Exposure to a putative chemical cue from *Gyrodactylus*-infected guppies alters behaviour and parasite intensity

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Abstract

In aquatic systems animals cannot rely on visual cues to gain information about their environment as water turbidity and water clarity continuously change. Many aquatic animals thus rely on chemicals in the water to forage, mate and perceive various threats. One threat which has often been overlooked in the literature is the threat of parasitism. Although parasitism does not cause immediate injury or death like predation, parasites can reduce overall fitness of aquatic animals. Evidence for the reliance of chemical communication in anti-predator behaviours has been well established, however only a few studies have observed the use of chemical cues for mitigating risk of parasitism. Furthermore, the link between chemical infection cues and future susceptibility to infections has not been studied. We aimed to determine if exposure to *Gyrodactylus turnbulli*-infected guppies (*Poecilia reticulata*) resulted in altered behaviour of uninfected conspecifics, and if prior exposure to this putative infection cue resulted in reduced transmission. Overall, the behavioural response to chemical cues released at mid and late stages of infection was subtle, inconsistent, and somewhat counterintuitive. Guppies spent less time in the center of the tank versus the periphery, indicating that fish responded to a chemical cue when it was added to the experimental tank, however, we did not detect any response in traditionally measured variables of stress such as freezing, darting, and sinking to the bottom of the tank. Males responded to the putative infection cues released by fish at later stages of infection by taking longer to start moving at the beginning of the trial. Exposure to continuous infection cues did not impact the overall behavioural activity of shoals, however, shoals exposed to putative infection from late stages of infection had a shorter inter-fish distance, which differs from previous research indicating that guppies preferentially avoid other fish when infection is added to the experimental set up. When guppies were experimentally infected after prior exposure to the chemical infection cue for 16 days, there was no impact on the epidemic prevalence over 40 days but the intensity was significantly reduced and epidemiological patterns of *G.turnbulli* infections in the shoal were significantly different. Together, these results demonstrate that guppies may respond to short-term and ongoing infection cues by altering specific behaviours, and that infection cues may reduce the intensity of *G.turnbulli* epidemics.

Résumé

Dans les systèmes aquatiques, les animaux ne peuvent pas se fier à des indices visuels pour obtenir de l'information sur leur environnement, car la turbidité et la clarté de l'eau changent constamment. De nombreux animaux aquatiques dépendent donc des produits chimiques présents dans l'eau pour trouver de la nourriture, s'accoupler et percevoir divers dangers. Une menace qui a souvent été négligée dans la littérature est le parasitisme. Bien que les parasites ne causent pas de blessures immédiates comme des prédateurs, ils peuvent causer une réduction importante de la performance biologique. La communication chimique dans les comportements anti-prédateur a été bien démontré dans la littérature scientifique, mais seulement quelques études ont observé l'utilisation des signaux chimiques pour atténuer le risque de parasitisme. De plus, le lien entre ces signaux d'infection chimique et la transmission des parasites n'a pas été étudié. Nous avons cherché à déterminer si les signaux chimiques des guppys (*Poecilia reticulata*) infectées par *Gyrodactylus turnbulli* provoque une modification du comportement des poissons non infectés et si ces signaux chimiques entraînent une réduction de la transmission. Dans l'ensemble, la réponse comportementale aux signaux chimiques libérés au milieu et à la fin des stades de l'infection était subtile, incohérente et contre-intuitive. Les guppys exposés à des signaux émis par des poisson infectés ont passé moins de temps au centre de l'aquarium que dans la périphérie. Cependant, nous n'avons décelé aucune réaction dans les variables de stress mesurées traditionnellement, comme le gel, le dard et le naufrage au fond du réservoir. Les mâles ont répondu aux signaux d'infection présumé émis par les poissons à des stades d'infection avancés en prenant plus de temps pour commencer à se déplacer au début de l'essai. Des bancs exposés à des signaux chimiques émis par des guppys dans les stades avancés de leur infection présentaient une distance plus courte entre les poissons, qui diffère des recherches précédentes indiquant que les guppies évitent les autres poissons lorsqu'une infection est ajoutée à l'installation expérimentale. Lorsque les guppies ont été infectées expérimentalement après une exposition antérieure aux signaux d'infection chimique pendant 16 jours, il n'y a eu aucun impact sur la prévalence de l'épidémie sur 40 jours, mais l'intensité a été considérablement réduite et les tendances épidémiologiques des infections à *G.turnbulli* étaient significativement différentes. Ensemble, ces résultats démontrent que les guppies peuvent réagir aux signes d'infection à court terme et en cours en modifiant quelques comportements spécifiques, et que des signaux chimiques d'infection peuvent réduire l'intensité des épidémies de *G.turnbulli*.

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Contribution of Authors

Katrina Di Bacco is the author of this manuscript-based thesis, written as per the guidelines of McGill University. The literature cited is included at the end of each chapter in this thesis. Dr. Marilyn E. Scott, my supervisor, is the co-author. I designed the study along with the help of Dr. Marilyn E. Scott. I was responsible for collecting, analyzing, and interpreting the data which included recording fish behaviour, analyzing the behaviour on an animal tracking software, monitoring parasitic infections on fish, and performing statistical analysis. I wrote the thesis with the comments and edits of Dr. Marilyn E. Scott. Dr. Andrew Hendry and Dr. Pierre Olivier Montiglio provided feedback on the study design and data interpretation.

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CHAPTER I – Introduction

In aquatic environments, gaining accurate information about the current state of the environment can allow a fish to appropriately allocate their energy (Godin and Smith 1988). However, fish which cannot gain information on when they are facing danger may be preyed upon and fish that inaccurately perceive threat of predation even when not in danger, will inappropriately allocate energy away from vital activities such as foraging or reproduction (Brown 2003; Lima and Bednekoff 1999). Fish cannot always rely on visual cues to detect danger as turbidity and sedimentation influence water clarity (Chivers et al. 2012; Hickman et al. 2004; McCormick and Manassa 2008). Thus, fish have evolved additional ways to gain reliable information about their surroundings, one of which is the reliance on chemical communication (Chivers et al. 2012).

Chemical communication is the process whereby one individual releases a chemical into the surrounding area, known as a chemical cue, and another individual perceives this chemical and may respond to it (Brönmark and Hansson 2012). A classic example is sex pheromone signaling, where one individual will release a chemical cue to attract or locate a mate and an individual of the opposite sex may respond and mate with the cue emitter (Breithaupt and Hardege 2012). Other well studied chemical cues are predator alarm cues, which are the chemicals released from punctured cells of injured fish into the surrounding water and elicit behavioural changes in conspecifics so they can evade predation (Mirza and Chivers 2003).

Predation and disease are the leading non-anthropogenic factors resulting in fish reduction of fitness (Huntingford et al. 2006; Raffel et al. 2008). Parasitism, although not as immediately fatal as predation, causes substantial mortality and loss of foraging and reproductive opportunities (Raffel et al. 2008). Avoiding parasites and pathogens is beneficial in increasing species survival, however, there may be negative trade-offs in avoiding infection such as increasing predation risk (Behringer et al. 2018; Wisenden et al. 2009). Therefore, gaining reliable information about infection risk at a certain time and location allows a fish to better balance these trade-offs (Behringer et al. 2018). In the case of ectoparasites, fish may be able to see large parasites or damaged skin on an infected conspecific or recognize altered behaviours especially at later stages of infections (Behringer et al. 2018). However, they may not be able to see signs of infection when the parasite is small, or if the infected conspecific does not yet show physical or behavioural symptoms of the disease (Rahn et al. 2015). In these cases, the most reliable information about a conspecific's infection status may be from chemical cues (Brönmark and Hansson 2012).

Research on behavioural responses to putative chemical infection cues is limited to a few examples. Juvenile rainbow trout (*Oncorhynchus mykiss*) altered their behaviour when exposed to chemical cues released while trematode cercariae penetrate the skin of conspecifics (Poulin et al. 1999). Bullfrog (*Rana catesbeiana*) tadpoles actively avoided the chemical cues from conspecifics that are infected with a fungal pathogen (Kiesecker et al. 1999). Finally, guppies (*Poecilia reticulata*) preferentially swam where chemical cues from healthy individuals have been released and avoid water containing chemical cues from individuals with a *G.turnbulli* infection (Stephenson et al. 2018).

The ectoparasite *Gyrodactylus* spp. is a small (<1mm) monogenean worm which is directly transmitted from one fish to another by physical contact and is known to cause damaging epidemics in lab, aquaculture and natural settings (Bakke et al. 2007). Such outbreaks are possible due to *Gyrodactylus'* reproductive life history traits such as birthing live young which can in turn give birth within 24 hours (Bakke et al. 2007; Bakke et al. 2002). During an outbreak, most of the population will become infected with the parasite but few individuals become severely infected (Van Oosterhout et al. 2008). Heavily infected fish may have lesions on their skin, clamped fins, excess mucus and will swim erratically, whereas fish infected with only a few worms are infectious but may not show any symptoms of disease (Cable and Harris 2002). *Gyrodactylus* parasites hook onto the surface of the skin (Bakke et al. 2007), potentially allowing for the continuous release of chemicals during an infection (Stephenson et al. 2018). This ectoparasite is therefore well suited for studying chemical infection cues as it is in the best interest of a fish to reliably detect infected conspecifics but continue to benefit from interacting with healthy fish (Chivers et al. 2012). In fact, avoidance of *Gyrodactylus*-infected individuals and *Gyrodactylus*-infected shoals has been observed in various species fish species (Croft et al. 2011; Hockley et al. 2014; Rahn et al. 2015), however the mechanism by which fish can distinguish healthy from infected individuals has not been extensively studied (Behringer et al. 2018).

Rationale and objectives

The literature on predator alarm cues has established that fish exposed to alarm cues dart and freeze more, seek refuge in less vulnerable microhabitats and shoal with conspecifics more (Brown 2003; Brown and Godin 1999). Observing these specific behaviours allowed researchers to speculate that fish may be using these alarm cues as a signal to hide from predators and avoid predation (Ferrari et al. 2010; Wisenden 2019). Evidence for this hypothesis was then confirmed when fish exposed to alarm cues were better able to evade predation than fish not exposed to alarm cues (Mirza and Chivers 2003).

Given that previous studies indicated that guppies avoided putative chemical infection cues released from *Gyrodactylus* infected guppies (Stephenson et al. 2018), the overarching purpose of this thesis is to bridge the behavioural responses observed with a hypothesis that a putative chemical infection cue may impact *Gyrodactylus* transmission. Filling these research gaps may allow for the link between the chemical released and the overall epidemiological outcomes in guppy populations to be developed, in a similar approach to the research on predator alarm cues.

The first step in establishing this link is to determine which behavioural responses are impacted by the presence of the putative infection cue, the consequences on epidemic profiles, and confirmation that responses were directly tied to release of a chemical from an infected guppy. Thus, the objectives of this thesis are to determine what specific behaviours of isolated and shoaling guppies are impacted by the presence of this chemical infection cue, and whether prior exposure to chemical infection cues alters *Gyrodactylus* transmission dynamics.

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CHAPTER II – Literature Review

1. *Gyrodactylus* spp. parasite

The monogenean ectoparasite, *Gyrodactylus* has over 400 described species and infects over 200 species of teleost fish, amphibians and mollusks (Cable et al. 2002; Harris et al. 2004). *Gyrodactylus* spp. are small ectoparasites (0.2 mm to 1mm) which feed on the mucus and epithelial tissue of their host (Bakke et al. 2007). This parasite can cause explosive outbreaks in populations due to its viviparous reproduction, short generation time and direct life cycle (Scott and Anderson 1984; Van Oosterhout et al. 2008).

Gyrodactylus has a fusiform body with a distinct opisthaphor, the main point of attachment, a translucent body, and a head on the anterior end (Bakke et al. 2007). The posterior opisthaphor is equipped with two large centrally positioned hooks called hamuli and 16 peripheral hooks (Harris 1986). When a worm first encounters a host, it attaches the peripheral hooks, then anchors with the larger hamuli to prevent dislodgement (Shinn et al. 2003). The parasites can then travel along the body of the host in a "inchworm-like" fashion by temporarily attaching their head with adhesive glands to the fish skin, then releasing their hamuli and anchoring them close to where their head is attached (Bakke et al. 2007). The transparent body contains an enlarged reproductive system and vital digestive organs (Bakke et al. 2007). The head of the worm contains a mouth connected to the gastric system by a pharynx and adhesive glands and sensory receptors (Bakke et al. 2007; Cable et al. 2002).

1a. *Gyrodactylus* reproductive biology

Gyrodactylus spp. are live bearers and can give birth to a daughter every 24 - 36 hours for their lifespan of four to twenty days (Bakke et al. 2007). This parasite has been compared to a "Russian Doll" as the first daughter of each worm is born pregnant and will subsequently give birth to a pregnant daughter within 24 hours if it is attached to a host (Bakke et al. 2007; Cable et al. 2002). All worms are born with female reproductive organs, then after the birth of the first daughter which arises asexually, worms develop male reproductive organs. Each subsequent daughter can then arise from parthenogenesis or sexual reproduction (Cable and Harris 2002). Generally, when the population density of parasites is low the worms rely on parthenogenesis and sexual reproduction is favored in high population densities to maintain genetic diversity

(Bakke et al. 2007). These reproductive strategies are suspected to be one of the drivers of success in speciation and evolutionary fitness for this genus (Schelkle et al. 2012).

1b. *Gyrodactylus* spp. transmission and epidemic profile

Gyrodactylus spp. has a direct lifecycle and is infectious throughout its entire life. When two hosts come in close contact (less than 0.5cm), a worm will dislodge from one host and jump onto the new host. During initial stages of infection, the parasite population may be low enough that the host immune system has not yet responded, and there is low competition for space and resources on the host (Buchmann 1999). Therefore, worms may be less likely to jump onto a new host as the cost of potential dislodgement outweighs the potential for a new host. However, once parasites begin to compete for resources on the host and the host immune response is activated (Buchmann and Lindenstrøm 2002), parasites may be more inclined to find new uninfected hosts, thus may be more likely to move from one host to another (Boeger et al. 2005).

Although a helminth macroparasite, the epidemic profile of *Gyrodactylus spp*. is more similar to microparasite infections (Scott and Anderson 1984; Tadiri et al. 2019). When parasites are first introduced on an individual or in a population, there is an initial exponential increase in number of worms, hosts will then develop an immune response or die from the infection (Van Oosterhout et al. 2008), decreasing the number of susceptible hosts and leading to a decline in infection prevalence (Tadiri et al. 2013). However, immunity to *Gyrodactylus* infections is not lifelong and, in some species, hosts can become susceptible in as little as four to six weeks (Scott 1985). Therefore, since parasites can persist at low levels in the population during interepidemic periods, once enough naive fish are born or when most fish lose their immunity another outbreak will occur in the population (Scott 1985).

1c. *Gyrodactylus* spp. pathology and impact on fish populations

The negative impact of *Gyrodactylus spp.* on host populations can cause substantial loss of fitness and mortality. For example, *Gyrodactylus salaris* poses serious threats to Norwegian stocks of Atlantic Salmon (*Salmo salar*) (Peeler and Thrush 2004). These parasites were introduced in the 1970's presumably as a consequence of salmon farming and are responsible for the loss of wild salmon in 45 rivers in Norway (Peeler and Thrush 2004). In wild populations of guppies (*Poecilia reticulata*), the recapture rate (a proxy for survival rate) was decreased by 19%

per additional worm found on an individual (Van Oosterhout et al. 2008). Symptoms of the infection on heavily infected fish include clamped fins, reduced swimming capabilities, increased chance of predation, paleness, and secondary bacterial infections (Bakke et al. 2007; Hockley et al. 2014a).

Control of *Gyrodactylus* in a laboratory or aquarium setting is generally feasible with products such as Levamisole, Melafix, Pimafix or saltwater baths (Hutson et al. 2018; Schelkle et al. 2015). The control of *Gyrodactylus* using natural treatments such as ginger and garlic can also be effective in treating fish in aquaria (Fridman et al. 2014; Levy et al. 2015; Schelkle et al. 2009; Schelkle et al. 2013). However, there is limited success and feasibility in treatment options for this parasite in wild populations (Denholm et al. 2016; Winger et al. 2007). For this reason, Canadian legislation lists *Gyrodactylus salaris* as an immediately notifiable pathogen (Canada 2017). Other species of concern in Canada are *G.colemanensis* and *G.salmonis*, which infect a variety of economically important salmonids such as Brooke trout (*Salvelinus fontinalis*), Rainbow trout (*Oncorhynchus mykiss*), Brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*) (Harris et al. 2008; You et al. 2011). Although not a known cause serious mortality in wild populations, it is suspected that farmed fish are more often infected with these parasites and the potential for aquaculture runoff to release many live parasites into rivers and streams is a concern (You et al. 2011).

1d. Immune response

The initial immune response to *Gyrodactylus* infections involve secretion of mucus on the skin in attempt to trap the parasite and limit its growth and movement (Buchmann and Lindenstrøm 2002; Gheorghiu et al. 2007). The amount of mucus secreted is negatively correlated with the parasite load (Buchmann and Bresciani 2006). Some species can rapidly respond to infections, for example, the epidermal layer thickens and there is an initial increase in mucus cell numbers within the first three days of infection for guppies (Gheorghiu et al. 2012). If the infection persists, the immune response will expose the parasites to increased levels of antibodies and phagocytic cells in attempt to increase worm mortality (Buchmann 1999; Stewart et al. 2017). The specific pathway of acquired immunity has not yet been fully described, however, a recent study suggests the role of Il-17/Th-17 may be important in the immune response of gyrodactylid infections in guppies as gene expression was differentially expressed when compared to controls (Konczal et al. 2020).

2. Trinidadian Guppy (*Poecilia reticulata*)

Guppies live approximately 2 years in captivity however their lifespan in the natural habitat of Trinidad is highly variable on selective pressures such as predation and parasitism (Reznick et al. 1997). They feed on a variety of invertebrates, algae, and detritus (Zandonà et al. 2015). The main predators of guppies in river systems in Trinidad are piscivorous fish such as, Hart's rivulus (*Rivulus hartii*) and pike cichlids (*Crenicichla alta*) (Templeton and Shriner 2004). However, avian and mammalian opportunists such as fishing bats (*Noctilio leporinus*), several species of kingfishers (*Chloroceryle spp*.) and fish-eating herons (Aves: Ardeidae) will also consume guppies (Templeton and Shriner 2004).

Guppies are sexually dimorphic, and the females bear live young. Females are larger (3 - 6 cm) and monochromatic (Reznick et al. 2001). Males are smaller (1.5 - 3.5cm), have a visible gonopodium that extends from the anal fin, and distinct orange and blue colour patterns. The orange coloration in males is comprised of carotenoids which are obtained from food consumed (Kolluru et al. 2006). Their bright colors signal that they are successful foragers to females (Hudon et al. 2003). However, males must trade-off attracting mates and not attracting predators with these colorations (Hudon et al. 2003). Therefore, male guppies with bright orange colorations have had to carefully balance this evolutionary trade-off between attracting many females and passing along their genes and being too conspicuous to predators (Kodric-Brown 1989).

Guppies breed two to three times per year however the female can store sperm for up to 10 months (López-Sepulcre et al. 2013). When a female becomes pregnant, the embryo remains in the ovary where it is fertilized. A placenta develops from the wall of the mother’s ovary which provides additional nutrients in addition to a yolk sac. The fry then travel down the female genital tract and are expelled (Thibault and Schultz 1978). The gestation period is between 21-30 days and a female usually gives birth to 5 - 40 fry with larger females producing larger broods (Reznick et al. 2001; Arendt and Reznick 2005). Females reach sexual maturity 8-10 weeks after birth whereas males reach maturity earlier at 6 - 8 weeks (Reznick et al. 2005). Males are considered bolder than females, meaning they are more likely to explore a new area or stimulus (Jacquin et al. 2016). Male boldness may be explained by pressures to mate and is associated male hormones (Harris et al. 2010).

2a. Guppy shoaling behaviour

Guppies begin shoaling with fry of the same size as soon as they are born. Shoaling is described as a social grouping of 2 - 20 individuals who remain in proximity to each other although the distance between fish and direction they are swimming is a dynamic process which is impacted by the time of day and various cues (Croft et al. 2004). Mature fish may begin venturing further from the shoal to increase their foraging success (Croft et al. 2004). When a guppy senses a perceived threat, their behaviour can vary from tightening the shoal, freezing, seeking refuge or swimming erratically (Botham et al. 2008; Elvidge et al. 2014). The tightness of the shoal is highly dependent on social, visual, and olfactory chemical cues, with distance between individuals being most influenced by presence of predators and familiarity of the fish with the rest of the shoal (Elvidge, Ramnarine et al. 2014). In laboratory tests, guppies preferentially shoal with familiar conspecifics and conspecifics that have similar diets (Budaev and Zhuikov 1998; Morrell et al. 2007)

2b. Guppies as model for evolutionary biology

Trinidadian guppies have been uses as a model in evolutionary biology since the 1960's (Farr 1975). Most of the Trinidadian stream and rivers are geographically isolated and have different water flow rates, microhabitats, predators and parasites, thus different populations of guppies have adapted to these various selection pressures (Farr 1975; Seghers 1974). Their short generation time has facilitated field experiments where local adaptation and evolution has been studied by moving guppies to populations with different selection pressures (Harris et al. 2010). Predation pressure was identified as the most significant selective pressure that led to differences in behaviour among guppy populations (Bashey 2006), as population with low pressure did not shoal as much and explored their surrounding areas more (Reznick et al. 1997; Botham et al. 2008; Hendry et al. 2006; Seghers 1974) and had different life history traits.

The various studies on guppy adaptation and evolution provided a foundation for the extensive knowledge on guppy behaviour, physiology and ecology, which has led to guppies being used a model for aquatic organisms in both laboratory and natural settings (Gotanda et al. 2013).

3. Guppy and *Gyrodactylus* infection model

In addition to predators, parasites also play a role in the evolutionary selection pressures of Trinidadian guppies. Infections can be deadly for the guppy host as the worms can damage too much skin, causing osmotic imbalances or the guppy will succumb to secondary bacterial infections (Bakke et al. 2007). Additionally, infected guppies have increased mortality rates from predation, as they are less efficient swimmers (Bakke et al. 2007; Houde and Torio 1992). The species of *Gyrodactylus* that infect guppies are *G.turnbulli* and *G.bullatarudis* (Harris 1986). *G.turnbulli* are generally found on the posterior end of the fish and have a longer hamulus (50 – 55 μm) and ventral bar (25 – 31 μm) than *G.bullatarudis* (46 – 50 μm and 20 – 25 μm, respectively) which is mostly found on the head and gills (Buchmann 1999; Harris 1986; Richards and Chubb 1996). Co-infection with the two species has been observed in wild populations in Trinidad (Harris and Lyles 1992).

3a. Resistance and tolerance to *Gyrodactylus*

In the field, resistance (ability to minimize infection), and tolerance (ability to maintain fitness at given infection level) differ among guppy populations (Jacquin et al. 2016; Tadiri et al. 2021). For example, some populations in the Aripo river lineage were more resistant or tolerant to serious infection than other lineages (Marianne and Turure rivers) (Pérez-Jvostov et al. 2015), indicating genetic heritability of resistance (Cable and Van Oosterhout 2007). Diversity of alleles in the major histocompatibility complex (MHC) has been found to decrease infection intensity when comparing genetically diverse wild populations of guppies from inbred domestic populations (Phillips et al. 2018; Smallbone et al. 2021). Also, the size of orange spots on males which are larger when a male eats a diet high in carotenoids is correlated with a lower parasite load (Kolluru et al. 2006), indicating that both genetic diversity within a population and quality of food in their habitat both play a role in tolerance and resistance (Pérez-Jvostov et al. 2015; Tadiri et al. 2021).

Sex may also play a role in resistance and tolerance to *Gyrodactylus* infections. Both male and female guppies are susceptible to *Gyrodactylus* infections, yet there may be differences in how likely they are to become infected or die from infection. Females tend to shoal more closely together, thus may be more likely to initially become infected by contact with an infected conspecific (Johnson et al. 2011; Richards et al. 2010). Larger fish are often found to have larger worm burdens and as females are generally larger than males, they are often observed to have a higher intensity of infection (Van Oosterhout et al. 2008). However, males may be more likely to have severe infection symptoms and die from a *Gyrodactylus* infection, as the relative damage to the skin surface area caused by one parasite is greater in males than in larger females (Richards et al. 2012; Tadiri et al. 2021). Additionally, androgens may play a role in a reducing the immune response as feminized and demasculinized males can tolerate infection better than control males (Dargent et al. 2015). Males may also be more vulnerable to predation when infected since their smaller size and lower tolerance to infection results in slower swimming speeds (Stephenson et al. 2016). Therefore, although larger females may have higher chance of becoming infected and have higher worm burdens (Van Oosterhout et al. 2008), recent literature suggests mortality from infection may disproportionately impact male guppies.

3b. Avoidance of infected conspecifics

Since *Gyrodactylus* infections are directly transmitted form one guppy host to another, one way to avoid infection would be to avoid infected conspecifics. Other aquatic animals avoid infected conspecifics (Karvonen et al. 2004; Kiesecker et al. 1999; Tobler and Schlupp 2008), thus understanding if guppies avoid *Gyrodactylus*-infected conspecifics would be relevant in understanding overall transmission dynamics (Tadiri et al. 2019). In a semi-natural setting, introduction of a *G.turnbulli*-infected individual into a shoal increased the distance between individuals (an index of shoal cohesion), whereas shoals accepted an uninfected fish into their group after 24 hours. This study concluded that shoals avoided new guppies added to the group if they were infected (Croft et al. 2011). In a more realistic system which incorporated water flow, if a member of the shoal was infected *G.turnbulli* for 3 days, shoal cohesion decreased, however as the water flow became stronger, the shoal became more cohesive (Hockley et al. 2014b). Thus demonstrating that parasitism plays a role in shoaling but other factors, such as water condition and risk of predation, may outweigh the potential benefit of conspecific avoidance (Hockley et al. 2014b; Reynolds et al. 2019). However, it is not known whether avoidance also occurs at later stages of infection when infected guppies are more likely to transmit the parasite to new hosts (Boeger et al. 2005).

Given the small size of *Gyrodactylus*, it would be difficult for a fish to see individual worms on an infected conspecific, especially at low levels of infections (Harris 1986; Rahn et al. 2015). At later stages of infections, guppies may avoid conspecifics based on social cues such as erratic swimming patterns or based on visual cues such as an increase in mucus and paleness. However, studies suggest that even at early stages of infection when symptoms are not yet severe, guppies avoid infected conspecifics (Behringer et al. 2018; Croft et al. 2011; Hockley et al. 2014b). Therefore, the role of other cues, such as chemical cues, should be further studied to determine how guppies detect infected individuals (Behringer et al. 2018).

4. Chemical ecology in aquatic environments

For aquatic organisms, chemoreception incorporates both olfaction and gustation to detect amino acids, amines, nucleotides, bile acids, aminosterols, sex steroids, and prostaglandins and perhaps other compounds (Derby and Sorensen 2008). An example of guppy reliance on chemoreception was demonstrated when fish reared in low light environments were able to switch from foraging based on visual cues to using chemoreception to find food (Chapman et al. 2010). Complex chemoreception among conspecifics has mainly been studied in the context of sex pheromones and predator alarm cues, which both play an important role in how fish behave, interact with their environment, and adapt (Brönmark and Hansson 2012). For a molecule to be considered a chemical cue, it must be released from one organism into the environment and perceived by another organism of a different species or the same species and may evoke a response in the cue receiver (Chivers et al. 2012).

4a. Predator alarm cues

Predator alarm cues are chemical cues which elicit a strong response in the cue receiver and have been well studied in comparison to other types of cues (Ferrari et al. 2010). These alarm cues are released from the epithelial cells of a fish when it is injured (Sorensen and Wisenden 2015; Stensmyr and Maderspacher 2012). When a fish is injured by a predator, various types of skin cells are ruptured, releasing this alarm cue which then indicates the presence of danger to conspecifics in the area (Brown et al. 2000). Alarm cues are received mainly in olfactory receptor cells of the fish mucosa (Bettini et al. 2009; Lane and Whitear 1982). Studies on chemical alarm cues in response to predation risk in guppies began when researchers observed that guppies exposed to the epithelium of a conspecific reacted by shoaling more tightly and seeking refuge at the bottom or periphery of the tanks (Brown 2003; Brown and Godin 1999). Additionally, guppies exposed to predator alarm cues increase the frequency of more complex behaviours such as freezing, which entails complete cessation of movement usually at the bottom of the substrate for 30 seconds, and darting, which entails swimming 5 times the body length in a quick motion (Brown et al. 2009; Lawrence and Smith 1989). These behaviours subsequently resulted in fewer fish being preyed upon (Larson and McCormick 2005; Mirza and Chivers 2003; Swaney et al. 2015).

These observations were then experimentally tested in wild guppies where fewer fish were captured in traps that contained conspecific skin extracts (Brown and Godin 1999) indicating that guppies actively avoided areas where this alarm cue was present. Guppies were also shown to respond to the chemical alarm cue in skin extracts more strongly if the cue was extracted from a guppy of the same population (Brown et al. 2010) suggesting that these cues are not only species specific but also population specific.

These cues may have evolved to attract secondary predators (Cashner 2004; Chivers et al. 2012; Chivers et al. 2007) or to increase survival of the injured fish's offspring (Chivers et al. 2012). Alternatively, conspecifics around the injured fish may have opportunistically responded to molecules in the skin which evolved for another function (Chivers et al. 2012; Halbgewachs et al. 2009)

4b. Predator alarm cue composition and properties

A candidate predator alarm cue is hypoxanthine-3-*N*-oxide that elicits a response in some but not all dace (*Leuciscus leuciscus*) and fat head minnows (*Pimephales promelas*) (Brown et al. 2000). The trigger is thought to be a nitrogen oxide functional group (Bazáes et al. 2013; Brown et al. 2000). A recent study provided evidence that a combination of mucus cell, club cell and bacterial lysates produce this alarm chemical cue, as bacteria on fish skin were transported into mucus cells and club cells in the epithelium in zebrafish (*Danio rerio*) and as the lysate of *Staphylococcus* elicited a behavioral response in the fish (Chia et al. 2019).

The duration and concentration of alarm cues is highly debated and variable depending on the species and water condition (Chivers et al. 2007, Chivers et al. 2013). For example, in marine habitats, chemical cues in coral reef damselfish (*Pomacentrus ambonensi*) were found to be active for less than 30 minutes, high UV and temperature reduced this time further (Chivers et al. 2013). However, in freshwater environments, fathead minnows and northern redbelly dace (*Phoxinus eos*) responded to alarm cues for between 3 and 6 hours (Wisenden et al. 2009b). It has been hypothesized that, for every 2cm^2 of fish skin surface area, a fish can produce a cue with a 2 m range and 2 hour duration (Wisenden 2008; Wisenden et al. 2009b).

4c. Protocol for producing predator alarm cues

The common method for producing predator alarm cues is to homogenize the skin and skeletal muscle of fish (Brown and Godin 1999). This homogenized tissue is then diluted to a desired concentration with water, filtered so any scales and large particles of tissue are removed, and frozen for future use (Brown and Godin 1999; Brown et al. 2009; Lawrence and Smith 1989). This protocol has the advantage that all experimental fish are exposed to the same concentration and content of chemical cue. Although loss of potency due to freezing has been described (Larson and McCormick 2005; Smith 1989), many studies have successfully elicited responses in test fish using low concentrations of previously frozen alarm cues (Brown et al. 2010; Brown and Godin 1999; Lawrence and Smith 1989; Stephenson 2016).

5. Chemical cues and infection

The role of conspecific chemical cues of fish infected with parasites has yet to be fully explored (Behringer et al. 2018; Stephenson et al. 2018; Wisenden et al. 2009a). One advantage of chemical cues is that they can be detected from a father range than other cues, thus limiting infection risk (Behringer et al. 2018). Further research into the chemical ecology of fear of infection is important as it may play a role in understanding parasite transmission dynamics (Behringer et al. 2018; Poulin et al. 1999; Rohr et al. 2009).

5a. Evidence of chemical infection cues in aquatic organisms

One common defense against pathogens is avoidance of infected conspecifics or of the parasite itself (Behringer et al. 2018). Thus, laboratory studies first began exploring how an organism could detect infected individuals. Bullfrog tadpoles (*Rana catesbeiana*) were placed in an arena with either chemical cues or visual cues of conspecifics infected with an intestinal fungal pathogen (Kiesecker et al. 1999). Avoidance of the infected individual was only observed when the tadpoles were exposed to chemical cues (Kiesecker et al. 1999).

There is evidence that some species of fish respond to cues released directly from the parasite and others only respond to cues released from infected conspecifics. Rainbow trout (*Oncorhynchus mykiss*) responded to the chemical cues of a conspecific during active infection with *Diplostomum spp.* cercariae by darting and freezing more, however they did not respond to cues from the parasite alone (Poulin et al. 1991). However, fathead minnows (*Pimephales promelas*) which had been previously infected with cercariae (*Ornithodiplostomum sp*.) reduced overall activity following exposure to water containing frozen and thawed cercariae, indicating that they perceived the threat of the parasite in the water and modified their behaviour (James et al. 2008). Therefore, in future study designs, one cannot discount the possibility that cues may be coming from either the pathogen or the infected individual.

5b. Putative cues released by *Gyrodactylus turnbulli* infection

There is evidence that *G.turnbulli* infected guppies release infection cues and that uninfected conspecifics respond to these cues however, the mechanism of action, the specific behavioural responses and the impact on transmission have yet to be fully established (Reynolds et al. 2018).

Guppies avoided the side of an experimental tank which contained chemical cues from guppies infected with *G.turnbulli* but only when the chemical cue producer was infected for more than 15 days. During this later stage of infection, guppies are considered to have the highest transmission risk (Stephenson et al. 2018). Fish did not avoid the side of the tank when exposed to visual cues of infected conspecifics at any stage of infection. Thus, this research suggests that guppies release and respond to chemical infection cues more than visual cues and that infected guppies may conceal infection status when transmission risk is low to maintain advantages of group living (Stephenson et al. 2018).

It is possible that the response of guppies to chemical cues is a response to a novel chemical odour, rather than specific response to the risk of infection. Guppies can become acclimated to chemical infection cues as they preferentially spent time on the side of an experimental tank with cues released from *G.turnbulli* infected conspecifics if they were continuously exposed to these infection cues as juveniles (Stephenson and Reynolds 2016). However, previously infected and recovered guppies spent less time associating with *G.turnbulli* infected fish, indicating that a previous exposure to infection may lead guppies to build a chemical infection cue repertoire (Reynolds et al. 2018). This recognition of infection chemicals may allow them to avoid infected individuals and thus avoid reinfection (Reynolds et al. 2018).

5c. Protocols for preparation of chemical infection cues

In the case of a cue released in response to *Gyrodactylus* infections, the established method is preparing conditioned water with an infected fish (Stephenson and Reynolds 2016). An infected guppy or a control guppy is placed in a small container for 24 hours where any chemical released into the water can accumulate (Stephenson and Reynolds 2016; Stephenson et al. 2018). The "conditioned" water from the tank is then frozen into aliquots for future use (Stephenson et al. 2018). This method has been used in the guppy-*Gyrodactylus* model but has the potential limitation of reduced potency due to freezing, which can be prevented by using freshly produced conditioned water.

6. Conclusion

Gaps in the literature on chemical infection cues include the chemical composition of infection cues, the impact of cues on guppy behaviour or immune response, and whether they impact transmission dynamics (Behringer et al. 2018; Reynolds et al. 2018; Stephenson et al. 2018; Stephenson and Reynolds 2016). This thesis addresses the impact of chemical infection cues on guppy behaviour and on transmission dynamics.

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CHAPTER III - Exposure to a putative chemical cue from *Gyrodactylus*-infected guppies subtly alters behaviour but impacts parasite transmission

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Abstract

The reliance on chemical communication is well established for evading predation in aquatic systems. However, only a few studies have found evidence that chemical cues released from animals infected with parasites alter a conspecifics behaviour. Furthermore, the link between these putative chemical cues and susceptibility to infection has not been studied. We aimed to determine if exposure to *Gyrodactylus turnbulli*-infected guppies (*Poecilia reticulata*) resulted in altered behaviour of uninfected conspecifics, and if prior exposure to this putative infection cue reduced transmission. We found subtle evidence that guppies respond to these chemical infection cues however these results are somewhat inconstant with previous research. Guppies exposed to cues released by infected guppies spent less time in the center of the tank versus the periphery, indicating that fish were responding to this chemical cue. Males responded to the putative infection cues released by fish at later stages of infection by taking longer to start moving at the beginning of the trial. Exposure to continuous infection cues did not impact the overall behavioural activity of shoals, however, in contrast to previous studies on guppy infection avoidance behaviour shoals exposed to guppies in later stages of infection had a shorter inter-fish distance. Finally, prior exposure to the infection cue for 16 days had no impact on the epidemic prevalence over 40 days but significantly reduced intensity and epidemiological patterns of *G.turnbulli* infections in the shoal. These results demonstrate that guppies may respond to short-term and ongoing infection cues by altering specific behaviours, and that infection cues may reduce the intensity of epidemics.

Introduction

Animals are continuously expending energy to mitigate the numerous stressors in their environment (Brownscombe et al. 2017). They must balance survival against expenditure of energy on stressors which will not result in morbidity or mortality (Lind and Cresswell 2005). Therefore, animals must be able to gain accurate information about the immediate risks they face to respond appropriately (Milinski and Heller 1978). In aquatic systems, relying solely on vision is unreliable as water turbidity (Brönmark and Hansson 2012), sedimentation and changing levels of light impact water clarity and visibility (Hickman et al. 2004). Therefore, aquatic animals also rely on chemicals for detecting risk in their habitat (Stephenson 2016). Chemical communication is more reliable than other forms of communication as chemicals can travel further distances and are uninterrupted by poor water clarity (Ferrari et al. 2010). Aquatic organisms use chemicals cues to forage, recognize spawning grounds, mate, and evade predation (Brönmark and Hansson 2012; Chivers et al. 2012). A well-studied example of these chemicals are predator alarm cues (Brown 2003). These chemicals are released from punctured epithelial cells when an aquatic organism is bitten by a predator, then are recognized by conspecifics and induce anti-predator behaviours, such as, hiding at the bottom of the substrate, darting back and forth, and freezing (Brown 2003; Mathuru 2016; Mirza and Chivers 2003). Shoaling fish will join a group and reduce the distance between conspecifics when exposed to these chemical alarm cues (Wisenden 2019). The behaviours associated with the response to these alarm cues increase predator avoidance and survival of surrounding fish in various species (Brown et al. 2004; Mirza and Chivers 2003).

Parasites and pathogens can also result in a reduction of fitness in aquatic organisms (Kodric-Brown 1989; Koprivnikar et al. 2006). Therefore, gaining reliable information about infection risk could improve fitness in aquatic animals (Wisenden et al. 2009). Evidence is emerging that infection cues are present in aquatic systems as chemical cues released by infected individuals can alter the behaviour of uninfected individuals (Behringer et al. 2018; Reynolds et al. 2018). Juvenile rainbow trout (*Oncorhynchus mykiss*) which were exposed to conspecifics actively being infected with cercariae of *Diplostomum* sp. darted and froze more (Poulin et al. 1999). This study suggests that the damage caused by cercarial penetration resulted in the release of chemical alarm cues from the fish epithelium, similar to the way that predator-induced damage stimulates release of predator alarm cues (Poulin et al. 1999, Poulin and FitzGerald 1989). In an experimental chemical choice test, bullfrog (*Rana catesbeiana*) tadpoles were either exposed to chemical cues of infected conspecifics via small holes in an opaque barrier separating the animals, or to visual cues where the animals were separated with a glass frame. The tadpoles responded only to the chemical cues and shifted their position to the side of a tank away from the chemicals of tadpoles infected with the fungus, *Candida humicola,* indicating that in this system chemical cues may be more reliable than visual cues (Kiesecker et al. 1999). Guppies (*Poecilia reticulata*) avoided the side of an experimental tank when exposed to chemical but not visual cues from conspecifics infected with the monogenean ectoparasite, *Gyrodactylus turnbulli,* but only at the late stages of infection when transmission risk was the highest (Stephenson et al. 2018). Thus, indicating that infected individuals may conceal their infection status during early stages of infection to continue to benefit from the advantages of living in groups (Stephenson et al. 2018). This model system is particularly interesting given that *G.turnbulli* parasites live on the surface of the skin (Harris 1986) and may release a chemical cue over a prolonged period (Stephenson et al. 2018), which contrasts predators or cercarial penetration where the release of the chemical would only be for a short period of time when the skin cells are ruptured (Poulin et al. 1999; Rohr et al. 2009).

Study System

The Trinidadian guppy (*P.reticulata*) is a small tropical fish, which lives in rivers, streams and ponds in Trinidad and its use as a model for evolutionary biology for many years has allowed for extensive knowledge of its biology and ecology (Gosline and Rodd 2008; Gotanda et al. 2013; Seghers 1974). The ectoparasite *Gyrodactylus turnbulli* infects wild guppies and is a well-known pest of domestic aquarium fish (Harris 1986; King and Cable 2007; Masud et al. 2019). This small (800 µm long and 300µm wide) monogenean (Harris 1986) lives on the fin and tail of guppies where it consumes epithelial and mucus cells (Harris and Lyles 1992), and is directly transmitted from one guppy to another via physical contact (Bakke et al. 2007). *G.turnbulli* are born with a fully formed daughter in their uterus and continue to give birth every 24 hours for the remainder of their lifespan of approximately 5 days (Cable and Harris 2002). Similar to other species of *Gyrodactylus* that are responsible for die-offs in salmon (Denholm et al. 2016; You et al. 2011), *G.turnbulli* causes substantial mortality in guppy populations. This mortality is mainly due to the damage to the epithelial barrier, secondary bacterial infections as well as increased susceptibility to predation (Harris and Lyles 1992; Scott and Anderson 1984). The rapid exponential increase in numbers on an infected fish, together with the direct transmission, generate epidemic outbreaks where the prevalence and worm intensity rapidly increase (Bakke et al. 2007; Buchmann 1999; Scott and Anderson 1984). As individuals die or recover from an infection, the prevalence and worm intensity decrease (Reynolds et al. 2018; Scott and Anderson 1984; Tadiri et al. 2019). Once a fish has recovered, they are refractory to re-infection for four to six weeks (Cable and Van Oosterhout 2007; Scott 1985). The parasite will then remain in the population at a low prevalence until enough susceptible hosts are present to cause another outbreak (Tadiri et al. 2019; Van Oosterhout et al. 2008).

Previous studies suggest that guppies do respond to putative infection cues released from *Gyrodactylus*-infected individuals (Stephenson et al. 2018). However, the specific changes in behaviour caused by an exposure to this infection cue are not well established. Additionally, the link between exposure to chemical infection cues to future infection susceptibility has not been studied (Reynolds et al. 2019). Filling these research gaps may provide an opportunity to generate hypotheses about the nature of this putative chemical cue and how this cue may impact guppy populations (Poulin et al. 1999; Rohr et al. 2009).

Objectives

The first aim of this study was to determine the role of the putative chemical cues released from stimulus guppies infected with *G.turnbulli* on the individual and shoaling behaviour of uninfected test guppies, and to determine whether outcomes were influenced either by the sex of the test fish or duration of infection on the stimulus. We hypothesized that exposure to the infection cue would reduce distance moved and darting but increase freezing of individuals (Poulin and FitzGerald 1989; Stumbo et al. 2012), and that individuals would move to the bottom of the tank and avoid the center of the tank (Poulin et al. 1991), strategies that would reduce the risk of infection with this directly transmitted parasite. We hypothesized that the inter-fish distance in shoals would be larger when the shoal was exposed to the infection cue, as guppies are known to avoid *Gyrodactylus*-infected conspecifics (Croft et al. 2011). We hypothesized that cues released at the peak of the infection (day 16) would have a stronger impact than cues released earlier in the infection (days 4 or 8) (Stephenson et al. 2018). As male guppies are exhibit bolder behaviours and are more susceptible to severe *G.turnbulli* infections than females (Dargent et al. 2015; Richards et al. 2010l; Richards et al. 2012; Stephenson et al. 2016), we hypothesized that males would be more likely to respond to infection cues than females. Our second aim was to test the hypothesis that prior exposure to the putative infection cue would lower infection levels when the parasite was introduced into a guppy shoal, as evidenced by the pattern of prevalence and intensity over a 40-day period.

Methods

1. *Animals and housing*

Guppies were housed in the McGill University Department of Biology greenhouse at 25°C ± 1 °C and a 12-h light-dark cycle and fed TetraMin® Tropical Food Flakes (Tetra Werke, Melle, Germany) daily. The guppies used in all experiments had been transported from the Turure river shed in Trinidad (GPS coordinates: 10.6903, -61.1638) in 2015, and bred for approximately 20 generations in a laboratory setting (Blondel et al. 2020). Guppies were held in 19L tanks with a box filter (filter floss, activated carbon, and gravel) at a density of 20 – 40 guppies per tank. A 20% water change was performed weekly and the water quality (pH, NO_2^- and NH_3/NH_4^+) was monitored every 2–3 days. Enrichment (plastic plants, clay pots) in the tanks also served as a refuge for young fry before they were removed and added to a tank with fry of similar size. Animal care approval was obtained according to McGill University Ethics Guidelines (AUP: 7547).

2. *Gyrodactylus turnbulli strain and infection protocol*

Parasites were isolated from a fancy guppy which was purchased from a supplier in Montreal (Quebec, Canada). To ensure the isolated strain only contained one species, 2 worms from the tail of one fancy guppy were transferred onto a lab reared guppy, and this guppy was then added to a tank with naive lab stock guppies. The parasite strain was maintained in 2 aquaria to which naive fish were added weekly (Scott and Anderson 1984; Tadiri et al. 2013). The parasite species was identified by measuring the total length of the hamulus (55.5±0.4µm) and the width of the ventral bar (29.6±0.5µm) on five parasites from three infected fish (Bakke et al. 2007; Harris 1986).

To ensure infection was established on experimental fish, an infected and a naive guppy were anesthetized in 0.02% tricaine methane-sulfonate (Finquel MS-222, Argent Laboratories, Redmond, WA, USA), buffered to a neutral pH with sodium bicarbonate. Once anesthetized, the fish were placed in a petri dish containing the MS-222 solution under a SMZ800 stereomicroscope (Nikon, Tokyo, Japan). A parasite on the donor fish scale was placed on the recipient fish surface and observed until it moved onto the recipient fish (Gheorghiu et al. 2012; Tadiri et al. 2013).

3. *Experiment 1: Exposure to an infection cue impacts behaviour of individual guppies*

The goal of this experiment was to determine if exposure to conditioned water from stimulus guppies with mid (8 days) to late stage (16 days) *G.turnbulli* infection would alter the individual behaviour of uninfected guppies.

Study design: Our 2 x 2 x 2 experimental design included two experimental groups (control or infected stimulus fish), two durations of infection on the female stimulus fish (8 and 16 days), and two sexes for the test fish (male and female). A total of 15 test fish of each sex were exposed to the infected stimulus fish at 8 and 16 days after infection of the stimulus fish, and 7 test fish of each sex were exposed to the control stimulus fish at each day. The behaviour of each test fish was recorded by an overhead video camera and by direct observation from the side of the experimental arena during the 10 min trial.

Preparation of stimulus cue: Eight days before the trial, female guppies isolated from the stock tanks were randomly assigned to two groups: infected stimulus fish that were experimentally infected with 2 - 3 individual *G.turnbulli* worms*,* and control stimulus fish that were anaesthetized but not infected. One day before the trial, 2 stimulus fish from each treatment group were anesthetized, their length was recorded, and the number of parasites was counted. Once recovered from anesthesia, they were placed in a plastic container with 1L of water for 24 hours which allowed any cues released to seep into the conditioned water (Stephenson et al. 2018). Fish were not fed during this 24-hour period.

Experimental arenas: The trials took place in a dark room (3m by 2m) which was artificially lit by two standing lamps in along the two side walls, and four florescent light fixtures attached to the ceiling near each corner of the room. The experimental tank was placed in the middle of this room. The observer was seated in a chair 1.5m away from the tank and minimized movement throughout the entire acclimation and trial period. The experimental arena was a rectangular 9.4L tank (L: 30 cm x W: 12.5 cm) and filled to a depth of 10cm. The arena was separated into a smaller (10 x 12.5 cm) and a larger (20 x 12.5 cm) compartment by a Plexiglas frame and plastic screen mesh. An air stone was placed in the smaller compartment to promote water movement into the larger compartment. The outside surface of glass of the smaller compartment was covered with black cardboard paper to darken the smaller compartment and minimize visual cues observed by the test fish much as possible. The outer surface of glass of the larger compartment was marked with white tape that separated the top, middle and bottom of the tank into three equidistant vertical sections.

Protocol: At the time of the trial, an uninfected test fish (male or female) was randomly selected from one of the six stock tanks which held adult Turure fish and then placed in the larger compartment where it acclimated to the new surroundings for 10 minutes (Croft et al. 2006). Then one of the two stimulus fish along with 100ml of the conditioned water were added to the smaller compartment. The trial was then video recorded for 10 minutes, and the vertical position of the test fish was recorded by the observer every 15 seconds (equidistant top, middle and bottom sections). After the trial ended, the test fish was anesthetized, the time of day, home tank, the length was recorded, and the fish was scanned to ensure that it was not infected with parasites. They were then placed in a new home tank, as test fish were only used in a trial once. After day 8 trials stimulus fish were placed back in their isolated tanks and reused for day 16 trials. The experimental arena was rinsed with 70% ethanol to ensure any residual cue or parasites were removed before the next trial. This experiment was completed between November 12 and December 4, 2020.

4. *Experiment 2: Prior exposure to an infection cue alters shoal tightness and reduces parasite transmission*

The goal of this experiment was to study the behavioural response of mixed sex guppy shoals to a putative infection cue and the impact of prior exposure to the cue on transmission after infection was introduced to the shoal.

Study design: The shoal behaviour phase used a 2 x 4 repeated design with two treatments (exposure to control or infected stimulus fish) and a sequence of 4 infection times (days 0, 4, 8 and 16). Test guppy shoals (2 female and 2 male guppies) were continuously exposed over 16 days to cues produced by infected or control conspecifics living in the same tank but separated by a permeable barrier. Each shoal (N = 8 per treatment) was recorded at 4 times points.

This was followed by a transmission phase using the same shoals; however, the stimulus fish were removed from the tank. One fish in each shoal was infected with *G.turnbulli* and the parasite numbers on each test fish in the shoal were recorded every 2-3 days for 40 days.

Experimental arenas: The above arenas were adapted by adding a permeable polyester barrier that prevented the parasites on the stimulus fish from crossing into the larger compartment. The barrier was held in position within a Plexiglas frame. The combination of the two barriers allowed for only faint shadows of the stimulus fish to be visible to the test fish. The behavioural trials took place in the dark room, with the same adjusted lighting as the first experiment.

Protocol: Prior to the beginning of the experiment, groups of 2 female and 2 male uninfected test fish (4-7 months old) were acclimated for 4 days in the larger compartment together with two adult uninfected stimulus females in the smaller compartment. After the acclimation period, the behaviour of the shoal was recorded for 10 min to provide baseline measures on experimental day 0. Then, the two adult stimulus fish from the smaller compartment were removed, anaesthetized and half the stimulus fish were infected with 2-3 *G.turnbulli* worms, the other half were used as control stimulus fish. The stimulus fish were then returned to the smaller compartment, and their infection was monitored every 3 days.

On experimental days preparations were made for video-recording the groups of uninfected test fish to improve the ability of the video software to record the fish. The experimental arena was transported into a dark room, the clay enrichment was removed from the tank, and the fish were left to acclimate for 15 minutes. Then, the video recorder was turned on from outside of the dark room and recorded for 10 min. After each trial, all test fish were anesthetized, their length was measured and visually scanned to ensure they were not infected. This experiment was completed between April 30 – May 14, 2021.

To determine whether the ongoing exposure to chemical cues from the two infected stimulus fish had an impact on parasite transmission, on experimental day 16, we introduced the parasite into the shoal by infecting one randomly selected female test fish with 3 *G.turnbulli*. To identify each fish when recording parasite data, the lengths and distinguishing features of each fish were recorded. The number of parasites on each test fish was counted every 3 days for 40 days. This experiment was completed between May 14 – June 25, 2021.

5. *Video tracking and definitions of behavioural variables*

For experiment 1, Ethovision XT 14.0 (Noldus, Wageningen, the Netherlands) animal tracking software was used to quantify behavioural parameters by sampling the recording 25 times per second. For Experiment 2, Ethovision XT 11.0 social interaction module was used to record the shoal characteristics at the same sample rate.

For both experiments, the *total distance moved* measured the length of the entire track for the 10-minute trial. The *latency to first move* was the time before the guppy first exceeded the threshold of 0.75cm/s. *The duration of "darting" events* was recorded using a multi-condition parameter that determined duration which the guppy was along the side of the wall and swimming at a higher velocity than 2.5cm/s. This was validated using the integrated visualisation feature on the software. The *duration of "freezing" events* was calculated as the cumulative duration when velocity averaged over 1 second was 0 cm/s. The *time spend in the center of the tank* was calculated based on a software defined center zone which equaled half of the total surface area of the experimental arena (125cm^2). The *vertical position* variables which were manually counted and averaged per treatment and sex group.

For experiment 2, the distance between the centre point of multiple subjects was used as a measure for shoal cohesion. The *average inter-fish distance* was calculated by averaging inter-fish distances between all four members of the shoal. The *duration in close proximity* (s) was defined as the average amount of time a subject spent within 0.5 cm of another subject (Green et al. 2012). This value was chosen as *G.turnbulli* are known to transfer when an infected fish is at least 0.5 cm away from another fish (Harris and Lyles 1992).

In both experiments, the subject loss due to misdetection by video-tracking software was less than 5%. If the subject loss exceeded 5% for an individual trial, the tracks were manually edited to add tracks for the portions that had not been detected (Green et al. 2012).

6. Infection variables

For the second phase of Experiment 2, the *prevalence* (percent of infected fish in each shoal) was averaged across shoals at each time point. The *intensity* (number of parasites) on each fish in the shoal was recorded and averaged on a given day, based on the number of fish in the shoal on a that day.

7. Statistical analysis

The raw data was exported from the Ethovision XT software into an excel file which was uploaded to R statistical software 4.1.1 (R Core Team 2020). Boxplots, scatter plots or stacked barplots (ggplot2 package) were created for each variable to provide visual representations of the data (Wickham 2016).

For experiment 1, Linear Models (LM) or Generalized Linear Models (GLM) were used to analyze behavioural variables using lm and glm functions (lme4 package) respectively (Bates et al. 2015). A Gamma distribution was used for all behavioural variables except for the *duration of freezing events* which was normally distributed. A chi-square analysis was completed for the vertical position variables (stats package) (R Core Team 2020). In all models, the treatment, day post infection exposure (D8 or D16) and sex of test fish were included as fixed effects. The Akaike information criterion (AIC) was calculated for all models, as this number indicates how well the model fits the data based on the deviance (Bolker et al. 2009), with smaller AIC scores indicating that a model is a better fit for the given data. The time when the trial was recorded, and interactions were included in a model if it significantly improved the model AIC. The length of the test fish, length of the stimulus fish and number of worms on the stimulus fish were also included in all initial models, however, did not significantly improve the models, thus, were excluded in the final analysis.

For experiment 2, as data for repeated times were reported for each tank, Linear Mixed Models (LMM) or Generalized Linear Mixed Models (GLMM) were used to assess behavioural data. In all models, the treatment, and duration of cue exposure (D0, D4, D8, D16) were included as fixed effects, tank was included as a random intercept in the model using lmer or glmer (lme4 package) (Bates et al. 2015). The time of day when the trial was recorded was included as a fixed effect if it significantly improved the model AIC. The number of worms on the stimulus fish, length of the test fish and stimulus fish were excluded in the final analysis as they did not improve the model. All models used normal distributions except for *total distance moved* and *latency to first move* which followed Gamma distributions.

Analysis of *prevalence* and *intensity* data was done separately for the increasing phase of infection (D4 - D18) and the declining phase of infection (D18 - D40). LMMs were used to analyse the prevalence data (log transformed). GLMMs were used to analyse the worm intensity, using a Poisson distribution (lme4 package) (Bates et al. 2015). In all models, treatment and day post infection were included as fixed effects and tank and average length of fish in the shoal as random intercepts, as larger fish can tolerate a higher number of worms (Van Oosterhout et al. 2008).

For both experiments, significance ($p < 0.05$) was tested using the summary function (base R package) and posthoc pairwise comparison tests were performed using the emmeans function (emmeans package) (Lenth 2021). Normality, independence, and homogeneity of variances were assessed to ensure the data met model assumptions (Bolker et al. 2009; Hartig 2021; Zuur and Ieno 2016).

Results

Experiment 1: Exposure to an infection cue impacts behaviour of individual guppies

Stimulus fish used to prepare the control conditioned water were larger (27.5±0.7mm) than infected stimulus fish (25.0±0.5 mm) ($t_{56.9} = -2.7$, $p = 0.009$), but the size of the stimulus fish did not impact test fish behavioural or location variables. Parasite numbers on the infected stimulus fish were higher on D16 (125.9±7.4) than D8 (44.2±1.8; $t_{32.6} = -10.6$, $p = 3.5 \times 10^{-12}$). No difference was detected in the length of the test fish that were exposed to the infection or control cue (22.0±0.4 mm).

Activity variables

Total distance moved, duration darting, and duration of freezing were not influenced by our experimental treatment during the 10-minute trial (Table 1), but the three activities differed by sex of the test fish and day of the trial. Males moved more, “darted” more and “froze” less than females (Figure 1). On D16, both male and female guppies moved more, “darted” more and “froze” less than on D8 (Figure 1).

Latency to first move was influenced by sex of the test fish but not the day of the trial and there were significant treatment*sex and treatment*sex*day interactions (Table 1). This interaction represented in Figure 2 shows that latency to first move was unaffected by guppy sex or treatment on D8. However, on D16, males rapidly responded to the control cue but had a longer latency to first move when exposed to the infected stimulus fish cues whereas female latencies were similar for infection and control cues (Table 1, Figure 2).

Location variables

Time spent in the center of the tank was significantly affected by treatment but not by sex or day of the trial (Table 1). Guppies exposed to the infection cue spent more time in the center of the tank whereas guppies exposed to the control cue spent more time in the periphery of the tank both on D8 and D16 (Figure 3).

All fish spent more than 50% of their time in the bottom third of the tank (Figure 4). Vertical position (top, middle, lower third to the tank) was unaffected by treatment, sex, or day of the trial (Figure 4).

Experiment 2: Prior exposure to an infection cue alters shoal tightness and reduces parasite transmission

Test fish exposed to the infection and the control cue were of similar length (16.8±0.3 mm), as were control and infected stimulus fish (19.4±0.7 mm). The number of parasites on the infected stimulus fish increased over time (D4: 19.1±2.7; D8: 31.1±7.2; D16: 49.4.2±19.9).

Activity, location, and social interactions during ongoing exposure to infection cues

Exposure to infection cues vs control cues did not alter how far shoals moved, duration of "freezing "events, latency to first move, or the proportion of time spent in the center of the tank. However, the duration of exposure to the cue significantly altered all behavioural variables (Table 2, Figure S1) indicating acclimation to ongoing cue exposure or habituation to the trial procedures. Compared with D0, the total distance moved by a shoal was lower on D4. Shoals were more likely to be in the center of the tank on D4 but more likely to be at the periphery on D8 and D16, compared with D0. Freezing events were higher on D4 and lower on D8 than D0. Latency to first move was lower on D8 than D0.

Shoals with ongoing exposure to infection cues had a shorter inter-fish distance on D16 compared to D0 and D4 based on comparison of least square means, but this was not seen in control shoals (Figure 5) and treatment was not significant in the model (Table 2). The duration of time spent in close proximity was not impacted by exposure to continuous infection cues but was affected to duration of exposure to the cues. Compared with D0, shoals spent less time in close proximity on D4 but more time in close proximity on D8 and D16, indication acclimation to the cue or increased familiarity with their shoal mates and experimental set up (Table 2, Figure 5).

Epidemic dynamics

Infection did not establish in one of the treatment tanks and in two of the control tanks, reducing the number of replicates to 7 and 6 respectively. Five guppies in the treatment group and four guppies in the control group died due to the infection over the 40-day period.

The prevalence and intensity of infection increased then decreased over 40 days regardless of whether shoals had previous exposure to an infection cue (Table 3, Figure 6). However, the temporal pattern differed by treatment. During the increasing phase of the epidemic (D4 to D18), parasite numbers increased more rapidly in the control shoals compared with shoals that had been exposed to the infection cue. During the declining phase (D18 to D40), a significant treatment*time interaction emerged (Table 4, Figure 7). In control shoals, intensity declined rapidly, plateaued, then declined further. In treatment shoals, intensity declined more slowly and began to increase. These results demonstrate that prior ongoing exposure to the infection cue dampened the epidemic following introduction of the parasite into the shoal.

Discussion

Key findings

The concept that infected hosts release chemical cues that warn conspecifics of the threat of infection has been studied in very few aquatic systems (Kiesecker et al. 1999; Poulin et al. 1999; Stephenson et al. 2018). In our study using the guppy skin ectoparasite, *G.turnbulli*, only three behavioural variables indicate that guppies were able to detect and respond to the putative infection cue. Both female and male guppies spent more time in the center of the tank. When the stimulus fish had been infected for 16 days but not for 8 days, male, but not female, guppies took longer to begin moving when in the presence of the putative infection cue compared with the control cue. Finally, shoal behaviour was altered by swimming tighter together, as indicated by the shorter inter-fish distance in response to late-stage infection cue. Despite the modest impacts on individuals and shoaling behaviour, prior prolonged exposure to the infection cue dampened the increase in intensity of infection after *G.turnbulli* was introduced to the shoal and dampened the subsequent decline in intensity relative to shoals that had no prior exposure to the infection cue. Together, these findings provide subtle evidence that guppy behaviour is altered by the release of chemical cues from infected guppies and provides evidence infected guppies release a chemical cue which not only alters epidemiological patterns of future infections but also provides a degree of protection against infection.

Comparison of behavioural responses to other chemical infection cues

In theory, chemical cues released by infected fish would warn conspecifics of a threat and lead to responses that would protect the conspecifics from infection. Evidence of the release of chemical cues has been reported during cercarial penetration of rainbow trout, where exposure to the putative cue led to more darting events and more time spent motionless in uninfected conspecifics (Poulin et al. 1999). Like during cercarial penetration, *G.turnbulli* hooks penetrate the fish skin presumably releasing chemicals into the water (Bakke et al. 2007), however unlike cercariae, *G.turnbulli* continue to live on the surface of the skin (Gheorghiu et al. 2012) and likely continuously puncture new epithelial cells. Due to these similarities with cercarial penetration, we expected guppy darting and freezing events to increase when guppies were exposed to chemical infection cues, however no evidence for these altered behaviours were found. Guppies exposed to a putative chemical cue released during late-stage *G.turnbulli* infection were recently shown to avoid the side of a tank near the source of previously frozen infection cues (Stephenson et al. 2018). In our study, guppies spent more time in the center of the tank when exposed to infection cues. When cues are released from individuals released at later stages of infections, males took longer to begin moving, indicating they detected this infection cue and may have been more cautious about moving (Piyapong et al. 2010), and shoals of fish had a reduced inter-fish distance. Thus, our data adds subtle evidence that infected fish do release a chemical cue that is received by conspecifics and elicits a shift in behaviour, specifically a preference for the center of the tank, a delay for male guppies in when they began moving, and tighter shoals.

Comparison of behavioural responses to predator alarm cues

In their natural habitat, guppies are more vulnerable to predation in open water than on the edge of ponds or rivers where they can find in vegetation (Elvidge et al. 2014). Thus, when guppies are exposed to predator alarm cues, they tend to move into less vulnerable positions, on the periphery or to the bottom of the ponds (Brown and Godin 1999). We initially hypothesized that guppies exposed to infection cues would also seek out the sides and bottom of the experimental tank. However, in individual guppy trials, guppies spent more time in the center of the tank compared to the periphery when exposed to infection cues collected at mid and late stages of infection. In contrast to predators, guppies may have more options for escaping an infected conspecific in the center compared with the periphery of the tank. Additionally, we did not observe alarm cue responses of freezing and darting during exposure to this putative infection cue (Brown et al. 2004). In other studies, a freezing event is defined as the cessation of movement for 30 seconds (Brown and Godin 1999; Lawrence and Smith 1989) and a darting event is described a burst in movement in a random direction of over 5 body lengths (Lawrence and Smith 1989), however we used a proxy measure of the cumulative duration of freezing and darting. Although we were not able to make direct comparisons with other studies for freezing and darting, the general trend should have detected differences if they were present. We also did not observe the characteristic alarm cue response of sinking to the bottom of the tank during exposure to this putative infection cue (Brown et al. 2004). Thus, we speculate that the composition of the infection chemical cue released in response to *Gyrodactylus* infection is not simply a diluted form of a predator alarm cue.

When guppies are exposed to predator alarm cues, they tend to shoal more tightly (Brown and Godin 1999; Chivers et al. 2012; Green et al. 2012; Poulin and FitzGerald 1989). Similarly, when some fish are exposed to trematode infections (Stumbo et al. 2012) or *Argulus* sp. ectoparasites (Poulin and FitzGerald 1989), they shoal more tightly and have a larger number of members in each shoal. This phenomenon seems intuitive for predator alarm cues, as when fish are closer together, a predator has a more difficult time selecting one individual to attack (Chivers et al. 2012). For parasites which are not transmitted via direct host contact, as the shoal moves closer together, the chance that each individual becomes infected with a parasite is reduced (Behringer et al. 2018; Poulin and FitzGerald 1989; Stumbo et al. 2012; Wisenden et al. 2009). However, in the case of *G.turnbulli* infections where transmission occurs as a result of direct physical contact, the risk of transmission would be higher in tight shoals, thus our results seem counterintuitive to previous research on infection avoidance. Guppies have been shown to actively avoid infected shoal mates (Croft et al. 2011) as well as chemical cues of infected shoal mates (Stephenson et al. 2018). Yet, we found evidence that a shorter inter-fish distance in response to the ongoing *G.turnbulli* cue when compared with earlier stages of infection. When guppies are exposed to chemical predator alarm cues, they are more vigilant to visual predator cues (Stephenson 2016). Thus, one possible explanation is that guppies had detected that the conspecifics they have been living with for 16 days were not infected since they did not have visual signs of infection such as clamped fins, paleness and excess mucus (Bakke et al. 2007; Hockley et al. 2014). These guppies are therefore more alert to infection potential and can determine that their shoal mates are not infected. They may then shoal with them more tightly to be protected from possible infection in the surrounding environment. However more studies are needed to gain more understanding on why guppies may be shoaling more tightly when exposed to infection cues.

Impact of infection duration on response to cue

Previous studies found that transmission risk is highest at later stages of infection (Boeger et al. 2005; Stephenson et al. 2018), and found evidence that avoidance of *G.turnbulli* chemical cues was highest when infection risk was highest (Stephenson et al. 2018). In our study, we also found that the duration of *G.turnbulli* infections played an important role in response of uninfected males inertia to begin moving and shoaling behaviour after exposure to the chemical cue. At later stages of infections, the parasite load on a fish is higher and damage to the epithelium is also increased (Bakke et al. 2007). Therefore, at later stages of infections, the amount of cue released and the concentration of cue in our experimental system may have been higher. We found that the parasite numbers of the stimulus fish did not impact our outcomes, however this study has not ruled out the possibility that this chemical cue can be arising from the parasite itself. The presence of dead cercariae in the water with fathead minnows reduced overall activity when fish had experienced prior cercarial penetration (James et al. 2008), therefore future directions should confirm if it is the chemicals released from the parasite or the extent of skin damage and/or immune by-products which results in the chemical cues which conspecifics respond to.

Males more responsive than females to cue

Male guppies are known to be bolder than females (Piyapong et al. 2010), and they are also thought to be more susceptible to severe gyrodactylid infections (Budaev and Zhuikov 1998; Richards et al. 2012). A shorter latency to begin moving has been used as a proxy for guppy boldness in previous research (Piyapong et al. 2010). We observed a longer latency to move when males, but not females, were exposed to later stage infection cues, thus indicating that male boldness may also have been reduced. Male guppies infected with *G.turnbulli* infections have been found to have reduced boldness, which reduces their reproductive fitness (Jacquin et al. 2016). It is thus conceivable that male guppies are more cautious than females to begin moving when exposed to chemical infection cues because they are more likely to suffer from severe infections (Elvidge et al. 2014; Jacquin et al. 2016). This reduction in boldness may be short lived, allowing them to avoid a transmission event. However if this reduced boldness lasts longer, there may be a reduction in reproductive success similar to a *G.turnbulli* infection. In contrast to our findings, Stephenson et al. (2018) found that guppy sex did not influence avoidance of the side of the tank where chemical infection cues were present. Therefore, it may be possible that only specific behaviours measuring boldness differ in response to infection cues. Future directions should thus consider if this altered behaviour in males correlates with a reduced risk of transmission or overall reproductive fitness.

Infection cue alters epidemic dynamics

The serial polyembryony and short generation time allow *G.turnbulli* numbers to increase rapidly on an individual and, when combined with direct transmission, allow prevalence and intensity in a population to rise quickly (Van Oosterhout et al. 2008). Once fish recover or die from infection, there are not enough susceptible individuals to sustain the parasite leading to the rapid decrease in prevalence and intensity (Scott and Anderson 1984). An outbreak will occur again when enough naive or susceptible guppies are in the population (Tadiri et al. 2019; Van Oosterhout et al. 2008).

In our experiment, the increase in prevalence was similar between experimental and control shoals, indicating that fish remained susceptible even when the shoal had been exposed to the infection cue and that transmission occurred within the shoal. On the other hand, the lower intensity during the increasing phase of the epidemic demonstrated that parasite reproduction and/or survival were reduced in response to the infection cue (Tadiri et al. 2019). If behavioural modifications alone resulted in lower infection intensity, we would expect that guppies exposed to chemical infection cues would have a greater inter-fish distance and thus a slower increase in prevalence, which was not the case. Given the rapid changes in the guppy epithelium (Gheorghiu et al. 2012) and in expression of immune related genes (Konczal et al. 2020) when a fish becomes infected, a question generated from these experiments is whether by-products from the immune response released in the water are responsible for the altered behaviour and reduction of parasite intensity in conspecifics.

Once infection was established in the shoal, the number of worms plateaued for the group exposed to the infection cue whereas the control group displayed a classic epidemic curve of quickly decreasing (Bakke et al. 2002; Van Oosterhout et al. 2008). We speculate that the initial reduction in parasite population growth in the group exposed to infection cues limited the infection duration of the first epidemic cycle as seen by the different pattern in epidemic profiles (Scott and Anderson 1984; Van Oosterhout et al. 2007). A future study monitoring long term behavioural and epidemic patterns in wild guppies when exposed to this chemical infection cue would allow for a better understanding about the degree to which this chemical infection cue impacts the transmission dynamics in wild populations.

Strengths and limitations

The design of this study allowed to compare results from well-established literature on predator alarm cues and chemical cues released from trematode infections, which are only released for a short period of time, to ongoing chemical cues released from an ectoparasite living on the skin of a fish. This study also highlighted specific behaviours impacted by these chemical infection cues such as positioning and shoaling which allowed for the prompting of hypotheses about how these chemicals may impact wild populations of guppies. Additionally, this study was the first to examine how prior exposure to chemical infection cues impacted transmission of *G.turnbulli*. Nevertheless, we acknowledge several limitations. Our sample sizes were relatively small and may have limited our power to detect additional effects of the infection cue. The guppy population used had been reared in the lab for many years, in the absence of any parasites or chemical cues (Blondel 2021). Thus, the behavioural responses to infection cues may have been dampened relative to wild fish with ongoing exposure to *G.turnbulli* which complicates making conclusions about these results (Kelley and Magurran 2003). Additionally, since the parasites were isolated from domestic guppies purchased from a pet supplier, we can make limited conclusions about how the behavioural and epidemiological patterns reported would differ if the parasite was from a wild source, especially given the gaps in the literature on how *G.turnbulli* strains found in domestic guppies differ from wild parasites. In experiment 1, we removed guppies from their home tank and studied their isolated behaviour and although we allowed the fish to acclimate for 10 minutes in the experimental arena (Croft et al. 2006), this change of environment and isolation from shoal mates may have been a greater stressor than any chemical cue in the water. The chemical composition and concentration of the chemical infection cue was not measured in this study, and it is possible that individuals and shoals were exposed to different composition or concentrations of the infection cue. Our design did not allow us to confirm that the cue was not released by the parasite. Finally, there were limitations in our Ethovision XT software as we were not able to track complex behaviours.

Conclusion

This study provided further evidence, although subtle and inconsistent with previous literature, that guppies respond to chemical cues released by infected fish. Additionally, it indicated that guppies exposed to infected fish chemical cues had a reduced infection intensity but shorter inter-epidemic period. Although the mechanism linking the behavioural differences and different epidemic dynamics are unclear, this study generated new observations and questions about how this putative infection cue may impact guppy-*Gyrodactylus* ecology.

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Tables and Figures

Table 1. Generalized linear model output for estimates (±SE) of fixed effects on individual fish behaviour when guppies are exposed to chemical cues for 10 minutes. These putative cues were released from guppies infected with *Gyrodactylus turnbulli* for 8 or 16 days. Variables which were not included in model represented by NI. $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$***

	Total distance moved (cm)	**Duration of “darting” events (%)**	**Duration of “freezing” events (%)**	**Latency to first move (s)**	**Duration in center of tank (%)**
Treatment	-0.13±0.20	-0. 31±0.26	4.9±6.5	-0.7±0.6	0.44±0.21*
Sex	0.41±0.19*	0.56±0.24*	-12.9±6.1*	-3.1±0.7***	-0.37±0.19
Day post exposure	-0.57±0.19**	-0.81± 0.24**	15.5±6.1*	0.5±0.7	-0.09±0.19
Time of day	-0.46±0.21*	-0.72±0.26**	NI	1.1±0.3**	NI
Treatment*Sex	NI	NI	NI	3.3±0.8***	NI
Treatment*Sex*Day	NI	NI	NI	-2.9±1.3*	NI

Table 2. Generalized linear mixed model output estimates (±SE) of fixed effects on shoal behaviour when groups of four guppies are continuously exposed to *Gyrodactylus turnbulli* infected guppies for up to 16 days. Tank used as random intercept. Variables which were not included in model represented by NI[1]. $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$***

	Total distance moved (cm)	**Duration of “freezing” events (%)**	**Latency to first move (s)**	**Duration in center of tank (%)**	**Average inter-fish distance (cm)**	**Duration in close proximity (%)**
Treatment	-0.06±0.11	-2.3±3.8	0.02±0.25	-0.06±0.14	-0.004±0.32	0.4±1.3
Duration of exposure[1]						
4 days	-0.20±0.07***	10.7±2.8***	-0.06±0.27	0.34±0.16*	0.44±0.37	-3.8±1.6*
8 days	0.13±0.07	-7.1±2.8*	-0.51±0.23*	-0.52±0.14***	-0.85±0.37*	5.1±1.6**
16 days	0.09±0.07	-5.7±2.8	-0.013±0.23	-0.44±0.14**	-1.2±0.37**	7.3±1.6***
Time of day	NI	NI	0.68±0.25**	0.39±0.14**	NI	NI

[1] Estimates compared to reference value (0 days of exposure)

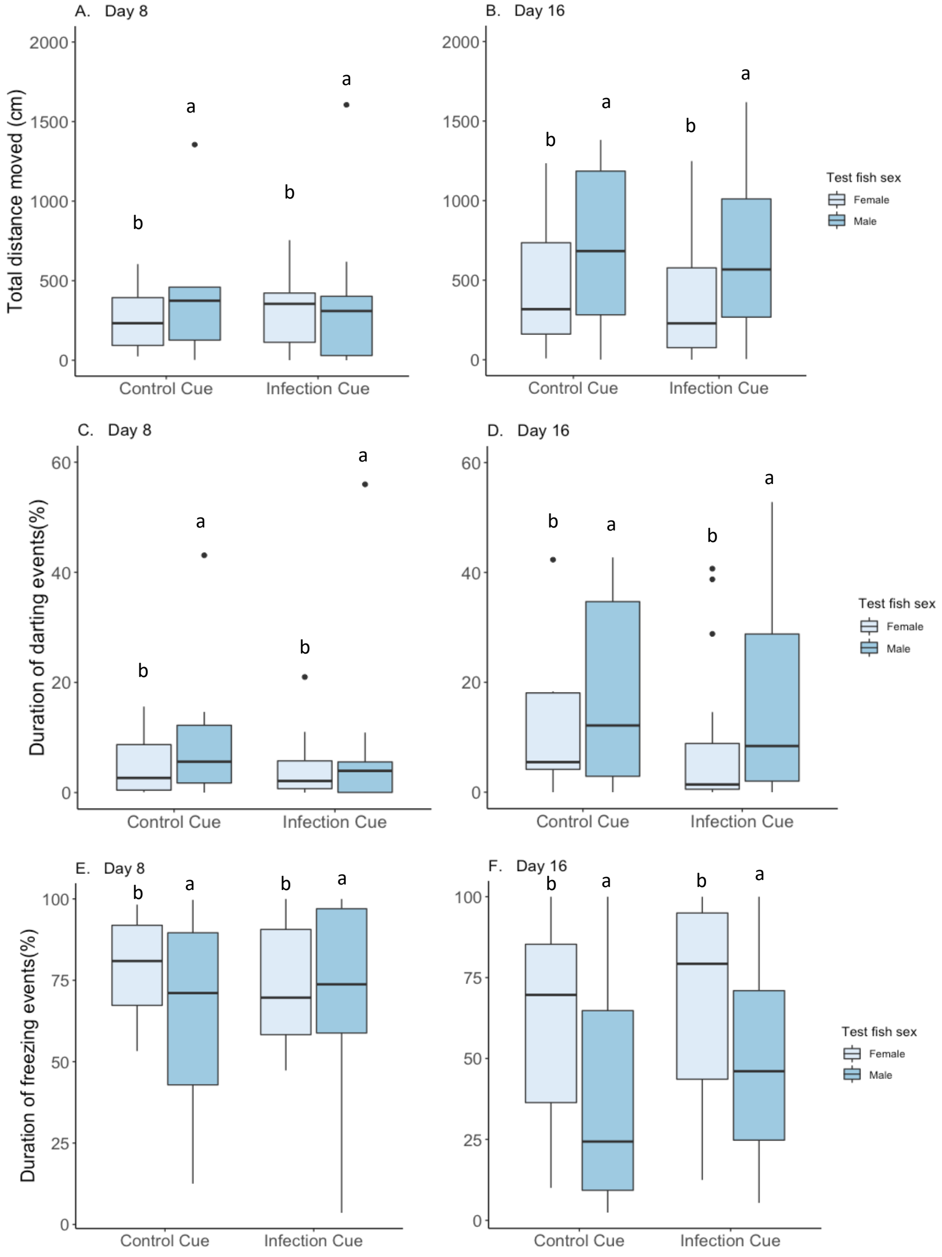
Table 3. Linear mixed model output estimates (±SE) of prevalence of *Gyrodactylus turnbulli* infection in guppies which were previously exposed to chemical cues released from *G.turnbulli* infected conspecifics. One linear model analyzed from day 4 to 18, then another model was used to analyse day 18 to 40. Tank and average shoal length used as random intercepts. p < 0.001***

	Prevalence D4 - D18	**Prevalence D18 - D40**
Treatment	-0.09±0.06	0.19±0.3
Day post infection	0.025±0.003***	-0.02±0.006***

Table 4. Generalized linear mixed model output estimates (±SE) for intensity of *Gyrodactylus turnbulli* worms in guppies which were previously exposed to chemical cues released from *G.turnbulli* infected conspecifics. One model analysed day 4 to 18, then another model analyzed day 18 to 40. Tank and average shoal length used as random intercepts. Variables which were not included in a model represented by NI. p < 0.001***

	Number of worms D4 – D18	**Number of worms D8 – D40**
Treatment	-0.66±0.09***	0.83±0.49
Day post infection	0.08±0.005***	-0.07±0.009***
Treatment*day of infection	NI	0.07±0.01***

Figure 1. Activity variables measured, total distance moved (A, B), duration of "darting" events as % of a 10 min trial (C, D), duration of "freezing" events as % of a 10 min trial (E, F) when an individual guppy was exposed to chemical infection cues for 10 minutes. These putative chemical cues were released from *Gyrodactylus turnbulli i*nfected conspecifics that had been infected for 8 days (A,C,E) or 16 days (B,D,F)[1].



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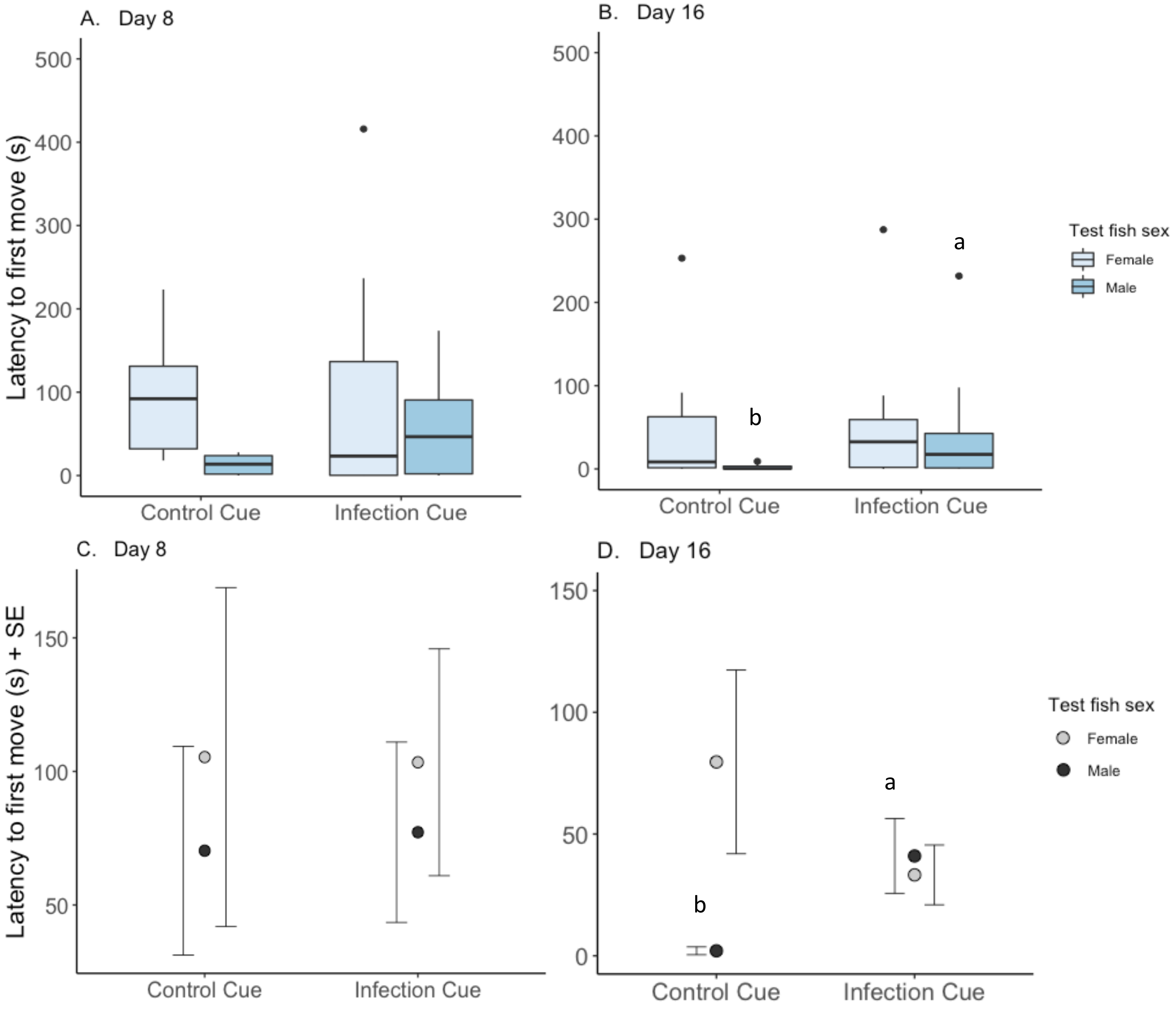
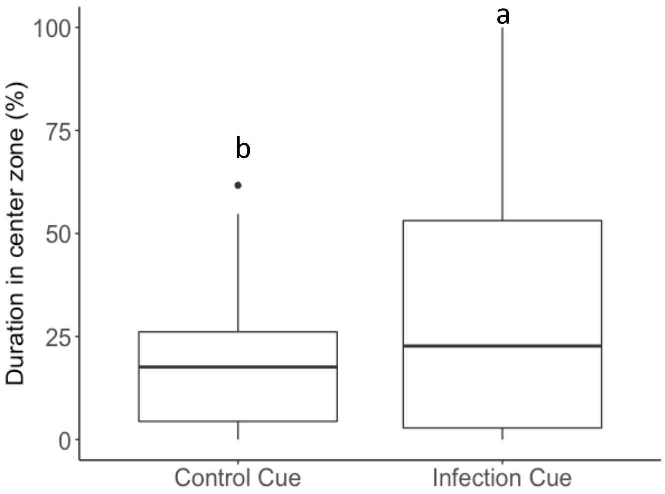
[1] Males are in dark blue and females in light blue. Different lowercase letters indicate significant difference between sexes within either the control or the infected cue exposure groups. For all variables except freezing, day 16 data were higher than day 8 data.

Figure 2. Boxplots for latency to first move when an individual guppy was exposed to water conditioned with chemical infection cues for 10 minutes. These putative chemical cues were released from guppies infected with *Gyrodactylus turnbulli* for days 8 (A) or 16 days (B). The significant interaction between treatment and sex is represented by least squared means±SE (C,D)[1].



[1] Females in the lighter colour (right SE bar) and males in the darker colour (left SE bar). Different lowercase letters indicate a significant effect of treatment in D16 male guppies.

Figure 3. Boxplot of time spent in the center zone compared to the periphery of the tank as % of a 10 min trial in individual guppies exposed to water conditioned with chemical cues for 10 minutes. These putative chemical cues were released from *Gyrodactylus turnbulli* infected conspecifics. No significant effect of day post infection or sex thus, day 8 and day 16 trials were pooled, and male and female trials were pooled[1].



[1] Different lowercase letters indicate a significant effect of treatment.

Figure 4. Stacked bar plots of the mean vertical position (top, middle, bottom) when individual guppies were exposed to water conditioned with chemical cues for 10 minutes. These putative chemical cues were released from *Gyrodactylus turnbulli* infected conspecifics that had been infected for either 8 (A,C) or 16 (B,D) days.

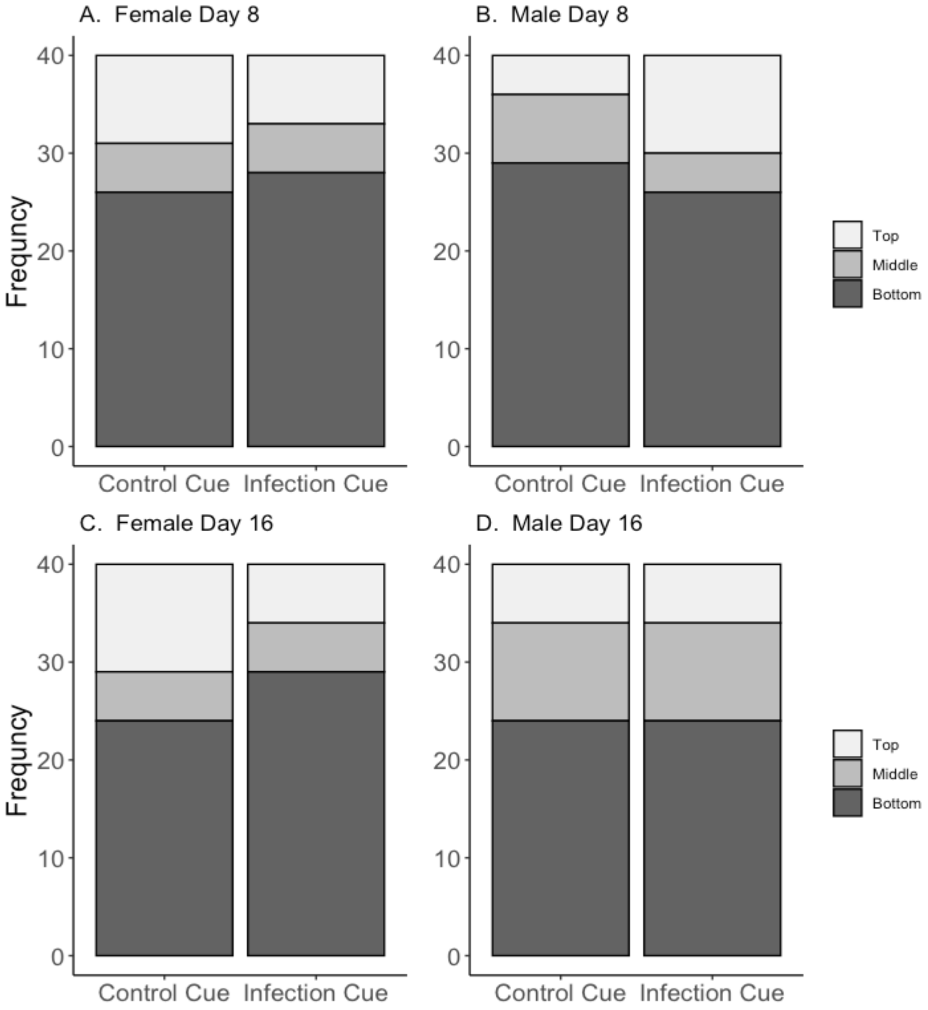
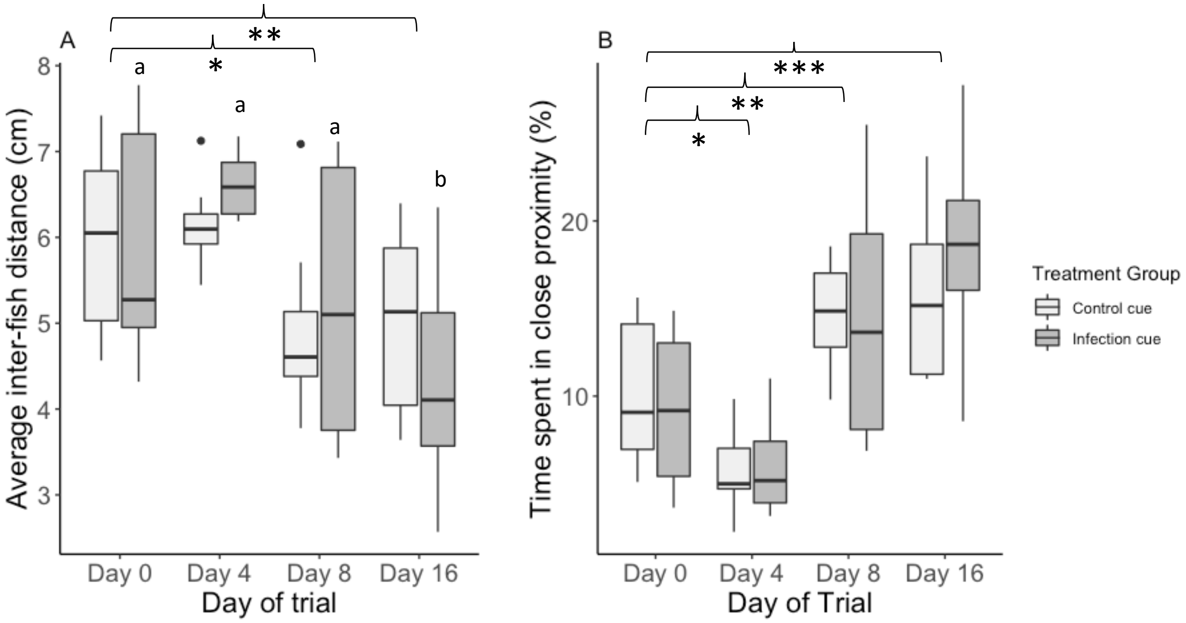


Figure 5. Boxplots of social interaction variables, average inter-fish distance (A) and duration in proximity less than 0.5cm (B) for shoals of guppies when exposed to continuous chemical cues from *Gyrodactylus turnbulli* infected conspecifics for up to 16 days. Day of trial corresponds to number of days test fish were exposed to chemical cues. Comparison bars represent significant effects of day of trial for pooled control and treatment groups. Lowercase letters indicate significant difference over time within a treatment group[1]. $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$***



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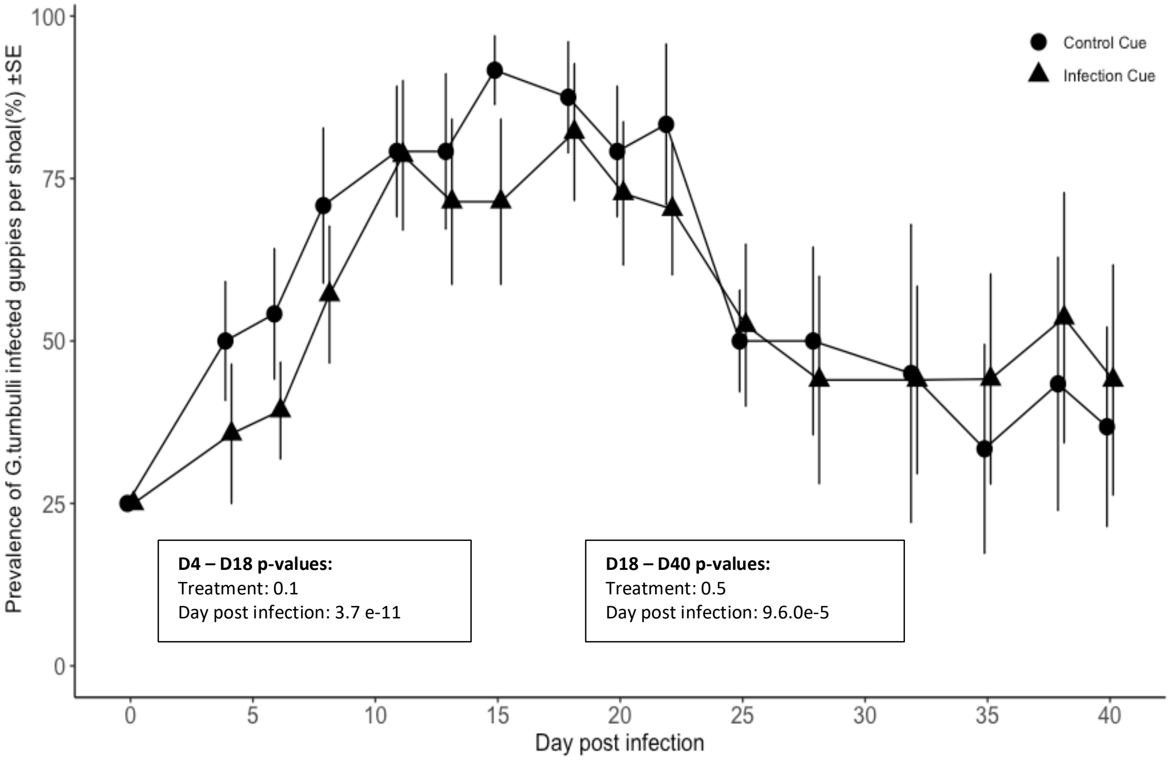
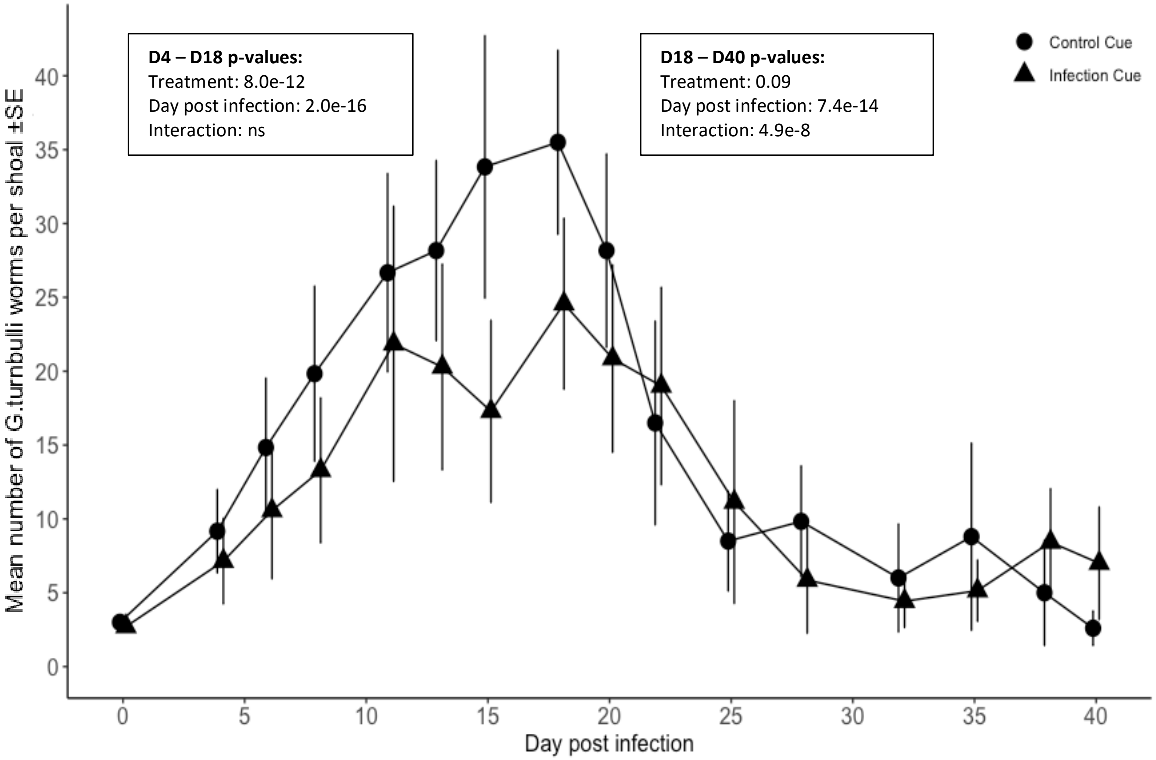
[1] Shoals exposed to putative infection cues are in dark grey, shoals exposed to control cues are in light grey. P-values demonstrate significant differences when compared to day 0 reference value for both treatment groups.

Figure 6. Prevalence (%) of *Gyrodactylus turnbulli* in guppy shoals over 40 days post infection. Shoals had previously exposed to chemical cues from *G.turnbulli*-infected fish or control fish for 16 days. P-values included for LMM day 4 – day 18 and day 18 – day 40[1].



[1] Data points for guppies exposed to infection cue are positioned slightly to the right to avoid SE bar overlap.

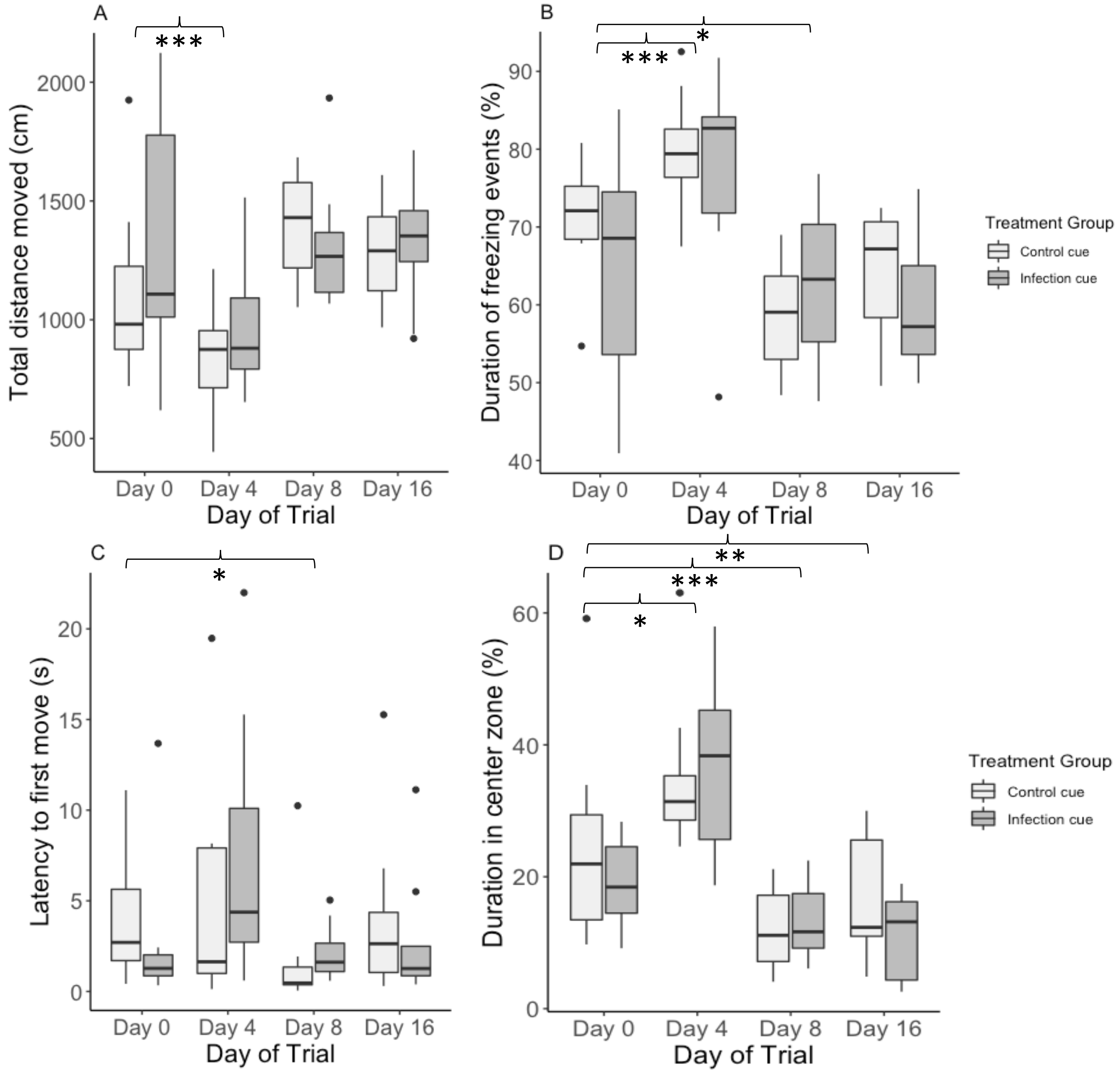
Figure 7. Intensity of *Gyrodactylus turnbulli* in guppy shoals over 40 days post infection. Shoals had previously been exposed to chemical cues from *G.turnbulli*-infected fish or control fish for 16 days. P- values included for GLMM day 4 – day 18 and day 18 – day 40[1].



[1]Data points for guppies exposed to infection cue are positioned slightly to the right to avoid SE bar overlap.

Supplemental Figure

Figure S1. Boxplots of behavioural variables for shoals, total distance moved (A), duration of "freezing" events (B), latency for first moved (C), duration in the center of the tank (D) when shoals of guppies were exposed to continuous chemical cues from *Gyrodactylus turnbulli* infected fish for up to 16 days. Comparison bars represent significant effects of day of trial for pooled control and treatment groups. Day of trial corresponds to number of days stimulus fish have been infected[1].



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[1] Shoals exposed to infection are in dark grey, shoals exposed to controls are in light grey. P-values demonstrate significant differences when compared to day 0 reference value for both treatment groups.

CHAPTER IV – General Discussion

Our first hypothesis that individual and shoaling of guppies exposed to infection cues would be altered by exposure to infection cues was supported but effects were different from what we had anticipated. We had initially hypothesised that guppies would dart and freeze more, and they would spend time in periphery of the tank. Instead, in response to the chemical infection cue, darting and freezing was unaffected and guppies spent more time in the center of the tank. Consistent with the literature (Stephenson et al. 2018) responses were detected during later stages of infection of the stimulus fish. Males took longer to begin moving when exposed to chemical cues from fish in late stages of infection and shoals were more tightly spaced, after prolonged exposure to the cue. Our second hypothesis that infection dynamics would differ in response to prior exposure to infection cues was also supported as the epidemic dynamics differed. Shoals with prior exposure to *Gyrodactylus* chemical cues had a lower infection burden during the increasing phase of an outbreak and showed the pattern of subsequent decline and rise diverged.

1. Nature of the putative infection cue

Although the aim of this thesis was not to determine the chemical composition of the cue, the results allow for comparisons, hypotheses, and speculations about what chemical(s) it may contain.

1a. Comparison with predator alarm cues

Given that previous studies found that fish exposed to trematode infection cues behaved in similar ways as if they were exposed to predator alarm cues (Poulin et al. 1999), much of the early literature assumed that the composition of this infection cue would be similar to predator alarm cues (Poulin et al. 1999). However, *Gyrodactylus* are anchored to the surface throughout the infection are whereas trematode infections rapidly penetrate layers of epidermal tissue (Koprivnikar et al. 2006). Therefore, it is possible that cues released from trematode infections are similar to predator alarm cues (Poulin et al. 1999), whereas *Gyrodactylus* infections elicit different types of chemical cues. Guppies exposed to the *Gyrodactylus* infection cue did not show the characteristic behaviours of exposure to alarm cues which include sinking to the bottom of the tank, hiding in the periphery of the tank, and increase in freezing and darting (Brown

2003; Brown and Godin 1999; Stephenson 2016). Even at low concentrations, alarm cues alter the behaviour of fish (Lawrence and Smith 1989), thus if this infection cue was simply a low concentration of the same chemicals as predator alarm cues, we would expect to see these characteristic behavioural differences. Therefore, we suspect the chemical composition of cues released by gyrodactylid infections may not be the same as predator alarm cues.

1b. Immune by-products

A question to arise from this thesis is how prior exposure to chemical infection cues can reduce worm burden in previously uninfected fish. It is possible that the chemical cues may be immune by-products. When a guppy is infected with *G.turnbulli*, the skin epithelial layer thickens and mucus composition changes (Gheorghiu et al. 2012). Additionally, the immune response includes activation of the host compliment and production of several cytokines which modulate the immune system by generating prostaglandins (Buchmann 1999; Buchmann and Lindenstrøm 2002; Lindenstrøm et al. 2004; Zhi et al. 2018). Finally, *Gyrodactylus* infections increase the cortisol levels of fish, which are speculated to be released into the surrounding water (Reynolds et al. 2018; Stoltze and Buchmann 2001). Therefore, the chemical infection cues to which guppies respond may be one or a combination of immune by-products released into the water by infected fish (Ángeles Esteban 2012). Given that in our experimental shoals, the increase in infection intensity was dampened by prior exposure to these chemical cues, it would be intriguing to determine if any immune by-products played a role in reducing worm burden. Future studies could expose fish to a combination of *Gyrodactylus* immune by-products such as acidic mucins, various prostaglandins, and cortisol to monitor if there are changes in behavioural response or reduction in future worm burdens. Alternatively, histology could be used to determine if prior exposure to the infection cue increased epithelial thickness and alter mucus composition as occurs during direct infection (Gheorghiu et al. 2012). A follow up study can determine if *Gyrodactylus* infection and exposure to *Gyrodactylus* chemical cues activates similar immune cells via confocal microscopy (Konczal et al. 2020). Together, these follow up studies will allow for better hypotheses regarding if the reduction in parasite burden observed in this study was a result an immune response or if this reduction was mediated by guppy behaviour.

1c. Skin microbiome

The fish microbiome has been shown to influence fish behaviour through metabolic processes (Soares et al. 2019). For example, a healthy gut microbiome normalizes basal stress levels (Gareau et al. 2008) as well as the production of metabolites, both which can influence chemical communication between fish conspecifics (Ezenwa and Williams 2014). However, the impact of the skin microbiome on social behaviour of animals has yet to been comprehensively studied (Soares et al. 2019). Studies on predator alarm cues revealed that a combination of bacterial lysates in the skin elicited a similar response to predator alarm cues, thus bacteria on the surface of the skin may play a role in alarm cue signaling (Chia et al. 2019).

Ectoparasites impact the abundance and species diversity of fish skin microbiome (Kashinskaya et al. 2021; Vasemägi et al. 2017). *G.turnbulli* infections increase the amount of mucus and composition of mucins within epithelial mucus cells (Gheorghiu et al. 2012). Mucus composition can alter the skin microbiome of fish infected with a marine monogenean ectoparasites (Fernández-Montero et al. 2021). Therefore, we speculate that the skin microbiome may play a role in the altered behavioural response to infection cues as guppies may be responding to the altered microbial population of their *G.turnbulli* infected conspecifics. A future study could treat *G.turnbulli* infected guppies with a broad spectrum antibiotic and expose chemical cues from this germ-free infected fish to uninfected conspecifics and monitoring their behavioural response (Chia et al. 2019). Comparison of the skin microbiome between infected and uninfected guppies would also provide further insights on this hypothesis.

1d. Parasite excretory/secretory products

One final possibility is that these chemical cues are released from *G.turnbulli* itself. This study was not designed to control for the possibility that excretory and secretory products, which *Gyrodactylus* releases on the fish skin and subsequently in the surrounding water, play a role in the behavioural or epidemic response observed (Reynolds et al. 2018). Gaps in the literature detailing if it would be possible for parasite excretory/secretory products to influence fish behaviour have been noted (Caña-Bozada et al. 2021), and other studies using this model have not specifically controlled for the possibility that the chemical cue may be arising from the parasite itself (Stephenson et al. 2018). Designing a study using the excretory/secretory products of live *Gyrodactylus* worms may be challenging as they do not survive long when detached form a host (< 24 hours) and manipulating the parasites to dislodge them from a host often kills them (personal observation). Therefore, a future study may include exposing uninfected fish to homogenized worms, in order to see if these products elicit a behavioural response or overall reduction in parasite burden (Caña-Bozada et al. 2021).

2. Ecological Implications

2a. Risk of predation

Although risk of predation seems to exert the strongest selective pressure for behavioural and physiological adaptation in guppies (Elvidge et al. 2014; Gotanda et al. 2013; Jacquin et al. 2016), fish must constantly balance anti-predator behaviour with parasite avoidance (Behringer et al. 2018). Therefore, when considering how cues released from *Gyrodactylus* infections may impact guppy behaviour in a natural setting, it would be inappropriate to exclude the impact predators may also have (Botham et al. 2008). We found that fish exposed to chemical infection cues spend more time in the center of an experimental tank, yet previous studies have demonstrated that fish exposed to visual or chemical alarm cues from predators avoid the center of the tank since this is the area where they would be most vulnerable to predation (Ferrari et al. 2010; Swaney et al. 2015). We suggest that the likelihood of close contact with an infected fish is lower in the center of the tank and thus spending more time in the center would lower the risk of infection, but increase their risk of predation (Lima and Bednekoff 1999). However, if guppies detect they are also at risk of predation, they will likely ignore the infection chemical cues as a trade-off for immediate survival (Koprivnikar and Penalva 2015; Pérez-Jvostov et al. 2012).

We found that guppies with prior exposure to chemical cues had a lower worm burden during the increasing phase of a *G.turnbulli* outbreak. Guppies with higher worm burdens are at higher risk of predation as they are less efficient swimmers (Van Oosterhout et al. 2008). Therefore, if guppies can detect this parasite induced chemical cues, they may have a reduced mortality both from reduction in infection intensity and from better ability to evade predators.

2b. Male fitness

A shorter latency to begin moving after exposure to a stimulus has been used as a proxy for fish boldness (Ehlman et al. 2015; Piyapong et al. 2010), as bolder fish will be less hesitant to start moving after a stimulus. Boldness in males has been attributed to better mating and foraging success (Piyapong et al. 2010), as well as higher survival rates from predators (Smith and Blumstein 2010). One intriguing finding in this study was that males, but not females, exposed to later stages of chemical infection cues took more time to start moving, perhaps indicating that exposure to the cue lowers their boldness. Fish that are less bold have reduced foraging success and reduced success in mating with females (Harris et al. 2010; Piyapong et al. 2010). Additionally, as foraging determines high quality carotenoids in the diet which is correlated with mating success (Kodric-Brown 1989), males exposed to the late stage of infection cue may have overall lower reproductive success. Therefore, we may be observing a trade-off between reproductive success and parasite avoidance, as males are generally more at risk of severe infection and mortality from *G.turnbulli* infections (Stephenson et al. 2016). However, males may only act less boldly for the short period of time needed to move away from the infection cue. Thus, future research should first confirm that male boldness is reduced, how long the change in boldness lasts, and whether ongoing chemical infection cues do in fact reduce overall reproductive success for males.

2c. Impact on transmission dynamics

We observed lower infection intensity during the increasing phase of an outbreak and that the pattern of the decreasing phase differed if shoals had previous exposure to chemical infection cues. Thus, in natural environments, we speculate that ongoing exposure to chemical cues will lower the overall worm burden and shorten the inter-epidemic period (Tadiri et al. 2019). The rate of water flow may play a role in how concentrations of this infection cue are perceived in a guppy's natural environment. Water flow along river and stream systems in Trinidad can be relatively slow, near almost stagnant pools, or can have high velocity near waterfalls (Blondel et al. 2019). Previous studies have observed a link between parasite transmission and water flow (Reynolds et al. 2019). In fast moving waters, *G.turnbulli* peak intensity was greater than in still water (Reynolds et al. 2019). Additionally, previous research has observed links between water flow rates, infection rates and guppy shoaling. In an infected shoal living in a low flow zone, as parasite load increased, the individuals in a shoal had a greater distance from each other. However, in fast moving water, as parasites increased, there was no difference in distance between shoal mates (Hockley et al. 2014; Reynolds et al. 2019). Our experimental tank which included only a small air stone, resembled a static to low flow regime (Hockley et al. 2014). In this regime we observed that chemical cues reduced parasite intensity during the increasing phase of an outbreak. It may thus be conceivable that in high flow regimes, the concentration of chemical infection cues is lower as the cues are swept away by the moving water. Thus, guppies are not exposed to ongoing chemical infection cues which may explain why the shoaling behaviour and peak parasite intensity differs between flow regimes (Reynolds et al. 2019). Future studies can include the exposing chemical infection cues to guppies at different water flow intensities, then introducing infection to the system and monitoring transmission over time.

3. Future directions and broader implications

Studying the ecology of chemical infection cues may help us understand how some organisms protect themselves from pathogens and could potentially lead to pharmaceutical benefits (Behringer et al. 2018). For example, as a reduction in parasite burden after exposure to chemical infection cues was observed in this study, characterising this chemical cue may allow for the adoption of chemical cues in fisheries or aquacultures which are struggling with uncontrolled *Gyrodactylus* spp. outbreaks (Denholm et al. 2016; Peeler and Thrush 2004). Additionally, as pollutants such as microplastics, petrol and elevated levels CO_2 enter aquatic systems, understanding of how foreign chemicals interfere with an organism's ability to communicate and survive will become crucial in conservation practices (Ferrari et al. 2010; Hamilton et al. 2017; Rohr et al. 2009).

Conclusion

Overall, this research has provided novel insights about the behavioural modifications and changes in epidemic patterns that guppies experience when exposed to chemical infection cues released by a *Gyrodactylus* infection. It has generated several hypotheses and new research questions, and we are optimistic that this work will inspire others to better understand this intriguing chemical infection cue.

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