system is extensively used here, the application of understanding is transferrable to other disease systems. Epidemiological networks, for example, can be applied to any animal group to model disease spread amongst and between populations, and make predictions of how disease may altar population dynamics. This thesis also highlights the need of a thorough understanding of the host-parasite system in question so to identify normal from abnormal behaviours that have significance in disease transmission processes. Parasites play an integral role in ecological systems by mediating population and food web dynamics (Cattadori et al. 2005; Lafferty et al. 2006; Britton 2013). Advances in molecular tools will be instrumental in revealing the mechanisms underpinning evolutionary adaptations of parasites and their hosts necessary for sustaining ecological integrity in changing environments.

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Appendix A

Assessing the effects of natural parasitism on the swimming performance of two coexisting poeciliid hosts[7]

A.1 Abstract

Multi-species fish assemblages may promote the dispersal of generalist parasites amongst potential hosts. The resulting impacts of each parasite taxon on its host are often investigated independently, with additive or synergistic effects of co-infection often overlooked. The current study therefore examines the impact of two coexisting parasite taxa on swimming endurance and predatory escape responses of two freshwater poeciliids, *Poecilia reticulata* and *Poecilia picta,* from sympatric and allopatric populations. Although a positive association was observed between *Gyrodactylus* and digenean intensities, hosts naturally infected with one, or both parasites, showed no significant impediment of swimming endurance or predatory escape responses. The prevalence and intensities of these parasites differed significantly between allopatric and sympatric populations: potentially driven, both directly and indirectly, by environmental salinity. This study reveals that natural parasitism levels do not necessarily cause detectable behavioural impacts on their fish hosts, and highlights how co-occurring parasites can influence one another.

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A.2   Introduction

Forming multi-species assemblages can provide anti-predatory and foraging benefits, which enhances survival (Sridhar et al. 2009; Srinivasan et al. 2010; Goodale et al. 2015). Conversely, such groups can promote the establishment success of invasive species (Camacho-Cervantes et al. 2014) and the dispersal of generalist parasites (Dargent et al. 2013). Exposure to a greater diversity of parasite taxa, each imposing variable pathology, may result in host physiological and behavioural modifications. For social hosts, such as fish, a wealth of literature demonstrates how naturally infected individuals exhibit atypical behaviours (e.g. changes in foraging: Godin and Sproul 1987; swimming performance: van Oosterhout et al. 2007). Such behavioural modifications not only compromise a host's survival, but also significantly influence disease propagation within natural populations.

Interactions between parasite taxa can have additive or synergistic impacts on a host. For example, wild three-spined sticklebacks, *Gasterosteus aculeatus,* harbouring *Schistocephalus solidus* pleroceroid larvae have heavier *Gyrodactylus* spp., hereafter *Gyrodactylus*, infections than fish without these cestodes (Barber and Scharsack 2010). The pathological consequence of gyrodactylid infection, including fin clamping, impairs host swimming performance (Cable et al. 2002), whereas *S. solidus* morphologically distorts the host's body and impedes fast-start escape responses, increasing its visibility and vulnerability to avian predators, respectively (Blake et al. 2006). Gyrodactylid infections also have significant negative impacts on their hosts (reviewed in Bakke et al. 2007), specifically swimming performance (see van Oosterhout et al. 2007; Stephenson et al. 2016), yet previous experimental work utilising these pathogens rarely considers how co-existing parasites might contribute to these observable host costs.

The current study focuses on two common poeciliid species, the freshwater Trinidadian guppy *Poecilia reticulata* and the brackish-dwelling swamp guppy *Poecilia picta,* which occur in both allopatry and sympatry. Being closely related species (Breden et al. 1999), these fish harbour similar parasite communities (see Cable et al. 2005; Dargent et al. 2013). Whilst there is limited ecological research focussing on *P. picta*, *P. reticulata* has been extensively studied in both the field and laboratory, and is a popular model organism for investigating epidemiology (Suanyuk et al. 2013; Smallbone et al. 2016b), behaviour (Templeton and Shriner 2003) and evolutionary ecology (O'Steen et al. 2002; Crispo et al. 2006). Previous studies utilising the *P. reticulata* system have focussed on how parasites from a single genus, *Gyrodactylus*, affect host life history and behavioural traits (e.g. van Oosterhout et al. 2003; Gotanda et al. 2013). This study tests a previous assumption that other common parasite taxa do not significantly affect host locomotion. Specifically, this study assesses how independent and co-occurring *Gyrodactylus* and digenean infections impact host swimming endurance and predatory escape responses. These two parasite taxa were the most dominant parasite groups identified infecting fish from the current study.

A.3   Materials and Methods

A.3.1   *Fish collection and ectoparasite screen*

A total of 170 *Poecilia reticulata* (standard length: 8.0 - 26.2 mm) and 134 *P. picta* (standard length: 10.1 - 26.2 mm) were collected from nine sites across five rivers in Tobago, June 2015 (Table A.1), from both allopatric (*P. reticulata* or *P. picta*) and sympatric (*P. reticulata* and *P. picta*) populations. Using seine nets (1 m x 1 m, mesh size 0.5 mm), fish were herded into small pools from which they were scooped up in a bucket containing 20 L river water (26±1 ºC), thus preventing parasite dislodgment through net contact, and transported to the laboratory. The presence and absence of

native and invasive snail species was recorded at each site, as well as water salinity (AquaCheck Salt Pool Test Strips) and water condition, categorised as either static or flowing; specific flow measurements were not attempted due to high spatial and temporal variability. Within 2 h post capture, individual fish were anaesthetised using 0.02% tricaine methanesulfonate (MS222) and screened to quantify ectoparasite infection intensities using a dissection microscope with fibre optic illumination. Each fish was then isolated in 250 ml river water (26±1 ℃) and the predatory escape response and swimming endurance tested within a 12 h period in outside external arenas close to their native rivers.

TABLE A.1: A summary of the sampling locations, fish (*Poecilia picta* and *Poecilia reticulata*) sample sizes, presence of native or invasive* snail species and water state (flow or static) from allopatric and sympatric populations captured across 9 sampling sites from five rivers in Tobago, June 2015.

Site	Grid Reference	Water salinity (ppm)	Water state	Present native or invasive* snail species	Sample size (n)	
					P.picta	*P.reticulata*
Dog River:						
DR1	0760013, 1242737	<380	Flow	*Tarebia granifera** *Neratina clenchi*	-	31
DR2	0760346, 1242080	<380	Flow	*Tarebia granifera**	29	30
DR3	0760812, 1242136	<380	Flow	*Tarebia granifera**	29	-
Golden Grove:						
GG1	0739713, 1235510	<380	Static	*Tarebia granifera* *Melanoides tuberculata** *Pomacea diffusa** *Pomacea urceus* *Marisa cornuarietis*	29	30
Goldsborough:						
G1	757935, 1240942	<380	Static	*Tarebia granifera**	2	27
G2	0757529, 1241553	<380	Static	*Tarebia granifera**	-	26
G3	758771, 1240639	<380	Static	*Tarebia granifera**	27	5
Lambeau: L1	743314, 1234311	4060	Static	NA	18	-
Speyside: S1	768653, 125453	<380	Flow	*Tarebia granifera** *Neratina clenchi*	-	21

A.3.2   Predatory escape response

The C-start predatory escape response of 75 *P. reticulata* and 45 *P. picta* was assessed during exposure to a startle stimulus within an experimental arena. The experimental setup comprised a white plastic tray (20 x 15 x 2 cm) filled with 3 cm depth river water at 26±1 ℃. At the beginning of a trial, each fish was transferred to the arena for a 5 min acclimation period before exposure to a novel stimulus: a 2 x 2 cm pebble dropped from 22 cm above the arena into the center of the tray. The C-start escape response of an individual was recorded using a Qumox SJ4000 HDDV camera, programmed at 30 frames per second, suspended in a central position above the arena. Video analyses were conducted using the computer software Tracker (Version 4.90), whereby each fish' velocity (mm s^{-1}) was calculated using the distance a test fish travelled (mm) during 30 ms immediately after stimulus exposure. Fish were returned to their 250 ml containers for a 10 min recovery period prior to swimming endurance trials.

A.3.3   Swimming endurance

A total of 90 *P. reticulata* and 50 *P. picta* were subject to swimming endurance tests, including all individuals that had previously been assessed for their predatory escape response. Swimming endurance was tested in an artificial flume (200 cm length x 4.5 cm width x 5 cm depth: Fig. A.1), with a flow rate of 42.9 ± 7 cm s^{-1}, calculated by measuring the mean time for a neutrally buoyant ball to drift 2 m for 10 replicates. This flow rate exceeded natural flow conditions, ensuring that test fish exhausted during a trial. A removable net at the terminal flume end prevented fish from entering the collection sump following placement into the flume. The net was removed following a 5 s period, which provided sufficient time for a fish to initiate rheotaxis (swimming into the flow).
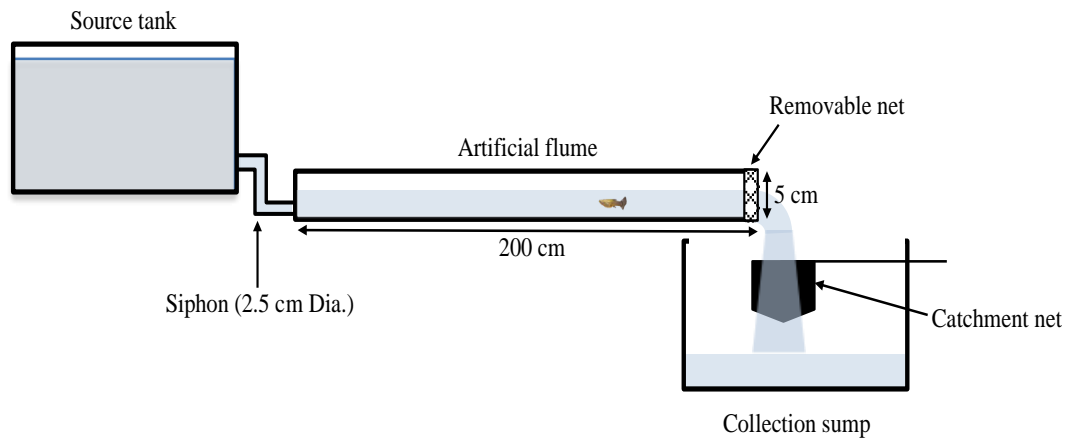
FIGURE A.1: 2D schematic of the artificial flume used to assess the effects of *Gyrodactylus* and digenean metacercariae intensities on the swimming endurance of *Poecilia reticulata* and *Poecilia picta* in Tobago. A 2 cm flow depth was maintained by recirculating water from the collection sump into the source tank.

During each trial, the time (seconds) fish spent swimming into the flow was recorded, and each trial terminated when a focal individual was washed out of the flume. Each fish was trialled five consecutive times to ensure the fish exited the flume due to exhaustion as opposed to exploratory behaviour. Swimming endurance was calculated by summing the total time (s) fish spent swimming into the flow during five consecutive trials.

A.3.4 Endoparasite screen

Following escape response and swimming endurance trials, each fish captured during the study was sacrificed using MS222 and dissected to record their parasite fauna. Parasites were identified to genus, with the exception of the digenean metacercariae larvae (hereafter digenean), which were not categorised beyond subclass taxon level, and it is predicted that several species were included within this grouping. Fish were measured according to their standard length (SL, mm) prior to removal of their internal organs, gills, eyes and exploration of the body cavity, which were screened to quantify

the prevalence and intensity of endoparasitic infections. Hosts were infected with at least eight parasite taxa representing five phyla. Subsequent analysis, however, focuses on the two most prevalent taxa: monogenean gyrodactylids (mean intensity: *P. reticulata* = 2.94, range: 1-14; *P. picta* = 4.39, range: 1-30) and digenean metacercariae (mean intensity: *P. reticulata* = 29.98, range: 1-128; *P. picta* = 32.51, range: 1-338).

A.3.5 *Statistical analysis*

Statistical analyses were conducted using R statistical software (version 3. 1. 3, R Development Core Team 2009). Using data of every fish collected during the trial, variables affecting both *Gyrodactylus* and digenean intensity were analysed using separate Generalised linear models (GLMs) with negative binomial distribution using the MASS package (Venables and Ripley 2002) and the glm.nb function with "log" link. Model robustness was assessed using residual plots. The dependent terms in these models were (1) *Gyrodactylus* intensity and (2) digenean intensity (the two most dominant parasite taxa in the current study), with fixed terms including host species (*P. reticulata* or *P. picta*), host SL, host sex, sampling site, the dominant species at the site (*P. reticulata*, *P. picta* or sympatric), and interactions between host SL x host sex, (1) digenean intensity x host SL, and (2) *Gyrodactylus* intensity x host SL. All terms were included in the original model. The model was refined using AIC goodness of fit, where individual variables were dropped independently and AIC identified. A model was classified as being more efficient when the AIC was > 4 less than that of the original model (Burnham et al. 2002; Richards et al. 2011). Using ΔAIC > 7 likely over estimates the number of variables that should remain in the model, whereas a ΔAIC > 2 under estimates important variables that should be retained. Therefore, the current analysis used an intermediate of ΔAIC > 4. The original models for both GLMs were the most appropriate and therefore all terms were retained.

GLMs were used to investigate the effects of *Gyrodactylus* and digenean intensity on the predatory escape response and swimming endurance of *P. reticulata* and *P. picta.* These analyses were performed on all fish that underwent swimming performance trials. As these individuals were tested prior to full parasite screens, their infection status at the time was unknown. Thus, fish with one, both or neither parasites were tested. Two global models were constructed, with Gaussian family and log error functions, and their robustness assessed using residual plots. The dependant terms in these models were (1) fish velocity and (2) swimming endurance, with fixed terms including *Gyrodactylus* intensity, digenean intensity, host species (*P. reticulata* or *P. picta*), host SL, host sex and sampling site. Interactions between host SL x *Gyrodactylus* intensity, host SL x digenean intensity, host sex x *Gyrodactylus* intensity, host sex x digenean intensity, host species x *Gyrodactylus* intensity, host species x digenean intensity and *Gyrodactylus* intensity x digenean intensity were also included in the models.

During model refinement, several equally well-supported models were identified based on comparisons of Akaike's Information Criterion (AIC). Thus, an information theoretic approach to multi-model inference was employed to assess the relative importance of each independent term in influencing dependent variables in each GLM (following methods in Burnham and Anderson 2002). As variables in each 'global' GLM were measured on different scales, model parameters were standardised to a mean of 0 and standard deviation 0.5 using the *arm* library (Gelman and Su 2013). The 'dredge' function within the *MuMIn* package (Bartoń 2014) was then used to generate a set of 'top' models, which fell within 4 AICc of the best model (see Burnham and Anderson 2002). Averaged parameter estimates from this top set of models were then calculated using the 'model.avg' function, and the relative importance of each parameter generated by summing the Akaike weights across the models in which the parameter occurred (Burnham and Anderson 2002). The closer the importance value

was to one, the greater relative importance that variable had in comparison to other model variables (Burnham and Anderson 2002). The variables were considered significant if the 95% confidence intervals did not bound zero.

A. 4   Results

A.4.1   *Gyrodactylus* intensity

Poecilia reticulata were infected with significantly lower gyrodactylid mean intensity than *P. picta* (Table A.2; Model 1). Significant site differences in *Gyrodactylus* intensity were also observed (Table A.2; Model 1), with River Lambeau (highly saline site; Table A.1) *P. picta* populations harbouring the lowest intensities. Allopatric populations had lower *Gyrodactylus* intensities compared to sympatric populations (Fig. A.2; Table A.2: Model 1). For both fish species, larger fish exhibited greater *Gyrodactylus* intensities. The interaction between digenean intensity and fish standard length did not significantly affect *Gyrodactylus* intensity (Table A.2: Model 1).

TABLE A.2: ANOVA model output assessing the factors influencing (1) *Gyrodactylus* and (2) digenean metacercariae intensity of *Poecilia reticulata* and *Poecilia picta.* Highlighted predictors indicate *P* values <0.05 and are therefore considered significant.

Model	Dependant term	Fixed term	df	F	P
1	*Gyrodactylus* intensity	Digenean intensity	1	120.107	≤0.001
		Host species	1	25.530	≤0.001
		Host standard length	1	11.849	≤0.001
		Host Sex	1	0.052	0.819
		Dominant host species	2	53.574	≤0.001
		Site	6	67.189	≤0.001
		Host standard length x Host sex	1	1.379	0.240
		Host standard length x digenean	1	0.196	0.658
2	Digenean intensity	*Gyrodactylus* intensity	1	141.991	≤0.001
		Host species	1	9.112	0.003
		Host standard length	1	153.146	≤0.001
		Host sex	1	0.700	0.403
		Dominant host species	2	26.653	≤0.001
		Site	6	23.410	≤0.001
		Host standard length x Host sex	1	3.795	0.051
		Host standard length x *Gyrodactylus*	1	5.512	0.019

A.4.2   Digenean metacercariae intensity

Significantly higher digenean intensities were observed infecting *Poecilia reticulata* than *P. picta* (Table A.2: Model 2), and similarly to *Gyrodactylus*, there were significant differences in digenean intensities between sites (Table A.2: Model 2). Allopatric *P. picta* populations harboured significantly lower metacercariae than those from *P. reticulata* only and sympatric populations (Fig. A.2, Table A.2: Model 2). Specifically, Lambeau *P. picta* harboured the least digeneans; likely associated with the absence of intermediate snail hosts (Table A.1). The interaction between *Gyrodactylus* intensity and host SL significantly affected digenean intensity, whereby larger fish harbouring more *Gyrodactylus* had significantly higher digenean intensities (Table A.2: Model 2).
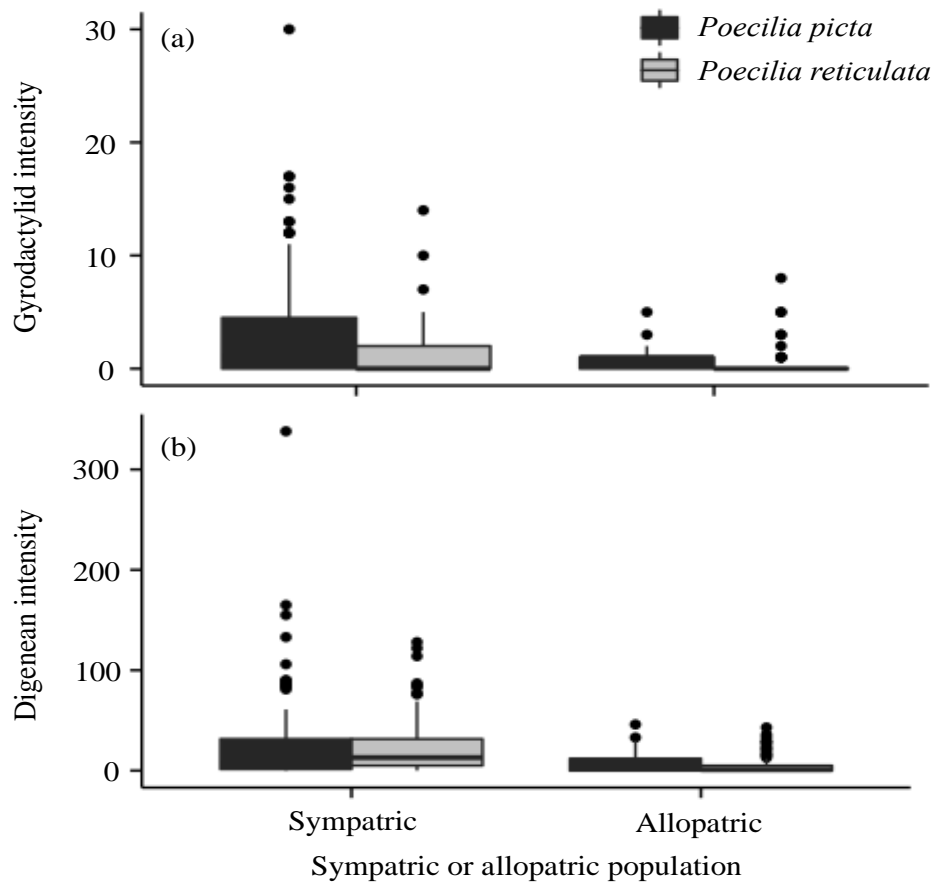

FIGURE A.2: Variation in (a) gyrodactylid, and (b) digenean intensity infecting *Poecilia reticulata* (grey) and *Poecilia picta* (black) in allopatric and sympatric sites. Black dots represent outliers; bars the upper and lower limits; the box the first and third quartile with median.

A.4.3   Predatory escape response

The predatory escape responses for hosts at each site are summarised in Table A.3. There was no significant correlation of *Gyrodactylus* or digenean intensity with the predatory escape responses of *P. reticulata* or *P. picta*. There were observed differences in escape responses between host species and sex, whereby *P. reticulata* exhibited a faster escape response than *P. picta* (Table A.4: Model 1). Additionally, female fish had quicker escape responses than males, irrespective of species (Table A.4: Model 2). There were also significant differences in predatory escape responses between sites (Table A.4: Model 2).

TABLE A.3: A summary of the mean swimming velocity and mean total time to exhaustion of *Poecilia picta* and *Poecilia reticulata* from allopatric and sympatric populations from 9 sampling sites across 5 rivers (Dog River, Goldsborough, Lambeau, Speyside and Golden Grove) in Tobago 2015.

	Site	Fish species	
		Poecilia picta	*Poecilia reticulata*
Predatory Escape Response: Mean swimming velocity (mm s^{-1} (range))	Dog River 1	-	0.47 (0.14 - 0.88)
	Dog River 2	0.39 (0.13 - 0.68)	0.51 (0.17 - 1.21)
	Dog River 3	0.40 (0.10 - 0.74)	-
	Goldsborough 1	-	0.67 (0.33 - 1.62)
	Goldsborough 2	-	0.59 (0.20 - 0.94)
	Goldsborough 3	0.34 (0.13 – 0.74)	0.48 (0.31 - 0.76)
	Speyside 1	-	0.33 (0.09 - 0.49)
Swimming Endurance: Mean total time to exhaustion (s(range))	Dog River 1	-	294.05 (34 - 1007)
	Dog River 2	88.75 (24 - 307)	185.26 (27 - 753)
	Dog River 3	69.42 (25 - 307)	-
	Goldsborough 1	-	111.28 (25 - 421)
	Goldsborough 2	-	90.83 (25 - 478)
	Goldsborough 3	85.76 (25 - 279)	103 (28 - 230)
	Speyside 1	-	65.79 (25 - 245)

A.4.4   Swimming endurance

There was no significant correlation of *Gyrodactylus* or digenean intensity with the swimming endurance of either host species. The most important variables influencing fish swimming endurance included sampling site (Table A.4), host SL, species and whether a fish originated from a site with flowing water (Table A.4: Model 2). Fish obtained from flowing environments had a significantly greater swimming endurance (mean ± SEM = 146.10 s ± 14.52, n=88) than those from static environments (mean ± SEM = 102.85 s ± 15.62, n=52; Fig. A.3). There was a positive association between swimming endurance and host SL for both species, with *P. reticulata* exhibiting significantly greater swimming endurance than *P. picta* (Table A.4; Fig. A.3).
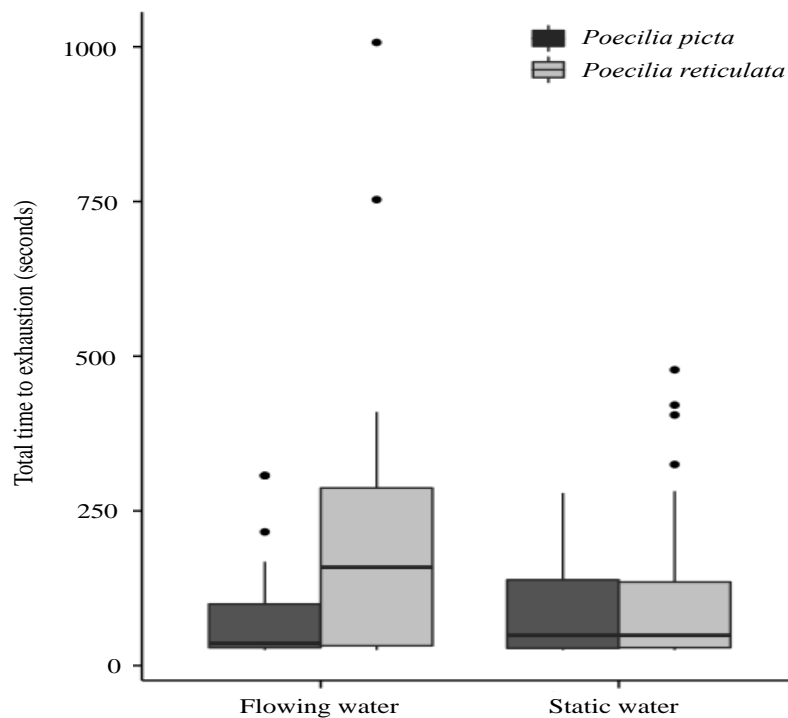

FIGURE A.3: Total swimming endurance of *Poecilia picta* (dark grey) and *P. reticulata* (light grey) originating from static or flowing water conditions. Black dots represent outliers; bars the upper and lower limits; the box the first and third quartile with median.

Model	Dependant Variable	Variable	Estimate	Adjusted SE	95% CI	Relative Importance
1	Predatory escape response	Intercept	0.46	0.07	0.32 – 0.60	
		Site:				1.00
		Dog River 2	-0.03	0.08	-0.19 – 0.14	
		Dog River 3	-0.06	0.12	-0.29 – 0.17	
		Goldsborough 1	0.21	0.09	0.03 – 0.38	
		Goldsborough 2	0.11	0.09	-0.06 – 0.29	
		Goldsborough 3	-0.07	0.10	-0.27 – 0.14	
		Speyside 1	-0.36	0.12	-0.59 - -0.13	
		Host species	0.12	0.07	-0.03 – 0.27	0.67
		Host standard length	0.00	0.00	-0.12 – 0.12	0.28
		Gyrodactylus intensity	0.02	0.05	-0.09 – 0.13	0.42
		Digenean intensity	-0.01	0.06	-0.13 – 0.11	0.35
		Host species x *Gyrodactylus* intensity	0.17	0.10	-0.04 – 0.38	0.16
		Host species x Digenean intensity	0.14	0.13	-0.11 – 0.40	0.08
		Host standard length x *Gyrodactylus* intensity	-0.22	0.14	-0.50 – 0.06	0.08
		Host standard length x Digenean intensity	-0.09	0.08	-0.24 – 0.06	0.01
		Host sex	-0.09	0.05	-0.18 – 0.01*	0.73
		Host sex x Host species	0.00	0.09	-0.20 – 0.19	0.08
		Host sex x *Gyrodactylus* intensity	-0.03	0.11	-0.25 – 0.19	0.05
		Host sex x Digenean intensity	0.14	0.14	-0.14 – 0.42	0.07
		Digenean intensity x *Gyrodactylus* intensity	0.25	0.17	-0.08 – 0.58	0.04
2	Swimming endurance	Intercept	242.53	38.57	166.94 – 318.12	
		Site:				1.00
		Dog River 2	-103.57	42.34	-186.56 - -20.58	
		Dog River 3	-176.92	60.79	-296.08 - -57.77	

Goldsborough 1	-142.71	89.36	-317.85 – 32.42	
Goldsborough 2	-142.17	89.00	-316.59 – 32.26	
Goldsborough 3	-139.94	56.60	-250.88 - -29.00	
Speyside 1	-194.47	49.68	-291.85 - -97.10	
Host species	81.08	38.68	5.28 – 156.88	0.88
Host standard length	77.71	29.01	20.85 – 134.57	1.00
Gyrodactylus intensity	-43.65	27.09	-96.75 – 9.45	0.73
Flow condition	-139.94	56.60	-250.88 - -29.00	0.50
Host species x *Gyrodactylus* intensity	-73.5	50.51	-172.49 – 25.49	0.35
Host standard length x *Gyrodactylus* intensity	40.69	49.65	-56.61 – 138.00	0.16
Host sex	-21.93	24.32	-69.61 – 25.49	0.28
Digenean intensity	14.70	32.65	-49.30 – 78.70	0.25
Digenean intensity x *Gyrodactylus* intensity	74.18	52.62	-28.94 – 177.30	0.08
Host sex x *Gyrodactylus* intensity	-27.06	53.01	-130.96 – 76.83	0.02

TABLE A.4: Summary of the standardised averaged model predictors assessing the affects of parasitism on the predatory escape response and swimming endurance of *Poecilia reticulata* and *Poecilia picta* from 9 sampling sites across 5 rivers (Dog River, Goldsborough, Lambeau, Speyside and Golden Grove) in Tobago 2015. Highlighted predictors indicate confidence intervals that do not bound zero and are therefore considered significant. CI = confidence intervals, SE = standard error. *Denotes approaching significance.

A.5 Discussion

The current study demonstrates that whilst the presence of particular parasites is important in influencing the co-occurrence of other parasite taxa, they do not necessarily have negative measurable impacts on a host. In this system, despite a positive association between two common parasites, *Gyrodactylus* and digeneans, the coexistence of these taxa did not significantly impede host predatory escape response or swimming endurance.

When hosts are infected with multiple parasite taxa, the efficacy and efficiency of specific immune responses towards individual infections may be reduced. Such immunosuppression by an initial parasite may increase host susceptibility to secondary pathogen infections (Busch et al. 2003; Bandilla et al. 2006; Pylkkö et al. 2006), which can be further enhanced following epidermal injury induced by the primary parasite. For example, epidermal damage caused by the feeding activity of *Gyrodactylus niloticus* on Nile tilapia (*Oreochromis niloticus*) increased host susceptibility to secondary streptococcal infections, resulting in significant mortality (Xu et al. 2007). Similarly, the current study showed that larger fish with higher *Gyrodactylus* intensities harboured more digeneans. This may be due to increased tegumental damage caused by *Gyrodactylus* feeding on the epidermal layer.

In addition to interactions between parasite taxa, environmental variation can significantly influence host-parasite associations (Smallbone et al. 2016a; Chapter 2). In the current study, a reduction in *Gyrodactylus* and digenean infections were observed within brackish environments, e.g. *P. picta* from Lambeau, whereby salinity was much higher than other sites. Salinity directly impedes some *Gyrodactylus* spp. (Bakke et al. 2007) and may also be population specific (Dargent et al. 2013). Additionally, salinity

may have indirectly influenced digenean populations by mediating intermediate host distributions (El-Darsh and Whitfield 1999).

Whilst parasitism can impede fish station holding in flowing rivers (van Oosterhout et al. 2007) and increase predation vulnerability in guppies (Stephenson et al. 2016), no observable impacts of natural base-line parasitism on either of these traits were found, consistent with the findings of Binning et al. (2014). This is not to say, however, that these low parasite intensities do not impact other traits including feeding, growth rates, mate choice and reproductive success. Individuals with particularly high parasite burdens may have already been removed from the population; either through predation or displacement downstream (van Oosterhout et al. 2007). Thus, only the 'healthiest' individuals, capable of tolerating low-level infections remaining below a threshold level for detectable impacts, were sampled in the current study.

A difference was observed, however, in swimming abilities between fish caught within static and flowing environments. Fish originating from standing water had significantly poorer swimming endurance compared to those inhabiting lotic environments, supporting Mohammed et al. (2014). Swimming performance is associated with the evolution of morphological attributes that enhance and optimise locomotion, particularly in energetically demanding environments. Fish in high-flow environments, for example, often evolve streamlined, stiff, muscular bodies with a larger caudal fin for propulsion (Langerhans 2008; Langerhans and Reznick 2009). Although such morphometrics were not collected in the current study, it may be that upstream guppies, which are frequently exposed to spate conditions associated with seasonal rains, may express such phenotypes, which aid more pronounced rheotaxis reducing their displacement probability (Mohammed et al. 2014).

Irrespective of host species, females and larger fish had quicker predatory escape responses with these differences likely due to a greater investment by females, compared to males, in anti-predatory strategies e.g. shoaling (Griffiths and Magurran 1997b) and growth (females are larger than males in the wild; Stephenson et al. 2015). Larger fish benefit in terms of enhanced swimming ability from a bigger caudal fin and reduced energy expenditure than smaller conspecifics (Videler and Wardle 1991; Breen et al. 2004). Furthermore, variation in predation regimes between different environments is likely to select for desirable traits that enhance survival in terms of escape responses. Female fish are likely under stronger selection pressures to evolve such traits considering they exhibit greater reproductive investment than males (Onjanguren et al. 2005).

To conclude, the current study shows how natural levels of parasitism do not have measurable consequences on host swimming endurance and predatory escape responses. Differences in *Gyrodactylus* and digenean intensities between allopatric and sympatric populations are likely driven, both directly and indirectly, by environmental variability in salinity (as in Dargent et al. 2013). Finally, positive associations between *Gyrodactylus* and digenean infections highlight the importance of considering coinfection when investigating host-parasite interactions.

A.6   Acknowledgements

Appendix B

Micro-evolutionary patterns of genetic variation and

virulence in the parasite *Gyrodactylus turnbulli*[8]

B.1   Abstract

Advances in comparative genomics have revolutionised the study of parasite evolution with respect to revealing molecular mechanisms underpinning adaptive traits. Annotating parasite gene repertoires can identify genetic polymorphisms relating to virulence, but also mutations important for subverting host immunity. The current study uses the Trinidadian guppy (*Poecilia reticulata*) - *Gyrodactylus turnbulli* host-parasite system to assess changes in *G. turnbulli* virulence and genotype frequencies during prolonged laboratory culture (2004-2016). Experimental infections conducted at three different points (2004, 200xx and 2016) showed a significant increase in parasite population growth rate, used here as a proxy for virulence. Annotation of the assembled parasite genomes predicted 14,367 gene regions. A total number of 19,581 single nucleotide polymorphisms (SNPs), of which 5,877 were found within 788 gene regions. Structural gene annotation between time points is on going. Links between parasite micro-evolutionary processes and increased virulence may be elucidated following functional annotation of the identified gene regions. In the future, comparative genomics of the parasite will be married with that of the host, aimed at revealing the precise molecular mechanisms involved in this host-parasite co-evolutionary arms race.

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[8] *This is an on going collaborative project between Michael Reynolds, Amy Ellison, and Jo Cable (Cardiff University), Justin Pachebat (Aberystwyth University) and Christoph Hahn (University of Graz). Willow Smallbone (Cardiff University) will provide data on the host. This preliminary report is included here to demonstrate a wider skill set achieved during this PhD.*

B.2   Introduction

All aspects of free-living organisms' ecology, behaviour and evolutionary processes are, to some degree, mediated by parasite exposure (Ezenwa et al. 2016). Additionally, parasites fulfil significant roles in moderating biodiversity and ecosystem functioning (Lafferty et al. 2006; Kuris et al. 2008; Hatcher et al. 2012). Their evolutionary origins have become a core interest in evolutionary biology given their importance in all ecological systems (reviewed in Jackson 2015). The development of molecular phylogenetics, for example, has made it possible to investigate parasite diversification (Moran and Poulin 2003), and advances in comparative genomics have revolutionised studies into the development of parasite complexity and host-parasite co-evolution (Otto et al. 2014; Laanto et al. 2017). Furthermore, annotating parasite gene repertoires can reveal the genetic basis underpinning adaptation (e.g. Miles et al. 2016; Wang et al. 2016), including micro-evolutionary mechanisms for subverting a host's immune response (Kent et al. 2005; Finley and McFadden 2006).

An antagonistic host-parasite co-evolutionary arms race exists between pathogen infectivity and host immunity (reviewed in Brockhurst et al. 2014). Under experimental conditions, for example, bacteriophages drive the diversity and dynamics of co-evolving bacterial populations (Bohannan and Lenski 2000; Buckling and Rainey 2002), whilst also showing a remarkable capacity for rapidly overcoming host immunity (Scanlan et al. 2010). The ability to infect hosts with pathogens under controlled conditions is an important tool in the study of disease, and often involves culturing pathogens *in vitro*. Such techniques provide experimental ease and flexibility compared to the use of pathogens that must first be isolated from naturally infected hosts. However, cultured pathogens can demonstrate a loss of virulence over time (see Ford et al 2002; Moreira et al. 2012; Petterson et al. 2015), and thus do not necessarily retain the same traits as

when occurring naturally. Maintaining a culture *in vivo,* if feasible, could therefore mitigate physiological modifications to a parasite of interest.

An iconic laboratory model for investigating host-parasite co-evolutionary mechanisms is the Trinidadian guppy (*Poecilia reticulata*) and its associated ectoparasite, *Gyrodactylus turnbulli.* These small (<1 mm) viviparous monogeneans exhibit short generation times (24 h at 24 °C; Scott and Nokes 1984), resulting in explosive population growth dynamics. Consequently, intense *G. turnbulli* infections impose severe pathology (see Cone and Odense 1984; Cable and van Oosterhout 2007) and ultimately host mortality (Scott and Anderson 1984). In practical terms, these parasites can be readily cultured on guppies, from which subsequent demographic trends can be monitored *in situ* at repeated time points, without the need to sacrifice a host (as in Smallbone et al. 2016; Mohammed et al. 2016). Additionally, evolutionary rates of the parasites are likely to exceed that of their hosts due to their short generation times; a general trend observed in other parasite taxa (Downton and Austin 1995; Light and Hafner 2007; Kochin et al. 2010). As a result, *G. turnbulli* may demonstrate rapid adaptive capabilities to both biotic and abiotic selection pressures. Using full genome sequencing, this study investigates the effects of long-term culture on genomic variation in *Gyrodactylus turnbulli* infecting Trinidadian guppies. Specifically, this study aims to (a) compare *G. turnbulli* micro-evolutionary processes at four time points between 2004 and 2016, (b) use trajectory experiments to assess changes in *G. turnbulli* virulence between these time points, and (c) elucidate links between parasite micro-evolutionary processes and shifts in virulence over time.

B.3 Materials and Methods

B.3.1 Gyrodactylus turnbulli culture

Parasite cultures (strain Gt3) were descendants of a single *Gyrodactylus turnbulli* worm isolated from an ornamental guppy in 1997, and subsequently maintained on naïve fish in 'culture pots' under standard conditions (24 °C; 12 h light: 12 h dark photoperiod). Each culture pot contained a minimum of four fish, collectively infected with *ca.* 40 gyrodactylid worms, which were subsidised biweekly with naïve fry to prevent parasite extinction. At four time points: March 2004, December 2008, December 2015 and June 2016, individual worms were harvested from the culture to form four pooled samples used for subsequent genetic analysis (see Table B.1).

TABLE B.1: A summary of the number of individual *Gyrodactylus turnbulli* worms harvested from a single strain at each time point, the total gDNA extract obtained from each pooled sample and sonication length of extracted gDNA.

Sub-culture	No. pooled worms	DNA extracted (ng gDNA)	Sonication (bp)
March 2004	335	184	400
December 2008	400	168	400
December 2015	300	293.4	400
June 2016	1000	326.4	400

B.3.2 DNA extraction and sequencing

Genomic DNA was extracted from the pooled *Gyrodactylus turnbulli* samples using the DNeasy blood and tissue kit (Qiagen, USA), according to the manufacturers protocol. Extract concentration was measured using a Qubit™ 3.0 Flourometer; confirming a mass of >100ng gDNA per sample necessary for subsequent library preparation. gDNA was quality assessed using an Agilent 4200 TapeStation system and sonicated to a desired fragment size of 400bp using a Covaris M220 Ultrasonic sample processor. Fragmented gDNA was sent to Aberystwyth University for TruSeq® Nano DNA Library prep and Illumina HiSeq 2500 sequencing.

B.3.3   Gyrodactylus turnbulli de novo genome assembly

Illmunia reads from each time point were pooled and trimmed (Trimmomatic; Bolger et al. 2014), from which putative non-target contigs were then removed prior to genome assembly. *De novo* assembly algorithms are continually developing and improving, and there are a variety of programmes now available. The most appropriate assembler depends on the genome in question. Here, a range of assemblers including ABySS (Simpson et al. 2001), Velvet 1.2.07 (Zerbino and Birney 2008), SparseAssembler (Ye et al. 2012), and SOAPdenovo (Luo et al. 2012) were used to assemble reads and produce a reference genome. Each assembler was run on a range of kmer values, from which the assembler that produced the highest N50 value was considered the most appropriate. Furthermore, the number and length of contigs produced by each assembler was compared to determine the most appropriate algorithm; in this case the draft genome assembled in Velvet 1.2.07 was best. Using BUSCO (Simão et al. 2015), the completeness of the draft reference genome was assessed.

B.3.4   Mapping reads to reference genome

Using PopPoolation2 (Kolfer et al. 2011), reads from each time point were mapped to the reference genome generated above. Ambiguously mapped reads were removed and single nucleotide polymorphisms (SNPs) called to calculate allele frequency differences between time points.

B.3.5   Gyrodactylus turnbulli trajectory experiment

Parasite trajectories on guppies were performed using descendants of the same ornamental fish population and parasite culture established in 1997, in March 2004 (n = 14; 7 male, 7 female), September 2004 (n = 10; 4 male, 6 female) and August 2016 (n = 31; 16 male, 15 female). Experimental infections entailed sacrificing a heavily infected

culture fish (>200 worms) and transferring two Gt3 worms via direct contact onto the caudal fin of a parasite-naïve sexually mature recipient fish, temporarily sedated using 0.02% trichaine methanesulfonate (MS222). Infected recipient fish were subsequently maintained in individual 1 L dechlorinated water and screened every other day, using a dissection microscope with fibre optic illumination, for a 17-day period to record *Gyrodactylus* intensity; defined as the total number of parasites infecting a host. The parasites demographic trajectory was used a proxy for virulence (as in Cable and van Oosterhout 2007), considering that positive correlations exist between a parasites reproductive rate and virulence (Elbert and Mangin 1997).

B.3.6   *Gyrodactylus turnbulli trajectory analysis*

All statistical analyses were performed using R statistical software. Using the *glmmADMB* package (Bolker *et al.* 2012), a Generalised Linear Mixed Effects Model (GLMM) was used to assess the significance of host standard length, sex, trajectory year and time (days since initial infection) on *G. turnbulli* intensity. An interaction between trajectory year and time was incorporated into the model, and fish identity included as a random factor to account for repeated measures. The model was fitted with a negative binomial family and log link structure, and refined through stepwise deletions of non-significant terms (Crawley 2007). Fish length was removed from the model, as it did not explain significant variation in *G. turnbulli* intensity. Only significant terms are reported here.

B.4   Preliminary results

B.4.1   *Genome sequencing and gene prediction*

Of the four genome assemblers tested, Velvet 1.2.07 produced the best draft assembly of *Gyrodactylus turnbulli.* The draft genome comprised 20,877 contigs, with a mean contig length of 3,827 (range = 201-341244) and N50 43668. The gene space was

predicted 80.9% complete and structural gene annotation predicted a total of 14,367 genes. A total number of 19,581 SNPs were identified, of which 5877 were found within 788 gene regions. Structural gene annotation between time points is on going.

B.4.2   *Gyrodactylus turnbulli experimental infections*

There was a significant interaction between infection day and year on *Gyrodactylus turnbulli* mean intensity (Day x year interaction, Table B.2). Specifically, higher *Gyrodactylus turnbulli* intensities were reached in August 2016 compared to March 2004 (GLMM: $z = 4.00$, SE = 1.03, $P < 0.001$) and September 2004 (GLMM: $z = 2.27$, SE = 1.13, $P = 0.023$). Similarly, *G. turnbulli* reached significantly higher intensities in September 2004 than March 2004 (GLMM: $z = 2.76$, SE = 0.568, $P = 0.005$). Parasite intensities increased over time (Day main effect, Table 1), peaking on days 11 and 15 for 2004 and 2016 trajectories, respectively (Fig. B.1).

TABLE B.2: GLMM output of variables explaining variation in Trinidadian guppy *Gyrodactylus turnbulli* intensity over a 17-day trajectory experiment. The values reported are from the removal point of non-significant terms from the model. Significant terms are highlighted in grey.

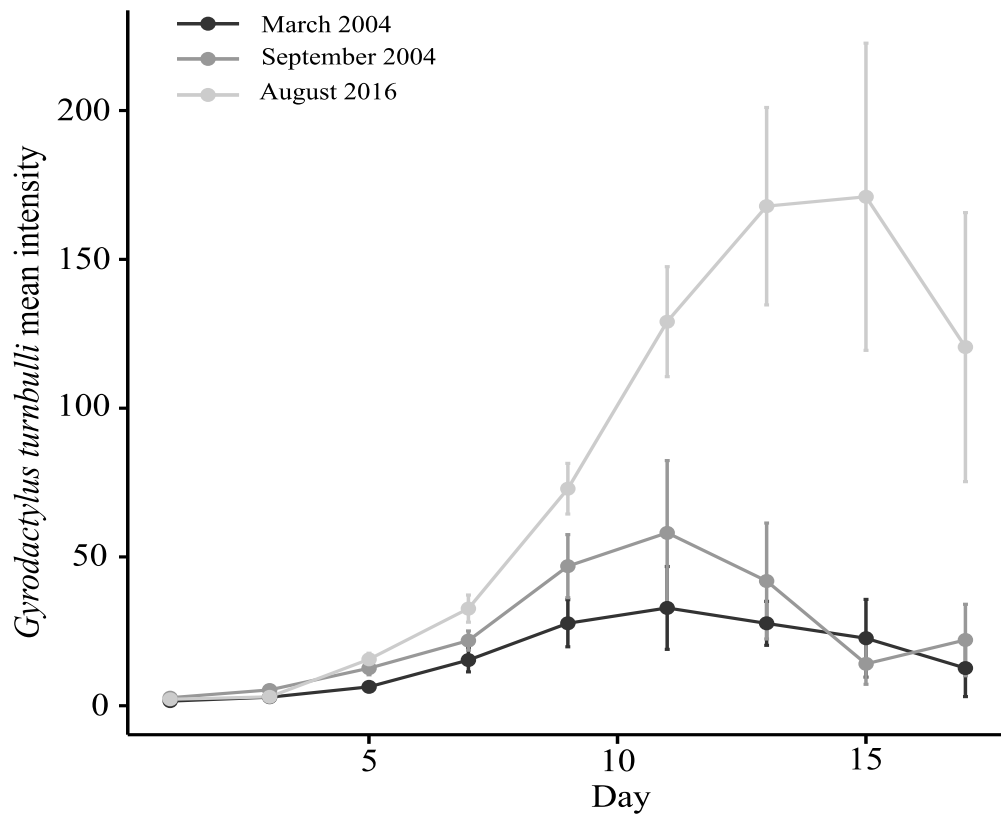| Variable	*df*	χ^2	*P* value
Day	1	56.0314	<0.0001
Fish SL	1	0.0825	0.7739
Sex	1	0.7608	0.3930
Year	2	70.7676	<0.0001
Day x Year	2	9.1699	0.0102
Fish SL x Year	2	0.1624	0.9220

FIGURE B.1: Trends in *Gyrodactylus turnbulli* intensity (mean ± SE) on Trinidadian guppies (*Poecilia reticulata*) at three time points: March 2004 (red), September 2004 (green) and August 2016 (blue).

B.4   Discussion

The current study presents preliminary evidence for changes in *Gyrodactylus turnbulli* genotype frequencies over time. Once functionally annotated, these results may elucidate links between parasite micro-evolutionary processes and shifts in virulence over time.

Trajectory data revealed that *G. turnbulli* reproductive rate, used here as a proxy for virulence, significantly increased over time. Parasite intensities in August 2016 reached *ca.* x3 fold higher compared to when ancestry host-parasite populations were used in a 2004 trajectories. Both host- and parasite mediated traits likely contributed to this

observation. Firstly, the longevity in which *G. turnbulli* had been in culture likely increased its virulence, as previously observed in serial passage experiments (see Locher et al. 2003; Chapuis et al. 2011). Repeat passage of a pathogen through the same host species can increase its virulence to that host, whilst attenuating virulence to alternative hosts (Albert 1998). As cultures were subsidised biweekly with naïve hosts, parasites would not have been exposed to antagonistic selection pressures, such as host immunity, which would have otherwise compromised the parasites reproductive ability, infectivity and transmissibility (see Cable and van Oosterhout 2007a).

Guppies show both innate and acquired immune responses to *G. turnbulli* infection (Cable and van Oosterhout 2007b). The innate immune response is probably activated at the onset of infection (van Oosterhout et al. 2008). Evidence for acquired resistance is typically observed 7-11 days post initial infection, whereby the parasite population begins to decline, as seen here in 2004 experimental trajectories. A decline in *G. turnbulli* intensity was not observed until day 15 in August 2016. Furthermore, parasites in this year reached significantly higher intensities compared to 2004. Considering the experimental fish were descendants of a population founded in 1997, an inbreeding depression may have resulted in increased susceptibility to *G. turnbulli* infection. Indeed, it has been shown that 'inbred' guppies are significantly more susceptible to infection in comparison to wild-type outbred fish (Smallbone et al. 2016). Here, a delayed reduction in the parasite population indicated that these potentially highly inbred individuals do retain an immune response to infection.

To summarise, this study provides experimental evidence for increased virulence in *G. turnbulli* over time. Experimental 2016 fish were more susceptible to infection compared to their 2004 ancestors, potentially associated with an inbreeding depression

and thus reduced immune efficiency. A total of 5877 SNPs were identified in 788 gene regions of the parasites genome. Following functional annotation of these genes, it is hoped that links between parasite micro-evolutionary processes and increased virulence may be revealed. In the future, comparative genomics of the parasite will be married with that of the host, aimed at revealing the precise molecular mechanisms involved in this host-parasite co-evolutionary arms race.