Gyrodactylid Biology, Transmission and Control

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The contents of Chapters 6-8 and Appendix IV-VII in this thesis are covered by a confidentiality agreement and conform to the ethical policy of WALTHAM Centre for Pet Nutrition.

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ABSTRACT

Infectious disease in aquaculture is omnipresent, but traditional treatments used against the causative agents often have broad anti-parasitic properties, are not 100 % efficacious, are toxic to humans, fish and the environment, and successful application is challenging. Hence, integrated disease management that combines an understanding of parasite life history and host welfare with treatment is highly recommended.

This thesis examined the life history characteristics of a ubiquitous genus of fish ectoparasites, *Gyrodactylus* species, and tested the efficacy of botanical treatments against gyrodactylids using the guppy (*Poecilia reticulata*) infected by *G. turnbulli* or *G. bullatarudis* as model, with the aim to work towards an integrated management plan for gyrodactylosis in the ornamental fish industry and the hobbyist market. The experiments conducted have shown that (1) gyrodactylids engage in sexual reproduction and on a population level show hybrid fitness and/or inter-strain competition when diverged genotypes are recombined; (2) parasite transmission is mainly driven by direct contact with potential hosts and on dead fish or after detachment gyrodactylids have a window of opportunity to re-attach of over 20 h; (3) extreme habitats provide guppies with a refuge from microbial and gyrodactylid infections in natural populations; (4) anti-parasitic treatment efficacy may be species specific using traditional methods; and (5) salt, garlic, cajuput oil and a combination treatment of the two commercially available treatments Melafix® and Pimafix® are efficacious alternatives for broad anti-parasitic aquarium treatments against gyrodactylids.

Due to their efficient reproduction, gyrodactylids are unlikely to be eradicated, as just a single, pregnant worm can trigger a new disease epidemic. However, combining the knowledge on pathogen life history characteristics and host-parasite interactions with effective treatment application will reduce evolutionary pressures on the pathogen and slow its virulence evolution. Overall, this increases the time available for treatment efficacies to be maintained in a manner which corresponds with new drugs being developed as treatment resistance arises.

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CHAPTER 1: GENERAL INTRODUCTION

Parasites are ubiquitous and make up the majority of living organisms on this planet. Indeed, infectious disease is a major factor in controlling natural populations and driving the evolution of species. Parasites impact natural dynamics in ecosystems, populations and individuals. This thesis focuses on infectious disease in aquatic environments, trying to understand the dynamics underlying parasite persistence in natural populations, and to advance effective disease management plans in aquaculture and the ornamental fish industry, in combination with botanical pathogen control. The following introduction includes work adapted from Schelkle et al. (2009) entitled 'treatment of the gyrodactylid infections in fish'.

1.1 Infectious disease in aquatic environments

Aquatic environments provide an ideal habitat for the survival of a wide range of adult and intermediate stages of parasites as water and the presence of species-rich zooplankton communities, which serve as hosts to intermediate parasite stages, aids their dispersal and transmission (Marcogliese 1995, Barber and Poulin 2002). Despite expected differences in disease epidemiology as a result (McCallum et al. 2004, Murray 2009), some parasite-induced costs to host life history, such as effects on survival and behaviour, and parasite traits, such as virulence and infectivity, are comparable to terrestrial host-parasite systems, thus allowing the use of aquatic models to address wider questions in, for instance, host-parasite evolution (e.g. Decaestecker et al. 2007), conservation (e.g. Faria et al. 2010) and the effect of infection on individual and population behaviour (e.g. Barber et al. 2000). Further, the presence and intensity of parasite infections in natural populations may even be used as indicators of overall aquatic ecosystem health (Marcogliese 2004, 2005, Blanar et al. 2009) and changes such as those attributed to climate change, habitat degradation and biological invasions (Marcogliese 2001, Okamura and Feist 2011, Poulin et al. 2011).

Fish are one of the key organisms in aquatic ecosystems with their evolution, growth, population recruitment success and mortality being strongly influenced by parasites (e.g. Barber and Poulin 2002, van Oosterhout et al. 2007, Lafferty 2008, Longshaw et al. 2010, Britton et al. 2011). It has been previously suggested that some fish populations seek refuge from parasites through adaptation to extreme environments to allow hosts to avoid infectious disease and increase the general health and fitness of the population (Tobler et al. 2007). The effect of the environment on host parasite assemblages is particularly notable in diadromous fish; parasites are lost due to changes in water chemistry when migrating between fresh and sea water (e.g. sea lice infecting salmon; Dill et al. 2009).

Wild fish populations are at higher risk of parasitic infection in areas where aquaculture is present (Tlusty 2002), as farmed fish maintained in open water systems may infect natural populations with both native and exotic pathogens (Naylor et al. 2001). *Vice versa*, farmed fish may become infected by 'wild' pathogens with the exchange of different parasites and parasite strains of varying global origin driving disease emergence in aquatic systems (Okamura and Feist 2011, Peeler and Feist 2011). A prominent example are salmon lice for which cross-transmission between wild fish populations and aquaculture stocks of parasites is not uncommon causing high economic costs and conservation concerns (Costello 2009).

1.2 Parasite life histories: How do reproduction and transmission contribute to disease persistence and epidemics?

The establishment, persistence and outbreak of disease varies across a host population and is dependent on host-specific factors (genetics, condition, sex, pregnancy, age, concomitant infections), parasite host specificity, life cycle complexity, virulence, reproduction and transmission. Due to their impact on human and livestock health, terrestrial parasites are comparatively well studied, allowing the use of intricate models to predict the spread and persistence of infectious disease in humans, domestic and wildlife populations (e.g. Keeling et al. 2001, Fulford et al. 2002, Li et al. 2004). For aquatic parasites, only a basic understanding of the life cycle is often available, with little knowledge on the effect sizes of transmission and reproduction parameters, despite the importance of such information in understanding evolutionary processes such as drug resistance (Criscione and Blouin 2004).

Parasites are generally highly fecund to account for high mortalities during their transmission stage with reproduction being strongly dependent on host condition (Calow 1983, Tinsley 2004, Seppälä et al. 2008). Hermaphroditic parasites also have the advantage of reproduction in the absence of mates and/or can invest in rapid population growth (also true for truly asexual organisms) without the necessity to mate (Schmid-Hempel 2011). In environmentally challenging circumstances, such as under immune attack by hosts, hermaphrodites may be more inclined to reproduce sexually leading to a mixing of the gene pool with potentially better adapted genotypes to the altered habitat conditions or offspring with improved transmission or dispersal chances (Cadet et al. 2004). Successful transmission from one host to another is generally more straightforward for directly than indirectly transmitted diseases; hence, fecundity for directly transmitted parasites tends to be much lower (Bush et al. 2001) and dispersal tends to depend on the availability and infectivity of as well as the immune health of both current and new host (Schmid-Hempel 2011).

With the increase in human population and animal domestication, it has become the norm to rear large densities of animals, for both food and companionship, in confined areas. Outside of their natural environment, these animals are particularly vulnerable to parasites as the increased stress experienced in intensive farming conditions impacts immune system functioning and the high host density benefits parasite transmission (Ashley 2007, Schmid-Hempel 2011). The ensuing problems associated with decreased animal welfare caused by infectious disease impact greatly on aquaculture productivity in large scale fish farms as well as small, private aquariums of enthusiasts.

1.3 Infectious disease and its control in food fish and ornamental aquaculture

Aquaculture is a growing industry with current projections indicating that, with the rise in the human population, an extra 29 million tonnes of fish per year will be needed by 2030 to keep up with demand (Cressey 2009). In 2009, global production of fish, crustaceans and molluscs was at 55.7 million tonnes and worth an estimated US $105.3 billion (FAO 2010). Although the trade in ornamental fish is almost negligible when compared to global food fish production, it is high in economic value. Alone in 2005 global exports and imports of ornamental fish were valued at US $ 237,636,000 and US $ 282,549,000, respectively (European Commission 2008).

Similar to terrestrial farming conditions, aquaculture is heavily affected by parasites (e.g. Scholz 1999, Barber and Poulin 2002, Bondad-Reantaso et al. 2005, Ashley 2007, Guo and Woo 2009). Infectious disease causes direct economic losses to the industry limiting its development and sustainability, increasing operating costs, restricting trade and impacting on biodiversity (Daszak et al. 2000, Whittington and Chong 2007, FAO 2010, Peeler and Feist 2011). It has been acknowledged that good husbandry conditions can improve health management considerably, but may not always prevent disease outbreaks (Ashley 2007). Prophylactic treatment is an option, but ultimately often only keeps disease at manageable low levels and does not completely eradicate it. In case of disease outbreaks, aquaculturists and fish keepers rely on a limited number of traditional treatments that are broadly anti-parasitic, but not 100% effective. For instance, saltwater is generally recommended against freshwater diseases and *vice versa*. Other common treatments include formalin, malachite green, hydrogen peroxide and metranidazole, all of which are highly toxic to host organisms, as well as humans, particularly at higher doses and prolonged, repeat exposures (Aldermann 2009).

As with all host-parasite systems, regular and continuous drug use exerts selection pressures on pathogens leading to disease evolution in aquaculture (Nowak 2007). Drug resistance is increasing, particularly resistance to antibiotics, with implications for human health (Cabello 2006, Verner-Jeffreys et al. 2009). Even if classical drug resistance is absent, pathogens will evolve to be more virulent or gain a transmission advantage leading to a gradual decline of drug efficacy and an increase

in disease emergence (Murray and Peeler 2005, Schneider et al. 2008, Mennerat et al. 2010, Pulkkinen et al. 2010, Peeler and Feist 2011). Reduced and/or partial effectiveness of currently used drugs, the exclusion of traditional drugs like formalin, malachite green and metranidazole from the Veterinary Medicine Directive under Annexes I, II or II of the European Council Regulation 2377/90 (primarily for food fish production in the European Union with similar bans affecting aquaculture in North America), as well as consumer pressure, mean that research into alternatives that are effective and safe for fish hosts, humans and the environment is vital (Alderman 2009).

1.4 Integrated infectious disease management and chemical control

The need to improve maintenance conditions in domestic fish populations to combat infectious disease has long been acknowledged, but may not always be the reality in aquaculture where pathogen treatment may be continuous and/or prophylactic (Tlusty 2002, Bondad-Reantaso et al. 2005, Ashley 2007). The idea of using knowledge of parasite and host life histories to effectively manage infectious disease is also not new with many existing drugs targeting certain periods or stages of the parasite life cycle. Equally, selective breeding for disease resistant fish is an option (Cnaani 2006, Salte et al. 2010). Generally, working under the concept that 'prevention is better than cure' is the best way to avoid epidemic infectious disease problems, but disease outbreaks are still common. This is also true when disease is released from low levels in populations through cessation of continuous, low level 'prophylactic' treatment; something that many aquarium owners experience when buying new fish from pet shops. In such situations, chemical control may still be necessary, ideally applied in an optimized way to avoid, or at least slow down, drug resistance (Débarre et al. 2012).

General drug discovery is experiencing a renewed interested in natural products as botanicals may potentially be good alternatives as anti-parasitic agents to replace traditional chemotherapy. Indeed, many of the current, approved drugs are derived from natural products (Schmahl 1989, Anthony et al. 2005, Li and Vederas 2009). New anti-parasitic treatments are expected to come from plant families that are clustered and not scattered in the phylogenetic tree (Zhu et al. 2011) and the use of whole plant structures may even provide mutual hyper-susceptibility characteristics against pathogens (e.g. Hu et al. 2010). Recent studies have found the testing of various botanical extracts against helminths in humans and cattle to be successful (Anthanasiadou et al. 2007, Mali and Mehta 2008), indicating they may also be effective against worms in aquatic environments. Future research should focus on pathogen specific drugs, rather than rely on broad anti-parasitic properties acting against a range of parasite genera. Even within specific genera, however, there may have to be multiple approaches to successful disease management, and this also applies to the specious group of gyrodactylids.

1.5 Gyrodactylids

Gyrodactylus spp. are ectoparasitic helminths that are omnipresent on teleost fish. Viviparous gyrodactylids (Fig. 1.1), the focus of this thesis, reproduce asexually, parthenogenetically and potentially sexually giving birth to live young (Cable and Harris 2002). Their reproductive system is further enhanced by a phenomenon termed hyperviviparity (Cohen 1977) in which the daughter of the hermaphroditic worm starts developing its own embryo whilst still in the womb of its mother. Such an acceleration in the life cycle means some species reproduce within 24 h of their own birth at their optimal temperature (*G. turnbulli* at 25 °C; Scott and Nokes 1984). Such rapid generation turnover and direct transmission allows for a high epidemic potential. Further, highly efficient reproduction and transmission on contact is an ideal requisite for evolution of new species, explaining the huge diversity of the genus *Gyrodactylus* (> 400 species described; Harris et al. 2004) for which host switching, rather than co-evolution, appears to be the norm (Bakke et al. 2007).

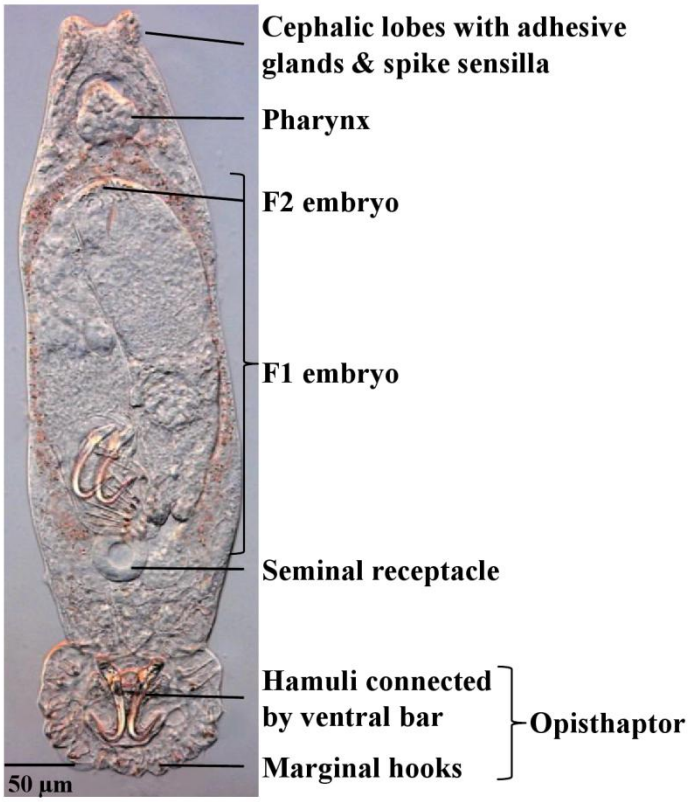

Fig. 1.1. Light microscope photograph of a viviparous, pregnant gyrodactylid. Note the developing attachment organ of the third generation worm (F2) in the second generation (F1) embryo (after Bakke et al. 2007).

Gyrodactylids are major drivers of evolution in natural fish populations: in Trinidadian guppies one extra parasite increases the likelihood of male fish to be flushed downstream in yearly flooding events by 19 %, so exposing guppies to predator rich environments to which they are maladapted (Magurran 2005, van Oosterhout et al. 2007). In Norway, the accidental introduction of *G. salaris* in the 1970s

with Baltic salmon (*Salmo salar*) resulted in up to a 90 % decline in some of the Atlantic salmon populations (Bakke et al. 2007). Currently, whole river courses are being routinely treated with rotenone as often as every two years, with treatment occurring over two consecutive days (Giuseppe Paladini, personal communication). Because of the general eco-toxicity of the treatment, the dose has to be tightly controlled and even concentration (i.e. efficacy) across and along the whole waterway can seldom be guaranteed contributing to the re-occurrence of epidemics (Kjærstadt and Arnekleiv 2011). In ornamental fish, gyrodactylids can be found from aquaculture production areas to pet shops (Kim et al. 2002, Thilakaratne et al. 2003, Whittington and Chong 2007, Hongslo and Jansson 2009) and can be equally destructive with up to 90 % mortalities in infected fish stocks (personal observations).

The high epidemic potential of gyrodactylid monogeneans due to their reproductive system, direct transmission and generalist host specificity makes them not only one of the most invasive parasite species worldwide (Bakke et al. 2007, King and Cable 2007, King et al. 2009), but also one of the most difficult ones to treat as just one pregnant worm is enough to initiate a new epidemic. Despite a large numberof treatments having been tested in various gyrodactylid systems, few show the potential to be highly effective on larger scales as most of the treatments have broad anti-parasitic properties, are less than 100 % efficacious and many are toxic to host, humans and the environment.

1.6 Treatment of gyrodactylid infections in fish*

A frequently asked, but rarely addressed, question is what is the most effective means of removing *Gyrodactylus* spp. from their hosts? With increasing interest in this large group of ectoparasitic worms, not only as important pathogens but also as model organisms (reviewed by Cable and Harris 2002, Bakke et al. 2002, 2007), it is timely to document the methods that have been used to remove gyrodactylids.

1.6.1 Problems with existing treatments

Some 99 compounds and treatment combinations have previously been used to treat gyrodactylid infections in research facilities, aquaculture and the hobbyist market (Table 1.1, end of chapter). Selecting the best of these treatments, however, is difficult, because few studies have compared compounds using the same methodologies and the majority of treatments have various associated problems. Formaldehyde, for instance, was found to be 100 % effective at eliminating *G. salaris* experimentally (Buchmann and Kristensson 2003), but under large scale aquaculture conditions it does not eradicate gyrodactylids completely (Rintamäki-Kinnunen and Valtonen 1996, Rowland et al. 2006). In some three spined stickleback (*Gasterosteus aculeatus*) populations it is only 10 % effective (personal observations). Due to its broad anti-parasitic properties formaldehyde is still commonly

*Adapted from: Schelkle B, Shinn AP, Peeler E, Cable J (2009) Dis Aquat Organ 86: 65-75.

used in aquaculture, even though it is classed as a human carcinogen (IARC 2004). Mutagenic and carcinogenic effects are also known for malachite green (Srivastava et al. 2004) which, like formaldehyde, is widely used as an anti-parasitic treatment, but its effectiveness against *Gyrodactylus* spp. has not been evaluated. Malachite green is now banned in food fish production in Europe (European Council Regulation 2377/90) and North America (U.S. Food and Drug Administration and the Canadian Food Inspection Agency) as it is retained in fish flesh (Srivastava et al. 2004).

Alternative treatments include rotenone, an indiscriminate ATPase inhibitor, which has been used to control *G. salaris* in Norway by killing all potential hosts (reviewed in Bakke et al. 2007), including other gill-breathing organisms. This is only partially effective and has potential negative effects on human health. Aqueous aluminium is another alternative that is being tested. Although aluminium appeared promising in laboratory studies (Soleng et al. 1999, 2005, Poleo et al. 2004), field trials in Norway using aluminium in combination with rotenone have been problematic because successful treatment of the whole river system requires maintaining a specific concentration without exceeding levels toxic to Atlantic salmon (Paul Shave, personal communication). Aluminium toxicity to fish increases under acidic water conditions (Birchall et al. 1989, Poleo 1995, Soleng et al. 1999), and elevated environmental aluminium levels can result in Alzheimer's disease (Doll 1993) as well as decreased agricultural and forestry productivity (Bi et al. 2001). Organophosphates, such as trichlorfon, are no longer in use against fish parasites as they cause irreversible effects in non-target species by phosphorylating acetylcholinesterase (see Kozlovskaya and Mayer 1984, Peña-Llopis et al. 2003, Costa 2006). Acute and chronic toxicity to other aquatic organisms has also been reported for benzimidazoles (e.g. Oh 2006), however, due to their low efficacy (see Table 1.1) these are not preferred over broad-antiparasitic treatments such as formalin.

In addition to efficacy, environmental and human health issues, the main problem associated with current gyrodactylid treatments is their toxicity to the host (Schmahl and Taraschewski 1987, Santamarina et al. 1991, Tojo et al. 1992, Scholz 1999, Ekarem et al. 2004, Srivastava et al. 2004). Even widely-used compounds, such as formaldehyde, may significantly change the host's gill structure and epidermis (Speare et al. 1997, Sanchez et al. 1998, Buchmann et al. 2004). For instance, although zinc exposure initially stimulates host mucus production, it then becomes depleted leaving the fish more susceptible to microbial infections (McGeer et al. 2000). If these fish are subsequently used for experimental infections without a sufficient recovery period they may show an abnormal response to infection (see review by Bakke et al. 2007). Host respiratory problems are also a common side effect of gyrodactylid treatments due to direct interference with gill function and indirectly via reduction of water quality (e.g. decrease in pH and/or dissolved oxygen: Smith and Piper 1972, Ross et al. 1985, Rowland et al. 2006). Toxicity in many cases is dose dependent, but may also be affected

by one or a combination of the following factors: temperature, pH, salinity, mechanism of delivery, species and exposure time (Schmahl et al. 1989, 1987, Scholz 1999), especially where multiple treatments are used.

Gyrodactylid treatments are applied either orally (with food) or topically (added to the water). Both methods usually lead to application of overly high doses to compensate for lack of control over drug administration (Scholz 1999), which may lead to environmental contamination. Oral administration of some drugs can also reduce host food consumption (e.g. piperazine, Fugotenil® and Neguvon®; Tojo and Santamarina 1998) which increases the dosage needed per unit of food for efficacy. Additionally, compounds that are partially effective as baths may not be effective if administered orally (e.g. trichlorfon; Santamarina et al. 1991, Tojo and Santamarina 1998). Moreover, resistance of parasites against antihelminthics has been reported, with Goven and Amend (1982) and Schmahl et al. (1989) finding resistance of gyrodactylids to dimethyl phosphonate and trichlorfon, respectively. Breeding parasite-resistant fish or developing a vaccine against gyrodactylosis would override many of the above problems and bypass the potential issue of drug resistance. Breeding experiments with wild Atlantic salmon (*Salmo salar*) that show resistance against *G. salaris* are currently under way in Norway (Salte et al. 2010), but we are unaware of any plans for vaccine development. In Norway destocking and drying of fish ponds was used successfully to eradicate *G. salaris* (see Mo 1994), however, this method is not suitable for establishments which receive potentially infected fish on a regular basis and infected fish would still have to be treated.

A major problem with many of the studies presented in Table 1.1 is that they used sub-sampling methods, such as mucus scrapings (e.g. Tojo et al. 1992, Tojo and Santamarina 1998) or partial examination of fish populations in field trials (e.g. Goven and Amend 1982). These are a crude and unreliable estimate of parasite infection, particularly where the gyrodactylid is not identified to species level (e.g. Tojo and Santamarina 1998, Chansue 2007) as different *Gyrodactylus* spp. show marked site specificities. Furthermore, the majority of compounds trialled successfully have been tested only once. For those with at least two independent studies, results have been variable, probably because of different methodologies. Hence, many seemingly successful compounds may not be effective when it comes to treating a different host-parasite system. Field studies are of particular importance as they provide an indication of how efficient treatments are on a large scale, potentially for use in the ornamental or food fish industries. There have, however, only been a few such studies (see Table 1.1) with only Rach et al. (2000) claiming 100 % efficiency for the compound they tested (hydrogen peroxide). Other authors defined a treatment in the field as successful if it just reduced the pathogen burden (e.g. Lewis 1967, Rintamäki-Kinnunen and Valtonen 1996).

1.6.2 Manual removal of gyrodactylids

Due to toxicity and/or difficulties in administrating (such as the need for prolonged exposure) no treatment detailed in Table 1.1 is entirely satisfactory or 100 % effective, although several treatments indicate 100 % efficacy against gyrodactylosis (e.g. formaldehyde and aqueous aluminium). In laboratory trials with small, hardy fish, the most effective means of removing gyrodactylids (at least those species that predominantly occur on the skin and fins) is a licensed UK Home Office procedure which requires the host to be lightly anaesthetised (0.02 % MS222, chlorobutanel, etc.) whilst the living parasites are removed manually. Following anaesthesia, the fish is transferred to a shallow dish, but kept fully immersed in dechlorinated water and screened using a stereo-microscope with fibre optic illumination. The latter is essential to prevent the host and parasites overheating during examination on the microscope stage. Parasites can be most easily detected by their movement, but it is still essential to scan all surfaces of the host. Small fish, such as guppies and sticklebacks, can be manipulated using a plastic disposable pipette tip. Once a parasite is detected, the worm can be removed and crushed using watchmaker's forceps. It is essential to ensure that the parasite is not released directly back into the water as even a damaged worm may re-attach to its host and successfully give birth. Once all parasites have been removed, the same screening procedure should be repeated until all fish in a population have been screened clear of parasites on three consecutive occasions, ideally by two independent, trained researchers and screens being approximately a week apart. If a parasite is found on any fish during this process, then the screening process should begin again for the entire host population.

For small fish, such as poeciliids, manual removal of parasites can be effective without any chemical intervention. For larger fish (or where gyrodactylids are abundant and/or show a preference for the gills), chemical treatment prior to screening, may be the only practical solution to remove parasites. In our laboratories we use levamisole, which is only available by veterinarian prescription and only reliable if applied to single fish that is closely monitored for signs of toxicity. Screening is still essential to ensure the fish are free of gyrodactylids following chemical treatment, with additional screening on subsequent occasions to ensure the experimental fish are free from infection. For larger fish, such as adult Atlantic salmon (*S. salar*), sub-sampling by examining mucus scrapings or selected fins may be the only practical way of screening, but this is not accurate. Mucus scraping is also stressful for the fish as it damages the fish's epithelium, thus increasing the risk for secondary infections. An alternative or ideally supplementary procedure to manual removal, but again only suitable for small fish, is to maintain each fish in isolation in a closed system with regular water changes. As fish become immune and gyrodactylids are shed, there is no opportunity for the parasites to transfer to new hosts. Even long periods of isolation are not always effective for certain host-parasite combinations (see King et al. 2008) and the accompanying high maintenance is extremely

time consuming and costly. Whatever the control method used (chemical, manual or host isolation), thorough screening is essential to ensure parasite extinction. The longer the interval between the three consecutive screens, the more reliable the procedure. A single (transparent, < 1 mm long) gyrodactylid missed in an earlier screen will have had ample time to reproduce *in situ* (as little as 24 h at 25 °C) generating a larger number of parasites which are less likely to be overlooked on subsequent screens.

1.6.3 Summary

In conclusion, although the majority of compounds tested against *Gyrodactylus* spp. are reportedly effective (see Table 1.1), 100 % efficacy has not been achieved without toxicity to hosts. Currently, the lack of comparative data makes the selection of a drug difficult. Leaving just one (hermaphrodite, viviparous) worm can be sufficient to initiate a new disease outbreak. For research projects, manual removal of skin gyrodactylids from small fish is an option, however, this is not feasible for gill parasites, larger hosts or for use in aquaculture. Further research into different treatments and their application on different species of parasite and host is necessary to combat the existing problems caused by *Gyrodactylus* spp. epizootics. The work conducted in this thesis primarily aimed to test these ideas utilizing a well established laboratory model consisting of *G. turnbulli* and *G. bullatarudis* on guppies (*Poecilia reticulata*).

1.7 The host-parasite system: *Gyrodactylus turnbulli* and *G. bullatarudis* infecting common guppies (*Poecilia reticulata*)

Gyrodactylus turnbulli and *G. bullatarudis*, next to *G. salaris*, are the most well studied gyrodactylids and are commonly used as a model system in combination with their guppy (*Poecilia reticulata*) host (Fig. 1.2A). Both parasite species occur in natural guppy populations in Trinidad, often co-infecting the same host (Harris and Lyles 1992), and are a strong selection pressure in infected fish populations (van Oosterhout et al. 2007, Fraser and Neff 2010). Parasitized fish in upstream, low predation areas are more likely to be flushed downstream into high predation areas where they are behaviourally mal-adapted for predation avoidance (Magurran 2005, van Oosterhout et al. 2007).

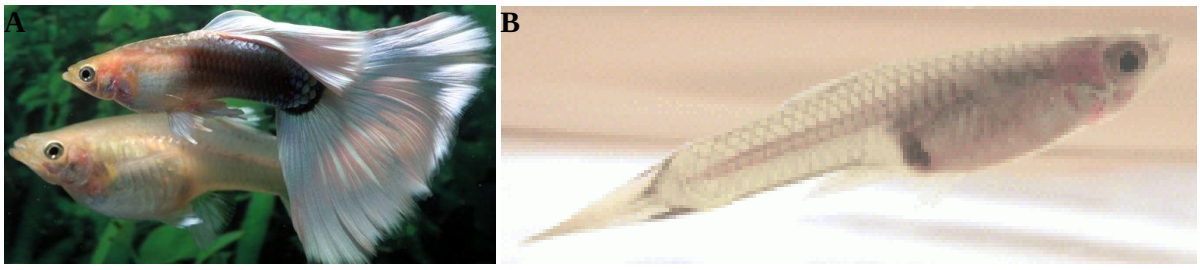

Fig. 1.2. (A) Ornamental male and pregnant female guppies (source: www.aqua-fish.net); (B) female guppy showing classic signs of fin clamping due to high gyrodactylid parasite burdens (Dr Jo Cable).

The potential of the guppy-gyrodactylid host system as a model for disease epidemiology was initially recognised by Scott and Anderson (1984) who established that the micro-parasite like behaviour of gyrodactylids allowed detailed study of disease persistence in socially interacting host populations. In guppies, shoaling behaviour is a strong driver of the maintenance of gyrodactylosis within populations with the frequency of social contact between fish determining disease epidemics rather than fish density itself (Johnson et al. 2011). Generally, transmission rates between females are elevated compared to males and are also influenced by host personality (Richards 2010, Richards et al. 2010).

At non-lethal infections, the main effect of parasite infection is on guppy behaviour: uninfected males have increased mating success compared to infected counterparts that spend more time foraging (Kolluru et al. 2009) and guppy social networks are disrupted by infected fish which are actively avoided in shoals (Croft et al. 2011). At high parasite loads, guppy swimming is thought to be severely affected due to fin clamping and impaired sight due to high burdens on the cornea (Bakke et al. 2007; Fig. 1.2B). The site specificity of the two *Gyrodactylus* spp. indicates fin clamping to be mainly a characteristic of *G. turnbulli* infections, whereas visual impairment is due to *G. bullatarudis* (Harris and Lyles 1992); however, even in single species infections, parasite populations may grow until the complete surface of the fish is covered (personal observation).

Guppies are one of the most popular fish in aquaculture and for aquarium hobbyists due to their colour diversity, high fecundity, robustness and ease of maintenance. Hence, their use in the laboratory as not only models of evolution, but also aquaculture and ornamental aquarium species makes them ideal for studies on disease issues in their 'domesticated' habitat in combination with *G. turnbulli* and *G. bullatarudis*. Of particular interest is disease control, particularly in light of the problems outlined earlier on in this chapter.

1.8 Thesis aims and hypothesis

This thesis aims to investigate gyrodactylid biology and control in order to work towards effective, integrated infectious disease management in aquaculture with a strong focus on the ornamental fish industry. By using a multidisciplinary approach including studies on parasite biology, host parasite interactions in natural guppy habitats and botanical efficacy testing, the following topics were addressed:

Firstly, I investigated gyrodactylid epidemiology to understand gyrodactylosis disease dynamics in more detail. Particularly, I set out to test whether gyrodactylids engage in sexual reproduction which has long been hypothesized in the literature (Chapter 2). I then continued to look into their transmission behaviour to assess the degree to which gyrodactylids contribute to successful

transmission events between guppies, hypothesizing that successful transmission depends on gyrodactylid initiative when close to a potential host (Chapter 3).

Secondly, I investigated the effect of altered water chemistry on *Gyrodactylus* spp. infections in natural guppy habitats or aquatic conditions they may experience in the wild. This involved a parasite survey of guppies in an extreme environment (Pitch Lake, Trinidad) and formally testing pitch water against gyrodactylid infected fish (Chapter 4). Further, I investigated the effect of salinity on *G. turnbulli* infections in guppies, since guppy populations in the wild often do occur in brackish water and because the use of salt is generally recommended in aquaculture and the aquarium industry against a range of freshwater parasites (Chapter 5).

Thirdly, I examined a range of botanicals against gyrodactylid infections against guppies (Chapter 6) including garlic (Chapter 7) and commercial treatments that are already marketed against fungal and bacterial infections (Chapter 8) hypothesizing that all of those might have a negative effect on gyrodactylid survival *in vitro* and *in vivo*.

Lastly, research that has been conducted as natural extension to the above projects, but not in direct context of the overall theme of the thesis, is presented in the appendix. This includes three published manuscripts, and one further manuscript draft (Appendices I-IV).

The main part of the thesis is organized into nine chapters: the general introduction, seven data chapters and a final discussion. Part of Chapter 1 and Chapters 2, 4 and 5 as well as Appendices I, II and III have been published in peer-reviewed journals. All other chapters (excluding general introduction and discussion) are written-up with the intention that they be submitted as manuscripts, which has inevitably led to some overlap between different chapters.

The published papers are (in order of their appearance in the thesis):

Schelkle B, Shinn AP, Peeler E, Cable J (2009) Treatment of gyrodactylid infections in fish. Dis Aquat Organ 86: 65-75. (Chapter 1)

Schelkle B, Faria P, Johnson M, van Oosterhout C, Cable J (2012) Mixed infections and hybridisation in monogenean parasites. PLoS One 7: e39506. (Chapter 2)

Schelkle B, McMullan M, Mohammed RS, Coogan MP, Gillingham E, van Oosterhout C, Cable J (2012) Parasites pitched against nature: Pitch Lake water protects guppies (*Poecilia reticulata*) from microbial and gyrodactylid infections. Parasitology 139: 1-8. (Chapter 4)

Schelkle B, Doetjes R, Cable J (2010) The salt myth revealed: treatment of gyrodactylid infections on ornamental guppies, *Poecilia reticulata*. Aquaculture 311: 74-79. (Chapter 5)

Schelkle B, Paladini G, Shinn AP, King S, Johnson M, van Oosterhout C, Mohammed RS, Cable J (2011) *Ieredactylus rivuli* n. gen. *et* sp. (Monogenea: Gyrodactylidae) from *Rivulus hartii* (Cyprinodontiformes: Rivulidae) in Trinidad. Acta Parasitol 56: 360–370. (Appendix I)

Mohammed RS, van Oosterhout C, Schelkle B, Cable J, McMullan M. Upstream guppies (*Poecilia reticulata*, Peters, 1859) go against the flow. Biota Neotropica, in press. (Appendix II)

Mohammed RS, McMullan MJ, Schelkle B, van Oosterhout C (2010) Colour variation of an individual of Hart's Rivulus (*Rivulus hartii*) found in a habitat rich in polycylic aromatic hydrocarbons in the Pitch Lake of Trinidad. Ecologia Balkanica 2: 61-63. (Appendix III)

Table 1.1. Treatments against infections of *Gyrodactylus* species. With the exception of orally administered treatments, other compounds below were applied directly to the water. [1]Signs of host toxicity; [2]Causes mortality in some or all hosts; [3]Mortality not indicated by author; [4-6]Infection intensity was determined [4]24h post-treatment by pelvic fin clips, [5]24h post-treatment by mucus scrapings, or [6]by other sub-sampling methods, as opposed to screening the entire fish; [7]*In vitro* tests included in study; [8]No primary literature available. ppt = parts per thousand; ppm = parts per million.

Compound	**Dose**	**Application**	***Gyrodactylus* spp.**	**Host**	**Efficacy**	**Reference**
1. Botanicals						
Garlic (*Allium sativum*) oil	1, 1.5, 2, 2.5, 3 ppt 3 ppt	24 h. Lab.study. 1 h. Field study.	*Gyrodactylus* spp.	*Oleochromis niloticus*	100 % effective within 4 h (2, 2.5, 3 ppt), 16 h (1.5 ppt) and 24 h (1 ppt).[3,6] Not effective. [3,6]	Abd El-Galil and Aboelhadid 2012
Crushed garlic (*Allium sativum*)	300 mg/L	Indefinite time. 27-33 °C. Field study.	*Gyrodactylus* spp.	*Oleochromis niloticus*	Not effective. [3,6]	Abd El-Galil and Aboelhadid 2012
Rotenone	Used extensively to treat *G. salaris* on salmonids in Norwegian rivers. Kills both fish and parasites. Linked to Parkinson's Disease.[7]					Pesticide Action Network UK 2008, GyroDB Net 2008, Caboni et al. 2004
Terminalia catappa extract	1.7, 3.4, 5.1 g/L	4 weeks. Lab. study.	*Gyrodactylus* sp.	*Carassius auratus*	Not effective (1.7 g/L) to 100 % effective after 14d (5.1 g/L).[6]	Chansue 2007
Tea tree oil and Tween 80	3, 10, 30 ppmv in 0.01 % Tween 0.0 1% Tween	48 h. 13 °C. Lab. study.	*Gyrodactylus* spp. (probably *G. gasterostei* and/or *G. arcuatus*)	*Gasterosteus aculeatus*	Reduced parasite mean from 12.6 worms/fish to 1.4 (30 ppmv).[6] Reduced parasite mean from 12.6 worms/fish to 5.9 and reduced prevalence by 45 %.[6]	Steverding et al. 2005
Piper guineense extract	0.5, 1, 1.5, 2 mg/L	96 h. Test solution renewed every 24 h. Lab. study.	*G. elegans*	*Carassius auratus auratus* (10 fish/treatment)	Dose dependent, but none 100 % effective.[2,6,7]	Ekanem et al. 2004
2. General Chemotherapeutics						
Acaprin (Acaprina®)	200 mg/L	3 h. 11 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	96 % effective.[1,4]	Tojo et al. 1993b

Actomar	1:10000	2.5 h. 12-13 °C. Lab. study.	*G. aphyae*	*Phoxinus phoxinus*	Effective.[8]	Cited in: Bakke and Sharp 1990
Agerin®	10, 500, 1000 ppm	Indefinite time, 2&1 h. 18 °C. Lab. study.	*Gyrodactylus* sp.	*Oreochromis niloticus*	Not effective.[2]	El-Khatib 2003
Albendazole (Oversol®)	25 & 200 mg/L	12 & 3 h, respectively. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective (200 mg/L) to 95.5 % effective (25 mg/L).[1,4,7]	Tojo et al. 1992
Aminosidine (Gabbrocol®)	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[5]	Tojo and Santamarina 1998
Aminosidine (Gabroral®)	200 mg/L	3 h. 11 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective.[4]	Tojo et al. 1993b
Amitraz	0.2, 0.4, 0.8, 1.6, 3.2 µM	48 h. 10 °C. Lab. study.	*G. gasterostei* and *G. acuatus*		Not effective (0.2 µM, 24 h) to 53 % effective (3.2 µM, 48 h).[7]	Brooker et al. 2011
Amprolium (Prolsal®)	200 mg/L	3 h. 11 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective.[4]	Tojo et al. 1993b
	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[5]	Tojo and Santamarina 1998
Benznidazole	200 mg/L	3 h. 11 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective.[4]	Tojo et al. 1993b
	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[5]	Tojo and Santamarina 1998
Bithionol	4, 20 & 60 mg/L	3h. 15°C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (4-10 fish/treatment)	98.6 % effective (4 mg/L) to 100 % effective (20 & 60 mg/L). [1,4,7]	Santamarina et al. 1991
	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[5]	Tojo and Santamarina 1998
Carnidazole (Spartrix®)	10 mg/L	3 h. 11 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective.[4]	Tojo et al. 1993b
Chlordimeform	0.2, 0.4, 0.8, 1.6, 3.2 µM	48 h. 10 °C. Lab. study.	*G. gasterostei* and *G. acuatus*		47 % effective (0.2 µM, 24 h) to 53 % effective (3.2 µM, 48 h).[7]	Brooker et al. 2011

Chloroquine	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[5]	Tojo and Santamarina 1998
Chloroquine diphosphate	200 mg/L	3 h. 11 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective.[4]	Tojo et al. 1993b
Clonidine Hydrochloride	0.2, 0.4, 0.8, 1.6, 3.2 µM	48 h. 10 °C. Lab. study.	*G. gasterostei* and *G. acuatus*		Not effective (0.2 µM, 24 h) to 60 % effective (3.2 µM, 48 h).[7]	Brooker et al. 2011
Closantel (Flukiver®)	0.25, 0.125 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (4-10 fish treatment)	99.6 % effective (0.125 mg/L) to 100 % effective (0.25 mg/L).[1,4,7]	Santamarina et al. 1991
Diethylcarbamazine	200 mg/L	3 h. 11 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective.[4]	Tojo et al. 1993a
	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[5]	Tojo and Santamarina 1998
Dimetridazole	200 mg/L	3 h. 11 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective.[4]	Tojo et al. 1993b
Diminazene aceturate (Berenil®)	200 mg/L	3 h. 11 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	98.6 % effective.[1,4]	Tojo et al. 1993b
Febantel (Rintal®)	2, 10 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (4-10 fish/treatment)	Not effective (2 mg/L) to toxic to host (10 mg/L: 4 trout dead after 1h, expt. terminated).[1,4,7]	Santamarina et al. 1991
Fenbendazole (Panacur®)	0.77, 1.5, 6.2, 12.5, 25 mg/L	12 h (additionally, 3 h for 25 mg/L). 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective (0.77 mg/L & 25 mg/L for 3h) to 100 % effective (>1.5 mg/L).[1,4,7]	Tojo et al. 1992
Flubendazole	25, 200 mg/L	12 & 3 h, respectively. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective.[4,7]	Tojo et al. 1992
	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[4]	Tojo and Santamarina 1998
Formaldehyde	2.5, 5, 10, 20, 40, 60 mg/L	18 h. Lab. study.	*G. aculeati*	*Gasterosteus aculeatus*	Not effective (2.5 mg/L) to 100 % effective (20, 40 & 60 mg/L).[3]	Buchmann and Kristensson 2003

Formalin	20, 25, 30 & 40 mg/L	24.1-26.9 °C or 13.2-15.7 °C. Field trial: applied to ponds.	*Gyrodactylus* sp.	*Bidyanus bidyanys*	Not effective (20, 25 mg/L) to sign. decrease (30, 40 mg/L).[2,6]	Rowland et al. 2006
Formalin; malachite green & formalin; salt bath	0.2 g/L; 4 g/L & 0.2 g/L; 1.5-2 %, respectively	Farm study: rotation of treatments.	Putatively *G. salaris*	*Salmo salar, S. trutta trutta & S. trutta lacustris*	Formalin partially effective (decreased of parasite burden); malachite green + formalin, &/or salt not effective.[3,6]	Rintamäki-Kinnunen and Valtonen 1996
Formalin (F) & Malachite Green (MG)	Recommended use in combination: 3.68 g MG in 1L F, used at 0.025 ml/L of water for 60 min, or 3.3 g MG in 1L F used at 0.015 ml/L of water as a prolonged immersion. Synergistic effect of compounds in combination, but toxic to both parasite and host.					Srivastava et al. 2004; Fishdoc 2008
HOE 092 V (triazine derivative)	0, 2, 5, 10, 15 µg/L	1, 2, 3 & 4 h. 22 °C. Lab. study.	*G. arcuatus*	*Gasterosteus aculeatus*	Not effective (0-5 µg/L) to effective after 4 h (10 µg/L) & 1 h (15 µg/L).[2,6,7]	Schmahl 1993
	10 mg/L	3 h.	*G. groschaffi*		Effective.[8]	Cited in: Schmahl 1993
Hydrogen peroxide	170, 280, 560 mg/L	3 x 30 min over 5 d. Field trials.	*Gyrodactylus* sp.	*Oncorhynchus mykiss*	100 % effective.[2,6]	Rach et al. 2000
	11, 16; 3, 6 mg/L	1 h; 24 h. 23.5 °C. Lab. study.	*Gyrodactylus* spp.	*Xiphophorus helleri* (51-75 fish/treatment)	Not effective.[2,6]	Russo et al. 2007
Hydrogen peroxide (35 % PEROX-AID)	50 mg/L	2 x 30 min over 2 alternate days. 12 °C. Lab. study.	*G. salmonis*	*Oncorhynchus mykiss*	Over 99 % effective.[6]	Bowker et al. 2012
Ivermectin (Ivomec®)	0.031 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (4-10 fish/treatment)	100 % host mortality.[2,4,7]	Santamarina et al. 1991
	0.1 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	96 % effective.[1,4]	Tojo et al. 1993a
Ivermectin + clorsulon (Ivomec-F®)	0.025, 0.25, 0.05 & 0.5 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (4-10 fish/treatment)	59 % effective (0.025 & 0.25 mg/L) to 100 % effective (0.05 & 0.5 mg/L).[1,4,7]	Santamarina et al. 1991
Ketoconazole	200 mg/L	3 h. 11 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective.[4]	Tojo et al. 1993b
Levamisol hydrochloride	0, 10, 20, 50 & 100 µg/L	25, 30, 60, 90 & 120 min. 20 °C. Lab. study.	*G. aculeati*	*Gasterosteus aculeat-us* (10 fish/treatment)	Decrease of parasite burden.[2,6]	Schmahl and Taraschewski 1987

Levamisol hydrochloride	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[5]	Tojo and Santamarina 1998
Levamisol hydrochloride (Citarin-L®)	100 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (4-10 fish/treatment)	Not effective.[4,7]	Santamarina et al. 1991
Malachite green	Recommended use: 0.5-0.8 ppm for 1d, or 0.015 ppm for prolonged immersion. Remains in fish tissue for up to 1 month. Highly toxic to fish: carcinogenic, mutagenic, chromosomal fractures, teratogenecity and respiratory toxicity. Now banned in the Europe and N. America for food fish. Most effective used together with formalin.[7]					Kou et al. 1988 (in: Liao et al. 1996), Liao et al. 1996, Srivastava et al. 2004
Mebendazole	0.1 mg/L	24 h. 23 °C. Lab. study.	*G. elegans*	*Carassius auratus* (10 fish/treatment)	100 % effective.[2,6]	Goven and Amend 1982
	25, 100 mg/L	12 & 3 h, respectively. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective (100 mg/L) to 95 % effective (25 mg/L).[4,7]	Tojo et al. 1992
	40 g/ kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[5]	Tojo and Santamarina 1998
	1 mg/L	24 h.	*G. bullatarudis*		Effective.[3,8]	Cited in: Schmahl 1993
	0.01 mg/L	24 h.	*G. elegans*		Effective. [3,8]	Cited in: Schmahl 1993
Mebendazole (meb) & trichlorfon (tri)	0.1-0.4 mg/L[1] meb & 0.46-1.8 mg/L tri. 0.5 mg/L meb & 2.3 mg/L tri.	24 h. 23 °C. Lab. study. Field tests.	*G. elegans*	*Carassius auratus* (10 fish/treatment) *Carassius auratus, Astronotus ocellatus, Pterphyllum scalare, Poecela velifera & Trichogaster trichopterus*	100 % effective.[2,6] 100 % effective.[2,6]	Goven & Amend 1982
Meglumine (Glucantime®)	200 mg/L	3 h. 11 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective.[4]	Tojo et al. 1993b
Methylene blue	Recommendations: 8-10 ppm, bath 1 d; 2 ppm, bath 3-5 d. Highly toxic to fish. Remains in fish tissue for up to 1 month. Carcinogenic.[7]					Liao et al. 1996

Metronidazole	200 mg/L	3 h. 11 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective.[4]	Tojo et al. 1993b
Metronidazole (Flagyl®)	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[5]	Tojo and Santamarina 1998
Naftalofos (Maretin®)	20 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective.[1,4]	Tojo et al. 1993a
Netobimin (Hapasil®)	2 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (4-10 fish/treatment)	Not effective.[4,7]	Santamarina et al. 1991
Niclofolan (Bilevon®)	0.025, 0.05, 0.2 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (4-10 fish/treatment)	91 % effective (0.025 mg/L) to 100 % effective (0.05 & 0.2 mg/L, but all host fish dead). [2,4,7]	Santamarina et al. 1991
Niclosamide	0, 0.05, 0.075, 0.1 µg/L	30, 60, 90 & 120 min. 20 °C. Lab. study.	*G. aculeati*	*Gasterosteus aculeatus* (10 fish/treatment)	Reduction of parasite burden.[6]	Schmahl and Taraschewski 1987
Niclosamide (Fugotenil®)	1.5 , 6 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	100 % effective.[1,4]	Tojo et al. 1993a
Niclosamide (Fugotenil®)	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not 100 % effective.[5]	Tojo and Santamarina 1998
Niridazole (Ambilhar®)	100, 200 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective (100 mg/L) to 100 % effective (200 mg/L).[4]	Tojo et al. 1993a
Nitroscanate (Lopatol 500®)	40, 6.25, 0.63, 6.25 g/kg feed	10 d, 2 d, 1 d, 1 d oral treatment, respectively. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	100 % effective.[5]	Tojo and Santamarina 1998
Nitroscanate (Lopatol®)	0.04, 0.07, 156.25 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (4-10 fish/treatment)	99 % effective (0.04 mg/L) to 100 % effective (0.07, 156.25 mg/L). [4,7]	Santamarina et al. 1991
Nitroxynil (Distomicine®)	30, 50 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective (30 mg/L) to 100% effective (50 mg/L)[1,4]	Tojo et al. 1993a
Nitroxynil	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss*	Not effective.[5]	Tojo and Santamarina 1998

				(20 fish/treatment)		
Octopamine Hydrochloride	0.2, 0.4, 0.8, 1.6, 3.2 µM	48 h. 10 °C. Lab. study.	*G. gasterostei* and *G. acuatus*		Not effective (0.2 µM, 24 h) to 67 % effective (3.2 µM, 48 h).[7]	Brooker et al. 2011
Organophosphate Dimethyl Phosphonate	0.2, 0.4, 0.8, 1.3. 3, 6, 12.5, 25, 50 mg/L	72 h. 18-20°C. Lab. study.	*G. elegans*	*Carassius auratus*	Not effective (0.2 mg/L) to 100% effective (50 mg/L).[3]	Goven et al. 1980
Oxfendazole	25, 200 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective. [4,7]	Tojo et al. 1992
Oxibendazole	25, 200 mg/L	12 & 3 h, respectively. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective (200 mg/L, 3 h) to 86 % effective (25 mg/L, 12 h). [1,4,7]	Tojo et al. 1992
	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[5]	Tojo and Santamarina 1998
Paraformalde-hyde	Ca. 10 g/L	Field trial: ponds sized 0.1-0.5acre, ~3 ft deep.	*G. elegans*	*Notemigonus crysoleucas*	Treatment was successful.[3,6]	Lewis 1967
Parbendazole	25, 200 mg/L	12 & 3 h, respectively. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective (200 mg/L, 3 h) to 91 % effective (25 mg/L, 12 h). [4,7]	Tojo et al. 1992
	40 g/kg feed	10 d oral treatment.15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[5]	Tojo and Santamarina 1998
Piperazine	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[5]	Tojo and Santamarina 1998
Piperazine citrate	200 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (4-10 fish/treatment)	Not effective.[4,7]	Santamarina et al. 1991
Piperazine di-hydrochloride	200 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (4-10 fish/treatment)	Not effective.[4,7]	Santamarina et al. 1991
Praziquantel	0, 1, 5, 10, 20, 50 µg/L	15, 30, 60, 90, 120 min, 4 h & 16 h. 20 °C. Lab. study.	*G. aculeati*	*Gasterosteus aculeatus* (10 fish/treatment)	Decrease of parasite burden.[1,6]	Schmahl and Taraschewski 1987
Praziquantel (Droncit®)	10 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (4-10 fish/treatment)	98 % effective.[4,7]	Santamarina et al. 1991

Praziquantel (Droncit®)	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[5]	Tojo and Santamarina 1998
Pyrantel pamoate (Trilombrin®)	50 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective.[4]	Tojo et al. 1993a
Quinacrine HCl (Atabrine®)	25, 100, 200 mg/L	3 h. 11 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	98 % effective (25 mg/L) to 100 % effective (100, 200 mg/L).[1,4]	Tojo et al. 1993b
Quinaldine	0, 26, 104, 260 mg/L	Ca. 20, 40 & 60 s. 20-22 °C. Lab. study.	*Gyrodactylus* sp.	*Carassius auratus, Cyprinus auratus*	Effective (26 mg/L, ca. 60 s, time needed to anaesthetise fish). [2,6]	Crigel et al. 1995
Rafoxanide (Ranide®)	50 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective.[4]	Tojo et al. 1993a
Ronidazole	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[5]	Tojo and Santamarina 1998
	200 mg/L	3 h. 11 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	84 % effective.[4]	Tojo et al. 1993b
Secnidazole	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[5]	Tojo and Santamarina 1998
Sodium percarbonate	10, 20, 40, 80, 160 mg/L	18 h. Lab. study.	*"G. derjavini" (sic G. derjavinoides)*	*Oncorhynchus mykiss*	Not effective (10, 20 mg/L) to 100 % effective (80, 160 mg/L).[3]	Buchmann and Kristensson 2003
Sod. sulpha-quinoxaline	200 mg/L	3 h. 11 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective.[4]	Tojo et al. 1993b
Suramin (Naganol®)	200 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective.[4]	Tojo et al. 1993a
Tetramisole (Nemicide®)	100 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	95 % effective.[1,4]	Tojo et al. 1993a

Tetramisole (Nemicide®)	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[5]	Tojo and Santamarina 1998
Thiabendazole (Triasox®)	10, 100 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective.[1,4,7]	Tojo et al. 1992
Thiophanate (Nermafax 20®)	200 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective.[4]	Tojo et al. 1993a
Thiophanate	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[5]	Tojo and Santamarina 1998
Toltrazuril	5, 10, 20, 30, 50 µg/L	0.3, 1, 2, 3, 4 & 6 h. 20 °C. Lab. study.	*G. arcuatus*	*Gasterosteus aculeatus* (10-15 fish/treatment)	Not effective (5, 10 µg/L) to effective after 2 h (50 µg/L).[2,6,7]	Schmahl and Mehlhorn 1988
	200 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective.	Tojo et al. 1993b
	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[5]	Tojo and Santamarina 1998
Trichlorfon	Up to 2 mg/L	24 h. 23 °C. Lab. study.	*G. elegans*	*Carassius auratus* (10 fish/treatment)	Not effective.[2,6]	Goven and Almend 1982
	0.25 mg/L	Continous.	*Gyrodactylus* sp.			Cited in: Schmahl 1993
Trichlorfon (Metrifonate)	0, 10, 50 µg/L	25, 30, 60 & 90 min. 20 °C. Lab. study.	*G. aculeati*	*Gasterosteus aculeatus* (10 fish/treatment).	Not effective.[3]	Schmahl and Taraschewski 1987
Trichlorfon (Neguvon®)	200 mg/L	3 h. 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (4-10 fish/treatment)	98% effective.[4,7]	Santamarina et al. 1991
	40 g/kg feed	10 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Not effective.[5]	Tojo and Santamarina 1998
Triclaben-dazol (Fasinex®)	6.2, 12.5, 25 mg/L	12 h (additionally, 25 mg/L for 3 h). 15 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* (5 fish/treatment)	Not effective (6.2 mg/L & 12 h, 25 mg/L & 3 h) to 100 % effective (25 mg/L &12 h).[7]	Tojo et al. 1992

Triclabendazole	40 g/kg feed	10 d & 5 d oral treatment. 15 °C. Lab. study.	*Gyrodactylus* sp.	*Oncorhynchus mykiss* (20 fish/treatment)	Time dependent: parasite reduction (5 d) to 100 % effective (10 d).[5]	Tojo and Santamarina 1998
Virkon S + heat	Low con-Centration	25, 30, 35 & 40 °C. Lab. study.	*G. salaris*	*Oncorhynchus mykiss* fin clips	Temperature dependent: parasite survival ranges from 119.7 min (25 °C) to 9 s (40 °C).[3]	Antilla et al. 2007
3. Metal Salts						
Aluminium	25, 50, 100, 200 µg/L	Continuous exposure (11-15 d) until fish died or recovered. 8 °C. Lab. study.	*G. salaris*	*Salmo salar* (each host infected with >50 parasites)	Not effective (25 µg/L) to 100 % effective after 3 d (200 µg/L).[2,6]	Poléo et al. 2004
Aluminium (& acidification)	92-250 µg/L at pH 5.0, 5.2, 5.6, 5.9	Continuous exposure (4-30 d). 12°C. Lab. study.	*G. salaris*	*Salmo salar* (15 fish/treatment)	No effect of pH, but dose dependent effect. At pH 5.0 effect of Al was enhanced: 100 % effective after 9 d.[2,6]	Soleng et al. 1999
	Al-rich & Al-poor water (pH 5.8) & control (pH 6.3)	Continuous exposure. 11-13 °C. Lab. study.	*"G. derjavini" (sic G. derjavinoides)* *G. macronychus*	*Salmo trutta* (10 fish/treatment) *Phonixus phonixus* (15 fish/treatment)	Al-rich water: 100 % effective after 3 d. Al-poor water: decrease in parasites.[3] Al-rich water: 100 % effective after 8 d. Al-poor water: not effective.[3]	Pettersen et al. 2006
Cadmium	5 µg/L	20 & 30d. 23°C. Lab. study.	*G. bullatarudis*	*Poecilia reticulata*	Not effective (after 5 & 15 d in 20 d trials) to decrease in parasite burden after 30 d.[1]	Carter 2003
	5 µg/L	60 d. 23 °C. Lab. study.	*G. turnbulli*	*Poecilia reticulata*	Not effective.[1]	Carter 2003
Copper	10, 20, 40, 80 µg/L	Continuous exposure until fish died/ recovered. 8 °C. Lab. study.	*G. salaris*	*Salmo salar* (each host infected with >50 parasites)	Not effective.	Poléo et al. 2004
Iron	25, 50, 100, 200 µg/L	Continuous exposure until fish died/ recovered. 8 °C. Lab. study.	*G. salaris*	*Salmo salar* (each host infected with >50 parasites)	Not effective.[2]	Poléo et al. 2004
Manganese	100, 200, 400, 800 µg/L	Continuous exposure (11-15 d) until fish died or recovered. 8°C. Lab. study.	*G. salaris*	*Salmo salar* (each host infected with >50 parasites)	Not effective.	Poléo et al. 2004

Zinc	50, 100, 200, 400 µg/L	Continuous exposure (11-15 d) until fish died or recovered. 8°C. Lab. study.	*G. salaris*	*Salmo salar*	Not effective (50 µg/L) to 100 % effective after 3 d (400 µg/L).[2]	Poléo et al. 2004
	8.3, 23.7, 38.1, 68.5, 129.5, 260.3 µg/L	1 week pre-exposure to required conc.; after infection continuous exposure until isolated fish dead or recovered (up to 29 d). 25 °C. Lab. study.	*G. turnbulli*	*Poecilia reticulata*	Decrease in peak parasite burden, but higher infection establishment (240 µg/L). Combined effects of parasite infection & Zn exposure more detrimental to host than parasite. [2]	Gheorgiu et al. 2006
4. Salt						
Salinity	5, 7.5, 10, 15, 20, 33 ‰ salinity	Continuous exposure. 1.4, 6 & 12 °C. Lab. study.	*G. salaris*	*Salmo salar* (fins of 6 fish for 33 ‰ salinity; for all others, 12 fish/treatment)	Not effective (5.0 ‰) to 100 % effective after a few minutes (33 ‰).[1,6]	Soleng and Bakke 1997
	33 ‰ salinity	5, 15, 30 & 60 min. 12 °C. Lab. study.	*G. salaris*	*Salmo salar* (15 fish/treatment)	Time dependent effect: no effect (5 min) to 100 % effective (60 min). [2,6]	Soleng et al. 1998
	5-20 ppt NaCl	4 d. 11 °C. Lab. study.	*G. derjavini*	*Oncorhynchus mykiss* (21-23 fish/treatment)	Not effective (5-7 ppt) to 100 % effective (10-20 ppt).[2,6]	Buchmann 1997

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CHAPTER 2: MIXED INFECTIONS AND HYBRIDISATION IN MONOGENEAN PARASITES*

2.1 Abstract

Theory predicts that sexual reproduction promotes disease invasion by increasing the evolutionary potential of the parasite, whereas asexual reproduction tends to enhance establishment success and population growth rate. Gyrodactylid monogeneans are ubiquitous ectoparasites of teleost fish, and the evolutionary success of the specious *Gyrodactylus* genus is thought to be partly due to their use of various modes of reproduction. *G. turnbulli* is a natural parasite of the guppy (*Poecilia reticulata*), a small, tropical fish used as a model for behavioural, ecological and evolutionary studies. Using experimental infections and a recently developed microsatellite marker, we conclusively show that monogenean parasites reproduce sexually. Conservatively, we estimate that sexual recombination occurs and that between 3.7-10.9 % of the parasites in our experimental crosses are hybrid genotypes with ancestors from different laboratory strains of *G. turnbulli*. We also provide evidence of hybrid vigour and/or inter-strain competition, which appeared to lead to a higher maximum parasite load in mixed infections. Finally, we demonstrate inbreeding avoidance for the first time in platyhelminths which may influence the distribution of parasites within a host and their subsequent exposure to the host's localized immune response. Combined reproductive modes and inbreeding avoidance may explain the extreme evolutionary diversification success of parasites such as *Gyrodactylus*, where host-parasite coevolution is punctuated by relatively frequent host switching.

2.2 Introduction

The widespread occurrence of sex is usually attributed to the fact that recombination generates new gene combinations, thereby increasing the rate of adaptive evolution whilst negating Müller's ratchet and the associated irreversible accumulation of deleterious mutations (Butlin 2002). If, however,sexual reproduction prevails in populations with small effective population sizes, consanguineous mating over several generations will result in severe inbreeding, potentially leading to inbreeding depression (Charlesworth and Willis 2009). The deleterious effects of inbreeding are predicted to be least noticeable in populations with a long history of inbreeding, as this tends to purge deleterious mutations in the population (Nei 1968, Ohta and Cockerham 1974, Kirkpatrick and Jarne 2000, Hedrick 2002). Conversely, the beneficial effects of outcrossing are expected to be most pronounced in the most inbred populations (Charlesworth and Willis 2009). This prediction is based on the assumption that in highly-inbred populations at least some loci will have been fixed for recessive deleterious alleles by drift, and that outcrossing will restore these loci to a heterozygous state (Barton 2001). Hybridisation may result in heterosis (or hybrid vigour), an increase in fitness in the F1 offspring (Charlesworth and Willis 2009). The benefits of outcrossing and hybrid vigour tend,

*Adapted from: Schelkle B, Faria PJ, Johnson MB, van Oosterhout C, Cable J (2012) PLoS One 7: e39506.

however, to decline in subsequent generations due to the breakdown of co-adapted gene complexes and epistatic gene interactions (Trouve et al. 1998, Charlesworth and Willis 2009).

Among micro- and macroparasites, hybridisation has been observed at an intra- (Badaraco et al. 2008, Seridi et al. 2008, Chargui et al. 2009) as well as inter- (Nolder et al. 2007, Steinauer et al. 2008, Barson et al. 2010) specific level. It can lead to the emergence of new diseases (Arnold 2004, Detwiler and Criscione 2010), with hybrid origins and/or current interbreeding events shown for viruses (e.g. Spanish flu, human rotavirus; Webster 2001, Laird et al. 2003), bacteria (e.g. *Haemophilus influenza*, see Smoot et al. 2002), fungi and oomycetes (Schardl and Craven 2003), protozoa (e.g. *Leishmania infantum*, *Trypanosoma cruzi*; see Machado and Ayala 2001, Chargui et al. 2009) and various schistosome species (e.g. Frandsen 1978, Brémond et al. 1989). Immediate consequences of hybridisation may involve increased pathogen fecundity, infectivity, virulence and transmission rates, wider host spectra, a shorter maturation time and phenotypic changes (Arnold 2004, Detwilder and Criscione 2010). For instance, hybrid vigour in F1 offspring of *Leishmania infantum*/*L. major* crosses led to increased resistance to immunity in an atypical vector (Volf et al. 2007). The fitness advantages of hybridization, however, are typically short-lived; most laboratory hybrids that show hybrid vigour lose their fitness in subsequent generations (Mutani et al. 1985, Rollinson et al. 1990, Trouve et al. 1998).

For gyrodactylids (a specious group of monogeneans), there is phylogenetic evidence that co-infecting species may hybridize before and after host switches (Zięt̨ara and Lumme 2002, Zięt̨ara et al. 2006, Kuusela et al. 2007, Barson et al. 2010). These ectoparasitic monogeneans are ubiquitous on teleost fish (Harris et al. 2004) and are largely transmitted between fish by direct host-host contact with a single parasite being sufficient to seed a population (Cable and Harris 2002). Most give birth to live young, which are already pregnant when born, a phenomenon termed hyperviviparity (Cohen 1977). The first born daughter is derived asexually by mitotic division when the mother is still an embryo; the second daughter can be produced by automictic parthenogenesis, and all other daughters (up to five in total recorded to date) are either produced via parthenogenesis or potentially sexual reproduction (Cable and Harris 2002). Hyperviviparity in combination with extreme progenesis allows these parasites to produce offspring in as little as 24 h (e.g. *Gyrodactylus turnbulli*; see Scott 1982) resulting in explosive population growth. It has been hypothesized that during epidemic population growth, sexual reproduction is more common than parthenogenesis due to crowding effects (Harris 1989, MacDonald and Caley 1975). For all monogeneans, while the occurrence of sexual recombination has been assumed (e.g. Zięt̨ara et al. 2006), it has never actually been proven.

In this study, we used a microsatellite marker to confirm sexual reproduction in monogeneans. This methodology was then applied to assess the effects of hybridising three *G. turnbulli* strains that have been isolated and inbred in the laboratory for 1, 8 and 12 years (circa 2×10^2 to 2×10^3 generations; assuming 2 days/generation). We then (conservatively) estimate the proportion of parasites that have a hybrid origin. By comparing the infection trajectories, maximum parasite burdens and duration of infections in parental and mixed populations, we provide evidence for hybrid vigour and/or inter-strain competition. By analysing time-to-infection for parasites transmitting to fish infected with same-strain or different-strain individuals, we show inbreeding avoidance behaviour within strains.

2.3 Material and methods

This work was conducted using the guppy (*Poecilia reticulata*) – *Gyrodactylus turnbulli* model system with all procedures conducted under UK Home Office licence (PPL 30/2357) regulations and approved by the Cardiff University Ethics Committee.

2.3.1 Source of animals and infection procedures

Guppies (*P. reticulata*, Peters) were originally isolated from a wild population from the River Tunapuna (20P, 678513E, 1177415N), Trinidad, and thereafter maintained in laboratory cultures at Cardiff for ca. 8 years. The fish were maintained at 25 ± 1 ºC with a 12 h light : 12 h dark cycle at a density of approximately 120 fish per 40 L aquarium, and fed at least twice daily on Aquarian® fish flakes plus *Artemia* and/or *Daphnia* spp. in addition to bloodworm once a week.

Three different *G. turnbulli* strains were used: *Gt3* (isolated from a pet shop guppy in Nottingham in 1997), *Gt1* (isolated from wild guppies in the Lower Aripo River, Trinidad, in November 2001) and *Gt8* (isolated from a pet shop guppy in Cardiff in March 2008), which are routinely maintained at Cardiff University at 25 ± 0.5 ºC on ornamental guppies. In this study, crosses are made between these lines and we use the term 'hybrid' or 'hybrid genotype' *sensu stricto*: "(1) a progeny of a cross between parents of different genotype; (2) heterozygote" (Lawrence 2005).

Experimental infections utilized donor guppies carrying > 20 parasites per fish from either of the three laboratory strains (*Gt1*, *Gt3* and *Gt8*) and which were anaesthetized with 0.02 % Tricaine Methanesulfonate (MS222, PharmaQ, UK). Guppies that were naïve to these parasites were also anaesthetized, sexed, measured and infected by bringing the infected donor guppy into contact with the recipient until the gyrodactylid moved from one fish to the other. To ensure infection by one parasite strain was not biased by prior exposure to another strain, half of the fish were first infected with one strain (i.e. first infection), then the other (i.e. second infection), so avoiding any 'priority effects'. Extreme care was taken to prevent cross-infection of individual fish with the wrong strain of

parasite by changing anaesthetic and glassware after each infection procedure. At various stages during Experiments 1 and 2, parasites were removed from anesthetized hosts with fine watchmaker's forceps and stored in 100 % ethanol for subsequent molecular analysis.

2.3.2 Experiment 1: Sexual recombination in gyrodactylids

To maximise our chances of detecting hybrid genotypes and to achieve high parasite infrapopulations (assuming sex may only occur at high parasite densities) (Harris 1989) we began the infection experiment with 10 parasites on each fish. In total, 24 guppies were infected with ten monogeneans each, five from two different parasite strains, resulting in 12 fish infected with *Gt3* and *Gt1* (parasite line *Gt3* x *Gt1*) and 12 fish infected with *Gt8* and *Gt1* (parasite line *Gt8* x *Gt1*). Fish were maintained in pairs in 1 L plastic pots to which two naïve fish were added the subsequent day (D1). Thereafter, water was changed every day and any deceased guppies were replaced with naïve fish. On D14, the parasite cultures were screened and the most heavily infected fish (1-2 fish per culture, infected with over 100 parasites) were euthanized with an overdose of MS222 and fixed in ethanol. A sample of parasites from these highly infected, fixed fish were removed with entomological pins and stored individually in fresh 100 % ethanol.

2.3.3 Experiment 2: Hybrid fitness and/or inter-strain competition

In this experiment, recipient fish (n = 101) were infected with either two parasites of the same strain (paternal parasite lines: *Gt1* x *Gt1*, *Gt3* x *Gt3* and *Gt8* x *Gt8*; hereafter the "pure parasite population") or two parasites from different strains (hybrid parasite lines: *Gt3* x *Gt1* and *Gt8* x *Gt1*, hereafter the "mixed parasite population"). The time-to-infection success (i.e. the time a parasite took to transfer from the donor fish to the recipient fish; see King and Cable 2007) was recorded for both parasites. The infected recipient fish (*Gt3*: n = 10, *Gt1*: n = 11, *Gt8*: n = 11, *Gt3* x *Gt1*: n = 38, *Gt8* x *Gt1*: n = 31) were maintained individually in 1 L pots.

On Day 1 (D1) all fish were screened following procedures by Schelkle et al. (2009, see Chapter 1.6) to check whether the infection had established (i.e. ≥ 2 parasites on a fish were regarded as successful establishment). From D1 onwards, all fish were screened every other day to follow the population dynamics of the parasites until the fish were screened clear for at least three times (Chapter 1.6). Additionally, on D7 half of the parasites (median: 6, range: 1-26) were removed with watchmaker's fine forceps from the anaesthetized host and any monogeneans from fish containing mixed parasite population were fixed for subsequent molecular analysis to assess the presence of hybrid genotypes on individual fish. Halving the parasite load at this stage ensured we had samples to genotype while still allowing the fitness experiment to continue on a like-for-like basis (i.e. all parasite infrapopulations were halved).

2.3.4 Microsatellite genotyping

The three parasite strains *Gt1*, *Gt3* and *Gt8* were previously genotyped at four microsatellite loci (Faria et al. 2011). However, only a single locus (TurB02) unambiguously discriminates between two of the three lines (i.e. was fixed for different alleles at two of the three lines). Hence, we use this locus to estimate the rate of sexual reproduction. *Gt3* and *Gt8* have different alleles to *Gt1* at the TurB02 locus, which allows us to detect potential hybrid genotypes between *Gt3* and *Gt1*, and *Gt8* and *Gt1*, but not *Gt3* and *Gt8*. Therefore, a mixed population of both latter strains was not included in this study.

In Experiment 1, a random sample of 48 gyrodactylids from 10 different mixed parasite cultures were screened to examine whether any hybrid (i.e. heterozygous) individuals were present, which would unambiguously establish sexual reproduction. In Experiment 2, 240 parasites (139 gyrodactylids isolated from 23 *Gt3* x *Gt1* infected fish, and 101 from 23 *Gt8* x *Gt1* infected fish) were successfully genotyped. Extraction was performed using the following lysis protocol: 50 µL of lysis buffer containing 1 x TE, 0.45 % Tween 20 and 9 µg of Proteinase K was added to a whole gyrodactylid and incubated at 65 °C for 30 min and then denatured at 95 °C for 10 min. PCR amplification was performed as described in Faria et al. (2011) using 5 µL Master Mix (Qiagen multiplex PCR kit), 2 pmol of each microsatellite primer (TurB02: forward ACGAGTGACAATAAAGCTGG, reverse ATCAATAGTTGAATGG) and 2 µL of DNA following manufacturer's instructions. The PCR cycling profile consisted of 15 min at 95 °C, followed by 40 cycles of 40 s at 95 °C, 90 s at 52 °C and 90 s at 72 °C, and a final extension for 30 min at 72 °C. PCR products were loaded on an ABI3130XL using size standard ROX-350 (Applied Biosystems) and chromatograms were analyzed using PeakScanner version 1.0 (Applied Biosystems).

Parasites were identified as being of hybrid or parental strains based on their genotypes. All parental strains were homozygous for the locus used: *Gt3* and *Gt8* parental genotypes presented only allele 244 bp, whereas *Gt1* presented the allele 246 bp. This enabled us to distinguish hybrid genotypes in crosses with *Gt8* x *Gt1* and *Gt3* x *Gt1*. Since just one microsatellite locus was used to differentiate between heterozygous or homozygous individuals, non-detection of hybrid genotype monogeneans could have occurred for five reasons: (1) One of the parasite strains did not establish after infection, but the parasite infection was still recorded as being established; (2) no sexual reproduction occurred; (3) no sexually produced parasites were sampled; (4) sexually reproduced parasites were homozygous for the microsatellite loci due to Mendelian segregation of alleles over several generations; and (5) sampled parasites were backcrossed individuals. Hence, hybridization rates quoted for both experiments below are significant underestimates and should be interpreted as a conservative estimate of sexual reproduction in our study system.

These five factors that potentially inflate the false-negative rate (i.e. the conclusion that no hybridisation occurred) also have implications for Experiment 2. Due to this potential non-detection of hybrid genotypes, analysis of the infection trajectories in Experiment 2 was only performed on replicates for which molecular analysis had revealed the presence of hybrid genotype parasites. These data were directly compared with the control treatments (parental strains only). Fish on which only parental genotypes were found, despite infection with two different strains, were discarded from the analysis. The rationale was that homozygous F2, F3, etc. and back-crossed individuals, and parasites that did not engage in sex, were indistinguishable from asexually produced individuals or non-establishment of one of the strains.

2.3.5 Statistical analysis

For Experiment 1, the occurrence of sex was assessed by scoring the presence of homozygous and heterozygous parasites on infected fish. Differences in cross-breeding success between parasite populations were assessed using a χ^2 contingency table test in Minitab v15. A direct comparison of hybrid genotype frequency with Experiment 2 could not be conducted due to the differences in timing of gyrodactylid collection in the experiments.

We investigated whether there was evidence for hybrid vigour and/or inter-strain competition in mixed parasite population by comparing the infection intensity trajectories, maximum parasite burdens and duration of infections in parental and mixed populations in Experiment 2. We used a combination of general linear models (GLMs) and a general linear mixed model (GLMM) based on restricted maximum likelihood (REML) analyses in R and ASReml-R (R v2.9.2) (R Development Core Team 2011). All models used a Gaussian error distribution and an identity link function. For mixed parasite populations only data from fish with known hybrids (confirmed by molecular analysis; n = 12) were considered. These data were compared and contrasted to the data of the pure lines (n = 32). For infection intensity trajectories and maximum parasite burden data were normalised by a natural logarithmic, ln(x), transformation within the model; data for the day of peak parasite burden and parasite loss did not need transformation. For the GLMM, animal ID (to account for repeated parasite counts on the same fish) and days post-infection (with a spline to account for non-linear effects) were added to the random model. Standardized residual distributions were assessed visually with histograms and normality plots plus Shapiro-Wilk normality tests for all models. Initial model terms are presented in Table 2.1. All model terms were assessed as significant with α=0.05 as critical value, and models were reduced with stepwise deletions (using the Log-Likelihood method for random terms in the GLMM). Finally, the minimal model for the GLMM was used to predict the infection trajectory on individual, isolated hosts.

Table 2.1. Model terms and interactions in the GLM and GLMM analyses used for Experiment 2. 2. * random terms in GLMM.

Infection trajectory measure (model)	**Dependent variable**	**Independent continuous (cont.)/ categorical (cat.) variable**
Infection intensity trajectory (GLMM)	Parasite intensity: Ln (x) transformed	Parasite population (cat.)
		Fish length (cont.)
		Fish sex (cat.)
		Days post-infection (cont.)
		Animal ID (cat.)*
		Spline (days post-infection) (cont.)*
Maximum parasite burden (GLM)	Maximum parasite burden: Ln (x) transformed	Parasite population (cat.)
		Fish length (cont.)
		Fish sex (cat.)
		Fish length X sex interaction
Day of maximum parasite burden (GLM)	Day of maximum parasite burden	Parasite population (cat.)
		Fish length (cont.)
		Fish sex (cat.)
		Fish length X sex interaction
Parasite loss (GLM)	Day of parasite loss	Parasite population (cat.)
		Fish length (cont.)
		Fish sex (cat.)
		Fish length X sex interaction

We also examined inbreeding avoidance behaviour within strains by evaluating time-to-infection for parasites transmitting to fish infected with same-strain or different-strain individuals. Parasite time-to-infection was analysed with a survival analysis in R v2.9.2 using Cox's Proportional Hazard and time-to-infection data for the second parasite to infect a fish. The initial model included two parameters: whether the second parasite infecting the recipient fish was a same or different strain individual compared to the first monogenean, as well as the strain origin of the second parasite. Parasite establishment was assessed with a binary logistic regression in Minitab using fish length, sex and parasite population as independent variables.

A χ^2 contingency table test in Minitab or Fisher's Exact Tests (available at http://www.physics.csbsju.edu/stats/contingency.html; Kirkman 1996) were used to detect (1) differences in recovery from parasite infection (i.e. screened clear from parasites on three subsequent screens, Chapter 1.6); (2) percentage of fish carrying hybrid genotypes or not; and (3) percentage of hybrid genotypes in the two mixed populations on fish with hybrids. Proportion of hybrid genotype gyrodactylids among all genotyped parasites and fish mortality were analysed using a binary logistic regression in Minitab including intended parasite population (pure or mixed) in the former, and intended parasite population, fish length and sex in the latter.

2.4 Results

2.4.1 Sexual reproduction and hybridisation

Gyrodactylid strains interbred freely, with 37.5 % and 34.6 % hybrid genotypes recovered at D14 from the *Gt3* x *Gt1* and *Gt8* x *Gt1* parasite lines, respectively, in Experiment 1 (high density starting infection). There was no apparent difference in hybrid genotype frequency between crosses (Contingency Table Test: χ^2 = 0.201, df = 1, p = 0.654). In Experiment 2 (low density starting infection), 12 fish were confirmed to carry hybrid genotype parasites: five (out of 32) initially infected with *Gt3* x *Gt1* (15.6 %) and seven (out of 31) initially infected with *Gt8* x *Gt1* (22.5 %). In this experiment significantly more hybrid genotype parasites were detected for the *Gt8* x *Gt1* population (10.9 %) than in the *Gt3* x *Gt1* population (3.7 %; Binary Logistic Regression: G = 3.841, df = 1, p = 0.05; Fig. 2.1).

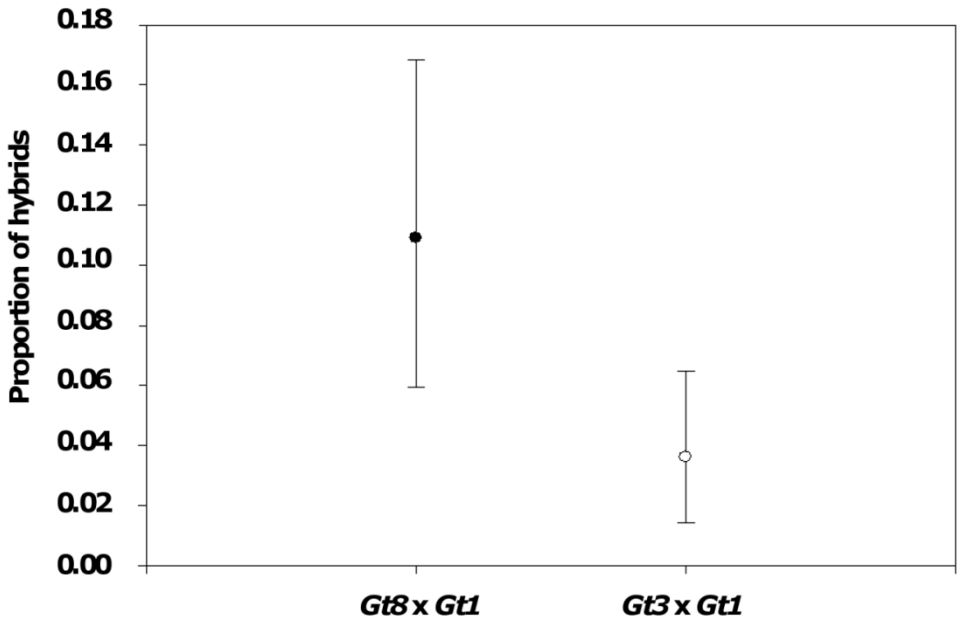

Fig. 2.1. Frequency of sexual reproduction in gyrodactylids. Proportion of hybrid parasites (± 95 % confidence interval) for the *Gt3* x *Gt1* and *Gt8* x *Gt1* parasite populations in Experiment 2. A significantly higher proportion of hybrids were detected in the *Gt8* x *Gt1* than in the *Gt3* x *Gt1* cross.

2.4.2 Fitness effects of mixed strain infections

The GLM and GLMM minimal models for each dependent variable analysed in Experiment 2 are presented in Table 2.2. Mixed gyrodactylid populations exhibited hybrid vigour and/or inter-strain competition at a population level (GLMM: Wald = 5.805, df = 1, p = 0.021). Fig. 2.2A shows the

initial growth phase after which there is no clear pattern between parasite populations due to the onset of fish immunity. Hence, in Fig. 2.2B we use predicted data from the GLMM to visualise different infection trajectories.

The apparent superiority of mixed infections was reflected in a higher parasite burden over time and increased maximum parasite burden, but not a longer duration of infection. Mixed infrapopulations reached their maximum parasite burden of 48.6 ± 15.2 (mean ± standard error of the mean) parasites per fish, which was significantly higher than the 44.2 ± 18.3 parasites in pure parental infrapopulations (GLM: $F_{1,40} = 7.024$, $p = 0.012$; Fig. 2.3A). However, this peak of infection occurred later in mixed populations at 8.3 ± 0.5 days compared to 6.7 ± 0.6 days in pure populations (GLM: $F_{1,32} = 4.963$, $p = 0.033$; Fig. 2.3B). Parasite infrapopulations were lost at 14.6 ± 1.2 and 15.5 ± 1.5 days post-infection in mixed and pure populations, respectively (GLM: $F_{1,34} = 1.103$, $p = 0.302$).

Table 2.2. Minimal models for the GLM and GLMM analyses in Experiment 2. For GLMs the term, then the residual degree of freedom are given.

a. Infection trajectory. Model type: GLMM (random terms = fish ID, days post-infection)			
Model term	Wald statistic (F)	d.f.	P
Parasite population	5.805	1	0.021
Fish length	12.7	1	0.001
Days post-infection	8.669	1	0.004
b. Maximum parasite burden. Model type: GLM			
Model term	Wald statistic (F)	d.f.	P
Parasite population	7.024	1, 40	0.012
Fish length	12.639	1, 40	<0.001
c. Day of peak parasite burden. Model type: GLM			
Model term	F statistics	d.f.	P
Parasite population	4.963	1, 32	0.033
Fish length	16.327	1, 32	<0.001
d. Day of parasite loss. Model type: GLM			
Model term	F statistics	d.f.	P
Fish length	13.343	1, 33	<0.001

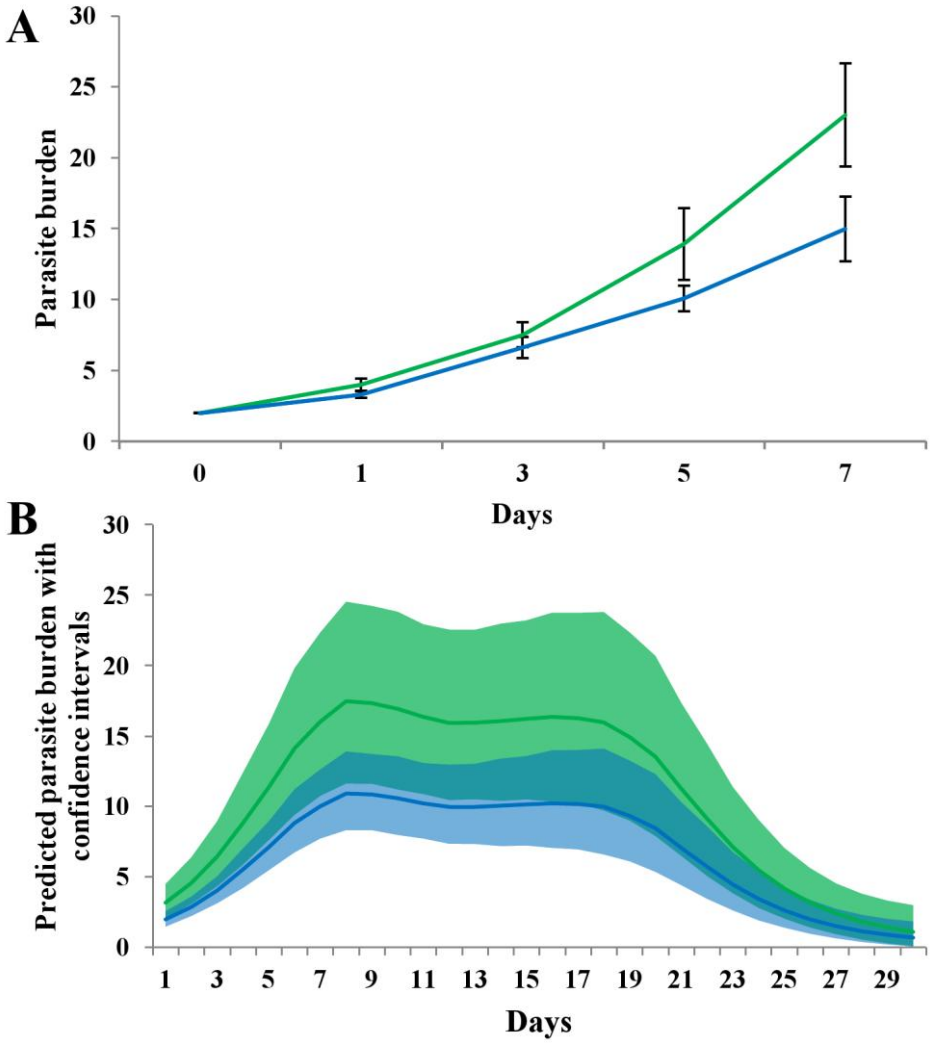

Fig. 2.2. Infection trajectory of pure and mixed *Gyrodactylus turnbulli* populations on individual guppies (*Poecilia reticulata*). (A) Mean parasite intensity in pure (blue) and mixed (green) parasite populations (± standard error of the mean; Experiment 2) in the initial phases of infection showing increased fitness in the mixed parasite population. Increased variation after Day 7 is due to the onset of the host's immune response and is not displayed in this graph. (B) Predicted mean parasite burden over time (controlled for fish standard length, ± 95% confidence intervals) showing that increased parasite fitness in mixed parasite populations leads to faster population growth and higher maximum parasite burdens. Halving of parasite loads on day 7 may have lead to an excaberation of the model predictions for pure and mixed populations, as the pure populations declined from day 7 onwards whereas mixed populations continued to grow for a further 1.5 days.

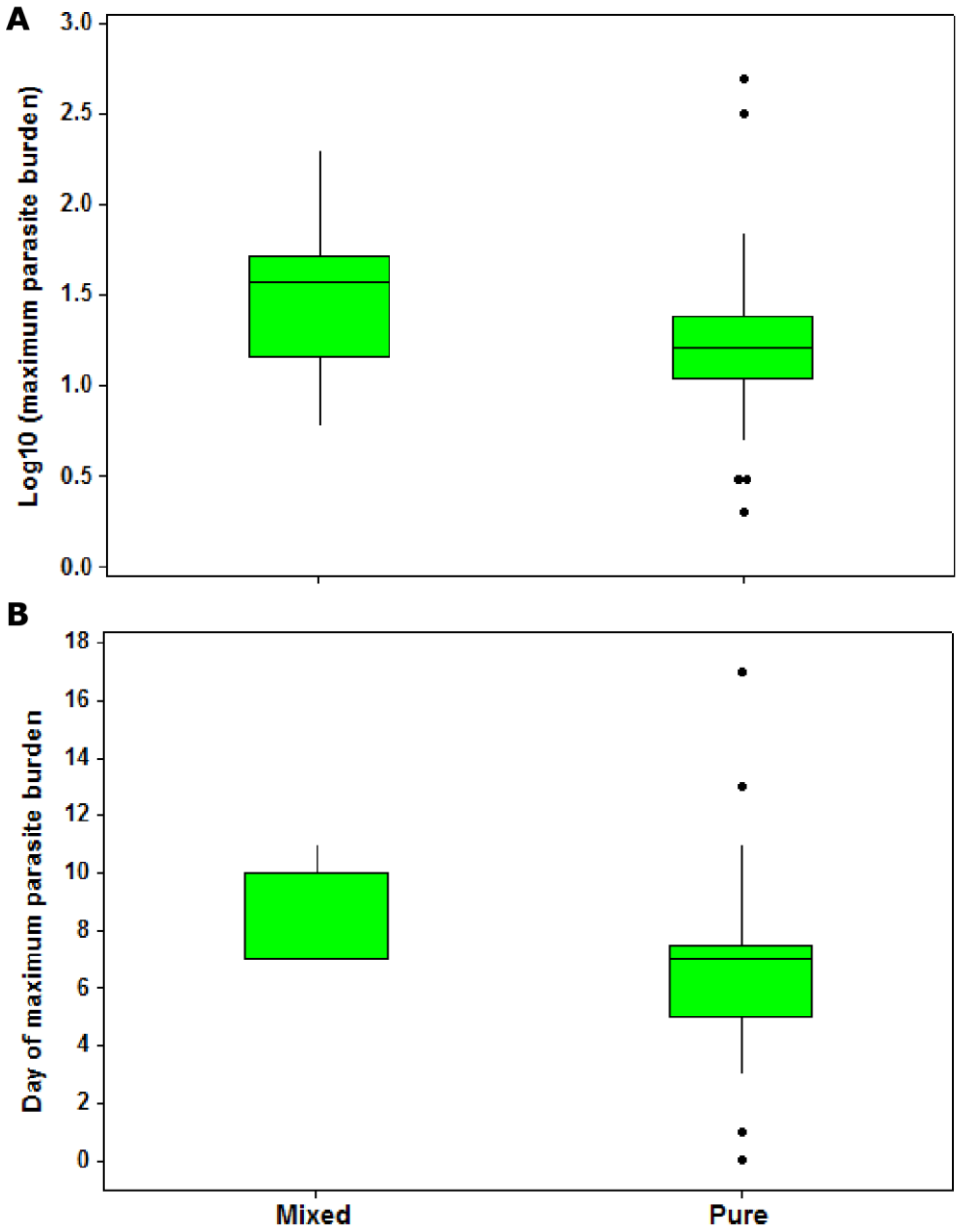

Fig. 2.3. Maximum parasite burden and day of maximum parasite burden in pure and mixed parasite populations. (A) Median maximum parasite burden (log_{10} transformed) and (B) median day of maximum parasite burden. Outliers are represented by dots; the bars are the lower and upper limits; and the box represents the 1st and 3rd quartile with the median. Significant differences between mixed and pure parasite populations for both A ($p = 0.012$) and B ($p = 0.033$).

The infection trajectory itself was also influenced by days post-infection (GLMM: Wald = 8.669, df = 1, $p = 0.004$) and host length (GLMM: $F_1 = 12.7$, $p = 0.001$). Larger hosts showed a higher maximum parasite burden (GLM: $F_{1,40} = 12.639$, $p < 0.001$) which was reached slightly later (GLM: $F_{1,32} = 16.327$, $p < 0.001$). Similarly, parasites were lost slightly later in large guppies (GLM: $F_{1,33} = 13.343$,

p < 0.001). There was no effect of host sex on maximum parasite burden, day of peak parasite burden, day of parasite loss or the infection trajectory.

2.4.3 Parasite infection, establishment success and host survival

In Experiment 2, infection success for parasites used to infect each fish was 100 % for all replicates in all treatments. Time-to-infection of the second monogenean was significantly slower if the parasite on the fish (from the first infection) was from the same strain (25.3 ± 2.2 s) than from a different strain (11.8 ± 5.6 s; survival analysis: z = -2.809, p = 0.005, n = 100; Fig. 2.4). Establishment success, measured on D1, increased with fish size (Binary Logistic Regression: G = 10.57, df = 3, p = 0.014 for overall test; Z = -2.07, p = 0.039 for fish length, odds ratio 0.62 (95 % confidence interval: 0.39-0.98)), but was not affected by parasite population (pure: 96.9 %, mixed: 89.9 %; Z = 0.92, p = 0.359 for parasite population, odds ratio 2.85 (95 % CI: 0.30-26.76)). There was no significant difference in fish mortalities between the parasite populations (Binary Logistic Regression: G = 5.125, df = 3, p = 0.163): 16.1 % mortality in pure and 25 % in mixed populations.

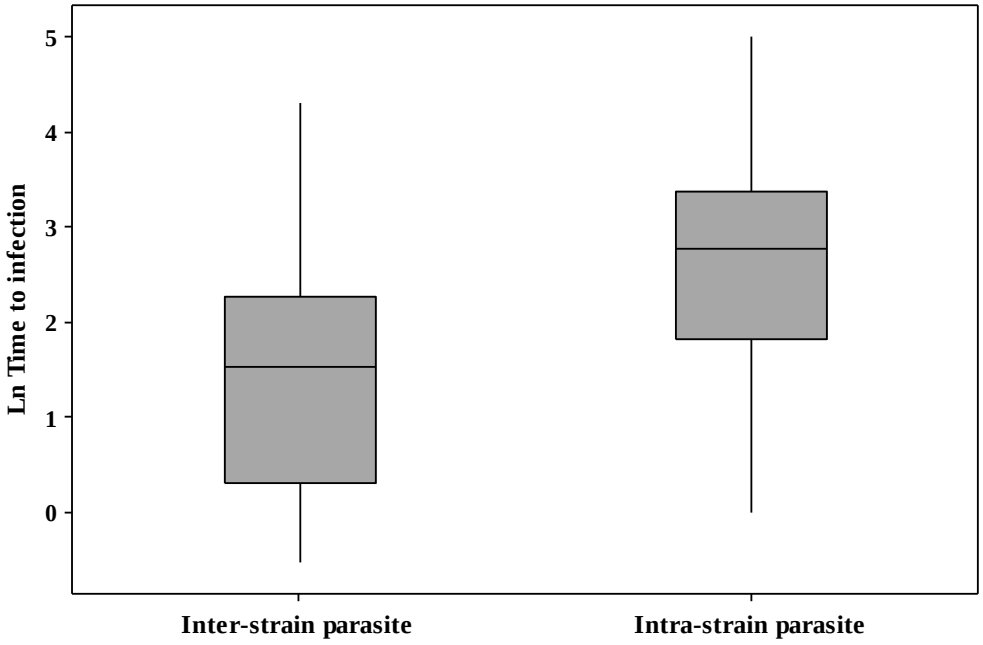

Fig. 2.4. Time-to-infection for inter- and intrastrain infections. Time-to-infection (s; natural log transformed) was significantly lower for secondary parasites infecting a fish already infected with a different (inter-)strain parasite rather than a same (intra-)strain parasite individual. The bars are the lower and upper limits; and the box represents the 1st and 3rd quartile with the median.

2.5 Discussion

This study unambiguously demonstrates, by making experimental crosses and using a microsatellite marker to identify sexually derived parasites (i.e. outbred or hybrid genotype offspring), the occurrence of sexual recombination in a monogenean parasite, *Gyrodactylus turnbulli*. Although it has long been assumed that sex does occur in monogenean hermaphrodite parasites based on their observed inter- and intra-specific mating behaviour and phylogenetic studies (MacDonald and Caley 1975, Cable and Harris 2002, Ziętara et al. 2006), there was no direct evidence for the production of viable sexually produced offspring. Importantly, we also show that outcrossing (hybridisation) between monogeneans of previously inbred populations and/or inter-strain competition significantly increases various fitness components of the parasites, resulting in a higher parasite burden over time and an increased maximum parasite burden, but not a longer duration of infection.

Previously, cytogenetic observations of decondensation of the sperm nucleus after the fusion of sperm and egg cell had suggested that sexual recombination occurs in gyrodactylids (Cable and Harris 2002). Release of male genetic material into the egg cytoplasm does not, however, necessarily equate to sexual recombination since any sperm material can be expelled from the egg. Sperm dependent parthenogenesisis displayed by, for example, the hermaphrodite flatworm *Schmidtea polychroa* (Beukeboom 2007). Further evidence of genetic exchange in the evolutionary history of gyrodactylids through sexual reproduction comes from previous phylogenetic studies (e.g. *G. salaris, Macrogyrodactylus* spp.; see Kuusela et al. 2007, Barson et al. 2010). The current study is the first to demonstrate conclusively sexual recombination in monogeneans in contemporary populations and establishes that, at least in the laboratory conditions of the present experiment, between 3.7 % and 10.9 % of all genotypes are recent hybrids with ancestors from two different inbred lines.

Confirmation of recombination in viviparous gyrodactylids has important consequences for the evolutionary history of this specious group of flatworms. Over 400 species of gyrodactylids have been described; and host-switching rather than co-evolution with the fish host appears to be the main mechanism of speciation (Bakke et al. 2002, 2007). Sexual reproduction between diverging populations can potentially facilitate the evolution of new species (Barton 2001, Seehausen 2004), particularly in combination with a switch to a host not previously populated by either of the parental species (Kuusela et al. 2007, Hayakawa et al. 2008). In more recent times, host switches and hybridisation events are further facilitated by changes in range distribution associated with climate change and global fish transport. The resulting co-occurrence of previously separated species in the same habitat (Harvell et al. 2002, Brooks and Hoberg 2007) and the new opportunities provided in these novel environments could augment the rate of hybridisation in the wild.

The occurrence and frequency of sexual reproduction in gyrodactylids may be species-specific and condition-dependent. Based on changes in haptor morphology over 20 generations of individually maintained parasites, Harris (1998) speculated that *G. gasterostei* reproduction is largely clonal. As explained in Section 2.3, our estimates of sexual reproduction for *G. turnbulli* are significantly downward biased given that we used only a single microsatellite locus to identify sexually reproduced offspring. Thereby, we will have missed hybrid genotype gyrodactylids that are homozygous for our marker locus due to, for instance, Mendelian segregation or back-crosses. The rates of sexual recombination varied by nearly a factor of three between our crosses. These differences may be due to the limitations of our methodology, the highly inbred *Gt3* parasites not showing any preference between same or different strain parasites as previously described for cestodes (Schjørring 2009), or they could reflect natural variation in the rate of outcrossing.

Even a low frequency of sex in hermaphrodites is sufficient to avoid the negative fitness consequences associated with inbreeding (Charlesworth and Willis 2009). Empirical data from the facultative sexual single-celled chlorophyte *Chlamydomonas reinhardtii* show that particularly in large hermaphrodite populations, sex will be maintained as a reproductive mode to overcome inbreeding depression (Peck and Waxman 2000, Colgrave 2002, MacCarthy and Bergman 2007). Mate preference for unrelated individuals is likely to evolve as this reduces the fitness costs of sexual reproduction with a related individual. We previously speculated about inbreeding avoidance occurring in *Gyrodactylus* species (Bakke et al. 2007) and empirical data from the current study are consistent with this argument, showing that the time-to-infection differs according to the relatedness of the novel gyrodactylid compared to the already existing infection. This suggests that these parasites may recognize conspecifics with similar genotypes. The mechanism for this remains unknown, but could be related to a chemical cue released through the parasite's glands or excretory products into fish mucus (Bakke et al. 2007). Humans, mice and fish are known to use chemical communication (i.e. olfaction) to make self-referential decisions of attractiveness based on genetic variation at the Major Histocompatibility Complex (MHC) (May and Hill 2004). Many vertebrates show preferences for partners with dissimilar genotypes, which are identified via MHC-related chemical cues (Milinski 2006). In invertebrates, behavioural mechanisms to avoid inbreeding are more commonly reported than the utilization of olfactory cues (e.g. insects: *Gryllus bimaculatus* and the subsocial spider, *Anelosimus* cf. *jucundus*; see Bonduriansky 2001, Bukowski and Avilés 2002, Tregenza and Wedell 2002), but there is also evidence that the innate immune system may be involved in mate choice (Milinski 2006). In the only other parasitic platyhelminth in which inbreeding avoidance has been examined, individuals of the cestode *Schistocephalus solidus* preferred siblings to unrelated parasites as mates (Schjørring and Jäger 2007). Hence, to our knowledge, the current study provides the first evidence of inbreeding avoidance behaviour in platyhelminths.

Once sexual reproduction occurs, the main fitness benefit in hybridising gyrodactylid populations appears to be the higher maximum parasite burdens and extended time of population growth before the host's immune response appears to cause an infra-population decline. Pure parasite populations started declining approximately seven days after initial infection, whereas mixed parasite infra-populations continued to grow on average for a further two days. These results are consistent with the hypothesis that: (i) hybrid genotypes are more tolerant (parasite damage limitation) and/or resistant (limitation of parasite burden) (Råberg et al. 2007) to the fish immune response allowing them to maintain a reproducing population on the host for longer than parental parasites; (ii) hybrid genotypes are better at evading the host's localised immune response (see van Oosterhout et al. 2008 for the effects of parasite mobility and distribution on parasite population dynamics); (iii) hybrid genotypes do not activate the immune response as rapidly as parental parasites, leading to delay in the onset of the host response; and/or (iv) they benefit from hybrid vigour (heterosis) and have increased fecundity relative to the inbred parental lines (see Cable and van Oosterhout 2007 for evidence of inbreeding depression in the *Gt3* line). Alternatively, the high fitness of mixed strain parasite populations could be caused by inter-strain competition, possibly resulting in increased virulence (Davies et al. 2002). However, mixed parasite populations did not cause higher host mortalities in the current study, indicating that despite increased growth rates in parasite populations, virulence of hybrid genotypes was not increased compared to parental populations. Further evidence against inter-strain competition is the lack of evidence for competitive exclusion among other monogeneans co-infecting the same host (Morand et al. 2002, Karvonen et al. 2007).

The exact reasons for the increased fitness of mixed parasite populations cannot be disentangled in this experiment, but warrant further study. Given the reduction in fitness of the *Gt3* line relative to recently wild-caught *G. turnbulli* (*Gt1*) (Cable and van Oosterhout 2007), we favour the hypothesis that hybridisation ameliorated the detrimental effects of inbreeding during the up to $\sim 2 \times 10^3$ generations in captivity, and that this resulted in hybrid vigour. There are implications of this study for the fitness of hosts and parasites in wild populations. In natural environments, a high parasite burden is associated with increased extrinsic mortality of this host, for example due to increased risk of downstream displacement during flash-flooding (van Oosterhout et al. 2007). This suggests that the increased parasite load in hybrid genotype infections could increase host mortality in the wild. Similarly, secondary (bacterial, fungal, etc.) infections may be more common with higher parasite burdens. In addition, other factors such as infectivity, transmission abilities and host specificity, which have not been considered for this experiment, could also be affected by hybrid vigour, and the effects of hybridisation on these life history traits and infection dynamics warrant further investigations.

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CHAPTER 3: SMALLER GUPPIES (*POECILIA RETICULATA*) AVOID ECTOPARASITE INFECTIONS FOR LONGER

3.1 Abstract

Effective infectious disease control requires detailed understanding of parasite life histories, including factors that govern successful transmission events. Here, we investigated the transmission behaviour of two co-infecting gyrodactylid monogeneans; *Gyrodactylus bullatarudis* and *G. turnbulli* infecting the guppy (*Poecilia reticulata*). Surprisingly, there is no difference in time-to-transmission from live to live hosts between the two parasite species (mean ± standard error of the mean: *G. bullatarudis* 44.4 ± 6.6 h; *G. turnbulli* 29.9 ± 7.1 h) and the similarities in dispersal are confirmed when investigating transmission from and parasite behaviour on dead hosts as well as survival *in vitro* which were all similar for both *G. bullatarudis* and *G. turnbulli*. Instead, transmission was affected by fish size: an increase of 1 mm in standard length of recipient fish led to guppies being infected on average 20 min faster. Hence, smaller fish may be less central in the contact network or more cautious around (infected) conspecifics. These findings add to the growing evidence that host behaviour rather than gyrodactylid specific traits facilitate transmission and disease persistence in fish shoals.

3.2 Introduction

Transmission is central to infectious disease dynamics. Understanding transmission parameters within a host-parasite system is essential for effective disease control management. Whereas early work assumed that all parasite transmission is constant (Anderson and May 1979, May and Anderson 1979), there is increasing awareness that transmission rates are dependent on a range of factors including the particular parasite species, the type of vector (for indirectly transmitted diseases), host behaviour and the environment (e.g. Stromberg 1997, Randolph and Katie 1999, Fenton et al. 2002, Krist et al. 2003). For instance, host behaviour itself will contribute to disease dispersal (Richards et al. 2010), but the parasites themselves can also alter host behaviour to increase transmission rates (Moore 2002). In addition, for micro-parasites with direct life cycles, the transmission rate is expected to be largely a consequence of host and parasite behaviour rather than environmental factors (Swinton et al. 2002). Our understanding of disease processes including transmission have been improved by theoretical modelling, but for model predictions to be accurately predicted, empirical data on infectious disease life history such as transmission rates are essential (May 2004).

Viviparous gyrodactylids, monogenean ectoparasites ubiquitous on teleost fish, lack a specific transmission stage. The direct life cycle of *Gyrodactylus* spp. in combination with their low host specificity compared to other parasite taxa and highly efficient hyperviviparous reproductive system, makes them one of most successful invaders worldwide (Cable and Harris 2002, Bakke et al. 2007,

Chapter 2). Their transmission may occur through (1) direct contact between conspecific or phylogenetically unrelated hosts both dead and alive, and (2) detached parasites in the water film, drifting or on substrate (Bakke et al. 1992, Soleng et al. 1999, King and Cable 2007). The relative importance of these transmission routes is unclear and may be context dependent (with the exception of low infection rates from dislodged, drifting parasites, Soleng et al. 1999). For instance, rather than detaching and seeking a new host by drifting in the water film or attaching to the substrate, *G. salaris* stays with its dead host for up to three days allowing it to maintain its fitness for when it is able to infect a new host (Olstad et al. 2006). In contrast, *G. turnbulli* migrates off the dead host into the water film within 1 h of its host's death, so potentially increasing its chances to encounter a surface feeding guppy (*Poecilia reticulata*) host (Cable et al. 2002a). The gyrodactylids' characteristic stretching movement (Bakke et al. 2007), sensors associated with the anterior glands (Watson and Rohde 1994) and locomotion (Brooker et al. 2011) enable them to re-attach to live hosts from substrate, and to transmit between live fish. Some gyrodactylids, such as *Ieredactylus rivuli* and *G. rysavyi*, can even propel themselves through water, but there are doubts about the efficiency of this locomotion behaviour in facilitating transmission (El-Naggar et al. 2004, Bakke et al. 2007, Appendix I).

Since many *Gyrodactylus* spp. coexist on the same fish species, often occupying different niche spaces on the host (e.g. Bakke et al. 1992, Harris and Lyles 1992, Raeymaekers et al. 2008), behaviour to enhance transmission can be expected to vary between parasite species. For instance, the timing of dispersal to other hosts may differ and depend on the health status of the fish. Early dispersal allows for a fast gain of unoccupied 'territory', but may be a riskier strategy as an estimated 35-39 % of parasites are lost during transmission events (Scott and Anderson 1984); in contrast, late dispersal might cause competitive interactions between parasites in highly dense populations for which there is mixed evidence in monogeneans (Karvonen et al. 2007). Indeed, older gyrodactylids may be the main dispersers (Dmitrieva 2003, Grano Maldonado 2011). On dead hosts, gyrodactylids are expected to stay as long as possible as, after detachment, embryos become malnourished in a relatively short time, so reducing the reproductive output of individuals (Cable et al. 2002b, Olstad et al. 2006). Further, parasites may survive for well over a day after detaching from the host (e.g. Cable et al. 2002b, Olstad et al. 2006, Chapter 5), although there may be differences in such life history characteristics between different parasite species infecting the same host to avoid competition. For example, since *G. turnbulli* moves relatively fast off the dead host (Cable et al. 2002a), will the co-infecting congener *G. bullatarudis* stay on the host to exploit the freed-up host resources? Alternatively, does *G. bullatarudis* rely on guppies to cannibalise their dead conspecifics for transmission, which in the wild occurs within 90-100 s (Cable, van Oosterhout, Mohammed, unpublished data)?

To address these questions of dispersal, we set out to assess factors of importance affecting transmission for two gyrodactylid species, *G. turnbulli* and *G. bullatarudis*, which commonly co-occur on the guppy (*P. reticulata*) both in the wild and in aquaculture (Cable 2011). The two species differ in their site preferences on infected fish with *G. bullatarudis* mainly occurring on the rostral region of its host, whereas *G. turnbulli* primarily infects the caudal fins and the peduncle (Harris and Lyles 1992). Infection dynamics of single species infections of both parasites appear similar overall (Richards and Chubbs 1996, 1998, Cable and van Oosterhout 2007a, King and Cable 2007, King et al. 2009, Chapter 5), but little is known about their interaction when co-infecting a fish. Presumably, at low infection intensities, which are common in the wild (Harris and Lyles 1992), the differential use of niche space avoids competitive interactions. However, evidence from other monogeneans indicates that intra-specific parasite communities do not interact even at high infection intensities (Morand et al. 2002, Karvonen et al. 2007). Importantly though, co-infecting *G. turnbulli* strains do show increased population growth when compared to single strain infections (Chapter 2). Work on *G. turnbulli* by Scott and Anderson (1984) and Cable et al. (2002a) determined some parameters of *in vitro* survival and behaviour on dead hosts which both are of importance for successful transmission when dislodged from fish; here, we expand this work to a direct comparison between the two co-infecting parasite species including host variables such as fish standard length and sex, to control for and investigate additional factors that may aid successful transmission in the guppy-*Gyrodactylus* host-parasite system. Specifically, we investigated the time-to-transmission between (1) live shoaling fish and (2) an infected, dead donor fish to a guppy shoal. Further, we investigated (3) time-to-detachment for parasites from dead hosts and (4) survival when detached from hosts.

3.3 Materials and methods

3.3.1 Source of animals and compounds

Ornamental guppies (*Poecilia reticulata*) were originally purchased from pet shops in the UK. They were maintained under a 12 h light : 12 h dark cycle at 25 ± 1 °C, and fed daily on fish flakes (Aquarian®, API) or during experiments exclusively with live *Daphnia* spp. plus frozen *Tubifex* at least once weekly. For the experiments, the fish were infected with gyrodactylid parasites isolated from pet shop guppies: *Gyrodactylus turnbulli* (*Gt3* strain isolated in 1997) and *G. bullatarudis* (two strains isolated in 2006 and 2010); these separate isolates of *G. bullatarudis* were required due to parasite culture extinctions.

At the beginning of the experiment, fish were sexed and measured under anaesthetic (0.02 % MS222, PharmaQ Ltd, UK). For parasite screens during the experiment, fish were captured in glass dishes, to avoid dislodging parasites using nets and they were simultaneously removed from and released back

into the original tanks to ensure equal duration of parasite exposure for all replicates. All screens were conducted using 0.02 % MS222 under cold light illumination (Chapter 1).

3.3.2 Experiment 1: Live transmission between guppies

To investigate the time-to-transmission between hosts for *G. turnbulli* and *G. bullatarudis* and whether or not this was affected by host sex and length, one donor (female or male) and two recipient guppies (one male, one female) were introduced into a 10 L aquarium (donors: n = 19 for each parasite species/treatment; total recipient fish: n = 76). The fish were then left for 48 h to familiarize with each other. After two days, the donor guppy was removed and exposed to either *G. turnbulli* or *G. bullatarudis* infected conspecifics allowing natural infection of the donor guppy to occur over a period of 2-3 d. The recipient guppies were maintained in separate 1 L pots throughout this time. Once the donor guppy had reached an infection load of ≥ 20 parasites, the fish was anaesthetized again, any superfluous parasites removed with forceps (if parasite load > 20), and returned back into its original tank with the male and female recipient. All fish were screened at 4 h, then every 24 h after the introduction into the tank. Once parasites had transferred to one or both of the recipient fish, the trial was terminated.

3.3.3 Experiment 2: Transmission from dead hosts

To test the time taken for parasites to transmit from recently deceased hosts to live guppies with and without access to the dead fish, three live recipient guppies, one male and two females were introduced into a 10 L plastic tank (total recipient fish: 216) for acclimatisation and familiarisation 24 h before the start of the experiment. On D0, fish destined to be dead donor hosts and infected with ≥ 50 *G. turnbulli* (n = 40) or *G. bullatarudis* (n = 32) were euthanized with an overdose of MS222, sexed and their standard length measured before their parasite burden was assessed and, if necessary, reduced to 50 parasite/fish with forceps. One dead fish (female or male) was then added directly into the middle of each experimental tanks containing the three live recipients which in half of the tanks did not have access to the euthanized fish as it was kept underneath a cage (Fig. 3.1) providing no opportunity for cannibalism and, hence, direct transmission.

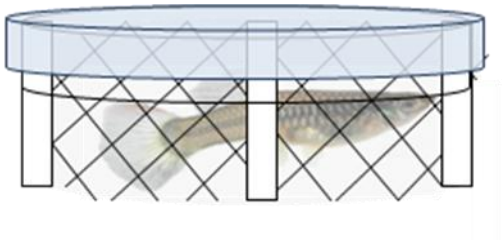

Fig. 3.1. Cage comprised of a Petri dish on plastic stilts supporting a fibre mesh to enclose parasite infected, dead guppy (*Poecilia reticulata*) used in Experiment 2.

Live hosts were screened every 4 h during the 12 h light period of the experiments, including directly after the lights were switched on and off (i.e. at 0, 4, 8, 12, 24, 28, 32, 36 and 48 h). The trial was stopped once transmission had occurred in the tanks.

3.3.4 Experiment 3: Parasite behaviour on dead hosts in vivo

To investigate how long *G. turnbulli* and *G. bullatarudis* take to detach from a deceased host, we infected guppies (*G. turnbulli*: n = 23; *G. bullatarudis*: n = 26) with a range of parasite burdens (*Gt*: mean: 52, range: 1-249; *Gb*: mean: 53, range: 1-205) and euthanized them by pithing. The corpses were immediately transferred to 25 mL dechlorinated aquarium water at 25 ± 0.5 °C in a 9 mm diameter Petri dish. The number and location of parasites were monitored hourly for 12 h and again 24 h after the host's death. After each check, any dislodged parasites were removed from the Petri dish to avoid pseudoreplication and the fish corpse was transferred to clean water to maintain optimal water conditions for the parasites.

3.3.5 Experiment 4: Parasite survival in vitro

To investigate the survival of parasites when detached from the host, *G. turnbulli* and *G. bullatarudis* worms were removed from heavily infected euthanized donor guppies and individually transferred in 200 µL of dechlorinated water into the wells of a 90 well microtitre plate using a Gilson pipette (Cable et al. 2002b). Parasites were assessed an hour after removal for death or altered behaviour (i.e. response to an insect pin closely wafted through the water near the worm) indicating damage during transfer, and any such worms were excluded from the experiment. All other parasites (*G. turnbulli*: n = 60; *G. bullatarudis*: n = 49) were checked hourly for the first 5 h, then every 2 h until all worms had died. Any worms that gave birth between observation periods were identified by their empty uterus, while their newborn daughters were visible pregnant as seen under a stereo-microscope (Cable and Harris 2002).

3.3.6 Statistical analysis

The time-to-transmission between live fish (Experiment 1) and the time-to-transmission from dead to live hosts (Experiment 2) were analysed using separate General Linear Models (GLMs) in R v2.13.2 (R Development Core Team 2011). Both dependent terms were natural log transformed prior to analysis to normalise residual distributions. In Experiment 1, parasite species, donor and recipient standard length (natural log transformed for donor), sex and final parasite load of the donor were included in the starting model. For Experiment 2, initial independent parameters included cage status, parasite species and donor and first infected sex as factors, plus standard lengths of all fish (natural log transformed except for second female recipient) as covariates. Models were fitted with a Gaussian error structure and a log (Experiment 1) or an identity (Experiment 2) link function. The standardised

residuals from each model were first checked visually for normality and homogeneity of variance using a histogram, Q-Q plots and fitted values. Models were then refined by stepwise deletions. Further, for Experiment 2, a binomial mass function to calculate probability that females are infected first was used to account for the female skewed sex bias in recipient fish.

With data distributions too skewed to fit any generalised linear models tested, a series of non-parametric tests were used to assess the time to detachment from dead hosts (Experiment 3) and host survival (Experiment 4) within R,. First, the time to detachment from dead hosts was analysed using a Kruskal-Wallis test, with parasite species as an independent variable. A Spearman's rank correlation analysis was then conducted to assess the relationship between the time-to-detachment of the parasite from the host and the length of the recipient host, as well as donor parasite load at the start of the experiment. Due to the low number of replicates used for this experiment, additional power analyses were conducted. *In vitro* data (Experiment 4) were analyzed with a non-parametric Cox proportional hazard to assess the effect of parasite species on the hour of host death. To present data consistently across experiments, means with the standard error are presented despite Experiment 3 and 4 being analysed with non-parametric statistical analyses.

3.4 Results

Time-to-transmission for parasites between live hosts (Experiment 1) was determined by recipient standard length, and not the parasite species or the fish sex: each 1 mm increase in standard length led to fish being on average infected 20 min earlier during the experiment (GLM: $F_{1,28} = 7.2$, $p = 0.012$; standard length: $T = -2.65$, $p = 0.013$; coefficient: -0.056; Fig. 3.2). Recipient standard length had no effect on time-to-transmission when donor fish were dead, nor did donor sex or parasite species ($p > 0.05$; Experiment 2). When dead hosts were caged and direct contact between the parasites prevented, parasites took much longer to transmit (mean ± standard error of the mean: 17.8 ± 1.8 h) than when fish were not caged (6.1 ± 0.9 h; GLM: $F_{1,70} = 49.0$, $p < 0.001$; Fig. 3.3). Both *Gyrodactylus turnbulli* (*Gt*) and *G. bullatarudis* (*Gb*) remained with dead hosts for similar times (*Gt:* 21.9 ± 1.3 h; *Gb*: 24.0 ± 1.0 h; $\chi^2 = 3.21$, $p = 0.073$; Experiment 3), the test having a statistical power of 0.45, and survived an average of 13.0 ± 1.2 h and 16.3 ± 0.9 h, respectively (Cox proportional hazard likelihood ratio test = 0, df = 1, $p = 0.965$), when detached from the host (Experiment 4).

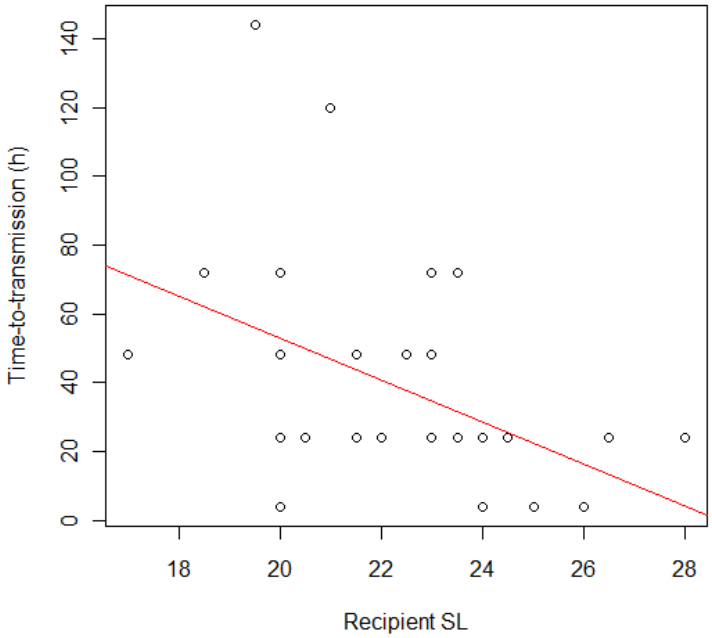

Fig. 3.2. Time-to-transmission of *Gyrodactylus bullatarudis* and *G. turnbulli* (h) from live to live hosts decreased with increasing recipient standard length (SL; mm) and fitted regression line.

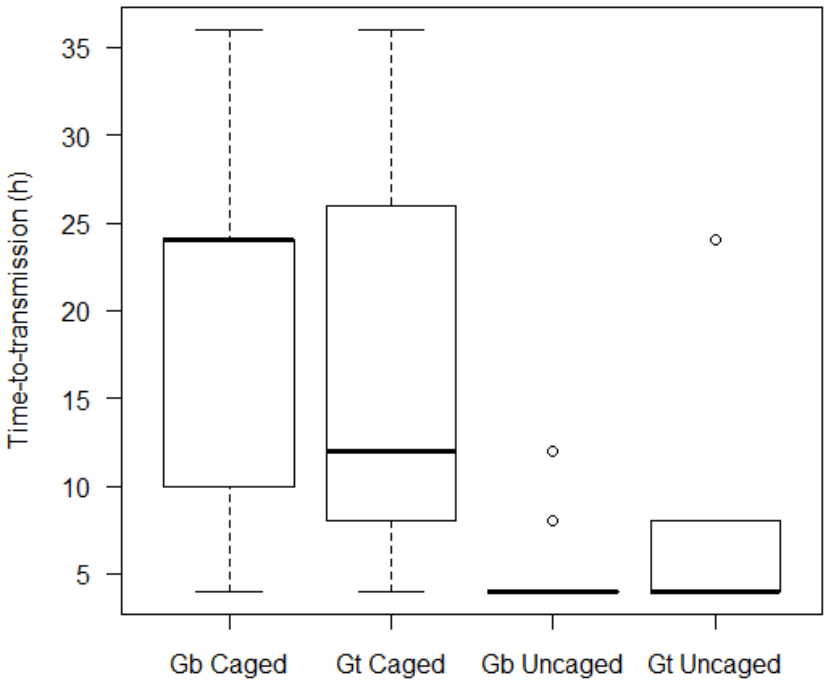

Fig. 3.3. Box and whisker plot for time-to-transmission (h) from dead to life hosts for *Gyrodactylus bullatarudis* (Gb) and *G. turnbulli* (Gt). Time-to-transmission was much longer for parasites on caged fish compared to gyrodactylids on uncaged fish. The dots represent outliers, the bars, the lower and upper limits, and the box represents the 1st and 3rd quartile with the median.

3.5 Discussion

This study suggests that parasite transmission success is dependent on characteristics of the recipient fish and not *Gyrodactylus* species. Time-to-transmission from a live, shoaling fish was significantly faster to larger fish compared to smaller fish. Further, when infected donors were dead, parasites transmitted three times faster when recipient fish had direct access to the corpse which confirms earlier studies on the relative low dispersal success rate of detached gyrodactylids (Soleng et al. 1999, Høgåsen et al. 2009). Parasite species and fish sex did not influence time-to-transmission from dead or alive fish to conspecifics, of time-to-detachment from infected corpses or *in vitro* survival of the parasites in any of the experiments. This adds to the growing evidence indicating that some host characteristics and behaviour are the main factors contributing to successful transmission of these parasites (Richards 2010, Croft et al. 2011, Johnson et al. 2011, Richards et al. 2012).

The similar transmission characteristics of the different *Gyrodactylus* spp. examined in the current study may be explained by little or no competitive exclusion during co-infection in gyrodactylids generally (Morand et al. 2002, Karvonen et al. 2007). Since inter-specific competition is negligible, the trade off between staying on increasingly crowded hosts, where parasites can secure further reproductive output, and dispersal, which is risky, appears to be most beneficial for both *G. bullatarudis* and *G. turnbulli* when transmission is delayed. Indeed, previous studies comparing *G. bullatarudis* and *G. turnbulli* infection in guppies showed similar characteristics between the two parasites or mixed evidence for species specific traits, such as *in vitro* survival (no difference between species: Chapter 5); maximum parasite load (no difference between species: Richards and Chubbs 1996, Cable and van Oosterhout 2007a); establishment on hosts (*G. turnbulli* more likely to successfully establish an infection: Richards and Chubbs 1996; no difference between species: Cable and van Oosterhout 2007a, King and Cable 2007, King et al. 2009, Chapter 5); infection trajectory of parasite population on individual fish (no difference between species: Cable and van Oosterhout et al. 2007a, Chapter 2); host mortality of individually maintained, infected guppies (*G. bullatarudis* causes higher mortality: Chapter 5; *G. turnbulli* causes higher mortality: Richards and Chubbs 1996; no difference between species: Cable and van Oosterhout 2007a) and in fish shoals (*G. turnbulli* causes higher mortality: Richards and Chubbs 1998). The relative low number of replicates does mean, however, for Experiment 3 means that in the current study statistical power may not have been strong enough to detect a real difference. Also, the current study cannot exclude a variation in transmission behaviour, primarily for the time-to-detachment from dead hosts for *G. turnbulli*. A previous study by Cable et al. (2002a) utilizing a different *G. turnbulli* strain showed parasites to leave dead hosts within an hour, which contradicts our results (mean ± standard error of the mean in current study: 21.9 ± 1.3 h for *G. turnbulli*). Hence, future work should focus on determining the importance of inter-strain variation in transmission success.

Of particular importance for successful transmission of parasites between live fish in our experiments was the size of the fish, with smaller guppies becoming infected later than larger counterparts. The delay in becoming infected may be particularly important in the wild where infection avoidance may be gained by the transient characteristics of guppy shoals (Croft et al. 2003a). For instance, smaller (i.e. younger) fish may be more cautious around infected fish, as presumably they would learn from their elders to avoid parasitized shoal members, although the avoidance of larger fish to evade being cannibalised or fin nipped may also play a role (Magurran 2005, Vukomanovic and Rodd 2007, Richards et al. 2010, Croft et al. 2011). Larger fish, particularly when having been infected with gyrodactylids before, may be less risk adverse, as they have already mounted a primary immune response previously to the parasites increasing their chances of survival during a renewed infection (Cable and van Oosterhout 2007b). However, fish used in the current study were naive, so have not had a previous experience of infection. Further, larger fish are more likely to accumulate high parasite burdens and subsequently die as a result, indicating that the consequences of contracting gyrodactylosis are far more detrimental to them than smaller fish (Cable and van Oosterhout 2007a).

The absolute increase in surface area of larger fish may also have contributed to the higher likelihood of catching a gyrodactylid infection, although this seems unlikely as the negative relationship between time-to-transmission and guppy size does not exist when donor fish are dead. Hence, guppy behaviour *per se*, for instance, increased movement of larger guppies such as in natural stream environments or an artificial flume (Croft, 2003b; Frances Hockley, personal communication), may account for the observed phenomenon. Indeed, recent studies show evidence for host behaviour being a strong driver of transmission in gyrodactylids by investigating shoaling behaviour and social network structure in the presence of infected conspecifics (Kolluru et al. 2009, Richards 2010, Croft et al. 2011, Richards et al. 2012), shoal size (Johnson et al. 2011) and fish personality (Richards 2010).

Overall, this study has shown that smaller guppies avoid contracting gyrodactylosis for longer than larger guppies and that *G. bullatarudis* and *G. turnbulli* infecting these fish are similar in their transmission behaviour and abilities. Hence, disease transmission among guppies is strongly dependent on host rather than parasite behaviour. Disease persistence in guppy populations is due to factors such as host sex, shoal size and cohesion, but the degree to which these contribute to gyrodactylid transmission is unclear.

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CHAPTER 4: PARASITES PITCHED AGAINST NATURE: PITCH LAKE WATER PROTECTS GUPPIES (*POECILIA RETICULATA*) FROM MICROBIAL AND GYRODACTYLID INFECTIONS*

4.1 Abstract

The Enemy Release Hypothesis proposes that in parasite depleted habitats, populations will experience relaxed selection and become more susceptible (or less tolerant) to pathogenic infections. Here, we focus on a population of guppies (*Poecilia reticulata*) that are found in an extreme environment (the Pitch Lake, Trinidad) and examine whether this habitat represents a refuge from parasites. We investigated the efficacy of pitch in preventing microbial infections in Pitch Lake guppies, by exposing them to dechlorinated water, and reducing gyrodactylid infections on non-Pitch Lake guppies by transferring them to Pitch Lake water. We show that (i) natural prevalence of ectoparasites in the Pitch Lake is low compared to reference populations, (ii) Pitch Lake guppies transferred into aquarium water develop microbial infections, and (iii) experimentally-infected guppies are cured of their gyrodactylid infections both by natural Pitch Lake water and by dechlorinated water containing solid pitch. These results indicate a role for Pitch Lake water in the defence of guppies from their parasites and suggest that Pitch Lake guppies might have undergone enemy release in this extreme environment. The Pitch Lake provides an ideal ecosystem for studies on immune gene evolution in the absence of parasites and long-term evolutionary implications of hydrocarbon pollution for vertebrates.

4.2 Introduction

Host parasite coevolution acts to maintain polymorphism and/or increase the rate of molecular evolution (Lively 1987, Dybdahl and Lively 1998, Lively and Jokela 2002, Decaestecker et al. 2007, Woolhouse et al. 2002, Paterson et al. 2010). With the majority of all free-living animals hosting at least one parasite taxon (Price 1980), populations without parasites represent a rare opportunity to understand the effects of host-parasite coevolution. Island populations may experience reduced selection pressures due to decreased parasite diversity, prevalence and intensity (e.g. Lindstroem et al. 2004), similar to invasive species that are released from their natural enemies in novel environments (Liu and Stiling 2006). A special case of "enemy release" may be experienced by populations in extreme environments in which hosts may find refuge from their parasites (Tobler et al. 2007). The adaptive forces in these hostile habitats are often well defined (Amils Pibernat et al. 2007), yet, their effects on parasites infecting hosts inhabiting such environments are rarely assessed even though they can have a major impact on the host-parasite relationships. Populations of guppies with little or no

*Adapted from: Schelkle B, Mohammed RS, Coogan MP, McMullan M, Gillingham EM, van Oosterhout C, Cable J (2012) Parasitology 139: 1-8.

parasites are rare (see Fraser and Neff 2010), but preliminary investigations indicate that the Pitch Lake may be one such habitat (Cable 2011).

Fig. 4.1. The Pitch Lake, Trinidad. Insert: a close up on one of the pools where guppies were collected for this study.

The Pitch Lake (Fig. 4.1) consists of a natural upwelling of oils that contain hydrocarbons, sulphur, metals and volcanic ash at concentrations usually considered toxic to living organisms (Peckham 1895, Ponnamperuna and Pering 1967, Burgess et al. 2005, World Health Organisation Concise International Chemical Assessment Document 2005, Marković et al. 2007). It is the largest asphalt lake in the world (ca. 0.8 km^2), resembling a volcano crater, located on the south west coast of Trinidad. Only four other asphalt lakes exist worldwide: the La Brea tar pits, the McKittrick tar pits, Carpinteria tar pits (all three California, USA) and Lake Guanoco (Venezuela). Although such lakes have been the focus of many paleoecological studies (e.g. Coltrain et al. 2004), little is published on their extant biodiversity (Kadavy et al. 1999, Ali et al. 2006, Schulze-Makuch et al. 2010) or on the evolution of species in this habitat (but see Tezuka et al. 2011). Despite its hostile environment, freshwater pools amongst the pitch folds of the encrusted Pitch Lake surface are a habitat for plants (e.g. *Nymphaea* and *Nitella*, personal observations), bacteria (e.g. *Pseudomonas*, see Ali et al. 2006,

Schulze-Makuch et al. 2010), invertebrates (e.g. dragon fly larvae, aquatic beetles, personal observations), the amphibian *Pseudis paradoxa* (personal observations) and fish (e.g. *Poecilia reticulata*, *Rivulus hartii*, see Burgess et al. 2005, and *Polycentrus schomburgkii*, personal observations).

The guppy (*P. reticulata*), a small, hardy freshwater fish, is found throughout Trinidad and Tobago as well as in Venezuela, Guyana and Surinam (Magurran 2005). Throughout their natural habitat, guppies are parasitized by the ectoparasitic, hermaphroditic gyrodactylids (for exceptions, see Martin and Johnson 2007, Fraser and Neff 2010). Despite being helminths, *Gyrodactylus* species are regarded as microparasites (Scott and Anderson 1984) due to their multi-modal, hyperviviparous reproductive system (Cable and Harris 2002, Bakke et al. 2007). In natural populations of guppies, gyrodactylids often occur at prevalences of up to 75 % (van Oosterhout et al. 2007). Such high parasite occurrence is mainly characteristic of lowland guppy populations, whereas the Trinidadian upland populations are occasionally free of gyrodactylids (Martin and Johnson 2007, Fraser and Neff 2010). The difference in gyrodactylid load may be explained by the fact that upland host populations are relatively small in census population size and have arisen from founder events, whilst being connected by a very limited amount of upstream migration (Barson et al. 2009). Furthermore, given that parasitized guppies are more likely to be flushed downstream (van Oosterhout et al. 2007) some upland populations appear to be parasite-free, either due to failure of parasite establishment or subsequent parasite population extinction. Hence, the guppies in some upland populations such as the Upper Naranjo have been shown to be devoid of gyrodactylids for almost a decade (personal observations) and can be regarded as naive to these parasites.

In the present study, we compared parasite burdens in the field with published data and assess the importance of the pitch environment for the health of guppies using experimentally infected fish. In Part 1, parasite infections are assessed for the Pitch Lake guppy population. Having confirmed the absence of *Gyrodactylus* spp. in the Pitch Lake, we hypothesized that Pitch Lake water had significant anti-parasitic properties. Therefore, in Part 2, we experimentally investigated the effect of Pitch Lake water on fungal and/or bacterial (from hereon: microbial) and helminth infections in guppies. Firstly, we exposed Pitch Lake guppies to aquarium water and *G. bullatarudis* infected fish to Pitch Lake water. Secondly, we exposed *G. turnbulli* infected, ornamental guppies directly to dechlorinated aquarium water containing solid pitch or to artificially produced pitch water of varying ages. By producing pitch water of varying ages, we also tested whether the antihelminthic compounds are chemically unstable or volatile to provide an overview of the anti-parasitic properties of Pitch Lake water.

4.3 Material and methods

This study was approved by the Cardiff University Animal Ethics Committee and regulated by a UK Home Office licence (PPL 30/2357).

4.3.1 Sampling sites

Guppies (SL: 9.1-22.4 mm) were collected from the Pitch Lake (grid reference UTM 20P: 650341.45 E, 1131668.93 N; Fig. 4.1) in 2004, and 2006-2008, and from the Upper Naranjo in 2008. Pitch Lake pools in which fish were caught varied widely in their water physio-chemistry (pH, temperature, salinity, dissolved oxygen and conductivity measured with an YSI pH 100 and YSI 85 Multi Probe) and differ from three riverine reference sampling sites (Table 4.1).

Table 4.1. Minimum and maximum water physio-chemistry values of the Pitch Lake and three riverine sites in Trinidad recorded in different seasons between 2003 and 2012.

	Pitch Lake	**Upper Naranjo**	**Mid Naranjo**	**Lower Aripo**
pH	2.8-8.4	7.6-9.35	7.4-7.6	7.1-8.8
Temperature °C	26.1-32.5	23.1-25.4	23.0-23.4	23.7-25.9
Salinity g/L	0.1-0.7	0.1-0.2	0.1	0.0-0.2
Conductivity µS	404-1649	120-402.5	210-235	232-328
Dissolved Oxygen mg/L	0.7-2.32	5.42-7.8	5.15-8.99	2.1-8.77

For the parasite prevalence analysis on Pitch Lake guppies (Part 1), variable numbers of live fish (n) collected in randomly chosen pools in February 2004 (n = 64), June 2006 (n = 54) and June 2007 (n = 25) were euthanized on site with an overdose (0.04 %) of MS222 and individually stored in 1.5 mL Eppendorfs containing 90% ethanol. In total, five representative pools of varying sizes were sampled, all of which contained guppies and which were spread across the lake. For Transfer Experiment 1 (Part 2), pitch water and live guppies were collected at the Pitch Lake in June 2008 (n = 10) and transported to the laboratory in Trinidad. The guppies originated from different pools that were all interconnected. A control sample of guppies (n = 10) for Experiment 1 plus 20 guppies for Experiment 2 (Part 2) were collected from the Upper Naranjo (692498.44 E, 118257.53 N), a tributary of the Aripo River that originates in the Northern mountain range of Trinidad and flows into the Caroni drainage.

4.3.2 Part 1: Natural parasite fauna

For the parasite analysis of Pitch Lake guppies, samples from 2004, 2006 and 2007 were analysed for gyrodactylid prevalence, mean intensity and range. The preserved fish were transferred to a Petri dish and completely immersed in ethanol. Using a dissecting microscope the surface of the fish was

scanned and any parasites recorded. The ethanol in which the fish had been originally fixed was also screened for any dislodged parasites. Gyrodactylid parasites were removed from their host using insect pins and transferred onto a microscope slide. Specimens were mounted in ammonium picrate glycerine after partial digestion (Harris et al. 1999) and examined for differences in haptor morphology to identify worms to species level.

4.3.3 Part 2: Experimental transfer

To monitor gyrodactylid infections in Experiments 1 and 2 guppies were anaesthetized with 0.02 % MS222 and their sex and standard length were recorded. Fish were screened for external parasites using a fibre optic light and a stereo-microscope according to the methods described in Chapter 1. Fish were maintained individually in 1 L pots throughout the duration of the experiment and were fed on Aquarian® fish flakes once daily. Additionally, uninfected fish were exposed to the same treatments in both experiments as mortality controls. For routine maintenance, fish and parasite cultures were kept at 25 ± 1 °C, a 12 h light : 12 h dark cycle and guppies were fed at least once daily with Aquarian® fish flakes.

In Experiment 1, we examined (a) the effect of replacing Pitch Lake water with dechlorinated aquarium water on the health of Pitch Lake guppies, and (b) the effect of original Pitch Lake water on the mean intensity of *G. bullatarudis* parasites (isolated from Lower Aripo guppies; Cable and van Oosterhout 2007) on guppies originating from the Upper Naranjo. To test the former, ten guppies from the Pitch Lake (with no gyrodactylid infection) were removed from the original Pitch Lake water and kept in aquarium water and maintained individually over 8 days. After 4 days of acclimatisation, fish were screened for signs of microbial infection daily until the experiment finished on Day 8. Controls originating from the Pitch Lake (n = 10) were maintained for the same period in the original pitch water. To examine the effect of Pitch Lake water on the presence of gyrodactylid parasites, wild, naive guppies from the Upper Naranjo were exposed to infected conspecifics from the Lower Aripo to acquire *G. bullatarudis* infections naturally (mean ± standard error of the mean: 11.9 ± 1 parasites per fish). Infected fish (n = 10) were then exposed to 25 % (v/v) original pitch water collected at the Pitch Lake and diluted with dechlorinated aquarium water. After 48 h, the Pitch Lake water concentration was raised to 50 % and maintained at this concentration until the end of the experiment. The stepped increase in Pitch Lake water concentration allowed the fish to acclimatize and prevent osmotic problems. Uninfected and infected control guppies (n = 10 each) were maintained in dechlorinated water. For Experiment 1, pitch water was used within 24 h of collection and trials were carried out at 21 ± 2 ºC.

Experiment 2 tested the efficacy of pitch and pitch water preparations against *G. turnbulli* (*Gt3*, isolated from pet shop guppies in 1997, see van Oosterhout et al. 2003) infections on ornamental guppies (total n = 84), acquired through exposure to infected conspecifics. Parasite loads on guppies were assessed by screening fish before and after the 8 day treatment period. The Pitch Lake water and pitch were collected in June 2009. In total, there were five treatments: (a) solid pitch, (b) fresh pitch water, (c) old pitch water, (d) ancient pitch water, and (e) aquarium water as a control. Pitch water was prepared from solid pitch and dechlorinated aquarium water 1-3 months after the field collection of pitch. Pitch was added to the bottom of a 1 L pot containing 250 mL dechlorinated water for the pitch treatment (32.5 g was regarded as safe for guppies after preliminary trials, and was moulded to a thickness of 0.5 cm to increase surface area: approx. 173 cm^2). For the three pitch water treatments, the pitch was removed from water after 24 h. Fresh pitch water was used directly after pitch removal, whereas old and ancient pitch water were left to stand for 41 and 82 days, respectively, at 15-25 ºC. These aged pitch water preparations resulted in the loss of unstable and volatile compounds narrowing down the possible chemical characteristics of the active compound before starting spectroscopic analyses, which were used to confirm the presence of volatile and/or unstable compounds. To identify any complex organic species which may be present in pitch and responsible for the observed anti-parasitic properties, proton and carbon Nuclear Magnetic Resonance (NMR) spectra were measured (Bruker Avance, 250, recording at ^{1}H 250 MHz; 62.5 MHz ^{13}C, 5 mm tube) on extracts of pitch samples, using deuterium oxide (heavy water, D_2O) and deuterated chloroform ($CDCl_3$) as extractants. Additionally, Infra Red (IR) spectra (Jasco 380, thin film) were analysed to identify organic functional groups.

Since not all fish were tested at the same time, the solid pitch treatment method was used in two small experiments before and after the main study to confirm that pitch efficacy against gyrodactylids remained constant throughout the experimental period despite the storage of pitch in plastic containers between individual sets of tests. Naive fish from a 10 year old inbred stock were infected by exposing individual fish to an infected donor for 3-4 days. For every set of tests, near equal number of replicates (15 ± 3 per treatment) were tested at 25 ± 1 ºC, with fish containers randomly placed within the experimental laboratory space including 27 control treatments.

4.3.4 Statistical analyses

Differences in the frequency of microbial infections and mortalities in Pitch Lake guppies exposed to aquarium water compared to their counterparts in Pitch Lake water (Experiment 1) were confirmed using a Fisher's Exact Test. The infection trajectories for Experiment 2 were assessed using a General Linear Mixed Model (GLMM). Treatment (pitch or aquarium water) and time were included in the model as independent variables with animal ID as random effect factor.

As a measure of efficacy of solid pitch and artificial pitch water treatments in Experiment 2 the relative decrease in parasite load (ΔE_t) was calculated to account for the initial variation in parasite loads between fish as well as the repeated measurement of parasite load before and after treatment on the same individual:

$\Delta E_t = (L_0 - L_t) / L_0$, for $L_t < L_0$, and

$\Delta E_t = 0$ for $L_t \geq L_0$,

where L_0 is the initial parasite burden at Day 0 and L_t is the parasite burden at Day t. When parasite load increased rather than decreased, the treatment was not efficient and was defined as $\Delta E_t = 0$.

A Generalized Linear Model (GLM) fitted with a quasibinomial error distribution and a logit link function was conducted to assess differences in efficacy with treatment, fish sex and standard length as independent variables plus a fish sex*standard length interaction. Standardized residuals for all models were assessed using a Shapiro-Wilk test additionally to visual examination of histograms and normality plots, and both random and fixed model terms were reduced using stepwise deletions based on the Log-Likelihood method with $\alpha = 0.05$. All data were analysed in R v. 2.9.2 (R Development Core Team 2011).

4.4 Results

4.4.1 Part 1: Natural parasite fauna

No helminth infections were found on the guppies sampled from the Pitch Lake population (n = 143), with the exception of three worms recovered on three individual fish in 2004 belonging to a newly described genus of the Gyrodactylidae, *Ieredactylus rivulus* (Appendix I), a parasite usually found on *Rivulus hartii*.

4.4.2 Part 2: Experimental transfer

Exposure of Pitch Lake guppies to aquarium water led to a 70 % increase in unspecified microbial infections. As a result, seven out of ten Pitch Lake fish were infected or died from the infection in aquarium water, compared to one out of ten in the control with original Pitch Lake water (Fisher's Exact Test: p = 0.01). The symptoms were consistent with fin rot and cotton-like growth characteristic of opportunistic *Saprolegnia* and *Flavobacterium* spp. infections. No further analyses were conducted to identify the specific infections.

When experimentally infected guppies from the Upper Naranjo were exposed to original Pitch Lake water they lost all their gyrodactylid parasites within 48 h of exposure, whereas the parasite

population on fish in aquarium water remained stable (GLMM: treatment t = 4.017, df = 18, p < 0.001; time t = -5.080, df = 71, p < 0.001; Fig. 4.2).

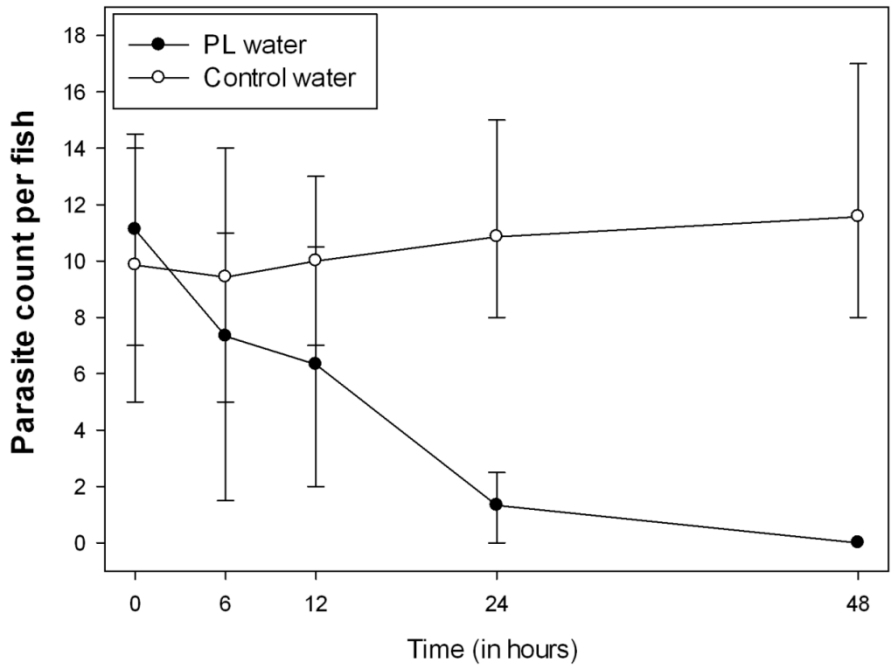

Fig. 4.2. *Gyrodactylus bullatarudis* parasite load (± standard error of the mean) on guppies (*Poecilia reticulata*) originating from the Upper Naranjo river exposed to original Pitch Lake (PL) water diluted to 25 % up to 12 h and 50 % between 12 and 48 h (closed circles) or de-chlorinated aquarium water (open circles) over time. N = 10 for each treatment.

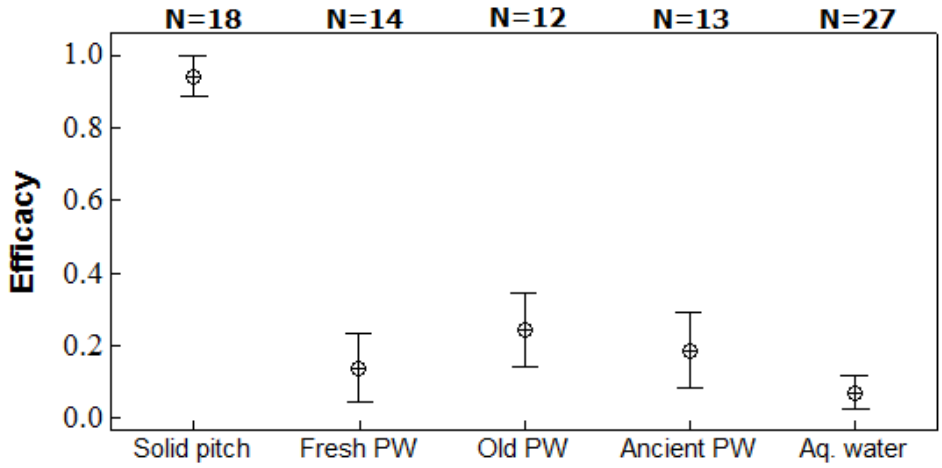

Fig. 4.3. Mean efficacy (± standard error of the mean) of solid pitch, fresh pitch water (PW), old pitch water, ancient pitch water and aquarium (aq.) water against *Gyrodactylus turnbulli* infections in ornamental strain guppies (*Poecilia reticulata*). Efficacy ($\Delta E_t = (L_0 - L_t) / L_0$, for $L_t < L_0$, and $\Delta E_t = 0$ for $L_t \geq L_0$): 1 = effective; 0 = not effective. N = number of replicates.

Table 4.2. Position of chemical bands (in parts per million, ppm), their associated peak shape and relative intensity compared to the internal standard as well as their suggested assignments based on the molecule's functional group from spectroscopic analysis (proton and carbon nuclear magnetic resonance: ^{1}H and ^{13}C NMR, respectively; infrared: IR) of pitch.

Position of Band (ppm)	Peak shape	Relative intensity	Chemical compound *(Suggested assignment to likely pitch components)*
^{1}H NMR			
-0.9-0.8	Broad	Medium	Methane *(trapped methane gas)*
0.5-1.5	Broad, featureless	Very strong	Hydrocarbon methylene *(simple hydrocarbons, hydrocarbon side-chains of substituted aromatics)*
2.0-2.5	Broad, multiple maxima	Weak	Methyl / methylene α to aryl, vinyl, carbonyls. *(methyl and polymethyl aromatics and polyaromatic hydrocarbons, unsaturated hydrocarbons, ketonic hydrocarbons, carboxylic acids)*
6.6-7.9	Broad, multiple maxima	Weak	Aromatic / heteroaromatic *(benzene, poly aromatic hydrocarbons, sulphur, oxygen and nitrogen heterocycles)*
10.5 (centre)	Very broad	Weak	Phenol hydroxyl / carboxylic acid *(phenols, cresols and phenolic heterocycles, long-chain carboxylic acids)*
^{13}C NMR			
29.7	Broad	Strong	Hydrocarbon methylene *(simple hydrocarbons)*
-4 (centre)	Very broad	Weak	Methane *(trapped methane gas)*
IR			
3696	Sharp	Weak	'Free' (non-H-bonded) O-H *(Phenols, phenolic heterocycles)*
3619	Sharp	Weak	'Free' (non-H-bonded) O-H *(Phenols, phenolic heterocycles)*
3403	Broad	Weak	Carboxylic O-H *(long chain carboxylic acids)*
2925	Broad	Very strong	Hydrocarbon C-H *(simple hydrocarbons, hydrocarbon side-chains of substituted aromatics)*
2855	Broad	Very strong	Hydrocarbon C-H *(simple hydrocarbons, hydrocarbon side-chains of substituted aromatics)*
1707	Sharp	Medium	Ketone *(aliphatic ketones in long chain simple hydrocarbon)*
1607	Broad	Strong	Aromatic C-C *(benzene, poly aromatic hydrocarbons, sulphur, oxygen and nitrogen heterocycles)*
1457	Broad	Medium	Hydrocarbon C-C *(simple hydrocarbons, hydrocarbon side-chains of substituted aromatics)*
1376	Broad	Medium	Hydrocarbon C-C *(simple hydrocarbons, hydrocarbon side-chains of substituted aromatics)*

In Experiment 2, the only treatment that was effective in removing parasites was the solid pitch in water treatment (94.4 % efficacy), whereas the efficacies of fresh (14.1 %), old (24.7 %) and ancient (19 %) pitch water treatments were not significantly different from the control (7.3 %) (GLM: treatment $F_{4,74} = 14.594$, $p < 0.001$; Fig. 4.3). Efficacy was also influenced by a fish sex*standard length interaction: efficacy increased with decreasing size in adult fish, but is constant in juveniles (GLM: fish sex*standard length $F_{2,74} = 3.966$, $p = 0.023$).

Spectroscopic analysis (proton ^{1}H and carbon ^{13}C Nuclear Magnetic Resonance, Infrared) indicated that while the majority of the pitch consists of simple hydrocarbons which did not transfer to the water phase, there were signals indicative of the presence of aromatic, phenolic and ketonic species, and trapped methane gas (Table 4.2). These were only observed following extraction into organic solvent ($CDCl_3$) with the levels of any species extracted into D_2O below the detection limits of the techniques by attempting to mimic the pitch water experiments.

4.5 Discussion

The water in the Pitch Lake in Trinidad is dominated by simple linear and branched hydrocarbons, and displays extreme chemical properties. Yet, it supports three species of freshwater fish (*Poecilia reticulata*, *Rivulus hartii* and *Polycentrus schomburgkii*). We find Pitch Lake guppies are not naturally infected with *Gyrodactylus* species (Part 1) and that gyrodactylid-infected control guppies lose their infections when transferred to Pitch Lake water (Part 2). Furthermore, Pitch Lake fish that are removed from Pitch Lake water rapidly develop microbial infections (Part 2).

Gyrodactylus species are highly prevalent in natural populations of guppies (Martin and Johnson 2007, van Oosterhout et al. 2007, Fraser and Neff 2010) making the observation of their absence in the Pitch Lake population unusual, particularly since hydrocarbons have been associated with an increase of gyrodactylids on infected fish (Kahn and Kiceniuk 1988, Khan 1990). In addition, McMullan (2010) did not detect any internal macroparasites in a sample of 25 guppies from Pitch Lake. A previously unknown monogenean genus, *Ieredactylus*, was, however, found which only occurs on guppies in the Pitch Lake (Appendix I). *Ieredactylus rivulus* normally infects Hart's Rivulus, a fish that can migrate over land; hence, the parasite is thought to be hardy and robust (Appendix I).

The extreme conditions in the Pitch Lake habitat may act as a defence for guppies against gyrodactylid and microbial infections originating from outside the Pitch Lake region. The isolation and genetic divergence of Pitch Lake guppies (Willing et al. 2010) indicates that this asphalt lake may act as a refuge, similar to the limestone caves in Mexico which are thought to be a refuge from

parasites for Atlantic mollies (*P. mexicana*, see Tobler et al. 2007). Such low parasite prevalence within guppy populations elsewhere in Trinidad has only been observed in small, relatively isolated upland populations (Martin and Johnson 2007, Fraser and Neff 2010). Possibly, the physical isolation in combination with the small population size could also have contributed to the lack of ectoparasites on the Pitch Lake guppies. On top of this, colonisation of the Pitch Lake by gyrodactylids will be severely hindered by the extreme environmental conditions that appear to be lethal, at least to the *Gt3* strain of these parasites, either through direct exposure to pitch components and/ or indirectly through increased host mucus production causing the parasites to be sloughed off (for review on fish mucus, see Shephard 1994). Ideally, we would have tested whether Pitch Lake guppies were susceptible to infection with *Gyrodactylus* spp., but as Pitch Lake fish die in normal aquarium water and gyrodactylids cannot survive in pitch water this was not possible.

In the Pitch Lake, guppy evolution appears to be primarily driven by the harsh environmental conditions. Although some dead guppies were observed in the pools with extreme pH (2.8 and 8.4; cf. Dunson et al. 1977), Pitch Lake guppies are adapted to this environment. Studies on the Major Histocompatibility Complex (MHC), a multigene family associated with immune functioning in vertebrates, show that the MHC polymorphism is low but remarkably diverged in the Pitch Lake guppy population compared to other Trinidadian guppy populations (McMullan 2010). A low MHC variation could account for the increased pathogen susceptibility of these fish when in aquarium water (six out of seven guppies were infected or dead at the end of the experiment), as could the multitude of mutagenic and teratogenic hydrocarbons in Pitch Lake water that are known to suppress the immune system (World Health Organisation Concise International Chemical Assessment Document 2005). We cannot rule out though that potential stress associated with changes in the water physio-chemistry caused chronic endemic infection in the guppies to become acute or opportunistic pathogens in the water to infect fish. An argument against this is that to maintain Pitch Lake guppies in the laboratory in dechlorinated aquarium water, they need to be continuously treated with anti-fungal and anti-bacterial medication (personal observations).

Exposing gyrodactylid infected fish to ‘original’ Pitch Lake water and solid pitch showed high anti-parasitic characteristics of the treatments, but artificial pitch water preparations were not effective in reducing ectoparasite burdens in guppies. This suggests that the anti-parasitic agent(s) within the Pitch Lake are volatile or unstable compound(s), supported here by spectroscopic analyses. Hence, the anti-parasitic pitch properties are probably not associated with simple hydrocarbon toxicity. It seems more likely that an unidentified chemical entity of high specific toxicity is transferred to the water at a concentration below Nuclear Magnetic Resonance detection limits, which is either unstable in water / air or is of low enough molecular mass that it is volatile and lost from the water (Pan and

Raftery 2007). The spectroscopic techniques used in the current study are mostly insensitive to simple inorganic ions, such as sulphides or selenides (Lawrence et al. 2000). Inorganic compounds such as sulphides or selenides are, however, stable ions, so they can be disregarded as potential anti-parasitic compounds due to its apparent instability or volatility (Lawrence et al. 2000).

The fast degradation of the active compound against parasites might explain the high efficacy of original pitch water and solid pitch against gyrodactylids: original pitch water in the lake is constantly enriched with soluble pitch compounds, so concentrations of unstable/volatile chemicals are likely to reach high levels. Indeed, the dissolved pitch compounds accumulate to a harmful concentration which required dilution for non-Pitch Lake guppies used in Experiment 1 (Part 2). The low efficacy of artificial pitch water preparations implies that water cycling in the Lake, as a consequence of the continual upwelling of fresh pitch (Peckham 1895, Ponnamperuma and Pering 1967), is essential for the enrichment of original pitch water to give it its anti-parasitic properties.

The discovery of a habitat with an apparently low parasite fauna such as the Pitch Lake opens up interesting possibilities for research on host-parasite coevolution and immunogenetics. By relaxing the parasite selection pressures, the adaptive evolution of the MHC genes is predicted to differ from that in other parasite-rich environments, although confounding effects of the environment (including mutagenic effects and immune-suppressing effects) need to be taken into consideration.

In conclusion, local water chemistry is essential for the survival of Pitch Lake guppies since these fish appear to depend heavily on its anti-parasitic properties. Volatile or instable compounds derived from pitch might be protecting these guppies from gyrodactylid and microbial infections, thus parasite-mediated selection may be relatively relaxed in the Pitch Lake environment. Further detailed analyses on changes in microbial community of pathogens with the corresponding changes in the direction of the evolutionary arms race in host immune genes are warranted to better understand the consequences of oil pollution on the macro-evolution of vertebrates.

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CHAPTER 5: THE SALT MYTH REVEALED: TREATMENT OF GYRODACTYLID INFECTIONS ON ORNAMENTAL GUPPIES, *POECILIA RETICULATA**

5.1 Abstract

Salt is commonly recommended as an inexpensive treatment against many fish parasites in freshwater fish culture; however, few studies have scientifically evaluated its efficacy. Amongst the monogeneans, salt has only been previously tested against *Gyrodactylus salaris* infecting Atlantic salmon (*Salmo salar*) and *G. derjavini* infecting rainbow trout (*Oncorhynchus mykiss*). Here we tested the efficacy of salt treatments against *G. bullatarudis* and *G. turnbulli* on guppies (*Poecilia reticulata*), both commercially important pathogens in the ornamental fish industry. *In vitro* survival of both parasites was negatively correlated with increasing salinities of 3, 5, 7 and 33 g/L. Parasite establishment on guppies maintained at 0, 3 and 7 g/L salinity decreased drastically for *G. turnbulli* from 94 % in the control to 78 and 0 % on fish in 3 and 7 g/L salinity, respectively. *G. bullatarudis* establishment was still 100 % at 3 g/L salinity but was reduced to 73 % in 7 g/L. Throughout an infection, parasite populations of both species increased faster on guppies in 3 g/L salinity compared to dechlorinated water, whereas population growth was severely affected at 7 g/L salinity. Overall a short duration, high concentration salt bath was most effective at treating gyrodactylid infections: 15 min exposure to 25 g/L salinity for adults or 5 min for juvenile fish removed 100% of *G. turnbulli* or 72 % of *G. bullatarudis*. The results reflect the generalist characteristics of the more tolerant *G. bullatarudis* compared to *G. turnbulli*, but have wider implications for treatment application: clearly one treatment regime does not suit all even within a genus.

5.2 Introduction

In freshwater aquaculture, salt is used against a range of pathogens from protozoans to helminths (Lio-Po and Lim 2002). It is less harmful to fish hosts compared to more traditional anti-parasitic treatments, such as formalin or malachite green (reviews by Srivastava et al. 2004, Chapter 1.6) and its low cost and availability make it the recommended treatment against a variety of fish diseases in ornamental fish keeping (e.g. www.fishdoc.com, www.fishkeeping.co.uk). Exposing freshwater organisms to saline conditions disrupts their osmoregulation, resulting in water loss and dehydration (Shephard 1994). Ectoparasites or free-living parasitic stages are more severely and rapidly affected by such disruption in osmoregulation compared to their fish hosts due to the parasite's increased surface area to volume ratio. Nevertheless, despite its wide practical use there have been very few empirical studies to test the efficacy of saline conditions on fish pathogens and most have focussed on *Flavobacterium columnare*, *Ichthyophthirius multifiliis* and *Saprolegnia* spp. (see Table 5.1). These studies indicate that the effectiveness of treatment depends strongly on the fish host, parasite strain and species, application scheme, temperature and salt concentration. For instance, salt is most

*Adapted from: Schelkle B, Doetjes R, Cable J (2010) Aquaculture 311: 74-79.

effective against White Spot disease when applied at a low dose over several days since it acts against the free-living theront stage (Tiemann and Goodwin 2001, Garcia et al. 2007, Mifsud and Rowland 2008). Continuous exposure of White Spot infected fish to saline conditions also ensures that theronts emerging at different times from encysted tomonts are killed, reducing the chances of re-infection (Mifsud and Rowland 2008).

For helminth ectoparasites salt baths are often the most practicable since a high dose, short duration treatment acts aggressively against the parasites. Salt not only causes direct osmotic problems to the parasites, but also strips the fish of its protective mucus layer which to a certain extent buffers the parasite from the external environment (reviewed by Burka et al. 1997, Bakke et al. 2007). Additionally, short duration chemical treatments forego the problem of increased mucus production in the fish host, a physiological response triggered in response to adverse environmental conditions (reviewed by Shephard 1994). Salt has only been tested against two helminth species (Table 5.1), both from the economically and ecologically important gyrodactylids. Soleng and Bakke (1997) and Soleng et al. (1998) focussed on salt treatments in the Atlantic salmon (*Salmo salar*)–*Gyrodactylus salaris* host–parasite system, whereas Buchmann (1997) investigated salinity against *G. derjavini* infecting rainbow trout (*Oncorhynchus mykiss*). Treatments were 0 to 100 % effective with concentration- and/or time-dependent effects apparent in all studies. However, both Atlantic salmon and rainbow trout are diadromous fish, migrating between marine and freshwater habitats. Salt treatment against helminths infecting purely freshwater fish has not been previously tested.

The guppy is a small, tropical freshwater fish that originates from Trinidad, and Central and South America and is popular as an aquarium fish because of its elaborate colours and ease of maintenance (Magurran 2005). Guppies are natural hosts to *G. bullatarudis* and *G. turnbulli* (see Harris and Lyles 1992), which under the confined conditions and the associated stress for fish in the aquarium industry find ideal conditions for increased transmission and population growth. Additionally, global fish transport may enable the parasites to encounter and infect alternative or reservoir hosts, which they usually would not come into contact with in their natural habitat, resulting in host switches (see King and Cable 2007, King et al. 2009). Gyrodactylids are difficult to control and existing treatments are associated with low efficacy, toxicity to the host, human health concerns and difficulties in application (Chapter 1.6). The old adage that ‘prevention is better than control’ remains, but once a disease outbreak occurs treatment is necessary to avoid economic losses and prevent animal suffering (Ashley 2007). This can be achieved by chemical control measures which may keep parasite prevalence in ornamental fish populations low and disease epidemics at a minimum.

Table 5.1. Efficacy of salt against freshwater fish diseases.

Parasite	Fish host	Treatment dose	Application	Efficacy	Reference
1. Bacteria					
Flavobacterium columnare	*Ictalurus punctatus* *Carassius auratus* *Morone saxatilis* *Acipenser oxyrinchus desotoi*	0, 1, 3 & 9 ‰	Continuous for 4 (*I. punctatus*, *C. auratus*) or 10 weeks (*M. saxatilis*, *A. oxyrinchus desotoi*).	Reduced mortalities in 1 ‰ salinity, no columnaris disease in 3 and 9 ‰ salinity.	Altinok and Grizzle 2001
F. columnare	*Oncorhynchus mykiss*	80 g/L	30 s.		Austin and Austin 1993 (cited in Altinok and Grizzle 2001)
F. columnare	*Oncorhynchus mykiss*	20 & 40 ‰	25 ºC. 20 ‰: one off 30 min salt bath. 40 ‰: 15min bath on day 1, 5 min baths on days 3 & 5.	No reduction in cumulative mortality.	Suomalainen et al. 2005
F. columnare		0.5, 1, 2, 3 & 4 %	*In vitro:* incubated in *Cytophaga* broth.	Effective at ≥ 1%.	Pacha and Porter 1968
F. columnare		5,10, 20 & 30 ‰	*In vitro* in Anacker & Ordal broth or agar, or in trypticase soy agar.	No growth at ≥ 10‰.	Bernardet 1989
Streptococcus isolates	*Oreochromis niloticus*	0, 15, 30 ‰	25 & 30 ºC.	Increase in fish mortalities with elevated temperature & salinities due to increased pathogen susceptibility.	Chang and Plumb 1996
F. psychrophilum and undefined bacteria and fungi	*Oncorhynchus mykiss* eggs	0.03 mg/L	*In vitro* incubated at 15 ºC on Ordahl's agar or trypticase soy agar (TSA).	Fewer bacterial colonies on enriched Ordahl's agar, but not on trypticase soy agar (TSA).	Wagner et al. 2008
2. Heterokonts					
Saprolegnia sp.	*Ictalurus punctatus*	1-5 g/L	Prolonged immersion.	Fungal growth inhibited at 5 g/L	Li et al. 1996 (cited in Altinok & Grizzle 2001)
Saprolegnia sp.	*Oncorhynchus tshawytscha* eggs	15000 ppm	Daily 15 min treatments.	Ineffective.	Waterstrat and Marking 1995
Saprolegnia sp.	*Oncorhynchus mykiss* eggs	30 g/L	15 min every other day until hatch.	Improved fry hatch, but did not prevent fungal growth (less effective than hydrogen peroxide and formalin).	Schreier et al. 1996

Saprolegnia parasitica	*Bidyanus bidyanus*	0, 2, 3 g/L	18 d. Continuous exposure. Lab. study.	At 2 and 3 g/L: no infection after harvest and 100 % survival.	Mifsud and Rowland 2008
Saprolegnia sp.	*Cyprinus carpio*	35 g/L	Flush treatment, twice daily.	No infections on eggs, increased hatching rate.	Khodabandeh and Abtahi 1995
3. Protozoa					
Cryptobia salmositica	*Onchorhynchus nerka*	Slow (6-8 d) or fast (1-2 d) change to 33 ‰ salinity	9 & 13 ºC.	No effect.	Bower and Margolis 1985
Ichthyophthirius multifiliis	*Bidyanus bidyanus*	0, 1, 2, 3 g/L	16 d. Continuous exposure. Lab. study.	At 2 and 3 g/L: theront & trophont free by D8 (17.3-21.3 ºC) and D6 (19.2-23.5 ºC).	Mifsud and Rowland 2008
I. multifiliis	*Rhamdia queen* fingerlings	4 g/L	30 d. Continuous exposure. Lab. study.	Reduces cumulative mortality after 4 d from 100 to 54 %.	Garcia et al. 2007
I. multifiliis	*Rhamdia queen* fingerlings	Oral : 0, 1.2, 2.5, 5, 6 %	30 d. Fed once daily. Lab. study.	Not effective.	Garcia et al. 2007
I. multifiliis	*In vitro*: trophonts	15-30 g/L	10, 15, 20 h. 20 ± 1 ºC.	Effective at ≥ 20 g/L	Lahnsteiner and Weismann 2007
I. multifiliis	*Oncorhynchus mykiss*	20 g/L.	18 ± 1 ºC. 5 treatments every 24 h.	Significant reduction of infestation level.	Lahnsteiner and Weismann 2007
I. multifiliis	*Cyprinus carpio*	20 g/L.	18 ± 1 ºC. 5 treatments every 24 h.	Salt concentration lethal to carp.	Lahnsteiner and Weismann 2007
I. multifiliis	*Bidyanus bidyanus, Macquaria ambigua, Maccullochella peeli, Tandanus tandanus*	5 g/L	19-26 °C or 11-18 °C. Continuous treatment.	Effective at D7 (19-26 °C) and at D14 (11-18 °C).	Selosse and Rowland 1990
I. multifiliis	*Rhamdia quelen*	0, 1, 2, 4 g/L	23 d. Continuous treatment.	2 and 4 g/L increased survival.	Miron et al. 2003
I. multifiliis	*Rhamdia quelen*	4 g/L	45 d. Continuous treatment.	100 % fish survival. Significant loss of white spots.	Miron et al. 2003
I. multifiliis	*Ictalurus punctatus*	1, 2, 3, 4, 5, 6 & 9 mg/L	22.5-27 ºC. 10 d. Continuous treatment.	Effective at 1-9 mg/L.	Allen and Avault 1970
I. multifiliis	*Ictalurus punctatus*	3 g/L	18-22 ºC. 20 d. Daily treatment after water changes.	Not effective.	Tieman and Goodwin 2001
I. multifiliis (Nordic strain)	*Oncorhynchus mykiss*	0, 3, 5, 7.5, 10, 15, 20 g/L	11.6ºC. Continuous treatment.	Theronts released at 5 g/L, but development time was doubled. At higher conc. life cycle inhibited.	Aihua and Buchmann 2001

I. multifiliis		10 g/L		Theronts still released in a German strain.	Wagner 1960 (cited in Altinok and Grizzle 2001)
Tetrahymena	*Poecilia reticulata*	0.5 %	Bath treatment plus Chinese herb mix diet.	Effective.	Ponpornpisit et al., 2000
Trichodina spp.		1-2 ‰.	1-2 d.Recommendation.		Lom 1995 (cited in Lio-Po and Lim 2001)
4. Helminths					
Gyrodactylus derjavini	*Oncorhynchus mykiss*	5-20 ‰.	11°C. 4d. Lab study.	No effect (5-7 ‰) to 100 % effective (> 7 ‰). Causes mortality in some or all hosts. Sub-sampling.	Buchmann 1997
G. salaris	fins of 6 *Salmo salar*: 33‰; 12 fish/treatment 5-20‰ salinity	5, 7.5, 10, 15, 20 & 33 ‰	1.4, 6 & 12 °C. Continuous exposure. Lab study.	No effect (5.0 ‰) to 100 % effective after a few minutes (33 ‰).	Soleng and Bakke 1997
G. salaris	*Salmo salar* (15 fish/treatment)	33 ‰	12 °C. 5, 15, 30 & 60 min. Lab study.	Time dependent effect: no effect (5 min) to 100 % effective (60 min).	Soleng et al. 1998
5. Crustaceans					
Argulus coregoni eggs		50 g/L	Bath.	Not effective.	Hakalahti-Sirén et al. 2008

In this study we tested various salt concentrations on the *in vitro* survival of *G. turnbulli* and *G. bullatarudis*. Further, the establishment of both parasite species on guppies maintained in saline waters of 3 and 7 g/L salinity was investigated and the efficacy of salt bath treatments of 15 and 25 g/L salinity was tested on guppies infected either with *G. turnbulli* or *G. bullatarudis*. The salt concentrations chosen are within the range of typical recommendations for freshwater aquarium owners for disease treatment (1–7 g/L continuous exposure for 1–2 days and 15–30 g/L for short duration salt baths) according to a variety of forums and information on websites for freshwater aquarists.

5.3 Materials and methods

5.3.1 Source of animals and compounds, and screening methods

Guppies (*Poecilia reticulata*) originating from a mixed pet shop stock were used and fed daily on Aquarian® (API) fish flakes and at least twice weekly with live *Daphnia* or frozen *Tubifex*. All fish were maintained under a 12 h light : 12 h dark cycle, at 25 ± 1 °C. For the experiments, fish were screened for parasites at regular intervals using 0.02 % MS222 and a cold light (see Chapter 1.6). Highly infected fish from *Gyrodactylus turnbulli* and *G. bullatarudis* laboratory cultures with no chance of survival were euthanized by a prolonged exposure to anaesthetic followed by pithing, and used as donor fish for Experiments 1 and 2. All procedures were carried out according to the UK Home Office licence regulations under project licence 30/1824.

G. turnbulli (*Gt3*) and *G. bullatarudis* populations have been isolated and maintained in laboratory culture on ornamental guppies since November 1997 and November 2008, respectively. Additionally, a small number of replicates (n = 31) infected with four different *G. turnbulli* strains originating from different fish stocks and isolated for different time periods were included for the high dose, short duration salt bath studies. Statistical analysis confirmed that isolation year of the parasite strain and the parasite strain itself did not have an effect on the susceptibility of parasites to salt. Aquarium salt (Aquarian® API) was used to make up salt water of 3, 5, 7, 15, 25 and 33 g/L salinity for all experiments.

5.3.2 Experiment 1: **In vitro** *parasite survival (0, 3, 5, 7 and 33 g/L salinity)*

Parasites were gently removed from the donor fish using an insect pin and transferred individually in 25 µL water into the wells of a 96 well plate using a Gilson pipette. Transfer was rapid to avoid parasites attaching to the pipette tip. One hour after parasites had been moved to the plates, they were observed for movement under a binocular microscope illuminated with a fibre optic source to ensure that removal from the fish host had not caused damage, potentially impairing parasite survival. At this

time point any dead or moribund worms (i.e. parasites that only moved after physical stimuli caused by stirring the water slightly in the near vicinity with an entomological pin) were excluded from the experiment (< 0.01 % of those transferred). Salt stock solutions were then added to make-up the required salt concentrations of 3, 5, 7 or 33 g/L salinity in the wells with a total of 150 µL volume of water per well. Dechlorinated aquarium water was added to the control treatments and the time of addition of treatments was defined as zero. From thereon, parasites (n = 79-98 per treatment for each species) were observed hourly for movement with time of death recorded when the parasite did not move even after the water near the worm was stirred gently with an entomological pin.

5.3.3 Experiment 2: **In vivo** *parasite survival (0, 3 and 7 g/L salinity)*

Over a 7 day period, guppies (n = 100, standard length (SL): 7-23.1 mm) were gradually habituated to their experimental salt concentrations by an incremental increase in salinity levels of 1 % starting 7 or 3 days ahead of the experiment for 7 and 3 g/L treatments, respectively. Guppies were maintained individually in 1 L pots throughout the habituation and experimental periods, and received water changes at least every other day. On Day 0 (D0) naive guppies were infected with two parasites each (either *G. turnbulli* or *G. bullatarudis*) by anaesthetizing the recipient fish and bringing the donor fish in close contact to enable parasites to transmit from one fish to the other. Time to infection was recorded and any infection in which the parasite failed to transmit within 120 s was aborted. Anaesthetic was prepared with the appropriate salt water concentration in which the fish was maintained. After infection, fish were screened on D1 to check whether parasites had established (i.e. whether at least one parasite was still attached to the host). Thereafter, fish were screened every other day until fish were either parasite free or had succumbed to infection.

5.3.4 Experiment 3: Efficacy of salt baths (15 and 25 g/L salinity)

Ornamental guppies (n = 96, SL: 6.5-26.4 mm) that acquired *G. turnbulli* or *G. bullatarudis* infections in previous experiments were randomly assigned to a 15 or 25 g/L salt bath treatment or a control treatment of dechlorinated aquarium water. The use of fish with unknown infection age and different initial parasite burdens aimed to simulate natural infections of individual fish within wild or cultured fish populations. Adult guppies (> 13 mm) were exposed to their respective treatments for 15 min, whereas juveniles received treatment for 5 min only to reduce osmotic stress. Parasite loads were recorded before and after treatment by screens of anaesthetized hosts.

5.3.5 Statistical analysis

Data for Experiment 1 were analysed with a non-parametric Cox proportional hazard model with an average hazard, time-to-death as independent variable, and parasite species and treatment as dependent variables. For Experiment 2, differences in infection trajectory between parasite

populations under different saline conditions were assessed with a Generalized Linear Mixed Model (GLMM) using restricted maximum likelihood analysis in ASReml-R and fitted with a Gaussian error structure and an identity link. *Gyrodactylus* species, salinity, day, fish sex and size were used as independent variables (excluding data for *G. turnbulli* infected guppies at 7 g/L salinity where there was 0 % establishment) and parasite load at any given day was used as dependent variable. The random model included day and fish ID as independent variables. Data were normalised by a natural log (ln) transformation and visual investigation of histograms gave an understanding of standardized residual distributions.

Parasite establishment and fish mortality were analysed using a General Linear Model (GLM) fitted with a binomial error structure and a logit link function. For both models gyrodactylid species, salinity, fish sex and standard length were used as independent variables. Efficacy of salt baths in Experiment 3 was calculated as followed:

$$E_t = (L_0 - L_t) / L_0 \quad \text{for } L_t < L_0, \text{ and}$$

$$\Delta E_t = 0 \quad \text{for } L_t \geq L_0$$

where E_t = efficacy of treatment; L_0 = parasite load before treatment; and L_t = parasite load after treatment. A GLM fitted with a quasi-binomial error structure and a logit link function was conducted to assess differences in efficacy with treatment, parasite species, parasite strain (nested within parasite species), fish sex and standard length as independent variables.

Significance of GLMM and GLM terms were assessed using $\alpha = 0.05$ as critical value and model reduction followed a stepwise log-likelihood deletion process. The contrast command was used to detect differences between factor levels. All data were analysed in R v. 2.9.2 (R Core Development Team 2010).

5.4 Results

Independent of species, parasite survival *in vitro* (Experiment 1) decreased with increasing salinity (Fig. 5.1). Mean survival at 25 ± 1 °C was reduced from 17.6 and 18 h in *G. turnbulli* and *G. bullatarudis* in dechlorinated aquarium water, respectively, to less than an hour in 33 g/L salinity (Fig. 5.2).

Infection trajectories for *G. turnbulli* infected guppies at 0 and 3 g/L and *G. bullatarudis* infected guppies at 0, 3 and 7 g/L salinity (Experiment 2) were influenced by salinity with 3 g/L saline conditions increasing gyrodactylid population growth and 7 g/L salinity decreasing population growth compared to the control (GLMM: $F_{2,570} = 5.775$, $P = 0.004$; Fig. 5.3A-C). There was no effect of *Gyrodactylus* species, day post-infection, fish sex and length on infection trajectory ($P > 0.05$).

Parasite establishment was lower in *G. turnbulli* than in *G. bullatarudis* (GLM: χ^2 = 35.018, p < 0.001, n = 99) and generally decreased with increasing salinity (GLM: χ^2 = 46.661, p < 0.001, n = 99): 94.4 %, 77.8 % and 0 % establishment for *G. turnbulli* and 100 %, 100% and 73.3 % for *G. bullatarudis* in 0, 3 and 7 g/L salinity, respectively. Host mortality was slightly higher for *G. bullatarudis* (36.7 %) than for *G. turnbulli* infected hosts (23.5 %; GLM: χ^2 =3.862, p < 0.049, n = 100), but was not affected by salinity (GLM: χ^2 = 5.766, p = 0.056, n = 100).

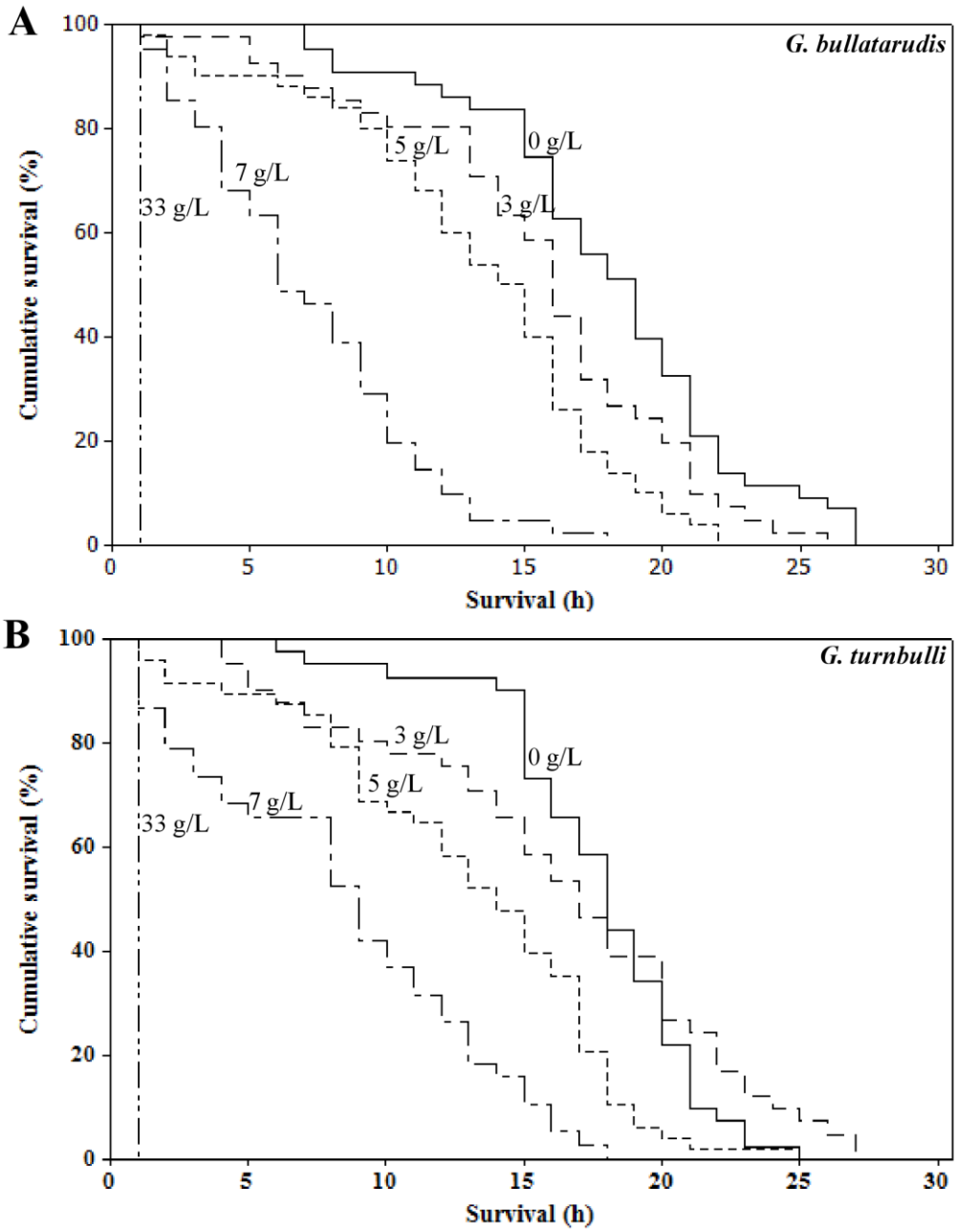


Fig. 5.1. Cumulative *in vitro* survival (%) of (A) *G. bullatarudis* and (B) *Gyrodactylus turnbulli* at 0 g/L (——), 3 g/L (– – –), 5 g/L (----), 7 g/L (– - – - –) and 33 g/L (– -- – -- –) salinities and 25 ± 1 ºC.

The effect of salt baths (Experiment 3) on parasite species was significantly different (GLM: χ^2 = 12.8, df = 1, $p < 0.001$, n = 96) with *G. bullatarudis* being generally more tolerant to salt treatments than *G. turnbulli* (Fig. 5.4). Efficacy of treatments increased with increasing salinity (GLM: $\chi^2 = 67.9$, df = 2, $p < 0.001$, n = 96; Contrasts: $p < 0.001$ for all tests) resulting in a 100 % efficacy of salt against *G. turnbulli* at 25 g/L, whereas *G. bullatarudis* was only reduced by 73.3 % (Fig. 5.4). Fish experienced no mortality throughout the salt bath treatments; however, routine monitoring of all fish after the experiment suggested elevated mortalities amongst juveniles in the three days following the experiment. Since initial deaths were not recorded, this observation could not be quantified.

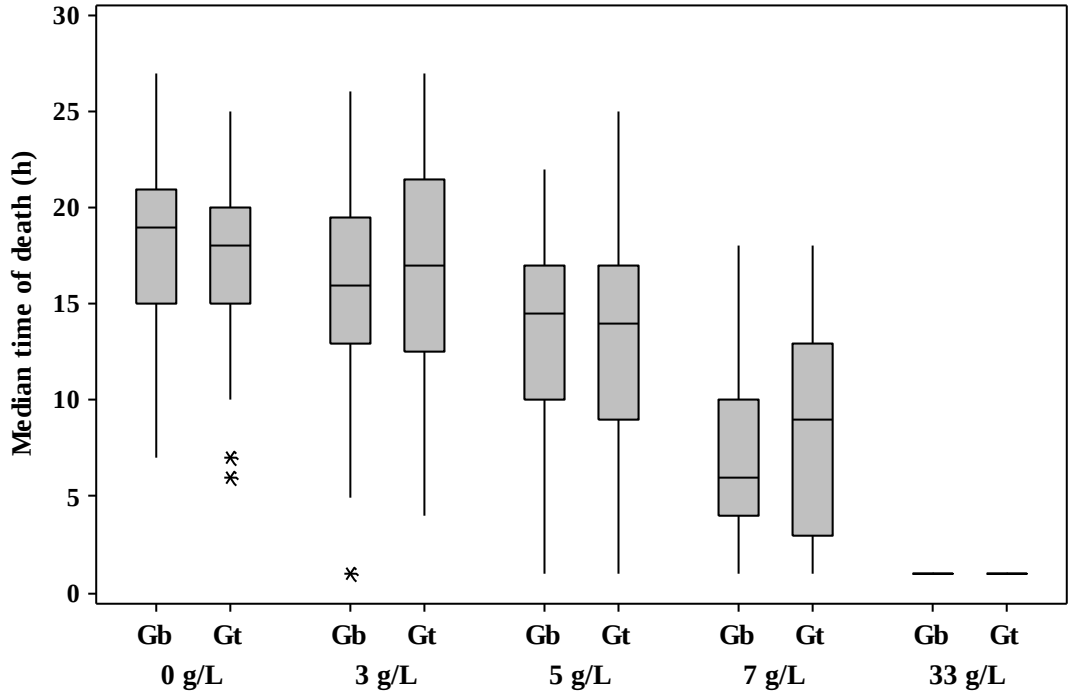
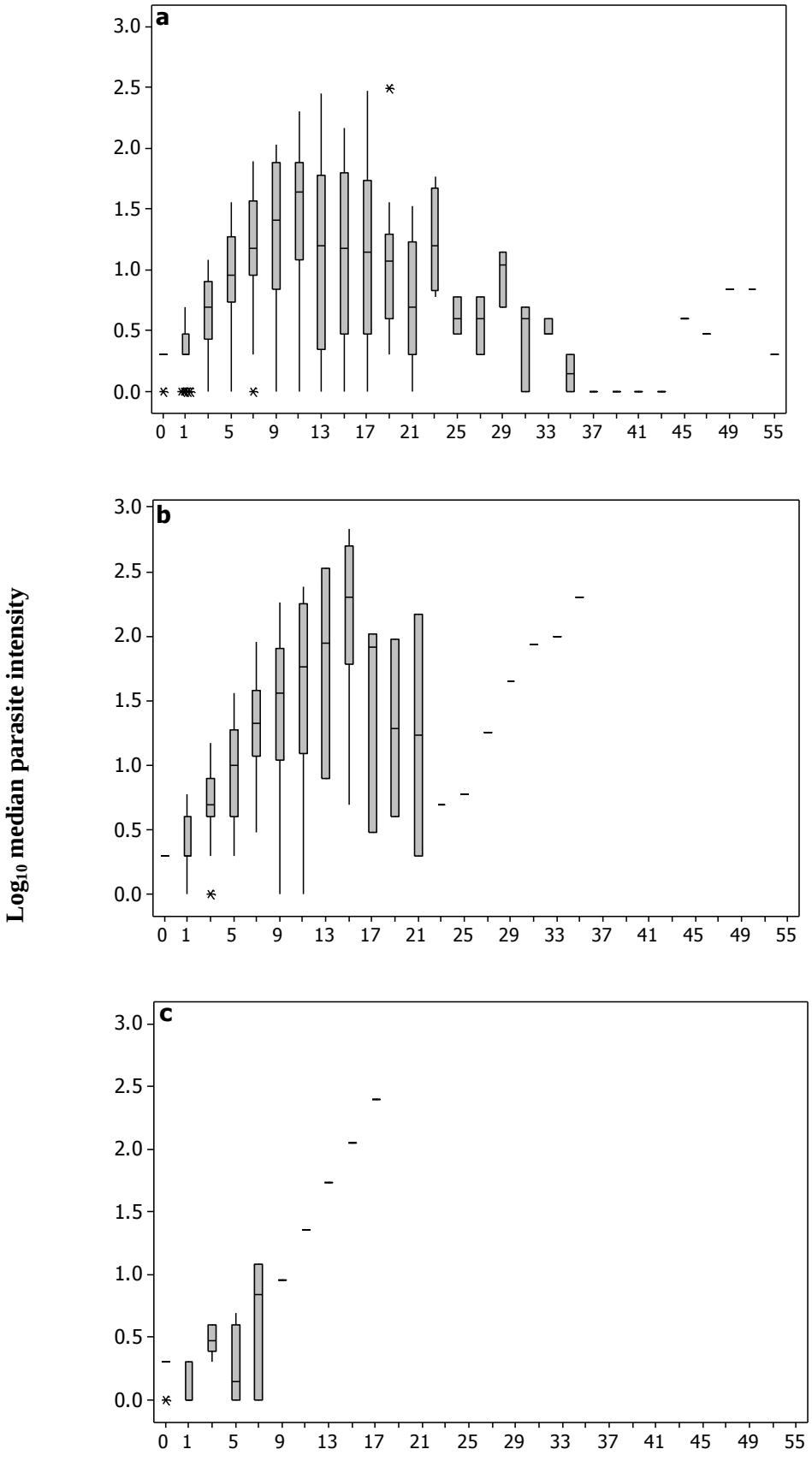


Fig. 5.2. Median time of death (h) of *Gyrodactylus bullatarudis* (Gb) and *G. turnbulli* (Gt) *in vitro* at 0, 3, 5, 7 and 33 g/L salinity. The stars represent outliers; the bars, the lower and upper limits; and the box represents the 1st and 3rd quartile with the median.

Fig. 5.3 (overleaf). Boxplots for $\log_{10}$ transformed median infection intensity trajectories of *Gyrodactylus bullatarudis* and *G. turnbulli* infected guppies (*Poecilia reticulata*) combined (no difference between parasite species confirmed by statistical analysis), at (a) 0 g/L, (b) 3 g/L and (c) 7 g/L salinity. In the control treatment (a, 0 g/L) one fish maintained its parasite population until Day 55, but the population size never increased to more than 7 parasites after Day 37. The stars represent outliers; the bars, the lower and upper limits; and the box represents the 1st and 3rd quartile with the median.


a
b
c
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 1 5 9 13 17 21 25 29 33 37 41 45 49 55
Log10 median parasite intensity
Days

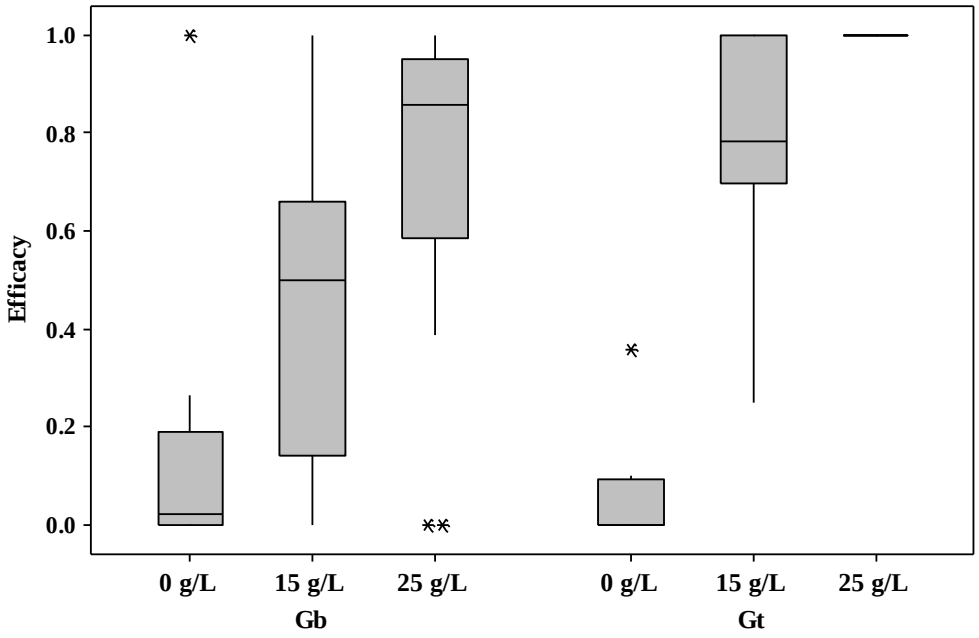


Fig. 5.5. Median efficacy of 25 and 15 g/L salt bath treatments against *Gyrodactylus bullatarudis* (Gb) and *G. turnbulli* (Gt) on guppies (*Poecilia reticulata*) compared to the control (0 g/L salinity). Efficacy: 1 = 100%; 0 = 0%. The stars represent outliers; the bars, the lower and upper limits; and the box represents the 1st and 3rd quartile with the median.

5.5 Discussion

Salt decreased the *in vitro* survival of both guppy parasite species, presumably due to disrupted osmoregulation, and hence, was similarly effective to other treatments tested *in vitro* against gyrodactylids (reviewed in Chapter 1.6). Establishment of *Gyrodactylus turnbulli* on guppies was prevented at 7 g/L salinity, but this same salt concentration only decreased *G. bullatarudis* establishment. Similarly, short duration salt baths were more effective against *G. turnbulli* (100 % efficacy at 25 g/L salinity) than in *G. bullatarudis* (73.3 % efficacy). Hence, *G. bullatarudis* is more tolerant of saline conditions than *G. turnbulli*. Comparing the *in vivo* and *in vitro* data revealed that the negative correlation between salinity and survival *in vitro* was not reflected in the *in vivo* infection trajectories. *In vivo*, parasite populations on fish in 3 g/L salinity reached larger population sizes than control populations on hosts maintained in dechlorinated water, whereas parasite populations on fish in 7 g/L salinity were severely impaired compared to the 0 g/L salinity controls.

Fish epidermis is protected by a mucus coat (reviewed by Shephard 1994) which provides a food source for gyrodactylids, and may also protect these parasites, at least partially, from the external environment (reviewed by Bakke et al. 2007). Since environmental stressors, such as changes in

salinity or parasite infection lead to an initial increase in fish mucus production (until mucus cells are depleted, Wells and Cone 1990), it is likely that parasites on hosts benefited from an increased mucus cover at 3 g/L salinity over the first few days of the experiment (Experiment 2). Assuming this initial increase in mucus production to be linear with salinity, mucus depletion then occurred more slowly and delayed at 3 g/L compared to the 7 g/L salinity. Thus, parasites on fish in 7 g/L salinity might have, at most, benefited from mucus during early infection stages, leading to an adverse effect on parasite population dynamics, similar to the situation observed when infected guppies were exposed to zinc (Gheorghiu et al. 2006, 2007). In fish maintained at 3 g/L salinity, however, acclimatisation to the saline environment may have occurred before mucus had been fully depleted with beneficial effects on parasite population dynamics.

In vivo establishment showed a similar trend to *in vitro* survival whereby increasing salinity led to a reduction in parasite establishment with *G. turnbulli* having a lower establishment rate than *G. bullatarudis* at both 3 and 7 g/L salinity. The smaller size of *G. bullatarudis* compared to *G. turnbulli* (Harris 1986) is potentially advantageous in saline conditions during host establishment since relatively more of its surface area may be enveloped by fish mucus compared to *G. turnbulli*. *G. bullatarudis* is also a generalist infecting a wider range of host species existing in a wider diversity of habitats, whereas *G. turnbulli* is more conservative in transmitting to new host species (reviewed in Bakke et al. 2007, King and Cable 2007, King et al. 2009). Both species may, however, naturally have a certain level of tolerance against salt since their primary hosts, guppies, can inhabit brackish water in their natural environment in which they often live sympatrically with mollies (*P. sphenops*, introduced species) and swamp guppies (*P. picta*, endemic species; see Froese and Pauly 2010). *G. turnbulli* and *G. bullatarudis* can infect both mollies and swamp guppies (King and Cable 2007, King et al. 2009). Short migrations between rivers using a brackish water hosts as a carrier in a saline environment may promote dispersal in guppy gyrodactylids similar to *G. salaris* on Atlantic salmon (*S. salar*, see Soleng et al. 1998, Peeler et al. 2004, 2006). Just one single, gravid gyrodactylid left on the hosts during a dispersal scenario can cause a subsequent disease epidemic in susceptible host populations. Similarly, only partially effective treatments may lead to new disease epidemics in aquaculture, where fish are exposed to high stressors and susceptibility to disease is increased.

Salt is a safer treatment option in aquaculture compared to other broad anti-parasitic treatments such as formalin or malachite green, despite reports of increased mortalities amongst fish (e.g. Buchmann 1997, Soleng et al. 1998). In the current study, no mortalities occurred during the short duration salt bath, but there appeared to be an increase in deaths of juveniles treated with salt baths in the days following the experiment indicating that dose and/or length of treatment were unsuitable for juveniles. Due to their smaller size, juvenile guppies would have been disproportionally affected by osmotic

changes compared to adults, potentially resulting in the increased post-experimental mortalities. Generally, guppies are regarded as a very hardy and robust fish species that tolerate salinity levels equal to sea water after a 3-day acclimation period for up to 7 days (Chervinski 1984). It is, however, questionable whether other fish might be able to tolerate salinities up to 7 g/L. Also, long-term consequences of exposure to unnaturally high saline environments in these fish are not studied yet, but it is known that salinity tolerance is dependent on genetics (Nakadate et al. 2003).

In comparison with other treatments, the efficacy of both continuous, low concentration saline conditions and short-term, high concentration salt baths is within the range of other treatments tested against gyrodactylids (reviewed in Chapter 1.6). Also, low dose, continuous saline conditions and short duration salt baths against *G. turnbulli* are as effective as a UK Home Office licenced procedure in which fish are treated with levamisole against gyrodactylids in research environments. Salt treatments against *G. bullatarudis* are, however, only partially effective. Despite being congeners, there is only a distant phylogenetic relationship between *G. turnbulli* and *G. bullatarudis* (see Cable et al. 1999). This leads to the conclusion that the generic application of treatments that have only been experimentally tested against one parasite species within a group of phylogenetically related organisms is unadvisable. Our study shows that the efficacy of a particular compound using a set application protocol against one parasite species does not postulate successful treatment of a second, congener parasite species. However, despite the reduced efficacy of salt baths against *G. bullatarudis* compared to the efficacy against *G. turnbulli* salt baths could potentially be a safe alternative for aquarium owners to treat fish against skin-infecting gyrodactylids. Exposure time to 25 g/L salt treatment can cautiously be increased if fish are kept under close observation for agitated behaviour which might further decrease the survival of the remaining *G. bullatarudis* parasites. Since *G. bullatarudis* population dynamics are characterised by sudden population crashes followed by extinction (personal observations), a population reduction to a low parasite intensity might cause the remaining parasites to go extinct. Using salt at a continuous low dose (7 g/L) as preventative treatment has negative effects on population dynamics, but does not prevent establishment in *G. bullatarudis* completely. Since *G. turnbulli* is more susceptible to salt and *G. bullatarudis* population dynamics are unstable, a 7 g/L salinity treatment over several days can control both parasite species by preventing the establishment of *G. turnbulli* individuals and reducing *G. bullatarudis* populations to unviable sizes. The possibility of a competitive release of *G. bullatarudis* over time through regular salt treatment of fish infected with both parasite species, however, cannot be excluded (see de Roode et al. 2004, Wargo et al. 2007).

In summary, efficacy of salt treatments is strongly dependent on treatment duration, strength and targeted parasite species. Application needs to be closely monitored, particularly in juvenile fish. Salt

baths are only partially effective in containing gyrodactylids, but might achieve effective disease control by reducing parasite populations to unviable population sizes leading to extinction. Exposing guppies to a low dose, continuous treatment over several days may prevent parasite populations establishing, particularly *G. turnbulli*. Generic application of salt in other host-parasite systems is unadvisable without preliminary tests to ensure the efficacy of the compound in the system and further research is needed to assess the long-term consequences of regular salt treatments against gyrodactylid epizootics.

5.6 References

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CHAPTER 6: *IN VITRO* AND *IN VIVO* EFFICACY OF CAJUPUT OIL AGAINST *GYRODACTYLUS TURNBULLI* INFECTING THE GUPPY (*POECILIA RETICULATA*)

6.1 Abstract

Infectious disease in aquaculture is economically costly and, from a conservation perspective, threatens susceptible fish populations. Commonly, ectoparasitic helminth infections are major culprits for high mortalities in farmed stocks, wild populations and ornamental fish, either by killing their hosts directly or by increasing their susceptibility to secondary infections. Available treatments tend to be low in efficacy, toxic to hosts and have negative consequences on human health and the environment. We tested 22 botanical treatments alongside 5 controls against *Gyrodactylus turnbulli in vitro* and *in vivo* infecting guppies (*Poecilia reticulata*). Survival of detached parasites was significantly reduced by all treatments with cajuput oil, bay rum oil, bladderwrack, octanoic acid, pine tree oil and barberry killing worms instantly. *In vivo*, cajuput oil was as effective as a licensed livestock de-wormer, levamisole, the latter of which is not routinely prescribed for use in fish; hence, cajuput oil has the potential to replace levamisole in UK research laboratories as well as in ornamental aquaculture.

6.2 Introduction

The freshwater ornamental fish market aims to rear fish cost-effectively leading to an intensification of aquaculture at which disease prevalence is inadvertently strongly promoted (Bondad-Reantaso et al. 2005, Ashley 2007). Short-term economic losses due to high disease prevalence can be detrimental to the industry which has been valued at US$ 238 million and US$ 283 million in exports and imports globally in 2005, respectively (European Commission 2008). Intensified aquaculture has, however, long-reaching consequences, such as the evolution of multi-drug resistance (e.g. Cabello 2006, Verner-Jeffreys et al. 2009), increased parasite virulence (Pulkkinen et al. 2010), changes in life-histories of pathogens (Mennerat et al. 2010) and translocation of parasite species with devastating consequences for naive fish populations (e.g. *Gyrodactylus salaris*; see Bakke et al. 2007). The use of chemotherapy against the 'weeds of aquaculture' (Cable and Harris 2003) has contributed to the problems of pathogen evolution (Nowak 2007, Mennerat et al. 2010, Pulkkinen et al. 2010); hence, the need for alternative treatment strategies. Options include optimization of anti-parasitic drug application (Débarre et al. 2009) and drug combination therapy as, for instance, recommended against *Saprolegnia* spp. infecting ornamental fish (FishDoc 2012). Treatment combinations are expected to increase anti-parasitic efficacy due to synergistic interactions between individual compounds. Similar synergistic effects are expected from the use of whole plant extracts that naturally combine a huge array of potentially effective compounds: these are being increasingly tested against a whole range of parasites (Anthony et al. 2005, Athanasiadou et al. 2007, Ansari and Inamdar 2010, Woods and

Knauer 2010) and applied in aquaculture not only as parasiticides, but also as immunostimulants and water conditioners (Citarasu 2010, Chakraborty and Hancz 2011)

In aquaculture, gyrodactylids are amongst the most invasive parasite species (Bakke et al. 2007, Guo and Woo 2009). These worms combine asexual, parthenogenetic and sexual reproduction with a 'Russian Doll' embryo system which enables them to reach epidemic population sizes within a short time period (Cable and Harris 2002, Chapter 2). Their species richness is largely due to species jumps, rather than co-evolutionary mechanisms, allowing them to exploit new niches (i.e. new fish species) (Ziętara and Lumme 2002, Harris et al. 2004). With global fish transport, geographical barriers between fish species are being broken down, increasing the risks of disease epidemics (Naylor et al. 2001, Murray and Peeler 2005). Treating gyrodactylid infections in fish is difficult, primarily because there is no completely effective medication on the market (Chapter 1.6, 5), even though tea tree oil has been shown to be a potential alternative against *Gyrodacylus* spp. infecting three-spined sticklebacks (*Gasterosteus aculeatus*; see Steverding et al. 2005). The current thesis has shown that salt (Chapter 5) is highly efficacous against *G. turnbulli* infecting guppies (*Poecilia reticulata*), a popular aquarium fish, which is commonly infected with gyrodactylids from its culturing sites in South East Asia to the consumer end point (Kim et al. 2002, Thilakaratne et al. 2003, Whittington and Chong 2007, Hongslo and Jansson 2009). The current study extends the previous work by testing 22 plant compounds on the same host parasite system, *G. turnbulli* on guppies.

6.3 Material and Methods

6.3.1 Ethical note

UK Home Office Licence (PPL 30/2357) Regulations and approval by the Cardiff University Ethics Committee regulated this work. Parasite infections on fish were monitored closely throughout the trials. If at any point fish behaviour and welfare appeared to be compromised by parasites, guppies were removed from the trial and treated with a parasiticide.

6.3.2 Source of animals and herbal compounds

Guppies (*Poecilia reticulata*) originated from an ornamental stock of inbred fish maintained at Cardiff University. They were kept at 25 ± 1 °C, on a 12 h light : 12 h dark cycle and fed at least once daily with Aquarian® fish flakes. These conditions were maintained for the experiment. The *Gt3* strain of *Gyrodactylus turnbulli* utilised was isolated from pet shop guppies in Nottingham in 1997. Additional *in vitro* trials assessed the survival of the *Gt1* strain, isolated from wild guppies in the Lower Aripo river (Trinidad) in 2001. Herbal compounds (n = 22) originated from various manufacturers and were either used in powder/dried leaf form (P) or as liquid plant extract (E): black walnut, bladderwrack,

blue cohosh, cascara sagrada, chamomile, fenugreek, gotu kola, uva ursi, white willow, yarrow (all E dissolved to equal parts in water and glycerin, HoneyCombs USA); bay rum and cajuput (both E, Berje, USA); green papaya (P, www.ranaturespowder.com/papaya_powder.html); berberine chloride (P) and octanoic acid (E) (Sigma-Aldrich, UK); barberry (www.globalherbalsupplies.com); goldenseal (P, Frontier, UK); myrrh resin and neem (both P, www.motherherbs.com); green tea (courtesy of Les Baillie and Will McCully, Welsh School of Pharmacy, Cardiff University); noni (P); pine needle oil (E, NOW foods, USA). Levamisole (marketed as Levacide by Norbrook®, UK) was included as an effective positive control and dechlorinated water as a negative control. We further controlled for two dissolvents, Tween 20 and Glycerine (Fisher Scientific, UK), which had to be used with some of the oil based treatments. *In vivo*, levamisole was only used on 11 fish, as the high efficacy of the negative control lead to statistically robust efficacy scorings so avoiding the need to use more animals in the current experiments (Table 6.1).

Working concentrations of compounds were chosen through a careful selection process that considered both toxicity to fish and efficacy *in vitro*. Thus, final concentrations for both *in vitro* and *in vivo* tests varied considerably between treatments (Tables 6.1, 6.2), but were applied in the same way as that levamisole is used against gyrodactylids in the Cardiff University laboratories (1 µL/mL for an initial 5 min exposure, followed by 0.1 µL/mL for 24 h). Further, commercialised products tend to use 1 % concentrations of effective compounds which is similar to concentrations used in the current study.

6.3.3 In vitro

In total, 22 compounds (plus five controls) were tested on isolated *G. turnbulli* individuals (n = 2297; Table 6.1). Trials followed the *in vitro* methods in Chapter 1.6. In brief, parasites were removed with insect pins from fin clips of infected guppy donors and a micropipette was used to transfer the parasites individually in 10 or 20 µL of water into wells of a 96 well microtitre plate. After 1 h, parasites were observed for abnormal behaviour and any parasites moribund (i.e. movement on gentle stirring of water next to the worm with an insect pin) or dead were discarded from trials (Cable et al. 2002). Thereafter, 90 or 180 µL of treatment was added to give a total volume of 100 or 200 µL, respectively; the lower volume was used if the solution was opaque and affected the observer's ability to view the parasites.

Table 6.1. Working concentration of compounds, sample size (N), mean, standard error (SE) of the mean, standard deviation (St Dev) and minimum (Min.) and maximum (Max.) survival (in h) for treatments tested *in vitro*. All concentrations refer to the concentration of the effective compound (i.e. solvent dilution effect is taken into consideration). Stock solutions were prepared either by weighing or measuring compounds by volume leading to different units; conversion to one unit would have required specification of the various constitutes' molar concentrations for which laboratory equipment was insufficient.

Treatment	Working concentration	N	Mean Survival	SE Mean	St Dev	Min. survival	Max. Survival
CONTROLS							
Water (*Gt3*)	-	198	16.26	0.56	7.90	1	31
Water (*Gt1*)	-	60	11.38	0.42	3.26	6	21
Levamisole	1.39 mg/mL	50	0.18	0.04	0.28	0.08	1
Levamisole	0.5 µg/mL	155	0.27	0.04	0.49	0	2
Glycerin	20 µL/mL	36	1.06	0.04	0.23	1	2
Glycerin	0.66 µL/mL	38	15.39	1.15	7.09	1	27
Glycerin	0.11 µL/mL	39	17.56	1.01	6.33	3	27
Tween 20	0.11 µL/mL	20	1.1	0.07	0.31	1	2
Crovol	0.11 nL/mL	17	15.94	1.23	5.23	2	23
PLANT COMPOUNDS							
Barberry	7.4 mg/mL	30	0.14	0.04	0.23	0.08	1
Barberry	14.8 mg/mL	30	7.00	0.47	2.57	2	10
Barberry	14.4 µg/mL	30	10.00	0.98	5.36	0.08	26
Barberry	7.41 µg/mL	29	7.97	1.20	6.47	1	29
Bay Oil + 0.01 % Tween	7.41 mg/mL	30	0.08	0	0	0.08	0.08
Bay Oil + 0.01 % Tween	0.07 mg/mL	30	0.08	0	0	0.08	0.08
Berberine Chloride	7.41 mg/mL	30	1.61	0.26	1.40	0.08	5
Berberine Chloride	0.07 mg/mL	32	2.60	0.39	2.22	0.08	8
Black Walnut	0.67 µL/mL	30	0.57	0.09	0.50	0	1
Black Walnut	0.22 µL/mL	30	2.50	0.76	4.17	0	16
Bladderwrack	0.67 µL/mL	29	0.10	0.06	0.31	0	1
Bladderwrack	0.22 µL/mL	32	1.00	0.06	0.31	0	2
Bladderwrack	0.11 µL/mL	42	3.38	0.60	3.88	1	19
Blue cohosh	0.67 µL/mL	29	0.59	0.09	0.50	0	1
Blue cohosh	0.22 µL/mL	30	0.90	0.07	0.40	0	2
Blue cohosh	0.11 µL/mL	42	2.57	0.30	1.99	1	7
Cascara sagrada	0.67 µL/mL	28	0.54	0.10	0.51	0	1
Cascara sagrada	0.22 µL/mL	30	0.87	0.08	0.43	0	2
Cascara sagrada	0.11 µL/mL	35	1.49	0.09	0.51	1	2
Chamomile	0.67 µL/mL	30	0.27	0.08	0.45	0	1

Table 6.1 continued.

Treatment	Working concentration	N	Mean Survival	SE Mean	St Dev	Min. survival	Max. survival
Chamomile	0.22 µL/mL	32	0.85	0.08	0.45	0	2
Chamomile	0.11 µL/mL	39	1.08	0.06	0.35	1	3
Fenugreek seed	0.67 µL/mL	24	0.96	0.04	0.20	0	1
Fenugreek seed	0.22 µL/mL	30	1.37	0.13	0.72	1	4
Goldenseal	7.41 mg/mL	30	0.83	0.13	0.74	0.08	4
Goldenseal	0.07 mg/mL	30	3.11	0.45	2.48	0.08	8
Gotu kola	0.67 µL/mL	29	0.93	0.41	2.19	0	12
Gotu kola	0.22 µL/mL	30	2.70	0.82	4.47	0	17
Green papaya	7.41 mg/mL	30	0.90	0.16	0.86	0.08	3
Green papaya	0.07 mg/mL	30	1.39	0.26	1.40	0.08	5
Green tea I	2.56 µg/mL	28	1.36	0.19	1.03	0	5
Green tea I	0.67 µg/mL	32	4.47	0.57	3.22	1	13
Green tea II	2.56 µg/mL	31	2.03	0.33	1.84	0	7
Green tea II	0.67 µg/mL	28	2.89	0.37	1.97	1	7
Myrrh resin	7.41 mg/mL	30	0.33	0.07	0.41	0.08	1
Myrrh resin	0.07 mg/mL	30	1.30	0.10	0.53	0.08	2
Neem	7.41 mg/mL	39	2.57	0.35	2.17	0.08	7
Neem	0.07 mg/mL	30	3.49	0.61	3.32	0.08	11
Noni	2.56 µg/mL	30	7.37	1.25	6.83	0	23
Noni	0.67 µg/mL	29	10.86	1.74	9.38	0	25
Octanoic acid	7.41 mg/mL	30	0.08	0	0	0.08	0.08
Octanoic acid	0.07 mg/mL	30	0.08	0	0	0.08	0.08
Pine needle oil with 0.01 % Crovol	3.37 nL/mL	16	0	0	0	0	0
Cajuput oil	7.41 mg/mL	30	0.11	0.03	0.17	0.08	1
Cajuput oil	0.07 mg/mL	30	0.54	0.10	0.53	0.08	2
Cajuput oil with 0.01 % Tween	7.41 mg/mL	30	0.08	0	0	0.08	0.08
Cajuput oil with 0.01 % Tween	0.07 mg/mL	30	0.08	0	0	0.08	0.08
Uva ursi	0.67 µL/mL	31	0.71	0.39	2.15	0	12
Uva ursi	0.22 µL/mL	30	0.57	0.09	0.50	0	1
Uva ursi	0.11 µL/mL	26	1.50	0.35	1.79	1	10
White willow	0.67 µL/mL	29	0.69	0.10	0.54	0	2
White willow	0.22 µL/mL	31	1.61	0.40	2.23	1	13
Yarrow	0.67 µL/mL	28	0.71	0.09	0.46	0	1
Yarrow	0.22 µL/mL	33	2.33	0.57	3.26	0	15

Table 6.2. Concentration (Conc.) of compounds, sample size (N), mean efficacy, standard error (SE) of the mean, standard deviation (St Dev) and minimum (Min.) and maximum (Max.) efficacy of treatments tested *in vivo* against *Gyrodactylus turnbulli* infecting guppies (*Poecilia reticulata*). All concentrations refer to the concentration of the effective compound and are given at the initial high concentration. Minimum and maximum efficacy for all treatments were zero and one, respectively, apart from barberry which was not effective at all (minimum and maximum both zero).

Treatment	Working concentration	N	Mean Efficacy	SE Mean	St Dev	Min. efficacy	Max. efficacy
CONTROLS							
Water control	-	64	0.12	0.04	0.28	0	1
Levamisole control	0.01 mL/mL	11	1.00	0.00	0.00	0	1
Glycerin control	0.01 mL/mL	10	0.43	0.13	0.41	0	0.92
Tween control	0.01 mL/mL	21	0.36	0.10	0.45	0	1
Crovol control	0.001 µL/mL	10	0.24	0.09	0.40	0	1
PLANT COMPOUNDS							
Barberry	1.33 mg/mL	20	0.00	0.00	0.00	0	0
Berberine chloride	0.67 mg/mL	20	0.68	0.10	0.46	0	1
Cajuput oil	2 mg/mL	20	0.35	0.11	0.49	0	1
Cajuput oil with Tween 20	0.003 µL/mL	20	0.90	0.07	0.31	0	1
Cascara sagrada	0.01 mL/mL	11	0.31	0.14	0.46	0	1
Chamomile	0.01 mL/mL	10	0.35	0.13	0.44	0	1
Goldenseal	0.67 mg/mL	20	0.33	0.10	0.43	0	1
Green papaya	0.67 mg/mL	20	0.22	0.09	0.39	0	1
Pine needle oil with Crovol	0.33 µL/mL	14	0.80	0.09	0.35	0	1
Uva ursi	0.01 mL/mL	11	0.64	0.15	0.51	0	1
West Indian bay oil with Tween	0.0003 µL/mL	17	0.53	0.13	0.51	0	1

6.3.4 In vivo

Guppies (n = 299; standard length (SL) = 5-32 mm; Table 6.2) were infected with gyrodactylids by exposure to infected fish over 3-4 d. Alternatively, infected guppies from behavioural experiments or fish from routinely maintained parasite cultures at Cardiff University requiring treatment were used (mean parasite intensity ± standard error of the mean: 35 ± 3; range: 3-500). Fish were screened for parasite numbers according to the methods described in Chapter 1.6. In brief, fish were anaesthetized with 0.02 % MS222 and parasites were counted on the fish using a dissection microscope and optic fibre illumination. Any fish with three parasites or more were used for the *in vivo* trials and transferred to 1 mL of water, immediately after which it received 100 µL of treatment. After 5 min, fish were screened for parasites again and transferred to 99 mL of water with the treatment solution for the next 24 h. Additional parasite screens were performed at 1 and 24 h. For any fish still infected after 24 h, the treatment procedure was repeated once or twice. Fish that acquired potentially lethal parasite burdens during the experiment were removed and treated with levamisole (n = 69).

6.3.5 Statistical analyses

Efficacy of *in vitro* treatments was assessed using a Cox proportional hazard survival analysis on maximum survival of individual worms (n = 2297) and using treatment as a dependent variable. Different concentrations of the same treatment were treated as separate groups as nesting is not possible in an unbalanced design within a survival analysis.

Efficacy of *in vivo* treatments was calculated based on the initial and final parasite burdens of infected fish to give an efficacy value of between 0 (not effective) and 1 (100 % effective) (Chapter 5). A non-parametric Kruskal-Wallis test established differences between treatment groups which was followed with individual Mann-Whitney tests. Multiple testing was controlled for with a modification to the Bonferroni procedure after Benjamini and Yekutieli (2001, see also Narum 2006) leading to a new α level of 0.009. All analyses were performed in R 2.13.2 (R Development Core Team 2012).

6.4 Results

All botanical treatments reduced parasite survival *in vitro* significantly with cajuput oil (with and without 0.01 % Tween 20), bay rum oil (with 0.01 % Tween 20), bladderwrack, pine needle oil and barberry killing tworms within an hour. *In vivo*, using a single treatment application, only cajuput oil (with 0.01 % Tween 20) was as effective as levamisole against gyrodactylids (Tables 6.1, 6.2).

6.4.1 In vitro

Parasites of the *Gt3* strain survived for up to 31 h in dechlorinated water (mean survival: 16 h) and *Gt1* strain worms for a maximum of 21 h only (mean survival: 11.4 h; survival analysis: $z = 6.255$, $p < 0.001$; Table 6.1, Appendix V.1). Levamisole (positive control) reduced parasite survival to about 0.5 h at all concentrations tested ($z > 23.526$, $p < 0.001$). Tween 20 (0.01 %) and glycerine (at the highest dose, 20 µL/mL) also reduced survival of worms significantly to about 1 h ($z \geq -14.764$, $p < 0.001$), but lower concentrations of glycerine (0.11-0.66 µL/mL) did not affect the parasites ($p > 0.05$). The botanical treatments were all effective against gyrodactylids reducing their overall survival ($p < 0.001$; excl. Noni 0.67 µg/mL: $p = 0.021$) with some being as effective as the lowest dose of levamisole (e.g. barberry 14.8 mg/mL) or even better (e.g. octanoic acid 7.41 mg/mL and tea tree oil 7.41 mg/mL).

6.4.2 In vivo

The 10 most effective compounds from the *in vitro* trials, cajuput oil (with and without 0.01 % Tween 20), uva ursi, cascara sagrada, chamomile, pine needle oil, green papaya, bay rum oil (with 0.01 % Tween 20), berberine chloride, barberry and goldenseal, were tested further *in vivo*. Octanoic acid was

excluded from *in vivo* testing, despite being highly effective *in vitro*, as it caused mortalities in fish post-treatment during preliminary trials.

Cajuput (with 0.01 % Tween 20), uva ursi and berberine chloride were all highly effective against gyrodactylids *in vivo*, but only cajuput oil was as effective as levamisole (Table 6.1, Fig. 6.1, Appendix V.2) and required only one treatment for complete parasite removal. Pine needle oil appeared effective after 5 min clearing 81 % of parasites at the first treatment application; however, the overall total after 24 h was reduced to 62 % indicating that parasites may be affected and dislodged from the host at the high initial treatment dose, but recover and re-attach when treatment concentration is reduced for the remaining 24 h.

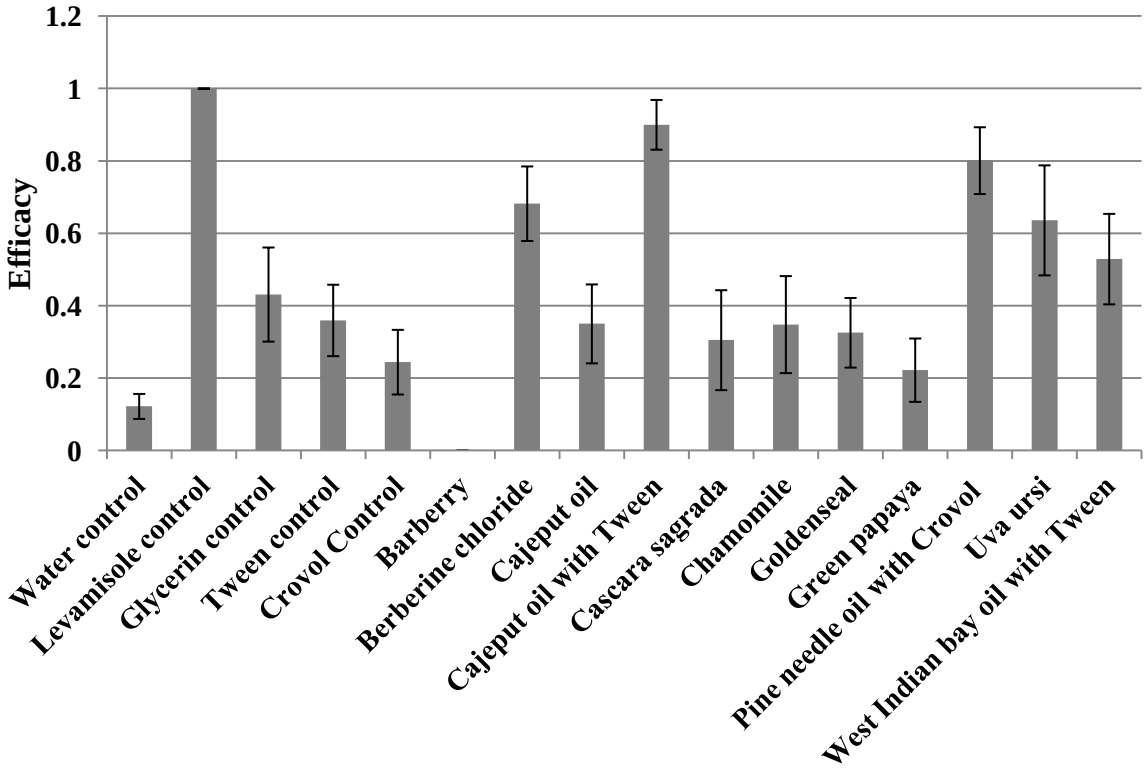

Fig. 6.1. Mean efficacy (± standard error of the mean of plant compounds and control treatments tested against *Gyrodactylus turnbulli* infecting guppies (*Poecilia reticulata*) with standard errors.

6.5 Discussion

After testing 22 botanical compounds *in vitro* and 10 compounds *in vivo* against *Gyrodactylus turnbulli* infections on guppies, we conclude that cajuput oil (with 0.01 % Tween 20) is as effective as a commercial lifestock de-wormer in the removal of gyrodactylids from guppies. Cajuput oil, derived from *Melaleuca leucadendron*, does not cause any adverse effects on the fish and only needs one

application to be 100 % effective under closely monitored conditions. In contrast, tea tree oil, a chemically similar oil from a closely related tree species, *M. alternifolia* (see De Colmenares 1998, Farag et al. 2004, Carson et al. 2006) used at the same concentration, was only partially effective against *Gyrodactylus* spp. infecting three-spined sticklebacks (Steverding et al. 2005). Differences in efficacy of treatments against different *Gyrodactylus* spp. has previously been shown using salt and may not be unusual for this specious genus (Chapter 5). The Steverding et al. (2005) study had, however, to be conducted at 13 °C which may also have affected treatment effectiveness.

The high efficacy of cajuput oil against gyrodactylids makes it an ideal candidate for commercial application; however, it should first be tested on fish shoals and, more importantly, on other fish species as the guppy is hardy and robust. Further, the use of combination treatments may prove useful in mixed *Gyrodactylus* spp. infections, which tend to be common both in wild and domestic fish populations (Cable 2011), and may have a synergistic effect on the parasites (Hu et al. 2010). Lastly, due to treatments being affected by factors such as water chemistry and temperature, combination treatment may expand efficacy across different environmental conditions.

Of the other treatments tested, which have all previously been unsuccessfully used against the putative agent of Hole-in-the-Head disease (*Spironucleus vortens*, see Appendix IV), none showed the same potential as cajuput oil. Pine needle oil significantly reduced parasite burden during the initial 5 min high concentration phase. Worms which became dislodged and moribund during treatment did not, however, always die. Parasite loads 24 h post-treatment indicate that during the low concentration exposure parasites recovered and re-attached, as the high number of worms could not be explained by parasite reproduction only. Hence, increased exposure time may benefit the efficacy of pine needle oil. However, increasing the concentration is not advised as guppies at higher concentrations during preliminary trials showed erratic behaviour when exposed to the oil. Pine oil has previously been shown to be anti-viral, anti-fungal, anti-protozoan and acaricidal (Hammer et al. 1999, Krauze-Baranowska et al. 2002, Macchioni et al. 2002, Koch et al. 2008, Molan et al. 2009), but this is the first study to test its efficacy against helminths.

In conclusion, cajuput oil (plus 0.01 % Tween 20) effectively cures *Gyrodactylus turnbulli* infections in guppies and, hence, has the potential to replace levamisole as a treatment in UK research laboratories. Pine needle oil is also effective, but requires further treatment evaluation at longer exposure. After further tests, both treatments may potentially be useful for commercialisation in aquarium fish.

6.6 References

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CHAPTER 7: *IN VITRO* AND *IN VIVO* EFFICACY OF GARLIC COMPOUNDS AGAINST *GYRODACTYLUS TURNBULLI* INFECTING THE GUPPY (*POECILIA RETICULATA*)

7.1 Abstract

Traditional compounds used to treat fish diseases (such as formalin and malachite green) can be more toxic to the hosts than their parasites. With the reviviscence in the use of herbal products, various botanicals have been heralded as cures for particular pathogens, but the efficacy of these compounds for parasitic worms is questionable. Here, we tested a range of garlic (*Allium sativum*) products against a major aquarium pathogen, *Gyrodactylus turnbulli*, infecting the guppy (*Poecilia reticulata*). All garlic products significantly reduced parasite mean survival time *in vitro*, from 13 h to < 1 h. In fully randomized trials, the number of parasites was also significantly reduced on infected fish exposed to garlic in different dried forms. Two garlic treatments (minced and granulated forms) reduced worm burdens by 66 and 75 %, respectively, after three doses, whereas Chinese freeze-dried garlic and allyl disulphide were 95 % effective after a single application. In fact, Chinese freeze-dried garlic was equally effective as levamisole, a licensed livestock de-wormer that is not prescribed for use in fish. Further research is required to assess the impacts of garlic treatments on fish behaviour.

7.2 Introduction

Fish keeping has become an increasingly popular hobby over the last few decades leading to a multi-million dollar industry accounting for US $238 and 283 million in global exports and imports, respectively, of aquarium fish in 2005 (European Commission 2008). Similar to food fish aquaculture, intensification of ornamental fish production has created ideal conditions for the rapid spread of infectious disease. Containment of infections in ornamental fish stocks relies heavily on broad spectrum anti-parasitic compounds, such as malachite green or the fixative formalin, which are harmful to both fish and humans (Srivastava et al. 2004, Wooster et al. 2005). Indeed, the use of malachite green for food fish was banned in the UK on recommendation from Defra in June 2002, as it is a known carcinogen (Srivastava et al. 2004). Legal application of formalin is expected to be reduced in the near future too, for environmental and human health reasons after a proposal to change the European Union Biocide Product Directive 98/8/EC (Wooster 2005, Commission of the European Communities 2009). For both malachite green and formalin, there is, however, currently no effective alternative. An inability to control infectious disease will continue to be of economic and conservation importance, particularly as aquaculture expands and fish parasites become increasingly resistant to commonly used drugs (Cabello 2006, Cressey 2009). There is also concern over the environmental effects of these chemicals on both freshwater and marine ecosystems (Cabello 2006). As current treatments often cause severe distress to fish, continued research into animal welfare is essential to prevent unnecessary suffering (Ashley 2007).

Traditionally, herbal remedies have been used for both humans and animals (e.g. garlic, reviewed by Rivlin, 2001) and potentially, the application of these remedies to a wider range of diseases may offer new treatments for parasitic infections (Anthony et al. 2005). Many previous studies have shown that garlic and its metabolites have antibacterial (e.g. Cavallito and Bailey 1944, Johnson and Vaughn 1969, Delaha and Garagusi 1985, Harris et al. 2000, 2001), antiprotozoal (e.g. Kramarenko 1951, Lun et al. 1994, Reuter et al. 1996, Millet et al. 2010, Gafaar et al. 2012), antifungal (e.g. Tansey and Appleton 1975, Ghannoum 1988, Lemar et al. 2002) and antiviral (e.g. Fenwick and Hanley 1985, Tsai et al. 1985) properties (reviewed by Harris et al. 2001, Williams and Lloyd 2012). The evidence for efficacy against helminths however is ambiguous (anti-helminthic effect: Riad et al. 2009, Strickland et al. 2009, Masamha et al. 2010, Abd El-Galil and Aboelhadid 2012; no effect: Burke et al. 2009, Worku et al. 2009).

Garlic's mode of action includes antioxidant properties, lipoprotein modification and inhibition of low density lipoprotein uptake and degradation by macrophages (Gonena et al. 2005); its activity against parasites, however, appears to be complex (reviewed by Kyung et al. 2012, Williams and Lloyd 2012). It is assumed that due to garlic's chemistry individual compounds may act in synergy even though some may break down rapidly (Freeman and Kodera 1995, Chung et al. 2007, Fujisawa et al. 2008a, b). Garlic also enhances immune system function in fish which may act in combination with direct effects on parasites (Martins et al. 2002, Sahu et al. 2007), and most importantly, drug resistance has so far not been reported.

In veterinary medicine, garlic is commonly used to treat endoparasites in a wide range of animals (from rodents to livestock, e.g. Strickland et al. 2009, Worku et al. 2009, Riad et al. 2009, Yusuf and Ekanem 2010, Dkhil et al. 2011, Velkers et al. 2011). With regard to aquatic parasites, hobby aquarists use garlic as a broad spectrum prophylactic anti-parasitic treatment, as well as an appetite stimulant, with articles in fish-keeping magazines advocating the addition of fresh garlic or garlic oil to feed in order to treat parasite infected tropical fish (e.g. Bartelme 2003). In Asia, garlic has been used as a fish treatment for many years, administered in food and as a tonic (Cortes-Jorge 2000, Jegede 2012), and there is a current resurgence of interest in the use of both garlic and other botanical compounds for fish farms (Haskell et al. 2004, Rodgers and Furones 2009). Indeed, garlic has been shown to eradicate a range of fish pathogens *in vitro* (Wei and Musa 2008, Peyghan et al. 2008). *In vivo*, garlic has been effective against the causative agent of whitespot (*Ichthyophthirius multifiliis)* in juvenile Nile tilapia (*Oreochromis niloticus*, see Soko and Barker 2005), but its efficacy appears to be life-stage specific (Buchmann et al. 2003). Crude garlic extracts eliminated *Trichodina* sp. infections in tilapia (Chitmanat et al. 2005) and allicin, a highly unstable garlic compound, which had previously

been shown to inhibit cysteine proteinase activity in *Entamoeba histolytica* (see Ankri et al. 1997, Ankri and Mirelman 1999), was also effective against *Aeromonas hydrophyla* infecting rainbow trout (Nya et al. 2010). Garlic added to feed also appeared to eliminate infections of *Capillaria* nematodes successfully in discus fish (Wattley 1999). There is also some evidence that garlic is effective against monogenean parasites reducing *Anacanthorus penilabiatus* infections in cultivated pacu (*Piaractus mesopotamicus*, see Martins et al. 2002) and gyrodactylosis in *O. niloticus* fry (Abd El-Galil and Aboelhadid 2012).

Gyrodactylids are ubiquitous, monogenean ectoparasites of teleost fish that are difficult to control with no treatment currently achieving 100 % efficacy, particularly across species (Chapters 1.6, 5). In the UK aquarium industry, up to 90 % of guppies (*Poecilia reticulata*), one of the most common aquarium fish, are usually parasitized with the pathogens *G. turnbulli* and *G. bullatarudis* (personal observations), both of which have the potential for explosive population growth (24 h doubling time at 25 °C for *G. turnbulli*, Scott 1982). As they are live-bearing hermaphrodites, just a single worm remaining can initiate a new epidemic. The only effective chemical treatment against these parasites is levamisole (Chapter 1.6) which is not routinely prescribed for fish (William H Wildgoose, personal communication). This study compares the efficacy of four garlic products and two garlic components as alternative treatments against *G. turnbulli* infections on guppies, *P. reticulata*, with the aim of determining whether any have the potential to be applied commercially.

7.3 Material and Methods

7.3.1 Source of animals and herbal compounds

The current study utilised an inbred ornamental stock of guppies (*Poecilia reticulata)* and a pet shop strain of *Gyrodactylus turnbulli (Gt3)*. An additional isogenic parasite strain, *Gt1*, originally isolated from guppies obtained from the Lower Aripo River, Trinidad, in November 2001 also served as negative control for *in vitro* experiments. All experiments were conducted at 25 ± 0.5 °C with a 12 h light : 12 h dark cycle and fish were fed twice daily on Aquarian® fish flakes.

Six garlic treatments including two garlic components were tested: Chinese freeze dried garlic powder and freeze dried garlic flakes (Cultech, Port Talbot, UK); minced garlic and garlic granules (ASDA Stores Ltd., UK); allyl alcohol and allyl disulphide (Sigma Aldrich, UK). *In vitro* concentrations were established using preliminary trials on a small number of guppies to ensure doses were safe for use in fish. *In vivo* concentrations were chosen based on the most effective *in vitro* concentrations, but may have needed further adjustment to maintain fish health and welfare, or to increase treatment efficacy if

it was safe for fish to do so. This selection process led to a range of concentrations being used which was not consistent between treatments (Tables 7.1, 7.2), but which lie within the range of those used in other studies both *in vitro* and *in vivo* (range: 0.08-500 mg/mL, e.g. Wei and Musa 2008, Millet et al. 2010, Nya et al. 2010, Abd El-Galil and Aboelhadid 2012). All treatments were prepared as concentrated stock solutions and stored in sealed containers overnight at 4 °C. Positive (levamisole: Levacide 7.5 % solution, Norbrook®, UK) and negative (dechlorinated water) controls were used, in addition to an emulsifier control for allyl disulphide, 0.01 % Tween 20.

7.3.2 In vitro

The effects of the six different garlic treatments were tested on individuals of *G. turnbulli* (n = 1172) isolated from fin clips or scales of infected *P. reticulata* donors, which were held on stock fish within the parasitology laboratory. Parasites were dislodged from host tissue using fine insect pins and transferred individually to wells of 96-well microtitre plates in 20 µL of dechlorinated water using a micropipette (following the methods of Cable et al. 2002a). Any worms that died or showed any abnormal behaviour (see Cable et al. 2002a, b) within 1 h of transfer were discarded from the trial. Thereafter, 180 µL of treatment was added to the wells containing a healthy worm leading to a final concentration of between 0.01 and 18 mg/mL (Table 7.1). To the controls, the same volume of water, levamisole or Tween 20 was added. The parasites were observed immediately after application of the treatment, 5 min later and thereafter every hour with their status recorded until they died.

7.3.3 In vivo

Fish acquired parasites naturally from other guppies through exposure to conspecifics or were available infected from behavioural experiments conducted in the laboratory (mean parasite load ± standard error of the mean: 35 ± 2, range: 2-285). Recipient fish (n = 349) were observed with a low power stereo-microscope (Nikon C-DSLS) and fibre optic illumination, and the number of attached parasites counted. Infected fish were isolated for use in trials and subsequently transferred to 1 mL of dechlorinated water in a glass dish, which is sufficient to fully immerse the juvenile fish in water, but confinement allows the fish to be continuously viewed with a stereo-microscope. Immediately after transfer, 100 µL of the treatment was applied (initial high dose, Table 7.2). After 5 min fish were screened again, then transferred with the initial treatment solution to a larger container with 99 mL of water (subsequent low dose). Two further observations of the fish under anaesthetic (0.02 % MS222) were carried out at 1 h and 24 h post-treatment to again count parasites. If fish were still infected 24 h after initial treatment, they received up to two subsequent treatments with a 24 h interval between each treatment which affected over a third of the replicates (n = 133).

7.3.4 Statistical analyses

In vitro data were analysed with a non-parametric Cox proportional hazard model with an average hazard, time-to-death as independent variable and treatment as dependent variable. For this analysis, which does not allow for nesting groups, treatments at different concentrations were tested independently.

Efficacy of *in vivo* treatments was expressed as $\Delta E_t = (L_0 - L_t) / L_0$, for $L_t < L_0$, and $\Delta E_t = 0$ when $L_t > L_0$ according to Chapter 5 to account for the initial variation in parasite load. Differences in efficacy were assessed with a Kruskal-Wallis test with treatment as independent variables and *post hoc* differences between groups assessed using Mann Whitney tests. Multiple testing was accounted for by Bonferroni corrections after Benjamini and Yekutieli (2001, see also Narum 2006) leading to an α-level of $\alpha = 0.010472$. All analyses were performed in R v.2.10.0 (R Development Core Team 2010).

7.3.5 Ethical note

This work was conducted under UK Home Office Licence (PPL 30/2357) Regulations with approval by the Cardiff University Ethics Committee. All fish utilised in these experiments were infected with *G. turnbulli* at levels well tolerated by guppies and were closely monitored throughout the trials. If the parasite burden appeared to be influencing fish behaviour and welfare, hosts were removed from trials and treated immediately with levamisole to clear them from parasite infection. Fish completely cleared of *G. turnbulli* received three separate screenings to confirm that they were clear of parasites before being returned into aquaria housing stock fish (Chapter 1.6).

7.4 Results

Chinese freeze-dried garlic and allyl disulphide (the latter applied with 0.01 % Tween 20) were the most effective treatments both *in vitro* and *in vivo*, clearing gyrodactylid infections on fish in a single application. Freeze-dried garlic was similarly effective, but required three treatment applications to reach the same efficacy (Tables 7.1, 7.2).

7.4.1 In vitro

In untreated controls (water), parasites of the *Gt1* and *Gt3* strains survived an average of 11.4 h and 13.7 h, respectively, with a maximum of 21 h and 31 h, comparable with previous experiments conducted at 25 °C on *Gyrodactylus turnbulli* (see Chapter 5). Most garlic treatments were as effective as levamisole in reducing parasite survival to an hour or less (Cox proportional hazard survival analysis: likelihood ratio test = 1525, df = 32, $p < 0.001$; Table 7.1, Appendix VI.1), apart

from freeze-dried garlic flakes at a concentration of 0.01 mg/mL and Chinese freeze-dried garlic powder at 0.09 mg/mL, both of which actually increased average survival time to over 15 h.

Table 7.1. Final concentration (Conc.), sample sizes (N), mean maximum survival (in h), standard error (SE) of the mean and minimum and maximum survival times (range in h) of *Gyrodactylus turnbulli* parasites exposed to various garlic treatments and concentrations *in vitro*.

Treatments *in vitro*	**Conc. (mg/mL)**	**N**	**Mean survival**	**SE Mean**	**Range of survival**
Water *Gt1* strain (control)	0	59	11.39	0.43	6-21
Water *Gt3* strain (control)	0	209	13.72	0.57	2-31
Levamisole (control)	18	50	0.18	0.04	0.08-1
	6	30	0.36	0.10	0.08-2
Tween 20 (control)	0.1	20	1.1	0.07	1-2
Allyl alcohol	10	30	0.08	0	0.08
	7.5	30	0.42	0.08	0.08-1
	5	30	1.07	0.07	0.08-2
	3.75	20	1.33	0.22	0.08-3
Allyl disulphide with 0.01 % Tween 20	0.5	20	0.08	0	0.08
	0.4	20	0.08	0	0.08
	0.1	20	0.27	0.08	0.08-1
	0.05	20	0.59	0.11	0.08-1
Chinese freeze-dried garlic powder	18	31	0.21	0.09	0.08-2
	9	30	0.08	0	0.08
	6	30	0.68	0.12	0.08-2
	0.09	38	15.46	1.31	0.08-27
Freeze-dried garlic flakes	18	32	0.08	0	0.08-0.08
	10	30	0.08	0	0.08-0.08
	6.67	29	0.08	0	0.08-0.08
	6	30	0.74	0.14	0.08-2
	1	30	0.36	0.08	0.08-1
	0.75	30	0.64	0.11	0.08-2
	0.5	30	0.45	0.08	0.08-1
	0.07	32	0.76	0.13	0.08-2
	0.01	38	16.09	1.34	0.08-27
Garlic granules	18	30	0.11	0.03	0.08-1
	6	33	0.08	0	0.08
	2.25	30	0.08	0	0.08
Minced garlic	18	36	0.31	0.07	0.08-1
	6	35	0.45	0.08	0.08-1

7.4.2 In vivo

When infected fish were treated with an initial high dose and subsequent low dose of Chinese freeze-dried garlic flakes (0.033 mg/mL), freeze-dried garlic flakes (1 mg/mL) and allyl disulphide (0.5 mg/mL), parasite survival was significantly reduced compared to the control with all three treatments equally effective (Kruskal-Wallis: χ^2 = 132.34, df = 11, p < 0.001) (Fig. 7.1, Table 7.2, Appendix VI.2). Efficacy was increased for all three treatments (mean efficacies of 0.964, 0.95 and 0.95, respectively) compared to the negative control (mean efficacy: 0.227) and was similar to the positive control levamisole (mean efficacy: 0.938). Garlic, however, induced slight damage to the fin edges, particularly at higher doses, but fish recovered well post-treatment in aquarium water. Any fin damage quickly healed within weeks of the trial being completed.

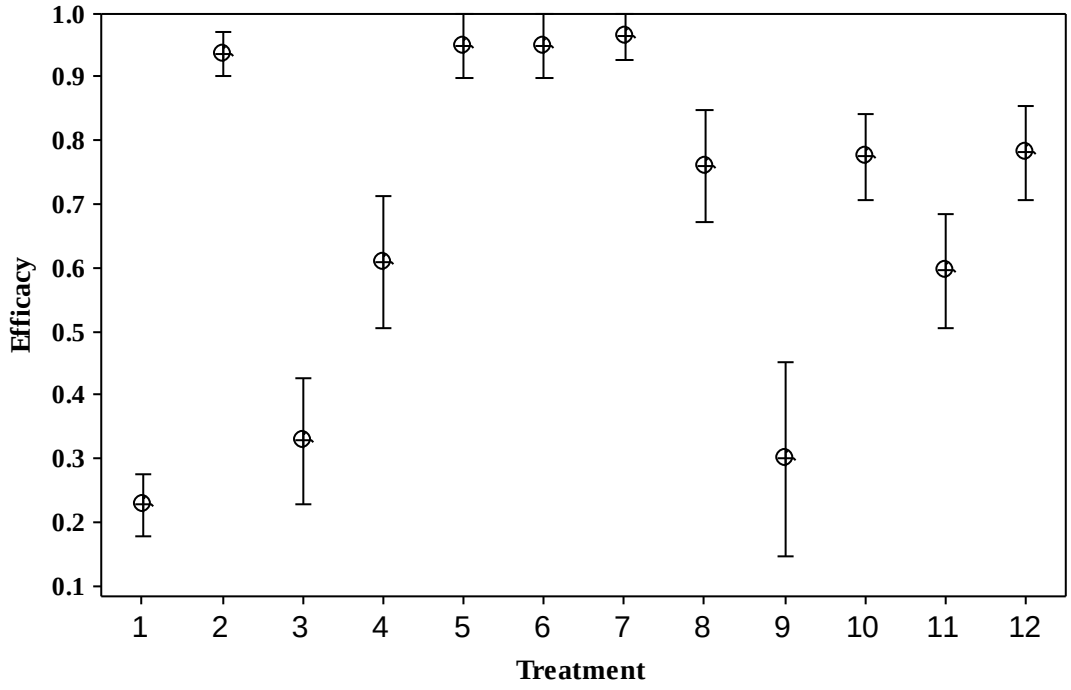

Fig. 7.1. Mean efficacy of treatments (± standard error of the mean) tested *in vivo* against *Gyrodactylus turnbulli* infecting guppies (*Poecilia reticulata*). (1) Water (negative control); (2) Levamisole (positive control); (3) 0.01 % Tween 20 (control); (4) Allyl alcohol 10 mg/mL; (5) Allyl disulphide 0.5 mg/mL + 0.01 % Tween 20; (6) Chinese freeze-dried garlic powder 0.033 mg/mL; (7)-(9) Freeze-dried garlic flakes 1 mg/mL, 0.5 mg/mL and 0.067 mg/mL; (10)-(11) Garlic granules 0.067 mg/mL and 0.033 mg/mL; (12) Minced garlic 0.067 mg/mL.

Table 7.2. Garlic treatments and their concentrations (Conc.) during the initial 5 min exposure used on *Gyrodactylus turnbulli* infected guppies (*Poecilia reticulata*) with their samples size (N), mean maximum efficacy (in h) and standard error of the mean. Efficacies ranged for all treatments between 0 and 1, with 0 being not efficacious and 1 having 100 % efficacy.

Treatment	Conc. (mg/mL)	N	Mean max. efficacy	SE Mean
Water (control)	0	60	0.2269	0.0498
Levamisole (control)	0.2	48	0.9375	0.0353
Tween 20 (control)	0.1	20	0.3275	0.0982
Allyl alcohol	10	23	0.609	0.104
Allyl disulphide + Tween 20	0.5	20	0.95	0.05
Chinese freeze dried garlic powder	0.033	20	0.95	0.05
Freeze dried garlic flakes	1	28	0.964	0.0357
	0.5	20	0.7599	0.0882
	0.067	10	0.3	0.153
Minced garlic	0.067	30	0.7817	0.0734
Garlic granules	0.067	40	0.775	0.0669
	0.033	30	0.5952	0.089

7.5 Discussion

The current study shows that different forms of garlic can significantly reduce the survival of gyrodactylids. Generally, treatments were more effective *in vitro*, this may be due to worms not being protected by the host's mucus layer, which provides a barrier to water borne compounds (reviewed by Shephard 1994, Burka et al. 1997, Bakke et al. 2007). Nevertheless, the *in vitro* tests were useful in providing a general indication of the suitability of a compound as a treatment and its effective concentration: ineffective *in vitro* botanicals therefore did not require *in vivo* testing, so reducing the number of animals used for the trials.

The most potent treatments against *G. turnbulli* were Chinese freeze-dried garlic powder, freeze-dried garlic flakes and allyl disulphide. The high efficacy of whole garlic preparations is potentially due to synergistic effects of garlic's constituents (Kyung et al. 2012, Williams and Lloyd 2012), but the effectiveness of the freeze-dried garlic treatments in this study may also be accounted for by the preparation process as it alters bioactivity (i.e. freeze drying, mincing, etc., Lemar et al. 2002). For instance, during the freeze-drying process one of the main active compounds in garlic, allicin, becomes concentrated (Ratti et al. 2007). Allicin degrades rapidly, though, hence might not be very efficacious (see Williams and Lloyd 2012). The degradation process may, however, be counteracted by one of allicin's breakdown products, allyl disulphide which, tested at 0.5 mg/mL, was as effective as levamisole against gyrodactylids.

The current study builds upon experiments by Abd El-Galil and Aboelhadid (2012) which indicate that garlic oil administered over 24 h may be effective against gyrodactylids infecting *Oreochromis niloticus* fry in a time and dose dependent manner, and that prolonged exposure may slow the spread of disease. This previous study and our data suggest that gyrodactylids are directly affected by the botanical rather than indirectly via enhanced host immune function, which in fish may take several weeks to develop (Martins et al. 2002, Sahu et al. 2007).

Garlic efficacy against gyrodactylids *in vitro* is dose dependent as shown by previous studies (e.g. Steverding et al. 2005); however, high doses of and/or prolonged treatment may impair fish health highlighting the importance of determining appropriate dosage requirements for the particular host-parasite system and the method of application (Chitmanat et al. 2005, Chapter 5). Furthermore, treatment may also affect fish behaviour: for example, Chitmanat et al. (2005) noted increased opercular movement and random swimming movements in *O. niloticus* fry following garlic treatment. With the exception of allyl alcohol which was applied at 10 mg/mL, all treatments in the current study were used at 1 mg/mL or less which is far below the lethal limit established by Abd El-Galil and Aboelhadid (2012) and Chitmanat et al. (2005). Such comparatively low doses also allow concentration adjustments which may help to counteract natural variation in garlic efficacy. Changes in effectiveness of garlic may be expected due to variable garlic biochemistry which depends on the local climate during plant growth and geographical origin (Burt 2004). Even minor concentration changes have to be monitored carefully, however, and hobbyists who currently use garlic *ad hoc*, must be made aware of the potential harm to their fish of using high garlic doses.

In the UK, garlic treatments are already commercially available, mainly as enhanced feed (e.g. Medikoi Wheatgerm and Garlic food™, Garlic Plus™ flakes, New Life Spectrum Thera A™ and Freshwater flakes with garlic™); however, there are no published data on the efficacy of these products on helminth infection in fish. This study supports the growing evidence of garlic's general anti-helminthic properties indicating that conserved garlic preparations and the garlic compound allyl disulphide can effectively reduce and, in some cases, eliminate infections of the aquarium pathogen *G. turnbulli*.

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CHAPTER 8: *IN VITRO* AND *IN VIVO* EFFICACY OF THE COMMERCIAL ANTI-BACTERIAL AND ANTI-FUNGAL TREATMENTS MELAFIX® AND PIMAFIX® AGAINST *GYRODACTYLUS TURNBULLI* INFECTIONS IN GUPPIES (*POECILIA RETICULATA*)

8.1 Abstract

The demand for both food and ornamental fish has led to a steep rise in aquaculture promoting the emergence and persistence of various infectious diseases. Total therapeutic control of disease outbreaks is rare, but plant compounds may herald potential alternatives effective against a range of pathogens. Melafix® and Pimafix® are based on cajuput (*Melaleuca leucadendron*) and West Indian bay (*Pimenta racemosa*) oils and marketed against bacterial and fungal diseases, respectively. The current study examined Melafix®, Pimafix® and their individual compounds' anti-helminthic properties against *Gyrodactylus turnbulli* infecting guppies (*Poecilia reticulata*). In particular, a combination treatment of Melafix® and Pimafix® was highly effective at reducing *in vitro* survival of parasites from 15 to 2 h, and eradicating 95 % of gyrodactylids *in vivo*. The high efficacy of this combination treatment is attributed to the high content of terpenes and phenols in the cajuput and West Indian bay essential oils and also due to the emulsifier Crovol PK 70. Hence, Melafix® and Pimafix® effectively reduce gyrodactylid burdens on fish thereby increasing the chances of efficient disease control in the aquarium industry.

8.2 Introduction

Overfishing has led to drastic declines in natural fish populations (Jackson et al. 2001) contributing to an > 50% increase in cultured fish for food production (Cressey 2009). The ornamental fish industry also depends increasingly on aquaculture (Tlusty 2002), as the harvest of visually pleasing, often endemic reef fish, affects (often endangered) fish populations directly and indirectly by habitat destruction (Andrews 1990, European Commission 2008). In both the food and ornamental trade, the need for fish is expected to increase further to satisfy the growing demands of consumers which can only be met with the expansion of aquaculture (Tlusty 2002, Cressey 2009). The high fish density in aquaculture does, however, impose additional stressors on individuals, thereby leading to increased susceptibility to diseases (Bondad-Reantaso et al. 2005, Ashley 2007). Development of drug resistance (e.g. Verner-Jeffreys et al. 2009), virulence evolution (Mennerat et al. 2010, Pulkkinen et al. 2010), emerging disease (Murray and Peeler 2005), climate change (Karvonen et al. 2010) and the ban of broad anti-parasitic, but highly toxic compounds such as malachite green and formalin (European Union Biocide Product Directive 98/8/EC, European Council Regulation 2377/90) further intensify the impact of a variety of highly prevalent parasites and pathogens on aquaculture (e.g. Scholz 1999, Kim et al. 2002, Thilakaratne et al. 2003, Hongslo and Jansson 2009). Hence, there is an

urgent need for alternative treatments that are effective and safe for fish, humans and the environment (Citarasu 2010, Chakraborty and Hancz 2011).

The screening of plant extracts has become increasingly important in the search for viable alternatives, with the expectation that individual compounds may have unsuspected anti-parasitic properties that, when combined, act synergistically and in a hyper-susceptible way (Anthony et al. 2005, Athanasiadou et al. 2007, Hu et al. 2010). Particularly essential oils, secondary metabolites produced by aromatic plants, whose relative concentration determine biological activity, have become popular research subjects against a whole range of diseases (Bakkali et al. 2008, Hammer and Carson 2011).

Two oils highly effective against fungi, bacteria, protozoa and other organisms such as mites are cajuput (*Melaleuca leucadendron*) and West Indian bay (*Pimenta racemosa*) (e.g. De Colmenares et al. 1998, Hammer et al. 1999, Valdes et al. 2008, George et al. 2010). The high efficacy of cajuput is not surprising as it is closely related to tea tree (*M. alternifolia*) whose anti-microbial effects are well studied (Carson et al. 2006, Hammer and Carson 2011, Zhu et al. 2011), with work on monogenean infections on fish also indicating strong anti-parasitic effects (Steverding et al. 2005, Chapter 6). Like cajuput, West Indian bay has a wide range of antimicrobial activity including anti-viral, anti-helminthic and larvicidal (Hammer et al. 1999, Burt and Reinders 2003, Saenz et al. 2004, Lee 2006, Yousif et al. 2007, Kim et al. 2008, Meneses et al. 2009, Si et al. 2009). Both oils may reduce the transmission of vector borne diseases (George et al. 2010, Greive et al. 2010) and show strain specific efficacy against microbes (Burt and Reinders 2003, Saenz et al. 2004); however, such effects may also be dependent on seasonal and geographical variation in essential oil constituents (Burt 2004). For instance, cajuput varies significantly in its chemotype depending on its origin, but overall is dominated by terpenes (Cuong et al. 1994, De Colmenares 1998, Farag et al. 2004, Pino et al. 2010). West Indian bay, on the other hand, appears to be dominated by a mixture of phenols and some terpenes (Nadal et al. 1973, McHale et al. 1977) with both chemical classes generally showing high anti-microbial efficacy (Gershenzon and Dudareva 2007, Hammer and Carson 2011).

Cajuput and West Indian bay oils are the essential ingredients in two commercially available treatments marketed for their anti-bacterial and anti-fungal properties, respectively, in the aquarium industry. Melafix® is based on oils of cajuput, whereas the active ingredient of Pimafix® is the oil of the West Indian bay tree (concentration: 1 %). There is some evidence that cajuput and West Indian bay tree extracts may be effective against parasitic worms (Steverding et al. 2005, Yousif et al. 2007); however, neither has been tested against helminths in an aquatic environment. This study aimed to test Melafix® and Pimafix® against gyrodactylids: ectoparasitic helminths that are ubiquitous on teleost

fish and have the potential to cause considerable economic damage in infected fish populations (Bakke et al. 2007). There is no completely effective treatment available against gyrodactylids (Chapter 1.6); hence, we investigated the anti-helminthic properties of the two commercial products and the individual essential oils *in vitro* and *in vivo* using the model system *Gyrodactylus turnbulli* infecting the guppy (*Poecilia reticulata*). Further, we used gas chromatography-mass spectrometry to identify and relative abundance of the individual compounds in cajuput and West Indian bay essential oils.

8.3 Material and Methods

8.3.1 Sources of animals and compounds

In vivo trials were conducted using an inbred strain of ornamental guppies (*Poecilia reticulata*) that have been maintained at Cardiff University since 1997, at 25 ± 1 °C, a 12 h light : 12 h dark cycle and fed at least once daily with Aquarian® fish flakes. Additionally, weekly feeds included live *Daphnia* spp. and frozen *Tubifex*. Parasites were of the *Gt3* strain of *Gyrodactylus turnbulli* which has been isolated from pet shop guppies in Nottingham in 1997.

Two commercially available treatments were tested *in vivo*: Melafix® and Pimafix® (both Aquarium Pharmaceuticals™, MARS Inc.). Their essential oils, cajuput oil and West Indian bay oil (Berje, Bloomfield, USA), were also tested in combination with the emulsifier Crovol™ PK 70 (Croda International Plc.; from hereon: Crovol) for which an additional control treatment was added. Water and levamisole (Levacide 7.5%, Norbrook, UK) were used as negative and positive controls. Both commercial products were tested at the concentration recommended by the manufacturer (Table 8.1) and the concentration of individual components was prepared to match their concentration in the commercial products. All treatments were initially prepared as a stock solution before being applied to the test subjects and levamisole was used at a concentration usually used in our laboratories (1 mL/L).

Samples of cajuput and West Indian bay oils (Berje, Bloomfield, USA) were used in gas chromatography-mass spectrometry analyses. Compounds run as standard were sourced from Fisher Scientific (UK) with the exception of α-Thujene which was obtained from Rose Chemicals (UK).

8.3.2 In vitro

All compounds were applied to individual *G. turnbulli* worms (n = 297; see also Table 8.1) that had been removed from fin clips of infected donor fish using an insect pin (Chapter 5). Following removal from the host, the parasites were transferred individually in 10 µL of water into wells of a 96-well

microtitre plate using a micropipette. Treatments (90 µL) were applied an hour after the worms were isolated and observed for abnormal behaviour before application (Cable et al. 2002).

Table 8.1. Final concentration of treatments (Conc., in µl/L), sample size (N), mean survival (h) with standard error of the mean (SE) and minimum (Min.) and maximum (Max.) survival of *Gyrodactylus turnbulli in vitro* exposed to Melafix® and Pimafix® treatments. Crovol was used relative to its amount in Melafix® (M), Pimafix® (P) and the combination treatment (M/P).

Treatment	Conc.	N	Mean	SE Mean	Min	Max
Levamisole	100	43	0.023	0.023	0	1
Water	-	56	14.875	0.863	3	28
Melafix®	132	23	2.826	0.306	1	7
Pimafix®	132	22	2.182	0.398	0	7
Melafix®/Pimafix®	132 (each)	19	2.263	0.404	1	7
Crovol & cajuput oil	4.8	23	5.174	0.794	1	15
Crovol & bay oil	6.9	22	2.591	0.537	0	8
Crovol & cajuput oil plus crovol & bay oil	4.8 plus 6.9	23	3.565	0.662	1	12
Crovol (M)	3.5	25	3.160	0.382	1	6
Crovol (P)	5.6	18	1.278	0.158	0	3
Crovol (M/P)	9.1	23	1.522	0.242	0	6

8.3.3 In vivo

Guppies (n = 176, standard length = 6.1-27 mm; see also Table 8.2) were infected with parasites by exposure to conspecifics (to mimic natural transmission) that carried the *Gt3* strain of *Gyrodactylus turnbulli* over 3-4 days. Screening for parasites before (Day 1) and after the experiment (Day 8) followed the protocol detailed in Chapter 1.6 whereby fish were anaesthetized with 0.02 % MS222 before their parasites were counted using a dissection microscope and optic fibre illumination. Fish with a parasite load of between three and 200 parasites were isolated and individually maintained in 1 L pots for the duration of the experiment, receiving daily water changes after which treatments at the same concentrations as *in vitro* (Table 8.1) were applied. For the *in vivo* trials, a negative control receiving no treatment, but daily water changes only, was also included. A positive control using levamisole was not included, as a 7 d exposure to levamisole would have been toxic to the fish. Individual, uninfected fish (n = 14) were maintained alongside the experimental fish to control for mortalities.

Table 8.2. Final concentration of treatments (Conc., in µL/L), sample size (N), mean efficacy and standard error of the mean (SE) of Melafix® and Pimafix® treatments against *Gyrodactylus turnbulli* infecting guppies (*Poecilia reticulata*). Efficacy range was 0-1 for all treatments, but for Crovol (M) which was 0-0.79.

Treatment	Conc.	N	Mean	SE Mean
Water	-	31	0.226	0.069
Melafix®	132	20	0.652	0.102
Pimafix®	132	20	0.320	0.088
Melafix®/Pimafix®	132 (each)	21	0.950	0.048
Crovol & cajuput oil	4.8	12	0.401	0.131
Crovol & bay oil	6.9	13	0.217	0.115
Crovol & cajuput oil plus crovol & bay oil	4.8 plus 6.9	18	0.422	0.097
Crovol (M)	3.5	12	0.200	0.090
Crovol (P)	5.6	13	0.205	0.097
Crovol (M/P)	9.1	12	0.416	0.129

8.3.4 Essential oil characterization and quantification

The essential oil samples were prepared for gas chromatography-mass spectrometry (GC-MS, Agilent 7890 GC coupled to an Agilent 5975C MS) analysis by diluting 0.2 g in 100 mL diethyl ether to which 200 µL of 2.5 % (m/v) 5-methylhexan-2-one was added. The samples (1 µL) were applied directly on to the GC column by cold-on-column injection, using an Agilent 7693 autosampler at 35 °C. The GC was equipped with two columns, a phenomenex non-polar DB5 (5 % diphenyl/ 95 % diemthyl siloxane) column and a phenomenex polar FFAP (nitroterphthalic acid modified polyethylene glycol) column. Both columns were 30 m in length, 0.25 mm diameter, with 0.25 µm film thickness, and were connected to an Agilent Deans switch which enabled the MS to connect automatically to the required column. Helium was used as carrier gas on each column.

The GC injector and oven temperature were initially held at 35 °C for 1 min, then increased to 240 °C at 3 °C / min and held for 5 min with a total run time of 77 min. The transfer line to the MS was heated to 220 °C. The MS ionisation was carried out by electron impact mode at 70 eV, source temperature 230 °C. The mass scan range was 40 to 550 atomic mass units, with a scan speed of 2.86 scans / s. Blanks were run between each sample to ensure complete clean-out of the column before each component analysis.

Chromatographs were analysed in the Enhanced Agilent MSD ChemStation software, with tentative identification of mass spectra peaks through comparison to Wiley and NIST 2.0 libraries. Identification of the peaks was completed by comparison of retention indices (RI) and mass spectra to authentic standards. RI were calculated on both polar and non-polar columns from the retention times

of n-alkanes (C_7 - C_{26}) by linear interpolation. Quantification was not carried out on the identified compounds; however, the relative abundance of each compound was calculated by comparison of peak area to the internal standard, 5-methylhexan-2-one. Using this quantification technique gives only a rough guide on the relative amount of compounds within one batch (Bicchi et al. 2008, Rubiolo et al. 2009, IOFI Working Group on Methods of Analysis 2011). Hence, for this study the constituents will be presented ranked, rather than with their relative abundance.

8.3.5 Statistical analysis

In vitro data were analysed using a Cox proportional hazard survival analysis with maximum survival of individual worms (n = 297) as the dependent variable. As nesting is not possible in a survival analysis protocol, all Crovol applications at different concentrations were treated as independent treatments.

Efficacy of *in vivo* treatments was calculated based on the initial and final parasite burdens of infected fish to give an efficacy value of between 0 (not effective) and 1 (effective, see Chapter 5). A non-parametric Kruskal-Wallis test established differences between treatment groups which was followed up with individual Mann-Whitney tests. Multiple testing was controlled for with a modification to the Bonferroni procedure after Benjamini and Yekutieli (2001, see also Narum 2006) leading to a new α level of $\alpha = 0.011377$. All analyses were performed in R 2.13.2 (R Development Core Team 2012).

8.3.6 Ethical note

This work was conducted under UK Home Office licence (PPL 30/2357) and approved by the Cardiff University Ethics Committee. All fish were closely monitored throughout the trials: if their behaviour and welfare appeared to be influenced negatively by parasites, fish were immediately treated with parasiticide to clear them from helminth infections with the treatment being recorded as ineffective.

8.4 Results

Both *in vitro* and *in vivo* a combination treatment of Melafix® and Pimafix® was highly effective against gyrodactylids reducing *Gyrodactylus turnbulli* survival to approximately 2 h *in vitro* and being 95 % efficacious *in vivo* (Tables 8.1, 8.2, Figs. 8.1, 8.2). Crovol (with and without essential oils), however, shows a similar efficacy to the commercial products indicating that the anti-helminthic properties might not be due to the essential oils. Both oils contain high amounts of terpenes and phenols.

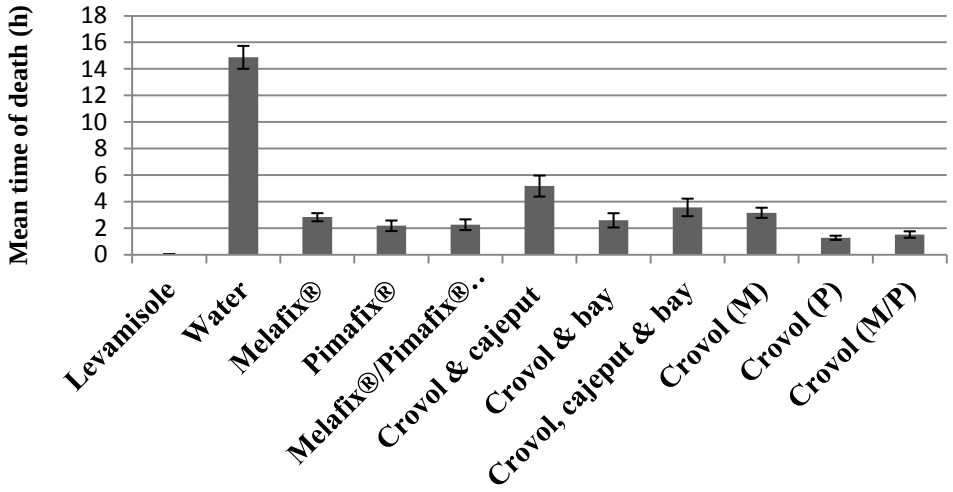

Fig. 8.1. Mean *in vitro* time of death of *Gyrodactylus turnbulli* (± standard error of the mean). Crovol was used relative to its amount in Melafix® (M), Pimafix® (P) and the combination treatment (M/P).

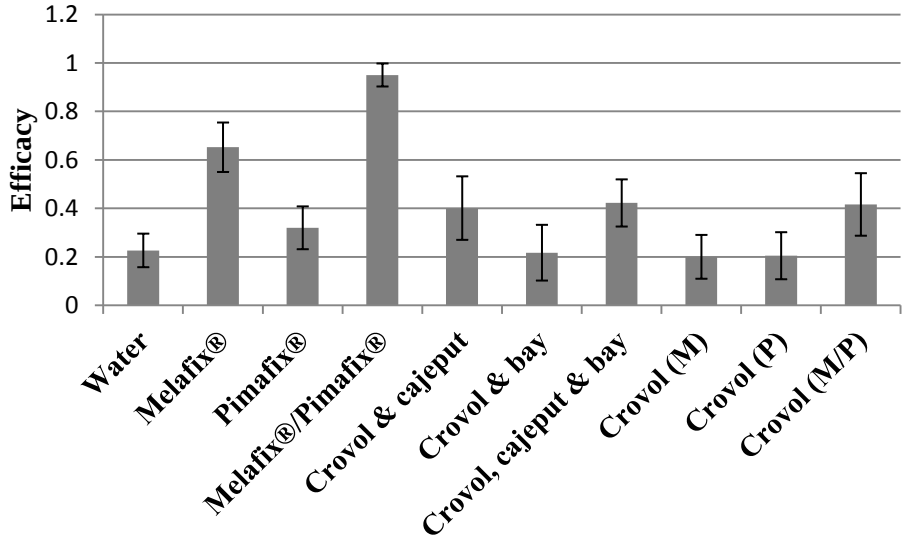

Fig. 8.2. Mean *in vivo* efficacy (± standard error of the mean; 0 = not effective, 1 = effective) of treatments tested *in vivo* against *Gyrodactylus turnbulli* infecting guppies (*Poecilia reticulata*).

8.4.1 In vitro

Parasites survived up to 28 h off the host in aquarium water (mean: 14.9 h) and were killed instantly when levamisole was applied (mean survival: 0.02 h). Overall, there was a significant difference in survival between treatments (survival analysis: Likelihood ratio test = 213.2, d.f. = 10, $p < 0.001$). All treatments significantly reduced survival time of worms when compared with aquarium water ($z \geq 4.217$; $p > 0.001$) and all were as effective as levamisole ($z \leq 1.926$, $p \geq 0.05$) except Crovol with

cajuput oil which was slightly less effective (z = 2.436, p = 0.015; Appendix VII.1). Crovol, at all concentrations tested, showed high efficacy against gyrodactylids. When essential oils were added there was a trend for efficacy to decrease (Tables 8.1, 8.2). Also, there appears to be a dose dependent relationship of Crovol efficacy.

8.4.2 In vivo

There was a significant difference in the efficacies of treatments (Kruskal Wallis: $\chi^2 = 50.9$, df = 9, $p < 0.001$) with Melafix® and the combination treatment Melafix®/Pimafix® were highly efficacious compared to the control (Mann Whitney tests: W = 167, p = 0.002 and W = 64.5, $p < 0.001$, respectively). No other treatments were significantly different from the control (Appendix VII.2).

8.4.3 Gas chromatography-mass spectrometry

Characteristics and quantities of oils are summarised in Table 8.3. The chemical compostion of cajuput oil was dominated by various terpenes consisting largely of 1,8-cineole and limonene, whereas West Indian bay contained mainly a mixture of the phenols eugenol and chavicol and the monoterpene myrcene.

8.5 Discussion

Both Melafix® and Pimafix® have been tested as 95 % effective against *Gyrodactylus turnbulli in vitro* and *in vivo* in this study. Tests on the emulsifier indicate Crovol itself have, however, shown some anti-helminthic effect. Consequently, the high efficacy of the Melafix®/Pimafix® combination treatment *in vivo* could be explained by not only a combined positive effect of the essential oils, but also by the overall amount of Crovol. The chemical compostion of cajuput oil was dominated by various terpenes consisting largely of 1,8-cineole and limonene, whereas West Indian bay contained mainly a mixture of the eugenol, myrcene and chavicol.

Table 8.3 (overleaf). Compounds identified in (A) cajuput and (B) West Indian bay oils and presented with their linear retention indices (LRI) for the non-polar (DB5) and polar (FFAP) gas chromatography-mass spectrometry columns, peak area on the FFAP column and ranked, relative abundance of compound. Ranks for DB5 column are not presented due to the co-elution of 1,8-cineole and limonene. Identification by comparison of mass spectrometry result with NIST or Wiley libraries (MS), direct comparison with pure standard (STD), comparison to previously published LRIs (LRI). Nd = not detected. * Tentative identifications. NA: not applicable (compound not detected on FFAP column.

(A) Component	**LRI DB5**	**LRI FFAP**	**Area**	**Rank**	**Identification**
1,8-Cineole	1029	1190	5731.71	1	MS, STD, LRI
4-Terpineol	1177	1587/1588	226.29	6	MS, STD, LRI
α-Pinene	928	1007	149.82	9	MS, STD, LRI
α-Terpinen	1013	1160/1161	67.42	11	MS, STD, LRI
α-Terpineol	1192	1686	197.75	7	MS, STD, LRI
α-Thujene	922	1013	10.94	15	MS, STD, LRI
β-Pinene	972	1086/1087	165.09	8	MS, LRI
Camphene	944	1044	trace	16	MS, STD, LRI
Camphor	1142	1488	57.14	13	MS, STD, LRI
Cymene*	1021	1253	586.18	3	MS, STD, LRI
γ-Terpinen	1055	1228	290.29	4	MS, STD, LRI
Limonene	nd	1181	968.86	2	MS, STD, LRI
Myrcene	987/988	1153	109.82	10	MS, STD, LRI
Phellandrene	1002	1146	56.38	14	MS, STD, LRI
Sabinene	968	1105	230.88	5	MS, STD, LRI
Terpinolen	1081	1263	61.08	12	MS, STD, LRI
(B) Component	**LRI DB5**	**LRI FFAP**	**Area**	**Rank**	**Identification**
1-Octen-3-ol	979/980	1446/1447	43.25	10	MS, STD, LRI
3-Octanol	nd	1390	21.05	14	MS, STD, LRI
3-Octanone	984	1244	46.85	9	MS, STD, LRI
4-Terpineol	1176/1177	1587	34.39	13	MS, STD, LRI
α-Farnesene	1512/1513	nd	NA	NA	MS, LRI
α-Pinene	nd	1007	13.50	17	MS, STD, LRI
α-Thujene	928	nd	NA	NA	MS, STD, LRI
Chavicol	1253/1254	2333	1421.99	3	MS, LRI
Cymene	1021	1252/1253	41.50	11	MS, STD, LRI
δ-Cadinene*	nd	1730	17.73	16	MS, LRI
Eugenol	1349/1350	2158/2159	8634.09	1	MS, STD, LRI
Limonene	1025	1179	168.83	4	MS, STD, LRI
Linalool	1098/1099	1544	166.09	5	MS, STD, LRI
Methyleugenol	1397	2010	150.83	6	MS, STD, LRI
Myrcene	988	1153/1154	1435.75	2	MS, STD, LRI
Ocimene	1044/1045	1240	51.03	8	MS, STD, LRI
Phellandrene	1002	1146/1145	19.75	15	MS, STD, LRI
trans-Caryophyllene*	1412/1413	1565	140.12	7	MS, LRI
Unknown (possibly 1,8-Cineole + another monoterpene)	NA	1187	37.95	12	

Previous studies on the survival of *G. turnbulli in vitro* reported similar mean survival times, but showed a higher effectiveness of compounds *in vitro* and *in vivo* when tested against the same host-parasite combination (Chapters 5, 6). Indeed, cajuput applied with Tween 20 using a slightly higher concentration and a different application method eradicated *G. turnbulli* infections completely and was as good as the laboratory de-wormer levamisole (Chapter 6). Such high effectiveness could not be repeated in the current study, which used a longer duration of exposure and a lower dose. The most effective treatment was the Melafix®/Pimafix® combination, with an efficacy of 95 %. Generally, it is desirable to have 100 % efficacy as just a single parasite remaining can lead to a new epidemic (Cable and Harris 2002). In the long term, repeated cycles of partially effective treatments may also lead to higher virulence and drug resistance (Gandon et al. 2001, Verner-Jeffreys et al. 2009, Pulkkinen et al. 2010).

The gas chromatography-mass spectrometry results confirm previous studies on the same oils from different sources (e.g. Farag et al. 2004, Kim et al. 2008), but also explain their anti-parasitic activity. Both terpenes and phenols are dominant in essential oils generally and are well known for their anti-microbial properties (Gershenzo and Dudareva 2007, Bakkali et al. 2008, Hammer and Carson 2011). Cajuput oil was dominated by 1,8-cineole (Farag et al. 2004, Pino et al. 2010). Important to note is that in cajuput oil 1,8-cineole co-occurs with limonene: the combination of these constituents may cause antagonistic, synergistic or additive interactions against microbes depending on the ratio and the limonene entantiomer present (van Vuuren and Viljoen 2007). Both the ratio of 1,8-cineole to limonene and the presence of limonene entantiomers can vary seasonally and geographically with cajuput (Burt 2004), but also depends on which *Melaleuca* spp. is used for extraction as several species of the same genus are referred to as cajuput (Craven 1999).

West Indian bay oil was dominated by eugenol, myrcene and the tentatively identified chavicol. Previous reports indicate the same constitution for bay oil (Nadal et al. 1973, Mc Hale et al. 1977) indicating great consistency in the composition of bay oils. Eugenol and chavicol are anti-microbial but there is little evidence of anti-microbial activity from myrcene (e.g. Caccioni and Guizzardi 1994, Blaszyk and Holley 1998, Walsh et al. 2003, Chang et al. 2008). To the knowledge of the authors, no study has investigated their anti-parasitic effects. Based on the tests utilizing the whole plant oil in this study against *G. turnbulli*, none of the three constituents has anti-parasitic properties, at least not at the concentrations used.

Overall, Melafix® and Pimafix® are effective as a combination treatment against gyrodactylids; however, the efficacy stems not only from the essential oils, but also the emulsifier Crovol. For a

seven day treatment, treatment dose might have to be increased slightly for 100 % efficacy which is desirable in both the short and long term.

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CHAPTER 9: GENERAL DISCUSSION

9.1 Summary

Parasites continue to threaten both humans and wildlife across the globe and urgent research is needed to understand the basic biology as well as novel treatment methods for controlling disease outbreaks. This thesis focuses on a host-parasite model in an aquatic ecosystem; the guppy-gyrodactylid system. The reproductive and transmission biology of these parasites and the host-parasite dynamics in an extreme environment that is naturally inhabited by the guppy (*Poecilia reticulata*) were investigated in the first part of this thesis. In the second part, the efficacy of botanical and traditional treatments *in vitro* and *in vivo* were tested on *Gyrodactylus turnbulli* and *G. bullatarudis* infected fish with the overall aim to work towards an integrated disease management plan for aquaculture and ornamental hobbyists. The results demonstrate that: (i) gyrodactylids engage in sexual reproduction and show hybrid fitness and/or inter-strain competition (Chapter 2); (ii) transmission is mainly driven by direct contact with potential hosts, and is host-size dependent (Chapter 3); (iii) extreme habitats provide a refuge from microbial and gyrodactylid infections (Chapter 4); (iv) treatment efficacy may be species specific (Chapter 5); and (v) salt, garlic, cajuput oil and a combination of two commercially available treatments Melafix® and Pimafix® are efficacious alternatives for broad anti-parasitic aquarium treatments against gyrodactylids (Chapters 5-8).

Many disease-control approaches require a detailed knowledge of parasite biology and often target the disruption of parasite life-cycles. For indirectly transmitted parasites in aquaculture (such as digeneans), this can generally be achieved through appropriate host maintenance. In the case of directly transmitted gyrodactylids, appropriate fish maintenance may be more difficult as transmission depends on physical contact between hosts which is very much a function of the shoaling behaviour that occurs in guppies (Croft et al. 2011, Johnson et al. 2011, Richards et al. 2012). Gyrodactylid transmission is therefore limited by the number of social interactions between potential guppy hosts (Croft et al. 2004, 2005, 2006, 2011, Richards 2010). For gyrodactylid parasites that remain on the host when it dies, there is only a 24 h window of opportunity to locate a new host (Chapter 3). When detached, drifting in the water/air interface can be a successful way of locating new hosts (Soleng et al. 1999). For example, *G. turnbulli* migrates into the water/air interface where there is a presumed increased likelihood of finding one of its surface-feeding guppy hosts (Cable et al. 2002). The infection risk by detached parasites, however, is expected to be relatively low (Høgåsen et al. 2009). The mode of transmission from dead hosts may also occur through cannibalism, an event observed in stagnant water in wild guppy populations in Tobago (Cable, Mohammed and van Oosterhout, personal communication).

The transmission-virulence trade-off hypothesis suggests that increased transmission rates lead to increased parasite induced mortality and *vice versa*, particularly in ectoparasites (Clayton and Tompkins 1994, Lipsitch and Moxon 1997, Day 2001, 2003, Ebert and Bull 2003). There is certainly evidence that aquaculture promotes virulence evolution (Nowak 2007, Mennerat et al. 2010, Pulkkinen et al. 2010) and, due to the high stocking density of animals in some systems, it follows that the transmission rate of parasites should also be increased. With viviparous gyrodactylids, however, transmission and virulence evolution might be indirectly enhanced through sexual recombination and/ or intra-specific competition and the resulting fitness consequences for the population (Chapter 2). After giving birth to the second daughter, gyrodactylids have the potential to reproduce sexually as all reproductive organs are fully developed by that time (Cable and Harris 2002). It is still unclear as to which reproductive modes prevail once worms are sexually mature, although this is expected to depend on environmental conditions (such as crowding, Harris 1989). Other selection pressures on reproduction such as anti-parasitic treatments cannot be excluded; the switch from parthenogenetic or asexual reproduction (expected to prevail at low selection pressures) to sexual recombination may result in new gene combinations with consequential positive effects on transmission and/ or virulence. Hence, anti-parasitic treatment should be applied in a high dose during acute disease outbreaks, to ensure that the treatment is highly effective and in doing so, reduce any subsequent selection for changes in parasite virulence and transmission. Furthermore, outbreaks should be addressed with species-specific treatments to avoid inadvertent selection of other parasites that may be prevalent at low levels but which may be 'selected' to become more virulent with time. For example, the highly effective botanical cajuput tested in Chapter 6, despite being effective against gyrodactylids has no apparent effect on the protozoan fish parasite of cichlids, *Spironucleus vortens* (see Paull and Matthews 2001, Appendix IV). Hence, treatment against gyrodactylids with cajuput is not expected to select for the suspected causative agent of Hole-in-the-Head disease in co-infected individuals.

Continuous exposure to treatments is advised against, as this may not only speed up virulence evolution, but may also lead to altered immune genes, as is the case for guppies living in an extreme environment in which they find refuge from pathogens (McMullan, Mohammed, Schelkle, Cable, van Oosterhout, unpublished data). Release from such environments leads to fish becoming diseased and dying (Chapter 4) or the opportunity for other parasites to utilize the previously uninfected fish temporarily, such as *Ieredactylus rivulus* infecting Pitch Lake guppies, with the potential for long term adaptation (Appendix I).

During this project, the only compounds that showed the potential to be effective treatments against gyrodactylids on guppies are salt (Chapter 5), cajuput oil (Chapter 6), garlic (Chapter 7), and a

combination treatment of two commercially available treatments Melafix® and Pimafix®, marketed against bacterial and fungal infections, respectively (Chapter 8). The high efficacy of the treatments tested may, however, be species-specific; salt, for instance, had a more negative effect on *G. turnbulli* than *G. bullatarudis*. In the case of co-infections, selective efficacy may lift niche competition and lead to competitive release (e.g. de Roode et al. 2004, Wargo et al. 2007). Hence, the selection of a particular treatment must always consider which parasites are present, as well as the ecology, co-infection biology and evolution of the disease causing agents.

The effectiveness of botanicals against parasites is often due to their volatile constituents which raises problems with reduced 'shelf-life' (Edris 2007, Hammer and Carson 2011). Furthermore, difficulties with potential treatments appear as drug development is an expensive, legislation-heavy process, and often putative drugs end up being abandoned, particularly in small industries such as the ornamental fish industry (Woods and Knauer 2010; Gary Jones, personal communication). As drug discovery and development may not be able to keep up with the evolutionary pressure pathogens are exposed to, an integrated approach to disease management is necessary to depress parasite evolution. Such approaches should include a high standard for animal maintenance focussing on 'prevention being better than cure'. In case of disease outbreaks in aquaria, all fish within a tank should ideally be individually treated with a highly effective treatment and an appropriate method (i.e. treatment baths for a short duration or solutions exposing fish to effective medication at a low dose for several days). Keeping fish in separate holdings, they should not be re-introduced into the tanks for a minimum of two days to ensure that all dislodged parasites that may potentially be in the tank are dead. Ideally, maintaining infected hosts separately should continue for up to two weeks in case fish are still infected with a low number of worms. A long quarantine allows for an immune response to be fully mounted after initial infection and hopefully rid infected hosts from any remaining parasites, as just one single, pregnant worm can initiate a new epidemic. Should an individual not be able to withstand the infection after partially effective treatments, isolation ensures that the parasites are not passed on to other fish anew. Addition of a small amount of salt (approx. 3g/L) into the water during quarantine and potentially after re-introduction of all fish into the main tank may also help towards impairing renewed parasite establishment, at least in the case of *Gyrodactylus turnbulli*, should treatments be only partially effective (Chapter 5).

The suggested procedure to eradicate gyrodactylids is work intensive, but may be a realistic compromise for aquarium owners seeing that gyrodactylids can maintain infections on individual fish for over 55 days (Chapter 5) and mass treatment of fish is often unsuccessful (personal observations). Further, the results from Chapters 5-8 suggest that, even with highly effective treatments, individual worms may remain on fish after treatment justifying the caution in preventing re-occurring epidemics.

In water recirculation systems, isolating infected tanks from the main cicuit is most important to prevent disease spread and, ideally, the fish should be treated against gyrodactylids in small, individual aquaria provided the parasites are still contained within the one tank. The suggestion of maintaining fish individually, however, is unrealistic once the disease has spread to several hundred fish. In case of *G. salaris*, the most effective way of dealing with a large number of infected stock is the complete disinfection and drying out of fish farms (Bakke et al. 2007). Mass treatment of fish or continuous exposure to low dose medication only reduces infectious disease to manageable levels for a short time causing time spans between treatments to be reduced and even greater problems regarding drug resistance in the future (Nowak 2007, Mennerat et al. 2010, Pulkkinen et al. 2010).

9.2 Suggested further work

The studies conducted for this thesis have successfully addressed specific questions on the reproductive biology, transmission and the effect of extreme environments on gyrodactylids, combining them with toxicological studies on the survival of helminths *in vitro* and *in vivo* using traditional anti-parasitic (salt, Chapter 5) and potential botanical alternatives (garlic and various other botanicals, Chapters 6-8). This work has highlighted the need for further in research all of these areas. For instance, now that sexual reproduction has, for the first time, been confirmed in viviparous gyrodactylids (Chapter 2), it opens up a range of research opportunities. For instance, a study to determine the relative effects of hybrid fitness and intra-specific competition is needed, but requires at least three similar parasite strains. Mixed infections need to be established utilizing two parasite species or strains as in Chapter 2, with one co-infection treatment using strains that can sexually reproduce and another treatment using strains that cannot interact in such way. Such experimental set up should give an indication whether the fitness effects observed in mixed parasite populations (Chapter 2) are due to hybridisation effects or intra-specific competition. Ideally, such an experiment should utilize several replicates of different strain combinations (or several sets of three strains). Further, to increase the likelihood of detecting hybrid genotypes, a larger number of molecular markers is required.

Mixed parasite strain infections have been shown to produce a different epidemiological outcome to single strain infections present on the host. The understanding of mixed infections determined in this study can therefore be used to understand the population level effects of mixed infections which are often neglected in host-parasite studies. With global fish transport and biological invasions facilitated through anthropogenic activities, the breakdown of geographical barriers between two inbred parasite strains is an extremely realistic scenario. Knock-on effects of such events may include changes to infectivity, transmission and virulence of infectious disease agents including characteristics only detectable when observed in fish populations as opposed to on individually maintained hosts.

Given that gyrodactylid sexual reproduction has been shown to occur, it is important to consider whether this mode of reproduction prevails once all reproductive organs are mature or whether sexual reproduction is context dependent. For example, under conditions of low stress, asexual or parthenogenetic reproduction should predominate (Tomlinson 1966, Crow 1994, Dawson 1995), whereas sexual recombination, a genetically costly strategy, may only be induced when environmental conditions are adverse to increase the chances of survival, fitness and transmission in the offspring (Colegrave 2002, D'Souza and Michiels 2010, Becks and Agrawal 2012). Changes in environmental conditions may include the use of sexual reproduction to gain a genetic advantage and reduce selection pressures when populations are crowded (see Harris 1989) or when water conditions are adverse, such as in Trinidad's Pitch Lake (Chapter 4). Gyrodactylids on guppies are exposed to adverse water conditions in aquaculture systems when treatments are applied or in polluted or estuarine habitats in the wild. Indeed, gyrodactylids (and other ectoparasites) are strongly affected by migrations of diadromous fish between freshwater and marine environments; *Gyrodactylus salaris* is most harmful to Atlantic salmon (*Salmo salar*) smolt, thus affecting population recruitment, but once the fish age and migrate into saltwater, the parasite is shed (Soleng and Bakke 1997, Bakke et al. 2007, Longshaw et al. 2010).

Parasite transmission in the *G. turnbulli* and *G. bullatarudis* guppy system is mainly due to host behaviour, which strongly supports current research on the degree to which social interactions, individual host behaviour and personality influence host contact (Barber et al. 2000, Barber and Dingemanse 2010, Richards 2010, Croft et al. 2011, Richards et al. 2012). Thus, environments in which fish are maintained should aim to decrease host population density significantly to reduce infectious disease transmission. This, however, requires space, impacts on overall productivity and may not be a solution if fish, through their shoaling activity, stay in close proximity to each other resulting in a density independent transmission of disease (Johnson et al. 2011).

Overall, disease prevention through the understanding of host and parasite biology may be a better solution when compared to drug treatment; however, disease outbreaks cannot always be successfully prevented. Environmentally 'friendly' treatments are therefore desirable and those botanicals identified to be effective against gyrodactylids may fulfil this criterion as natural extract degradation is faster than that recorded for synthetic compounds. All those potential treatments identified here need to be further trialled before their use is recommended. Firstly, the effectiveness of treatments such as cajuput should be established for other host parasite systems and with co-infected fish to ensure clear understanding of how mixed parasite communities may be affected by species specific treatments. Extended testing may also establish whether botanicals have toxicological effects on other

fish species, as the guppy is known to be a very hardy and robust fish. Secondly, the effect of treatments on fish behaviour, including feeding response and social interactions, needs investigating with the latter potentially affecting transmission positively if fish aggregate more as a result of stressful, but ineffective treatment.

9.3 Conclusions

The eradication of many infectious diseases is impossible as suggested by advances in theoretical modelling of infectious disease which links the success of parasite extinctions to the basic reproductive number (R_0; describes how many new infections arise from one infected individual) and the presence of 'superspreaders' in a population (see Roberts and Heesterbeek 2003, Heffernan et al. 2005). In fact, extinction of parasites is not desirable in many ecological systems as they play an important role in host population dynamics and food webs (e.g. Hudson and Dobson 1989, Cattadori et al. 2005, Lafferty et al. 2006). For the purpose of animal health and high productivity in 'anthropogenic' (i.e. farm or domesticated) environments, however, infectious diseases need to be better managed (Débarre et al. 2012). Improved maintenance can be achieved via a thorough understanding of the basic biology, ecology and disease epidemiology of the parasite in question which allows us to ensure a sustainable long term use of captured fish populations, for both food and ornamental purposes. This thesis has contributed to such knowledge in the gyrodactylid-guppy system, but it is hoped that the application of this understanding can also be transferred to other disease systems.

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APPENDIX I: *IEREDACTYLUS RIVULI* N. GEN. *ET* SP. (MONOGENEA: GYRODACTYLIDAE) FROM *RIVULUS HARTII* (CYPRINODONTIFORMES: RIVULIDAE) IN TRINIDAD*

I.1 Abstract

A new genus and species of Gyrodactylidae, *Ieredactylus rivuli* gen. *et* sp. nov. (Platyhelminthes, Monogenea), is described from the skin of Hart's Rivulus (*Rivulus hartii* Boulenger), a cyprinodontiform fish collected from streams of the Caroni and Oropouche drainages and the Pitch Lake in Trinidad (prevalence all localities: 16.7-94.6 %; mean parasite intensity 1-9 parasites/fish; range 1-34) with the type originating from a tributary of the Aripo River. This viviparous monogenean is distinctive from other genera of Gyrodactylidae by its split ventral bar membrane, the shape of its male copulatory organ, the presence of two conical accessory pieces associated with the hamulus root and two differently shaped marginal hook sickles. Its unique rDNA sequence shows the closest ITS2 similarity (70 %) to *Gyrodactyloides andriaschewii* Bychowsky *et* Poljansky, 1953. The presence of *I. rivuli* gen. *et* sp. nov. in the Pitch Lake indicates an adaptation to extreme environmental conditions such as high temperatures, hydrocarbons and adverse pH. Guppies may potentially serve as temporary hosts. The parasite displays distinct behaviours, including a characteristic 'swimming-like' movement. The ecology and phylogeny of *I. rivuli* gen. *et* sp. nov. is discussed in relation to the diversity of other gyrodactylids in Trinidad.

I.2 Introduction

In Trinidad and Tobago, only *Gyrodactylus bullatarudis* Turnbull, 1956 and *Gyrodactylus turnbulli* Harris, 1986 from the guppy *Poecilia reticulata* Peters, and *Gyrodactylus pictae*, Cable, van Oosterhout, Barson et Harris, 2005 from the swamp guppy *Micropoecilia* (=*Poecilia*) *picta* (Regan) have been recorded in genus Gyrodactylidae (Platyhelminthes, Monogenea). Currently, due to the lack of parasite surveys nothing is known of the gyrodactylid fauna from non-poeciliid fish in Trinidad and Tobago (see Harris et al. 2004).

One of the most abundant predators of guppies in Trinidad is Hart's Rivulus (*Rivulus hartii* Boulenger). Its diet is principally represented by invertebrates, but it will also feed on small guppies in areas of shared habitat (Endler 1978), potentially allowing parasite transmission between host species. Host switching is reportedly the major driver of speciation amongst the Gyrodactylidae (see Ziętara and Lumme 2002) leading to conjecture that interacting host species, including predator-prey (e.g. *R. hartii* - *P. reticulata*), might share related parasite species. Individuals of *R. hartii* (locally known as the Jumping Guabine) can travel short distances across land allowing them access to a range

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of waterways (Froese and Pauly 2012). This expands the contact network between this species and other indigenous fish and therefore raises the potential for host switching (Bakke et al. 2007).

In the current study, *R. hartii* specimens collected in streams and Trinidad's Pitch Lake, co-inhabited by guppy (*P. reticulata*) populations, were examined for parasites. A monogenean parasite was recovered, the opisthaptoral armature of which differs from the three known Trinidadian species of *Gyrodactylus*. Further, unique behavioural, morphological and molecular differences identified this parasite as belonging to a new gyrodactylid genus.

I.3 Material and Methods

I.3.1 Origin of samples

Specimens of *Rivulus hartii* (standard length 12.5-49.1 mm, weight 0.028-1.366 g) were sampled in 2004 (n = 9) and 2006 (n = 5) from Pitch Lake, southern Trinidad (Grid. Ref.: UTM 20P - 650341.45 E, 1131668.93 N), in 2006 (n = 37) and 2008 (n = 6) in the Naranjo tributary of the Upper Aripo River in northern Trinidad (UTM 20P - 692498.44 E, 118257.53 N), and in 2010 (n = 41) from the Upper Guanapo River (UTM 20P - 689796.25 E, 1183101.4 N). In addition, 118 specimens of *Poecilia reticulata* (standard length 4.9-29 mm, weight 0.003-0.489 g) collected from the Pitch Lake over the period 2004 and 2006 were screened for gyrodactylids.

Fish were euthanised with an overdose of anaesthetic MS222 (Sigma, Poole, UK) and stored individually in 90 % ethanol until examined under a dissection microscope using optic fibre illumination. Attached gyrodactylids were carefully removed from the host using entomological pins and transferred into 0.5 mL Eppendorf tubes containing 90 % ethanol; no other ectoparasites were detected.

I.3.2 Light microscopy

From the monogenean material collected, a total of 22 specimens was prepared as either whole mounts (n = 5) or partially digested (n = 17) for morphological analysis. Undigested, whole mounts were prepared by rinsing alcohol preserved specimens in distilled water, staining for 5 min in Mayer's paracarmine, differentiated in 50 % acid alcohol and then dehydrated through an alcohol series to 100 % alcohol and cleared in clove oil. Specimens were then slide mounted in Pertex (Histolab, Gothenburg, Sweden). Partially digested mounts (which included the type specimens) were prepared as described by Paladini et al. (2009) before being mounted in ammonium picrate glycerine. Seven specimens, including the two specimens collected from *P. reticulata*, had their opisthaptors excised

which were subsequently prepared for morphological identification using the digestion protocol cited above, whereas the corresponding body was fixed in 90% ethanol for subsequent molecular analyses.

The morphology of parasites was studied using an Olympus BH2 compound microscope at ×40 and ×100 oil immersion, a JVC KY-F30B 3CCD Zeiss AxioCam MRc digital camera with an ×0.75 lens and KS300 ver. 3.0 image analysis software (Carl Zeiss Vision GmbH 1997). A total of 30 point-to-point measurements were made on the opisthaptor based on the methods for 25 measurements described by Shinn et al. (2004) with an additional five measurements (HAPL: hamulus accessory piece length measured from the root to the tip of the accessory piece; HAPW: hamulus accessory piece width measured as the width at the root; DBL×W: dorsal bar length × width; DBAPL: dorsal bar attachment point length) to provide a comprehensive account of the new gyrodactylid genus. The ventral bar membrane was measured in the median point across the whole length of both membrane parts. Whole body measurements (12 in total) included the total body length × width (TBL×W), opisthaptor length × width (HL×W), anterior pharynx length × width (APL×W), posterior pharynx length × width (PPL×W), pharynx process length (PProL), male copulatory organ length × width (MCOL×W) and the length of the male copulatory organ principal spine (MCOPS). All measurements are given in micrometres (μm).

I.3.3 Scanning electron microscopy (SEM)

Five alcohol preserved specimens were prepared for SEM analysis of the opisthaptoral hard parts. Specimens were placed in a drop of distilled water on a 12 mm diameter round glass coverslip (Chance Propper Ltd., Warley, UK), which in turn was attached to a glass slide using a drop of water. Tissue surrounding the opisthaptoral hooks was removed by adding 2.5 μL of 10× digestion buffer consisting of 75 mM Tris-HCl pH 8.0, 10 mM EDTA, 5 % sodium dodecyl sulphate (SDS) and proteinase K to a final concentration of 100 μg/mL (Harris et al. 1999) and incubating at 55 °C for 10 min. The specimens were then re-hydrated with a drop of distilled water and examined under a dissection microscope. If necessary, a further 2.5 μL of 10× digestion buffer was added, and the sample was re-incubated for an additional 10 min. The dried digested specimen remained superficially adhered to the coverslip and, while viewed under a stereomicroscope, was washed 3-4 times with distilled water to produce tissue-free hook preparations. The specimen was then air-dried and the coverslip was attached to a 0.5” aluminium pin stub (Agar Scientific Ltd., Essex, UK) using double-sided carbon tape and sputter-coated with gold prior to examination with a LEO 1450VP scanning electron microscope at 20 kV.

I.3.4 Molecular identification

DNA from seven gyrodactylids (five from *R. hartii*, two from *P. reticulata*) was individually extracted in 15 µL TE buffer including 3 µg of proteinase K and 0.45 % Tween 20 by incubating the mixture at 65 °C overnight and neutralising it at 95 ºC for 10 min. rDNA was amplified using a forward P3b and a reverse P4 primers (Cable et al. 2005) which anneal to the 18S and 28S, respectively. Amplifications were carried out in a Perkin Elmer thermocycler (9700) using an initial denaturation of 95 ºC, followed by 35 cycles of 94 ºC for 30 s, 50 ºC for 1 min, 72 ºC for 2 min and a final extension of 72 ºC for 10 min. PCR products were purified using Exonuclease I and SAP (Shrimp Alkaline Phosphatase) (BioLabs) and both strands were sequenced using BigDye (ver. 3.1; Applied Biosystems) on an ABI3100 sequencer. Strands were manually aligned and corrected using the program BioEdit (Hall 1999).

The consensus sequences from the seven individuals were aligned with EMBLALIGN: Align_000605 using CLUSTAL X (Jeanmougin et al. 1998) following the criteria detailed by Matějusová et al. (2003), generating a single haplotype. The family Gyrodactylidae currently includes 31 genera (Bakke et al. 2007, Vianna et al. 2007, Přikrylová et al. 2009) but of these, sequences are only available for 7 genera. Thus the haplotype of the unknown specimen was aligned with the following sequences from GenBank: *Acanthoplacatus* sp. (AF465784); *Diplogyrodactylus martini* Přikrylová, Matějusová, Musilová, Gelnar et Harris 2009 (AM943008); *Fundulotrema foxi* (Rawson 1973) Kritsky et Thatcher 1977 (GQ918278); *Fundulotrema porterensis* King et Cone 2009 (FJ845514); *Fundulotrema prolongis* (Hargis 1955) Kritsky et Thatcher 1977 (GQ918279); *Fundulotrema stableri* (Hathaway et Herlevich 1973) Kritsky et Thatcher 1977 (AY099505); *Gyrdicotylus gallieni* Vercammen-Grandjean, 1960 (AJ001843); *Gyrodactyloides bychowskii* Albova 1948 (AJ249348); *Gyrodactylus anguillae* Ergens 1960 (AB063294); *Gyrodactylus arcuatus* Bychowsky 1933 (AJ001839); *Gyrodactylus branchicus* Malmberg 1964 (AF156669); *Gyrodactylus bullatarudis* (AJ011410); *Gyrodactylus derjavinoides* Malmberg, Collins, Cunningham et Jalali 2007 (AJ132259); *Gyrodactylus macronychus* Malmberg 1957 (AJ407893); *Gyrodactylus poeciliae* Harris et Cable 2000 (AJ001844); *Gyrodactylus pseudonemachili* Ergens et Bychowsky 1967 (AJ567674); *Gyrodactylus salaris* Malmberg 1957 (Z72477); *Gyrodactylus stephanus* Müller 1937 (FJ845515); *Gyrodactylus truttae* Gläser 1974 (AJ132260); *Gyrodactylus turnbulli* (AJ001846); and *Macrogyrodactylus polypteri* Malmberg 1957 (AJ567672). For the ITS1 analysis, three *Dactylogyrus* species were included: *Dactylogyrus vastator* Bychowsky 1933 (AJ564159), *Dactylogyrus vranoviensis* Ergens 1956 (AJ564163) and *Dactylogyrus zandti* Bychowsky 1933 (AJ564165; ITS2 sequences not available for this genus). The aligned sequences were imported into MEGA version 4.0 (Tamura et al. 2007) and phylogenetic analysis was carried out using the Neighbor-Joining algorithm

(NJ) and Maximum Likelihood (ML) with bootstrap support n = 2000. Bayesian analysis was also implemented using MrBayes 3.1 (Ronquist and Huelsenbeck 2003) with $5x10^6$ Markov chain Monte Carlo (MCMC) generations.

I.4 Results

Monogenea van Beneden 1858

Gyrodactylidae Cobbold 1864

I.4.1 Ieredactylus *gen. nov.*

Body fusiform. Prohaptor with a single pair of cephalic lobes each bearing posterior glands and a spike sensillum. No eye spots. Spherical pharynx consisting of two bulbs (anterior and posterior), the anterior bearing 8 processes opens ventrally. Oesophagus short, branching into two simple, blind ended intestinal crura which extend beyond the ootype. Male copulatory organ (MCO) ventrally positioned, bulbous with a central principal spine which originates from the base of the bulb as two spines that merge at the top and face a single row of 8-10 closely arranged triangular spines. Two excretory bladders present, in-line with the MCO. *Vesicula seminalis* positioned posterior to the MCO. Female reproductive system consists largely of tubular uterus and usually contains an F1 embryo. F2 embryo observed in 3 and no embryo in 4 specimens (whole mount and partially digested preparations). No vagina. Viviparous. Opisthaptor trapezoidal-shaped, demarcated from trunk, bears a single pair of hamuli with conical accessory pieces associated with the roots. Ventral bar with small processes bearing, posteriorly, a bi-lobed, spatulate membrane. Sixteen marginal hooks directed towards the posterior edge of the opisthaptor. Marginal hook sickles of two different morphologies. The numbering system of the marginal hook position follows that described by Malmberg (1970). Pair 3 and 4 of the marginal hook sickles are noticeably more angular, whereas the remaining sickles are smoothly rounded. All marginal hook sickles bear a prominent, circular process (‘button’) on their heel which remains after proteolytic digestion of the soft tissue, and is likely a point of muscle attachment. Marginal hook shafts long and flexible compared to species of *Gyrodactylus*. Presence of two muscular pads adjacent to the hamuli, similar to the ‘muscular adhesive disks’ observed by Přikrylová et al. (2009) in *Diplogyrodactylus martini*.

I.4.2 Ieredactylus rivuli *gen.* et *sp. nov.*

Measurements for whole specimens and body parts are presented in Table I.1. Further, there are 8 pharynx processes and the male copulatory organ (MCO) is positioned off centre to the mid-line of the parasite and posterior to posterior pharyngeal bulb; armed with a principal spine and a single row of 8-10 smaller spines. Excretory bladders present. Dorsal bars have elongated attachment points on hamuli. The ventral bar is butterfly-shaped, the bar membrane bifurcated with each wing attached at

the mid-point of the ventral bar proper, extending tangentially and increasing in width with round ends. Two marginal hook types both with broad bases, approximately divided into equal toe and heel regions. The sickle shaft is marked by a deep groove on each side of the hook along which the filament loop operates. Sickle shaft proper for marginal hook pairs 1, 2, 5-8 is proportionately broad, gently angled at three points, forward sloping, terminating at a point well beyond the limit of the toe; sickle tip sharply deflected. Inner blade face of the sickle gently curved. Square heel with prominent circular muscle attachment point on the upper edge. Marginal hook pairs 3 and 4 show a sickle shaft broader than those of pairs 1, 2, 5-8; sharply angled at three points, terminating at a point well beyond the limit of the toe; sickle tip not as sharply deflected as that of pairs 1, 2, 5-8. Inner blade face of the sickle is also similarly angular (Figs. I.1, I.2).

Table I.1. Morphological measurements (mean ± 1 standard deviation followed by the range in parentheses; in micrometres) of *Ieredactylus rivuli* gen. *et* sp. nov. from *Rivulus hartii* collected from the Pitch Lake and the Naranjo tributary, Trinidad, in 2004, 2006 and 2008. Measurements were taken from 17 specimens.

Measurement	***Ieredactylus rivuli* gen. *et* sp. nov. from Trinidad (n = 27)**	
Total body length	675.5 ± 78 (470–805)	
Total body width	118.5 ± 22 (70–160)	
Opisthaptor length × width	94.8 ± 9.7 (70–111) × 80.7 ± 12.7 (58–102)	
Anterior pharynx length × width	8.7 ± 2.2 (5.5–13.3) × 38.4 ± 7.2 (22.8–50.1)	
Posterior pharynx length × width	17.3 ± 3.2 (10.8–21.5) × 48.8 ± 9.9 (27.3–65.6)	
Pharynx processes length	21.4 ± 2.7 (13.1–23.8)	
MCO length × width	12.1 ± 1.2 (10.3–14.3) × 12.1 ± 1.5 (9.9–14)	
MCO principal spine	14.1 ± 1 (11.7–15.3)	
Hamulus (H)		
H aperture	16.6 ± 1.4 (14.8–19.4)	
H proximal shaft width	9.3 ± 0.5 (8.1–10.0)	
H point length	29.1 ± 0.7 (28.0-30.0)	
H distal shaft width	4.6 ± 0.4 (3.8–5.1)	
H shaft length	34.8 ± 1.1 (33.4–37.3)	
H inner curve length	2.2 ± 0.4 (1.3–2.7)	
H aperture angle (°)	30.0 ± 1.8 (26.8–32.6)	
H point curve angle (°)	5.7 ± 1.2 (3.3–7.5)	
Inner H aperture angle (°)	34.6 ± 2.1 (31.3–39.1)	
H root length	22.3 ± 1 (21.1–24.5)	
H total length	55.4 ± 2 (52.7–59.6)	
H accessory piece length × width	11.5 ± 1.2 (9.1–13.6) × 5.3 ± 0.4 (4.8–6.3)	
Dorsal bar (DB)		
DB total length	12.0 ± 1.2 (9.4–13.4)	
DB width	2.0 ± 0.2 (1.5–2.3)	
DB attachment point length	10.2 ± 0.6 (9.5–11.5)	
Ventral bar (VB)		
VB total width	17.6 ± 0.8 (16.4–19.2)	
VB total length	19.9 ± 0.8 (18.2–21.3)	
VB process-to-mid length	3.1 ± 0.3 (2.6–3.5)	
VB median length	6.0 ± 0.5 (5.1–7.1)	
VB process length	0.9 ± 0.3 (0.5–1.8)	
VB membrane length	12.5 ± 0.6 (11.4–13.5)	
Marginal hook (MH)	**Pairs 1, 2, 5–8**	**Pairs 3 & 4**
MH total length	46.7 ± 2.6 (39.9–49.8)	41.6 ± 3.3 (35.8–46.1)
MH shaft length	41.6 ± 2 (38.0–45.3)	36.6 ± 3.3 (30.7–42.0)
MH sickle length	5.4 ± 0.3 (4.9–5.9)	4.6 ± 0.2 (4.3–5.0)
MH sickle proximal width	3.5 ± 0.1 (3.3–3.8)	3.6 ± 0.2 (3.3–3.9)
MH toe length	2.0 ± 0.1 (1.8–2.3)	2.1 ± 0.1 (1.9–2.3)
MH sickle distal width	4.1 ± 0.2 (3.7–4.5)	3.8 ± 0.3 (3.2–4.3)
MH aperture	6.3 ± 0.2 (5.9–6.7)	5.6 ± 0.3 (5.0–6.2)
MH instep / arch height	0.4 ± 0.1 (0.3–0.4)	0.3 ± 0 (0.25–0.4)

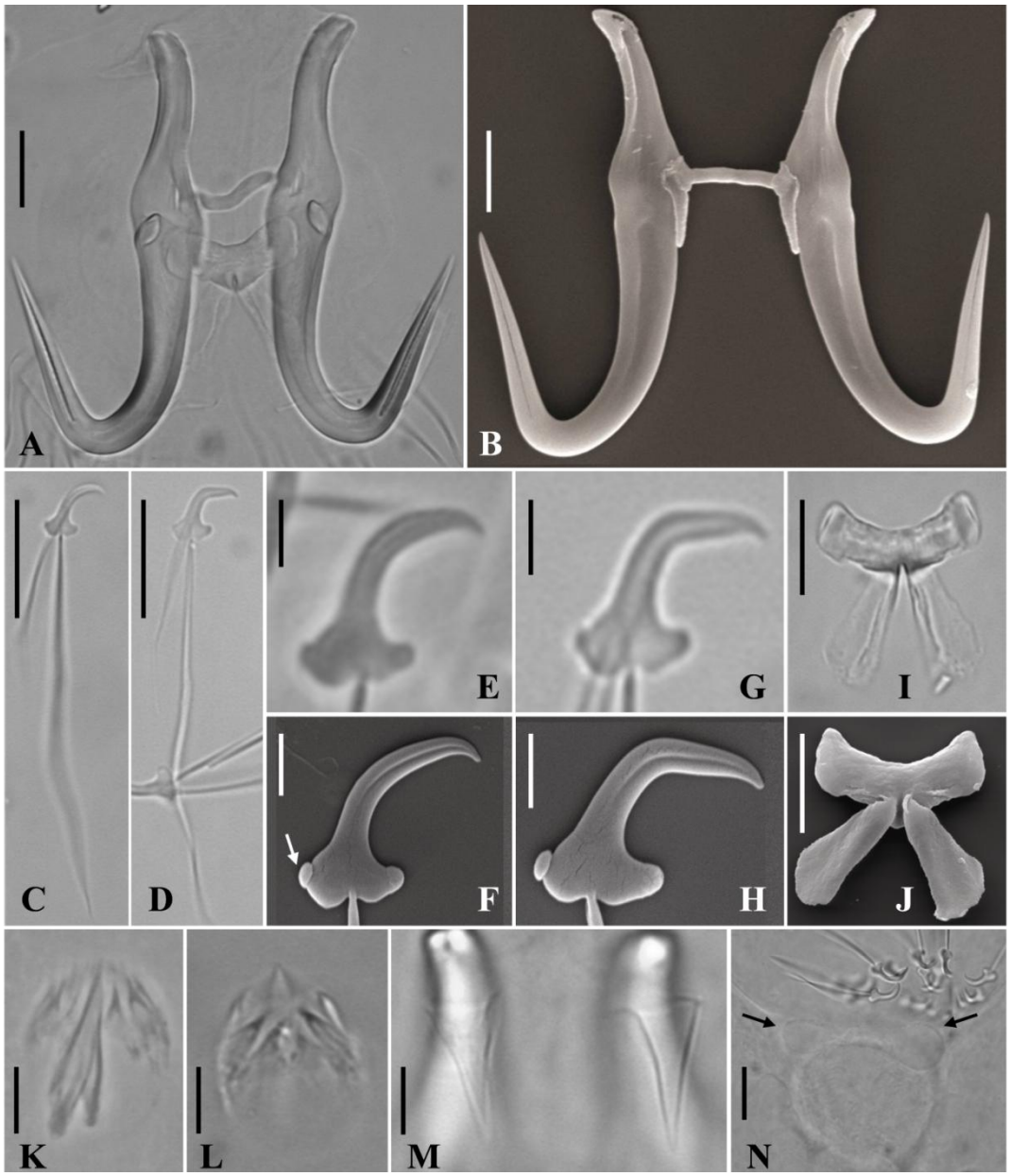

Fig. I.1. Light and scanning electron micrographs of *Ieredactylus rivuli* gen. *et* sp. nov. from *Rivulus hartii* Boulenger from Trinidad. A – opisthaptoral central hook complex showing the hamuli, the accessory pieces on the hamulus root, the dorsal and the ventral bar (ventral view); B – hamuli (dorsal view); C – marginal hook representing pairs 1, 2, 5-8; D – marginal hook representing pairs 3 and 4; E, F – marginal hook sickle representing pairs 1, 2, 5-8. Note the prominent muscle attachment point at the level of the heel (arrow); G, H – marginal hook sickle representing pairs 3 and 4; I, J – ventral bar, showing the split membrane; K, L – dorsal and ventral views of the male copulatory organ (MCO) showing the principal spine originating from the base of the bulb and facing a single row of 8-10 closely arranged triangular spines; M – triangular hamulus accessory pieces associated with the roots; N – detail of a flattened oval structure (arrows) positioned posterior to the uterus, presumably part of the reproductive system. Scale bars: A-D, I, J, N = 10 µm; E-H = 2 µm; K-M = 5 µm.

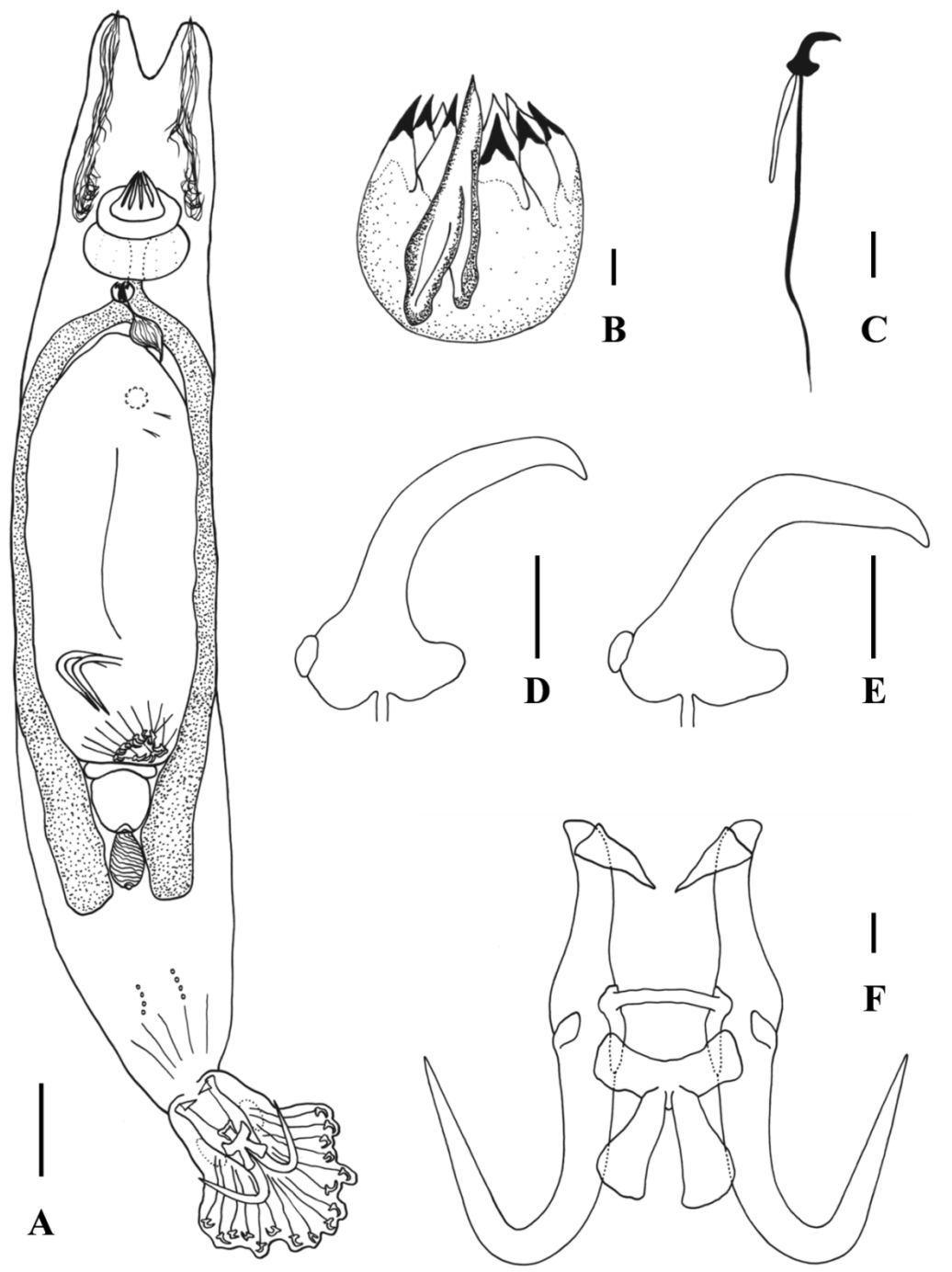

Fig. I. 2. Drawings of *Ieredactylus rivuli* gen. *et* sp. nov. from *Rivulus hartii* Boulenger from Trinidad. A – whole parasite in ventral view; B – male copulatory organ (MCO); C – marginal hook representing pair 3 and 4, showing the soft and long shaft; D – marginal hook sickle representing pairs 1, 2, 5–8; E – marginal hook sickle representing pairs 3 and 4; F – opisthaptoral central hook complex. Scale bars: A = 50 µm; B, D, E = 2 µm; C, F = 5 µm.

I.4.3 Taxonomic summary

Type and only species: *Ieredactylus rivuli* gen. *et* sp. nov.

Type host: Hart's Rivulus *Rivulus hartii* Boulenger, 1890 (Cyprinodontiformes, Rivulidae).

Site on the host: Skin and fins.

Type locality: Mid-Naranjo tributary of the Aripo river (Grid. Ref.: UTM 20P - 692498.44 E, 118257.53 N), Trinidad.

Other reported localities: Guanapo river (689796.25 E, 1183101.4 N) and Pitch Lake (650341.45 E, 1131668.93 N), Trinidad.

Type material: Holotype (acc. no. NHMUK 2011.9.19.1) and 15 paratypes (acc. nos. NHMUK 2011.9.19.2-16) are deposited in the parasitic worm collection at The Natural History Museum, London. Additionally, 6 paratypes (acc. nos. AHC 35253-8) are deposited in the gyrodactylid collection held at the South Australian Museum.

DNA reference sequence: The 980 bp amplified fragment consisting of partial 18S (17 bp) ITS1 (331 bp), 5.8S (156 bp), ITS2 (421 bp) and partial 28S (55 bp) is deposited in GenBank under acc. no. HQ738514.

Etymology: The genus is named after the Arawak (an indigenous tribe of the West Indies) name for Trinidad *i.e.* "Iëre". The species is named after the fish host on which this parasite was first encountered.

General: The species profile including taxonomic details is provided on www.monodb.org (Shinn et al. 2011).

I.4.4 Remarks

Within the family Gyrodactylidae, *Diplogyrodactylus martini*, *Macrogyrodactylus simentiensis* Přikrylová et Gelnar 2008, *Gyrodactylus chologastris* Mizelle, Whittaker et McDougal 1969 and *Gyrodactylus heterodactylus* Rogers et Wellborn 1965 have been reported as having two types of marginal hook sickles (Rogers and Wellborn 1965, Mizelle et al. 1969, Přikrylová and Gelnar 2008, Přikrylová et al. 2009), whilst *Gyrodactylus milleri* Harris et Cable 2000 possesses three different marginal hook sickle morphologies (Rubio-Godoy et al. 2010). *Gyreteroncus* spp. were reported to

have marginal hooks of different sizes and shapes and their MCO to have many small spinelets (Euzet and Birgy 1988), but this genus was never formally described. Indeed, after re-examination of Euzet's drawings, Přikrylová et al. (2009) claim that the species reported by Euzet and Birgy (1988) as *Gyreteroncus* sp. from *Polypterus senegalus senegalus* Cuvier is identical to *D. martini*, having a tubular and unarmed MCO, and the same marginal hook morphology. Although a dorsal bar is present in Euzet's drawings, Přikrylová et al. (2009) discussed the lack of this feature in *D. martini*. Confusion of all these genera with *Ieredactylus* gen. nov., however, is unlikely as apart from the two marginal hook types, all the other diagnostic features are different. The new genus also features a 'muscular adhesive disk-like' zone next to the hamuli, around the ventral bar attachment points, characteristic of those also seen, for example, in *Diplogyrodactylus*, *Gyrdicotylus* Vercammen-Grandjean 1960 and *Macrogyrodactylus* Malmberg 1956 (see Přikrylová et al. 2009). There was no evidence of species variation based on haptor morphology of specimens collected from both *Rivulus hartii* and *Poecilia reticulata* (although only two parasites were recovered from the guppy host).

I.4.5 Molecular characterisation of Ieredactylus rivuli *gen.* et *sp. nov.*

Overall, the ITS2 region of *Ieredactylus rivuli* gen. *et* sp. nov. showed the greatest genetic similarity (70 % similarity with 47 % coverage) to *Gyrodactyloides bychowskii*. All three phylogenetic analyses (Neighbor Joining, Maximum Likelihood and Bayesian) of the Gyrodactylidae genera based on ITS2 data, grouped *I. rivuli* gen. *et* sp. nov. with *Gyrodactyloides bychowskii*, *Gyrdicotylus gallieni*, *Macrogyrodactylus polypteri* and *Diplogyrodactylus martini*. ITS2 Bayesian analysis (data not shown) and Maximum Likelihood (Fig. I.3) tree topologies were almost identical. The major difference between the three analyses was the position of *Acanthoplacatus* sp.

ITS1 analyses were conducted to assess the relationship of the new genus with *Dactylogyrus* (as no ITS2 sequences of the latter are currently available). As predicted the ITS1 results were highly variable (e.g. Cable et al. 1999) and alignments are problematic; but in all cases *Ieredactylus* gen. nov. clearly clustered within the Gyrodactylidae (Figs. I.3, I.4).

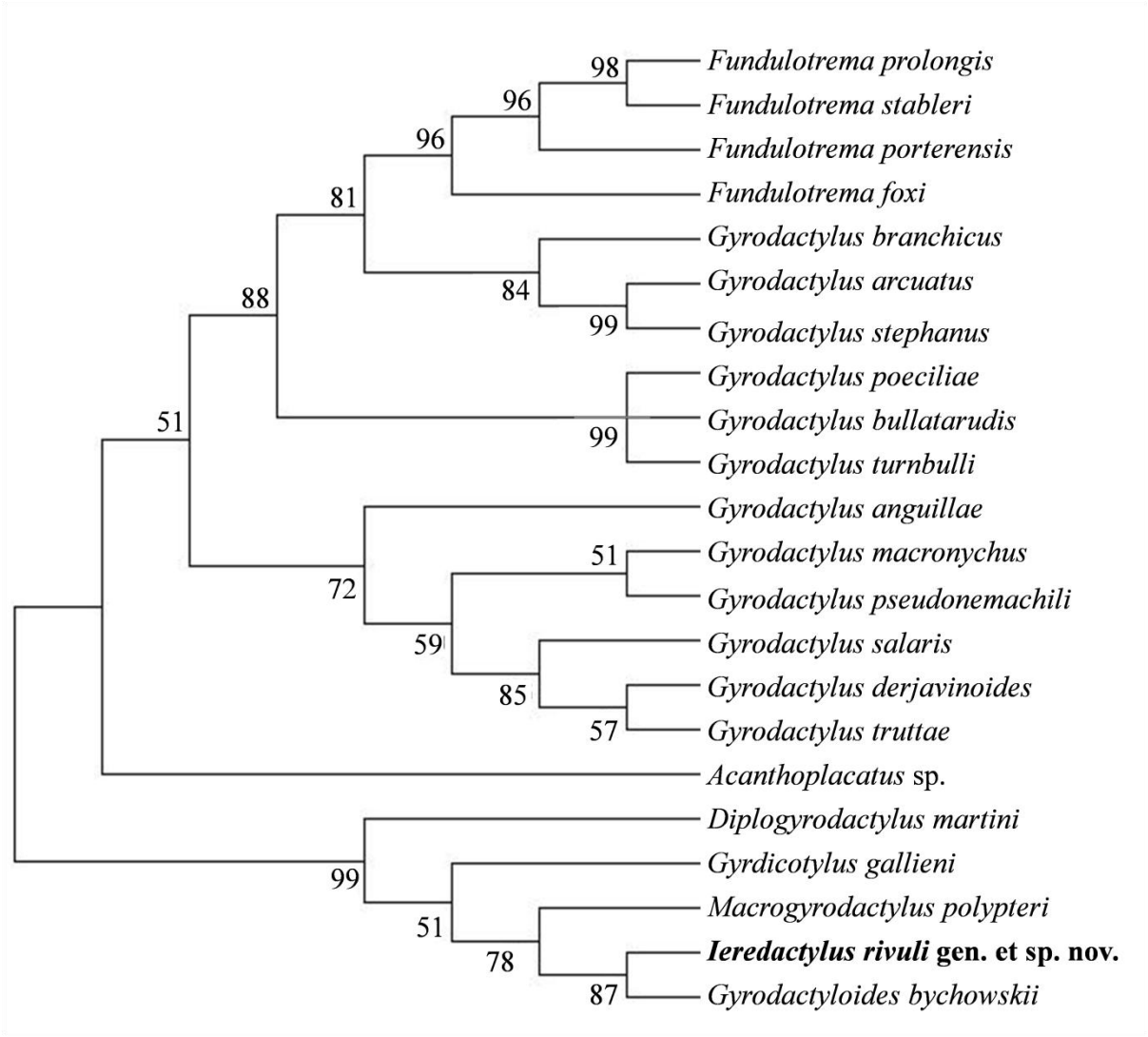

Fig. I.3. Maximum Likelihood (ML) tree showing the relationship of *Ieredactylus rivuli* gen. *et* sp. nov. to the other gyrodactylid genera for which there are GenBank rDNA Internal Transcribed Spacer 2 sequences (421 bp) available (see material and methods for individual accession numbers for all species listed).

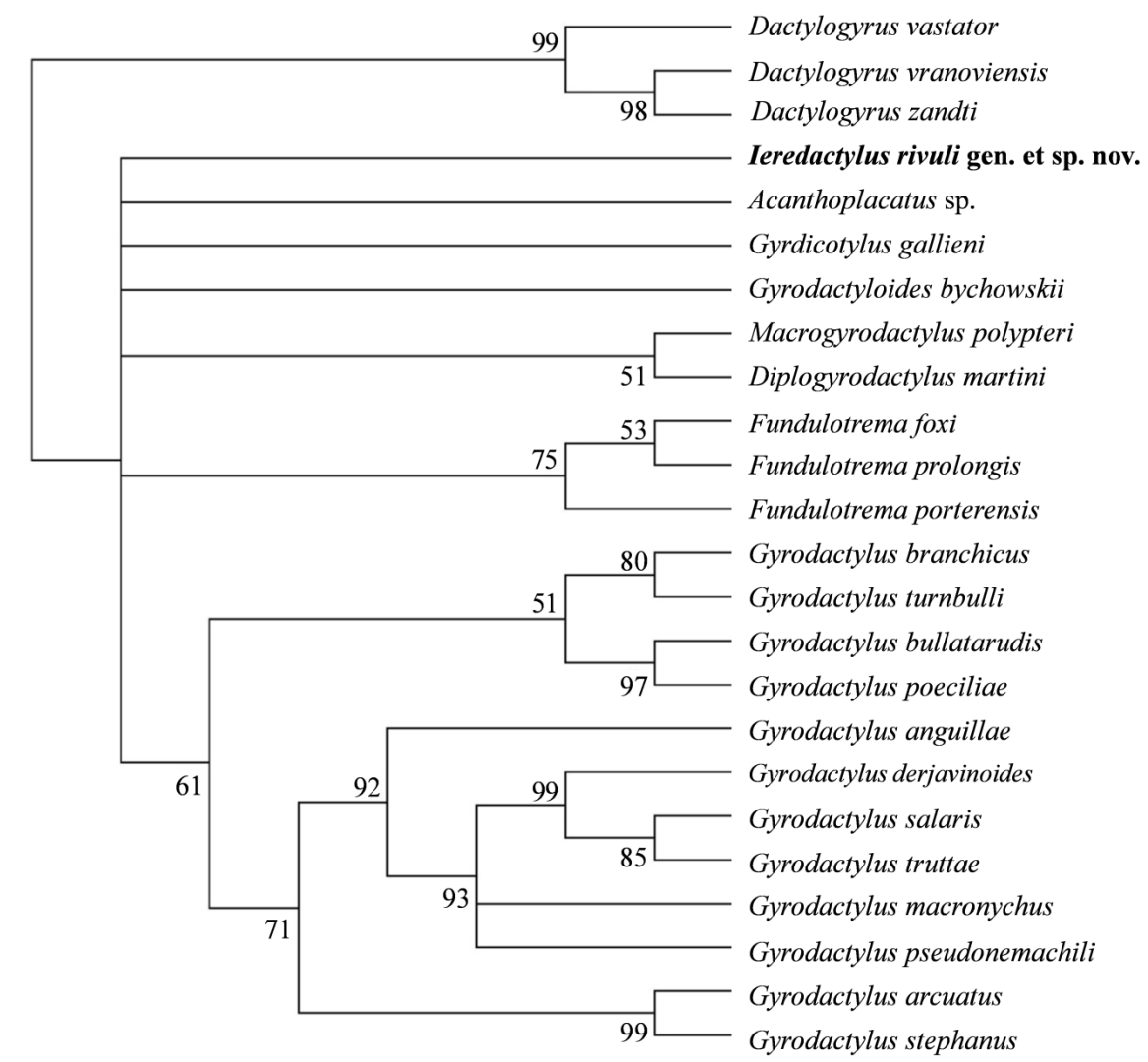

Fig. I.4. Neighbor-Joining (NJ) tree showing the relationship of *Ieredactylus rivuli* gen. *et* sp. nov. to the other gyrodactylid genera for which there are GenBank rDNA Internal Transcribed Spacer 1 sequences (331 bp) available (see material and methods for individual accession numbers for all species listed).

I.4.6 Ecological observations

Ieredactylus rivuli gen. *et* sp. nov. occurred at a prevalence of 16.7–94.6 %, with a mean parasite intensity of 1–9 parasites/fish on Hart's Rivulus in three different fish populations (Table I.2). This parasite can also naturally infect *P. reticulata*; however, this was only observed in 2004 on Pitch Lake guppies at a prevalence of 4.7 % and a mean intensity of 1 parasite/fish. Although we have screened nearly 5000 guppies from the rest of Trinidad (Cable J, van Oosterhout C, unpublished data), this new genus has not been recovered from guppy populations elsewhere in Trinidad.

When detached from its host, the new species was observed to move unidirectionally, characteristically twisting the body in a repeated figure of eight movement causing the parasite to move erratically, up or down in the water column (similar to the 'swimming behaviour' of *Gyrodactylus rysavyi* Ergens 1973 described by El-Naggar et al. 2004). Occasionally the parasite was observed motionless with its opisthaptor upright in the water film, similar to *G. turnbulli* (see Cable et al. 2002). In comparison to the *Gyrodactylus* species found on guppies, *I. rivuli* gen. *et* sp. nov. is a distinctly larger parasite.

Table I.2. Prevalence, mean intensity and range of *Ieredactylus rivuli* gen. *et* sp. nov. from *Rivulus hartii* collected in 2004, 2006, 2007 and 2008. Details of Guanapo samples collected in 2010 are not available (n/a). For the Pitch Lake, specimens collected from *Poecilia reticulata* are presented for 2004 only: the new genus has not been recovered from guppies elsewhere in Trinidad nor on the Pitch Lake guppies collected in 2006.

Year	No. of specimens	Location	Prevalence (%)	Mean intensity	Range
		Rivulus hartii			
2004	9	Pitch Lake	33.3 (3/9)	2.3	2–3
2006	5	Pitch Lake	20 (1/5)	9	9
2006	37	Mid-Naranjo	94.6 (35/37)	8.2	1–34
2008	6	Mid-Naranjo	16.7 (1/6)	1	1
2010	41	Guanapo	n/a	n/a	n/a
		Poecilia reticulata			
2004	64	Pitch Lake	4.7 (3/64)	1	-

I.5 Discussion

The new genus *Ieredactylus* gen. nov. consists of a distinct viviparous gyrodactylid with a unique ITS rDNA sequence, and featuring a split ventral bar membrane, a MCO which differs from the *Gyrodactylus*-type in having the principal spine longer than the whole bulb (see Table I.1) and the smaller spines not clearly delineated from the bulb (Figs. I.1K-L, I.2B), the presence of two accessory pieces attached to the roots of the hamuli, and two types of marginal hooks. Both marginal hook sickles show the presence of a "button" on the heel (Figs. I.1E-H, I.2D-E), which was resistant to the proteolytic digestion alongside other the hard parts of the opisthaptor. A partial split in the ventral bar membrane has previously been observed in *G. poeciliae* (see Harris and Cable 2000), however, not to the degree of a complete split as is characteristic for the newly described genus. This cross-shaped ventral bar membrane is a feature that is occasionally seen on species belonging to the genus *Dactylogyrus* (*i.e. Dactylogyrus crucifer* Wagener 1857 or *D. zandti*). Molecular analyses, however, clearly position *Ieredactylus rivuli* gen. *et* sp. nov. within the family Gyrodactylidae, most similar to

the genera *Gyrodactyloides*, *Macrogyrodactylus*, *Gyrdicotylus* and *Diplogyrodactylus* (see Figs. I.3, I.4).

The arrangement of the two types of marginal hook sickles can be divided in pairs 1, 2, 5-8, which are smoothly rounded (similar to *Gyrodactylus* spp.), and pairs 3 and 4 sharply angular. Differences in sickle shape within a full set of marginal hooks have previously been recorded for *Diplogyrodactylus martini* (see Přikrylová et al. 2009), *Gyrodactylus chologastris* (see Mizelle et al. 1969), *G. heterodactylus* (see Rogers and Wellborn 1965), *G. milleri* (see Rubio-Godoy et al. 2010) and *Gyreteroncus* spp. (see Euzet and Birgi 1988), although one unidentified species of the latter has been suggested to be identical with *D. martini* (see Přikrylová et al. 2009). The size difference between the two sickle types in *Diplogyrodactylus* is larger compared with *Ieredactylus* gen. nov. and the ratio of the number of large type to the smaller type hooks is 10 to 6, whereas *Ieredactylus* gen. nov. has 12 large and 4 smaller marginal hooks.

The prominent "button" on the heel of the marginal hook sickles is a consistent feature and is not lost during the digestion process which appears to be the case for most species of *Gyrodactylus.* A similar feature, however, is retained in a new *Gyrodactylus* species, currently under description, which infects the skin and gills of the south European toothcarp *Aphanius fasciatus* (Valenciennes) inhabiting hypersaline pools (Paladini G, Huyse T and Shinn AP, unpublished data). This "button" most likely serves as a muscle attachment point, as might the conical accessory pieces associated with the roots of the hamuli, which disappear after proteolytic digestion.

The bulb of the MCO of *Ieredactylus* gen. nov. is in shape similar to *Gyrodactylus* spp., but the size and the arrangement of the principal spine differ from this genus. In *Ieredactylus* gen. nov. the MCO principal spine originates from the base of the bulb, where appears to be slightly bifurcated, and it extends to the top as a single spine, having a total size longer than the rest of the bulb (Figs. I.1K, I.2B; Table I.1). These features had been observed in other gyrodactylids, i.e. *Gyrodactyloides andriaschewi* Bychowsky et Poljansky 1953 and *Gyrodactyloides petruschewskii* Bychowsky 1947 in which the MCO has a similar principal spine and four smaller spines, and *Laminiscus gussevi* (Bychowsky et Poljansky 1953) Pálsson et Beverley-Burton 1983 possessing one similar principal spine and ten smaller spines (see figures in Pálsson and Beverley-Burton 1983).

Preliminary laboratory experiments indicate *I. rivuli* gen. *et* sp. nov. does not transmit from Hart's Rivulus to what is believed to be its temporary host, the guppy (*P. reticulata*). A remarkable feature of *I. rivuli* gen. *et* sp. nov. is that, unlike other monogeneans examined (McMullan M, Schelkle B, Mohammed RS, Coogan MP, Gillingham E, van Oosterhout C, Cable J, unpublished data), it has

adapted to the extreme physio-chemical conditions in the Pitch Lake which is inhabited by Hart's rivulus, but also a potential temporary host of *I. rivuli*, the guppy. This asphalt lake is a natural upwelling of oil characterised by high hydrocarbon content and the habitat is thought to act as a refuge for teleosts from most parasites (McMullan et al., unpublished data). Nevertheless, some parasites, such as *I. rivuli* gen. *et* sp. nov., appear to have evolved tolerance to these extreme conditions, enabling it to exploit hosts and conquer a niche in this unique habitat. In addition to infections on Hart's Rivulus, three guppies (2.5 %) from Pitch Lake were also infected with *I. rivuli* gen. *et* sp. nov., even though these fish were devoid of all other gyrodactylids.

Ieredactylus rivuli gen. *et* sp. nov. seems to be a robust monogenean adapted to a host known for terrestrial locomotion and inhabiting hostile environments. Further studies are necessary to assess its potential role as a pathogen which can infect other hosts in different environmental conditions.

I.6 References

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APPENDIX II: UPSTREAM GUPPIES (*POECILIA RETICULATA* PETERS, 1859) GO AGAINST THE FLOW*

II. 1 Abstract

Guppies (*Poecilia reticulata* Peters, 1859) in lakes and from captive-bred populations are predicted to show little rheotaxis compared to conspecifics in a stream environment that are regularly exposed to flash floods associated with involuntary downstream migration. Here we test this hypothesis using an artificial stream, examining guppies of two wild riverine populations, one lake population, and one ornamental strain. Guppies from the most upstream riverine habitat show the most pronounced rheotaxis and are less likely to be swept downstream during flooding events. However, there is no significant difference between guppies from the lowland riverine habitat, the Pitch Lake and ornamental strain. We propose that station-keeping behaviours are most strongly selected in the upstream population because large spatial differences exist in ecology and environment between up- and downstream habitats. Given that these sites are separated by barrier waterfalls that prevent compensatory upstream migration, natural selection operates particularly strong against upstream guppies that have been displaced downstream during flooding events.

II.2 Introduction

Many freshwater fish species have an innate response to orientate their bodies in water currents, a phenomenon known as positive rheotaxis (Northcutt 1997). Unlike species without active swimming abilities or other station-keeping adaptations (e.g. Blake et al. 2007), this innate swimming response prevents the inevitable extinction of closed populations subject to dominant downstream migration (cf. Müller 1954 'drift paradox'). Rheotaxis also maximizes perception of chemical cues, interception of prey, and minimizes energy expenditure (Montgomery et al. 1999). From an evolutionary perspective, rheotaxis allows animals to maintain a position within a stream (station-keeping) which avoids potential fitness costs involved with emigration (McCormick et al. 1998).

Guppies (*Poecilia reticulata* Peters, 1859) can be found in a wide range of habitats, from riverine environments to lakes (Deacon et al. 2011).This species is also common in the aquarium trade; they have been bred and kept in captivity since the 1920s (Deacon et al. 2011). The hydrodynamic environment the fish in wild populations encounter is dramatically different. In the mountainous region of the Caroni Drainage in Trinidad, the fish are exposed to seasonal flash-flooding events, coinciding with the wet-season rains (van Oosterhout et al. 2007a). In contrast, the guppies from ornamental strains in aquaculture and those occurring in natural lakes never encounter high water

*Adapted from: Mohammed RS, van Oosterhout C, Schelkle B, Cable J, McMullan M (2012) Biota Neotropica, in press.

velocities or flash-flooding. For example, the Pitch Lake in Trinidad is a flat crater with pitch and asphalt folds that create several freshwater pools. It is approximately 0.8 km^2 and guppies in this habitat experience little or no water currents. Here we hypothesize that guppies have adapted to the hydrodynamic conditions typical for their habitat. In particular, we predict that the guppies in the Pitch Lake may have lost their innate rheotaxic behaviours. Similarly, we predict that due to relaxed natural selection in captivity (van Oosterhout et al. 2007b) ornamental strain guppies will show little station-keeping behaviour. In contrast, guppies from a riverine habitat are predicted to show more pronounced rheotaxis and station-keeping.

II.3 Materials and methods

II.3.1 Experimental animals and procedure

The behaviour of 60 adult guppies from three populations in Trinidad were studied: the Upper Naranjo (UN: Grid Ref. UTM 20P 693443, 1183935), the Lower Aripo (LA: 694432, 1178141) and the Pitch Lake (PL: 650459, 1131727). Additionally, ornamental strain guppies (OS, n = 20) belonging to the Istanbul strain were tested for their rheotactic or station-keeping behaviours. The UN is a small upstream tributary of the Aripo River. The mean water flow rate of upstream sites is significantly higher than in the downstream sites (upstream ≈ 8.7cm/s, downstream ≈ 5.5cm/s, see Reznick et al. 2001). This set of populations was chosen to test the hypothesis that riverine fish populations that experience seasonal floods in the wild (i.e. the UN and LA populations) display stronger station-keeping behaviours than guppies from a natural or captive environment with little or no natural water currents (i.e. the PL and the OS guppies). All fish were collected at the end of the dry season (March-June) in 2009 where the water depth and flow rate was comparable to Reznick et al. (2001) observations.

In total, 20 guppies per population with approximately equal sex ratio and similar size range were used: standard length SL = 12-25 mm, (mean ± standard deviation: SL=18.3 ± 1.2 mm). Guppies were maintained in four 80 L aquaria in groups of 35-60 fish per tank. They were screened for parasites following the protocol described in van Oosterhout et al. (2003) and Chapter 1.6. These screens were conducted because guppies infected with *Gyrodactylus* spp. are more likely to be swept downstream than uninfected counterparts (van Oosterhout et al. 2007a). Briefly, guppies were anaesthetised with 0.02 % MS222 and using a stereo-microscope and fibre optic illumination, gyrodactylids were removed with watchmaker's tweezers. Fish were clean of all ectoparasites and showed no symptoms of disease in the two weeks prior to the experiment.

The behaviour of guppies was recorded in an artificial stream (length x width x depth = 112.2 x 12 x 4.0 cm^3). The water flow rate was 15.4 ± 1.2 cm/s, comparable to their mean critical swimming speed (Syriatowicz and Brooks 2004). The artificial stream was divided into 11 segments of 10.2 cm each, with a downstream weir which led to a small pool. The focal fish was released into the sixth segment in the middle of the stream. Its position was recorded at 5 s intervals over a period of 240 s. The experiment was terminated after 240 s or when the focal fish went across the weir into the pool (i.e. swept downstream). Post release, all monitoring was done via video to avoid disturbance to the fish.

The incidence of guppies being swept downstream was noted, and the average position of a guppy in the river and its mobility (i.e. average distance swum in 5 s intervals) during its time in the stream was calculated. A previous study using an artificial stream showed that guppies were not attracted to conspecific chemosensory cues (Archard et al. 2008). Hence, we used tank water that was recycled throughout the experiment. The water temperature was 27.0 ± 1.0 °C and recordings were made between 0700-1700h under indirect natural daylight in June 2009.

II.3.2 Statistical analyses

A binary logistic regression analysis (logit) with a dichotomous dependent variable ("swept downstream" or "kept station") was used to test whether the incidence that a guppy was swept downstream was explained by the origin of population, sex and SL. The model had three predictors: 'population' and 'sex' as fixed factors and 'SL' as covariate. The model was fitted using an iterative re-weighted least squares algorithm to obtain maximum-likelihood estimates of all parameters. The log-likelihood was used to test whether the coefficients of the predictors were significantly different from zero. A logit link function was used to calculate the odds ratio and its 95 % confidence interval (CI). Differences in the mean position of guppies in the stream between populations, sexes and SL were tested using a General Linear Model (GLM). We also used a GLM to compare the mobility of guppies among populations, sexes and SL. In these models, Population and Sex were fixed factors, and SL was the covariate. We checked whether the data were appropriate for parametric analysis and confirmed homogeneity of variances and normal distributions of residuals. All tests were conducted in Minitab 12.1.

II.3 Results

Guppies from the UN were significantly less likely to be swept downstream than their LA counterparts (Binary Logistic Regression: Z = -2.47, p = 0.014), mean and 5-95 % confidence interval (CI) odds ratio = 0.11 (0.02-0.64). However, there was no significant difference in the likelihood of fish being displaced downstream between the other populations (OS guppies: Z = -1.83, p = 0.068; PL

guppies: Z = -1.25, p = 0.212) (Fig. II.1). The sex and SL of guppies did not explain variation in the probability being swept downstream (Z = 0.47, p = 0.636 and Z = 0.17, p = 0.862, respectively).

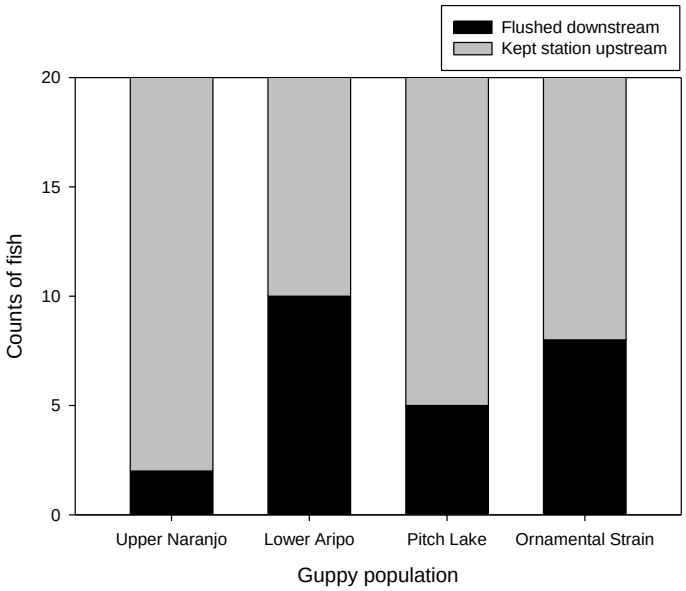

Fig. II.1. Number of fish that remained stationary and retained their position in the artificial stream (grey bars) and fish swept downstream over the weir into the pool (black bars) in the four populations. UN guppies were significantly less likely to be swept downstream than LA guppies (see text).

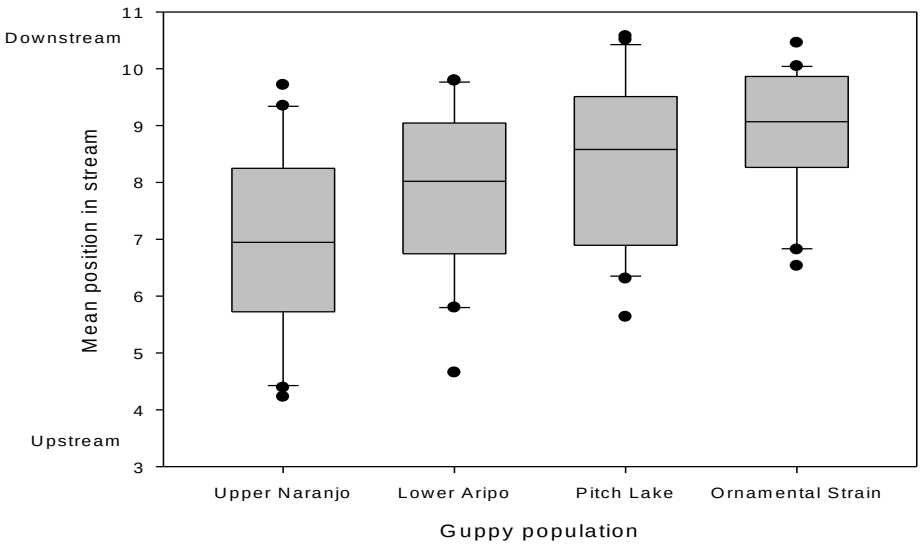

Fig. II.2. Box plot showing the average position of guppies in the artificial stream. Dots represent outliers, bars show the lower and upper limits and the box represents the first and third quartile value with the median. There was a significant difference between the mean positions of fish among populations (see text).

There was a significant difference in the mean position of fish among populations ($F_{3,74}$ = 4.32, p = 0.007), with the UN being on average most upstream, and the OS the furthest downstream (see Fig. II.2). Fish size (SL) and sex did not affect the position of fish within the artificial river ($F_{1,74}$ = 0.16, p = 0.692, and $F_{1,74}$ = 1.87, p = 0.161). There were significant differences in mobility between the fish of the four populations (GLM: $F_{3,74}$ = 3.71, p = 0.015) without the inclusion of sex and SL data. However, SL did not explain variation in mobility between the fish (GLM: $F_{1,74}$ = 0.61, p = 0.438), and there was no difference between the sexes (GLM: $F_{1,74}$ = 0.89, p = 0.414). The UN fish were significantly less mobile than fish of the other three populations, moving on average 6.0 cm per 5 s, compared to 11.4, 9.1 and 9.3 cm per 5 s for the LA, PL and OS guppies, respectively.

II.4 Discussion

Guppies from the upstream population, the Upper Naranjo (UN) were significantly less likely to be flushed downstream than Lower Aripo (LA), Pitch Lake (PL) and ornamental strain (OS) guppies. We hypothesised that wild fish, experiencing seasonal floods (i.e. the UN and LA populations) should display stronger rheotaxis or station-keeping behaviours than guppies in habitats with little or no natural water currents (i.e. the PL and the OS guppies). The results are inconsistent with our hypothesis, and suggest that the level of rheotaxis of guppies in populations that are not subjected to seasonal flooding is similar to that of guppies occurring in (lowland) rivers which are regularly in spate-conditions.

First, we consider the hypothesis that the relatively reduced level of station-keeping observed in the high-predation LA guppies can be explained by a trade-off between escape-response versus swimming endurance. The UN guppies live in a low predation environment, whereas the LA has high predator pressure on the guppies (van Oosterhout 2007c). Selection favours enhanced escape-response in high predation sites, a behaviour known as fast-start evasion response or c-start (Ghalambor et al. 2004). Could adaptations favouring the c-start compromise rheotaxis and swimming endurance in the high-predation LA? To answer this question we need to consider the station-keeping behaviour of the Pitch Lake and the ornamental guppies, which originate from habitats with little or no predation. Similar to the low-predation UN guppies, these populations do not experience strong selection for c-start. Nevertheless, Pitch Lake and ornamental guppies are equally prone to being swept downstream as the LA guppies. This suggests that the hypothesised trade-off between escape-response versus swimming endurance in the LA cannot be held responsible for reduced station-keeping performance in all three populations (i.e. LA, PL and OS).

Reduced predator fauna has been shown to increase the number of guppies that occupy the fast flowing regions of the river (Kodric-Brown and Nicoletto 2005). Flow rates in upstream sites are, on

average, greater than in downstream sites (Reznick et al. 2001). Furthermore, upstream guppies that are not discouraged (by piscivorous predators) from deeper or faster flowing regions of the river are presumably more likely to develop peduncle muscle in response to exposure to high flow rates (e.g. Nicoletto 1996). Therefore, increased rheotactic behaviour in the UN may be a plastic response to reduced predator fauna in a fast flowing river. Darden and Croft (2008) found that in high predation (lowland) sites, predation risk is greater in the deeper regions of a river. Interestingly, the authors also found that, in response to male presence, females will move into deeper waters, thereby increasing their predation risk (Darden and Croft 2008). The authors argue that this behaviour may increase the risk of female predation, but that this cost is balanced by a reduced level of harassment from males. It is conservable that in low predation (upstream) sites males do not suffer such increased predation risk in high flow regions and therefore only those males able to display and maintain their position in faster flowing regions of the river pass on their genes (e.g. Kodric-Brown and Nicoletto 2005). The findings in the present study and those in previous studies (Kodric-Brown and Nicoletto 2005, Darden and Croft 2008) suggest that a reduction in predator fauna in upstream sites may drive both phenotypic plasticity (in development of peduncle muscle) and selection toward increased rheotactic behaviour in the UN guppies.

Fish that are displaced from the UN during seasonal flood events may be prevented from returning upstream by barriers to gene flow such as waterfalls (Crispo et al. 2006, van Oosterhout et al. 2007a). Compensatory upstream migration in the lowlands may allow the return of displaced fish that have not been swept over such barriers (see Barson et al. 2009, Willing et al. 2010). In addition, distinct differences exist in predator and parasite faunas between upland and lowland habitats (Endler 1980, Reznick et al. 2001, Cable and van Oosterhout 2007). Several translocation experiments have shown that guppies are particularly well-adapted to cope with the local biotic and abiotic environmental conditions (e.g. Gordon et al. 2009). For example, guppies that evolved in a low-predation upland habitat have reduced anti-predator responses such as shoaling behaviour (Huizinga et al. 2009). Furthermore, the males tend to be more colourful, which make them vulnerable to visually-hunting predators that are common in the lowland environment (Endler 1995). Consequently, selection will favour behavioural responses that increase site fidelity (Winker et al. 1995, Aparicio and De Sostoa 1999). It is therefore likely that in the low-predation upland population of the UN, natural selection has promoted flush avoidance behaviour and positive rheotaxis. In contrast, even after downstream displacements during floods, lowland guppies of the LA population will find themselves in a similar, high-predation habitat to which they are adapted. We propose that the combination of a larger flow rate in the upland habitats in combination with the dramatic fitness consequences for upland guppies that are unable to resist flash-flooding has resulted in strong selection for station-keeping in the UN. This could explain why the UN guppies show the highest level of rheotaxis.

Croft et al. (2003) showed sex-biased dispersal in guppies, demonstrating a significant bias for upstream movement by males but not females. In addition, they found a positive correlation between body length and distance moved in females. van Oosterhout et al. (2007a), on the other hand, showed that males with parasite infections were more likely to be swept downstream during wet-season floods than females. The current study did not detect differences in rheotaxic behaviour between the sexes, and the size of fish did not explain differences in this behaviour. Instead, most variation was explained by the population origin of the fish.

Although our data can be explained by differences in selection pressures between populations, we cannot rule out that these results can be explained also by proximate (mechanistic) differences between populations. Future anatomical, behavioural and genetic studies into rheotaxis of guppies seem warranted, as due to the strong gradient in selection pressure the expression of this behaviour should vary predictably across the environment.

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APPENDIX III: COLOUR VARIATION OF AN INDIVIDUAL OF HART'S RIVULUS (*RIVULUS HARTII*) FOUND IN A HABITAT RICH IN POLYCYCLIC AROMATIC HYDROCARBONS IN THE PITCH LAKE OF TRINIDAD*

III.1Abstract

Typical coloured *Rivulus hartii* have been documented in South America and the twin island state of Trinidad and Tobago in habitats ranging from rock pools to slow moving streams. After repeated sampling at the Pitch Lake, an area rich in polycyclic aromatic hydrocarbons in Trinidad, a single individual was found within a pool of the lake with abnormal colouration. The fish exhibited normal behavior and showed no signs of pathology.

III.2 Main text

Rivulus hartii or Hart's rivulus is one of the larger members of the Killifish family (Cyprinodontidae family) in the ornamental trade. They have a natural distribution in Trinidad and Tobago and drainages along the northern coast of Venezuela, where they inhabit streams, swamps, ponds and rock pools at the bases and above waterfalls (Axelrod and Schultz 1983). Adult males are light brown with blue-green hints. Longitudinal rows (approximately eight rows) of red spots from the operculum to the caudal peduncle are prominent. The caudal fin has yellow stripes to the top and bottom (Fig. III.1). Females also have similar, but less intense coloration. The adult fish show clear sexual dimorphism when the fish reaches about 30 mm total length (TL). Males develop a white fringe on the caudal fin, both dorsally and ventrally, whereas females have a black fringe at the periphery of the caudal fin (Axelrod and Schultz 1983). Females above 50 mm TL and about 50-80 % of all males have a dark pigmented spot on the dorsal part of the caudal peduncle which is also visible in juveniles. There is hardly any size difference between sexes of this species (Axelrod and Schultz 1983).

Fig III.1. Normal coloured *Rivulus hartii*.

*Adapted from: Mohammed RS, McMullan MJ, Schelkle B, van Oosterhout C (2010) Ecologia Balkanica 2: 61-63.

In Trinidad and Tobago Hart's rivulus has a wide distribution across both islands (Kenny 1995, Phillip 1998). Phillip (1998) showed their existence in south west Tobago and we have observed specimen from both central (Gilpin Trail, UTM 20P: 759065.37 E, 1249820.56 N) and northern regions (UTM 20P: 767634.95 E, 1253366.84 N and 766369.16E, 1253366.84N) of the island in forested areas with seasonal streams. They show a greater diversity in habitat types in Trinidad, however, deviating from the typical freshwater habitat mentioned above. We have recorded them in small streams within less than 10 m from sea water at salinities between 3-5 ‰, at both the west (Iros Bay, UTM 20P: 640103.04 E, 1123072.10N) and east coastlines (Mayaro, UTM 20P 719148.75 E, 1137293.55 N) in Trinidad. The most interesting site they have been recorded at, however, is the Pitch Lake (UTM 20P: 650341.45 E, 1131668.93 N) of Trinidad.

The Pitch Lake can be described as relatively flat crater that closely resembles a large asphalt car park with pitch folds creating several freshwater pools, furrows and connecting drains. The lake is approximately 0.8 km^2. The perimeter is fringed with marshes and the pools surrounding the lake have several aquatic plants such as lilies (*Nymphaea* sp.) and algae (*Nitella* sp.). Thus far, Kenny (1995) has documented three species of fish for the lake; namely, *Poecilia reticulata* (guppy), *Polycentrus schomburgkii* (Guyana leaf fish) and *Rivulus hartii* (Hart's rivulus). *Rivulus hartii* has the highest density amongst the three species within the lake (personal observations by authors). We have documented pools with fish having mean temperatures of 31 °C, pH of 4.94, conductivity of 1328 µS and salinity of 0.6 ‰.

During June 2009 a specimen of Hart's rivulus was collected from the Pitch Lake showing coloration unlike the description above (Fig. III.2). After reviewing several pieces of literature on *Rivulus* and Killifish coloration it was determined that this natural color morph is unique. The individual collected is 41 mm TL, however, the sex cannot be determined due to the unusual color of the fish. The individual lacks the peduncle spot and seems to have a pink and white hue. Two mottled darker patches are also visible on the dorsal posterior area. The markings along the body are dull orange, only a faint orange band is visible on the caudal fin. With the exception of color, the morphology and attributes of our specimen is in keeping with what can still be taxonomically called a *Rivulus hartii*. When viewed at 4x magnification, minute dots of dark pigment are seen within the scales. No signs of pathology have been observed. The fish's health, behavior and feeding are the same compared to other *Rivulus hartii* collected at the same time in the lake. During captivity, the specimen was kept in both illuminated and un-illuminated conditions to observe changes in the intensity of coloration. This yielded no change in the fishes' hue or pattern.

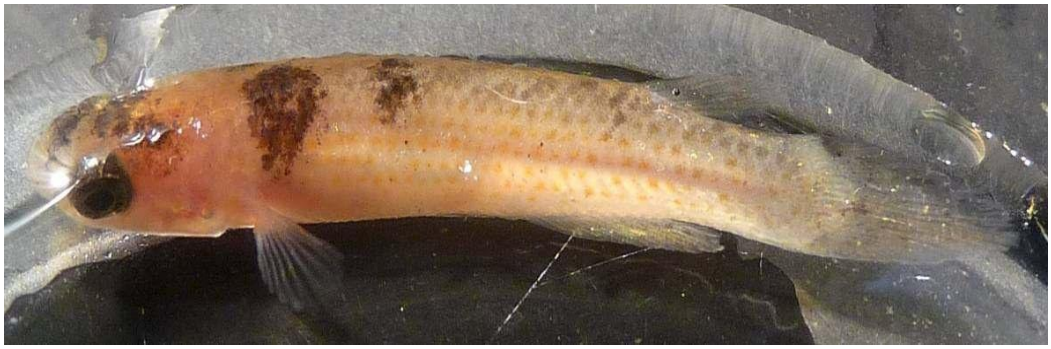

Fig. III.2. Unique coloured *Rivulus hartii* from the Pitch Lake, Trinidad.

Harrington and Rivas (1958) noted color variation of *Rivulus marmoratus* (collected in Cuba), reflective of the habitat and substrate; light coloration of fish collected in areas of light colored sediments and darker colored fish collected in environments with dark leaf litter substrates. This contradicts our findings for this specimen considering the substrate at the Pitch Lake is mostly black or dark colored. Other *Rivulus hartii* collected within the lake do conform to this adaptation, however (personal observation by authors), but this is temporary with individuals changing color intensity when the substrate is altered.

In Trinidad only the catfish *Rhamdia quelen* has ever shown a color morph in extreme habitat. Romero et al. (2004) and Kenny (1995) documented several pale catfish in cave systems and their associated drainages in Northern Range of Trinidad. The catfish documented had variable eye diameters and barbell lengths. This reduction in pigmentation and other changes of morphology was attributed to an adaption to reduced exposure to sunlight; in dark conditions pigmentation is not important. This situation does not occur at the Pitch Lake, as this habitat is exposed to sunlight with little cover by vegetation. Changes in faunal community and individuals within oil impacted habitat have previously been noted (Agard et al. 1993) in marine assemblages. Rather, we hypothesis that due to large natural oil seeps, this habitat is rich in polycyclic aromatic hydrocarbons (PAHs) which are mutagenic and carcinogenic compounds of crude oil. Consequently, one or more loss-of-function mutations may have occurred in the melanin pathway, resulting in the abnormal colour morph. Further investigations into the mutational load of *Rivulus* and guppies are warranted to examine the impact of PAHs on the genetics of wild fish populations. Following this we are now seeking explanations for our observations. This is the first of a series of our ongoing investigations into the dynamics of the ichtyological fauna of the Pitch Lake.

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APPENDIX IV: EFFICACY OF HERBAL COMPOUNDS AGAINST THE PUTATIVE AGENT OF HOLE-IN-THE-HEAD DISEASE, *SPIRONUCLEUS VORTENS*

IV.1 Abstract

Protozoan parasites in aquaculture cause huge economic losses, but effective treatment is hampered by the development of drug resistance and ban of traditional treatments. Hence, further research into alternative treatments is required. Here, we tested 19 botanical extracts *in vitro* against *Spironucleus vortens*, a diplomonad fish parasite and putative agent of Hole-in-the-Head disease in cichlids. None of the treatments tested reduced the growth rate of the parasite. However, the observed biphasic growth pattern of *S. vortens* differs from previous experiments, with log phase 1 being faster than log phase 2 in the current study. This may be explained by the exclusion of bile from the Keister's modified TYI-S-33 culture medium or the higher cell density used during inoculation in the current study, both of which warrant further study as they appear to disrupt the 'atypical' growth pattern characteristic of *S. vortens* populations.

IV.2 Introduction

Parasitic, piscine diplomonads are a major problem in food and ornamental aquaculture as they cause high mortalities in many fish species (Poynton et al. 1995, Sterud et al. 1998, Paull and Matthews 2001, Guo and Woo 2004, Andersson et al. 2007, Williams et al. 2011). *Spironucleus* species are associated with high pathogenicity in mainly immunocompromised hosts, including cichlids and cyprinids, with systemic infection being linked to Hole-in-the-Head disease (Paull and Matthews 2001, Williams et al. 2011). Recent years have seen increased interest in the basic biology, genetics and biochemistry of this genus, utilizing largely *in vitro* cultures in an attempt to find suitable drug targets for this group of protozoan pathogens (Roxström-Lindquist et al. 2010, Millet et al. 2010, 2011 a,b, Jørgensen et al. 2011). New treatments against this genus are urgently needed, particularly due to the ban of metronidazole for food fish and outdoor aquaculture within the European Union and the USA (L82/14 CRE 1998, Payne et al. 1999).

The use of herbal biomedicines has recently become popular in aquaculture due to controversial broad parasiticides in the aquarium and food fish industry, and the development of drug resistance in other effective treatments (Inglis 2000, Anthony et al. 2005, Cabello 2006, Sorum 2006, Citasaru 2010, Chakraborty and Hancz 2011). Botanical alternatives to traditional medicines have been successfully employed against terrestrial and aquatic parasites *in vitro* and *in vivo*, mainly using herbal extracts (Steverding et al. 2005, Bakkali et al. 2008, Hammer and Carson 2011). Indeed, garlic and allium-derived compounds were highly effective against *Spironucleus vortens* (see Millet et al. 2011c), with vinyl dithiins and ajoene having the highest inhibitory effect of the compounds tested (minimum

inhibitory concentration 83 and 107 µg/mL, respectively; Millet et al. 2011c). The mode of action of garlic-derived compounds in *S. vortens* is associated with disrupted intracellular redox balance due to antioxidant failure, ultimately leading to cell death (Williams et al. 2012). The current study aimed to expand this previous work to investigate a range of botanical extracts against *Spironucleus vortens in vitro*, in order to elucidate other potential alternative treatments against the parasite.

IV.3 Material and methods

IV.3.1 *Culture origin and compounds*

Spironucleus vortens (ATCC 50386) was isolated from *Pterophyllum scalare* in Florida in 1991 (Poynton et al. 1995). The parasite culture is maintained at Cardiff University's School of Biosciences in Keisters's modified TYI-S-33 medium according to the protocol detailed in Williams et al. (2012) and adapted from Millet et al. (2010). Specifically, the more recent culturing protocol requires cultures to be maintained at 24 °C instead of 19 °C and bile is excluded from the culture medium. Botanicals (19 in total) previously tested against the fish ecto-parasitic *Gyrodactylus turnbulli* were used at a similar concentration to the previous study (see Table 6.1 in Chapter 6). Metronidazole and water were used as positive and negative controls, respectively, and Glycerin and Crovol PK 70 were controlled for due to their function as a solvent for some of the compounds. All compounds were filtered (Sartorius: Minisart® filters, 0.2 µm pore size) before use to ensure all treatments were sterile.

IV.3.2 *Growth in Bioscreen C*

The growth of *S. vortens* populations was monitored in 100 well honeycomb plates in a Bioscreen C (Oy Growth Curves Ab Ltd.) automated optical density measurement system. To each well, 287 µL culture medium, 10 µL *S. vortens* culture at a final density of 8.6×10^5 cells/mL in the wells and 3 µL stock concentration of the test compound was added. The Bioscreen C was set to take optical density readings from each well every 15 min with wells being shaken 10 s before each measurement at a low amplitude. Temperature was kept constant at 24 °C and readings were taken with a wideband filter over 120 h. For each treatment, up to five replicates were run (Table IV.1).

Optical density measurements were used to calculate exponential growth rates, doubling times and final yields for both log phases (see Millet et al. 2010). Growth curves were assessed for each individual treatment; any abnormal growth curves resulting from mistakes during well plate preparation were discarded leading to only two replicates for some treatments (Table IV.1).

IV.3.3 Statistical analyses

Log phases were standardized as between 8.25 - 30.5 h for log phase 1 and 30.5-50 h for log phase 2 (see Millet et al. 2011b) since there was great consistency between log phases in growth curves of individual treatments (Fig. IV.1). Growth rates for log 1 and log 2 of each growth curve and final yields were compared between treatments using an ANOVA. To assess differences in the growth rates between the two log phases a Wilcoxon rank sum test was executed. Distribution of standardized residuals was assessed visually using histograms and normality plots and homogeneity of variance and auto-correlation were tested by a Fligner-Killeen and a Durben Watson test, respectively. Differences between treatment groups were assessed with Tukey Kramer *post hoc* analyses. All analyses were conducted in R 2.13.2 (R Development Core Team 2012)

IV.4 Results

Mean growth rate and doubling time of *Spironucleus vortens* populations in both log phases and the mean final yield in each treatment and all controls are presented in Table IV.1. Taken all data across treatments together, *S. vortens* population growth was faster during log 1 compared to log 2 phase (Wilcoxon rank sum test: $W = 11344$, $p < 0.001$) with average growth rate being about 0.049 ± 0.001 OD/h and 0.015 ± 0.001 OD/h, respectively.

There were significant differences between *S. vortens* population growth rates in the first log phase (ANOVA: $F_{24,84} = 11.67$, $p < 0.001$): most importantly, treatments containing oregano and pine needle oils, octanoic acid and the crovol control significantly reduced population growth when compared to the negative control, as did the positive control, metronidazole, when compared to all treatments (Tukey Kramer: $p < 0.02$). However, these differences, except for reduced growth in the metronidazole treated cultures compared to all other cultures (Tukey Kramer: $p < 0.001$) and in the black walnut treatment compared to the negative control (Tukey Kramer: $p = 0.014$), were no longer apparent in the second log phase (ANOVA: $F_{24,84} = 4.67$, $p < 0.001$).

Comparisons of final yields showed differences to the positive control, but not between other treatments (ANOVA: $F_{24,84} = 46.19$, $p < 0.001$): differences exist between all treatments and metronidazole, but there is no difference to the positive control. Indeed, there was a trend for higher final yield in some of the treatments compared to *S. vortens* populations in water and culture medium only (Table IV.1).

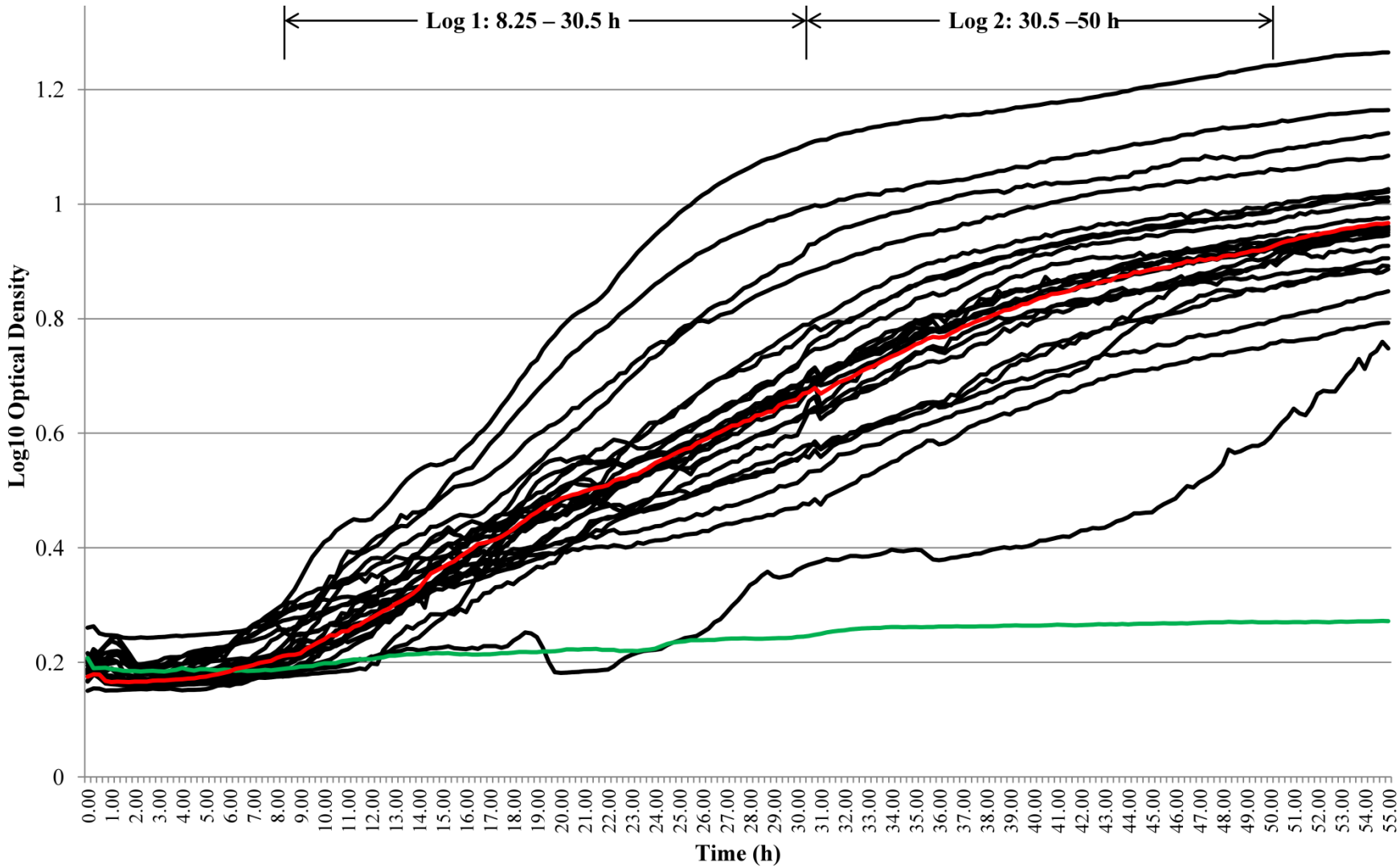

Fig. IV.1. Log optical density growth curves for *Spironucleus vortens* populations exposed to various botanical treatments. Negative control (water) is shown in red, positive control (metranidazole) in green.

Table IV.1. Compounds tested, their concentration, growth rate and doubling time in log phase 1 and log phase 2 and final yield of *Spironucleus vortens* culture growth in modified Keister's TYI-S-33 medium. N = number of replicates.

Compound	Final conc. in well	N	Growth rate Log 1	Doubling Time Log 1	Growth rate Log 2	Doubling Time Log 2	Final yield
CONTROLS							
Dechlorinated Water	-	18	0.052	13.329	0.016	42.354	0.930
Crovol Control	0.001 µL/mL	4	0.032	21.597	0.022	31.601	0.816
Glycerin at 1%	0.1 µL/mL	5	0.060	11.599	0.015	44.927	0.879
Metronidazole	10 µg/mL	5	0.012	57.857	0.005	145.283	0.117
BOTANICALS							
Barberry Root Powder	0.06 mg/mL	2	0.053	13.013	0.024	29.396	0.889
Berberine Chloride	0.06 mg/mL	5	0.047	14.601	0.014	50.236	0.904
Black Walnut (in Glycerin)	0.02 mL/mL	5	0.051	13.724	0.016	42.526	0.897
Bladderwrack (in Glycerin)	0.01 mL/mL	3	0.056	12.337	0.015	46.902	0.887
Blue Cohosh (in Glycerin)	0.02 mL/mL	4	0.054	12.887	0.014	48.696	0.952
Cascara Sagrada (in Glycerin)	0.01 mL/mL	5	0.066	10.512	0.007	98.138	1.020
Chamomile (in Glycerin)	0.01 mL/mL	5	0.063	11.050	0.012	57.401	0.946
Gotu Kola (in Glycerin)	0.02 mL/mL	4	0.066	10.561	0.010	72.765	0.949
Green Papaya	0.06 mg/mL	3	0.046	15.084	0.014	49.753	0.892
Green Tea 1	0.06 mg/mL	4	0.045	15.386	0.012	57.201	0.930
Green Tea 2	0.06 mg/mL	4	0.057	12.233	0.017	40.751	0.903
Myrrh Leaves	0.06 mg/mL	2	0.053	13.159	0.016	43.324	0.917
Neem Leaves	0.06 mg/mL	3	0.055	12.557	0.018	39.304	0.872
Noni	0.06 mg/mL	4	0.052	13.261	0.016	42.645	0.898
Octanoic Acid	0.06 mg/mL	2	0.033	21.115	0.024	28.732	0.905
Oregano	0.33 µL/mL	4	0.028	25.015	0.021	33.720	0.873
Pine Needle Oil (in Crovol)	0.33 µL/mL	4	0.023	29.786	0.024	29.116	0.810
Uva Ursi (in Glycerin)	0.01 mL/mL	4	0.056	12.328	0.016	42.460	0.858
White Willow (in Glycerin)	0.02 mL/mL	3	0.059	11.747	0.008	83.596	1.005
Yarrow (in Glycerin)	0.02 mL/mL	4	0.059	11.835	0.006	116.546	1.030

IV.5 Discussion

None of the botanicals tested *in vitro* against *Spironucleus vortens* in this study reduced the pathogen's population growth as effectively as metronidazole. However, oregano oil, pine needle oil and octanoic acid significantly depressed initial population growth. Such initial difference in growth, without affecting final yield, may indicate active compounds being metabolized during log phase1 after which no constraints to further microbial division exist. Further, since both oregano and pine needle oil were applied with crovol, which by itself has a negative effect on the parasite population, the delayed *S. vortens* culture growth may be caused by the emulsifier alone and not due to the essential oils.

The concentration of treatments used in the current study are comparable to those previously applied against helminths (see Chapter 6) and are higher than generally recommended against other protozoan diseases in fish (e.g. Millet et al. 2010). In practice, treatment concentration may be limited by how toxic a solution is to the fish host. For instance, oregano is highly toxic to guppies when applied in a water bath at concentrations as low as 0.3 µL/mL (personal observation), but oral administration may not induce the same stressors for the fish as a bath treatment.

The inefficacy of the botanicals tested against *S. vortens* in the current study is evident from population growth curves. Apart from populations exposed to octanoic acid, for which log phase 1 shows an extremely irregular growth pattern, all *S. vortens* cultures continue to show biphasic growth which is usually disrupted when effective treatments are applied (Millet et al. 2011b). Interestingly, the current study shows a reversal to the "atypical" growth pattern observed by Millet et al. (2011b) with growth in log phase 1 being faster than that of log phase 2. The reversal of the growth pattern observed in the current study is most likely due to the exlusion of bile from the culture medium compared to Millet et al. (2011b). Bile inhibits *S. vortens* growth (Sangmaneedet and Smith 2000), hence, the previous observation by Millet et al. (2011b) of lower growth in log phase 1 compared to log phase 2 may be due to bile degradation during log phase 1, thus leading to increased parasite growth in log phase 2. Further, initial Bioscreen C well inoculations in the current study started at a higher cell density than Millet et al. (2011b; 8.6 x 10^5 cells/mL in the current study compared to approximately 0.1 x 10^5 cells/mL) thus causing crowding early on in population growth leading to potentially slower growth in log phase 2 compared to log phase 1. High initial cell density may also have lead to an overall decrease in doubling time which for the current study is nearly six times slower than previously reported: 13.3 h during log phase 1 in the control treatment compared to 1.79 h reported by Millet et al. (2011b). The growth rate in the current experiment is therefore far slower than that which has been reported for other ciliated protozoa (Lynn 2008), particularly when considering that the present study utilizes laboratory cultures which are generally not restricted by nutrients (Lynn 2008).

Overall, this study showed no effect of botanicals on *S. vortens* growth *in vitro* indicating that further research is required to find alternative treatments against the parasite. In spite of this, the present study indicates that both the addition of bile and the initial cell inoculum strongly may affect the 'atypical' population growth characteristic of *S. vortens*, both of which warrants further study.

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APPENDIX V: TREATMENT EFFICACY COMPARISONS FOR BOTANICAL EXPERIMENTS (CHAPTER 6)

V.1 *In vitro*

Table V.1 (overleaf). *In vitro* treatment efficacy comparisons for botanical experiments (Chapter 6). Significant differences are highlighted in bold. Dechlor - water control (*Gt3*); LevH - Levamisole (1.39 mg/mL); LevL - Levamisole (0.005 µg/mL); GlyH - Glycerin (0.66 µL/mL); GlyL - Glycerin (0.66 µL/mL); Gly1% - Glycerin (0.11 µL/mL); Tween 20 0.01 % - Tween 20 control (0.11 µL/mL); CroCo39C - Crovol control for pine needle oil (0.11 nL/mL); 7H - Barberry (7.4 mg/mL); 7.1H - Barberry (14.8 mg/mL); 7.1L - Barberry (14.4 µg/mL); 7L - Barberry (7.41 µg/mL); 12H - West Indian bay oil (7.41 mg/mL); 12L - West Indian bay oil (0.07 mg/mL); 4H - Berberine chloride (7.41 mg/mL); 4L - Berberine chloride (0.07 mg/mL); 25H - Black Walnut (0.67 µL/mL); 25L - Black Walnut (0.22 µL/mL); 27H - Bladderwrack (0.67 µL/mL); 27L - Bladderwrack (0.22 µL/mL); 27-1 % - Bladderwrack (0.11 µL/mL); 20H - Blue Cohosh (0.67 µL/mL); 20L - Blue Cohosh (0.22 µL/mL); 20-1 % - Blue Cohosh (0.11 µL/mL); 22H - Cascara sagrada (0.67 µL/mL); 22L - Cascara sagrada(0.22 µL/mL); 22-1 % - Cascara sagrada (0.11 µL/mL); 23H - Chamomile (0.67 µL/mL); 23L - Chamomile (0.22 µL/mL); 23-1 % - Chamomile (0.11 µL/mL); 29H - Fenugreek seed (0.67 µL/mL); 29L - Fenugreek seed (0.22 µL/mL); 8H - Goldenseal (7.41 mg/mL); 8L - Goldenseal (0.07 mg/mL); 24H - Gotu kola (0.67 µL/mL); 24L - Gotu kola (0.22 µL/mL); 3H - Green Papaya (7.41 mg/mL); 3L - Green Papaya (0.07 mg/mL); 30H - Green Tea I (2.56 µg/mL); 30L - Green Tea I (0.67 µg/mL); 31H - Green Tea II (2.56 µg/mL); 31L - Green Tea II (0.67 µg/mL); 9H - Myrrh resin (7.41 mg/mL); 9L - Myrrh resin (0.07 mg/mL); 10H – Neem (7.41 mg/mL); 10L - Neem (0.07 mg/mL); 34H - Noni (2.56 µg/mL); 34L - Noni (0.67 µg/mL); 6H - Octanoic acid (7.41 mg/mL); 6L - Octanoic acid (0.07 mg/mL); 39C - Pine needle oil (3.37 nL/mL); 11H - Cajuput oil (7.41 mg/mL); 11L - Cajuput oil (0.07 mg/mL); 11H + 0.01 % Tween 20 - Cajuput oil + 0.01% Twn (7.41 mg/mL); 11L + 0.01 % Tween 20 - Cajuput oil + 0.01 % Tween 20 (0.07 mg/mL); 21H - Uva ursi (0.67 µL/mL); 21L - Uva ursi (0.22 µL/mL); 21-1 % - Uva ursi (0.11 µL/mL); 28H - White willow (0.67 µL/mL); 28L - White willow (0.22 µL/mL); 26H - Yarrow (0.67 µL/mL); 26L - Yarrow (0.22 µL/mL).

		10L	11H	11H + 0.01 % Tween	11L	11L + 0.01 % Tween	12H	12L	20-1 %	20H	20L	21-1 %	21H	21L
10H	z	-1.594	13.529	14.593	6.838	14.593	14.593	14.593	**0.005**	3.830	4.091	2.152	0.518	3.830
	p	0.111	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.996	**0.000**	**0.000**	**0.031**	0.604	**0.000**
10L	z		14.129	15.135	7.872	15.135	15.135	15.135	1.622	4.910	5.268	3.473	1.601	4.910
	p		**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.105	**0.000**	**0.000**	**0.001**	0.109	**0.000**
11H	z			1.408	-6.601	1.408	1.408	1.408	-13.730	-7.437	-8.670	-10.441	-8.986	-7.437
	p			0.159	**0.000**	0.159	0.159	0.159	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
11H + 0.01 % Tween	z				-7.823	**0.000**	**0.000**	**0.000**	-14.799	-8.481	-9.797	-11.520	-9.874	-8.481
	p				**0.000**	1.000	1.000	1.000	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
11L	z					7.823	7.823	7.823	-6.944	-1.834	-2.363	-4.215	-4.224	-1.834
	p					**0.000**	**0.000**	**0.000**	**0.000**	0.067	**0.018**	**0.000**	**0.000**	0.067
11L + 0.01 %Tween	z						**0.000**	**0.000**	-14.799	-8.481	-9.797	-11.520	-9.874	-8.481
	p						1.000	1.000	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
12H	z							**0.000**	-14.799	-8.481	-9.797	-11.520	-9.874	-8.481
	p							1.000	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
12L	z								-14.799	-8.481	-9.797	-11.520	-9.874	-8.481
	p								**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
20-1 %	z									3.869	4.147	2.178	0.518	3.869
	p									**0.000**	**0.000**	**0.029**	0.604	**0.000**
20H	z										-0.245	-1.844	-2.425	**0.000**
	p										0.806	0.065	**0.015**	1.000
20L	z											-1.798	-2.396	0.245
	p											0.072	**0.017**	0.806
21-1 %	z												-1.023	1.844
	p												0.306	0.065
21H	z													2.425
	p													**0.015**

		22-1 %	22H	22L	23-1 %	23H	23L	24H	24L	25H	25L	26H	26L	27-1 %
10H	z	1.829	3.667	4.032	4.352	2.881	4.091	1.306	-1.334	3.830	-1.508	4.042	-0.368	-2.221
	p	0.067	**0.000**	**0.000**	**0.000**	**0.004**	**0.000**	0.192	0.182	**0.000**	0.132	**0.000**	0.713	**0.026**
10L	z	3.241	4.719	5.204	5.601	3.769	5.268	2.504	0.209	4.910	-0.030	5.155	1.153	-0.458
	p	**0.001**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.012**	0.834	**0.000**	0.976	**0.000**	0.249	0.647
11H	z	-11.658	-7.147	-8.592	-9.724	-5.707	-8.670	-9.537	-13.708	-7.437	-13.344	-7.810	-13.067	-15.608
	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
11H + 0.01 % Tween	z	-12.758	-8.161	-9.712	-10.910	-6.552	-9.797	-10.517	-14.716	-8.481	-14.330	-8.891	-14.102	-16.621
	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
11L	z	-5.014	-1.760	-2.349	-2.811	-1.399	-2.363	-4.133	-7.530	-1.834	-7.423	-1.928	-6.755	-8.932
	p	**0.000**	0.078	**0.019**	**0.005**	0.162	**0.018**	**0.000**	**0.000**	0.067	**0.000**	0.054	**0.000**	**0.000**
11L + 0.01 % Tween	z	-12.758	-8.161	-9.712	-10.910	-6.552	-9.797	-10.517	-14.716	-8.481	-14.330	-8.891	-14.102	-16.621
	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
12H	z	-12.758	-8.161	-9.712	-10.910	-6.552	-9.797	-10.517	-14.716	-8.481	-14.330	-8.891	-14.102	-16.621
	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
12L	z	-12.758	-8.161	-9.712	-10.910	-6.552	-9.797	-10.517	-14.716	-8.481	-14.330	-8.891	-14.102	-16.621
	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
20-1 %	z	1.857	3.701	4.086	4.428	2.896	4.147	1.315	-1.357	3.869	-1.531	4.089	-0.378	-2.266
	p	0.063	**0.000**	**0.000**	**0.000**	**0.004**	**0.000**	0.189	0.175	**0.000**	0.126	**0.000**	0.705	**0.023**
20H	z	-2.342	**0.000**	-0.253	-0.434	**0.000**	-0.245	-2.092	-4.673	**0.000**	-4.714	**0.000**	-3.951	-5.555
	p	**0.019**	1.000	0.801	0.664	1.000	0.806	**0.036**	**0.000**	1.000	**0.000**	1.000	**0.000**	**0.000**
20L	z	-2.381	0.236	-0.010	-0.196	0.189	**0.000**	-2.052	-4.985	0.245	-4.997	0.257	-4.185	-6.077
	p	**0.017**	0.814	0.992	0.845	0.850	1.000	**0.040**	**0.000**	0.806	**0.000**	0.797	**0.000**	**0.000**
21-1 %	z	-0.453	1.774	1.770	1.774	1.425	1.798	-0.493	-3.219	1.844	-3.304	1.933	-2.367	-4.161
	p	0.650	0.076	0.077	0.076	0.154	0.072	0.622	**0.001**	0.065	**0.001**	0.053	**0.018**	**0.000**
21H	z	0.721	2.365	2.374	2.379	2.025	2.396	0.542	-1.439	2.425	-1.575	2.499	-0.758	-1.991
	p	0.471	**0.018**	**0.018**	**0.017**	**0.043**	**0.017**	0.588	0.150	**0.015**	0.115	**0.012**	0.448	**0.047**

		27H	27L	28H	28L	29H	29L	30H	30L	31H	31L	34H	34L	39C
10H	z	1.872	4.300	3.616	1.848	4.224	2.546	2.234	-2.626	0.428	-0.745	-6.492	-8.889	1.109
	p	0.061	**0.000**	**0.000**	0.065	**0.000**	**0.011**	**0.025**	**0.009**	0.668	0.456	**0.000**	**0.000**	0.267
10L	z	2.489	5.496	4.740	3.244	5.362	3.867	3.536	-0.944	1.874	0.772	-4.814	-7.109	1.486
	p	**0.013**	**0.000**	**0.000**	**0.001**	**0.000**	**0.000**	**0.000**	0.345	0.061	0.440	**0.000**	**0.000**	0.137
11H	z	-3.768	-8.948	-8.035	-11.249	-8.124	-10.555	-10.379	-15.239	-12.216	-13.251	-17.738	-19.957	-2.250
	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.024**
11H + 0.01 % Tween	z	-4.350	-10.101	-9.096	-12.342	-9.235	-11.660	-11.459	-16.227	-13.261	-14.269	-18.625	-20.801	-2.604
	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.009**
11L	z	-0.919	-2.414	-2.251	-4.783	-2.008	-4.094	-4.145	-8.919	-5.949	-7.031	-11.996	-14.249	-0.548
	p	0.358	**0.016**	**0.024**	**0.000**	**0.045**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.584
11L + 0.01 % Tween	z	-4.350	-10.101	-9.096	-12.342	-9.235	-11.660	-11.459	-16.227	-13.261	-14.269	-18.625	-20.801	-2.604
	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.009**
12H	z	-4.350	-10.101	-9.096	-12.342	-9.235	-11.660	-11.459	-16.227	-13.261	-14.269	-18.625	-20.801	-2.604
	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.009**
12L	z	-4.350	-10.101	-9.096	-12.342	-9.235	-11.660	-11.459	-16.227	-13.261	-14.269	-18.625	-20.801	-2.604
	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.009**
20-1 %	z	1.875	4.364	3.656	1.873	4.278	2.583	2.263	-2.671	0.430	-0.761	-6.572	-8.998	1.109
	p	0.061	**0.000**	**0.000**	0.061	**0.000**	**0.010**	**0.024**	**0.008**	0.667	0.447	**0.000**	**0.000**	0.267
20H	z	**0.000**	-0.219	-0.309	-2.231	**0.000**	-1.651	-1.779	-5.752	-3.289	-4.220	-8.736	-10.691	**0.000**
	p	1.000	0.826	0.757	**0.026**	1.000	0.099	0.075	**0.000**	**0.001**	**0.000**	**0.000**	**0.000**	1.000
20L	z	0.125	**0.037**	-0.089	-2.244	0.267	-1.586	-1.726	-6.236	-3.433	-4.482	-9.466	-11.646	0.075
	p	0.900	0.971	0.929	**0.025**	0.789	0.113	0.084	**0.000**	**0.001**	**0.000**	**0.000**	**0.000**	0.940
21-1 %	z	0.946	1.897	1.565	-0.376	2.007	0.279	0.075	-4.426	-1.615	-2.678	-7.873	-10.087	0.566
	p	0.344	0.058	0.117	0.707	**0.045**	0.780	0.940	**0.000**	0.106	**0.007**	**0.000**	**0.000**	0.571
21H	z	1.449	2.472	2.207	0.765	2.557	1.250	1.077	-2.299	-0.200	-1.013	-5.196	-6.944	0.905
	p	0.147	**0.013**	**0.027**	0.444	**0.011**	0.211	0.281	**0.021**	0.841	0.311	**0.000**	**0.000**	0.365

		3H	3L	4H	4L	6H	6L	7.1H	7.1L	7H	7L	8H	8L	9H
10H	z	3.903	2.445	2.015	-0.229	14.593	14.593	-4.475	-7.214	12.599	-6.774	5.230	-0.849	9.193
	p	**0.000**	**0.014**	**0.044**	0.819	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.396	**0.000**
10L	z	5.138	3.782	3.380	1.309	15.135	15.135	-2.735	-5.382	13.256	-5.002	6.373	0.703	10.069
	p	**0.000**	**0.000**	**0.001**	0.191	**0.000**	**0.000**	**0.006**	**0.000**	**0.000**	**0.000**	**0.000**	0.482	**0.000**
11H	z	-9.291	-10.620	-11.014	-13.178	1.408	1.408	-16.635	-18.967	-1.032	-18.390	-8.101	-13.504	-4.384
	p	**0.000**	**0.000**	**0.000**	**0.000**	0.159	0.159	**0.000**	**0.000**	0.302	**0.000**	**0.000**	**0.000**	**0.000**
11H + 0.01 % Tween	z	-10.429	-11.722	-12.106	-14.221	**0.000**	**0.000**	-17.578	-19.854	-2.421	-19.282	-9.279	-14.527	-5.676
	p	**0.000**	**0.000**	**0.000**	**0.000**	1.000	1.000	**0.000**	**0.000**	**0.015**	**0.000**	**0.000**	**0.000**	**0.000**
11L	z	-2.806	-4.185	-4.588	-6.757	7.823	7.823	-10.484	-12.948	5.628	-12.454	-1.546	-7.208	2.276
	p	**0.005**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.122	**0.000**	**0.023**
11L + 0.01 % Tween	z	-10.429	-11.722	-12.106	-14.221	**0.000**	**0.000**	-17.578	-19.854	-2.421	-19.282	-9.279	-14.527	-5.676
	p	**0.000**	**0.000**	**0.000**	**0.000**	1.000	1.000	**0.000**	**0.000**	**0.015**	**0.000**	**0.000**	**0.000**	**0.000**
12H	z	-10.429	-11.722	-12.106	-14.221	**0.000**	**0.000**	-17.578	-19.854	-2.421	-19.282	-9.279	-14.527	-5.676
	p	**0.000**	**0.000**	**0.000**	**0.000**	1.000	1.000	**0.000**	**0.000**	**0.015**	**0.000**	**0.000**	**0.000**	**0.000**
12L	z	-10.429	-11.722	-12.106	-14.221	**0.000**	**0.000**	-17.578	-19.854	-2.421	-19.282	-9.279	-14.527	-5.676
	p	**0.000**	**0.000**	**0.000**	**0.000**	1.000	1.000	**0.000**	**0.000**	**0.015**	**0.000**	**0.000**	**0.000**	**0.000**
20-1 %	z	3.963	2.480	2.044	-0.238	14.799	14.799	-4.541	-7.315	12.790	-6.866	5.310	-0.867	9.335
	p	**0.000**	**0.013**	**0.041**	0.812	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.386	**0.000**
20H	z	-0.558	-1.735	-2.078	-3.892	8.481	8.481	-7.167	-9.336	6.612	-8.983	0.518	-4.332	3.769
	p	0.577	0.083	**0.038**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.604	**0.000**	**0.000**
20L	z	-0.348	-1.681	-2.070	-4.132	9.797	9.797	-7.808	-10.229	7.755	-9.803	0.873	-4.618	4.553
	p	0.728	0.093	**0.038**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.383	**0.000**	**0.000**
21-1 %	z	1.516	0.179	-0.211	-2.272	11.520	11.520	-6.076	-8.573	9.549	-8.160	2.733	-2.797	6.385
	p	0.130	0.858	0.833	**0.023**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.006**	**0.005**	**0.000**
21H	z	2.185	1.175	0.881	-0.662	9.874	9.874	-3.598	-5.574	8.285	-5.309	3.105	-1.084	5.868
	p	**0.029**	0.240	0.379	0.508	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.002**	0.278	**0.000**

		9L	CroCo39C	Dechlor	Dechlor-1T	Gly-1 %	GlyH	GlyL	LevH	LevL	Tween 20 0.01 %
	z	2.528	-8.404	-14.586	-8.414	-11.756	4.336	-10.685	13.342	6.567	3.376
10H	p	**0.011**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.001**
	z	3.849	-6.838	-11.649	-6.164	-9.631	5.569	-8.610	13.904	7.696	4.528
10L	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
	z	-10.562	-18.625	-27.625	-21.497	-23.375	-9.492	-22.423	-2.058	-8.866	-8.440
11H	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.040**	**0.000**	**0.000**
11H + 0.01 % Twn	z	-11.666	-19.453	-28.315	-22.366	-24.173	-10.669	-23.243	-3.576	-10.157	-9.506
	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
	z	-4.106	-13.365	-20.650	-14.756	-17.297	-2.691	-16.315	5.402	-1.362	-2.579
11L	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.007**	**0.000**	**0.000**	0.173	**0.010**
11L + 0.01 % Twn	z	-11.666	-19.453	-28.315	-22.366	-24.173	-10.669	-23.243	-3.576	-10.157	-9.506
	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
	z	-11.666	-19.453	-28.315	-22.366	-24.173	-10.669	-23.243	-3.576	-10.157	-9.506
12H	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
	z	-11.666	-19.453	-28.315	-22.366	-24.173	-10.669	-23.243	-3.576	-10.157	-9.506
12L	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
	z	2.564	-8.488	-14.915	-8.574	-11.928	4.408	-10.843	13.605	6.708	3.415
20-1 %	p	**0.010**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.001**
	z	-1.661	-10.284	-14.609	-10.227	-12.919	-0.369	-12.076	6.420	0.913	-0.569
20H	p	0.097	**0.000**	**0.000**	**0.000**	**0.000**	0.712	**0.000**	**0.000**	0.361	0.569
	z	-1.598	-11.035	-16.871	-11.531	-14.336	-0.125	-13.385	7.751	1.393	-0.374
20L	p	0.110	**0.000**	**0.000**	**0.000**	**0.000**	0.900	**0.000**	**0.000**	0.164	0.708
	z	0.267	-9.591	-15.071	-9.712	-12.715	1.813	-11.744	9.738	3.504	1.305
21-1 %	p	0.790	**0.000**	**0.000**	**0.000**	**0.000**	0.070	**0.000**	**0.000**	**0.000**	0.192
	z	1.240	-6.855	-9.502	-5.964	-8.655	2.408	-7.908	8.152	3.609	2.005
21H	p	0.215	**0.000**	**0.000**	**0.000**	**0.000**	**0.016**	**0.000**	**0.000**	**0.000**	**0.045**

		22-1 %	22H	22L	23-1 %	23H	23L	24H	24L	25H	25L	26H	26L	27-1 %
21L	z	-2.342	**0.000**	-0.253	-0.434	**0.000**	-0.245	-2.092	-4.673	**0.000**	-4.714	**0.000**	-3.951	-5.555
	p	**0.019**	1.000	0.801	0.664	1.000	0.806	**0.036**	**0.000**	1.000	**0.000**	1.000	**0.000**	**0.000**
22-1 %	z		2.245	2.343	2.435	1.770	2.381	-0.129	-2.960	2.342	-3.054	2.469	-2.060	-3.987
	p		**0.025**	**0.019**	**0.015**	0.077	**0.017**	0.897	**0.003**	**0.019**	**0.002**	**0.014**	**0.039**	**0.000**
22H	z			-0.243	-0.415	**0.000**	-0.236	-2.028	-4.495	**0.000**	-4.546	**0.000**	-3.796	-5.316
	p			0.808	0.678	1.000	0.814	**0.043**	**0.000**	1.000	**0.000**	1.000	**0.000**	**0.000**
22L	z				-0.182	0.196	**0.010**	-2.028	-4.926	0.253	-4.942	0.265	-4.131	-5.995
	p				0.855	0.845	0.992	**0.043**	**0.000**	0.801	**0.000**	0.791	**0.000**	**0.000**
23-1 %	z					0.325	0.196	-2.029	-5.273	0.434	-5.251	0.458	-4.408	-6.580
	p					0.745	0.845	**0.042**	**0.000**	0.664	**0.000**	0.647	**0.000**	**0.000**
23H	z						-0.189	-1.687	-3.605	**0.000**	-3.686	**0.000**	-3.030	-4.169
	p						0.850	0.092	**0.000**	1.000	**0.000**	1.000	**0.002**	**0.000**
23L	z							-2.052	-4.985	0.245	-4.997	0.257	-4.185	-6.077
	p							**0.040**	**0.000**	0.806	**0.000**	0.797	**0.000**	**0.000**
24H	z								-2.311	2.092	-2.431	2.171	-1.547	-3.013
	p								**0.021**	**0.036**	**0.015**	**0.030**	0.122	**0.003**
24L	z									4.673	-0.229	4.901	0.930	-0.681
	p									**0.000**	0.819	**0.000**	0.352	0.496
25H	z										-4.714	**0.000**	-3.951	-5.555
	p										**0.000**	1.000	**0.000**	**0.000**
25L	z											4.928	1.117	-0.397
	p											**0.000**	0.264	0.691
26H	z												-4.149	-5.867
	p												**0.000**	**0.000**
26L	z													-1.706
	p													0.088

		27H	27L	28H	28L	29H	29L	30H	30L	31H	31L	34H	34L	39C
21L	z	**0.000**	-0.219	-0.309	-2.231	**0.000**	-1.651	-1.779	-5.752	-3.289	-4.220	-8.736	-10.691	**0.000**
	p	1.000	0.826	0.757	**0.026**	1.000	0.099	0.075	**0.000**	**0.001**	**0.000**	**0.000**	**0.000**	1.000
22-1 %	z	1.154	2.516	2.071	0.071	2.578	0.775	0.535	-4.261	-1.270	-2.409	-7.856	-10.182	0.685
	p	0.248	**0.012**	**0.038**	0.943	**0.010**	0.439	0.592	**0.000**	0.204	**0.016**	**0.000**	**0.000**	0.493
22H	z	**0.000**	-0.211	-0.299	-2.141	**0.000**	-1.585	-1.712	-5.524	-3.162	-4.058	-8.431	-10.317	**0.000**
	p	1.000	0.833	0.765	**0.032**	1.000	0.113	0.087	**0.000**	**0.002**	**0.000**	**0.000**	**0.000**	1.000
22L	z	0.130	**0.047**	-0.078	-2.209	0.275	-1.558	-1.699	-6.160	-3.388	-4.427	-9.372	-11.533	0.078
	p	0.897	0.962	0.938	**0.027**	0.783	0.119	0.089	**0.000**	**0.001**	**0.000**	**0.000**	**0.000**	0.938
23-1 %	z	0.210	0.245	0.082	-2.273	0.480	-1.546	-1.696	-6.684	-3.575	-4.728	-10.094	-12.463	0.125
	p	0.833	0.806	0.935	**0.023**	0.632	0.122	0.090	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.901
23H	z	**0.000**	-0.167	-0.245	-1.702	**0.000**	-1.261	-1.374	-4.395	-2.530	-3.249	-6.860	-8.391	**0.000**
	p	1.000	0.867	0.806	0.089	1.000	0.207	0.169	**0.000**	**0.011**	**0.001**	**0.000**	**0.000**	1.000
23L	z	0.125	**0.037**	-0.089	-2.244	0.267	-1.586	-1.726	-6.236	-3.433	-4.482	-9.466	-11.646	0.075
	p	0.900	0.971	0.929	**0.025**	0.789	0.113	0.084	**0.000**	**0.001**	**0.000**	**0.000**	**0.000**	0.940
24H	z	1.164	2.137	1.846	0.185	2.236	0.744	0.556	-3.313	-0.901	-1.823	-6.495	-8.465	0.712
	p	0.244	**0.033**	0.065	0.853	**0.025**	0.457	0.578	**0.001**	0.368	0.068	**0.000**	**0.000**	0.477
24L	z	2.390	5.197	4.493	2.992	5.092	3.586	3.271	-1.140	1.630	0.551	-5.028	-7.388	1.430
	p	**0.017**	**0.000**	**0.000**	**0.003**	**0.000**	**0.000**	**0.001**	0.254	0.103	0.582	**0.000**	**0.000**	0.153
25H	z	**0.000**	-0.219	-0.309	-2.231	**0.000**	-1.651	-1.779	-5.752	-3.289	-4.220	-8.736	-10.691	**0.000**
	p	1.000	0.826	0.757	**0.026**	1.000	0.099	0.075	**0.000**	**0.001**	**0.000**	**0.000**	**0.000**	1.000
25L	z	2.472	5.193	4.536	3.085	5.105	3.654	3.355	-0.854	1.785	0.752	-4.584	-6.814	1.487
	p	**0.013**	**0.000**	**0.000**	**0.002**	**0.000**	**0.000**	**0.001**	0.393	0.074	0.452	**0.000**	**0.000**	0.137
26H	z	**0.000**	-0.231	-0.322	-2.346	**0.000**	-1.735	-1.865	-6.047	-3.452	-4.428	-9.122	-11.165	**0.000**
	p	1.000	0.818	0.747	**0.019**	1.000	0.083	0.062	**0.000**	**0.001**	**0.000**	**0.000**	**0.000**	1.000
26L	z	1.999	4.375	3.744	2.091	4.317	2.728	2.434	-2.114	0.742	-0.359	-5.915	-8.246	1.193
	p	**0.046**	**0.000**	**0.000**	**0.037**	**0.000**	**0.006**	**0.015**	**0.034**	0.458	0.720	**0.000**	**0.000**	0.233

		3H	3L	4H	4L	6H	6L	7.1H	7.1L	7H	7L	8H	8L	9H
21L	z	-0.558	-1.735	-2.078	-3.892	8.481	8.481	-7.167	-9.336	6.612	-8.983	0.518	-4.332	3.769
	p	0.577	0.083	**0.038**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.604	**0.000**	**0.000**
22-1 %	z	2.110	0.665	0.245	-1.966	12.758	12.758	-5.997	-8.620	10.721	-8.178	3.416	-2.530	7.343
	p	**0.035**	0.506	0.807	**0.049**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.001**	**0.011**	**0.000**
22H	z	-0.536	-1.666	-1.996	-3.736	8.161	8.161	-6.890	-8.983	6.351	-8.652	0.498	-4.163	3.618
	p	0.592	0.096	**0.046**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.619	**0.000**	**0.000**
22L	z	-0.333	-1.653	-2.038	-4.078	9.712	9.712	-7.719	-10.119	7.685	-9.700	0.874	-4.560	4.516
	p	0.739	0.098	**0.042**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.382	**0.000**	**0.000**
23-1 %	z	-0.180	-1.652	-2.081	-4.365	10.910	10.910	-8.388	-11.021	8.736	-10.523	1.168	-4.887	5.225
	p	0.857	0.099	**0.037**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.243	**0.000**	**0.000**
23H	z	-0.426	-1.326	-1.589	-2.967	6.552	6.552	-5.513	-7.213	5.061	-6.976	0.396	-3.321	2.876
	p	0.670	0.185	0.112	**0.003**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.692	**0.001**	**0.004**
23L	z	-0.348	-1.681	-2.070	-4.132	9.797	9.797	-7.808	-10.229	7.755	-9.803	0.873	-4.618	4.553
	p	0.728	0.093	**0.038**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.383	**0.000**	**0.000**
24H	z	1.808	0.658	0.322	-1.445	10.517	10.517	-4.772	-7.005	8.749	-6.677	2.858	-1.914	6.006
	p	0.071	0.511	0.748	0.149	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.004**	0.056	**0.000**
24L	z	4.831	3.494	3.101	1.068	14.716	14.716	-2.898	-5.598	12.842	-5.219	6.051	0.479	9.698
	p	**0.000**	**0.000**	**0.002**	0.285	**0.000**	**0.000**	**0.004**	**0.000**	**0.000**	**0.000**	**0.000**	0.632	**0.000**
25H	z	-0.558	-1.735	-2.078	-3.892	8.481	8.481	-7.167	-9.336	6.612	-8.983	0.518	-4.332	3.769
	p	0.577	0.083	**0.038**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.604	**0.000**	**0.000**
25L	z	4.844	3.567	3.191	1.251	14.330	14.330	-2.537	-5.095	12.508	-4.744	6.009	0.686	9.496
	p	**0.000**	**0.000**	**0.001**	0.211	**0.000**	**0.000**	**0.011**	**0.000**	**0.000**	**0.000**	**0.000**	0.493	**0.000**
26H	z	-0.586	-1.824	-2.184	-4.094	8.891	8.891	-7.521	-9.787	6.948	-9.406	0.545	-4.550	3.963
	p	0.558	0.068	**0.029**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.586	**0.000**	**0.000**
26L	z	3.999	2.633	2.231	0.137	14.102	14.102	-3.877	-6.550	12.180	-6.149	5.245	-0.449	8.969
	p	**0.000**	**0.008**	**0.026**	0.891	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.653	**0.000**

		9L	CroCo39C	Dechlor	Dechlor-1T	Gly-1 %	GlyH	GlyL	LevH	LevL	Tween 20 0.01%
	z	-1.661	-10.284	-14.609	-10.227	-12.919	-0.369	-12.076	6.420	0.913	-0.569
21L	p	0.097	**0.000**	**0.000**	**0.000**	**0.000**	0.712	**0.000**	**0.000**	0.361	0.569
	z	0.762	-9.611	-15.858	-9.929	-13.031	2.461	-12.002	11.179	4.422	1.806
22-1 %	p	0.446	**0.000**	**0.000**	**0.000**	**0.000**	**0.014**	**0.000**	**0.000**	**0.000**	0.071
	z	-1.595	-9.969	-13.890	-9.765	-12.411	-0.354	-11.600	6.125	0.869	-0.550
22H	p	0.111	**0.000**	**0.000**	**0.000**	**0.000**	0.724	**0.000**	**0.000**	0.385	0.582
	z	-1.570	-10.944	-16.621	-11.375	-14.178	-0.112	-13.236	7.664	1.386	-0.361
22L	p	0.116	**0.000**	**0.000**	**0.000**	**0.000**	0.911	**0.000**	**0.000**	0.166	0.718
	z	-1.559	-11.651	-19.167	-12.792	-15.606	0.075	-14.560	8.979	1.829	-0.224
23-1 %	p	0.119	**0.000**	**0.000**	**0.000**	**0.000**	0.940	**0.000**	**0.000**	0.067	0.822
	z	-1.269	-8.285	-10.608	-7.587	-9.896	-0.278	-9.247	4.745	0.667	-0.449
23H	p	0.205	**0.000**	**0.000**	**0.000**	**0.000**	0.781	**0.000**	**0.000**	0.505	0.653
	z	-1.598	-11.035	-16.871	-11.531	-14.336	-0.125	-13.385	7.751	1.393	-0.374
23L	p	0.110	**0.000**	**0.000**	**0.000**	**0.000**	0.900	**0.000**	**0.000**	0.164	0.708
	z	0.733	-8.212	-11.960	-7.674	-10.565	2.063	-9.715	8.716	3.461	1.617
24H	p	0.464	**0.000**	**0.000**	**0.000**	**0.000**	**0.039**	**0.000**	**0.000**	**0.001**	0.106
	z	3.569	-7.079	-12.116	-6.399	-9.970	5.251	-8.936	13.404	7.285	4.278
24L	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
	z	-1.661	-10.284	-14.609	-10.227	-12.919	-0.369	-12.076	6.420	0.913	-0.569
25H	p	0.097	**0.000**	**0.000**	**0.000**	**0.000**	0.712	**0.000**	**0.000**	0.361	0.569
	z	3.638	-6.598	-10.873	-5.738	-9.171	5.234	-8.195	12.943	7.128	4.328
25L	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
	z	-1.747	-10.676	-15.569	-10.834	-13.572	-0.390	-12.687	6.807	0.971	-0.594
26H	p	0.081	**0.000**	**0.000**	**0.000**	**0.000**	0.697	**0.000**	**0.000**	0.331	0.553
	z	2.711	-7.865	-13.224	-7.514	-10.898	4.399	-9.870	12.711	6.414	3.515
26L	p	**0.007**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**

		27H	27L	28H	28L	29H	29L	30H	30L	31H	31L	34H	34L	39C
27-1 %	z	2.705	6.372	5.411	3.973	6.135	4.620	4.223	-0.552	2.458	1.276	-4.759	-7.276	1.602
	p	**0.007**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.581	**0.014**	0.202	**0.000**	**0.000**	0.109
27H	z		-0.110	-0.166	-1.118	**0.000**	-0.829	-0.912	-2.892	-1.673	-2.150	-4.618	-5.646	**0.000**
	p		0.912	0.868	0.263	1.000	0.407	0.362	**0.004**	0.094	**0.032**	**0.000**	**0.000**	1.000
27L	z			-0.125	-2.366	0.240	-1.683	-1.823	-6.506	-3.592	-4.679	-9.795	-12.044	0.066
	p			0.901	**0.018**	0.810	0.092	0.068	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.948
28H	z				-1.959	0.333	-1.359	-1.499	-5.613	-3.060	-4.026	-8.688	-10.706	0.101
	p				0.050	0.739	0.174	0.134	**0.000**	**0.002**	**0.000**	**0.000**	**0.000**	0.920
28L	z					2.443	0.680	0.453	-4.245	-1.303	-2.412	-7.861	-10.191	0.666
	p					**0.015**	0.496	0.650	**0.000**	0.193	**0.016**	**0.000**	**0.000**	0.505
29H	z						-1.807	-1.938	-6.296	-3.589	-4.603	-9.443	-11.559	**0.000**
	p						0.071	0.053	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	1.000
29L	z							-0.202	-4.859	-1.958	-3.057	-8.309	-10.567	0.494
	p							0.840	**0.000**	0.050	**0.002**	**0.000**	**0.000**	0.621
30H	z								-4.487	-1.693	-2.756	-7.881	-10.072	0.546
	p								**0.000**	0.090	**0.006**	**0.000**	**0.000**	0.585
30L	z									2.826	1.709	-4.009	-6.339	1.724
	p									**0.005**	0.088	**0.000**	**0.000**	0.085
31H	z										-1.088	-6.435	-8.672	1.000
	p										0.277	**0.000**	**0.000**	0.317
31L	z											-5.419	-7.657	1.285
	p											**0.000**	**0.000**	0.199
34H	z												-2.226	2.785
	p												**0.026**	**0.005**
34L	z													3.404
	p													**0.001**

		3H	3L	4H	4L	6H	6L	7.1H	7.1L	7H	7L	8H	8L	9H
27-1 %	z	5.990	4.526	4.093	1.877	16.621	16.621	-2.483	-5.387	14.695	-4.972	7.317	1.212	11.293
	p	**0.000**	**0.000**	**0.000**	0.061	**0.000**	**0.000**	**0.013**	**0.000**	**0.000**	**0.000**	**0.000**	0.226	**0.000**
27H	z	-0.280	-0.873	-1.046	-1.948	4.350	4.350	-3.647	-4.789	3.334	-4.651	0.260	-2.191	1.891
	p	0.779	0.383	0.296	0.051	**0.000**	**0.000**	**0.000**	**0.000**	**0.001**	**0.000**	0.795	**0.028**	0.059
27L	z	-0.399	-1.782	-2.185	-4.327	10.101	10.101	-8.124	-10.621	8.004	-10.167	0.867	-4.824	4.686
	p	0.690	0.075	**0.029**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.386	**0.000**	**0.000**
28H	z	-0.227	-1.447	-1.803	-3.684	9.096	9.096	-7.077	-9.320	7.188	-8.951	0.889	-4.141	4.253
	p	0.821	0.148	0.071	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.374	**0.000**	**0.000**
28L	z	1.970	0.576	0.169	-1.982	12.342	12.342	-5.966	-8.617	10.330	-8.176	3.241	-2.536	7.044
	p	**0.049**	0.565	0.866	**0.047**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.001**	**0.011**	**0.000**
29H	z	-0.611	-1.899	-2.274	-4.265	9.235	9.235	-7.819	-10.166	7.231	-9.759	0.568	-4.734	4.127
	p	0.541	0.058	**0.023**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.570	**0.000**	**0.000**
29L	z	1.287	-0.105	-0.510	-2.649	11.660	11.660	-6.526	-9.059	9.633	-8.628	2.552	-3.183	6.350
	p	0.198	0.917	0.610	**0.008**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.011**	**0.001**	**0.000**
30H	z	1.441	0.101	-0.290	-2.350	11.459	11.459	-6.111	-8.570	9.486	-8.165	2.660	-2.873	6.319
	p	0.149	0.920	0.772	**0.019**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.008**	**0.004**	**0.000**
30L	z	6.149	4.778	4.370	2.288	16.227	16.227	-1.835	-4.540	14.365	-4.169	7.400	1.657	11.145
	p	**0.000**	**0.000**	**0.000**	**0.022**	**0.000**	**0.000**	0.066	**0.000**	**0.000**	**0.000**	**0.000**	0.098	**0.000**
31H	z	3.219	1.860	1.462	-0.621	13.261	13.261	-4.530	-7.071	11.330	-6.676	4.451	-1.188	8.142
	p	**0.001**	0.063	0.144	0.534	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.235	**0.000**
31L	z	4.316	2.964	2.565	0.502	14.269	14.269	-3.439	-5.998	12.377	-5.622	5.539	-0.083	9.212
	p	**0.000**	**0.003**	**0.010**	0.616	**0.000**	**0.000**	**0.001**	**0.000**	**0.000**	**0.000**	**0.000**	0.934	**0.000**
34H	z	9.482	8.247	7.876	6.056	18.625	18.625	2.272	-0.265	16.948	**0.005**	10.617	5.435	14.020
	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.023**	0.791	**0.000**	0.996	**0.000**	**0.000**	**0.000**
34L	z	11.740	10.513	10.142	8.363	20.801	20.801	4.565	2.076	19.183	2.318	12.875	7.711	16.270
	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.038**	**0.000**	**0.020**	**0.000**	**0.000**	**0.000**

		9L	CroCo39C	Dechlor	Dechlor-1T	Gly-1 %	GlyH	GlyL	LevH	LevL	Tween 20 0.01%
	z	4.600	-6.911	-12.903	-6.340	-10.135	6.517	-9.012	15.747	9.040	5.204
27-1 %	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
	z	-0.834	-5.711	-6.725	-4.882	-6.524	-0.181	-6.094	3.045	0.425	-0.303
27H	p	0.404	**0.000**	**0.000**	**0.000**	**0.000**	0.856	**0.000**	**0.002**	0.671	0.762
	z	-1.696	-11.354	-17.788	-12.093	-14.900	-0.170	-13.915	8.067	1.418	-0.420
27L	p	0.090	**0.000**	**0.000**	**0.000**	**0.000**	0.865	**0.000**	**0.000**	0.156	0.675
	z	-1.370	-10.264	-14.898	-10.284	-13.039	-0.019	-12.166	7.062	1.344	-0.264
28H	p	0.171	**0.000**	**0.000**	**0.000**	**0.000**	0.985	**0.000**	**0.000**	0.179	0.792
	z	0.667	-9.631	-15.786	-9.904	-13.019	2.302	-11.996	10.675	4.150	1.698
28L	p	0.505	**0.000**	**0.000**	**0.000**	**0.000**	**0.021**	**0.000**	**0.000**	**0.000**	0.090
	z	-1.819	-10.996	-16.415	-11.359	-14.123	-0.407	-13.204	7.144	1.022	-0.614
29H	p	0.069	**0.000**	**0.000**	**0.000**	**0.000**	0.684	**0.000**	**0.000**	0.307	0.539
	z	-0.013	-10.000	-15.952	-10.327	-13.321	1.589	-12.327	9.890	3.356	1.088
29L	p	0.990	**0.000**	**0.000**	**0.000**	**0.000**	0.112	**0.000**	**0.000**	**0.001**	0.277
	z	0.190	-9.586	-14.954	-9.674	-12.664	1.735	-11.703	9.671	3.425	1.237
30H	p	0.850	**0.000**	**0.000**	**0.000**	**0.000**	0.083	**0.000**	**0.000**	**0.001**	0.216
	z	4.838	-6.127	-10.791	-5.218	-8.844	6.630	-7.804	15.185	8.920	5.414
30L	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
	z	1.944	-8.282	-13.410	-8.033	-11.234	3.581	-10.247	11.744	5.487	2.822
31H	p	0.052	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.005**
	z	3.041	-7.355	-12.110	-6.810	-10.137	4.713	-9.146	12.905	6.724	3.803
31L	p	**0.002**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
	z	8.285	-2.394	-5.083	-0.250	-4.237	10.004	-3.237	17.786	12.251	8.541
34H	p	**0.000**	**0.017**	**0.000**	0.802	**0.000**	**0.000**	**0.001**	**0.000**	**0.000**	**0.000**
	z	10.540	-0.372	-2.313	2.394	-1.877	12.337	-0.843	20.243	14.782	10.588
34L	p	**0.000**	0.710	**0.021**	**0.017**	0.060	**0.000**	0.399	**0.000**	**0.000**	**0.000**

		3H	3L	4H	4L	6H	6L	7.1H	7.1L	7H	7L	8H	8L	9H
39C	z	-0.167	-0.520	-0.623	-1.160	2.604	2.604	-2.180	-2.867	1.989	-2.791	0.155	-1.308	1.126
	p	0.867	0.603	0.533	0.246	**0.009**	**0.009**	**0.029**	**0.004**	**0.047**	**0.005**	0.877	0.191	0.260
3H	z		-1.390	-1.794	-3.949	10.429	10.429	-7.787	-10.299	8.357	-9.846	1.265	-4.458	5.062
	p		0.165	0.073	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.206	**0.000**	**0.000**
3L	z			-0.405	-2.552	11.722	11.722	-6.457	-9.000	9.702	-8.566	2.651	-3.092	6.431
	p			0.685	**0.011**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.008**	**0.002**	**0.000**
4H	z				-2.141	12.106	12.106	-6.058	-8.608	10.099	-8.181	3.055	-2.689	6.833
	p				**0.032**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.002**	**0.007**	**0.000**
4L	z					14.221	14.221	-4.068	-6.704	12.279	-6.297	5.216	-0.596	9.010
	p					**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.551	**0.000**
6H	z						**0.000**	-17.578	-19.854	-2.421	-19.282	-9.279	-14.527	-5.676
	p						1.000	**0.000**	**0.000**	**0.015**	**0.000**	**0.000**	**0.000**	**0.000**
6L	z							-17.578	-19.854	-2.421	-19.282	-9.279	-14.527	-5.676
	p							**0.000**	**0.000**	**0.015**	**0.000**	**0.000**	**0.000**	**0.000**
7.1H	z								-2.688	15.790	-2.357	9.005	3.424	12.654
	p								**0.007**	**0.000**	**0.018**	**0.000**	**0.001**	**0.000**
7.1L	z									18.152	0.282	11.496	6.033	15.080
	p									**0.000**	0.778	**0.000**	**0.000**	**0.000**
7H	z										-17.581	-7.147	-12.622	-3.383
	p										**0.000**	**0.000**	**0.000**	**0.001**
7L	z											11.025	5.645	14.553
	p											**0.000**	**0.000**	**0.000**
8H	z												-5.700	3.818
	p												**0.000**	**0.000**
8L	z													9.417
	p													**0.000**

		9L	CroCo39C	Dechlor	Dechlor-1T	Gly-1 %	GlyH	GlyL	LevH	LevL	Tween 20 0.01%
	z	-0.497	-3.489	-3.940	-2.879	-3.893	-0.107	-3.635	1.794	0.249	-0.183
39C	p	0.619	**0.000**	**0.000**	**0.004**	**0.000**	0.915	**0.000**	0.073	0.803	0.855
	z	-1.300	-11.071	-17.472	-11.746	-14.595	0.247	-13.604	8.458	1.874	-0.062
3H	p	0.194	**0.000**	**0.000**	**0.000**	**0.000**	0.805	**0.000**	**0.000**	0.061	0.951
	z	0.092	-9.947	-15.939	-10.277	-13.281	1.692	-12.283	9.961	3.463	1.179
3L	p	0.927	**0.000**	**0.000**	**0.000**	**0.000**	0.091	**0.000**	**0.000**	**0.001**	0.238
	z	0.496	-9.609	-15.458	-9.827	-12.878	2.113	-11.879	10.406	3.926	1.540
4H	p	0.620	**0.000**	**0.000**	**0.000**	**0.000**	**0.035**	**0.000**	**0.000**	**0.000**	0.124
	z	2.633	-7.970	-13.373	-7.701	-11.021	4.354	-9.996	12.868	6.433	3.451
4L	p	**0.008**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.001**
	z	-11.666	-19.453	-28.315	-22.366	-24.173	-10.669	-23.243	-3.576	-10.157	-9.506
6H	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
	z	-11.666	-19.453	-28.315	-22.366	-24.173	-10.669	-23.243	-3.576	-10.157	-9.506
6L	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
	z	6.503	-4.525	-8.318	-3.056	-6.861	8.307	-5.835	16.708	10.677	6.904
7.1H	p	**0.000**	**0.000**	**0.000**	**0.002**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
	z	9.032	-2.254	-5.221	0.053	-4.225	10.900	-3.148	19.286	13.455	9.182
7.1L	p	**0.000**	**0.024**	**0.000**	0.958	**0.000**	**0.000**	**0.002**	**0.000**	**0.000**	**0.000**
	z	-9.641	-17.897	-26.786	-20.622	-22.582	-8.517	-21.620	-0.911	-7.791	-7.585
7H	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.362	**0.000**	**0.000**
	z	8.602	-2.470	-5.526	-0.269	-4.471	10.415	-3.396	18.596	12.849	8.808
7L	p	**0.000**	**0.013**	**0.000**	0.788	**0.000**	**0.000**	**0.001**	**0.000**	**0.000**	**0.000**
	z	-2.563	-12.107	-18.912	-13.109	-15.817	-1.076	-14.830	7.105	0.419	-1.196
8H	p	**0.010**	**0.000**	**0.000**	**0.000**	**0.000**	0.282	**0.000**	**0.000**	0.675	0.232
	z	3.165	-7.390	-12.363	-6.893	-10.259	4.868	-9.249	13.207	6.937	3.919
8L	p	**0.002**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**

		9L	CroCo39C	Dechlor	Dechlor-1T	Gly-1 %	GlyH	GlyL	LevH	LevL	Tween 20 0.01 %
	z	-6.361	-15.216	-23.206	-17.176	-19.474	-5.063	-18.498	2.882	-3.977	-4.610
9H	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.004**	**0.000**	**0.000**
	z		-9.978	-15.899	-10.291	-13.286	1.602	-12.294	9.899	3.370	1.099
9L	p		**0.000**	**0.000**	**0.000**	**0.000**	0.109	**0.000**	**0.000**	**0.001**	0.272
	z			-1.493	2.535	-1.265	11.563	-0.350	18.592	13.604	10.139
CroCo39C	p			0.135	**0.011**	0.206	**0.000**	0.726	**0.000**	**0.000**	**0.000**
	z				6.702	**0.049**	18.732	1.516	30.435	23.790	14.886
Dechlor	p				**0.000**	0.961	**0.000**	0.130	**0.000**	**0.000**	**0.000**
	z						15.390	1.146	24.340	18.534	12.963
Gly-1 %	p						**0.000**	0.252	**0.000**	**0.000**	**0.000**
	z							-14.363	8.703	1.702	-0.284
GlyH	p							**0.000**	**0.000**	0.089	0.777
	z								23.271	17.411	12.076
GlyL	p								**0.000**	**0.000**	**0.000**
	z									-8.025	-7.505
LevH	p									**0.000**	**0.000**
	z										-1.698
LevL	p										0.090

V.2 *In vivo*

Table V.2. *In vivo* treatment efficacy comparisons for botanical experiments (Chapter 6). Significant differences are highlighted in bold (α level after Bonferroni corrections: 0.009). Treatment concentrations as listed in Table 6.2.

		Levamisol control	**Glycerin 1%**	**Crovol control**	**Tween control**	**Cajeput**	**Uva Ursi**	**Cascara sagrada**	**Chamomile**	**Green Papaya**	**Cajeput oil with Tween**	**Pine needle oil with Crovol**	**Bay oil with Tween**
Water control	**w**	11	184	278.5	493.5	507.5	161	284.5	254	565.5	95	75.5	317
	p	**< 0.001**	**0.003**	0.305	0.012	0.043	**< 0.001**	0.133	0.037	0.223	**< 0.001**	**< 0.001**	**< 0.001**
Levamisol control	**w**		110	104.5	203.5	181.5	82.5	110	115.5	209	121	115.5	137.5
	p		**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.035	**< 0.001**	**< 0.001**	**< 0.001**	0.309	**0.009**	**0.009**
Glycerin 1%	**w**			63	111	104	32	61	60	128	16	23	64
	p			0.301	0.802	0.864	0.097	0.676	0.737	0.1718	**< 0.001**	**< 0.001**	0.271
Crovol Control	**w**				90.5	88	29.5	50.5	47.5	100.5	22	21	56.5
	p				0.498	0.541	0.0050	0.737	0.572	1	**< 0.001**	**0.003**	0.111
Tween control	**w**					212.5	77.5	124	118.5	242.5	75	70.5	142.5
	p					0.952	0.101	0.719	0.912	0.332	**< 0.001**	**< 0.001**	0.245
Cajeput oil	**w**						78.5	115.5	112.5	224	90	80	139.5
	p						0.138	0.806	0.924	0.441	**< 0.001**	0.025	0.289
Uva ursi	**w**							84	85.5	161	81	75.5	103.5
	p							0.093	0.083	0.018	0.08568	0.952	0.602
Cascara sagrada	**w**								59	120	33	33.5	69
	p								0.941	0.636	**< 0.001**	0.014	0.203
Chamomile	**w**									128	25	29.5	68.5
	p									0.398	**< 0.001**	**< 0.001**	0.206
Green Papaya	**w**										44	40	113
	p										**< 0.001**	**< 0.001**	0.049
Cajeput oil with Tween	**w**											190	233
	p											0.025	0.013
Pine needle oil with Crovol	**w**												139.5
	p												0.386

		Berberine Chloride	Barberry	Goldenseal
Water Control	**W**	250	770	440
	P	< **0.001**	0.04087	**0.005**
Levamisol control	**W**	154	220	203.5
	P	0.019	< **0.001**	< **0.001**
Glycerin 1%	**W**	53	160	109
	P	0.031	< **0.001**	0.693
Crovol Control	**W**	48	130	83.5
	P	0.013	0.0127	0.436
Tween control	**W**	132	300	208.5
	P	0.028	**0.001**	0.977
Cajeput oil	**W**	137	270	194.5
	P	0.057	**0.004**	0.880
Uva ursi	**W**	110	180	149.5
	P	1	< **0.001**	0.085
Cascara sagrada	**W**	63	150	102
	P	0.036	**0.005**	0.734
Chamomile	**W**	59	160	108.5
	P	0.025	**0.001**	0.965
Green Papaya	**W**	96	260	164
	P	**0.002**	0.010	0.278
Cajeput oil with Tween	**W**	258	380	343
	P	0.040	< **0.001**	< **0.001**
Pine Needle oil with Crovol	**W**	143	270	223
	P	0.923	< **0.001**	**0.003**
Bay oil with Tween	**W**	150	260	260.5
	P	0.496	< **0.001**	0.234
Berberine Chloride	**W**		340	283
	P		< **0.001**	0.018
Barberry	**W**			100
	P			< **0.001**

APPENDIX VI: TREATMENT EFFICACY COMPARISONS FOR GARLIC EXPERIMENTS (CHAPTER 7)

VI.1 *In vitro*

Table VI.1. Cox proportional hazard survival analysis results for garlic *in vitro* experiment (Chapter 7): comparison between individual treatments. Significant results are highlighted in bold. 1.1 – Control *Gt1* strain; 1.2 – Control *Gt3* strain; 2.1-2.9 Freeze dried garlic flakes 18, 10, 6.67, 6, 1, 0.75, 0.5, 0.0667 and 0.01 mg/mL; 3.1-3.2 – Minced garlic 18 and 6 mg/mL; 4.1-4.3 – Garlic granules 18, 6 and 2.25 mg/mL; 5.1-5.4 – Chinese freeze dried garlic powder 18, 9, 6 and 0.09 mg/mL; 6.1-6.2 – Levamisole 18 and 6 mg/mL; 7.1-7.4 – Allyl alcohol 10, 7.5, 5 and 3.75 mg/mL; 8.1-8.4 – Allyle disulphide + Tween 20 0.5, 0.4, 0.1 and 0.05 mg/mL; 9.1-9.3 – Tween 20 100, 50 and 0.1 mg/mL.

	1.2	**2.1**	**2.2**	**2.3**	**2.4**	**2.5**	**2.6**	**2.7**	**2.8**	**2.9**
1.1	**z = -3.952 p < 0.001**	**z = 16.374 p < 0.001**	**z = 16.222 p < 0.001**	**z = 16.14 p < 0.001**	**z = 12.029 p < 0.001**	**z = 13.976 p < 0.001**	**z = 12.577 p < 0.001**	**z = 13.473 p < 0.001**	**z = 12.119 p < 0.001**	**z = -3.755 p < 0.001**
1.2		**z = 18.994 p < 0.001**	**z = 18.803 p < 0.001**	**z = 18.699 p < 0.001**	**z = 14.564 p < 0.001**	**z = 16.506 p < 0.001**	**z = 15.093 p < 0.001**	**z = 15.985 p < 0.001**	**z = 14.688 p < 0.001**	z = -1.094 p = 0.295
2.1			z < 0.001 p = 1	z < 0.001 p = 1	**z = -5.958 p < 0.001**	**z = -3.16 p = 0.002**	**z = -5.135 p < 0.001**	**z = -3.811 p = 0.001**	**z = -6.079 p < 0.001**	**z = -17.684 p < 0.001**
2.2				z < 0.001 p = 1	**z = -5.869 p < 0.001**	**z = -3.111 p = 0.002**	**z = -5.057 p < 0.001**	**z = -3.753 p < 0.001**	**z = -5.986 p < 0.001**	**z = -17.535 p < 0.001**
2.3					**z =-5.822 p < 0.001**	**z = -3.085 p = 0.002**	**z = -5.016 p < 0.001**	**z = -3.722 p < 0.001**	**z = -5.935 p < 0.001**	**z = -17.456 p < 0.001**
2.4						**z = 2.866 p = 0.004**	z = 0.888 p = 0.374	**z = 2.218 p = 0.027**	z = -0.021 p = 0.983	**z = -13.615 p < 0.001**
2.5							**z = -2.005 p = 0.045**	z = -0.658 p = 0.511	**z = -2.936 p = 0.003**	**z = -15.427 p < 0.001**
2.6								z = 1.349 p = 0.177	z = -0.924 p = 0.355	**z = -14.12 p < 0.001**
2.7									**z = -2.277 p = 0.023**	**z = -14.954 p < 0.001**
2.8										**z = -13.705 p < 0.001**

	3.1	**3.2**	**4.1**	**4.2**	**4.3**	**5.1**	**5.2**	**5.3**	**5.4**	**6.1**	**6.2**	**7.1**
1.1	**z = 14.635 p < 0.001**	**z = 13.775 p < 0.001**	**z = 15.892 p < 0.001**	**z = 16.445 p < 0.001**	**z = 16.222 p < 0.001**	**z = 15.006 p < 0.001**	**z = 16.222 p < 0.001**	**z = 12.354 p < 0.001**	**z = -3.308 p < 0.001**	**z = 16.317 p < 0.001**	**z = 13.86 p < 0.001**	**z = 16.222 p < 0.001**
1.2	**z = 17.281 p < 0.001**	**z = 16.375 p < 0.001**	**z = 18.473 p < 0.001**	**z = 19.083 p < 0.001**	**z = 18.803 p < 0.001**	**z = 16.374 p < 0.001**	**z = 17.667 p < 0.001**	**z = 14.887 p < 0.001**	**z = -0.486 p = 0.627**	**z = 19.169 p < 0.001**	**z = 16.444 p < 0.001**	**z = 18.803 p < 0.001**
2.1	**z = -2.911 p = 0.004**	**z = -3.956 p < 0.001**	z = -0.512 p = 0.609	z < 0.001 p = 1	z < 0.001 p = 1	**z = -2.320 p = 0.02**	z < 0.001 p = 1	**z = -5.513 p < 0.001**	**z = -17.434 p < 0.001**	z = -1.533 p = 0.125	**z = -3.597 p < 0.001**	z < 0.001 p = 1
2.2	**z = -2.862 p = 0.004**	**z = -3.891 p < 0.001**	z = -0.504 p = 0.615	z < 0.001 p = 1	z < 0.001 p = 1	z = -2.284 p = 0.022	z < 0.001 p = 1	**z = -5.43 p < 0.001**	**z = -17.29 p < 0.001**	z = -1.503 p < 0.133	**z = -3.542 p < 0.001**	z < 0.001 p = 1
2.3	**z = -2.836 p = 0.005**	**z = -3.856 p < 0.001**	z = -0.499 p = 0.617	z < 0.001 p = 1	z < 0.001 p = 1	z = -2.265 p = 0.024	z < 0.001 p = 1	**z = -5.386 p < 0.001**	**z = -17.212 p < 0.001**	z = -1.487 p = 0.137	**z = -3.513 p < 0.001**	z < 0.001 p = 1
2.4	**z = 3.376 p < 0.001**	**z = 2.3 p = 0.02**	**z = 5.408 p < 0.001**	**z = 5.999 p < 0.001**	**z = 5.869 p < 0.001**	**z = 3.761 p < 0.001**	**z = 5.869 p < 0.001**	z = 0.483 p = 0.629	**z = -13.354 p < 0.001**	**z = 5.132 p < 0.001**	**z = 2.44 p = 0.015**	**z = 5.869 p < 0.001**
2.5	z = 0.393 p = 0.694	z = -0.683 p = 0.495	**z = 2.622 p = 0.009**	**z = 3.182 p = 0.001**	**z = 3.111 p = 0.002**	z = 0.844 p = 0.399	**z = 3.111 p = 0.002**	**z = -2.4 p = 0.016**	**z = -15.175 p < 0.001**	**z = 1.998 p = 0.046**	z = -0.454 p = 0.65	**z = 3.111 p = 0.002**
2.6	**z = 2.484 p = 0.013**	z = 1.4 p = 0.161	**z = 4.586 p < 0.001**	**z = 5.172 p < 0.001**	**z = 5.075 p < 0.001**	**z = 2.864 p = 0.004**	**z = 5.057 p < 0.001**	z = -0.407 p = 0.684	**z = -13.863 p < 0.001**	**z = 4.21 p < 0.001**	z = 1.553 p = 0.12	**z = 5.075 p < 0.001**
2.7	z = 1.08 p = 0.28	z < 0.001 p = 1	**z = 3.269 p = 0.001**	**z = 3.839 p < 0.001**	**z = 3.753 p < 0.001**	z = 1.5 p = 0.134	**z = 3.753 p < 0.001**	**z = -1748 p < 0.001**	**z = -14.701 p < 0.001**	**z = 2.727 p = 0.006**	z = 0.201 p =0.84	**z = 3.753 p < 0.001**
2.8	**z = 3.46 p < 0.001**	**z = 2.364 p = 0.018**	**z = 5.518 p < 0.001**	**z = 6.123 p < 0.001**	**z = 5.986 p < 0.001**	**z = 3.844 p < 0.001**	**z = 5.986 p < 0.001**	z = 0.512 p = 0.609	**z = -13.442 p < 0.001**	**z = 5.262 p < 0.001**	**z = 2.501 p = 0.012**	**z = 5.986 p < 0.001**
2.9	**z = 16.057 p < 0.001**	**z = 15.25 p < 0.001**	**z = 17.226 p < 0.001**	**z = 17.748 p < 0.001**	**z = 17.535 p < 0.001**	**z = 16.404 p < 0.001**	**z = 17.535 p < 0.001**	z = 13.916 p < 0.001	z = 0.435 p = 0.664	**z = 17.639 p < 0.001**	**z = 15.326 p < 0.001**	**z = 17.535 p < 0.001**
3.1		z = -1.125 p = 0.261	z = 2.349 p = 0.261	**z = 2.934 p = 0.003**	**z = 2.862 p = 0.004**	z = 0.488 p = 0.625	**z = 2.862 p = 0.004**	**z = -2.893 p = 0.003**	**z = -15.801 p < 0.001**	z = 1.669 p = 0.095	z = -0.865 p = 0.387	**z = 2.862 p = 0.004**
3.2			**z = 3.391 p < 0.001**	**z = 3.987 p < 0.001**	**z = 3.891 p < 0.001**	z = 1.557 p = 0.119	**z = 3.891 p < 0.001**	**z = -1.814 p = 0.07**	**z = -14.993 p < 0.001**	**z = 2.857 p = 0.004**	z = 0.209 p = 0.834	**z = 3.891 p < 0.001**

	7.2	**7.3**	**7.4**	**8.1**	**8.2**	**8.3**	**8.4**	**9.1**	**9.2**	**9.3**
1.1	**z = 13.632 p < 0.001**	**z = 11.233 p < 0.001**	**z = 9.587 p < 0.001**	**z = 15.14 p < 0.001**	**z = 15.14 p < 0.001**	**z = 13.582 p < 0.001**	**z = 11.966 p < 0.001**	**z = 14.678 p < 0.001**	**z = 13.289 p < 0.001**	**z = 10.38 p < 0.001**
1.2	**z = 16.150 p < 0.001**	**z = 13.692 p < 0.001**	**z = 11.922 p < 0.001**	**z = 17.456 p < 0.001**	**z = 17.456 p < 0.001**	**z = 15.872 p < 0.001**	**z = 14.205 p < 0.001**	**z = 16.991 p < 0.001**	**z = 15.570 p < 0.001**	**z = 12.590 p < 0.001**
2.1	**z = -3.607 p < 0.001**	**z = -6.8 p < 0.001**	**z = -7.531 p < 0.001**	z < 0.001 p = 1	z < 0.001 p = 1	**z = -2.115 p = 0.034**	**z = -4.128 p < 0.001**	z = -0.663 p = 0.507	**z = -2.488 p = 0.013**	**z = -6.171 p < 0.001**
2.2	**z = -3.551 p < 0.001**	**z = -6.698 p < 0.001**	**z = -7.445 p < 0.001**	z < 0.001 p = 1	z < 0.001 p = 1	**z = -2.089 p = 0.037**	**z = -4.078 p < 0.001**	z = -0.655 p = 0.512	**z = -2.457 p = 0.014**	**z = -6.097 p < 0.001**
2.3	**z = -3.521 p < 0.001**	**z = -6.643 p < 0.001**	**z = -7.399 p <0.001**	z < 0.001 p = 1	z < 0.001 p = 1	**z = -2.075 p = 0.038**	**z = -4.05 p < 0.001**	z = -0.651 p = 0.515	**z = -2.44 p = 0.015**	**z = -6.057 p < 0.001**
2.4	**z = 2.423 p = 0.015**	z = -0.806 p = 0.42	**z = -2.377 p = 0.017**	**z = 5.28 p < 0.001**	**z = 5.28 p < 0.001**	**z = 3.268 p = 0.001**	z = 1.266 p = 0.205	**z = 4.666 p < 0.001**	**z = 2.902 p = 0.004**	z = -0.802 p = 0.422
2.5	z = -0.451 p = 0.652	**z = -3.696 p < 0.001**	**z = -4.838 p < 0.001**	**z = 2.788 p = 0.005**	**z = 2.788 p = 0.005**	z = 0.709 p = 0.478	z = -1.317 p = 0.188	**z = 2.146 p = 0.032**	z = 0.337 p = 0.736	**z = -3.39 p < 0.001**
2.6	z = 1.556 p = 0.12	z = -1.7 p = 0.089	**z = -3.135 p = 0.002**	**z = 4.54 p < 0.001**	**z = 4.54 p < 0.001**	**z = 2.499 p = 0.012**	z = 0.479 p = 0.632	**z = 3.915 p < 0.001**	**z = 2.129 p = 0.033**	z = -1.602 p = 0.109
2.7	z = 0.207 p = 0.836	**z =-3.044 p = 0.002**	**z = -4.272 p < 0.001**	**z = 3.365 p < 0.001**	**z = 3.365 p < 0.001**	z = 1.297 p =0.195	z = -0.729 p = 0.466	**z = 2.728 p = 0.006**	z = 0.925 p = 0.355	**z = -2.805 p = 0.005**
2.8	**z = 2.485 p = 0.013**	z = -0.799 p = 0.424	**z = -2.386 p = 0.017**	**z = 5.368 p < 0.001**	**z = 5.368 p < 0.001**	**z = 3.331 p < 0.001**	z = 1.302 p = 0.192	**z = 4.747 p < 0.001**	**z = 2.96 p = 0.003**	z = -0.795 p = 0.426
2.9	**z = 15.103 p < 0.001**	**z = 12.859 p < 0.001**	**z = 11.296 p < 0.001**	**z = 16.487 p < 0.001**	**z = 16.487 p < 0.001**	**z = 15.016 p < 0.001**	**z = 13.486 p < 0.001**	**z = 16.051 p < 0.001**	**z = 14.738 p < 0.001**	**z = 11.993 p < 0.001**
3.1	z = -0.864 p = 0.387	**z = -4.247 p < 0.001**	**z = -5.329 p < 0.001**	**z = 2.542 p =0.011**	**z = 2.542 p =0.011**	z = 0.386 p = 0.7	z = -1.711 p = 0.087	z = 1.875 p = 0.061	z < 0.001 p = 1	**z = -3.852 p < 0.001**
3.2	z = 0.215 p = 0.83	**z = -3.158 p = 0.002**	**z = -4.391 p < 0.001**	**z = 3.463 p < 0.001**	**z = 3.463 p < 0.001**	z = 1.336 p = 0.181	z = -0.751 p = 0.453	**z = 2.809 p = 0.005**	z = 0.952 p = 0.34	**z = -2.889 p = 0.004**

	4.2	**4.3**	**5.1**	**5.2**	**5.3**	**5.4**	**6.1**	**6.2**	**7.1**
4.1	z = 0.515 p = 0.606	z = 0.504 p = 0.614	z = -1.788 p = 0.074	z = 0.504 p = 0.615	**z = -4.963** **p < 0.001**	**z = -16.98** **p < 0.001**	z = -0.944 p = 0.345	**z = -3.058** **p = 0.002**	z = 0.504 p = 0.615
4.2		z < 0.001 p = 1	z = -2.337 p = 0.0194	z < 0.001 p = 1	**z = -5.551** **p < 0.001**	**z = -17.501** **p < 0.001**	z = -1.548 p = 0.122	**z = -3.623** **p < 0.001**	z < 0.001 p = 1
4.3			**z = -2.284** **p = 0.022**	z < 0.001 p = 1	**z = -5.430** **p < 0.001**	**z = -17.290** **p < 0.001**	z = -1.503 p = 0.132	**z = -3.542** **p < 0.001**	z < 0.001 p = 1
5.1				**z = 2.284** **p = 0.022**	**z = -3.278** **p = 0.001**	**z = -16.149** **p < 0.001**	z = 1.059 p = 0.289	z = -1.308 p = 0.191	**z = 2.284** **p = 0.022**
5.2					**z = -5.43** **p < 0.001**	**z = -17.29** **p < 0.001**	z = -1.503 p = 0.133	**z = -3.542** **p < 0.001**	z > 0.001 p =1
5.3						**z = -13.657** **p < 0.001**	**z = 4.633** **p < 0.001**	z = 1.961 p = 0.05	**z = 5.43** **p < 0.001**
5.4							**z = 17.381** **p < 0.001**	**z = 15.071** **p < 0.001**	**z = 17.29** **p < 0.001**
6.1								**z = -2.491** **p = 0.013**	z = 1.503 p = 0.133
6.2									**z = 3.542** **p < 0.001**

	7.2	**7.3**	**7.4**	**8.1**	**8.2**	**8.3**	**8.4**	**9.1**	**9.2**	**9.3**
4.1	**z = -3.066 p = 0.002**	**z = -6.241 p < 0.001**	**z = -7.047 p < 0.001**	z = 0.45 p = 0.652	z = 0.45 p = 0.652	z = -1.645 p = 0.100	**z = -3.647 p < 0.001**	z = -0.205 p = 0.837	z = -2.015 p = 0.044	**z = -5.680 p < 0.001**
4.2	**z = -3.633 p < 0.001**	**z = -6.848 p < 0.001**	**z = -7.571 p < 0.001**	z < 0.001 p = 1	z < 0.001 p = 1	z = -2.128 p = 0.033	**z = -4.152 p < 0.001**	z = -0.667 p = 0.505	z = -2.502 p = 0.012	**z = -6.206 p < 0.001**
4.3	**z = -3.551 p <0.001**	**z = -6.698 p < 0.001**	**z = -7.445 p < 0.001**	z < 0.001 p = 1	z < 0.001 p = 1	z = -2.089 p = 0.037	**z = -4.078 p < 0.001**	z = -0.655 p = 0.512	z = -2.457 p = 0.014	**z = -6.097 p < 0.001**
5.1	z = -1.294 p = 0.196	**z = -4.560 p < 0.001**	**z = -5.691 p < 0.001**	**z = 2.045 p = 0.041**	**z = 2.045 p = 0.041**	z = -0.045 p = 0.964	**z = -2.070 p = 0.039**	z = 1.397 p = 0.162	z = -0.418 p = 0.676	**z = -4.159 p < 0.001**
5.2	**z = -3.551 p < 0.001**	**z = -6.698 p < 0.001**	**z = -7.445 p < 0.001**	z < 0.001 p = 1	z < 0.001 p = 1	**z = -2.089 p = 0.037**	**z = -4.078 p < 0.001**	z = -0.655 p = 0.512	**z = -2.457 p = 0.014**	**z = -6.097 p < 0.001**
5.3	z = 1.954 p = 0.051	z = -1.291 p = 0.12	**z = -2.795 p = 0.005**	z = 4.879 p < 0.001	z = 4.879 p < 0.001	z = 2.852 p = 0.004	z = 0.841 p = 0.401	z = 4.26 p < 0.001	**z = 2.484 p = 0.013**	z = -1.236 p = 0.216
5.4	**z = 14.851 p < 0.001**	**z = 12.599 p < 0.001**	**z = 11.036 p < 0.001**	**z = 16.253 p < 0.001**	**z = 16.253 p < 0.001**	**z = 14.777 p < 0.001**	**z = 13.244 p < 0.001**	**z = 15.815 p < 0.001**	**z = 14.499 p < 0.001**	**z = 11.746 p < 0.001**
6.1	**z = -2.498 p = 0.013**	**z = -6.072 p < 0.001**	**z = -6.886 p < 0.001**	z = 1.313 p = 0.189	z = 1.313 p = 0.189	z = -0.974 p = 0.33	**z = -3.171 p = 0.002**	z = 0.601 p = 0.548	z = -1.379 p = 0.168	**z = -5.404 p < 0.001**
6.2	z = 0.005 p = 0.996	**z = -3.249 p = 0.001**	**z = -4.509 p < 0.001**	**z = 3.178 p = 0.002**	**z = 3.178 p = 0.002**	z = 1.113 p = 0.266	z = -0.907 p = 0.364	**z = 2.541 p = 0.011**	z = 0.742 p = 0.458	**z = -2.987 p = 0.003**
7.1	**z = -3.551 p < 0.001**	**z = -6.698 p < 0.001**	**z = -7.445 p < 0.001**	z < 0.001 p = 1	z < 0.001 p = 1	z = -2.089 p = 0.037	z = -4.078 p < 0.001	z = -0.655 p = 0.512	**z = -2.457 p = 0.014**	**z = -6.097 p < 0.001**
7.2		**z = -3.25 p = 0.001**	**z = -4.45 p < 0.001**	**z = 3.183 p = 0.002**	**z = 3.183 p = 0.002**	z = 1.112 p = 0.266	z = -0.915 p = 0.36	**z = 2.545 p = 0.011**	z = 0.74 p = 0.46	**z = -2.989 p = 0.003**
7.3			z = -1.645 p = 0.1	**z = 6.018 p < 0.001**	**z = 6.018 p < 0.001**	**z = 4.009 p < 0.001**	**z = 1.998 p = 0.046**	**z = 5.408 p < 0.001**	**z = 3.642 p < 0.001**	z = -0.081 p = 0.935
7.4				**z = 6.848 p < 0.001**	**z = 6.848 p < 0.001**	**z = 5.063 p < 0.001**	**z = 3.273 p = 0.001**	**z = 6.306 p < 0.001**	**z = 4.736 p < 0.001**	z = 1.433 p = 0.152

	8.2	**8.3**	**8.4**	**9.1**	**9.2**	**9.3**
8.1	z < 0.001 p = 1	z = -1.909 p = 0.056	**z = -3.73** **p < 0.001**	z = -0.598 p = 0.55	**z = -2.245** **p = 0.025**	**z = -5.582** **p < 0.001**
8.2		z = -1.909 p = 0.056	**z = -3.73** **p < 0.001**	z = -0.598 p = 0.55	**z = -2.245** **p = 0.025**	**z = -5.582** **p < 0.001**
8.3			z = -1.849 p = 0.065	z = 1.316 p = 0.188	z = -0.34 p = 0.734	**z = -3.737** **p < 0.001**
8.4				**z = 3.151** **p = 0.002**	z = 1.509 p = 0.131	z = -1.898 p = 0.058
9.1					z = -1.655 p = 0.098	**z = -5.019** **p < 0.001**
9.2						**z = -3.4** **p < 0.001**

VI.2 *In vivo*

Table VI.2. Mann Whitney U tests for garlic *in vivo* experiment (Chapter 7): comparison of efficacies between individual treatments. Significant results are highlighted in bold (α-level after Bonferroni corrections: 0.010472). 1 – Water control; 2 – Levamisole 0.2 mg/mL; 3 – 0.01 % Tween 20; 4-6 – Freeze dried garlic flakes 1, 0.5 and 0.07 mg/mL, respectively; 7 – Minced garlic 0.07 mg/mL; 8-9 – Garlic granules 0.07 and 0.033 mg/mL; 10 – Chinese freeze dried garlic powder 0.03mg/mL; 11 – Allyl alcohol 10mg/mL; 12 – Allyle disulphide 0.5 mg/mL + 0.01 % Tween 20.

	2.1	**2.2**	**2.3**	**3.1**	**4.1**	**4.2**	**5.1**	**6.1**	**7.1**	**8.1**	**9.1**
1.1	**W = 150.5 p < 0.001**	**W = 223.5 p < 0.001**	W = 281.5 p = 0.704	**W = 349 p < 0.001**	**W = 470.5 p < 0.001**	**W = 519.5 p < 0.001**	**W = 114.5 p < 0.001**	**W = 295.5 p < 0.001**	**W = 402.5 p < 0.001**	**W = 114.5 p < 0.001**	W = 527.5 p = 0.0329
2.1		**W = 373.5 p = 0.006**	**W = 231.5 p < 0.001**	W = 533 p = 0.0133	W = 650.5 p = 0.079	**W = 577.5 p = 0.001**	W = 274.5 p = 0.803	W = 667.5 p = 0.92	**W = 429.5 p = 0.005**	W = 274.5 p = 0.803	**W = 490 p < 0.001**
2.2			W = 147.5 p = 0.024	W = 283.5 p = 0.711	W = 352.5 p = 0.351	W = 336.5 p = 0.421	**W = 132.5 p = 0.013**	**W = 325.5 p = 0.001**	W = 250.5 p = 0.576	**W = 132.5 p = 0.013**	**W = 304 p = 0.003**
2.3				W = 81 p = 0.015	**W = 105 p = 0.005**	W = 103 p = 0.101	**W = 35 p < 0.001**	**W = 87 p < 0.001**	W = 79.5 p = 0.113	**W = 35 p < 0.001**	W = 96 p = 0.857
3.1					W = 553 p = 0.478	W = 515 p = 0.269	W = 217 p = 0.024	**W = 531 p = 0.003**	W = 383 p = 0.426	W = 217 p = 0.024	**W = 456 p < 0.001**
4.1						W = 716 p = 0.09	W = 330 p = 0.091	W = 804 p = 0.028	W = 536.5 p = 0.166	W = 330 p = 0.091	**W = 612 p < 0.001**
4.2							W = 186 p = 0.004	W = 456 p < 0.001	W = 339.5 p = 0.917	W = 186 p = 0.004	W = 398 p = 0.034
5.1								W = 486 p = 0.856	**W = 308.5 p = 0.01**	W = 200 p = 1	**W = 348 p < 0.001**
6.1									**W = 733.5 p < 0.001**	W = 474 p = 0.856	**W = 828 p < 0.001**
7.1										**W = 151.5 p = 0.01**	W = 306 p = 0.041
8.1											**W = 348 p < 0.001**

APPENDIX VII: TREATMENT EFFICACY COMPARISONS FOR MELAFIX® AND PIMAFIX® EXPERIMENTS (CHAPTER 8)

VII.1 *In vitro*

Table VII.1 Cox proportional harzard survival analysis for Melafix®/Pimafix® experiments (Chapter 8): treatment comparisons. Significant results are highlighted in bold. 1 = Melafix® (M); 2 = Pimafix® (P); 3 = Melafix®/Pimafix® combination; 4 = Crovol (M) control; 5 = Crovol (P) control; 8 = Crovol and cajuput oil; 9 = Crovol and bay oil; 10 = Water control; 11 = Levamisole control; 12 = Crovol (M/P) control; 13 = Crovol, cajuput oil & bay oil.

Treatment		**2**	**3**	**4**	**5**	**8**	**9**	**10**	**11**	**12**	**13**
1	**z**	0.587	0.797	-0.514	3.146	-3.089	-0.169	-8.567	1.518	2.511	-1.405
	p	0.557	0.425	0.607	**0.002**	**0.002**	0.866	**< 0.001**	0.129	**0.012**	0.160
2	**z**		0.212	-1.093	2.515	-3.571	-0.734	-8.918	1.340	1.85	-1.94
	p		0.832	0.274	**0.012**	**< 0.001**	0.463	**< 0.001**	0.180	0.064	0.052
3	**z**			-1.299	2.295	-3.734	-0.937	-9.014	1.273	1.615	-2.127
	p			0.194	**0.022**	**< 0.001**	0.349	**< 0.001**	0.203	0.106	**0.033**
4	**z**				3.62	-2.672	0.324	-8.305	1.665	3.046	-0.936
	p				**< 0.001**	**0.008**	0.7458	**< 0.001**	0.096	**0.002**	0.349
5	**z**					-5.748	-3.179	-10.574	0.517	-0.834	-4.298
	p					**< 0.001**	**0.001**	**< 0.001**	0.605	0.405	**< 0.001**
8	**z**						2.919	-6.392	2.436	5.392	1.781
	p						**0.003**	**< 0.001**	**0.015**	**< 0.001**	0.075
9	**z**							-8.531	1.563	2.582	-1.222
	p							**< 0.001**	0.118	**0.010**	0.222
10	**z**								4.217	10.607	7.83
	p								**< 0.001**	**< 0.001**	**< 0.001**
11	**z**									-0.784	-1.926
	p									0.433	0.054
12	**z**										-3.806
	p										**< 0.001**

VII.2 *In vivo*

Table VII.2. Mann Whitney U test results for Melafix®/Pimafix® *in vivo* experiments (Chapter 8): treatment comparisons. Significant results are highlighted in bold (α-level after Bonferroni corrections 0.011377). 4 = Pimafix®; 5 = Melafix®; 6= Melafix®/Pimafix®; 7= Crovol (as in Melafix®); 8= Crovol (as in Pimafix®); 9= Crovol (as in Melafix®/Pimafix®); 10= Crovol (as in Melafix®) & Cajuput oil; 11= Crovol (as in Pimafix®) & West Indian bay oil; 12= Crovol (as in Melafix®/Pimafix®) & Cajuput oil & West Indian bay oil; control = water control.

		11	**12**	**4**	**5**	**6**	**7**	**8**	**9**	**Control**
10	**W**	108	117	143	100	54	101	108	83	154
	P	0.175	0.812	0.624	0.250	**0.001**	0.177	0.188	0.956	0.116
11	**W**		78	105	69	30	76	82	61	214
	P		0.064	0.306	0.016	**<0.001**	0.918	0.895	0.175	0.860
12	**W**			224	142	60	156.5	169.5	128	199
	P			0.328	0.167	**<0.001**	0.074	0.065	0.874	0.024
4	**W**				118	37	145	156	114	265
	P				0.021	**<0.001**	0.293	0.296	0.543	0.234
5	**W**					118	187	197.5	160	167
	P					**0.005**	**0.006**	**0.008**	0.260	**0.001**
6	**W**						244	253.3	219	64.5
	P						**<0.001**	**<0.001**	**0.001**	**<0.001**
7	**W**							80	55	192
	P							0.922	0.177	1
8	**W**								61	208.5
	P								0.188	1
9	**W**									154
	P									0.116