HOST SPECIFICITY AND LOCAL ADAPTATION IN GYRODACTYLIDS

Tracey Anne King

This thesis is submitted for the degree of Doctor of Philosophy

School of Biosciences, Cardiff University
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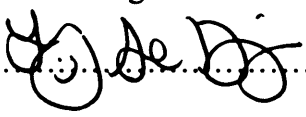
Signed  (candidate) Date 4/1/2009

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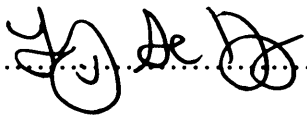
Signed  (candidate) Date 4/1/2009

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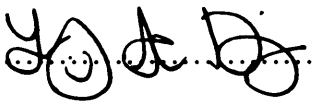
Signed  (candidate) Date 4/1/2009

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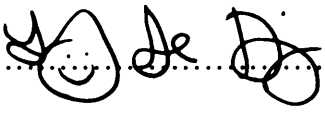
Signed  (candidate) Date 4/1/2009

ABSTRACT

The monogenean fish ectoparasites, *Gyrodactylus* spp. have been studied extensively as model organisms, but two key areas, to date, have received little attention; host specificity and local adaptation, and these form the central theme of this thesis.

Host specificity was examined for two tropical parasite species infecting the guppy; *Gyrodactylus turnbulli* and *G. bullatarudis*. Contrary to the expectation that *G. turnbulli* is a strict specialist, this parasite can infect a range of hosts under both laboratory and semi-natural conditions. Furthermore, the congener species, *G. bullatarudis*, can infect and reproduce on a temperate fish host, the three-spined stickleback. This thesis also identified different transmission strategies of these two species, which are affected by temperature. Whereas *G. turnbulli* migrates away from a dead host at high burdens, *G. bullatarudis* stays with a dead host, even though survival times for both species are similar. Arising from these host specificity studies was the discovery that *G. lomi*, a parasite of chub, could persist as long-term infections on isolated fish, and two new gyrodactylid species, *G. zebrae* n. sp. and *G. danio* n. sp., are described from zebrafish.

Finally, local adaptation theory was examined for *G. gasterostei*, which infects the three-spined stickleback throughout England and Wales but not in the Hebridean Islands. This study ascertained that Hebridean host populations were no more susceptible to *G. gasterostei* than their mainland counterparts. No evidence of local adaptation was found due to overriding temporal effects. However, local differentiation between populations in their susceptibility and resistance was detected, together with sticklebacks having a much longer immunological memory than previously considered.

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CHAPTER 1: INTRODUCTION

1.1 *GYRODACTYLUS*

1.1.1 Overview

The monogenean ectoparasites, *Gyrodactylus* spp. are ubiquitous parasites of teleost fish, considered by Kennedy (1994) to be amongst the most invasive of fish parasites, due to their unique viviparous reproduction and exponential growth rate. These parasites have a direct life cycle, reproducing *in situ* on the host and lack a specialised transmission stage, so are capable of transferring to new hosts at any time during their life cycle (Boeger *et al.*, 2005). This genus is the most economically important group of monogeneans (Bakke *et al.*, 2002), they pose a conservation threat to endangered fish stocks (Leberg and Vrijenhoek, 1994; Hedrick *et al.*, 2001) and have been of interest to researchers since the 19th Century (reviewed in Cable and Harris, 2002).

The use of gyrodactylids as model organisms was first realised primarily due to their unique mode of reproduction, particularly their viviparity (e.g. Braun, 1966). This momentum gathered pace with their use as an epidemiological model (Scott and Anderson, 1984) but came to the fore when their status as important fish pathogens was confirmed following introduction of *G. salaris* into East Atlantic salmon stocks and its catastrophic effects on these fish populations in Norway. Even more recently, gyrodactylids have been used as a model system to assess the impact of parasites on conservation practices (van Oosterhout *et al.*, 2007; Faria *et al.*, submitted). As such this genus is now among the most well studied taxon amongst the monogeneans. Much of this work has been summarised in the last six years, with three major reviews on gyrodactylids: Bakke *et al.* (2002), Cable and Harris (2002), and the most comprehensive to date by Bakke *et al.* (2007). In addition, there has been the recent development of a dedicated database, www.gyrodb.net, aimed at providing a common resource for species descriptions and taxonomic literature on *Gyrodactylus* species (see Harris *et al.*, 2004, 2008). This Introduction Chapter sets out to briefly summarise what is known about the basic biology of this specious genus, before reviewing two research areas which form the central theme of this thesis. Finally, the aims and layout of the data chapters are briefly described.

1.1.2 Taxonomy

The Monogenea are typically divided into two sub-classes: the Monopisthocotylea Odhner, 1912 and the Polyopisthocotylea Odhner, 1912 (see Bentz *et al.*, 2003), differentiated by their

attachment organ (the opisthaptor or haptor). The Monopisthocotylea haptor is a single unit bearing hooks, whilst the haptor of the Polypisthocotylea is more complex, some species bearing suckers and/or clamps. In addition, there are broad differences in feeding modes, the Monopisthocotylea being typically tissue grazers causing significant damage, whilst the Polyopisthocotylea tend to be blood feeders with comparatively little damage (Roberts, 2001). The two sub-classes diverged before the emergence of the modern fishes (Kearn, 1994), and the Gyrodactylidae are traditionally affiliated with the monopisthocotyleans, although this taxonomic position is controversial (Boeger *et al.*, 2003). It was suggested by Boeger *et al.* (2003) that the Gyrodactylidae originated via a host switch from a marine ancestor to freshwater catfishes (*Loricariidae*) in South America. Previously, Harris (1983) had described the first oviparous gyrodactylid, *Oogyrodactylus farlowellae*, from the South American catfish, *Farlowella amazonum*, noting a close resemblance between the immature reproductive system of this species and the mature system in *Gyrodactylus*. However, it is still an issue of debate as to whether the viviparous gyrodactylids evolved from oviparous gyrodactylids (Bakke *et al.*, 2002).

The genus *Gyrodactylus* is one of 23 genera of the family Gyrodactylidae, with 19 viviparous genera and four oviparous genera (Bakke *et al.*, 2007), although two of the viviparous genera are synonyms and thus 17 viviparous genera are considered valid (Bakke *et al.*, 2007). The viviparous gyrodactylids occur on all bony fish from the Anguilliformes onwards (Bakke *et al.*, 2002), the most primitive fish host being the chondrostean, *Polypterus*, although it has been suggested by Bakke *et al.* (2002) that this host actually acquired gyrodactylids via a secondary infection. Although there is high species richness, their morphological diversity is conserved (Bakke *et al.*, 2002). Currently, there are 409 valid *Gyrodactylus* species described (Harris *et al.*, 2004). However, this number may represent only a small fraction of the true number of species, given the assumption that most gyrodactylids have been recorded from a single host, that some hosts support more than one gyrodactylid and that there is an estimated 23,000 fish host species (Bakke *et al.*, 2002). Therefore, the actual number of species could be as high as 20,000, making this a hyperdiverse genus among the monogeneans and probably one of the largest parasite taxons on the planet (Bakke *et al.*, 2002, 2007).

Traditionally, the taxonomy of the genus *Gyrodactylus* was predominantly based on morphology, although this required considerable expertise by researchers. Hence, their taxonomy was revolutionised with the advent of molecular markers allowing the construction of phylogenetic relationships, and relatively simple identification of a species. However, it is widely recognised that although the molecular tools are extremely useful a combination of

morphological, molecular and ecological data is optimal for species descriptions (Harris *et al.*, 2004).

1.1.3 Morphology

In common with other monogeneans, the genus *Gyrodactylus* is characterised by a posterior opisthaptor (Fig. 1.1A). This attachment structure comprises a central pair of "fish-hook" shaped hamuli connected by a dorsal and ventral bar. There are 16 peripheral marginal hooks (Fig. 1.1B) which allow the parasite to distribute its load on the host's epidermis (Kearn, 1994). Despite the apparent severe appearance of the hamuli on first observations, these are not actively involved in attachment (Shinn *et al.*, 2003). Rather than piercing the host's epidermis, Lester (1972) described the mode of attachment as tension on muscles associated with the marginal hooks, thus causing the hamuli to sink into the host epidermis thereby compressing epidermal cells. The marginal hooks act as the principle mode of attachment, whilst the hamuli act in preventing dislodgement. Thus, the alternating action of these two muscle systems (hamuli and marginal hooks), achieve attachment via the marginal hooks with the hamuli acting as tension. Variations in the size and shape of the hamuli, marginal hooks, dorsal and ventral bars have been utilised as characteristics in identifying different species.

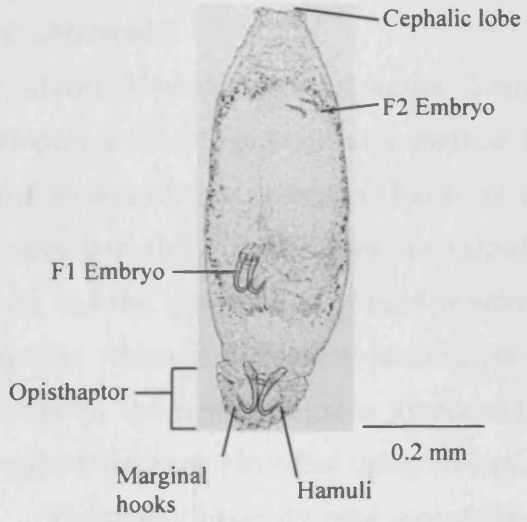

Fig. 1.1A: Light micrograph of *Gyrodactylus salaris* (Micrograph provided by Dr Jo Cable, Cardiff University)

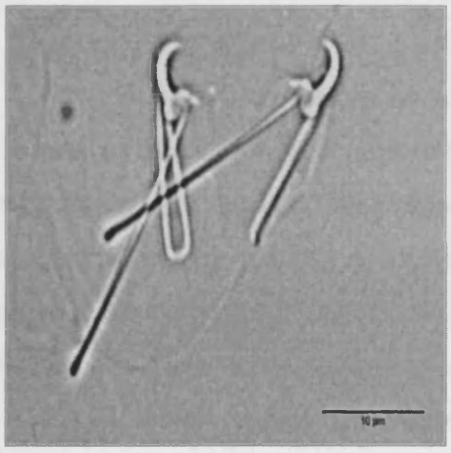

Fig. 1.1B: Marginal hooks of *Gyrodactylus lomi*

Much of what is now known of the taxonomy of the genus *Gyrodactylus* originated from the work of Malmberg (1970). He sub-divided this genus, based on the structure of the excretory systems, into six subgenera: *G. (Gyrodactylus)*, *G. (Mesonephrotus)*, *G. (Paranephrotus)*, *G.*

(Metanephrotus), *G. (Neonephrotus)* and *G. (Limnonephrotus)*. Within each of these subgenera, *Gyrodactylus* species were allocated to 20 species groups based on their marginal hook morphology, host identity and site of infection. However, identification based on morphology alone has proved problematic, in part due to the level of expertise that is required for identification and discrepancies in measurement of the marginal hooks that can arise during specimen preparation. Further confounding factors are the considerable intra-species variation (see Harris, 1998a) and marginal hook variation that can arise due to abiotic factors such as water temperature and seasonality (e.g. Ergens and Gelnar, 1985; Mo, 1991; Harris, 1998a; Geets *et al.*, 1999; Dávidová *et al.*, 2005; reviewed in Bakke *et al.*, 2007).

More refined methods of *Gyrodactylus* species identification have been proposed, principally as a response to the *G. salaris* epidemic, in order to differentiate between the highly pathogenic *G. salaris* from the non-pathogenic species occurring on salmonids. Such methods include chaetotaxy (the use of silver nitrate in mapping surface sensory structures) described by Shinn *et al.* (1998a, 1998b) and the use of statistical classifiers, such as feed-forward neural networks, on morphological measurements obtained via Scanning Electron Microscopy (Shinn *et al.*, 2000) and light microscopy (McHugh *et al.*, 2000).

1.1.4 Molecular

The advent of molecular techniques during the 1990s arose predominantly as a means of developing a reliable identification method for *G. salaris* in the on-going attempt to prevent the further spread of this pathogen (Bakke *et al.*, 2007). Molecular markers have driven forward advances into fields such as species identification, particularly by identifying hitherto cryptic species and the construction of host-parasite phylogenies (Criscione *et al.*, 2005). For example, *G. salaris* which is difficult to distinguish morphologically from other salmon gyrodactylids, particularly, the non-pathogenic gyrodactylid, *G. thymalli* which infects grayling (*Thymallus thymallus*), has been identified using molecular markers (Hansen *et al.*, 2003).

The most frequently used molecular markers are based on the genes and spacers of the ribosomal DNA gene cassette (rDNA). The first marker to be developed was from the V4 region of the 28S gene (Cunningham *et al.*, 1995), having been chosen as this region is highly conserved allowing the development of universal primers. Sequence variation of the V4 region was used to distinguish *G. salaris* from *G. truttae* and *G. derjavinoides* although it could not distinguish *G. salaris* from *G. thymalli* (see Cunningham *et al.*, 1995). These results prompted the development of markers based on other regions of the rRNA gene array, such as the Internal Transcribed

Spacers (ITS) and 5.8S gene, via restriction fragment length polymorphism (RFLP) and sequence analysis (Cunningham, 1997; Cable *et al.*, 1999). However, ITS markers do not discriminate between *G. salaris* and *G. thymalli* (see Cunningham, 1997; Matějusová *et al.*, 2001) and hence, Sterud *et al.* (2002) proposed the use of the intergenic spaces (IGS) as a viable alternative marker. Nevertheless, ITS sequences have been invaluable for all other species identification, with approximately 100 species listed in GenBank. ITS markers have helped to clarify phylogenetic relationships, two significant findings being the existence of two paraphyletic clades of *G. salaris*: *G. salaris* (*sensu stricto*) Malmberg, 1957 and a Danish variant from rainbow trout (*Oncorhynchus mykiss*) (see Lindenstrøm, 2003) and Cable *et al.*'s (1999) finding that *Gyrodactylus* can be split into two distinct groups. Using ITS1 sequences, Cable *et al.* (1999) discovered that species groups such as *G. arcuatus* have short ITS1 sequences (ranging from 300 to 500 base pairs), whilst others such as the *G. wageneri* species group have long ITS1 sequences (ranging from 610 to 630 base pairs).

Although, ITS sequences have no doubt been invaluable in establishing phylogenetic relationships, there is still considerable debate as to whether molecular analyses support the taxonomic groups proposed by Malmberg (1970). Ziętara *et al.* (2002) concluded that 5.8S sequences do provide evidence for divergence between the subgenera, but there were discrepancies at the species level caused by morphological and molecular variation. However, it was noted by Matějusová *et al.* (2003) that these conclusions were somewhat premature, with Ziętara *et al.*'s (2002) results based on sequences from just 10 out of the 400 described species.

Mitochondrial DNA is commonly used as a phylogeographic marker, and the cytochrome oxidase gene (COI) is the marker of choice for DNA barcoding studies. On face value, mitochondrial DNA would appear to be an optimal marker given that the biology of *Gyrodactylus* is female dominated. Indeed, Meinilä *et al.* (2002) described this marker as being "superoptimal" for *Gyrodactylus*, particularly as during asexual reproduction, the genome would act as a single linkage group. Thus, there has been the development of primers which are capable of amplifying approximately 820 base pairs of the mitochondrial COI gene from *G. salaris* by Meinilä *et al.* (2002), who detected a difference of 24% between *G. salaris* and the closely related *G. lavareti*. This is significant considering that this difference is eight-fold than that detected by ITS sequences (3%) between the two species. A major step forward in resolving the on-going difficulties in differentiating between *G. salaris* and *G. thymalli*, came with Hansen *et al.*'s (2003) findings, using COI, that there appeared to be several lineages of both *G. salaris* and *G. thymalli* which suggested that there had been multiple introductions into Norway. Although it

shows much promise as a marker, the widespread use of COI for *Gyrodactylus* has been delayed due to the lack of universal primers (Meinilä *et al.*, 2002). However, recent developments have included the sequencing of whole mitochondrial genomes. Currently three genomes have been sequenced for salmonid gyrodactylids; *G. salaris* (see Huyse *et al.*, 2007); *G. thymalli* (see Plaisance *et al.*, 2007) and *G. derjavinoides* (see Huyse *et al.*, 2008).

1.1.5 Reproduction

Interest in *Gyrodactylus* species arose primarily due to their unusual reproductive biology, particularly their viviparity. The significance of the *in utero* F1 embryo, was initially overlooked by von Nordmann (1832) who attributed hooks found within the central region of the body, whilst describing *G. elegans*, as stomach hooks. Their true significance was subsequently realised by von Sieboldt (1849). The reproductive strategy of *Gyrodactylus* species has been a major factor in their success in colonising teleost hosts. *Gyrodactylus* species are viviparous, the parent contains an F1 embryo *in utero*, which in turn has a developing embryo (F2) within it. Thus, the parent gives birth to fully formed offspring which attach themselves *in situ* on the host, alongside the parent. This method of viviparity is exceptional amongst viviparous organisms and has been termed "hyperviviparity" by Cohen (1977), although it is commonly best described using the analogy of a "Russian Doll". Although this hyperviviparity allows reproductive rate to be extremely rapid, the drawback is the obvious loss of fecundity caused by the cost of retaining an embryo *in utero* (Kearn, 1998), but by adopting a "Russian Doll" strategy, these costs are easily overcome.

Reproduction in *Gyrodactylus* follows a specific pattern, with each successive generation having different origins (Cable and Harris, 2002; see Fig. 1.2). The first born daughter develops as a ball of cells within the embryonic parent, whilst second born and subsequent daughters develop from oocytes, these entering the uterus following the birth of the preceding daughter (Cable and Harris, 2002). As described by Cable *et al.* (1998), the female reproductive system is greatly simplified, comprising an Egg Cell Forming Region (ECFR), the uterus and vitelline cells. The ECFR lies posteriorly to the uterus and serves as both a seminal receptacle (sperm storage) and ovary. All structures are lined by syncytia rather than conventional cellular epithelia (Cable *et al.*, 1998). The ovary consists of a thin lining to the chamber, where a single maturing oocyte occupies the central region of the ECFR. Following birth, the large central oocyte enters the uterus in the region of the posterior cap cell and divides almost instantaneously.

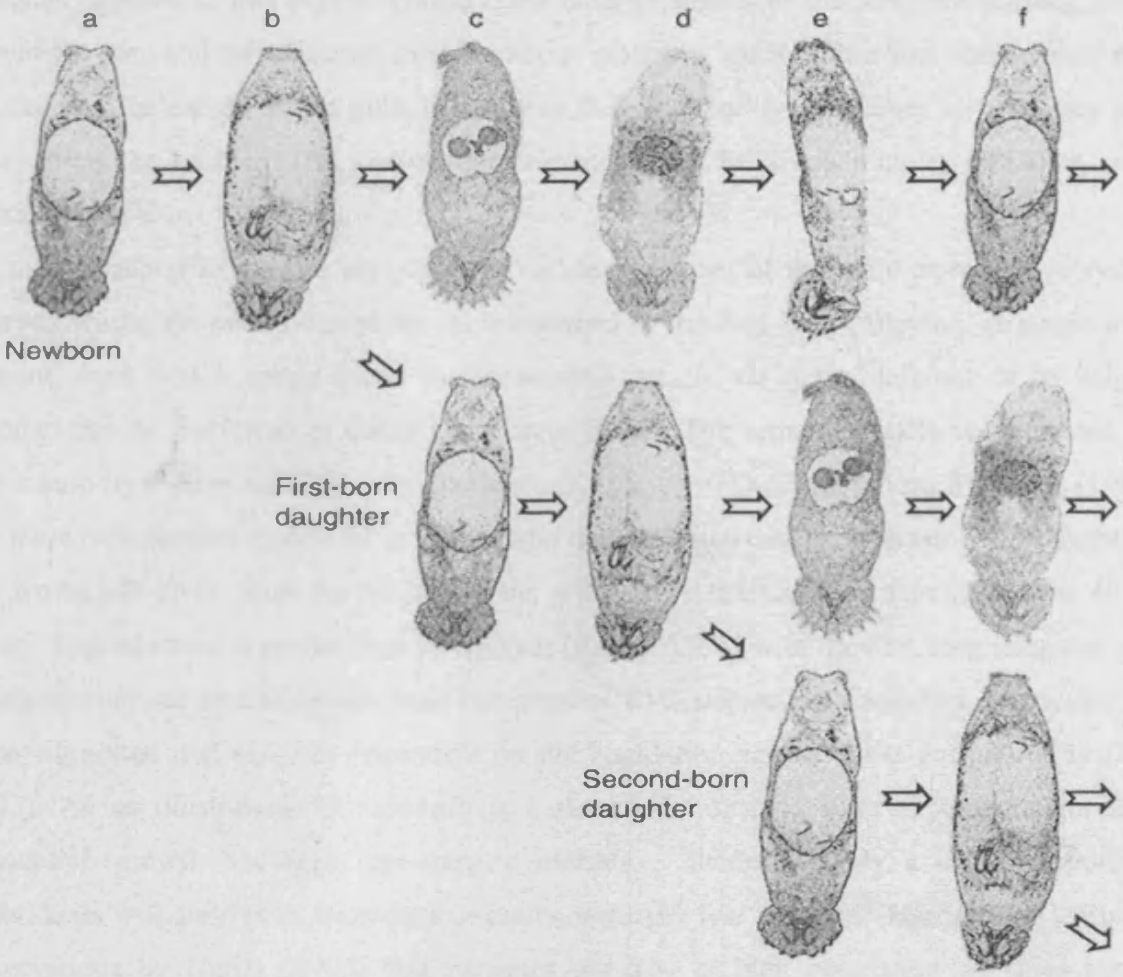

Fig. 1.2: Micrographs of the different reproductive modes and stages of development in *Gyrodactylus*. a-f represent the life cycle stages of a newborn parasite where: a = newborn; b = Uterus containing developing F1 and F2 embryos; c = Uterus contracting following birth; d to f = Development of a subsequent F1 embryo (after Bakke *et al.*, 2007).

Embryo development is characterised by duet cleavage and "blastomere anarchy", in which F1 cells are freely distributed within the uterus, associating and disassociating with each other until a fairly late stage of development (Cable and Harris, 2002). The F2 embryo develops within the F1 while the latter is still an undifferentiated ball of cells. Both embryos are thought to derive their nutrition directly from the parent (Cable *et al.*, 1996), rather than from vitelline cells as in other monogeneans. The F1 remains *in utero* until it is fully grown, with the near term embryo folded double within the uterus, with the embryo haptor and anterior attachment glands clearly visible in the proximal portion of the parent's uterus just above the ECFR. Birth occurs very quickly involving participation of both mother and daughter. The birth pore lies just below the pharynx of the mother (Jones *et al.*, 1998), but its position is only revealed when a small portion of the

daughter appears in this region. Quickly, the anterior region of the daughter is freed from the parental worm, and the offspring uses it anterior glands to attach to the host immediately next to its mother. The daughter then pulls herself free from the mother and moves several body lengths away from the mother. The mother remains contracted for some minutes, but then resumes normal behaviour.

Gyrodactylus species are protogynous, development of the male reproductive system is delayed whilst the parent completes its investment in the first born offspring. A single testis is present, from which sperm travel to the seminal vesicle via a vas deferens or by migrating through tissues (reviewed in Cable and Harris, 2002). The seminal vesicle is connected to the penis bulb by a short duct, the pars prostatica (Kritsky, 1971). Observations by Harris (1985) on the male reproductive system of *G. gasterostei* demonstrated that the penis develops shortly after the worm has given birth for the first time, with active spermatozoa appearing after 40 to 50 hours. Reproduction is predominantly asexual (Harris, 1993) with the first born daughter always being asexual and sexual reproduction being restricted to subsequent daughters. Previously, it has been suggested that sex was dependent on the population age structure and mortality (Harris, 1993). As an illustration, *G. turnbulli* is a short lived species, with exponential increase in population growth but high, age-specific mortality. Therefore, only a small proportion of individuals will survive to reproduce sexually, typically less than 1% (Harris, 1993). However, observations by Harris (1993) that parasites copulate at high population densities has been contradicted by recent studies which indicate that insemination of *G. turnbulli* occurs frequently even at low densities (Cable and Scott, unpublished). In contrast, *G. salaris*, experiences low mortality with sexual reproduction reportedly being common, as more individuals survive to be capable of sexual reproduction (approximately 10 to 15%; Harris, 1993). Another species, *G. gasterostei*, behaves similarly to *G. turnbulli* at 15°C but a reduction in temperature to 10°C results in a decrease in mortality, thus sexual reproduction appears to be rare with clonal reproduction the norm (Harris, 1998b).

The use of asexual and sexual reproductive strategies could be correlated with both host specificity and phenotypic variation (Harris, 1993). For species, such as *G. salaris,* that have a wide host range, sexual reproduction may predominate (Bakke *et al.*, 2002), as well as in species that have high levels of morphological variation (see Geets *et al.*, 1999). Alternatively, asexual reproduction may predominate as a means of preventing hybridisation, thus promoting the fixation of mutations and adaptive mechanisms (Boeger *et al.*, 2003). One obvious downside of this, is that asexual clones face the risk of extinction if they cannot develop adaptations to

overcome new host defences, as would evolve via sexual reproduction. Furthermore, asexual reproduction allows deleterious alleles to remain in the population (Boeger *et al.*, 2003). In most species, there is probably a balance between sexual and asexual reproduction, but the factors controlling this are yet to be evaluated. However, all these studies regarding the importance of sexual and asexual reproduction are speculative as although transfer of sperm has been demonstrated (e.g. Harris *et al.*, 1997) there is no conclusive evidence yet of recombination in these parasites.

1.1.6 Immunity

Fish populations typically show heterogeneity in their response to parasitic infection and as such can be classified in one of three ways based on the outcome of an infection (Bakke *et al.*, 2002). Susceptible fish are those on which parasite population growth increases, as the host is unable to mount an effective immune response, and thus the infection is usually fatal. Responding fish experience an initial parasite population growth which peaks and then declines in response to the fish mounting an effective immune response. Finally, some fish can be innately resistant and the parasite does not reproduce. The proportion of fish in each of these categories varies between host populations and species (Lindenstrøm, 2003). In common with other vertebrates, the immune system of fish comprises two lines of defence: innate (non-specific) and acquired (specific) immunity, which can either operate sequentially or simultaneously as seen in the complement system (Bone and Moore, 2008).

1.1.6(i) Innate immunity

The non-specific immune response is rapid (Wegner *et al.*, 2007) involving physical barriers (such as the epidermis), chemical defences (cytokines, precipitins, nitric oxide and histamines) and generalised responses (phagocytosis and inflammation). With repeated exposure to the same parasite, innate immunity does not become more effective (Bone and Moore, 2008). With regard to *Gyrodactylus* infections, the actual mechanisms by which the immune system of fish responds is yet to be fully understood (Buchmann *et al.*, 2003). Non-specific responses of fish have been suggested to be the major line of defence by numerous studies (reviewed by Bakke *et al.*, 2007). Lester (1972) observing infections of *Gyrodactylus alexanderi* on the three-spined stickleback (*Gasterosteus aculeatus*), noted that a mucoid layer was shed every one to two days by healthy fish, and thus any gyrodactylids attached to this layer would be removed. Although Lester (1972) suggested that this action was not a direct response, it could be elicited via a stimulus from

gyrodactylid activity, such as attachment or feeding. Therefore, this shedding is not considered as a humoral response but rather a local tissue response. Subsequently, Scott (1985a) also considered that the host response (guppies) to gyrodactylid was predominantly non-specific. Lindenstrøm and Buchmann (2000) reported mucus cell hyperplasia, followed by a decline, in rainbow trout infected with *G. derjavinoides* and suggested that the increased mucous cell density was a direct result of immune activity, but with the subsequent decline being caused by cell depletion.

Buchmann (1999) described the skin immune response in fish, which he noted has parallels with mammalian systems. Ectoparasitic activity on the host epidermis triggers the production of cytokines (IL-1 and possibly TNF and INF) from epithelial cells, which in turn stimulate the secretion of mucus from mucous cells. Fish mucus contains cytokines, immunoglobulin, C-reactive protein, lectins, lysozyme, haemolysins and complement (Yano, 1996). Studies by Harris *et al.* (1998) and Buchmann (1998a) demonstrated that host complement produced via the alternative pathway (via complex carbohydrates on the surface of the pathogen; Bakke and Harris, 1998) will kill *G. salaris in vitro.* Whether complement affects other *Gyrodactylus* species has yet to be evaluated. Parasites may also be killed via cytokines which direct the action of leucocytes at the site of inflammation, releasing noxious substances (Buchmann, 1999).

1.1.6 (ii) Acquired immunity

For higher vertebrates, acquired immunity involves two types of lymphocytes, B-cells and T-cells, the activity of both being under the control of the Major Histocompatibility Complex and in this form of immunity, there is increased protection to an organism with exposure to the same parasite/pathogen (Bone and Moore, 2008). Moreover, it is slower than the innate response and can take several weeks to develop (Wegner *et al.*, 2007). There is currently no evidence for the involvement of specific antibodies by a host in response to *Gyrodactylus* infections (Lindenstrøm, 2003). Studies on the genetic basis of immunity via the Major Histocompatibility Complex genes (MHC), particularly the Class IIB genes, indicate that individual fish show considerable variation in their allelic diversity in these genes, resulting in variable parasite resistance (Kurtz *et al.*, 2004; van Oosterhout *et al.*, 2006). Although genetic factors are a major contributor to the efficiency of the immune response, stress (caused by overcrowding, isolation and photoperiod) can have an important role in facilitating gyrodactylid infections.

Experimentally, it was shown that exposure of salmonids to hydrocortisone acetate (an immunosuppressor) increased host susceptibility to *G. salaris* (see Harris *et al.*, 2000).

Immune responses specific against gyrodactylid infection are uncommon (Lindenstrøm, 2003). Richards and Chubb (1996) found no evidence of a species specific immune response to either *G. turnbulli* or its congener, *G. bullatarudis* on guppies. Madhavi and Anderson (1985) were the first to present evidence of innate and acquired immunity in guppies infected with gyrodactylids. Although they made no specific attempt to describe the basis of the response, their study showed clear differences between host individuals in the response to gyrodactylid infection, which had to have a genetic basis. Furthermore, they demonstrated that ornamental guppies had a refractory period of 6 weeks, during which time they were resistant to gyrodactylid infection, a similar finding was made by Scott (1985b) of a refractory period of 4-6 weeks in ornamental stocks. More recently, Cable and van Oosterhout (2007a) carried out a study on wild guppy populations and found that prior exposure improved immunocompetence in a susceptible population (Upper Aripo). In addition, they found that the refractory period in wild guppies was longer, with some being resistant for up to 53 days (Cable and van Oosterhout, 2007a).

Of course, typically parasites do not occur as single species infections, but in the wild, hosts may carry a range of parasite co-infections and cross species immunity has been suggested by Buchmann *et al.* (1999) who found that susceptibility to the parasitic ciliate, *Ichthyophthirius multifilis*, differed between naïve rainbow trout and those that had been exposed to *G. derjavinoides*. For susceptible hosts, gyrodactylid infections can prove fatal. To date, it is unknown how gyrodactylids cause host mortality. In their review on parasite pathogenicity, primarily on *G. salaris*, Bakke *et al.* (2007) refer to two likely causes: (i) either damage inflicted by gyrodactylids allows the entry of a secondary pathogen, or (ii) it causes a disruption in the osmotic balance.

1.1.7 Transmission

In view of their direct life cycle and lack of a specialised transmission stage, *Gyrodactylus* spp. are reportedly capable of continuous transmission and able to infect new hosts at any time during their life cycle, thus ensuring that they have access to new host resources (Boeger *et al.*, 2005). Presently, there are four acknowledged transmission routes of gyrodactylids to their hosts: (i) direct transfer between live fish; (ii) direct transfer between fish and detached parasites on the substrate; (iii) transfer between fish and gyrodactylids in the water column, and (iv) contact between live and dead infected fish (see Bakke *et al.*, 1992; Soleng *et al.*, 1999a).

Gyrodactylus spp. had been considered incapable of swimming but this has since been disproved with the discovery that *G. rysavyi*, which occurs on the Nile catfish, is capable of unidirectional swimming movements for up to 8 s (El-Naggar *et al.*, 2004). Transmission is a risky strategy (Scott, 1985a) and therefore it may be expected that gyrodactylids adopt a transmission strategy to maximise their chances of finding suitable hosts. Therefore, such strategies may be related to host behaviour and ecology (Bakke *et al.*, 2007). For example, Atlantic salmon (*Salmo salar*) are predominantly solitary fish, with fry and parr occurring close to the substrate in shallow, fast flowing water (Baglinière and Champigneulle, 1986). One of its associated gyrodactylids, *G. salaris*, has been shown experimentally to remain with a dead host, attributed in part to the solitary nature of their host (Olstad *et al.*, 2006). In contrast, some species move off a dead host, such as: *G. rarus* and *G. cryptarum* which infect the nine-spined stickleback (*Pungitius pungitius*) and Atlantic cod (*Gadus morhua*), respectively (Malmberg, 1970); *G. gasterostei* which occurs on the three-spined stickleback (*Gasterosteus aculeatus*) (see Cable *et al.*, 2002a); and *G. turnbulli* which infects the guppy (*Poecilia reticulata*) (see Cable *et al.*, 2002b). For these *Gyrodactylus* species, detachment from a dead host, may be attributed to the behaviour of their respective hosts, which all occur in shoals, and therefore the likelihood of successful transmission to a new host is increased.

1.1.8 Epidemiology

As noted by Bakke *et al.* (2002), there may be host factors on innately resistant fish that affect gyrodactylids even in the early stages of infection prior to any observations of a population decline in reproductive rate of *G. salaris*. In addition to this microenvironment control over gyrodactylid infections, there are also a range of macroenvironmental factors which are involved in controlling gyrodactylid population dynamics (Bakke *et al.*, 2007). Some of these factors are natural, such as water temperature and salinity, whilst others are artificial and include pollutants such as metals and petroleum hydrocarbons (Bly *et al.*, 1997). Of these, Bakke *et al.* (2007) considered that the most important of these abiotic factors was most likely to be temperature, followed by water chemistry and water quality, although of course there are many factors that are yet to be evaluated.

1.1.8 (i) Temperature

Fish are poikilothermic, thus, their immune system has to be able to cope with a range of environmental conditions (Tort *et al.*, 2004). It is thought that at low temperatures, the acquired

immune system is suppressed, although this appears to be offset by the actions of the innate immune system, although the mechanisms that control this are yet to be fully understood (Le Morvan *et al.*, 1998). However, greater clarification is needed as to the extent to which the innate immune system is also compromised at low temperatures (Nikoskelainen *et al.*, 2004). Seasonal changes such as fish migration or spawning place heavy physiological demands which in turn can lead to immunosuppression and thus promote increased infection potential for parasites (Tort *et al.*, 2004).

With regard to gyrodactylids, temperature is the major factor that affects their population growth rate (Dávidová *et al.* 2005), reproduction and mortality (Scott and Anderson, 1984; Jansen and Bakke, 1991; Dávidová *et al.*, 2005; Bakke *et al.*, 2007). There have been a number of studies on the effect of temperature on gyrodactylids and from these there appears to be a common trend. At higher temperatures, population growth rate and reproductive rate are increased, whilst at lower temperatures their survival is prolonged. Thus, it would appear that gyrodactylids have to make a trade-off in maximising either their fitness or survival. However, as noted by Scott and Nokes (1984), although there may be optimal temperatures for key life history traits such as fecundity and survival, these must be balanced in line with the optimal temperature range of the host.

These studies on temperature have been restricted to just a few *Gyrodactylus* species, but all have shown a similar trend. Effects of temperature on reproductive rate have been studied for *G. gasterostei* (see Harris, 1982); *G. salaris* (see Jansen and Bakke, 1991) and *G. turnbulli* (see Scott and Anderson, 1984; Scott and Nokes, 1984). All found that reproductive rate increased significantly with an increase in temperature. In addition, it would also appear that the time between births is affected by temperature (Scott and Nokes, 1984; Harris, 1998b) and temperature directly affects gyrodactylid development. Harris (1998b) noted that for *G. gasterostei*, embryogenesis is prolonged at lower temperatures. This may explain seasonal variation found in the size of the hamuli and marginal hooks (Ergens and Gelnar, 1985; Harris, 1998b; Bakke *et al.*, 2007), with haptoral parts being smaller during the warmer seasons than those collected during the winter (Dávidová *et al.*, 2005).

Effects on temperature on population growth can be significant. Studies by Gelnar (1987, 1991) found that the mean maximum parasite burden increased two fold and the time taken to maximum load almost halved with a six degree increase from 12 to 18°C for *G. katharineri* (see Gelnar, 1987). However, for *G. gobiensis*, the reverse occurred whereby parasite numbers increased with a gradual decrease in temperature from 18 to 12°C (Gelnar, 1991).

Temperature also affects survival of gyrodactylids, generally low temperatures prolonging survival. Although maximum mean instantaneous per capita birth rate for *G. turnbulli* is highest at 27.5°C, its maximum survival is several degrees lower at 21°C (Scott and Nokes, 1984). This trend was previously noted for *G. alexanderi* by Lester and Adams (1974) where maximum survival *in vitro* was 28 days at 15°C but was extended considerably to 71 days at 7°C. Another species infecting the same host as *G. alexanderi*, the three-spined stickleback, *G. gasterostei* also had prolonged survival *in vitro* with a reduction of temperature increasing from 66 h at 15°C to 103 h at 4°C (Cable *et al.*, 2002a). This prolonged survival could be explained by a slowing down of the parasite's metabolism but this is at the cost of reproduction. With regards to *in vitro* survival, such studies have been restricted to only four gyrodactylid species as reviewed by Cable *et al.* (2002a), namely *Macrogyrodactylus polypteri* (see Khalil, 1964 as cited by Cable *et al.*, 2002a); *G. alexanderi* (see Lester and Adams, 1974), *G. turnbulli* (see Scott and Anderson, 1984; Scott and Nokes, 1984) and *G. gasterostei*. More recently, *in vitro* studies by Olstad *et al.* (2006) on *G. salaris* indicated that it was able to survive for only one day at 18°C but for four days at 3°C.

Temperature can also influence gyrodactylid transmission and abundance (Bakke *et al.*, 2007). Hosts may become more active and of course, host immunity is affected by changes in temperature, both of which can facilitate parasite transfer (Scott and Nokes, 1984). Increasing water temperatures were shown by Soleng *et al.* (1999a) to promote transfer of *G. salaris* from donor to recipient *Salmo salar* hosts, with maximum transmission of 4.9% occurring at the highest temperature tested 12.2°C. Bakke *et al.* (1991a) had previously demonstrated an effect of temperature on transmission of *G. salaris* to the European eel. Soleng *et al.* (1999) suggested that this increased transmission with increased temperature may explain why *G. salaris* colonises salmon parr more during the spring/summer months. This may also explain seasonal variation in abundance often found for gyrodactylids (Bakke *et al.*, 2007). Lamková *et al.* (2007) showed seasonal changes in the parasite abundance for chub, with monogeneans being most abundant in April and June, a period which may correlate with immunosuppression caused by host breeding activity. Furthermore, temperature may have an effect on the distribution of gyrodactylids on a host as alluded to by Anthony (1969) studying *G. elegans* on the goldfish, who noted that at lower temperatures, more gyrodactylids occurred on the body than the gills, whilst the reverse trend was noted for high temperatures

1.1.8 (ii) Water chemistry

The effects of salinity on *Gyrodactylus* have been restricted to just two species, *G. salaris* (see Soleng and Bakke, 1997; Soleng *et al.*, 1998) and *G. derjavinoides* (see Buchmann, 1997). Studies on the response of *G. salaris* to salinity focused on assessing whether this species can disperse through brackish water. It can transfer from freshwater to 5‰ salinity and this is correlated with temperature (Soleng and Bakke, 1997). However, at 7.5‰ salinity, parasite population growth rate declined and in sea water (33‰), the parasite could not survive. This concentration dependant survival led the authors to conclude that brackish waters could be utilised in the parasite's dispersal. In a subsequent study, transmission of *G. salaris* was studied from salmon smolt to parr at varying salinities of 0, 7.5, 10, 20 and 33‰ at a constant temperature of 12°C but with varying lengths of exposure (Soleng *et al.*, 1998). Their results indicated that the parasite was able to transfer at all concentrations tested, although this was time dependant as after 60 min exposure to full strength sea water the parasite could not transfer. Buchmann (1997) found that at 5‰ salinity, *G. derjavinoides* was able to survive for 4 days.

Although deemed pollutants, studies on heavy metals have been included in this section, as for gyrodactylids their use has been in determining their potential as an alternative to the much harsher, indiscriminate control treatments, such as rotenone. Again, such studies appear to be restricted predominantly to *G. salaris* and more recently to the tropical *G. turnbulli* (reviewed by Bakke *et al.*, 2007). Studies on the use of heavy metals have focused on aqueous aluminium, the most common contaminant of waterways in Northern Europe (Bakke *et al.*, 2007). An initial study by Soleng *et al.* (1999b) on the effects of exposure of Atlantic salmon to aqueous aluminium found that it was concentration dependent, a concentration of 202 µg/l eliminating *G. salaris* infections after 4 days. However, subsequent findings by Soleng *et al.* (2005) noted that although numbers of *G. salaris* were reduced following exposure of Atlantic salmon parr for one month to aqueous aluminium, these effects were not irreversible, as following termination of exposure, parasite growth assumed. Therefore, as noted by Soleng *et al.* (2005) further studies are needed into the mechanisms of aluminum resistance in *G. salaris*.

Poléo *et al.* (2004) extended the range of heavy metals tested on *G. salaris* to include zinc, copper, iron and manganese in addition to aluminium at 4 varying concentrations and although again aluminium proved to be effective (although not at the lowest concentration tested), zinc, proved to be effective at all concentrations and therefore may have promise as a potential control treatment. Zinc has also proved effective against *G. turnbulli* having toxic

effects on this parasite at 30 and 120 μg/l, and although it did not appear to affect generation time, it did affect *in vitro* survival with a two hour decrease in survival time (Gheorghiu *et al.*, 2007).

1.1.8 (iii) Pollutants

To date, the effects of pollutants on gyrodactylids is very much restricted to the use of heavy metals (see Section 1.1.8.(ii)), other environmental pollutants such as sewage, pesticides and petroleum hydrocarbons (Khan and Thulin, 1991) have yet to be evaluated. Pollutants may have a detrimental effect on parasites by reducing their abundance due to high host mortality, or if the parasite cannot respond as well to the pollutant as the host (Lafferty and Kuris, 1999). Alternatively, pollutants may cause host immunosuppression, allowing parasites to proliferate (Khan and Thulin, 1991). Khan and Kiceniuk's (1988) study on the parasite fauna of cod following exposure to crude oil on the water's surface found that an unidentified gyrodactylid infecting the gills, increased in prevalence and intensity. Trichodinids also increased in prevalence and intensity on cod that had been exposed to crude oil following the Exxon Valdez oil spill in Alaska (Khan, 1990). It has been suggested that pollutants, particularly crude oil, cause immunosuppression by acting on the innate immune system, namely mucous, by impairing its defensive action by either over-stimulation or coagulation (Burton *et al.*, 1972 as cited by Khan, 1987).

1.2 HOST SPECIFICITY

1.2.1 Overview

The significance of host specificity was first recognised by von Ihering during the 1900s, when its role was considered in parasite diversification (reviewed by Brooks and McLennan, 1993), but it is now regarded as a key parasite life history trait (Adamson and Caira, 1994; Poulin and Mouillot, 2003). It is also an important factor in parasite ecology (Poulin and Mouillot, 2003), evolution, speciation (Brooks and McLennan, 1993; Tompkins and Clayton, 1999; Poulin and Mouillot, 2003) and emerging infectious diseases (Timms and Read, 1999; Little *et al.*, 2006; Poulin and Keeney, 2008). Furthermore, it has been implicated as a basis of the red queen hypothesis, in which parasites mediate genetic variation by specialising on common host genotypes, thereby giving rare host genotypes an advantage (Ebert, 2000). Therefore, understanding the mechanisms that control host specificity and the evolutionary advantage to a

parasite of being either strictly host specific (specialist) or utilising a variety of hosts (generalists) is fundamental to understanding and controlling the impact of parasites.

1.2.2 Defining and measuring host specificity

An essential pre-requisite to understanding host specificity is defining and measuring this phenomenon (Poulin and Mouillot, 2004), but herein lies a problem. There is currently no universally accepted measure of host specificity but rather a continuum of indices ranging from the simplistic, based on the number of hosts a parasite is recorded, to more elaborate measures incorporating factors such as host ecology and phylogeny. Based on assumptions that monogeneans are highly host specific (Bychowsky, 1933), Hargis (1957) proposed defining host specificity in terms of host phylogeny, ranging from species-specificity (one parasite, one host species) to supraspecificity ("a natural group" of monogeneans infecting "a natural grouping of fish species"). Although, these terms suggest that monogeneans cannot infect across fish species, Hargis (1957) was ahead of his time in recognising the importance of host phylogeny. Subsequently, Euzet and Combes (1980) defined specificity based on the number of host species ranging from oioxenic (strict) to euryxenic (wide), but with an additional term to allow for intermediate parasites that infected closely related hosts (stenoxenic). Rohde (1980) proposed several more complex indices based on the reciprocal number of hosts and the "evenness of infection of the host". In contrast, Humphrey-Smith (1989) took the most simplistic view with specialists defined as parasites that infect a single host, whilst generalists infect several hosts. Thus, definitions of host specificity have oscillated between complex and simple.

A major problem with simplistic measures of host specificity, based purely on host range, is the assumption that a parasite will use all of its hosts equally (Poulin and Mouillot, 2005). Can a parasite that has a strong preference for one host but utilises many hosts rarely, be categorised in the same way as a parasite that has no strong predilection for a particular host? Furthermore, distinction is needed between those parasites infecting closely related hosts and those that are more distantly related (Poulin and Mouillot, 2005). Thus recent indices of host specificity have reverted to some of the traditional views about host specificity taking into account ecological and phylogenetic factors (e.g. Poulin and Mouillot, 2003, 2005). Lymbery (1989) suggested that host preference, in addition to host range, must be considered when defining host specificity. He defined host specificity as the restriction of a parasite to a particular number of host species, taking into account the prevalence/intensity of infection on each host. This definition is still widely cited in the literature, however, many researchers still refer to generalists and specialists

without defining them. Clearly any definition of host specificity is going to be a compromise, simplistic measures are the most workable providing their limitations are taken into account. Although Poulin and Mouillot (2005) proposed a host specificity index, this relies on having a wealth of information for each host-parasite combination which is rarely available.

A further consideration is that there are differing degrees of host specificity within and between parasite species (Brooks and McLennan, 1993; Little *et al.*, 2006). Specialisation within a parasite population to a particular sympatric host genotype can lead to the process of local adaptation, whilst within parasite populations it can lead to different strains (Little *et al.*, 2006). Categorising parasites as specialists or generalists is also problematic, as some species may employ both strategies during their life cycle (Brooks and McLennan, 1993). The empirical testing of host specificity is potentially a minefield, with reviews on host specificity noting that due to limited sample sizes, the number of "true" generalists is actually low (Poulin and Keeney, 2008), with many having been redefined as specialists when tested at a local level (Norton and Carpenter, 1998). McCoy *et al.* (2001) tested the host range of the ectoparasite, *Ixodes uriae*, hitherto considered a generalist on the basis of its very broad host range of over 50 hosts. Their study subsequently found that it was actually specialised amongst different host species. Previously, host specificity was used as a diagnostic tool, particularly for the Monogenea, based on the identity of the host (Lambert and El-Gharbi, 1995), although this led to misleading results due to unknown host range and species crypticity. Advances in molecular biology have revealed many morphologically cryptic generalist species to actually be distinct specialist species. This highlights again the problems of Poulin and Mouillot's (2005) index, in that host specificity cannot be defined unless all information is at hand, as host range may be of little value as atypical and accidental hosts are typically not included (Tripet and Richner, 1997).

1.2.3 What are the mechanisms controlling host specificity?

There are a number of factors that have been postulated in the evolution and control of host specificity. Host specificity may have a genetic basis (reviewed by Little *et al.*, 2006), in which a particular feature in a parasite's ancestor may constrain its descendant's ability to infect a range of hosts (Desdevises *et al.*, 2002). It may also be determined by a range of physical factors such as host ecology, behaviour, physiology and immune response, and/or external environment (Brooks and McLennan, 1993; Lambert and El-Gharbi, 1995).

Traditionally, the main factors controlling host specificity were considered to be based on the dispersal ability of the parasite or the degree to which a parasite is adaptively constrained to a

particular host by its morphology or the need for a particular resource (the latter termed "resource tracking" by Kethley, 1970; Read and Hafner, 1997; Timms and Read, 1999). These factors could be distinguished via empirical testing whereby removing the barriers to dispersal should result in a parasite being able to infect atypical hosts, whilst parasites that are adaptively constrained should have lower fitness on atypical hosts (Timms and Read, 1999).

Typically, parasites are over dispersed, with only a small proportion of the host population being heavily infected (Váquez *et al.*, 2005). Therefore, it may be expected that there will be a correlation between the availability of hosts and whether a parasite will be a specialist or generalist. Hosts that are highly abundant represent a reliable, predictable resource and thus favour specialist parasites, whilst low abundance and reliability will favour generalist parasites (McCoy *et al.*, 2001; Poulin and Keeney, 2008). On a wider scale, Norton and Carpenter (1998) proposed that more generalists should be found in tropical regions due to greater environmental heterogeneity and that with higher species richness, no one species dominates. In contrast, specialists should be expected to occur more in temperate regions due to lower species richness and thus the likelihood that one host species will be prevalent. Resource availability can also be deemed in terms of host size, as specialists will occur on predictable resources such as larger body size (Ward, 1992; Simková *et al.*, 2001). For generalists, a study by Simková *et al.* (2001) found no correlation with host size thus leading them to the conclusion that this could be explained by generalists foregoing host size and focusing their resources against host resistance. Host immunity may be the central factor in the evolution and control of host specificity. A study by Møller *et al.* (2005) on bird fleas and their avian hosts found that the occurrence of specialist and generalist parasites was determined by the strength of the host's immune response. The greater the immune response, the more likely a parasite will specialise, whilst generalists exploited hosts that had a weak immune response. Clearly, further studies are needed to evaluate the importance and relevance of each of these factors.

1.2.4 Specialists vs. Generalists – What is the evolutionary advantage?

Although it would appear that generalists have a greater evolutionary advantage over specialists, adopting such a strategy could result in a trade-off between a greater range of hosts but at the cost of lower fitness/abundance on these hosts (Ebert, 2000). Although few studies have shown this empirically (Sasal *et al.*, 2004), those that do support a "Generalist vs. Specialist trade-off" hypothesis typically involve selection or serial passage experiments (Ebert, 2000). Infecting a range of hosts means that a parasite will come into contact with different host environments,

including immune responses, resulting in variable selective pressures (McCoy *et al.*, 2001). Therefore, generalists may be restrained in the extent of their host range and may develop a preference for a particular host which serves as the optimal host in terms of fitness, whilst other hosts, on which fitness is lower, are utilised for maintaining genetic variation (Norton and Carpenter, 1998).

Specialists are faced with an increased risk of extinction, due to limited niche space, exposure to a single host's immune system (although this could also be deemed as an advantage) or risk of host extinction. Restricted opportunities for diversification could lead to parasite mortality as specialists are unable to adapt to a new host's morphology or immune system (Combes, 1995; Sasal *et al.*, 1999; Stireman, 2005). However, on the plus side, specialists are better adapted to exploit a single host more profitably (Jaenike, 1990), compared to generalists which have to exploit a range of hosts. In addition, generalists although able to utilise a range of hosts, face the risk of infecting hosts that cannot support them (referred to as "false" and "casual" hosts by Odening 1976) and may be outcompeted by specialists that are better adapted at exploiting a particular niche space (Price, 1980; Palm and Klimpel, 2007).

There has been much debate in the literature about the evolutionary origins of generalists and specialists akin to the "chicken and egg" phenomenon, but this issue is still unresolved. In brief and based on host phylogenies (Stireman, 2005), generalist parasites are considered to have evolved from specialists, assuming specialists have higher extinction rates and diversify less than generalists. However, other studies have found either no such evolutionary direction or the complete reverse (Stireman, 2005). In his review, Stireman (2005) concluded further information is required to solve this evolutionary puzzle and that a universal rule may not exist, as each parasite species needs to be considered individually. As a case in point, there are two conflicting hypotheses as to whether parasitic birds have become more specialised or more generalised to their brood hosts (Rothstein *et al.*, 2002). On one hand, it is argued that due to the co-evolutionary arms race, such birds have become specialists as they develop more refined counter-defences, whilst on the other hand, based on their host's phylogenies, they have actually become more generalised. Overall, Rothstein *et al.* (2005) could find no evidence to reject either hypothesis.

To add to this confusion, the validity of the actual concept of specialists and generalists has been questioned. As noted by Thompson (1982, cited by Sukhdeo and Hernandez, 2005), all organisms must specialise to some extent and due to the limited parameters by which parasites are assigned as specialists or generalists, such terms are completely artificial. Furthermore, it may

be more appropriate to question why a parasite should choose to be a generalist rather than a specialist (Thompson, 1982 cited by Sukhdeo and Hernandez, 2005).

1.2.5 Speciation

An understanding of host specificity is closely linked to speciation processes. There are two key mechanisms of parasite speciation; co-evolution and host switching. The former is considered the most common, but to be demonstrated, parasite phylogenies must be congruent with host phylogenies, the 'Farenholz Rule' (Desdevises *et al.*, 2002). However, it is difficult to prove due to the wealth of information needed on host and parasite phylogenies, host range and specificity. The most well known examples of co-evolution are those of chewing lice (Hafner and Nadler, 1988; Read and Hafner, 1997) and swiftlet lice (Page *et al.*, 1998). The second method of parasite speciation is host switching (also known as "ecological transfer"; Kearn, 1994), which may occur within a short period of evolutionary time and the most widely cited example is that of the Apple maggot fly, *Rhagoletis pomonella* (see Coyne and Orr, 2004; Jiggins and Bridle, 2004).

1.2.6 Monogenean host specificity

Generally, the monogeneans are considered to be among the most host specific of all parasite (Hargis, 1957; Poulin, 1992; Tinsley and Jackson, 1998; Whittington *et al.*, 2000), some species are less host specific than others (Sasal *et al.*, 1999) and thus 30% are thought to be strict specialists (as reviewed by Bakke *et al.*, 2007). Their high host specificity has been thought to be due to their simple life cycle (Rohde, 1978; 1979; Simková *et al.*, 2001). Although highly host specific, and thus predicted not to undertake host switching, they have a strong capacity for speciation following a successful host switch (Brooks and McLennan, 1991; Secord and Kareiva, 1996).

Of the Monogenea, the genus *Gyrodactylus*, is considered to have the widest host range, although there is considerable variation between species (Bakke *et al.*, 2002). With regard to speciation within this genus, although there is some evidence in support of co-evolution, such as the species group, *G. pleuronecti* (see Bakke *et al.*, 2002) and *G. pictae* (see Cable *et al.*, 2005), it is thought that co-evolution is the exception rather than the rule (Bakke *et al.*, 2002). The main mechanism of speciation is considered to be host switching as suggested by Harris (1993) and this has been confirmed by molecular evidence (Cable *et al.*, 1999; Ziętara and Lumme, 2002). It is thought that glaciation events, specifically those of the Pleistocene, facilitated host switching, due to glacial refugia containing mixed freshwater fish host species, thus allowing the transfer of

gyrodactylids to unrelated hosts. This is illustrated by the minnow (*Phoxinus phoxinus*) which is host to 14 species of *Gyrodactylus*, more than any other fish host (Bakke *et al.*, 2002) and by the *G. wageneri* "trout stream assemblage" proposed by Cable *et al.* (1999), whereby related gyrodactylids were found to infect unrelated hosts.

A number of factors have been proposed for the control of host specificity for gyrodactylids. Buchmann (1998b) proposed the importance of host epidermis, particularly host mucus in giving chemical cue for recognition as a suitable host. Whittington *et al.* (2002) proposed that the anterior adhesive glands played a central role in host specificity due to its role in detecting suitable hosts and point of first contact with host epidermis.

Although gyrodactylids are highly amenable to studies of their host specificity, to date such studies have been restricted to a few species: *G. salaris*, *G. derjavinoides*, *G. gasterostei*, *G. turnbulli* and *G. tularosae*. Most of these studies have focused on *G. salaris*, thought to be an aberrant species due to its wide host range and whether it can infect other atypical hosts such as the European eel (see Bakke *et al.*, 1991a), sticklebacks and flounder (see Soleng and Bakke, 1998) or its occurrence on other salmonids (e.g. Bakke *et al.*, 1991b; Bakke *et al.*, 1992; Olstad *et al.*, 2007). *G. derjavinoides* is a commonly encountered gyrodactylid infecting salmonids (as reviewed by Bakke *et al.*, 2007). *G. gasterostei*, which infects the three-spined stickleback, is considered both a generalist (see Matějusová *et al.*, 2000) and a specialist (see Gläser, 1974; Harris, 1998b (but see Bakke *et al.*, 2007)). Studies by Harris (1982) showed that *G. gasterostei* could experimentally infect the nine-spined stickleback (*Pungitius pungitius*) and the common minnow (*Phoxinus phoxinus*).

The tropical species, *G. turnbulli* has been the subject of two studies, determining whether this hitherto assumed strict specialist could infect the fish genus *Poeciliopsis*. Leberg and Vrijenhoek (1994) found that although this species could not infect sexual stocks of *Poeciliopsis* via casual contact, one of the clonal stocks, where a genetic change had occurred was susceptible to this, for them, exotic parasite. A further study by Hedrick *et al.* (2001) demonstrated that it could infect the endangered Gila topminnow (*Poeciliopsis o. occidentalis*) and that infections could be acquired via casual contact. However, it is questionable whether the gyrodactylid used by Hedrick's study was actually *G. turnbulli*, as based on their description of its site preference, it is more likely that this parasite was the congener species, *G. bullatarudis* (see Chapter 2). Moen and Stockwell (2006) tested the assumption of strict host specificity of *G. tularosae* and found that although it had a distinct preference for its assumed optimal host, the White Sands pupfish (*Cyprinodon tularosa*) it was able to infect another related host, the sheepshead minnow

(*Cyprinodon variegatus*). Therefore, given that over 400 *Gyrodactylus* species have been described, host specificity of this speciose genus is an area that is currently very much neglected.

1.3 LOCAL ADAPTATION

1.3.1 Overview

The degree of host specificity is considered to be an important component of co-evolutionary dynamics (Gandon, 2002) leading to the phenomenon of local adaptation, a prediction of the red queen hypothesis (Lively and Dybdahl, 2000). This hypothesis, as proposed by van Valen (1973), is one of the leading theories that have been postulated for the evolution and prevalence of sexual reproduction, particularly in organisms capable of asexual reproduction. The supposition of this hypothesis is that parasite mediated selection against local, common host genotypes leads to sexual reproduction in order for the host to generate counter defences (Lively *et al.*, 2004). Parasites are typically thought to be ahead in the co-evolutionary arms race as they have much shorter generation times than their hosts (Kaltz and Shykoff, 1998). Therefore, according to the red queen hypothesis, parasites should have co-evolved so that they have higher fitness on their sympatric hosts than allopatric hosts, thus leading to the process of local adaptation (as described by Williams, 1966 reviewed by Kawecki and Ebert, 2004).

1.3.2 When should local adaptation occur?

A major pre-requisite for local adaptation to occur is that the environment should show spatial heterogeneity (Gandon and Van Zandt, 1998; Gandon and Michalakis, 2002). Simulation models show several additional factors with migration rates of both host and parasites being a strong determinant (Gandon, 2002) of whether or not local adaptation occurs. If migration rates of both host and parasite are low to intermediate, then local adaptation is predicted to occur when parasite migration is greater than the hosts. Host and parasite migration rates that are similar to each other or are high should result in local adaptation not occurring (Gandon *et al.*, 1996, 1998; Lively, 1999; Gandon, 2002). However, such models are based on negative frequency dependent selection (also termed "matching-alleles model" by Gandon and Michalakis, 2002). In addition, these assumptions are based on the environment also changing over time, in order that selection can act (Gandon and Van Zandt, 1998). However, for environments that are spatially variable but remain constant over time, local adaptation is predicted to only occur if migration of the parasite is very low (Gandon and Van Zandt, 1998). Other factors that are predicted to promote the occurrence of local adaptation are high parasite virulence (Lively, 1999; Gandon and Michalakis,

2000), although this may be counterintuitive as high virulence could kill the host and thus the parasite (the suicide king hypothesis, see Dybdahl and Storfer, 2003) and fast generation times.

Although local adaptation theory has been demonstrated in simulation models, its occurrence when tested empirically is far from universal (Lively, 1999; Gandon and Michalakis, 2002; Greischar and Koskella, 2007). In their meta-analysis of 54 empirical tests of local adaptation, Greischar and Koskella (2007) found that 18 demonstrated local adaptation. Initially, local adaptation was experimentally tested using a plant-herbivore system (Edmunds and Alstad, 1978, Kaltz and Shykoff, 1998) and the majority of studies are based on this system (Greischar and Koskella, 2007), with subsequent studies tending to focus towards host-parasite systems (Kawecki and Ebert, 2004). The most common fitness measures used to detect local adaptation are parasite life history traits such as infectivity, whereby sympatric parasites should infect their sympatric hosts more successfully than allopatric ones. The other main measure is population growth rate, as many parasites display exponential growth rate (Kawecki and Ebert, 2004). To date, many of these studies have used parasites that are horizontally transmitted (i.e. to conspecifics), although local adaptation has been demonstrated in *Nosema granulosis* which is vertically transmitted (from parent to offspring) (Hatcher *et al.*, 2005). One of the most widely cited studies of local adaptation in a host-parasite system is by Lively and Dybdahl (2000) who used a snail-digenean system. Their results supported local adaptation (a prediction of the red queen hypothesis) and rejected an alternative 'trade off' hypothesis, in which common host genotypes trade off their competitive ability against parasite susceptibility, and thus should be more susceptible to both sympatric and allopatric parasites than rare host genotypes (Lively and Dybdahl, 2000). However, the validity of these hypotheses is still an area of contention (Woolhouse and Webster, 2000).

Ebert (1994) proposed that local adaptation should be regarded as an ecological "rule", however, empirical studies have demonstrated conflicting results, ranging from evidence in support (e.g. Lively and Dybdahl, 2000) or against local adaptation (e.g. Kalbe and Kurtz, 2006), to those that show local maladaptation whereby sympatric parasites actually do better on allopatric hosts (e.g. Oppliger *et al.*, 1999). Findings of local maladaptation have been explained by the fact that parasite responses to their hosts are time lagged (Nee, 1989; Gandon and Michalakis, 2002; Hatcher *et al.*, 2005), thus the proportions of common and rare host genotypes that a parasite comes into contact with can differ, allowing allopatric combinations to be more favourable periodically as shown by mathematical models (Morand *et al.*, 1996; Dybdahl and Storfer, 2003; Kawecki and Ebert, 2004; Lively *et al.*, 2004). Therefore, Ebert's (1994)

supposition that local adaptation should be upheld as a general rule, is clearly not robust (Morand *et al.*, 1996).

1.3.3 Local adaptation and glacial refugia

Several studies have suggested that the strength of local adaptation is not affected by genetic distance or geographical distance (Morand *et al.*, 1996; Thrall *et al.*, 2002; Lively *et al.*, 2004). Although whether local adaptation is affected by the marked gradients in genetic diversity resulting from post-glacial colonisation is unknown. Hewitt (1999) predicted that populations in northern Europe show less genetic diversity than populations in the south. This prediction was based on the assumption that southerly populations would have recolonised from glacial refugia after the Pleistocene glaciation with a subset of the population migrating northwards, thus reducing the effective population size. Founder events at the leading edge would result in a loss of alleles and increased homozygosity. To date, this prediction has been upheld for terrestrial organisms, ranging from trees to mammals (reviewed by Hewitt, 1999), and also for some aquatic organisms, such as the European eel (Maes and Volckaert, 2002) and Atlantic salmon (Consuegra *et al.*, 2002). However, this view of refugial populations having greater genetic diversity is controversial. Petit *et al.* (2003) suggested that the most genetically diverse populations do not occur in the south, but are found at intermediate latitudes due to the mixing of separate refugial populations. Likewise, Comps *et al.* (2001) observed that genetic diversity in the European beech was lower in refugia than in recently colonised regions. Most phylogeographic studies on post-glacial recolonisation have focused solely on genetic diversity but as suggested by Widmer and Lexer (2001), loss of diversity depends on the different estimators used, such as the proportion of polymorphic loci or allelic richness. For instance, Comps *et al.* (2001) found no evidence of reduced genetic diversity but they did find higher allelic richness in refugia. Obviously, the best approach is to measure genetic diversity using a range of different methods (Widmer and Lexer, 2001).

1.3.4 Empirical testing and measuring of local adaptation

Ideally, the most appropriate method of testing local adaptation in animal host-parasite systems is via reciprocal cross-infection experiments (Gandon and Van Zandt, 1998), otherwise genetic variation in host susceptibility and parasite virulence could lead to misleading results (Thrall *et al.*, 2002; Lively *et al.*, 2004). Gandon and Van Zandt (1998) proposed that in order to overcome discrepancies in genetic variation in both hosts and parasites, information from cross inoculation

experiments should be pooled and that local adaptation should be interpreted as "a general pattern that describes adaptive structure of both host and parasite".

Distinctions should be made between those studies that use parasite infectivity as a measure of local adaptation and those that use parasite virulence, in which the degree of host mortality and pathogenicity is compared to a predicted optimum (Dybdahl and Storfer, 2003). In addition, the host specificity of the parasite should be known, as parasites that are highly host specific are more likely to show local adaptation (Gandon, 2002; Lively *et al.*, 2004). Generalist parasites should demonstrate a lesser degree of local adaptation than specialists, due to reduced species specific selection (Lajeunesse and Forbes, 2002). The distinction between specialist and generalist parasites is one that is often ignored (see Section 1.2.2) and therefore disparity in the literature over the occurrence of local adaptation could be explained in part by variation in the parasite's host range (Lajeunesse and Forbes, 2002).

To date, most empirical studies of local adaptation in host-parasite systems have focused on plant-herbivore systems and invertebrates (Greischar and Koskella, 2007), the most well known study being Lively and Dybdahl (2000). In contrast, relatively few studies involve vertebrates (but see Jackson and Tinsley, 2005) with fish host studies being restricted to endoparasites with indirect life cycles such as Ballabeni and Ward (1993) who demonstrated local adaptation of the digenean, *Diplostomum phoxini* to its second intermediate host, the common minnow (*Phoxinus phoxinus*). No studies have explicitly tested for local adaptation using gyrodactylids despite these appearing to be ideal model organisms, due to their narrow host specificity, short generation time and ease of laboratory maintenance.

1.4 MODEL SYSTEMS

This thesis largely focused on three species of *Gyrodactylus* (*G. gasterostei*, *G. bullatarudis* and *G. turnbulli*) from two fish hosts, the three-spined stickleback (*Gasterosteus aculeatus*) and the guppy (*Poecilia reticulata*) respectively. Both of these host species are small fish that are readily available, hardy and amenable to laboratory studies with short generation times. In addition, the gyrodactylids studied are known to be excellent model organisms and relatively easy to maintain. These ectoparasites can be readily counted on restrained or anaesthetised hosts, so this allows non-invasive sampling and the entire infection trajectories of individual worms can be monitored throughout their life. The other fish-gyrodactylid systems described in this thesis resulted from incidental infections.

1.4.1 Poeciliid-Gyrodactylus turnbulli and G. bullatarudis

The guppy (*Poecilia reticulata* Peters, 1859) is a tropical livebearer, that is sexually dimorphic, with the female being larger than the male (Froese and Pauly, 2008). Its native habitat is the Caribbean Basin and South America, but due to its popularity as a aquarium fish and use as a biological control agent, it now has a global distribution and has been recorded in 41 countries outside of its native habitat (FIGIS, 2008). It has a temperature range between 18-28°C (Froese and Pauly, 2008). Guppies have a depauperate parasite fauna which are dominated by two species of *Gyrodactylus*; *G. turnbulli* and *G. bullatarudis* (Lyles, 1990), which have a long evolutionary history with their host, possibly dating back several million years (Cable *et al.*, 2005).

Gyrodactylus turnbulli Harris, 1986

Despite *G. turnbulli* having been used widely as a model organism of population dynamics (e.g. Scott, 1982; Scott and Anderson, 1984; Harris, 1989), site specificity (Harris, 1988, see Fig. 1.3A), host resistance (Cable and van Oosterhout, 2007a,b) and toxicology studies (Gheorghiu *et al.*, 2007), its host range had never been previously evaluated, having been hitherto considered to be a strict specialist (Harris *et al.*, 2004). It can be distinguished from its congeneric species, *G. bullatarudis* by the presence of distinct downward projecting lugs in its dorsal bar (Fig. 1.3B).

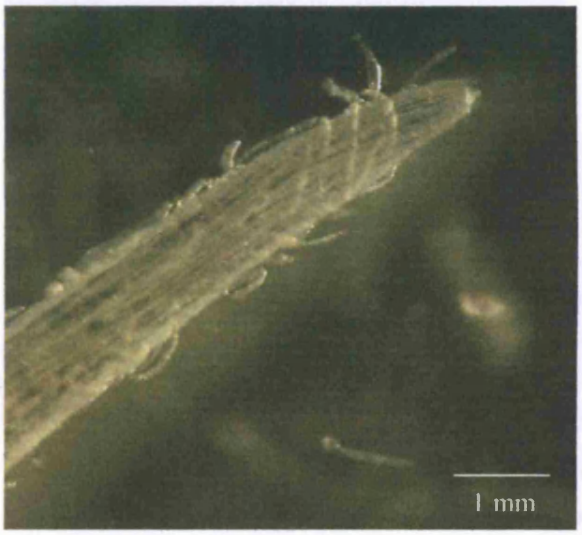

Fig. 1.3A: Caudal fin of *Poecilia reticulata* infected with *Gyrodactylus turnbulli*

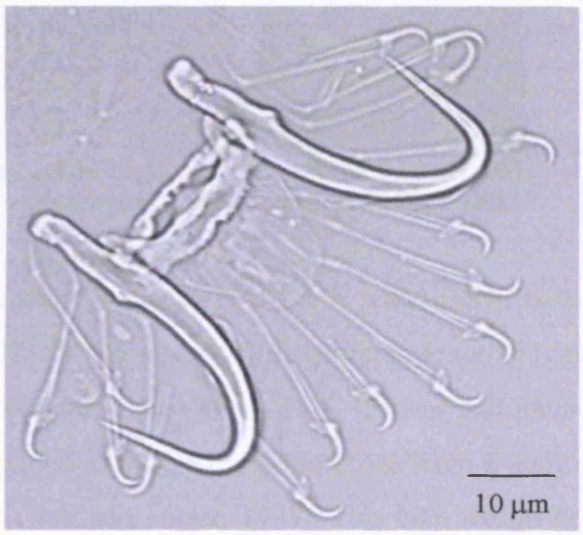

Fig. 1.3B: Light micrograph of hamuli and marginal hooks of *Gyrodactylus turnbulli*

Gyrodactylus bullatarudis Turnbulli, 1956

In comparison to the wealth of studies on *G. turnbulli*, relatively little is known about *G. bullatarudis*. Studies in the 1980s by Scott and colleagues (Scott 1982, 1985a,b, 1987; Scott and Anderson, 1984; Scott and Nokes, 1984; Scott and Robinson, 1984) that described the use of *G.*

bullatarudis were actually found subsequently to have been using *G. turnbulli*, see Harris (1986). According to Harris *et al.* (2004), this species is a generalist, although again its actual host range has never been evaluated. To date, experimental studies on this parasite appear to be restricted to those of Richards and Chubb (1996, 1998) who evaluated long-term survival and host resistance. Also, Cable and van Oosterhout (2007b) directly assessed the virulence of a wild strain of *G. bullatarudis* and found it to be intermediate between that of a wild and inbred strain of *G. turnbulli*, the latter being most pathogenic. *G. bullatarudis* reportedly has a distinct site preference for the rostral region (Fig. 1.4A) cf. the caudal region of *G. turnbulli* (see Harris and Lyles, 1992) although this is by no means a diagnostic characteristic. Morphologically, it can be discriminated from *G. turnbulli* by the presence of a distinct notch in its dorsal bar (Fig. 1.4B).

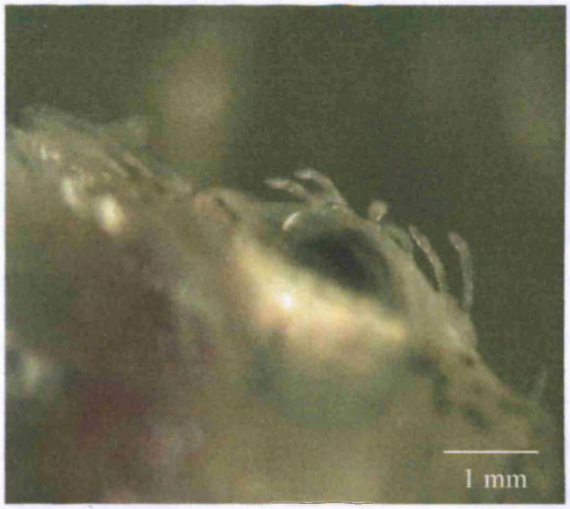

Fig. 1.4A: *Gyrodactylus bullatarudis* on the corneal surface of *Poecilia reticulata*

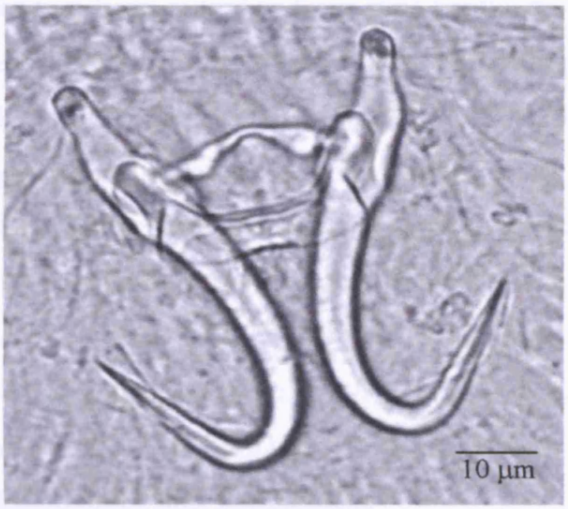

Fig. 1.4B: Light micrograph of hamuli and marginal hooks of *Gyrodactylus bullatarudis*.

1.4.2 Gasterosteus aculeatus-Gyrodactylus gasterostei

The three-spined stickleback (*Gasterosteus aculeatus* Linnaeus, 1758) is a widely distributed teleost that has a holarctic range (Boughman, 2007). It is a small fish (maximum length 11cm) occurring in temperate countries with a water temperature range of 4 - 20°C (Froese and Pauly, 2008). This teleost displays high phenotypic divergence of freshwater populations from a marine ancestor, caused by post-glacial adaptive radiation (Bell and Foster, 1994). This high freshwater diversity resulted in the description of 40 species as of 1910 (Mattern, 2007) until it was synonymised as a single species and is now considered to be a species complex (Foster *et al.*, 2003). Typically, three morphs are recognised based on the number of lateral plates: complete, partial and low plated (Mattern, 2007). The most widely described and studied phenotypes in the literature are populations from British Columbia, due to the distinct bethnic-limnetic species pairs that occur in the Strait of Georgia (McPhail, 1994). More recently, there have been calls for the

conservation of such phenotypically unique populations, following the collapse and extinction of particular benthic-limnetic species pairs in British Columbia, due to anthropogenic activities (Taylor *et al.*, 2006). It is a classic model for evolutionary, ecological and biological studies (Bell and Foster, 1994).

The three-spined stickleback has a diverse natural parasite fauna, which includes seven *Gyrodactylus* species, one of the most prevalent being *Gyrodactylus gasterostei*, Gläser, 1974 (Figs. 1.5A and B). Of the three species used as a model organism for this thesis, *G. gasterostei* is the most recently evolved species, its current distribution pattern in the UK postulated to be related to the post-glacial recolonisations of its host (*Gasterosteus aculeatus*) into SE England from the Channel River drainage basin (Harris *et al.*, unpublished). This parasite is found throughout England and Wales, its most northern limit believed to be the central lowlands of Scotland (Harris *et al.*, unpublished) and has never been recorded from NW Scotland. There is currently contention as to whether this species is a specialist or generalist. According to the original species description by Gläser (1974) and Harris (1998b), it is a specialist. However, according to Matějusová et al. (2000) it is a generalist, being recorded from several host species. Just under bright field illumination using a stereo microscope, live specimens of *G. arcuatus* and *G. gasterostei* can be discriminated by the presence of excretory bladders in the shoulder region of *G. arcuatus* which are absent in *G. gasterostei* (P.D. Harris, personal communication). *G. gasterostei* has been used to study gyrodactylid reproduction (Harris, 1985, 1998b; Cable *et al.*, 2002a), *in vitro* and *in vivo* survival and feeding (Cable *et al.*, 2002a).

Fig. 1.5A: Light micrograph of *Gyrodactylus gasterostei*.

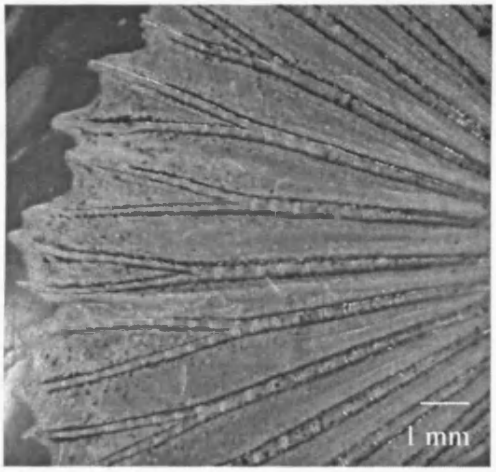

Fig. 1.5B: *Gyrodactylus gasterostei* on the caudal fin of *Gasterosteus aculeatus*.

1.5 THESIS AIMS AND LAYOUT

This thesis aimed to examine two research areas of *Gyrodactylus* species, namely host specificity and local adaptation, which have previously received very little attention. The thesis is presented as seven data chapters and has been compiled so that each chapter is self contained and potentially publishable. Chapters 2 and 7 are published in the International Journal of Parasitology and Journal of Parasitology, respectively. Chapter 3 has been submitted for publication and is under review. There is therefore some repetition between chapters. Two chapters have an appendix (Chapters 2 and 4) which represent incomplete data sets.

Chapter 2 tests the hypothesis that *G. turnbulli* is a strict specialist on its host, the guppy. Using a similar methodology, Chapter 3 assesses whether *G. bullatarudis* is a generalist with regard to host range. Chapter 4 tests the hypothesis that there is a difference in transmission behaviour of *G. bullatarudis* compared to its congener, *G. turnbulli in vivo* on dead hosts, whilst Chapter 5 tests the hypothesis that temperature affects *G. bullatarudis in vitro* behaviour. Chapters 6 and 7 arose from these host specificity studies, whereby incidental infections were found to occur on zebrafish (Chapter 6) and chub (Chapter 7). Chapter 6 describes two new gyrodactylid species from zebrafish (author's contribution was performing the experimental infections of *G. zebrae* n. sp. and conducting some of the microscopy, the other contributors to this chapter are given in the footnote of Chapter 6). Chapter 7 reports the first occurrence of isolated chub maintaining long-term infections with *G. lomi*. The last data chapter (Chapter 8) aims to test the hypothesis of local adaptation theory using the *G. gasterostei*-three-spined stickleback model, specifically assessing whether island host populations are more susceptible than mainland populations to this parasite. Finally, Chapter 9 overviews all the findings from this study and discusses some potential areas for future research.

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CHAPTER 2: EXPERIMENTAL INFECTIONS OF THE MONOGENEAN *GYRODACTYLUS TURNBULLI* INDICATE THAT IT IS NOT A STRICT SPECIALIST.

2.1 ABSTRACT

Parasites represent a threat to endangered fish species, particularly when the parasite can host switch and the new host is vulnerable. If the parasite is highly host specific then successful host switching should be a rare occurrence; however, the host range of many parasites which are assumed to be specialists has never been tested. This includes the monogenean *Gyrodactylus turnbulli*, a well studied ectoparasite found caudally on its known host, the guppy, *Poecilia reticulata*. In this study, we monitored parasite establishment and reproduction on a range of poeciliids and more distantly related fish. Individually maintained fish were experimentally infected with a single parasite and monitored daily to establish whether *G. turnbulli* could survive and reproduce on other fish species. *G. turnbulli* can infect a wider range of hosts than previously considered, highlighting the fact that host specificity can never be assumed unless experimentally tested. Our findings also have significant implications for parasite transmission to novel hosts and provide further insight into the evolutionary origins of this ubiquitous group of fish pathogens. Previous molecular evidence indicates that host switching is the key mechanism for speciation within the genus *Gyrodactylus*. Until recently, most *Gyrodactylus* spp. were assumed to be narrowly host specific, however, our findings suggest that even so-called specialist species, such as *G. turnbulli*, may represent a threat to vulnerable fish stocks. In view of the potential importance of host switching under artificial conditions, we propose to describe this as 'artificial ecological transfer' as oppose to 'natural ecological transfer', host switching under natural conditions.

2.2 INTRODUCTION

Fish, despite being the most diverse vertebrate group (>30,000 species) have largely been neglected with regard to the conservation impact of pathogens, even though there are currently 1,143 threatened fish species (IUCN, 2006) and, after amphibians, freshwater fish may be the most threatened vertebrate group (Bruton, 1995). One of the most notorious fish pathogens is the monogenean ectoparasite, *Gyrodactylus salaris,* which poses a conservation threat to vulnerable East Atlantic salmon populations (Verspoor *et al.*, 2005). Kennedy (1994) considered *Gyrodactylus* spp. to be amongst the most invasive fish parasites, due to their viviparous reproduction and exponential growth rate. They are ubiquitous on teleosts and host switching is

considered the key mechanism of speciation with over 400 described *Gyrodactylus* species (Harris *et al.*, 2004).

Global translocation of fish promotes the transfer of pathogens to endemic and farmed fish. Much of the research on introduced fish diseases has focused on salmonid aquaculture, a global industry worth US$3.1 billion per annum (Gooley, 1998). *G. salaris*, which has devastated Norwegian salmon stocks since its accidental introduction in the 1970s, has caused losses in excess of US$500 million (Bakke *et al.*, 2007). Its continued spread across Europe is a cause of great concern, particularly where there is the potential for disease transfer between escaped farmed salmon and vulnerable wild East Atlantic salmon populations (Gross, 1998). *Gyrodactylus* spp. also inflict heavy losses to other commercial non-salmonids, such as carp farms (e.g. Schmahl and Mehlhorn, 1988). Although the Monogenea are considered to be among the most host specific of all parasites, *Gyrodactylus* spp. have the widest host range but with considerable variation between parasite species (Bakke *et al.*, 2002).

Disease introduction via the ornamental fish industry has not received the same attention as it has for food fish, even though the ornamental fish industry is worth US$7.2 billion per annum worldwide, with the USA and UK being among the largest importers of ornamental fish (Andrews, 1990). Amongst the most popular tropical fish species is the guppy (*Poecilia reticulata*, see Piementa Leibowitz *et al.*, 2005), which has been translocated worldwide as an ornamental and also as a biological control agent with 41 recorded introductions outside its native habitat (FIGIS, 2008). In their natural environment (Caribbean basin and South America), guppies have a relatively depauperate parasite fauna which is dominated by the ectoparasitic worms, *G. bullatarudis* and *G. turnbulli* (Cable and van Oosterhout, 2007a,b). The former parasite is reportedly a generalist and the latter a specialist (Harris *et al.*, 2004), based on their occurrence on a range of hosts or a single host species, respectively (e.g. Sasal *et al.*, 1999). Paradoxically, Leberg and Vrijenhoek (1994) and Hedrick *et al.* (2001) claimed that *G. turnbulli* could experimentally infect *Poeciliopsis* species. Due to the global distribution of its normal host (the guppy), if *G. turnbulli* were capable of host switching to atypical hosts, this could have detrimental conservation and economic implications, considering the known pathogenicity of this parasite on the guppy (Scott and Anderson, 1984). However, despite *G. turnbulli* having been used widely as a model organism of population dynamics (e.g. Scott, 1982; Scott and Anderson, 1984; Harris, 1989), site specificity (Harris, 1988), host resistance (Cable and van Oosterhout, 2007a) and toxicology studies (Gheorghiu *et al.*, 2007), its host range has never been investigated. This study experimentally investigates the host range of an isogenic strain of *G.*

turnbulli via artificial infections on a range of poeciliids and other phylogenetically distant fish hosts to ascertain if this gyrodactylid is a strict specialist or is capable of host switching.

2.3 MATERIALS AND METHODS

2.3.1 Host origins and maintenance

The origin of fish species used in the current study is shown in Table 2.1 with the relationships between each species represented by Fig. 2.1. We selected the sister species of *Poecilia reticulata* (*P. picta*) and another poeciliid, *P. sphenops,* as they both form heterospecific shoals with the guppy in its native habitat (Trinidad and Tobago). The remaining poeciliids and *Danio rerio* are commonly kept in aquaria, and therefore were selected to test the potential for host switching in the pet trade. In addition, common temperate species were chosen to test whether the tropical parasite, *G. turnbulli*, could host switch to native UK fish.

Species	n	Origin	Host standard length (mm)
Poecilia reticulata (guppy)			
Ornamental Stock (OS)	28	Aquarium supplier, laboratory maintained since 1994	17 - 18.5
Tobago Stock	21	Wild caught, Goldsborough, Tobago, 2001-2004 (lab. bred F3/F4 generations)	17 - 33.5
Poecilia picta (Swamp guppy)			
Tobago Stock	21	Wild caught, Goldsborough, Tobago, July 2003 (lab. bred F3/F4 generations)	15 - 24
Trinidad Stock	6	Wild caught, Aripo River, Trinidad, July 2004 (lab. bred F3/F4 generations)	11 - 14.5
Poecilia sphenops (Molly)			
Ornamental Stock	28	Aquarium supplier	11.5 - 39
Trinidad Stock	11	Wild caught, drainage ditch linked to the Tacarigua River, Trinidad, July 2003 (lab. bred F3/F4 generations)	19 - 46.5
Xiphophorus hellerii (Green Swordtail)	19	Aquarium supplier	21 - 40
Xiphophorus maculatus (Hi Fin Platy)	20	Aquarium supplier	14 - 38.5
Danio rerio (Zebrafish)	21	Aquarium supplier	28.5 - 34
Phoxinus phoxinus (Common minnow)	15	Wild caught, Roath Park, Cardiff, UK, 2004	28 - 42
Gasterosteus aculeatus (Three-spined stickleback)	21	Wild caught, Roath Park, Cardiff, UK, 2004	18 - 30.5
Pungitius pungitius (Nine-spined stickleback)	17	Wild caught, Roath Park, Cardiff, UK, 2004	22.5 - 42
Salmo salar (Atlantic salmon)	13	Cynrig Hatchery, Abercynrig, Brecon, UK, 2005	52 - 73.5

Table 2.1: Origin of fish species infected with *Gyrodactylus turnbulli* during the current study

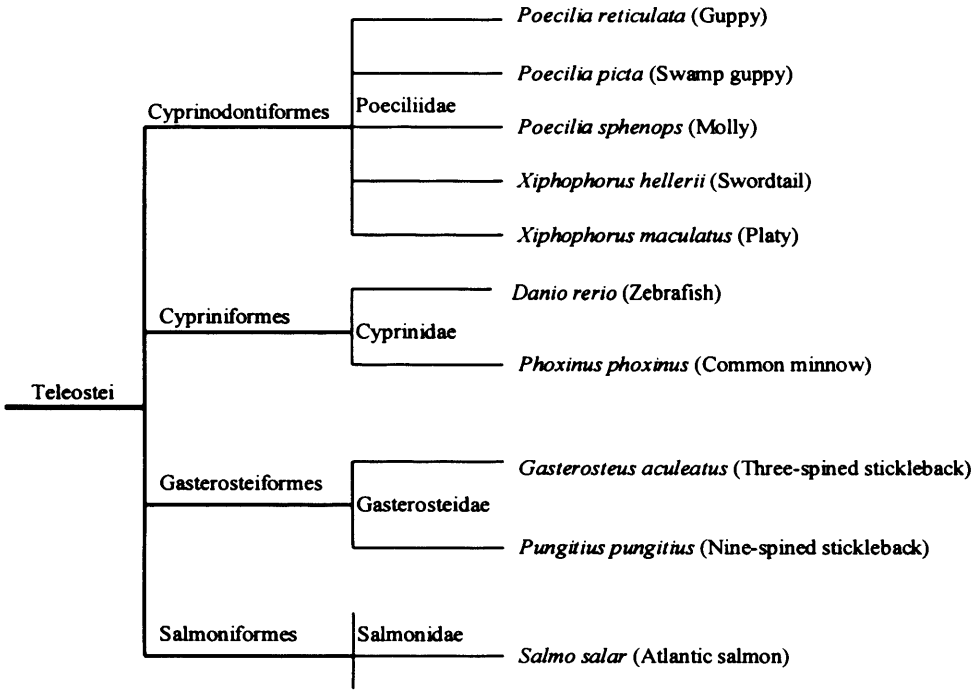

Fig. 2.1: Schematic diagram of relationships of fish species used in the current study (data from FishBase, 2006; Breden *et al.*, 1999)

Prior to infection, all tropical fish were maintained in aquaria at 25±0.5°C and fed daily on Aquarian® fish flakes. Temperate fish were kept at 15±0.5°C and fed daily on a mixture of live *Daphnia* and *Artemia*, and frozen bloodworm (*Chironomous* spp.). Atlantic salmon (*Salmo salar*) parr were maintained in a flow through system at 13±0.5°C and fed daily on pelleted food (Ewos). During experimental infections, the majority of fish were maintained individually in 1.1 litre jars of dechlorinated water at 25±0.5°C. Atlantic salmon were maintained individually in 10 litre aquaria with a constant air supply at 12±1.0°C. All laboratory bred fish were naïve to *Gyrodactylus turnbulli*, and commercially supplied and wild caught fish were uninfected when obtained, but were still held in parasite-free conditions for at least 3 months prior to infection.

2.3.2 Parasite cultures

All experimental infections were performed with an isogenic strain of *G. turnbulli* (*Gt3*) originally isolated in October 1997 from Nottingham aquarium stocks of *P. reticulata*, and identified as *G. turnbulli* following the methods of Harris *et al.* (1999). This strain was

maintained in laboratory culture on an inbred ornamental stock of guppies. Cultures were monitored on a daily basis and twice weekly additional naïve fish were introduced to maintain parasite numbers.

2.3.3 Experimental infections

Donor guppies were euthanised and individual gyrodactylids removed using insect pins using well established procedures as described by Scott (1982). Individual recipient fish were anaesthetised with tricaine methanesulfonate (MS222) buffered to a neutral pH with $NaHCO_2$, either 0.01 or 0.02% MS222 depending on the species. These fish were transferred to a Petri dish containing dechlorinated water and examined under a stereo-microscope with fibre optic illumination. An individual gyrodactylid removed from its donor host and attached by its opisthaptor to an insect pin was then held directly over the caudal fin of the potential host and time of attachment recorded. If after 2 min the parasite had not attached to the caudal fin, it was presented to other potential attachment sites along the length of the fish, with its position and time of attachment recorded. If after 5 min, the parasite had failed to attach, this was recorded as a failed attempt. This procedure was repeated with a second parasite but if no attachment occurred, this particular fish was abandoned. Parasite behaviour, including probing activity (the flattening of the cephalic lobes on to the surface of the fish followed by immediate withdrawal), was monitored continuously throughout the infection process. Host standard length and, where possible, gender were recorded; thereafter each fish was maintained individually.

All fish which had been successfully infected with *G. turnbulli* (Day 0) were examined the following day. Any fish found to be parasite free on Day 1 was re-infected in order to rule out the possibility that the gyrodactylid may have been old or damaged and therefore unable to establish. Subsequently, all fish were examined every 24 h with embryo development (shape and size of the *in utero* F1 attachment hooks) being recorded until more than three gyrodactylids were found on the host. Thereafter, just the number and position of gyrodactylids were recorded daily until the fish had shed all their parasites and were recorded free of gyrodactylids for three consecutive days. *P. reticulata* infections were terminated after Day 17 (following the methods of van Oosterhout *et al.*, 2003). Salmonids were infected with a modified infection protocol as preliminary trials revealed that infections (n = 20) with a single gyrodactylid were unsuccessful. A heavily infected guppy corpse was held in direct contact with an anaesthetised salmon parr for 1 min. Fish which had been successfully infected were monitored an hour after infection and then every 2 h until the fish had shed all their parasites.

Experimental infections were set up over several months and, in order to control for inherent parasite variation, fish from at least four different species (and always including *P. reticulata*) were infected on any one day with parasites from the same donor host. In total, 241 experimental fish were infected during this study.

2.3.4 Statistical analysis

Infection success (i.e. fish successfully infected on Day 0) was recorded for all hosts. For those fish species with <100% infection success, Fisher's Exact Test was used to test whether the frequency of successfully infected fish differed from their closest relative and one of the control stocks (OS guppy). This test was also used to analyse differences in establishment success (i.e. survival of the parasite 24 h after experimental infection) by comparing the presence or absence of infections for all fish on Day 1.

Bartlett's Test and the Anderson-Darling Test indicated heterogeneity of variance and non-normal distribution of the data for attachment times, maximum parasite load, day of maximum parasite load, duration of infection and parasite reproductive rate, which could not be rectified by transformation. As our data sets were also of an unbalanced design and the assumptions of analysis of variance were violated, non-parametric analyses were performed. Attachment time of *G. turnbulli* was compared between poeciliids and non-poeciliids (excluding *Salmo salar*) using the Mann-Whitney test and then between all fish species (excluding *Salmo salar*) using a Kruskal-Wallis test. This test was also used to assess differences in maximum parasite load, day of maximum load and maximum duration of infection between poeciliid stocks (*Poecilia reticulata* was excluded from maximum duration of infection analysis as these infections were terminated at Day 17). *Post hoc* tests were performed using a Steel-Dwass test (Neuhäuser and Bretz, 2001) as an alternative to the usual *post hoc* Mann-Whitney tests with Bonferroni correction, which was too conservative considering the large number of pairwise comparisons.

The reproductive rate of *G. turnbulli* was calculated up to Day 9 (the time point at which most responding fish mounted a response to infection) but was statistically compared only up to Day 5 due to differing sample sizes. The reproductive rate was calculated using the formula $\text{Ln}(N_t + 0.1)-\text{Ln}(N_{t-1} + 0.1)$ (see van Oosterhout *et al.*, 2003) where N_t is the number of parasites on the host at day t, and N_{t-1} is the number of parasites recorded the previous sampling day. To avoid taking the natural logarithms of zero, $N_t + 0.1$ was used. Parasite population growth rate per day (up to Day 5) were compared between the two guppy stocks using a Mann-Whitney test,

and between all poeciliids using a Kruskal-Wallis test, with *post hoc* Steel-Dwass tests. Data analyses were performed using Minitab vs. 14 and KyPlot vs. 5 (for Steel-Dwass tests). Fisher's Exact tests were computed using a web-based programme available at http://bardeen.physics.csbsju.edu/stats/exact.html.

2.4 RESULTS

2.4.1 Infection success

All fish species tested were successfully infected on Day 0 with *Gyrodactylus turnbulli* with the exception of the Atlantic salmon (Table 2.2). The only significant difference in infection success of the non-salmonids was between *Poecilia reticulata* (OS) and *Gasterosteus aculeatus,* and between *G. aculeatus* and its closest relative, *Pungitius pungitius* ($P < 0.05$, Fisher's Exact Test).

Species	% Infection success	% Establishment success
Poecilia reticulata (guppy)		
Ornamental Stock (OS)	100 (28/28)	89.3 (25/28)
Tobago Stock	100 (21/21)	71.4 (15/21)
Poecilia picta (Swamp guppy)		
Tobago Stock	90.5 (19/21)	78.9 (15/19)
Trinidad Stock	100 (6/6)	83.3 (5/6)
Poecilia sphenops (Molly)		
Ornamental Stock	89.3 (25/28)	68.0 (17/25)
Trinidad Stock	100 (11/11)	81.8 (9/11)
Xiphophorus hellerii (Green Swordtail)	100 (19/19)	94.7 (18/19)
Xiphophorus maculatus (Hi Fin Platy)	95 (19/20)	73.7 (14/19)
Danio rerio (Zebrafish)	95.2 (20/21)	20.0 (4/20)
Phoxinus phoxinus (Common minnow)	100 (15/15)	13.3 (2/15)
Gasterosteus aculeatus (Three-spined stickleback)	52.4 (11/21)	0 (0/21)
Pungitius pungitius (Nine-spined stickleback)	100 (17/17)	0 (0/17)
Salmo salar (Atlantic salmon) parr	0 (0/13)	0 (0/13)

Table 2.2: Infection and establishment success of *Gyrodactylus turnbulli* on different host species. Percentage of fish successfully infected on Day 0 is shown in the second column with numbers of fish in parentheses. Establishment (*i.e.* survival of parasite 24 h after experimental infection with a single, or in the case of salmon parr, multiple parasites) is shown in the third column. Although, salmon parr were not infected by exposure to individual parasites, all fish were infected when exposed to multiple parasites simultaneously

2.4.2 Attachment

Gyrodactylus turnbulli showed little or no hesitation in attaching to poeciliids. Initial attachment was via their anterior adhesive glands. The opisthaptor was then detached from the pin and then immediately reattached to the host while simultaneously releasing the temporary anterior attachment; the entire process taking < 1 s. Some individual parasites would probe the surface of

a host once or twice before attaching, but this activity occurred infrequently and could not be compared statistically.

Figure 2.2 shows the mean time taken for *G. turnbulli* to attach to different fish species, with a range from <1 s to 5 min, the time at which the trial was ended. The shortest mean attachment time was recorded for *Poecilia sphenops* (Trinidad stock) at 18.5 s, which was nearly half that for the controls, *P. reticulata* (OS at 35 s and Tobago stock at 63 s). Attachment times differed between fish species (Kruskal-Wallis, $H = 24.28$, $df = 11$, $p = 0.012$). *Post hoc* testing only identified a significant difference between *P. reticulata* (OS) and *Pungitius pungitius* (Steel-Dwass, $p < 0.05$), but there was a significant difference between poeciliids and non-poeciliids (Mann-Whitney, $W = 19642.5$, $p = 0.0108$).

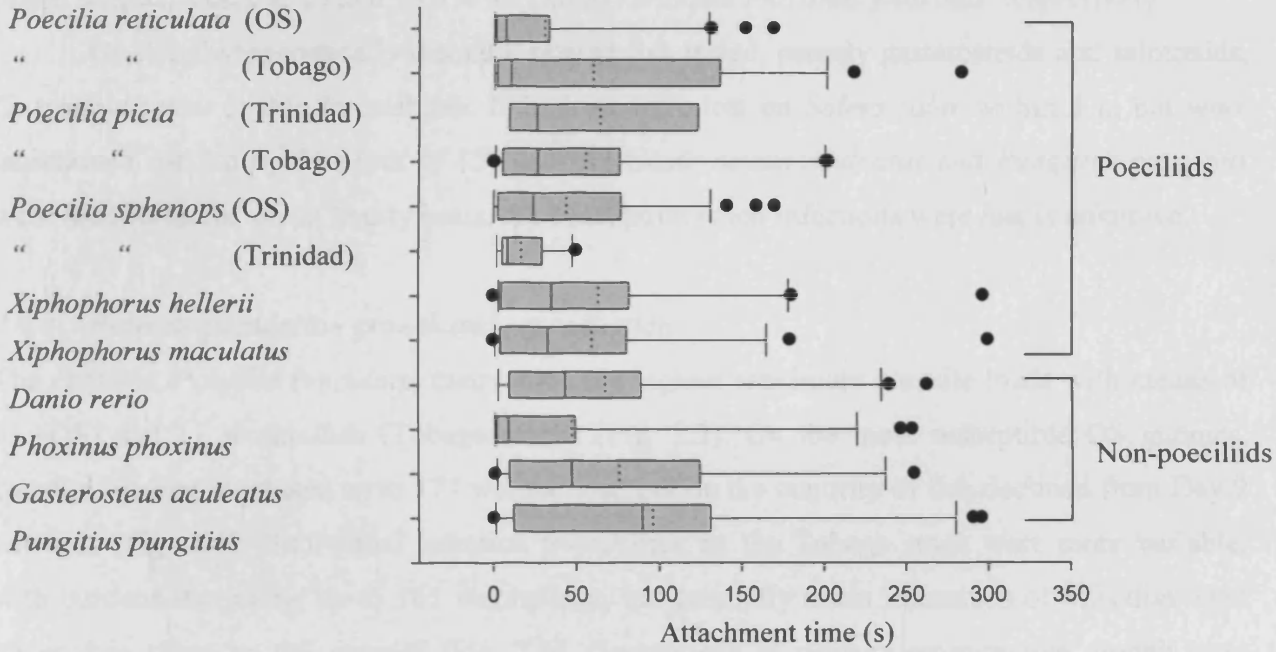

Fig. 2.2: Boxplot of attachment time (s) for *Gyrodactylus turnbulli* on different fish species showing the median, first and third quartile and outlier values with mean indicated by dotted line

Behaviour of parasites on gasterosteids was highly variable. On *P. pungitius*, parasites occasionally probed the host's surface (max. 3.6 probes/min) before attaching, but the mean attachment time of 100 s was the longest recorded for any fish species. On *Gasterosteus aculeatus*, some individual parasites actively tried to avoid contact with their potential host, but if contact did occur some of these parasites contracted and died almost immediately. On the remaining temperate species, *Phoxinus phoxinus*, some worms probed up to 60 times/min before

attaching, but with a relatively short mean attachment time of 49.9 s compared to 72 s on the tropical cyprinid *Danio rerio*. For *Salmo salar*, infections with single worms failed, but using the modified infection protocol with multiple worms, all fish were infected within 60 s.

2.4.3 Establishment success

Table 2.2 shows the proportion of each fish species that were still infected on Day 1 and surprisingly, *G. turnbulli* can be maintained on fish species other than its known host, *Poecilia reticulata*. Establishment was highest on *Xiphophorus hellerii* (94.7%) but did not differ statistically from the other poeciliids, ($p > 0.05$, Fisher's Exact Test) which ranged from 68 to 89.3% with almost as much intra- as inter-specific variation. Establishment on non-poeciliids was limited to the cyprinids and was significantly lower than on *P. reticulata* (OS) ($p < 0.0001$, Fisher's Exact Test), at 20 and 13.3% for *Danio rerio* and *Phoxinus phoxinus,* respectively.

On the phylogenetically distantly related fish tested, namely gasterosteids and salmonids, *G. turnbulli* was unable to establish. Infections were lost on *Salmo salar* within 3 h, but were maintained for 2 h by five (out of 13) fish. As *Gasterosteus aculeatus* and *Pungitius pungitius* were not monitored on an hourly basis, the exact point when infections were lost is unknown.

2.4.4 Parasite population growth and reproduction

The controls, *Poecilia reticulata,* maintained the highest maximum parasite loads with means of 45 (OS) and 11 worms/fish (Tobago stock) (Fig. 2.3). On the most susceptible OS guppies, parasite burdens increased up to 177 worms/host, but on the majority of fish declined from Day 9 onwards (Fig. 2.4). Individual infection trajectories on the Tobago stock were more variable; with burdens increasing up to 165 worms/host, but generally mean intensities of infection were lower than those on OS guppies (Fig. 2.5). Comparison of parasite reproductive growth rates between OS and Tobago guppy stocks (Fig. 2.6) showed a significant difference on Days 2, 4 and 5 ($W = 714.5$, $p = 0.0016$; $W = 643.0$, $p = 0.0365$ and $W = 640.0$, $p = 0.0022$, respectively) .

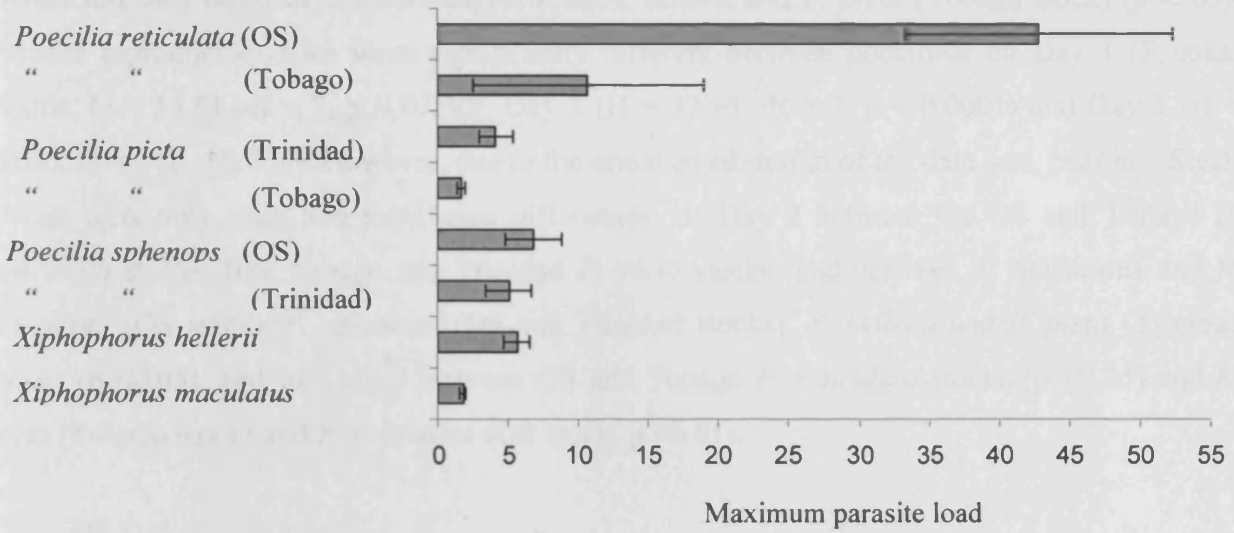

Fig. 2.3: Maximum parasite load (mean±SE) for each poeciliid species infected with a single specimen of *Gyrodactylus turnbulli*

Median maximum parasite loads were significantly different between poeciliids (Kruskal Wallis, $H = 66.51$, $df = 7$, $p < 0.0001$) as was day of maximum parasite load ($H = 47.69$, $df = 7$, $p < 0.0001$). *Post hoc* Steel-Dwass tests identified significant differences for day of maximum parasite load between *P. reticulata* (OS) and all other poeciliids, *X. hellerii* and *X. maculatus,* and in addition, between *X. hellerii* and *P. picta* (Tobago stock) for maximum parasite load ($p< 0.05$). Despite being the guppy's sister species, *P. picta* had amongst the lowest maximum parasite load (5 worms/host) and shortest mean duration of infection (4 d) (Fig. 2.7) comparable only to the most distantly related poeciliid to *P. reticulata, X. maculatus* (5 worms/host for 3 d). Light microscope observations showed delayed embryological development of *G. turnbulli* on *P. picta* and *X. maculatus.* Whereas parasite development on the other poeciliids was marked by discernible changes in the development of the F1 hooks between Day 0 and Day 1 (see Cable and Harris, 2002), for the majority of parasites on *P. picta* and *X. maculatus*, embryo development was only detected between Days 1 to 3.

P. picta and *X. maculatus* only maintained infections for a maximum of 12 or 11d respectively, whilst the remaining poeciliids, *P. sphenops* (OS and Trinidad stocks) and *X. hellerii*, both sustained the parasite for similar periods reported for the control fish (24 d; see Madhavi and Anderson, 1985), some individuals maintaining infections for over 20 d (*P. sphenops*, 20 and 21 d and *X. hellerii*, 24 d) (see Fig. 6). Median duration of infection (excluding *P. reticulata*) was significantly different ($H = 19.38$, $df = 5$, $p = 0.002$), although *post hoc* Steel-

Dwass test only detected a difference between *X. hellerii* and *P. picta* (Tobago stock) ($p<0.05$). Parasite reproductive rates were significantly different between poeciliids on Day 1 (Kruskal Wallis, $H = 15.51$, $df = 7$, $p = 0.030$), Day 2 ($H = 37.95$, $df = 7$, $p < 0.0001$) and Day 5 ($H = 20.46$, $df = 7$, $p = 0.005$). However, due to the unbalanced design of the data sets, *post hoc* Steel-Dwass tests only identified significant differences for Day 2 between the OS and Tobago *P. reticulata* stocks; (the Tobago and Trinidad *P. picta* stocks; and between *X. maculatus* and *P. reticulata* (OS stock), *P. sphenops* (OS and Trinidad stocks), *X. hellerii* and *P. picta* (Trinidad stock) ($p <0.05$), and for Day 5 between OS and Tobago *P. reticulata* stocks ($p <0.05$) and *P. picta* (Tobago stock) and *P. reticulata* (OS stock, $p <0.01$).

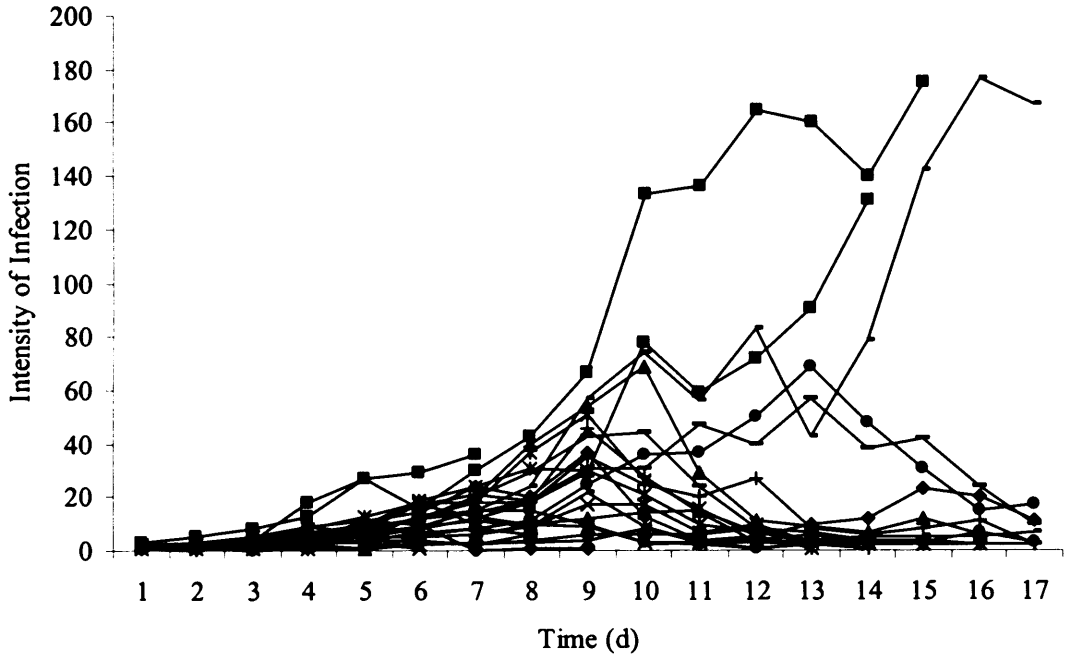

Fig. 2.4: Intensity of *Gyrodactylus turnbulli* infection for individual *Poecilia reticulata* (ornamental stock) infected on Day 0 with a single worm

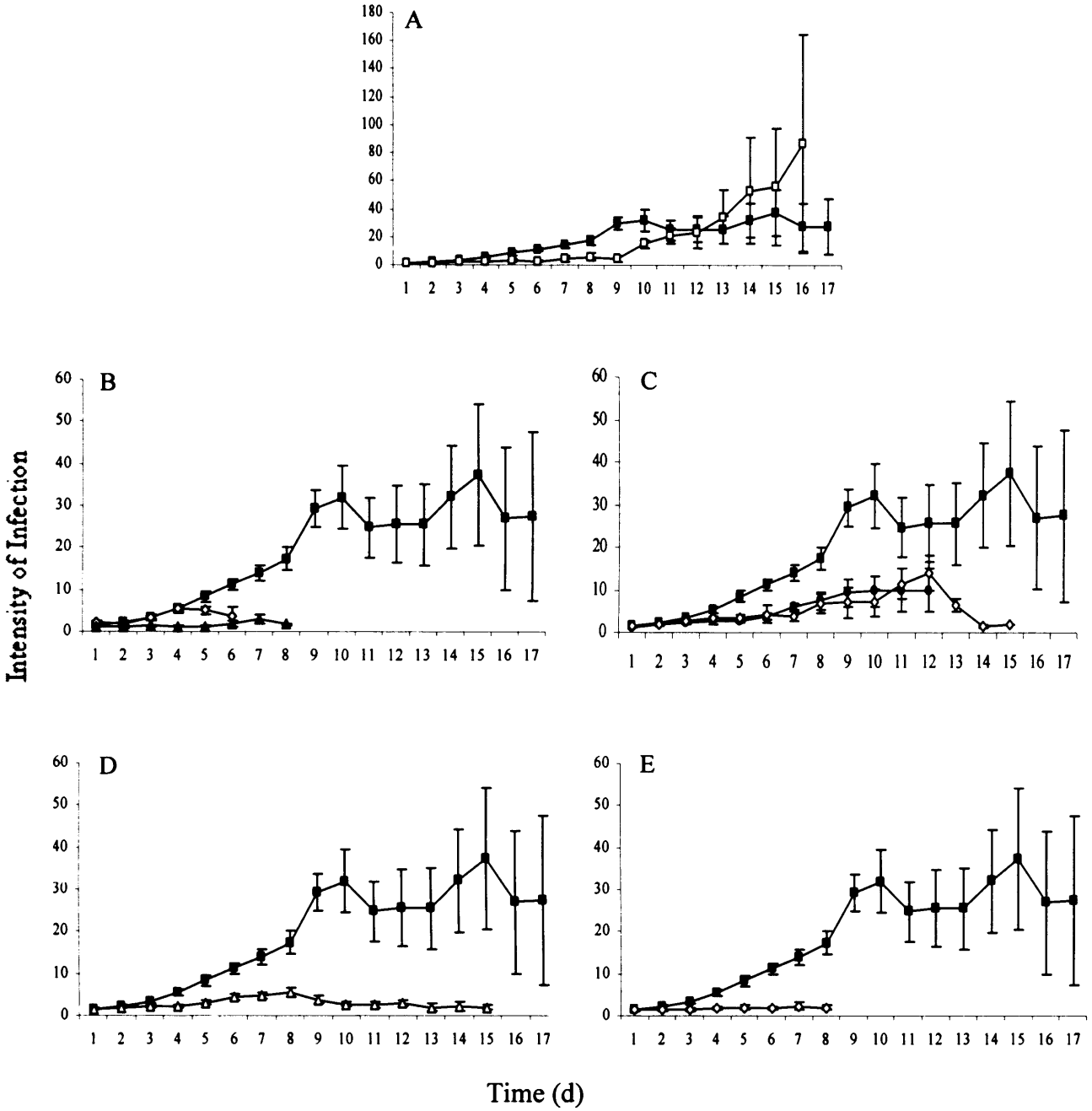

Fig. 2.5: Intensity of infection (mean ±SE) for *Poecilia reticulata*, ornamental stock (closed square) compared with (A) *P. reticulata* Tobago stock (open square); (B) *P. picta*, Trinidad (open diamond) and Tobago (closed triangle) stocks; (C) *P. sphenops*, ornamental (closed star) and Trinidad (open diamond) stocks; (D) *Xiphophorus hellerii* (open triangle); and (E) *X. maculatus* (open diamond). Not shown are the single fish of *P. reticulata* (Tobago stock), *P. picta* (Tobago stock), *P. sphenops* (Trinidad stock), *X. hellerii* and *X. maculatus*, which held an infection for 17, 11, 21, 24 and 12 d, respectively

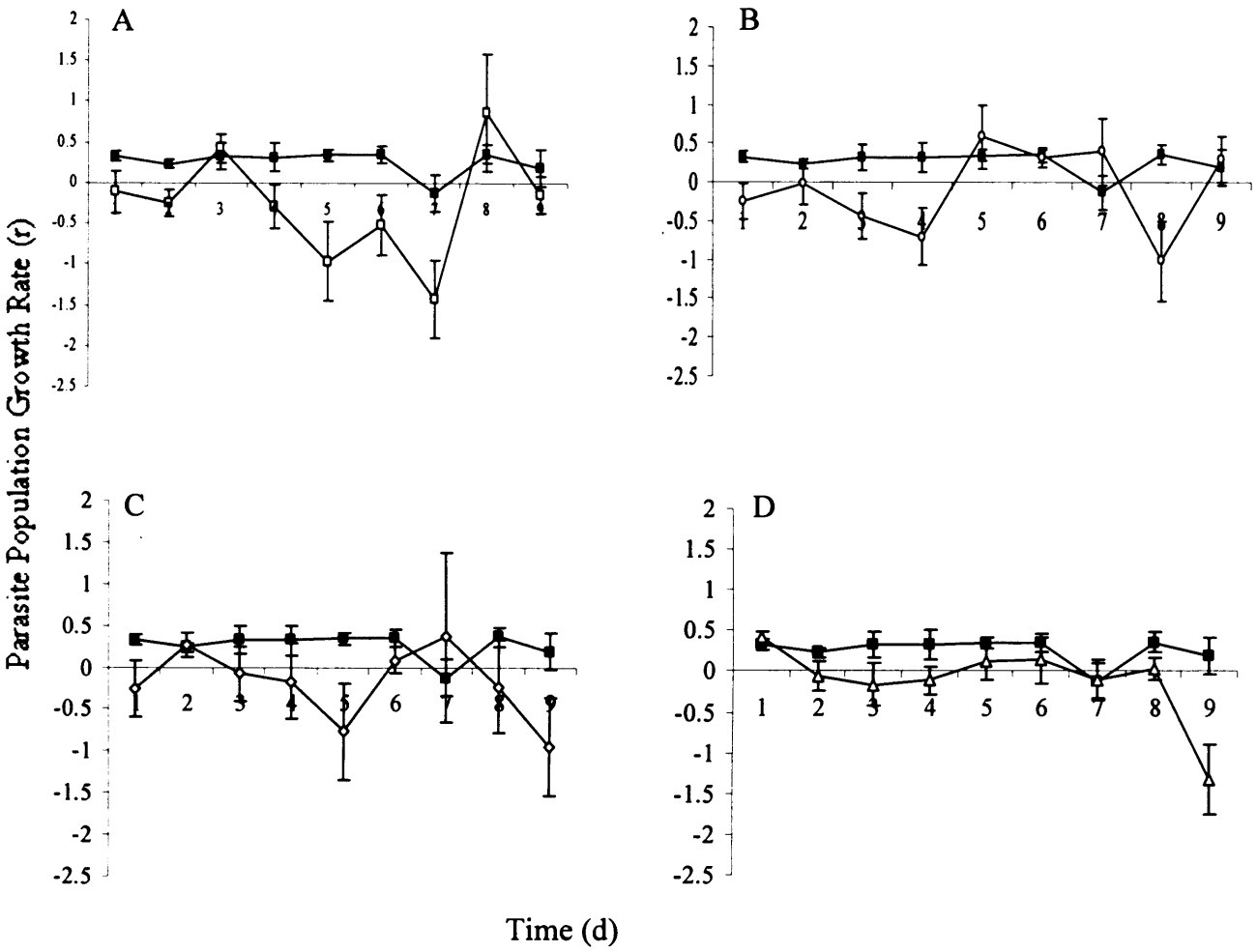

Fig. 2.6: Parasite population growth rate (r) (mean ±SE) up to Day 9 for *Poecilia reticulata*, ornamental stock (closed square) and (A) *P. reticulata*, Tobago stock (open square); (B) *P. sphenops*, ornamental stock (open star); (C) *P. sphenops* Trinidad stock (open diamond); and (D) *Xiphophorus hellerii* (open triangle). Positive values indicate that the mean parasite population is increasing, while negative values show that the population is declining by per day

On non-poeciliids, *G. turnbulli* either failed to establish on Day 1 or was lost within two days and therefore differences in maximum parasite load, day of maximum infection and duration of infection were not analysed.

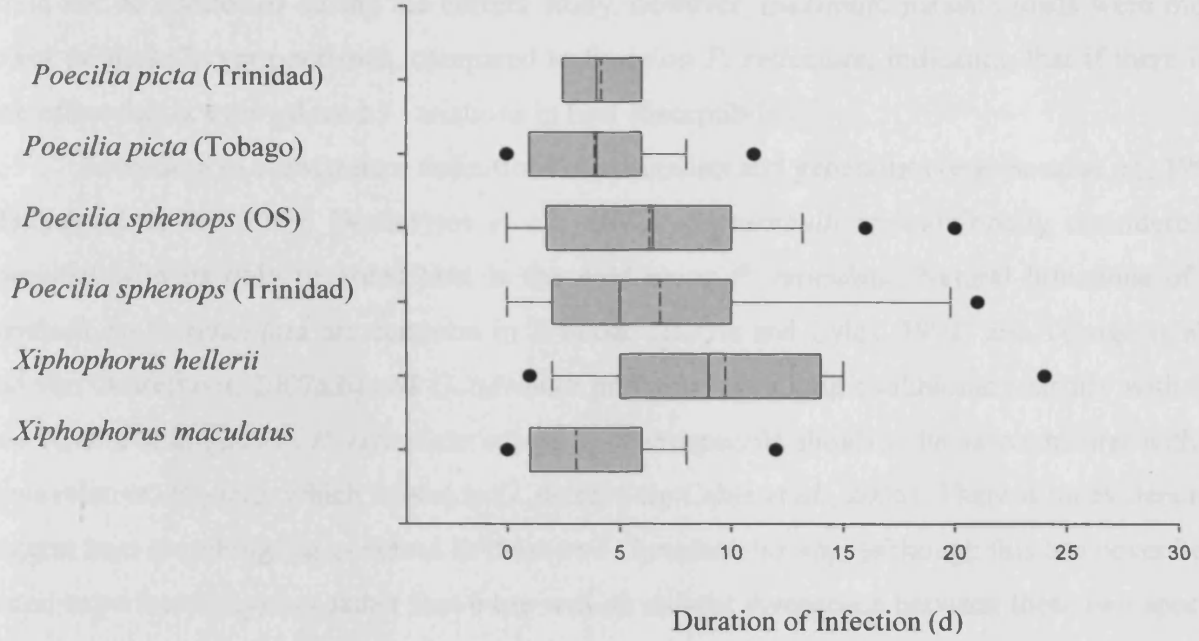

Fig. 2.7: Boxplot of duration of infection (days) for *Gyrodactylus turnbulli* on poeciliid species (excluding the controls, *Poecilia reticulata*) showing the median, first and third quartile and outlier values with mean indicated by dotted line

2.5 DISCUSSION

This is the first experimental study to confirm that the hitherto presumed specialist of *Poecilia reticulata, Gyrodactylus turnbulli* (see Harris *et al.*, 2004), can infect a range of different hosts, at least under aquarium conditions. Ten fish species were successfully infected with *G. turnbulli*, and *Poecilia sphenops*, rather than *P. reticulata*, was the host most quickly infected. Furthermore, establishment success of *G. turnbulli* was similar on five different poeciliid species and, as previously observed (van Oosterhout *et al.*, 2003; Cable and van Oosterhout, 2007a), there was considerable intraspecific variation related to differences in the proportion of resistant, responding and susceptible fish within each host stock (see Bakke *et al.*, 2002).

Based on host phylogeny, we predicted that *P. picta*, the sister species to *P. reticulata* (see Breden *et al.*, 1999), would be the optimal surrogate host for *G. turnbulli* compared to more distantly related poeciliids. However, *G. turnbulli* appeared to prefer *P. sphenops* and *Xiphophorus hellerii* over *P. picta* both in terms of attachment time, longer duration of infection and higher maximum parasite load. Furthermore, the mechanism underlying host specificity of these parasites is completely unknown. Both *P. sphenops* and *X. hellerii* are larger than *P. reticulata* and host size is likely to have influenced the data as larger guppies sustain higher

parasite loads than juvenile fish (Cable and van Oosterhout, 2007b). Size variation across species could not be controlled during the current study, however, maximum parasite loads were much lower on these larger poeciliids, compared to those on *P. reticulata*, indicating that if there is a size effect this is over-ridden by variations in host susceptibility.

According to the standard definitions of specialists and generalists (e.g. Sasal *et al.*, 1999; Matějusová *et al.*, 2000; Desdevises *et al.*, 2002), *G. turnbulli* is traditionally considered a specialist with its only recorded host in the wild being *P. reticulata*. Natural infections of *G. turnbulli* on *P. reticulata* are common in Trinidad (Harris and Lyles, 1992) and Tobago (Cable and van Oosterhout, 2007a,b) and *G. turnbulli* probably has a long evolutionary history with this host (Cable *et al.*, 2005). *P. reticulata* occurs in heterospecific shoals in its native habitat with its close relative, *P. picta*, which is host to *G. pictae* (see Cable *et al.*, 2005). There is no evidence to suggest host switching has occurred in these two *Gyrodactylus* spp. (although this has never been tested experimentally) but rather that there was an ancient divergence between these two species (Cable *et al.*, 2005). The current study emphasises the value of empirical data as opposed to predicting the host range of parasites based on field records alone and, inadvertently, highlights the importance of accurate parasite identification. Previous experimental infections by Leberg and Vrijenhoek (1994) and Hedrick *et al.* (2001) suggested that *G. turnbulli* could also infect *Poeciliopsis* species (Poeciliidae). However, these authors did not explain how their parasites were identified, which is of particular importance, considering the guppy is also host to *G. bullatarudis*, a reported generalist that occurs rostrally on the host, which many authors have confused with *G. turnbulli* (see Harris, 1986). Unfortunately, no specimens are available to confirm the identity of the parasites used in earlier studies, but considering the wider host range of *G. bullatarudis* (see Chapter 3) and the fact that Hedrick *et al.* (2001) describe this gyrodactylid as a predominantly gill species (which is definitely not the case for *G. turnbulli*), we suspect the parasite they used was *G. bullatarudis*. Furthermore, the parasite strain used by Hedrick *et al.* (2001) may have originated from a pet shop supplier rather than a feral population (parasites for this study were collected by Helen Rodd, personal communication) and so there is currently no experimental evidence of whether wild variants of *G. turnbulli* can transfer to other hosts, although we have identified this parasite from commercially obtained *P. sphenops* (unpublished). Bakke *et al.* (1991, 1992) and Olstad *et al.* (2006) have suggested that laboratory cultures of *G. salaris* are less host selective and therefore more likely to infect atypical hosts. In the current study, an isogenic strain of *G. turnbulli* was used, and whilst we acknowledge that laboratory culturing is likely to have influenced our results, it demonstrates that even with

restricted parasite genetic variation there is a high potential for host switching. Thus, the data reported here could even be a conservative estimate of the host range of *G. turnbulli.*

Establishment of *G. turnbulli* on non-poeciliids was low (≤20%), but cyprinids potentially could serve as reservoir hosts. In an aquarium setting or tropical fish farm, only *Danio rerio* used in the current study, is likely to come into contact with *P. reticulata*, but such interactions could have important disease implications. Reservoir hosts which transport the parasite but limit or fail to support reproduction are often overlooked in aquatic environments, despite their recognised importance in transferring pathogens to susceptible hosts (van Damme and Vandepitte, 1980; Lafferty and Gerber, 2002; Dobson, 2005). The most intensively studied gyrodactylid, *G. salaris*, paradoxically is upheld as an aberrant parasite with its unusually wide host range (Bakke *et al.*, 2002). However, unless experimentally tested (see above) host range can not be inferred from natural parasite distributions. Further research is likely to show that other gyrodactylids have a more extensive host range and, considering the ubiquitous nature of these parasites (Harris *et al.*, 2004), this has important implications for vulnerable fish species. Control measures aimed solely at the primary host (such as targeting *Salmo salar* to control *G. salaris*) are futile if the parasite can quickly re-establish via reservoir hosts (reviewed in Bakke *et al.*, 2002).

Due to the global aquarium trade, *P. reticulata* has been transported around the world, with feral populations in Australia (Dove and Ernst, 1998). In Europe, guppies were introduced to Italy (Holcik, 1991) and the U.K. (cited in Harris, 1986), and an established feral population has been reported in the Netherlands (FIGIS, 2008). With such worldwide translocation of the host, it is highly likely that *G. turnbulli* has also been transported globally, increasing opportunities for host switching to atypical hosts. As *Gyrodactylus* spp. have a direct life cycle and no specialised transmission stage, they are likely to be amongst the most successful invaders with an exotic host (cf. Torchin *et al.*, 2003). At present, the optimal mechanism of *G. turnbulli* transfer is unknown, although it is believed to occur via direct contact between hosts and it has been demonstrated that this parasite is capable of surviving in, and transferring to new hosts from, the water film (Cable *et al.*, 2002).

Evidence for host switching within the genus *Gyrodactylus* is largely derived from molecular studies (e.g. Cable *et al.*, 1999; Ziętara and Lumme, 2002) and is traditionally referred to as ecological transfer whereby a host acquires parasites from a phylogenetically, unrelated host that occupies the same environment. One of the best known examples is transfer of parasites within the "trout stream assemblage" and the pathogenic *G. salaris* may have originated from just such a host switch from grayling to salmon (Bakke *et al.*, 2007). Harris (1986) forewarned that

introductions of *P. reticulata* should be undertaken with care, until more was known about the potential for speciation and host switching of their associated gyrodactylids to native fish species. The current study reiterates this warning as guppies released into waterways (intended or accidental) could come into contact with temperate fish species, such as *Phoxinus phoxinus*, which we have shown here can act as a reservoir host for *G. turnbulli*, at least at 25°C (temperature occasionally reached in European waters, but also associated with ornamental ponds and waste water outlets). However, perhaps of greatest concern is the potential transfer of guppy gyrodactylids to other tropical fish species in the pet trade. Occurrences of host switching are well documented, but there is a distinct divide between those transfers that occur under natural conditions (e.g. Bensch *et al.*, 2000) and those that occur between hosts that would not normally encounter each other in the wild. The latter includes host switches in safari parks (e.g. Roelke-Parker *et al.*, 1996) zoos (e.g. Richman *et al.*, 1999), agricultural (e.g. Coyne and Orr, 2004) and aquaculture, including the ornamental fish industry (current study). On this basis, we propose to describe host switching between animals under natural habitats as "natural ecological transfer" and host switching occurring in artificial conditions as "artificial ecological transfer".

The current study supports studies such as Ward (1992) and Van Driesche and Hoddle (1997) in questioning how we define host specificity and optimal hosts. Based on a combination of factors, such as infection rates, survival, reproduction, population growth and parasite virulence, the most susceptible host may not necessarily be the optimal host for long term parasite survival. *P. reticulata* does appear to be the most susceptible host to *G. turnbulli* (cf. Hedrick *et al.*, 2001 and discussion above), but other poeciliids, such as *P. sphenops*, may be able to sustain the parasite for longer periods at lower burdens. Although our knowledge of the host range of *G. turnbulli* remains a conservative estimate, the fact that this parasite can survive on reservoir hosts, such as cyprinids, has important implications for other so-called specialist parasites.

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APPENDIX: EXPERIMENTAL POOLED INFECTIONS WITH THE MONOGENEAN ECTOPARASITE, *GYRODACTYLUS TURNBULLI*: IS HOST SWITCHING A LABORATORY ARTIFACT?

2.7 ABSTRACT

Parasites which are highly host specific (specialists) are generally considered to be unlikely to host switch, however, the host range of many so-called specialists has yet to be fully evaluated. Recently, it was demonstrated that the monogenean ectoparasite, *Gyrodactylus turnbulli* is not a strict specialist and is capable of infecting a range of different hosts, other than its assumed host, the guppy (*Poecilia reticulata*) under laboratory conditions. With an ever increasing number of novel host-parasite combinations being experimentally tested, it has been noted that host specificity barriers can be broken down under laboratory conditions and therefore whether such results have any ecological context is debatable. The propensity for *G. turnbulli* to switch to novel hosts under semi-natural conditions is not without precedent, as this parasite has previously been reported from *P. sphenops* in fish farms in Sri Lanka. The aim of the current study was therefore threefold: i) to determine if *G. turnbulli* can transfer to atypical hosts under semi-natural conditions; ii) to assess, when given a choice of hosts, whether *G. turnbulli* displays a preference for its assumed optimal host, *P. reticulata*, and iii) if *G. turnbulli* can be maintained on atypical hosts in the absence of *P. reticulata*. The results of the current study indicate that this parasite is capable of transferring to atypical hosts and that, surprisingly, initial transmission when given a choice of hosts, is random. Furthermore, there is evidence that this parasite is capable of reaching a lethal burden on an atypical host. In view of these findings, host range cannot be inferred from natural distributions without experimental testing and even so-called specialist species should be considered as a potential threat to vulnerable fish stocks.

2.8 INTRODUCTION

Parasites which occur on a single host (specialists) are considered unlikely to switch to a new host, although those that do might be more likely to speciate than generalist parasites, which utilise a number of hosts (Brooks and McLennan, 1993). One such parasite group that are considered to speciate primarily via host switching are the Monogenea (Brooks and McLennan, 1993; Cable *et al.*, 1999; Ziętara and Lumme, 2002), traditionally considered to be highly host specific. Within the Monogenea, the genus *Gyrodactylus* was considered narrowly host specific, however, a review by Bakke *et al.* (1992) found that only 30% of species were specialists and therefore the current known host range of individual species may be a gross underestimate,

especially as the number of species could be as high as 20,000 (Bakke *et al.*, 2002). One such species that had hitherto been assumed to be a specialist is *G. turnbulli* which occurs on the guppy (*Poecilia reticulata*). Experimental infections carried out by King and Cable (2007) found that this parasite, when artificially introduced to a range of atypical hosts, could infect and be maintained on other poeciliid species, such as *P. sphenops* and *Xiphophorus hellerii*.

Due to advances in experimental and molecular techniques, there is an ever growing number of experimental studies which aim to test the host specificity of parasites, particularly given their credence in being able to predict novel host-parasite combinations which could lead to emerging infectious diseases (Poulin and Keeney, 2008). In their review, Poulin and Keeney (2008) added a cautionary note stating that novel host-parasite combinations which are demonstrated to be viable in the laboratory may not occur in the wild due to a range of factors which would prevent such a combination ever occurring. Previously, a study by Thilakaratne *et al.* (2003) on parasitic infections in Sri Lankan ornamental fish farms recorded *G. turnbulli* on *P. sphenops*, a species which King and Cable (2007) found to be a suitable surrogate host for this parasite. Aquarium conditions can lead to overcrowding and poor water conditions which in turn creates stress, lower immunity and therefore create favourable conditions for transfer of a parasite to a novel host. The aim of the current study was to carry out pooled infections with *G. turnbulli* to ascertain whether this parasite is capable of switching to atypical hosts and to rule out the possibility that the previous findings of King and Cable (2007) were a laboratory artifact. A three-fold approach was taken to test whether i) *G. turnbulli* is capable of transferring to atypical hosts under semi-natural conditions; ii) when *G. turnbulli* is given a choice of hosts (*P. reticulata*, *P. sphenops* and *X. hellerii*) will it prefer its assumed optimal host (*P. reticulata*), and iii) if *G. turnbulli* can survive and be maintained on *P. sphenops* and *X. hellerii* in the absence of *P. reticulata*.

2.9 MATERIALS AND METHODS

Origins and screening of hosts, together with the maintenance of both hosts and parasite culture (an isogenic strain, *Gt3*) are identical to the methodology described by King and Cable (2007) and are therefore not repeated here.

2.9.1 Experimental design and infections

All experiments were conducted between 2006-2007, the methodology for each experiment is outlined below.

2.9.2 Experiment One: Transfer of G. turnbulli under semi-natural conditions

A donor guppy was left with a heavily infected guppy, maintained in 50ml of dechlorinated water until the donor had acquired a moderate (~20 gyrodactylids) or heavy (~100 gyrodactylids) infection (usually within 4 to 24 h). The donor was then placed into 10l aquaria of dechlorinated water that contained five individuals of a recipient species (*Poecilia picta, P. sphenops*; *Xiphophorus hellerii* and *Danio rerio*). Due to the unavailability of fish, infections with *P. picta* were only conducted with moderate parasite burdens. To control for inherent parasite variation, a guppy control was performed along side each novel host trial. All recipient fish were then screen 4h after introduction of the infected donor, and every 24h thereafter until one or more recipient fish were recorded as infected. Once a recipient fish was found to be infected, it was immediately removed from aquaria and maintained individually in a 1l jar of dechlorinated water, its infection was recorded and monitored daily until it had screened clear of gyrodactylids for three consecutive screenings. However, for infections with *P. picta*, a modified protocol was used whereby as a recipient fish became infected, it was replaced with a naïve fish, therefore this data was not been pooled with that for *P. reticulata*, *P. sphenops*, *X. hellerii* and *D. rerio*. For each host species, n = 15 (moderate burden) or n = 20 (for heavy burden). Sample size for *P. picta* was n = 29 (moderate burden).

2.9.3 Experiment Two: Host preference of G. turnbulli

Following the findings of King and Cable (2007) that *P. sphenops* and *X. hellerii* were the most suitable surrogate hosts after the assumed optimal host, *P. reticulata* to maintain an infection of *G. turnbulli*, five fish of each of the three species (plus one *P. reticulata* that would be the future donor) were placed into 120l aquaria containing dechlorinated water and allowed to habituate for one week. In addition, two fish of each species were added to act as reserves in the event of any recipient dying during experimental procedures. In total 21 fish (plus the guppy donor which was removed after transmission) were maintained per tank. The reserves were maintained in 1l jars, weighed down by gravel and covered with a fine mesh to prevent accidental transfer of the parasite (see Fig. 2.8). In order to control for host size, F1 juveniles of *P. sphenops* and *X. hellerii* were used that had been size matched to *P. reticulata*. After habituation, fish were removed and

elastomer dye marked in order to identify each host individual, and treated with Binox® to prevent fungal/bacterial infection that may have occurred due to the marking procedure. After marking, the fish were then allowed to recover for a further week prior to experimental procedure i.e. including infection.

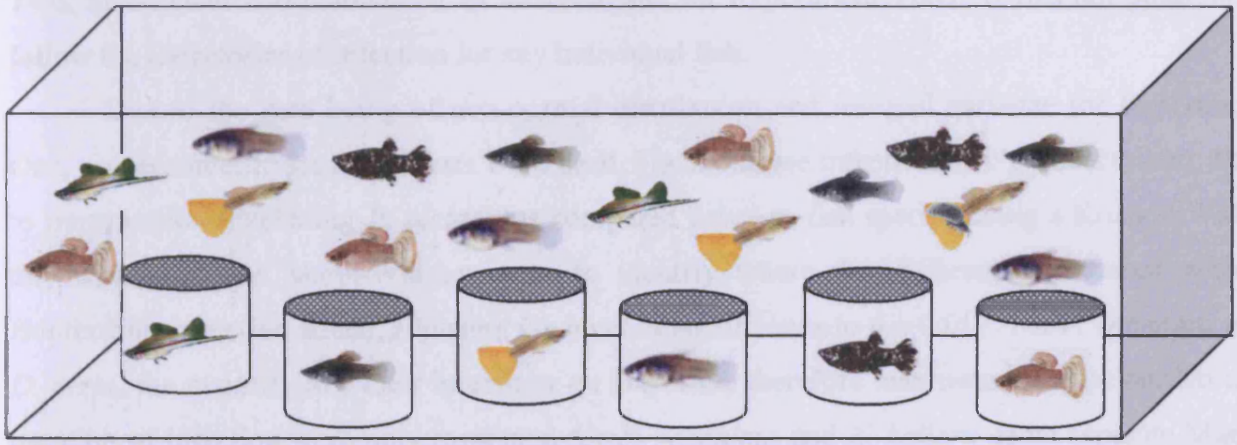

Fig. 2.8: Schematic diagram indicating the experimental set-up for Experiment Two: Host preference

During infection of the donor, all fish were removed from the tank and maintained in 1l jars of dechlorinated water in full view of each other, infection of the donor following the same procedure as outlined for Experiment One. Once the donor had a burden of approximately 20 gyrodactylids, all fish were reintroduced to the aquaria and then screened 24h and every 24h thereafter until the first recipient had become infected, at which point the donor was removed. After this time, all fish were screened every 48h with infections monitored until all fish had screened clear of gyrodactylid infection for three consecutive screenings.

To control for transmission not occurring due to resistant hosts or poor condition of the parasites, several guppies were infected at the same time as the guppy donor for each experimental replicate with the same number of parasites. These controls were maintained individually until such time as a recipient host maintained in aquaria became infected (n = 4, 1 control per replicate).

2.9.4 Experiment Three: Host switching of G. turnbulli

An individual *P. sphenops* or *X. hellerii* was left with a heavily infected guppy in 1l jars of dechlorinated water until the recipient had acquired an infection of approximately 30 gyrodactylids. This infected fish was then added to a 10l tank of dechlorinated water containing 5

conspecifics. All fish were then screened one week after introduction and every week thereafter, with the addition of naïve fish twice weekly to maintain the parasite, until such time that the parasite culture went extinct (n = 1 per host species).

2.9.5 Statistical analyses

Statistical analyses could only be performed on data from Experiment One, as for Experiment Two, insufficient fish maintained an infection and for Experiment Three, it was not possible to follow the trajectories of infection for any individual fish.

Due to the data being of non-normal distribution and unequal variance for Experiment One, non-parametric statistical tests were used. For moderate infections (20 gyrodactylids), time to transmission (excluding *P. picta*) was compared between fish species using a Kruskal-Wallis test with *post hoc* Mann-Whitney tests to identify where the differences occurred with a Bonferroni correction added, adjusting the level of significance to $p = 0.017$. For *P. sphenops* and *D. rerio*, the majority lost their infections on Day One, therefore maximum parasite burden and duration of infection were only compared for *P. reticulata* and *X. hellerii* using separate Mann-Whitney tests. However, due to the small sample sizes (n = 5), and thus low statistical power, these results should be treated with caution. For high infections (100 gyrodactylids), due to the unbalanced data set, the only variable that could be statistically tested was whether there was a significance difference in the frequencies of each fish species that became infected. This was not tested for the moderate gyrodactylid burdens, as the same number (five) of fish became infected for each species. Infection success was tested using a 4 x 2 Fisher's Exact Test available from the web-based programme http://www.physics.csbsju.edu/stats/exact.html.

2.10 RESULTS

2.10.1 Experiment One

2.10.1(i) Infection success (high burden, 100 gyrodactylids)

Infection success was highest for the control group, *P. reticulata* at 46.7%, this then reduced to 33.3% for *P. sphenops* and 13.3% for both *X. hellerii* and *D. rerio*. However, with the small sample sizes, this was not statistically different (Fisher's Exact Test, $p > 0.05$). For infections with moderate burdens (20 gyrodactylids), five infected recipients were removed and maintained individually for each species.

2.10.1(ii) Transmission time

At moderate burdens, median time for transfer of *G. turnbulli* to different host species was significantly different (Kruskal-Wallis, H = 15.44, p = 0.001 excluding *P. picta*), being fastest for *P. reticulata* (mean 4h), followed by 20h for *X. hellerii*, 29.6h for *P. sphenops*, 36h for *P. picta* and longest for *D. rerio* at 124.8h (Fig. 2.9). Subsequent *post hoc* tests identified the differences as being between *P. sphenops* and *D. rerio* (Mann-Whitney, W = 15.0, p = 0.0107) and *X. hellerii* and *D. rerio* (Mann-Whitney, W = 15.0, p = 0.0088. At high burdens, transmission was again fastest for *P. reticulata* with a mean of 6.3h compared to 24h for both *P. sphenops* and *X. hellerii*. As predicted, transmission time was longest for the non-poeciliid, *D. rerio* (mean of 84h).

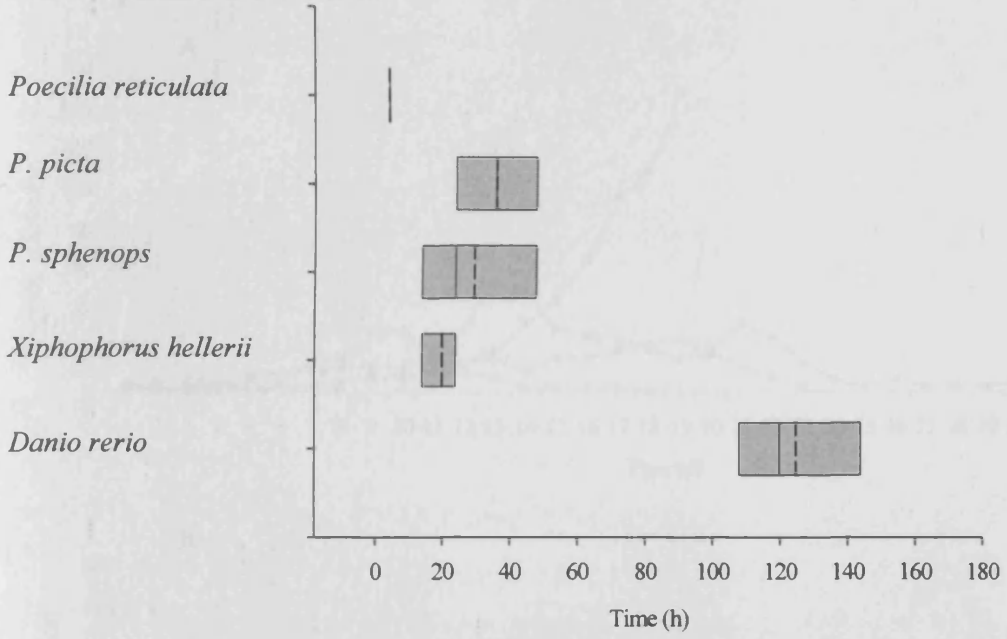

Fig. 2.9: Boxplot of attachment time (h) for *Gyrodactylus turnbulli* on different fish species (Experiment One – 20 gyrodactylids) showing the median, first and third quartile and outlier values with mean indicated by dotted line

2.10.1(iii) Maximum parasite load and duration of infection

For moderate burdens of the initial donor (20 gyrodactylids), the highest parasite burden (147 worms) was recorded for one individual *P. picta* with duration of infection being similar to its sister species, *P. reticulata* (range 8-23 d) with one *P. picta* maintaining an infection for 36 d (see Fig. 2.10). Maximum parasite load for *P. reticulata* reached 76 worms for one individual of *P. reticulata*, with duration of infection on this host ranging from 10-25 d. For *X. hellerii*, duration of infection ranged from 7-12 d, with a maximum burden of 41 worms for one individual. The majority of *P. sphenops* lost their infections on Day One, the remaining individual maintaining

the parasite for six days with a peak burden of 11 worms (see Fig. 2.11). The most distantly related host species, *D. rerio*, did not maintain infections beyond Day Two. Maximum parasite load and maximum duration of infection were not significantly different between *P. reticulata* and *X. hellerii* (Mann-Whitney tests, $p > 0.05$).

For high burdens on the initial donor, trajectories of infection were only followed for three individuals of *P. reticulata*. Parasite load peaked at 95 worms for one individual, whilst duration of infection for two fish was similar (14 and 16 d), the remaining individual lost its infection at Day One. Infection duration was similar for *P. sphenops*, where two individuals maintained infections for 12 and 14 d, with infections peaking for one individual at 23 worms. For *X. hellerii*, infections peaked at 25 worms and a duration of infection of 16 d whilst for *D. rerio*, the parasite did not establish.

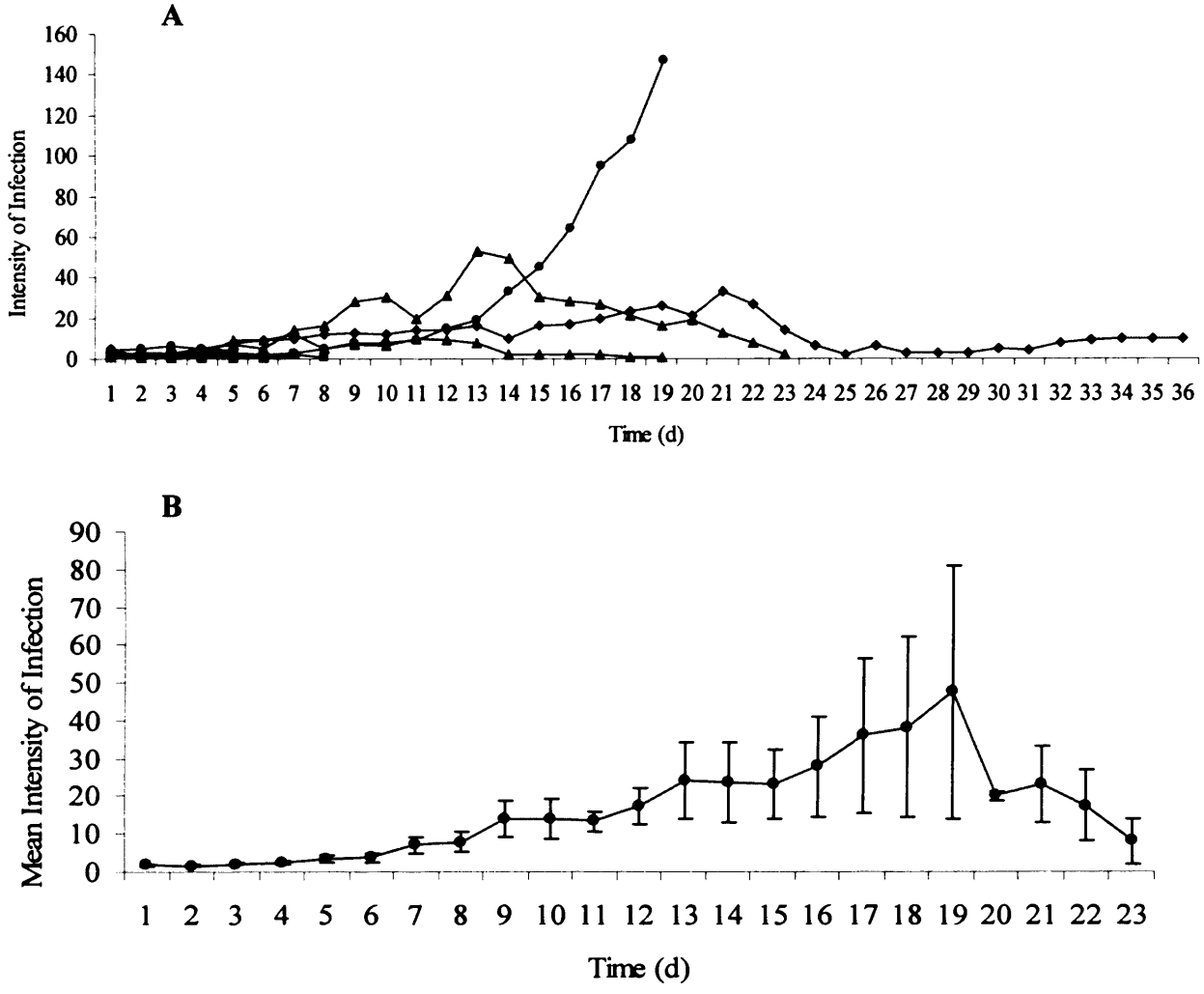

Fig. 2.10: Trajectories of infection of *Gyrodactylus turnbulli* on *Poecilia picta* (Experiment One – 20 gyrodactylids) indicating A) Intensity of infection of individual fish and B) Mean intensity of infection (±SE)

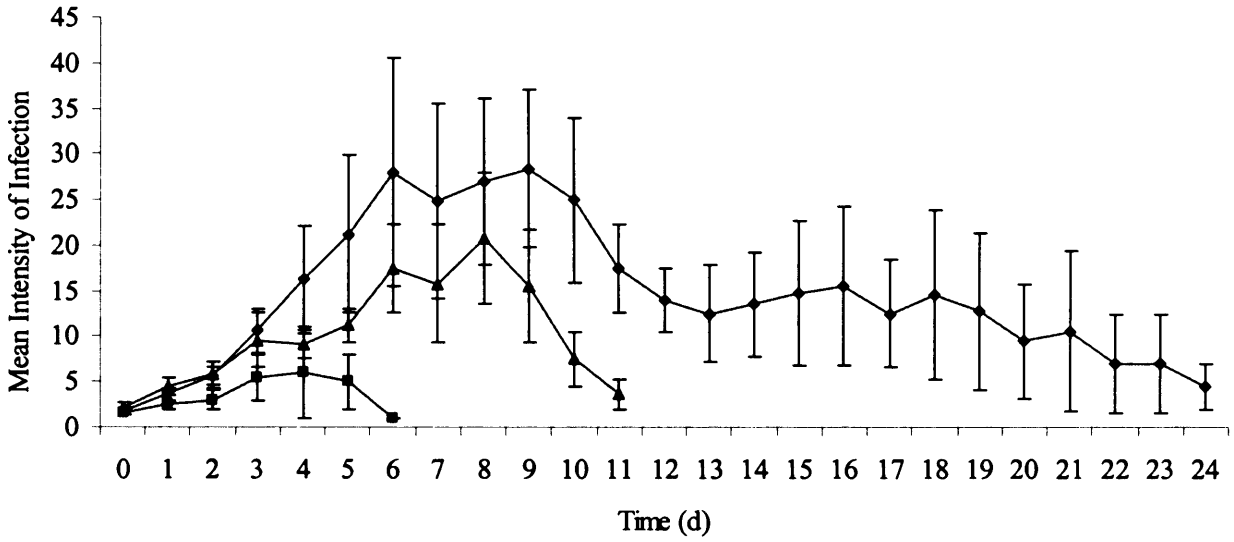

Fig. 2.11: Mean intensity of infection (±SE) of *Gyrodactylus turnbulli* on *Poecilia reticulata* (closed diamond); *P. sphenops* (closed square) and *Xiphophorus hellerii* (closed triangle) for Experiment One – 20 gyrodactylids

2.10.2 Experiment Two: Host preference

As no statistical analyses could be performed on this data, only preliminary results are given. In total, 4 replicates were performed and for half of the replicates, the time to first transmission was 48h, whilst for the remaining two replicates, first transmission occurred after 72h. Surprisingly, the control (*P. reticulata*) was not the predominant species to become infected first but rather initial transmission to a host species appeared to be random. *Xiphophorus hellerii* was among the first species to become infected in 3 out of 4 replicates.

For one experimental tank, the parasite went extinct after first transmission had occurred (after 72h). For the remaining three replicates, the highest burdens occurred on the control, *P. reticulata*, peaking at 175 worms for one individual. With regards to atypical hosts, maximum burdens were much lower, *P. sphenops* individuals became infected as time progressed, achieving a maximum burden of 25 worms on one individual. Although *X. hellerii* was predominantly the first species to become infected after introduction of the infected guppy donor, low level infections were maintained peaking at a maximum of 6 worms. Maximum duration of infection for *X. hellerii* was 14 d, this individual then lost its infection before being reinfected.

2.10.3 Experiment Three: Host switch

The control group, *P. reticulata* maintained an infection with the regular addition of naïve fish and removal of heavily infected or resistant fish for the duration of the experiment. Interestingly,

in the absence of *P. reticulata*, both *P. sphenops* and *X. hellerii* maintained an infection for 2 and 4 weeks, respectively, before the parasite went extinct. For both hosts, infection was typically low (max 12 worms/host for *X. hellerii*), however, in one instance for *P. sphenops*, the initial donor achieved a burden of 80 worms and died shortly afterwards.

2.11 DISCUSSION

The current study has demonstrated that *G. turnbulli* is capable of transferring to atypical hosts under semi-natural conditions. Therefore the findings of King and Cable (2007) that this parasite is not a strict specialist, do not appear to be a laboratory artifact, particularly given the finding that this parasite can be maintained in the absence of its optimal host. Furthermore, when given a choice of hosts, initial transmission of *G. turnbulli* to a host species appears to be random. Whilst infections persist on *X. hellerii* at a low level, this is not so clear cut for *P. sphenops*, where it would appear that although low level infections are maintained, for susceptible fish, lethal burdens can be achieved, the first evidence that this parasite is capable of killing an atypical host. In their study of parasite infections on ornamental fish farms in Sri Lanka, Thilakaratne *et al.* (2003) found that monogeneans were the most prevalent parasites, even though such fish are routinely treated with various chemical compounds, such as formalin and malachite green. Their finding of *G. turnbulli* on *P. sphenops* and the findings of the current study show that this species is a suitable surrogate host for this parasite. However, contrary to the findings of King and Cable (2007), where *P. picta* had one of the lowest maximum parasite loads for *G. turnbulli* under pooled conditions (Experiment One in current study) this host species supported parasite loads and duration of infection, similar to its sister species, *P. reticulata*. Due to a lack of fish, *P. picta* was not included in tests on host preference and host switching and therefore further work is needed to determine whether this species is a suitable surrogate host.

Aquarium fish can represent a major source of potential invasive species (McDowall, 2004) and their associated parasites, but for temperate countries such as the United Kingdom, it is assumed that the threat is tempered by the fact that tropical fish will fail to establish due to the lower ambient water temperatures (McDowall, 2004; Copp *et al.*, 2005). However, in countries where ambient conditions are more favourable, such as occur in regions of the United States (McDowall, 2004), this threat must not be underestimated. In their study, Leberg and Vrijenhoek (1994) found that a clonal hybrid of *Poeciliopsis* spp. was vulnerable to infection with *G. turnbulli* (but see King and Cable, 2007) and therefore, as reviewed by Bakke *et al.* (2007), a genetic alteration in this particular host might have lead to an atypical parasite being able to

exploit it. Therefore, the potential of parasites, such as *Gyrodactylus* spp. to have detrimental affects on vulnerable fish populations should not be underestimated

In the current study, we have demonstrated that *G. turnbulli* is capable of being maintained for up to one month on *X. hellerii* and have therefore, further highlighted the fact that host range cannot be inferred from natural distributions and that this parasite could represent a threat to vulnerable fish populations.

2.12 Future Work

From the current work, there are still a number of issues that need to be addressed and the results given here are only preliminary. Work that needs to be urgently undertaken include:

- Increasing sample sizes of all three experiments described here.
- Although these experimental studies have shown that *G. turnbulli* can occur on atypical hosts, it needs to be clarified whether it occurs in the wild. The guppy (*P. reticulata*) occurs in heterospecific shoals with its sister species, *P. picta* but it is unknown whether *G. turnbulli* can infect *P. picta* in the wild. In addition, further experimental studies are needed of *G. turnbulli* on *P. picta* (host preference and host switch).
- Following introduction of *P. sphenops* to Trinidad and Tobago (Cable, personal communication) and introduction of *X. hellerii* to other areas, both species are known to occur in sympatry with *P. reticulata*. Therefore, work should be undertaken to detect the presence of *G. turnbulli* on such populations
- With regard to host preference, further replicates are needed to complete this study. This will then hopefully give further information as to why *G. turnbulli* occurs on atypical hosts. Currently two scenarios have been hypothesised for the current study, that *G. turnbulli*:
    - Moves to an atypical host at random, but has lower fitness i.e. lower infection rate, lower growth rate and will then move on to its optimal host (*P. reticulata*) where it achieves higher fitness OR,
    - It stays on whichever host species it comes into contact with, having similar infection rates and population growth.
- With regard to host switching, further bioassays are needed to ascertain whether this parasite can be maintained in the absence of its preferred host. Currently, only one

isogenic strain (*Gt3*) has been used. Future work would involve hybridising all current laboratory strains of *G. turnbulli* to create hybrid vigour and this resultant strain would be used in serial passage cultures on *P. sphenops* and *X. hellerii*. Should the parasite be able to be maintained for a period of several months, it is hypothesised that as the parasite becomes adapted to a new host, it should then have lower fitness on its hitherto optimal host, the guppy. This would be tested by fixing parasites for morphological and molecular analysis, together with a cross infection bioassay to monitor establishment and population growth on a combination of hosts.

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CHAPTER 3: EXPERIMENTAL INFECTIONS WITH THE TROPICAL MONOGENEAN, *GYRODACTYLUS BULLATARUDIS*: POTENTIAL INVADER OR EXPERIMENTAL FLUKE?

3.1 ABSTRACT

Introduced exotic species have the potential to spread their associated parasites to native species which can be catastrophic if these hosts are immunologically naïve to the novel parasite. The guppy (*Poecilia reticulata*) has been disseminated worldwide outside of its native habitat and therefore could be an important source of infection to native fish species. Its parasite fauna is dominated by the ectoparasitic monogeneans, *Gyrodactylus turnbulli* and *G. bullatarudis*. The current study tested the host specificity of *G. bullatarudis* by experimentally infecting a range of isolated fish hosts, including temperate species. Surprisingly, the parasite was capable of establishing and reproducing, for several days, on the three-spined stickleback (a temperate species) when transferred directly to this host. We also established that *G. bullatarudis* could be transmitted under aquarium conditions at both 25°C and 15°C. At the higher temperature, the parasite was even capable of reproducing on this atypical host. The implications of these findings are discussed in terms of host specificity, host switching and climate change.

3.2 INTRODUCTION

Exotic species can have catastrophic effects on native species (Manchester and Bullock, 2000), adversely affecting biodiversity dynamics, reducing available resources and potentially introducing novel parasites to immunologically naïve hosts. Although the majority of parasites carried by exotic species are predicted to fail to infect native species (Torchin *et al.*, 2003), the few that become established may become invasive, potentially resulting in high virulence to native, naïve species. Some of the most well known examples of introduced pathogens affecting native fauna relate to avian and mammal hosts. For example, avian pox and malaria have detrimentally affected the native bird fauna of Hawaii; whilst African rinderpest and canine distemper virus have adversely affected keystone predators in the Serengeti (as reviewed by Lafferty *et al.*, 2005).

Pathogen introduction via exotic species is probably even more widespread amongst freshwater fish, due to anthropogenic activities, such as aquaculture, shipping, restocking of waterways and their use as live baits and biological control agents. In Europe alone, there have been 40 exotic fish species recorded (Elvira and Almodóvar, 2001). The effect of introduced fish pathogens is well known on commercially important food fish (such as the occurrence of

furunculosis and infectious salmon anaemia virus in salmonids). However, the effect of pathogens introduced via aquarium fish due to deliberate or accidental introductions can be just as catastrophic to native species. For instance, the Asian fish tapeworm (*Bothriocephalus acheilognathi*) initially spread via carp aquaculture, is now prevalent in Hawaii where it has been dessimated to native fish fauna via non-native poeciliids (Font, 2003). It has been predicted that parasites of aquatic hosts that have a direct life cycle should establish fairly easily (Bauer, 1991), but to be a successful invader they should also have the ability for dispersal, high reproductive potential, asexual reproduction and a broad host range (reviewed by Kennedy, 1994). One such group of pathogens which fulfills almost all of these criteria are the monogenean ectoparasites, *Gyrodactylus* spp. which Kennedy (1994) considered to be one of the most invasive groups of fish parasites due to their life history and mode of reproduction. This genus currently has just over 400 described species, the most well known being *G. salaris* which was accidentally introduced into Norway during the 1970s and subsequently decimated East Atlantic salmon strains, which had previously never encountered this parasite (Bakke *et al.*, 2002).

Disease introductions to temperate native species via tropical aquarium fish are assumed to fail due to low water temperatures (Copp *et al.*, 2005). However, feral populations of tropical fish, such as the guppy (*Poecilia reticulata*) can survive in areas where suitable conditions exist, such as industrial effluent (as occurred in the River Lee, Essex (Maitland, 2004). In previous studies, Leberg and Vrijenhoek (1994) and Hedrick *et al.* (2001) found that a guppy ectoparasite, *Gyrodactylus turnbulli* could infect vulnerable populations of the genus *Poeciliopsis*. One clonal lineage of a *P. monacha-lucida* hybrid (Leberg and Vrijenhoek, 1994) and the endangered Gila topminnow (*P. occidentalis*) (Hedrick *et al.*, 2001) were both susceptible to gyrodactylid infection. However, it should be noted that King and Cable (2007) speculated that the parasite used in both of these previous studies was more likely to have been a congener species, *G. bullatarudis*.

The guppy has been disseminated worldwide due to its popularity as an aquarium fish and its use as a biological mosquito control. It is currently recorded in 41 countries outside of its native habitat of Trinidad and South America, including several countries in Europe where feral populations are established (FIGIS, 2008). The guppy's parasite fauna is dominated by *Gyrodactylus* spp., being infected by two congeneric species, *G. turnbulli* and *G. bullatarudis*, which have distinct site preferences on their host (Harris and Lyles, 1992). Unlike *G. turnbulli*, which has been used extensively in empirical studies, comparatively little is known about *G. bullatarudis*. Previous studies by Scott and colleagues in the 1980s on *G. bullatarudis* were

subsequently found by Harris (1986) to have been using *G. turnbulli*. Actual studies on *G. bullatarudis* are restricted to those on the host response (e.g. Richards and Chubb, 1996; van Oosterhout *et al.*, 2006; Cable and van Oosterhout, 2007a,b and long-term survival (Richards and Chubb, 1998).

Previously, King and Cable (2007) experimentally tested the host specificity of *G. turnbulli* which had been assumed to be a strict specialist and found that it had a much broader host range than previously predicted. *G. bullatarudis* has always been considered a generalist (Harris *et al.*, 2004) although it has only been recorded from the aquarium trade on three poeciliids (*Poecilia reticulata* and *Xiphophorus hellerii*; see GyroDb [Harris *et al.*, 2008, www.gyrodb.net) and *X. maculatus*, see Kim *et al.* (2002) and from wild *P. sphenops* (Kritsky and Fritts, 1970). The wide-held believe that this parasite has a broad host range (Harris *et al.*, 2004) has never been experimentally tested, and it is unknown whether its host range extends to non-poeciliids. Therefore, the aim of the current study was to experimentally test the host range of *G. bullatarudis* using a range of aquarium fish and to evaluate whether this tropical parasite could infect and be maintained on several UK temperate species.

3.3 MATERIALS AND METHODS

The methods described in this study are similar to those used in a previous study on the host specificity of *G. turnbulli* (see King and Cable, 2007) and therefore only a brief summary is given below.

3.3.1 Host origins and maintenance

The origin of fish species used in the current study is given in Table 3.1. All laboratory bred fish were naïve to *G. bullatarudis*. Commercially supplied and wild caught fish were uninfected when obtained, with the exception of *Leuciscus cephalus* which was naturally infected with *G. lomi* (see Chapter 7). These fish, when screened clear were given a recovery period of two months prior to infection with *G. bullatarudis*. Wild caught fish, with the exception of *Salmo salar,* were habituated from 15 to 25°C in 2° increments over 5 days and then maintained at that temperature for a week prior to experimental infections. This procedure was also used when habituating guppies from 25 to 15°C. Atlantic salmon parr (*Salmo salar*) were maintained at 10.5°C in 10l aquaria during experimental procedures.

Species	n	Origin	Host standard length (mm)
Poecilia reticulata (Guppy)	28	Aquarium supplier, laboratory maintained since 1994.	8.0 - 28.5
Poecilia picta (Swamp guppy)	20	Wild caught from Trinidad and Tobago 2003/2004. Lab. bred F3/F4 generations.	8.5 - 21.5
Poecilia sphenops (Molly)	14	Aquarium supplier.	19.0 - 45.0
Xiphophorus hellerii (Green swordtail)	22	Aquarium supplier.	14.5 - 35.0
Xiphophorus maculatus (Hi Fin Platy)	17	Aquarium supplier.	13.0 - 47.5
Danio rerio (Zebrafish)	20	Aquarium supplier.	25.5 - 34.0
Leuciscus cephalus (Chub)	15	Wild caught, Roath Park, Cardiff, UK, 2007.	26.0 - 31.0
Gasterosteus aculeatus (Three-spined stickleback)	20	Wild caught, Roath Park, Cardiff, UK, 2004 - 2007.	22.0 - 33.0
Pungitius pungitius (Nine-spined stickleback)	19	Wild caught, Roath Park, Cardiff, UK, 2004 - 2007.	22.0 - 38.0
Salmo salar (Atlantic salmon)	16	Cynrig Hatchery, Abercynrig, Brecon, UK 2006.	52.0 - 78.0

Table 3.1: Origin of fish species infected with *Gyrodactylus bullatarudis* in the current study

3.3.2 Parasite cultures

All experimental infections were performed with strains of *G. bullatarudis* isolated in 2004 and 2007 from commercial stocks of *P. reticulata*, and identified following the methods of Harris *et al.* (1999). These strains were maintained in laboratory culture on an inbred ornamental stock of guppies. Cultures were monitored on a daily basis and screened every other day, with heavily infected and immune fish removed and additional naïve fish introduced to maintain parasite numbers.

3.3.3 Individual infections

Briefly, donor guppies were euthanized, individual gyrodactylids removed and presented via an insect pin to the caudal fin of an individual recipient fish previously anaesthetised with either 0.01 or 0.02% MS222 depending on the species. The parasite was presented for 2 min to the caudal fin, after which time, it was presented to other potential attachment sites along the length of the fish, with position and time of attachment recorded. If after 5 min, the parasite had failed to

attach, this was recorded as a failed attempt. The procedure was repeated with a second parasite but if no attachment occurred, this particular fish was abandoned. Parasite behaviour, including probing activity (see King and Cable, 2007), was monitored throughout the infection process. Host standard length and, where possible, gender were recorded; thereafter each fish was maintained individually.

Fish successfully infected with *G. bullatarudis* (Day 0) were examined the following day (Day 1). Those found to be parasite free on Day 1 were re-infected in order to rule out failure to establish being due to the gyrodactylid being old or damaged. Subsequently, fish were examined every 24 h with embryo development recorded until more than three gyrodactylids were found on the host. Thereafter, the number and position of gyrodactylids were recorded daily until the fish had shed all their parasites and were recorded free of gyrodactylids for three consecutive days. *P. reticulata* infections were terminated after Day 17 (see King and Cable, 2007). Salmonids were infected using the modified protocol described by King and Cable (2007).

Experimental infections were carried out between 2004 and 2007 and, in order to control for inherent parasite variation, fish from at least four different species (and always including *P. reticulata*) were infected on any one day with parasites from the same donor host. In total, 191 individual fish were infected during this study.

3.3.4 Pooled infections

For UK native fish on which *G. bullatarudis* were found to infect and reproduce, pooled infections were conducted at 25°C (within the preferred temperature of *Poecilia reticulata;* Froese and Pauly (2008)) and assumed optimum temperature for fecundity of guppy gyrodactylids (Scott and Nokes, 1984) and at 15°C, a water temperature common for *Gasterosteus aculeatus*. For pooled infections, five sticklebacks were maintained in 10l aquaria containing dechlorinated water. A moderately infected (ca. 20-25 parasites), size matched guppy donor, was introduced and all fish were screened 4h after introduction and every 24h thereafter. The experiment was terminated as soon as an individual stickleback was found to be infected, the individual being removed, isolated and the subsequent infection monitored. All pooled infections, conducted during 2007, were matched with guppy control tanks, with three replicates carried out for each temperature tested (i.e. n = 15 for each fish species).

3.3.5 Statistical analysis

Infection success (defined as whether an individual fish was successfully infected on Day 0) was recorded for all hosts, together with establishment success (the survival of the parasite 24 h after experimental infection). Fisher's Exact Tests were used to test for differences by comparing the presence or absence of infections for all fish on Days 0 and 1.

Transformations did not result in equal variances and normally distributed residuals for attachment times, maximum parasite load, day of maximum parasite load, maximum parasite reproductive growth rate (defined as the highest parasite reproductive between consecutive screenings), and maximum duration of infection. Differences in worm-attachment times between poeciliids and non-poeciliids (excluding *Salmo salar*) were therefore analysed using the Kruskal-Wallis test. This test was also used to analyse differences between poeciliid stocks for the other variables. *P. reticulata* was excluded from the maximum duration of infection data set, as these infections had been terminated at Day 17. *Post hoc* tests were performed using a Steel-Dwass test (Neuhäuser and Bretz, 2001), a non-parametric equivalent of the Tukey test.

The reproductive rate of *G. bullatarudis* was calculated using the formula $Ln(N_t + 0.1)$-$Ln(N_{t-1} + 0.1)$ (see van Oosterhout *et al.*, 2003) where N_t is the number of parasites on the host at day t, and N_{t-1} is the number of parasites recorded the previous sampling day. To avoid taking the natural logarithms of zero, $N_t + 0.1$ was used. Data analyses were performed using Minitab vs. 14 and KyPlot vs. 5 (for Steel-Dwass tests). Fisher's Exact tests were computed using a web-based programme available at http://bardeen.physics.csbsju.edu/stats/exact.html. For pooled infections, variables such as maximum load and duration of infection could not be statistically tested due to the small sample sizes.

3.4 RESULTS

3.4.1 Individual infections: Infection and establishment success

There was no significant difference in infection and establishment success of *Gyrodactylus bullatarudis* on the five species of poeciliids tested (Fisher's Exact Test, $p > 0.05$). All poeciliids had 100% infection success, with the exception of an individual *Poecilia sphenops* (see Table 3.2). For non-poeciliids, infection success for *Leuciscus cephalus* and *Gasterosteus aculeatus* was high (100% and 95%, respectively) but significantly lower for *Pungitius pungitius* and *Danio rerio* (84.2% and 70%, respectively; Fisher's Exact Test, $p = 0.039$). *Gyrodactylus bullatarudis* could only establish on a few individuals of *D. rerio* and *P. pungitius*, and not on *L. cephalus* at all. However, there was over 50% establishment success on *Gasterosteus aculeatus* (Table 3.2).

Species	% Infection success (numbers in parentheses)	% Establishment success (numbers in parentheses)
Poecilia reticulata	100 (28/28)	85.7 (24/28)
Poecilia picta	100 (20/20)	85 (17/20)
Poecilia sphenops	92.9 (13/14)	69.2 (9/13)
Xiphophorus hellerii	100 (22/22)	68.2 (15/22)
Xiphophorus maculatus	100 (17/17)	88.2 (15/17)
Danio rerio	70 (14/20)	14.3 (2/14)
Leuciscus cephalus	100 (15/15)	0 (0/15)
Gasterosteus aculeatus	95 (19/20)	52.6 (10/19)
Pungitius pungitius	84.2 (16/19)	18.8 (3/16)
Salmo salar	68.8 (11/16)	0 (0/16)

Table 3.2: Infection (percentage of fish infected on Day 0) and establishment success (percentage of fish infected after 24h) of *Gyrodactylus bullatarudis* on different host species

For *Salmo salar*, using the modified protocol, 11 of the 16 fish were successfully infected within one min, with a maximum of eight worms attaching to one individual. However, most infections were lost within the first hour. The individual fish with eight worms maintained these for 3 h before the parasites were lost.

3.4.2 Individual infections: Attachment

Attachment time of *G. bullatarudis* on each fish species ranged from a mean of 12 to 119s (Fig. 3.1). For the poeciliids, mean attachment time was fastest for the most distantly related poeciliid to the control (*Poecilia reticulata*), *Xiphophorus maculatus,* at 12s. The most closely related poeciliid to the control group, *P. picta* had a mean attachment time (18s) which was almost half that for *P. reticulata* (37.5s). The other poeciliids tested had a mean attachment time longer than the control at 53 and 56s for *P. sphenops* and *X. hellerii,* respectively. However, median attachment times between each poeciliid species were not significant (Kruskal-Wallis, $H = 7.37$, $df = 4$, $p = 0.118$).

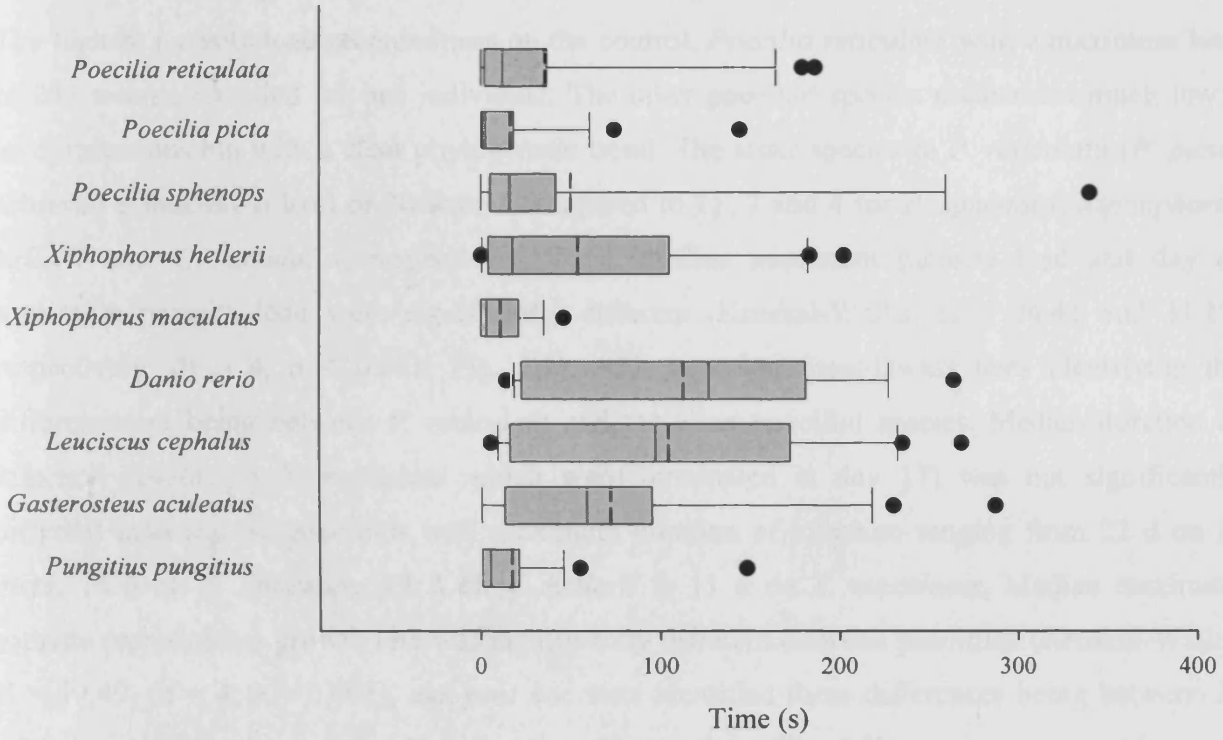

Fig. 3.1: Boxplot of attachment time (s) for *Gyrodactylus bullatarudis* on different fish species (excluding *Salmo salar*) showing the median, first and third quartile and outlier values. The mean is indicated by the dotted line

For the non-poeciliids, as expected, mean attachment was considerably longer with the exception of *Pungitius pungitius*, which had a mean attachment time of 18s, the same as that for *Poecilia picta*. For *Gasterosteus aculeatus*, *Leuciscus cephalus* and *Danio rerio* mean attachment times were 75, 108 and 119s, respectively. Median attachment times between each non-poeciliid species (except *Salmo salar*) were significantly different (Kruskal-Wallis, $H = 27.70$, $df = 3$, $p < 0.001$), the difference being between *Pungitius pungitius* and the other hosts (*post hoc* Steel-Dwass test, $p < 0.05$).

The behaviour of the parasite during experimental infection varied between the poeciliids and non-poeciliids. Parasite probing was rarely observed on the poeciliids, in fact there was no noticeable change in the behaviour of individual worms in the presence of a new host. On the non-poeciliids, individual worms probed the host and/or substrate continuously, and in some cases displayed hyperactivity (rapid thrashing of the body) when introduced to an atypical host.

3.4.3 *Individual infections: Parasite load, duration of infection and maximum parasite reproduction growth rate*

The highest parasite load recorded was on the control, *Poecilia reticulata* with a maximum load of 243 worms recorded for one individual. The other poeciliid species maintained much lower level infections but with a clear phylogenetic trend. The sister species to *P. reticulata* (*P. picta*) achieved a maximum load of 20 worms compared to 11, 7 and 4 for *P. sphenops*, *Xiphophorus hellerii* and *X. maculatus*, respectively. Thus, median maximum parasite load and day of maximum parasite load were significantly different (Kruskal-Wallis, H = 34.45 and 31.12, respectively, df = 4, $p < 0.001$; Fig. 3.2), with *post hoc* Steel-Dwass tests identifying the differences as being between *P. reticulata* and the other poeciliid species. Median duration of infection (excluding *P. reticulata* which were terminated at day 17) was not significantly different between the poeciliids with maximum duration of infection ranging from 22 d on *P. picta*, 14 d on *P. sphenops*, 13 d on *X. hellerii* to 11 d on *X. maculatus*. Median maximum parasite reproductive growth rate was significantly different between poeciliids (Kruskal-Wallis, H = 19.49, df = 4, p = 0.001), and *post hoc* tests identified these differences being between *P. reticulata* and *P. sphenops*, and *X. hellerii* and *X. maculatus* (Fig. 3.3).

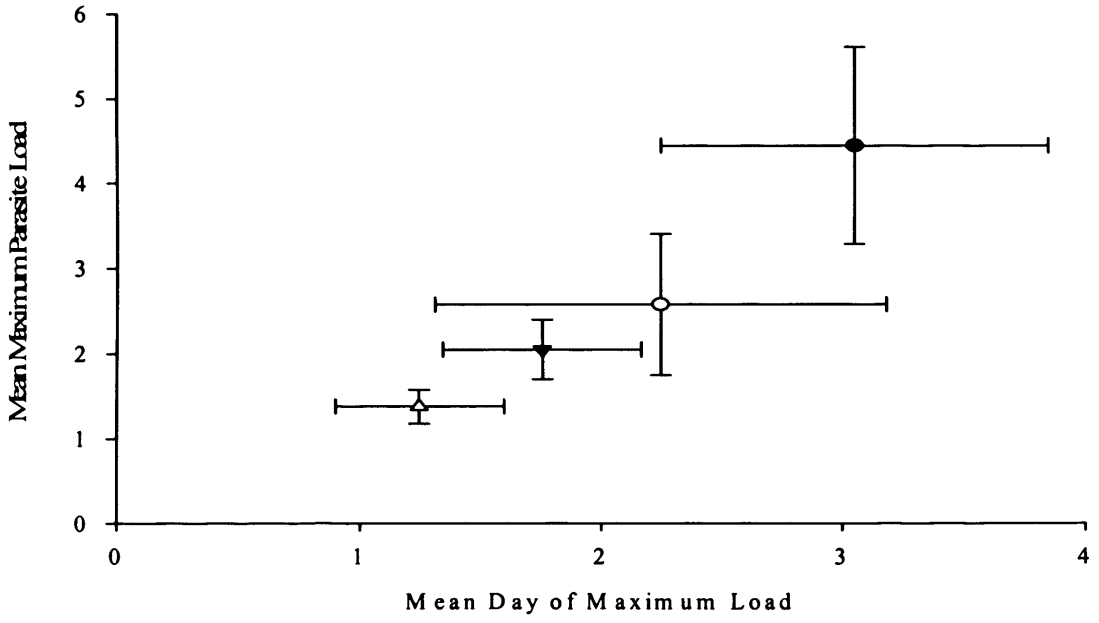

Fig. 3.2: Biplot of mean maximum parasite load against mean day of maximum load (±SE) for *Poecilia picta* (closed circle); *P. sphenops* (open circle); *Xiphophorus hellerii* (closed inverted triangle) and *X. maculatus* (open triangle). Data for *P. reticulata* (control) not shown as these control infections had been terminated at Day 17

The majority of non-poeciliids, could not maintain infections beyond Day 1, with the exception of two individuals each of *Pungitius pungitius* and *D. rerio* which remained infected for 2 and 5d, respectively. Surprisingly, on *G. aculeatus*, the parasite reproduced on six (out of 20) hosts with a maximum parasite load of five worms and a maximum duration of infection of 8d. The pattern of parasite growth on *G. aculeatus* was different to *Poecilia reticulata*, whereby the majority responded to infection after Day 9, but with parasite growth increasing on the remaining susceptible fish (Fig. 3.4A). In contrast, parasite growth on *G. aculeatus* was more random, with only a small proportion of hosts maintaining an infection (Fig. 3.4B).

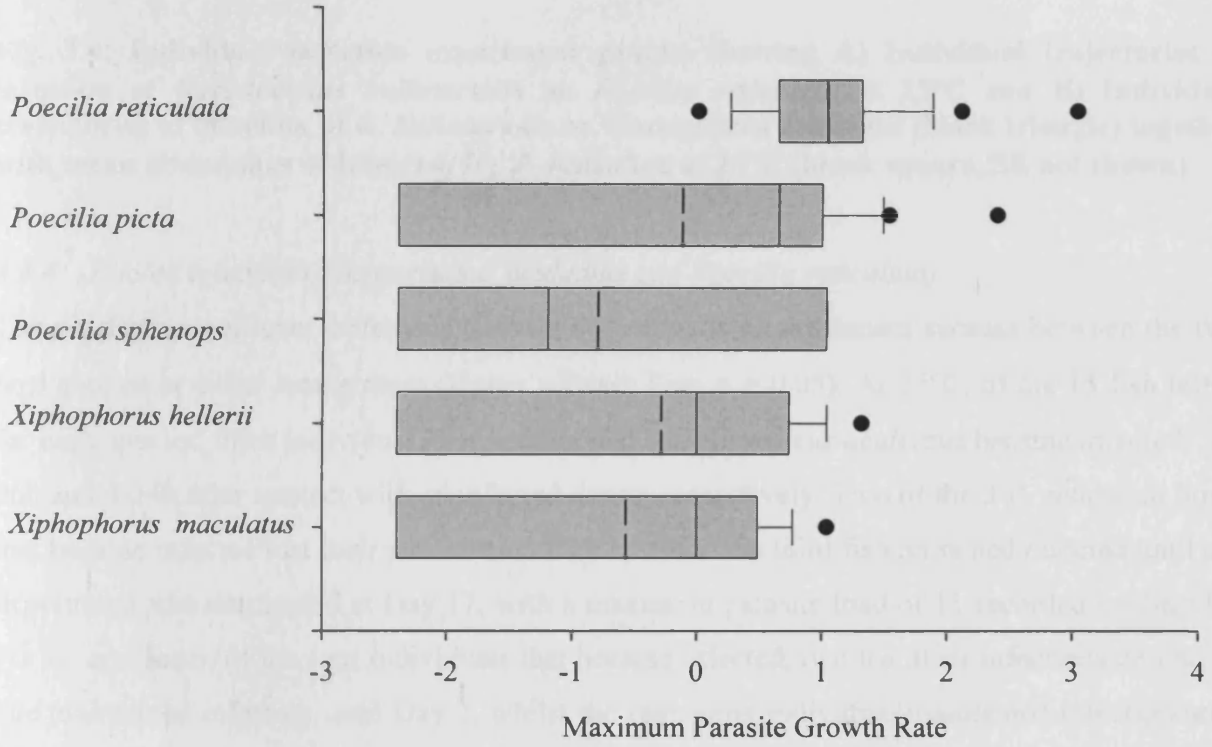

Fig. 3.3: Boxplot of maximum parasite growth rate (R) for *Gyrodactylus bullatarudis* on each poeciliid species showing the median, first and third quartile and outlier values. The mean is indicated by the dotted line

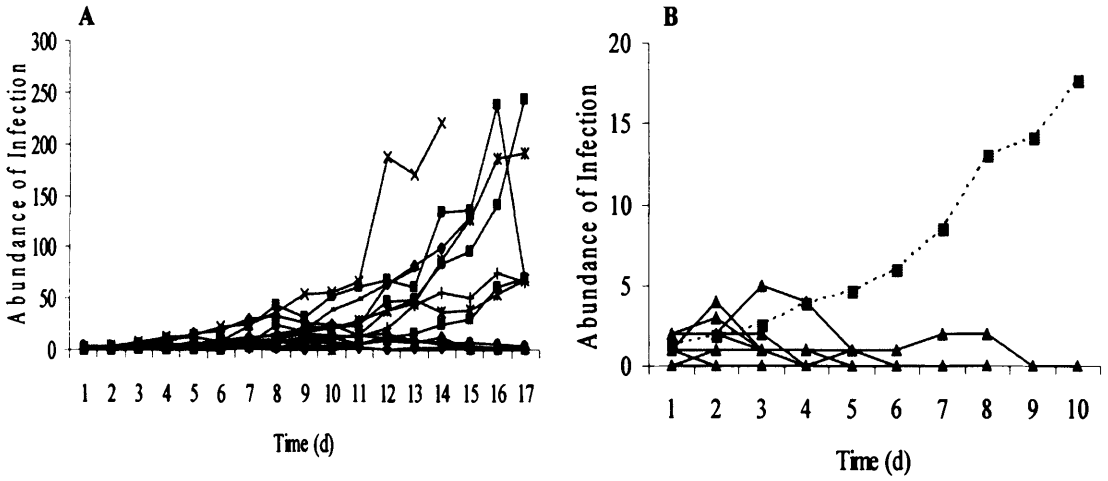

Fig. 3.4: Individual infection experiment graphs showing A) Individual trajectories of infection of *Gyrodactylus bullatarudis* on *Poecilia reticulata* at 25°C and B) Individual trajectories of infection of *G. bullatarudis* on *Gasterosteus aculeatus* (black triangle) together with mean abundance of infection for *P. reticulata* at 25°C (black square, SE not shown)

3.4.4 Pooled infections (Gasterosteus aculeatus and Poecilia reticulata)

There was no significant difference between infection or establishment success between the two host species at either temperature (Fisher's Exact Test, $p > 0.05$). At 25°C, of the 15 fish tested for each species, three individual *P. reticulata* and 4 *Gasterosteus aculeatus* became infected, 24-96h and 4-24h after contact with an infected donor, respectively. Two of the 3 *P. reticulata* hosts that became infected lost their parasites on Day 1, whilst the third fish remained infected until the experiment was terminated at Day 17, with a maximum parasite load of 11 recorded on Day 15. For *G. aculeatus*, of the four individuals that became infected, two lost their infections on Day 1, one maintained infection until Day 2, whilst the remaining individual maintained infection until Day 3. The maximum parasite load was two worms recorded on Day 1. At 15°C, infection success of the two host species was also similar, infection occurring after 4-24h for four of the *P. reticulata* and after 24h for all 5 *G. aculeatus*. None of the four *P. reticulata* maintained their infections post-Day 1. Four of the five *G. aculeatus* also lost their infections on Day 1, with one individual (with an initial burden of four worms) maintaining its infection until Day 2.

3.5 DISCUSSION

This is the first experimental study to test the host range of *Gyrodactylus bullatarudis*. The parasite was able to successfully infect all ten fish species tested and, most importantly, was able to reproduce on a temperate species, *Gasterosteus aculeatus*. With regard to the poeciliids, and in contrast to our previous study on *Gyrodactylus turnbulli* (see King and Cable, 2007), there appeared to be a phylogenetic trend amongst the poeciliids (see Fig.3.2). After the type host, *Poecilia reticulata*, its sister species, *P. picta* appeared to have the greatest potential to be a suitable surrogate host for *G. bullatarudis*. Both fish species occur in heterospecific shoals in the wild (Cable *et al.*, 2005) and there is ongoing research to ascertain whether *G. bullatarudis* infects *P. picta* in the wild. The remaining poeciliids tested (*P. sphenops*, *Xiphophorus hellerii* and *X. maculatus*) maintained low levels of *G. bullatarudis,* but up to 22 days for *P. picta*. Surprisingly, *G. bullatarudis* was also able to establish on the two gasterosteids tested and was able to reproduce and survive for up to day eight on *Gasterosteus aculeatus*. One possible explanation as to why this tropical parasite could transfer and be maintained on a temperate fish species, is that at the higher temperature tested (25°C), the innate immune response of *G. aculeatus* was compromised. Similarly, Wegner *et al.* (2008) postulated that the high parasite loads recorded in their experimental populations of *G. aculeatus* may have been due to environmental stress, such as the high water temperature (24.3°C). However, this does not explain why *Gyrodactylus bullatarudis* and not *G. turnbulli* was able to infect this host (see King and Cable, 2007).

Parasite host specificity is receiving an increasing amount of attention due to the recent experimental testing of novel host-parasite combinations. However, such data should be viewed with caution as laboratory conditions can lead to breakdown of host specificity and such combinations may have little or no ecological context (Poulin and Keeney, 2008). Whilst we acknowledge that our individual infection experiments could represent an extreme example of a breakdown of host specificity, pooled infections demonstrated that this parasite can naturally transfer between live fish. *G. bullatarudis* could infect *Gasterosteus aculeatus* both at 25°C and more importantly, at 15°C which is within the natural temperature range of this fish host. Infection and establishment success of *G. bullatarudis* on the two host species were similar at both temperatures. Thus, this tropical parasite can potentially infect and be maintained, albeit for a short time, on a temperate host, in a scenario where aquarium fish are released into waterways. Most fish populations are heterogeneous in their response to gyrodactylid infection, with often a

proportion of the hosts being able to maintain a long-term infection (Bakke *et al.*, 2002). Therefore, should a stickleback population contain a significant proportion of susceptible fish, there is a risk that tropical parasites could infect these temperate fish in the wild.

Feral guppy populations have become established in European countries, which are within the natural range of *Gasterosteus aculeatus*. For instance, guppies are established in Niederaussem, Cologne, Germany, occurring in heated discharge from a power plant (Fred Poesner, personal communication) and in the Mijares River in Spain following accidental release from aquarists (Elvira and Almodóvar, 2001). Although such feral populations are believed to be now extinct in the UK, they may recolonise in the future. The effect of climate change and subsequent global warming could lead to altered host-parasite assemblages, with parasites adapted to current environmental conditions being precluded and increasing water temperatures leading to new host-parasite combinations, via the increased potential for host switching (Brooks and Hoberg, 2008). This is of particular importance as the primary mode of speciation in *Gyrodactylus* spp. is host switching (Bakke *et al.*, 2002). In addition, the predicted increase in the use of nuclear power could lead to further heated waste water outlets thereby providing suitable conditions.

To date, studies on gyrodactylid host specificity have been limited to a few species, focusing on *G. salaris* even though it is considered an anomaly with its broad host range (Bakke *et al.*, 2007). Current assumptions of strict host specificity have been shown to be incorrect for species such as *G. turnbulli* (see King and Cable, 2007) and *G. tularosae* (see Moen and Stockwell, 2006). However, our finding that a parasite (such as *G. bullatarudis*) is capable of switching from a tropical to a temperate environment, may not be without precedent. Kennedy *et al.* (1987) infected and maintained a *Gyrodactylus* sp. from the goldfish (*Carassius auratus*) on the guppy; furthermore, this parasite was then able to reproduce and be transferred to other guppy stocks. However, it is not known at what temperature this parasite was maintained on its original host. Nevertheless, the current results have important ramifications as to legislation with regard to ornamental fish, although extensive legislation is in place with regard to commercially important food fish, such as salmonids, the same does not apply for ornamental fish. Furthermore, most of the general public are unaware of current legislation with regard to the prohibition of keeping certain species of fish and as such, most of what is being currently done to protect UK native fish relies on the goodwill of those involved in the ornamental fish trade (Copp *et al.*, 2005).

Our present knowledge of gyrodactylid host range may be a gross underestimate, given current assumptions that most species are narrowly host specific. The current study demonstrates that parasites from a tropical fish species can transfer to UK native fish and further reiterates the point that assumptions of host specificity cannot be made without empirical tests (King and Cable, 2007). Although most exotic parasites are predicted to fail to establish, niches do exist for novel parasites (Kennedy, 1994). Furthermore, there may be a considerable time delay before a novel parasite becomes invasive, as it may take many generations for a parasite to adapt to a new host/environment. As such, the true nature of the threat may only become apparent as the new host-parasite combination co-evolves over time with potentially the parasite becoming more virulent over successive generations (Dybdahl and Storfer, 2003). Therefore, the potential of *Gyrodactylus* species to cause disease on novel host species should not be underestimated, especially as these parasites fulfill all the criteria for a successful invader and their primary mode of speciation is host switching.

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CHAPTER 4: DIFFERENT TRANSMISSION STRATEGIES OF TWO CO-INFECTING GYRODACTYLID SPECIES (*GYRODACTYLUS BULLATARUDIS* AND *G. TURNBULLI*) ON DEAD HOSTS.

4.1 ABSTRACT

Gyrodactylus species are viviparous monogeneans, with a direct life cycle that reproduce *in situ* on their hosts. The guppy (*Poecilia reticulata*) is infected by two congeneric *Gyrodactylus* species; *G. turnbulli*, a comparatively low-virulent species which migrates into the water film when it leaves a dead host, and *G. bullatarudis*, a more virulent species whose transmission strategy was previously unknown. Here we show that the transmission strategies on recently dead hosts differed between both parasite species and appeared to be density dependent. At low burdens, both species remained with a dead host, whilst at higher burdens *G. turnbulli* migrated away from the guppy corpse whilst *G. bullatarudis* remained with its dead host. Given that the parasite's life span is considerably reduced when it is detached from its host, this remarkable difference in transmission strategy between both parasite species is considered further. We propose three hypotheses that could explain why *G. turnbulli* parasites leave their hosts at high burden. We discuss: (*i*) differences between the two species in their tolerance to crowding effects, (*ii*) different genetic bet hedging strategies in relation to differences in genetic relatedness of worms on a single host, and (*iii*) different efficiencies in transmission routes. We hypothesize that different transmission routes may have evolved in a high- and low-predation environment, and propose a number experiments that could test the various hypotheses.

4.2 INTRODUCTION

The genus *Gyrodactylus* is a specious group with currently over 400 described species and an estimated total number of 20,000 (Bakke *et al.,* 2002). They are viviparous monogeneans that are ubiquitous on teleost fish (Harris *et al.*, 2008). Despite their occurrence on a wide range of hosts, *Gyrodactylus* spp. have a remarkably conserved morphology. In view of their direct life cycle and lack of a specialized transmission stage, *Gyrodactylus* spp. are reportedly capable of continuous transmission and can infect new hosts at any time during the life cycle thus ensuring that they have access to new host resources (Boeger *et al.*, 2005).

There are presently four acknowledged transmission routes of gyrodactylids to their hosts: (A) direct transfer between live fish; (B) direct transfer between fish and detached parasites on the substrate; (C) transfer between fish and gyrodactylids in the water column, and (D) contact between live and dead infected fish (see Bakke *et al.*, 1992; Soleng *et al.*, 1999). Transfer is

assumed to occur by gyrodactylid characteristic locomotive behaviour (see Bakke *et al.*, 2007), but at least one *Gyrodactylus* sp. (*G. rysavyi* that parasitises the Nile catfish) is capable of unidirectional swimming movements (El-Naggar *et al.*, 2004). The distinct gyrodactylid transmission strategies may be related to host behaviour (Bakke *et al.*, 2007), although other factors may also be important. Atlantic salmon (*Salmo salar*) are predominantly solitary fish, with fry and parr occurring close to the substrate in shallow, fast flowing water (Baglinière and Champigneulle, 1986). One of its associated gyrodactylids, the infamous *G. salaris*, has been shown experimentally to remain with a dead host, attributed in part to the solitary nature of salmon (Olstad *et al.*, 2006). However, other species have been shown to move off a dead host. These include: *G. rarus* and *G. cryptarum* which infect the nine-spined stickleback (*Pungitius pungitius*) and Atlantic cod (*Gadus morhua*), respectively (Malmberg, 1970); *G. gasterostei* which occurs on the three-spined stickleback (*Gasterosteus aculeatus*) (see Cable *et al.*, 2002a); and *G. turnbulli* which infects the guppy (*Poecilia reticulata*) (see Cable *et al.*, 2002b). These *Gyrodactylus* species parasitise hosts that form shoals and transmission is thought to occur by host-host contact (transmission route A). Remaining on a diseased host is likely to be a maladapted strategy, which suggests that leaving dead hosts may actually increase the likelihood of successful transmission to a new host through transmission routes B and C.

Individual parasites that move off their host can survive, but their life span is considerably reduced. For example, *in vitro* survival of *G. gasterostei* was approximately 15% of the maximum life span *in vivo* (Cable *et al.*, 2002b) and this trend has been demonstrated across species (e.g. Lester and Adams, 1974; Scott and Anderson, 1984; Olstad *et al.*, 2006). Reduced survival has been attributed to a lack of available nutrients causing the parasite to starve, whereas parasites which have access to nutrients on dead hosts maintain their viability for longer (Olstad *et al.*, 2006). Genetic bet hedging theory suggests that apparently maladaptive behaviours such as moving off the host may reduce arithmetic mean fitness components (e.g. maximum life span of the parasite), but this behaviour may actually increase the worm's *geometric* mean fitness (i.e. the number of offspring reproduced across generations, calculated as the n^{th} root of their product) (Philippi and Seger, 1989). This is particularly appropriate for clonally and asexually reproducing parasites such as gyrodactylids, as all clones will be lost if the host is predated and all worms follow the single-best strategy (i.e. staying on the host).

G. turnbulli is a well studied organism (e.g. Cable and van Oosterhout, 2007a,b; van Oosterhout *et al.*, 2003, 2006, 2007a,b). Its distinct transmission strategy of hanging in the water film has been attributed by Cable *et al.* (2002b) to being an adaptive response to its surface

feeding host. In comparison, relatively little is known of the biology of *G. bullatarudis* (but see Cable and van Oosterhout, 2007b) which co-infects the guppy (studies by Scott in the 1980s on a parasite described as *G. bullatarudis* were subsequently identified as *G. turnbulli*; see Harris, 1986). This study compares *in vitro* survival of the two gyrodactylid species (*G. bullatarudis* and *G. turnbulli*), assesses their transmission strategy from dead hosts and discusses the implications for the evolution and biology of these species.

4.3 MATERIALS AND METHODS

4.3.1 Origin and maintenance of fish hosts

This study utilised an ornamental stock of guppies (*Poecilia reticulata*) originally obtained from a Nottingham aquarium supplier and subsequently maintained in the laboratory for generations, which were known to be parasite free i.e. naïve to infection. The fish were maintained in 96 l aquarium tanks at 25°C with a light regime of 12L: 12D and fed daily with Aquarian® flakes.

4.3.2 Parasite cultures

Gyrodactylus bullatarudis was recovered from an ornamental stock of *P. reticulata* obtained from an aquarium supplier in December 2006. The parasite was identified following the methods of Harris *et al.* (1999). The *Gt3* strain of *G. turnbulli* was an isogenic line isolated from guppies obtained from a Nottingham aquarium supplier and continuously maintained in laboratory culture since October 1997. Maintenance of these parasite strains followed the methods of King and Cable (2007), with the exception that the *G. bullatarudis* cultures required screening every 48 h with supplementary water changes and the addition of naïve fish every 2 days to maintain a viable culture. For all experimental studies, parasites were viewed using a stereo-microscope with fibre optic illumination, with experiments carried out between 2007 and 2008.

4.3.3 In vitro survival and behaviour

Individual worms of *G. bullatarudis* and *G. turnbulli* were removed from heavily infected euthanized guppies and transferred to the wells of microtitre plates containing 200 µl of dechlorinated water at 25±0.5°C. As described in Cable *et al.* (2002b) and Olstad *et al.* (2006), any worms which died in the first hour were excluded from subsequent analysis. All worms were monitored every hour for the first 5 h and thereafter every 2 h until all worms had died. While alive, the location of each parasite was recorded as in the water film, attached to the side,

attached or detached to the bottom of the well, or moribund. If an individual could not be located in the well at any time point, due to a blind spot caused by refractive light in the wells, it was recorded as not found. Any worms that had given birth were recorded, with the mother and daughter being distinguished based on embryo development: the mother having a contracted uterus and the daughter having a developing F1 embryo within its uterus. Sample sizes were N = 60 parasites for each species.

4.3.4 Behaviour on dead hosts

Infected guppies with a range of burdens, classed as either low (less than 50 worms) or high (greater than 50) (n = 16 per burden/parasite species) were euthanised by pithing of the brain. These corpses were immediately transferred to individual Petri dishes (9 cm diameter) containing 25 ml of dechlorinated water at 25±0.5°C and were monitored at hourly intervals for 12 h after the host's death and then again, 24h after the host's death. To avoid the possibility of body fluids from the dead host killing the parasites in the first 12 h, the corpse was transferred to clean dechlorinated water at each observation period. In order to avoid pseudoreplication, any worms which were found detached, on the bottom on the Petri dish or in the water film were removed after counting. The numbers and location of parasites in the Petri dish and remaining with the host were recorded until all parasites had died.

4.3.5 Statistical analyses

Bartlett's Test and the Anderson-Darling test indicated heterogeneity of variance and non-normal distribution of the data. Hence, non-parametric analyses (Mann-Whitney tests) were used to test whether maximum survival different between parasite species both *in vitro* and *in vivo*.

The proportions (mean and SE) of worms that were dislodged from the host were compared between of *G. turnbulli* and *G. bullatarudis*. The guppies were therefore infected with a low (<50 worms) or high burden (>50 worms). The numbers of worms that were attached to the host and detached were counted every hour over a 12 h time period after the start of the infection. Statistical significance between the treatments in the proportion of worms that become detached from the host were tested using a Chi-square test (or Fisher Exact test if expected values were N < 5).

The percentage of *Gyrodactylus turnbulli* and *G. bullatarudis* worms attached on different positions of the host were analysed for guppies infected with a low (<50 worms) and high (>50 worms) parasite burden. The numbers and positions of worms were counted every hour over a 12

hour time period after the start of the infection, and the mean and 95% CI of percentage coverage were calculated across individuals over the 12h period. Statistical significance was observed when the 95% CI did not overlap. Similarly, the percentage worms attached on different positions of the host were compared for the first and last three hours of the infection (0-3 and 10-12h, respectively). The mean and 95% CI of percentages (see Table 4.1) were calculated across individuals over this 3h period, and differences in distribution between both parasites were tested using a Fisher Exact test.

Gb high		**0-3h**		**10-12h**	
		mean	95%CI	mean	95%CI
Tail	(T)	11.8	9.0	18.3	12.3
Lower Body	(LB)	10.3	8.3	16.9	14.3
Upper Body	(UB)	17.7	9.1	25.2	13.7
Anal fin	(A)	3.1	3.6	4.8	5.8
Dorsal fin	(D)	2.0	2.4	2.2	2.7
Pectoral fin	(Pect)	8.9	9.2	7.1	10.4
Pelvic fin	(Pelv)	5.2	8.6	3.1	5.9
Head	(H)	40.9	19.2	22.4	16.7

Gt high		**0-3h**		**10-12h**	
		mean	95%CI	mean	95%CI
Tail	(T)	31.2	18.3	31.4	19.8
Lower Body	(LB)	27.8	10.6	36.2	26.0
Upper Body	(UB)	18.8	12.4	14.8	12.8
Anal fin	(A)	2.3	3.3	2.2	4.3
Dorsal fin	(D)	0.9	2.6	2.1	8.4
Pectoral fin	(Pect)	3.1	4.5	2.1	4.8
Pelvic fin	(Pelv)	0.5	1.8	0.3	1.1
Head	(H)	15.3	17.6	10.8	17.2

Table 4.1: Percentage of *Gyrodactylus turnbulli* (Gt) and *G. bullatarudis* (Gb) worms attached on different positions of the host, for guppies infected with a high (>50 worms) parasite burden at the first three hours of the infection (0-3h) and the last three hours (10-12h). The values in the tables represent the mean and 95% CI calculated across individuals over the 3h period (0-3 and 10-12h)

4.4 RESULTS

4.4.1 In vitro survival and behaviour

At 25°C, maximum survival times of *Gyrodactylus bullatarudis* and *G. turnbulli* were similar (26h and 31h, respectively; see Fig. 4.1), with no significant difference in median maximum survival time (Mann-Whitney, $W = 2985.0$, $p > 0.05$).

The median amount of time spent at various locations in the well was significantly different between species, with *G. turnbulli* more commonly observed in the water film (55.7%) compared to *G. bullatarudis* (21.7%) (Mann-Whitney, $W = 575.0$, $p < 0.0001$). In contrast, *G. bullatarudis* was more likely to be found attached to the bottom of the well (32.6%), unlike *G.*

turnbulli (7.8%) (Mann-Whitney, W = 2509.5, p < 0.0001). Both species also differed in the median amount of time spent attached to the side of the well and detached in the well (Mann-Whitney, W = 611.0 and 1432.0, p = 0.0199 and 0.0002, respectively).

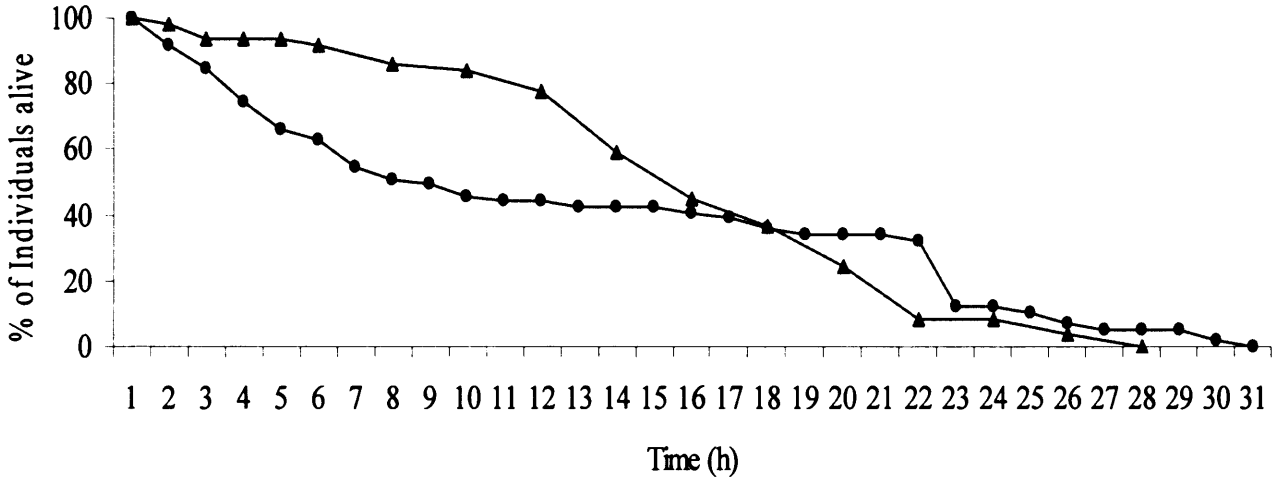


Fig. 4.1: Survival curves representing the percentage of individual *Gyrodactylus bullatarudis* (▲) and *G. turnbulli* (•) worms alive at 25°C over time. Interpolation has been used to correct for time differences in screening

4.4.2 In vivo behaviour on dead hosts

Figure 4.2 shows the proportion (mean and SE) of *G. bullatarudis* and *G. turnbulli* worms that were dislodged from the host. Guppies were infected with either a low (<50 worms) or high burden (>50 worms). Except for t=0 h, highly significant differences exist between the treatments in the proportion of worms that become detached from the host in all observation periods (Chi-sq.≥36.136, df =3, p<0.001). *G. turnbulli* appears to leave its host when at high parasite burdens, but not at lower burdens. In contrast, *G. bullatarudis* does not change its behaviour but stays with its hosts at both high and low burdens.

Figure 4.3 shows that the differences in distribution between the parasites species on the host are most pronounced when the parasite load is low, with proportionally more *G. bullatarudis* infecting the head and upper body, while *G. turnbulli* is most prevalent on the tail and lower body. At higher loads, the distributions become more similar and more equally spread over the host.

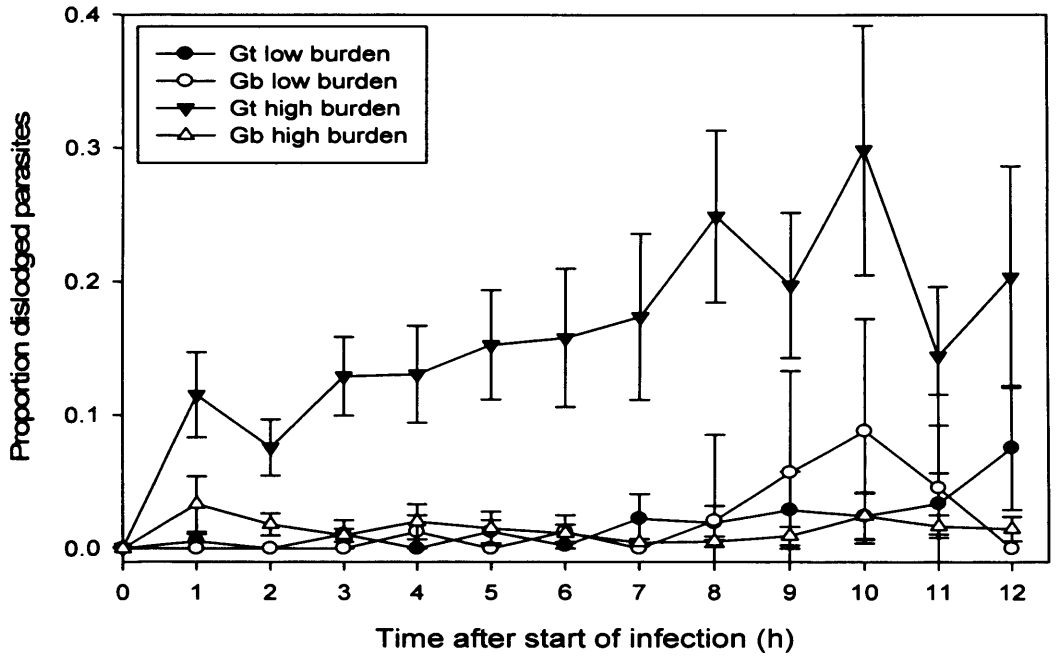
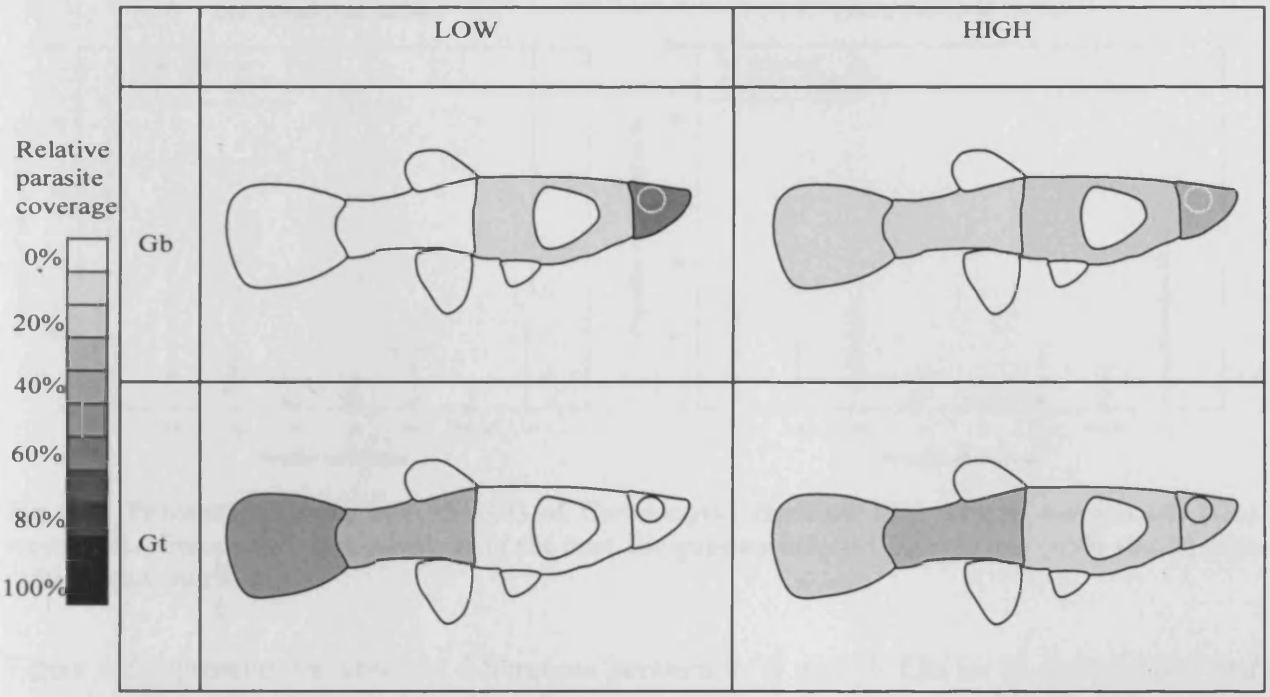


Fig. 4.2: Proportion (mean and SE) of *Gyrodactylus turnbulli* (Gt) and *G. bullatarudis* (Gb) worms that were dislodged from guppies infected with a low (<50) or high burden (>50 worms). The numbers of worms that were attached to the host and detached were counted every hour over a 12 h time period immediately following death of the host

Figures 4.4A and 4.4B show that *G. bullatarudis* has a markedly different distribution early (0-3h) and late (10-12h) in the infection. The distribution between time periods 0-3h and 10-12h differs by 44.8% for *G. bullatarudis* and only by 19.6% for *G. turnbulli* (Fisher Exact test: $p < 0.0001$).



0-12h		Mean	Mean
Position		**Gb Low**	**Gb High**
Tail	(T)	4.7	14.7
Lower Body	(LB)	9.7	12.8
Upper Body	(UB)	12.2	23.1
Anal fin	(A)	1.8	4.6
Dorsal fin	(D)	1.5	2.0
Pectoral fin	(Pect)	3.2	7.9
Pelvic fin	(Pelvic)	1.1	4.3
Head	(H)	66.2	30.7
		Gt Low	**Gt High**
Tail	(T)	53.3	27.8
Lower Body	(LB)	30.8	33.2
Upper Body	(UB)	4.7	14.6
Anal fin	(A)	2.0	1.9
Dorsal fin	(D)	1.6	1.0
Pectoral fin	(Pect)	0.4	2.6
Pelvic fin	(Pelvic)	0.1	0.3
Head	(H)	7.1	18.6

Fig. 4.3: Diagrammatical representation of the mean percentage of *Gyrodactylus bullatarudis* (Gb) and *G. turnbulli* (Gt) worms attached on different positions on the host at low (<50) and high (>50 worms) burdens from 0-12 h. The values in the table represent the mean and across individuals over the 12 h period

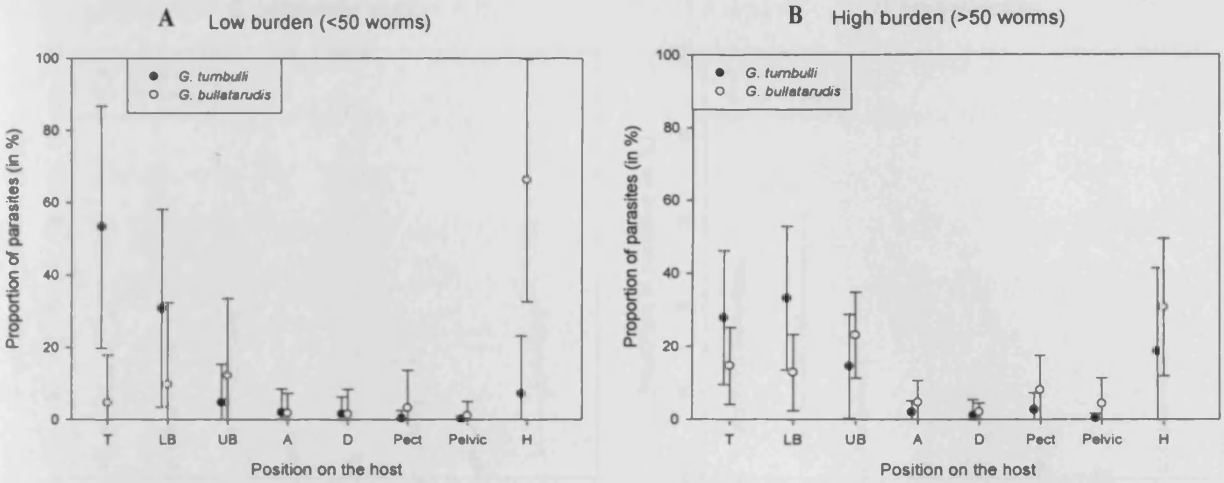
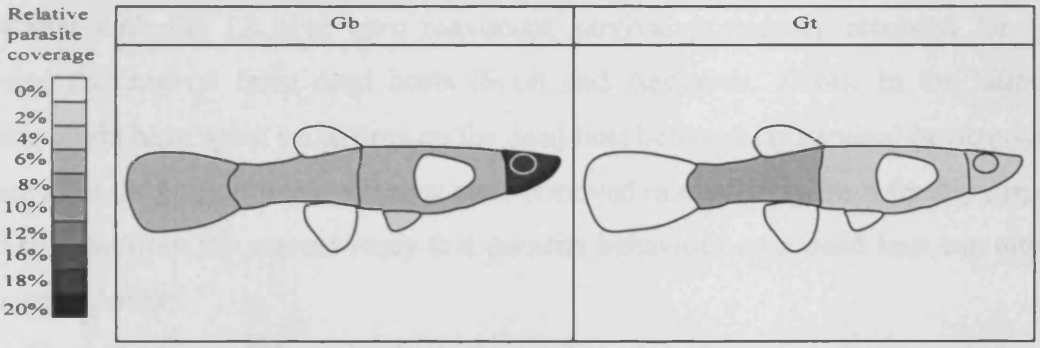


Fig. 4.4: Percentage (mean and 95%CI) of *Gyrodactylus turnbulli* (Gt) and *G. bullatarudis* (Gb) worms attached on different positions of the host, for guppies infected with A) low (<50) and B) high (>50 worms) burden

Figure 4.5 represents the absolute differences between 0-3h and 10-12h for *G. bullatarudis and G. turnbulli* worms at high (> 50 burdens), whilst Figure 4.6 shows motility of both parasite species.



Position	Gb absolute differences (between 0-3 and 10-12h)	Gt absolute differences (between 0-3 and 10-12h)
Tail (T)	6.4	0.2
Lower Body (LB)	6.5	8.4
Upper Body (UB)	7.5	4.0
Anal fin (A)	1.7	0.1
Dorsal fin (D)	0.2	1.2
Pectoral fin (Pect)	1.8	1.0
Pelvic fin (Pelv)	2.1	0.2
Head (H)	18.5	4.5

Fig. 4.5: Diagrammatical representation of the absolute differences in parasite distribution (see table underneath) between 0-3h and 10-12h for *Gyrodactylus bullatarudis* (Gb) and *G. turnbulli* (Gt) when infecting guppies at high (>50 worms) parasite burden. Absolute difference calculated as the difference between the mean percentage of worms between 0-3 and 10-12h

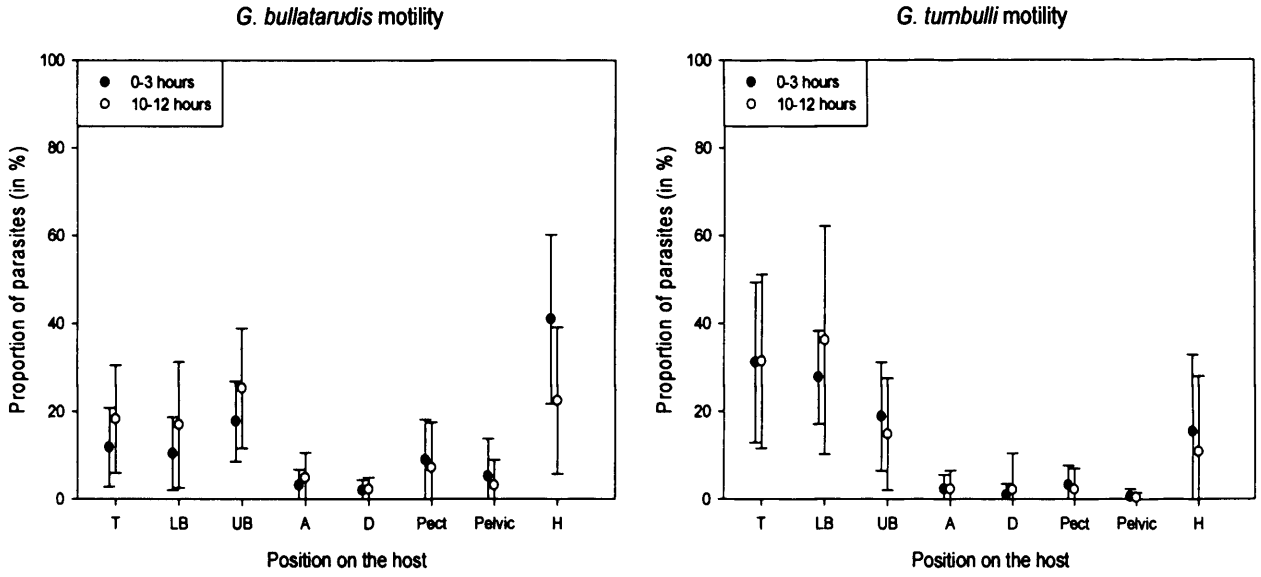


Fig. 4.6: Percentage (mean and 95%CI) of *Gyrodactylus turnbulli* and *G. bullatarudis* motility i.e. movement of the parasite on the dead host

4.5 DISCUSSION

The maximum *in vitro* survival times of the tropical gyrodactylids, *Gyrodactylus bullatarudis* and *G. turnbulli*, at 25°C were similar (26 and 31h, respectively). These values are probably not comparable with the 12 h *in vitro* maximum survival previously recorded for *G. turnbulli* following its removal from dead hosts (Scott and Anderson, 1984). In the latter study, the parasites might have spent some time on the dead host before their survival *in vitro* was assessed, whereas as in the current work parasites were removed immediately from freshly dispatched fish. We know also from the current study that parasite behaviour on a dead host can alter with time and parasite density.

G. turnbulli appears to leave its host when at high parasite burdens but not at lower burdens. By contrast, *G. bullatarudis* does not change its behaviour and stays with its hosts at both high and low burdens. The differences in distribution between the parasite species are most pronounced when the parasite load is low, with *G. bullatarudis* infecting the head and upper body, while *G. turnbulli* is most prevalent on the caudal fin and lower body, as previously recorded by Harris (1988; Harris and Lyles, 1992). At higher loads, the distributions become more similar with a more uniform coverage over the host. The differences in distribution between the parasites species are more pronounced for *G. bullatarudis* than for *G. turnbulli* with the distributions differing by 44.8% for *G. bullatarudis* and only by 19.6% for *G. turnbulli* between the start and the end of the 12 h observation period. We can thus conclude that both parasite species show a behavioural plastic response in reaction to high parasite burden, and that this

behaviour differs markedly between both species. Whereas *G. bullatarudis* appears to avoid the localised immune response by moving along the host's body at high densities, *G. turnbulli* shows less mobility on the host, but leaves the host by transferring to the water column and substrate. This finding shows that the transmission strategies of individual gyrodactylid species do not only reflect their host's ecology, as suggested by Bakke *et al.* (2007), but rather, have evolved to increase particular transmission routes of each parasite species. Given that the parasite's life span is considerably reduced when it is detached from its host (e.g. Cable *et al.*, 2002b), this remarkable difference in transmission strategy between parasite species needs to be further examined on live hosts. In the meantime, we propose and discuss three hypotheses that could explain why *G. turnbulli* parasites leave cadavers at high burdens.

(*i*) Differences in tolerance to crowding effects

The fact that the two gyrodactylid species only display different behaviours at high parasite loads suggests that they may have different tolerances to crowding. As yet, the mechanisms that control the host's immune response to *Gyrodactylus* infections are not fully understood (Buchmann *et al.*, 2003). However, such responses are thought to be predominantly non-specific (Lester, 1972; Scott, 1985). Furthermore, the guppy immune response is not species specific to *G. turnbulli* and *G. bullatarudis*, although Richards and Chubb (1996) suggested *G. bullatarudis* was more sensitive to the host's immune response than *G. turnbulli*. Both species have distinct site preferences (Harris and Lyles, 1992) with *G. bullatarudis* having a preference for the rostral region, particularly at high densities, the cornea - an immunologically naïve site. This preference may be an adaptation for this species in avoiding the host's localised immune response. In contrast, *G. turnbulli* occurs predominantly in the caudal region but at high burdens, may migrate anteriorly, possibly to escape the host response. However, by moving anteriorly, *G. turnbulli* may face increasing competition from *G. bullatarudis* for resources and risk of exposure to the host's localised immune response evoked by its potential competitor (Richards and Chubb, 1996). Therefore, in order to escape such crowding effects, *G. turnbulli* may risk leaving the host. However, there is no evidence for intra- and interspecific competition for resources among other monogeneans (Rohde, 1977, 1979; Morand *et al.*, 2002).

(*ii*) Different in genetic bet-hedging strategies

For environments which fluctuate temporally, it cannot be predicted which genotypes will be best adapted at any point in time. By increasing genetic diversity, organisms can prevent "putting all

their eggs in one basket" and so genetic bet-hedging has been proposed to explain why some females adopt polyandrous practices (Seger and Brockman, 1987; Yasui, 1998; Grafen, 1999; Fox and Rauter, 2003). Although bet-hedging may not have any immediate effects short-term (lower mean fitness, Grafen, 1999), such a strategy long-term will result in reduced variance in subsequent generations and therefore geometric mean fitness is increased (Flaxman, 2000; Fox and Rauter, 2003).

A genetic bet-hedging strategy may be employed during transmission of different *Gyrodactylus* species that have variable reproductive modes. Gyrodactylid reproduction is complex, involving a variety of different reproductive modes but much remains unknown (reviewed by Cable and Harris, 2002). What is certain is that the first-born daughter from this viviparous, sequential (progyny) hermaphrodite is always asexual and that the second-born daughter can arise parthenogenetically. The parasites have a short life span (approximately 4 days at 25°C for *G. turnbulli*, see Scott and Nokes, 1984) and can potentially persist as asexual clones (Harris, 1998). However, nothing is known yet about how reproductive strategies might vary across species. It has been speculated that virulent species, such as *G. salaris* and *G. bullatarudis* (see Harris, 1993) might employ sexual reproduction more frequently than less virulent species, such as *G. turnbulli* (see Cable and van Oosterhout, 2007b). Assuming *G. turnbulli* is more clonal than its congener, *G. bullatarudis*, then migration into the water column at high burdens may represent a bet-hedging strategy for this species. Transmission is risky, with Scott and Anderson (1984) estimating that for guppy gyrodactylids, approximately 40% of worms fail to transfer. By adopting a bet-hedging strategy whereby only a proportion of worms leave the host, the low probability of infecting a new host is counterbalanced by leaving some asexual clones on the original host and thus the risk of migration is negated by increased geometric mean fitness. In contrast, if *G. bullatarudis* undertakes predominantly sexual reproduction, then there would be no obvious benefit to the individual in risking migration away from the host.

(*iii*) Different efficiencies in transmission routes.

Transmission strategies may have evolved in response to predation pressure and the corresponding window of opportunity. In the guppy's native habitat, the Aripo River, Trinidad, field studies have indicated that in the lower part of this river where high guppy predation occurs, *G. bullatarudis* is the more common species, although *G. turnbulli* is more prevalent. Therefore, due to the high predation pressure, there will be a very narrow window of opportunity for *G. turnbulli* to transmit via direct contact and therefore it will maximise infection success by

migrating into the water column. In contrast, in the upper reaches of the Aripo River, predators are mostly absent and *G. bullatarudis* is much more common than *G. turnbulli*. In such an environment, it is more likely that cannibalisation of guppies will occur as dead fish will quickly attract the attention of conspecifics (Scott and Anderson, 1984). Cannibalisation of cadavers can occur very quickly in the wild. Indeed, Cable, Mohammed & van Oosterhout (unpublished) found that the time taken for individual dead hosts to be detected and cannibilised by guppies in a small tributary in Tobago (stagnant water to $<1 m/m^2$ flow rate with guppy density of $20\text{-}75/m^2$) ranged from 10 to 900s (mean 95.6s).

To conclude, we have postulated several hypotheses as to why these two gyrodactylids adopt different transmission strategies, but a combination of factors may be responsible. For example, lack of migration of *G. bullatarudis* may be the result of a trade-off. This species is a generalist (Chapter 3) and therefore might have to make a greater investment in counter-acting different host responses. It is also more virulent than *G. turnbulli* (see Cable and van Oosterhout, 2007b). Therefore, *G. bullatarudis* may trade-off infection ability against reduced investment in transmission. Certainly, during experimental infections, *G. bullatarudis* is much more difficult to dislodge from its host and it transfers more reluctantly to a new host than *G. turnbulli* (personal observations). Further studies are needed to clarify the factors involved for these different transmission strategies, such information could be gained by examining the transmission of these two gyrodactylids on live hosts and co-infection studies. If competition does occur between these two species then this may elucidate further why these species adopt such different transmission strategies.

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APPENDIX: IS TRANSMISSION OF TWO *GYRODACTYLUS* PARASITES EXPLAINED BY THEIR SPECIES-SPECIFIC TRANSMISSION STRATEGIES?

4.7 ABSTRACT

Gyrodactylus bullatarudis and *G. turnbulli* are conspecific parasites that co-exist on the same host, the guppy (*Poecilia reticulata*). Their capacity to co-exist, even though they are competing for the same resource, could be explained by their distinct site preferences on the host. The more benign species, *G. turnbulli* has a clear preference for the caudal region of the host's body, whereas the highly virulent *G. bullatarudis* remains in the rostral region. Furthermore, studies *in vitro* and *in vivo* on recently dead hosts have shown that these two species adopt different transmission strategies. *G. turnbulli* migrates away from a dead host, although this migration appears to be density dependent whilst *G. bullatarudis* remains with the corpse. In the current study, transmission of *G. bullatarudis* and *G. turnbulli* between mixed sex groups of live hosts was examined to determine: i) if there were differences in the time to first transmission between the two parasite species, and ii) if transmission was faster between male or female hosts. Contrary to expectation based on data from dead hosts (see Chapter 4), there was no significance difference in the time to first transmission between parasite species. Although the data suggests that females become infected faster than males, this was non significant.

Traditionally, it has been accepted that parasites should evolve to become less virulent, as by killing or evoking a faster immune response from their host, this would result in reducing their own fitness (Anderson and May, 1979). However, the view that parasites make a trade-off in their virulence has been challenged (as reviewed by Alizon and van Baalen, 2005) and this remains a contentious issue. Andrė *et al.* (2003) suggested that the trade-off in parasite virulence is modified by the host's immune strength, whereby hosts that respond strongly to infection result in parasites being more virulent, but having a shorter duration of infection.

The guppy, *Poecilia reticulata*, is infected by two congeneric species of *Gyrodactylus*; *G. bullatarudis* and *G. turnbulli*, with the former being more virulent (Cable and van Oosterhout, 2007b). Previously, Scott and Anderson (1984) suggested that direct contact between live hosts was the main mode of transmission for *G. turnbulli*. Both parasite species differ in their site preference, *G. bullatarudis* occurring mostly in the rostral region of the host, whilst *G. turnbulli* is found mainly in the caudal region (Harris and Lyles, 1992). Recent research (Chapters 4 and 5) has found that in addition, these two parasites also differ in their behaviour both *in vitro* and *in*

vivo on dead hosts, although their maximum survival time is similar. *G. turnbulli* migrates into the water film (Cable *et al.*, 2002b), although this behaviour appears to be density dependent (Chapter 4). In contrast, *G. bullatarudis* remains with its dead host but does not feed off the corpse unlike *G. salaris* (see Olstad *et al.*, 2006). It is therefore more probable that *G. bullatarudis* remains with a dead host in order to maximize its transmission success when the cadaver is cannibalised by live hosts, which are surface feeders, although this window of opportunity is extremely short.

Personal observations of the behaviour of both parasite species in culture indicate that transmission of *G. turnbulli* between live hosts occurs more rapidly than for *G. bullatarudis*. Given the distinct transmission behaviours of both species, it was hypothesized that between live hosts, transmission of *G. turnbulli* should be much faster than for *G. bullatarudis*, given the propensity of the latter to remain with a host until such time as that host dies or a very high burden is reached. Recently, it has been demonstrated that comparisons between single sex shoals in transmission of *G. turnbulli*, indicated that transmission occurred fastest between males than females (Richards-Hobbs *et al.*, personal communication 2008).

Experimental infections were carried out using either a male or female donor (n = 10 for each sex for each parasite species) which was left with a heavily infected guppy. When the donor was infected with approximately 20 gyrodactylids, the fish was then added to a 10l tank of dechlorinated water at the same time as one male and one female recipient (all fish having been familiarised beforehand in order to avoid antagonistic interactions). All fish were then screened under 0.02% MS222, 4h after introduction and every 24h thereafter until such time as one of the recipients became infected, at which point, the experiment was terminated. Statistical analyses were carried out using Fisher's Exact Tests to compare differences in the sex of the donor and the sex of the first recipient to become infected. Two-tailed, non-parametric Mann-Whitney tests were used to compare time to first transmission and final burden of the donor both within and between parasite species. To determine whether sex of the donor, sex of the recipient and the final burden of the donor were related for each parasite species, a binary logistic regression was used whereby sex of the donor was listed as either 0 for male and 1 for female, sex of the recipient was used as a factor and crossed with the final burden of the donor as a covariate. Minitab vs. 12 was used to carry out Mann-Whitney tests and binary logistic regression whilst Fisher's Exact Tests were carried out using the web-based programme http://www.physics.csbsju.edu/stats/exact.html.

Comparisons within and between parasite species showed that there was no evidence of a sex bias for a recipient host, neither was there a significant difference in the time for first transmission (Fisher's Exact Tests and Mann- Whitney tests, both $p>0.05$), although the data suggested that mean time to first transmission was slightly faster for females than males (see Fig. 1). However, for *G. turnbulli*, there was a significant difference between male and female donors in their final burden before transmission occurred (Mann-Whitney, $W = 77.5$, $p = 0.0404$) but was non significant for *G. bullatarudis* (Mann-Whitney, $p > 0.05$). There was also a significant difference between the final burden of *G. bullatarudis* and *G. turnbulli* infected male donors (Mann-Whitney, $W = 114.5$, $p = 0.0484$) but not between female donors. With regard to sex differences in the sex of the donor, sex of the recipient host and the final burden of the donor, this was significant for *G. turnbulli* ($Z = -2.00$, $p = 0.045$) but not for *G. bullatarudis*. However, it is noted that these p values are close to 0.05 and therefore should be interpreted with caution.

Although the current study did not find any statistical differences in time to first transmission between the two parasite species, *G. turnbulli* did tend to follow the predicted trend of transferring more rapidly (the majority occurring within 4 to 24h), whilst first transmission for the majority of *G. bullatarudis* individuals occurred after 48h. However, there was a difference in the final burden of the male donors between *G. bullatarudis* and *G. turnbulli*, in that *G. bullatarudis* achieved higher burdens before transmission occurred. However, before any definite conclusions can be inferred, it is suggested that the sample sizes be increased to 25 or 30 for each sex/parasite species in order to increase the statistical power. Furthermore, screening should be carried out at more frequent intervals (1, 2 and 4h) after introduction of the donor.

Richards-Hobbs *et al.* (personal communication, 2008) found in their study of transmission of *G. turnbulli* within single sex shoals, that transmission occurred fastest to males, due to the tendency of males for more frequent contact, and therefore increased transmission opportunities. In contrast, the current study using mixed sex groups suggests that transmission is marginally faster from female donors than male donors but further work is needed before this conclusion can be confirmed.

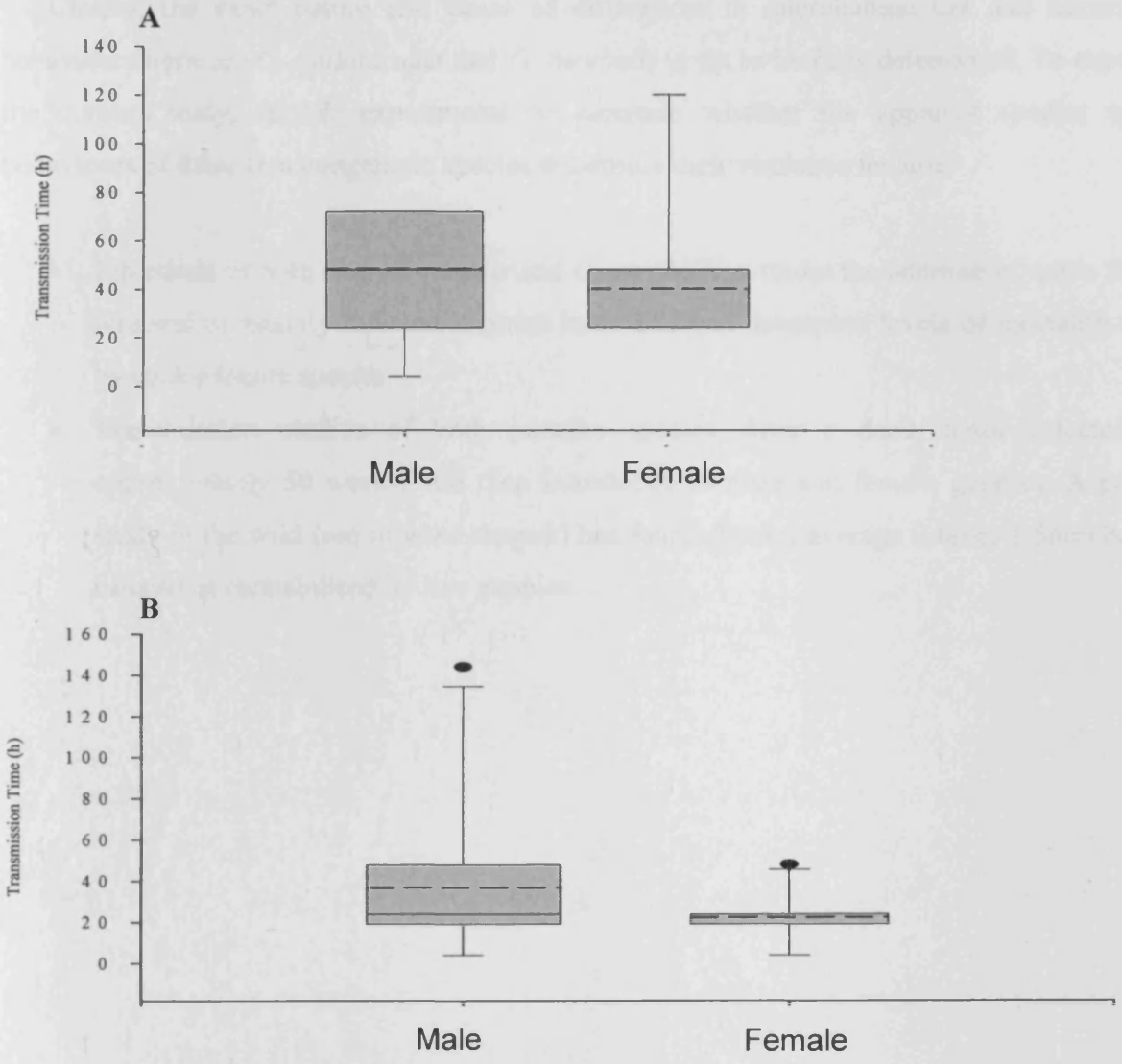

Fig. 4.7: Boxplots showing the time to first transmission for male and female guppies for *Gyrodactylus bullatarudis* (A) and *G. turnbulli* (B)

It is unknown why these two parasite species should exhibit such distinct differences in site preference and transmission behaviours, although there are a range of plausible explanations. Site preferences may develop between co-infecting gyrodactylid species to reduce competition, as a method to avoid the host response which could be induced by the congeneric gyrodactylid, and/ or as a method of reproductive isolation (Bakke *et al.*, 2007). However, Richards and Chubb (1996) demonstrated that the guppy's host immune response to gyrodactylids is not species specific and therefore it is unlikely that niche partitioning is a response to this factor and it is unlikely that it is also a response to prevent hybridisation (Bakke *et al.*, 2007).

Clearly, the exact nature and cause of differences in microhabitat use and transmission behaviours between *G. bullatarudis* and *G. turnbulli* is yet to be fully determined. To expand on the current study, further experiments to ascertain whether the apparent species specific behaviours of these two congeneric species determine their virulence include:

- Infections of both *G. bullatarudis* and *G. turnbulli*, without the addition of naïve fish and removal of heavily infected/immune individuals to determine levels of mortality caused by each parasite species
- Transmission studies of both parasite species from a dead donor infected with approximately 50 worms and then introduced to male and female guppies. A previous study in the wild (see *in vitro* chapter) has found that on average it takes 1.5min before a cadaver is cannabilised by live guppies

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CHAPTER 5: EFFECT OF TEMPERATURE ON *GYRODACTYLUS BULLATARUDIS IN VITRO* SURVIVAL AND BEHAVIOUR

5.1 ABSTRACT

Previous studies have shown that the two common guppy (*Poecilia reticulata*) parasites, *Gyrodactylus turnbulli* and *G. bullatarudis* can survive for approximately 1 day off their host at 25°C. However, they display different transmission strategies; *G. turnbulli* actively migrates away from the corpse into the water film, while *G. bullatarudis* remains with its host. The current study showed that this behaviour in *G. bullatarudis* can be influenced by temperature; fewer individuals remained attached to the substrate as temperature decreased. However, even at the highest temperature tested, *G. bullatarudis* remained more often on the substrate than *G. turnbulli.* Surprisingly, however, the reproductive rate also increases at higher temperatures. We discuss the conundrum why parasites leave their host at apparently optimal conditions for reproduction, focusing on the costs and benefits of active migration, and the possibility of accidental parasite dislodgement.

5.2 INTRODUCTION

There are several recognised abiotic factors that have an impact on population dynamics of the monogenean ectoparasites, *Gyrodactylus* spp. Of these, the most significant is water temperature (Bakke *et al.*, 2007), which affects all life history traits such as reproduction; mortality (Scott and Anderson, 1984; Jansen and Bakke, 1991; Dávidová *et al.*, 2005; Bakke *et al.*, 2007) and population growth rate (Dávidová *et al.* 2005). A number of studies have ascertained the effect of temperature on gyrodactylids, primarily on the most well known member of this specious group, *G. salaris* (see Jansen and Bakke, 1991) but also *G. gasterostei* (see Harris, 1982; Harris, 1998; Cable *et al.*, 2002a); *G. katherineri* (see Gelnar, 1987) and *G. gobiensis* (see Gelnar, 1991). From such studies, a common trend has appeared in that there appear to be optimum temperatures for different life history traits, for example, at higher temperatures, population growth rate and reproductive rate is increased. At lower temperatures survival is prolonged, attributed in part to a reduction in metabolic activity. Thus, there appears to be a trade-off for gyrodactylids in maximising their fitness against their survival, although this is tempered by the optimal temperature range of the host (Scott and Nokes, 1984).

With regards to *in vitro* survival of *Gyrodactylus* spp., studies are restricted to just four species as reviewed by Cable *et al.* (2002a), namely *Macrogyrodactylus polypteri* (see Khalil, 1964 as cited by Cable *et al.*, 2002a); *G. alexanderi* (see Lester and Adams, 1974), *G. turnbulli*

(see Scott and Anderson, 1984; Scott and Nokes, 1984) and *G. gasterostei* (see Cable *et al.*, 2002a). The most recent *in vitro* study by Olstad *et al.* (2006) on *G. salaris* corroborated the trend of prolonged survival at low temperatures by surviving for only one day at 18°C but for four days at 3°C.

The guppy (*Poecilia reticulata*) is infected by two congeneric species of *Gyrodactylus*; *G. turnbulli* and *G. bullatarudis*. There is a wealth of data on *G. turnbulli* (as reviewed by Bakke *et al.*, 2007) but relatively little is known about the more pathogenic species, *G. bullatarudis* apart from studies by Richards and Chubb (1996, 1998) and Cable and van Oosterhout (2007). Scott and Nokes (1984) studied the effect of temperature on *G. turnbulli* and found that optimal temperatures for reproductive rate, *per capita* birth rate and survival were different. For instance, at 27.5°C, mean maximum instantaneous *per capita* birth rate is optimal but a reduction in temperature of 6°C is the optimal temperature for survival (Scott and Nokes, 1984). Furthermore, Cable *et al.* (2002b) demonstrated that *G. turnbulli* has a unique transmission strategy of migrating into the water film. However, it is unknown how temperature affects gyrodactylid behaviour; one study by Anthony (1969) alluded to a change in distribution of *G. elegans* on its host, the goldfish, with temperature. At low temperatures, more gyrodactylids were found on the body compared to the gills whilst the reverse trend occurred at higher temperatures.

It has been recently demonstrated that *G. bullatarudis* on dead hosts, has a distinct transmission strategy compared to its congener, *G. turnbulli* (see Chapter 4). Whereas, *G. turnbulli* migrates into the water film, *G. bullatarudis* remains with a dead host. Furthermore, these two species have a similar *in vitro* maximum survival time at 25°C, at 31h and 26h, respectively. As *in vitro* on the effect of temperature have been carried out for *G. turnbulli* (see Scott and Nokes, 1984), the aim of the current study was to ascertain how *in vitro* behaviour and survival of *G. bullatarudis* are affected at 4, 15 and 25°C, respectively.

5.3 MATERIALS AND METHODS

Gyrodactylus bullatarudis was recovered from an ornamental stock of *Poecilia reticulata* obtained from an aquarium supplier in December 2006. The parasite culture was maintained by the regular addition of naïve guppies (see Chapters 3 and 4).

Individual worms of *G. bullatarudis* were removed from heavily infected euthanised guppies and transferred to the wells of microtitre plates containing 200 µl of dechlorinated water at 4, 15 and 25±0.5°C (N=60). Any worms which died in the first hour were excluded from

subsequent analysis, resulting in samples sizes of 59 (4°C), 60 (15°C) and 49 (25°C) for analysis. All worms were monitored hourly for the first 5 h and then every 2 h thereafter until all worms had died. The location of each parasite, whilst alive, was recorded as being either in the water film, attached to the side, attached or detached to the bottom of the well, or moribund. If, due to a blind spot caused by refractive light in the wells, an individual was not located in the well at any time point, it was subsequently recorded as not found. Any births were recorded, the mother (contracted uterus) and daughter (developing F1 embryo in uterus) being distinguished based on embryo development. Bartlett's Test and the Anderson-Darling test indicated heterogeneity of variance and non-normal distribution of the data; therefore non-parametric analyses were carried out using Minitab vs. 12.1. The number of hours that an individual worm spent either in the water film or all other locations was calculated to the last time point at which each individual worm was recorded alive and then compared within and between temperatures, using Kruskal-Wallis tests. Probit analysis was used to calculate the time at which 50% of individuals died (LT_{50}) *in vitro*.

5.4 RESULTS

5.4.1 Survival and birth rate

The survival curves representing the percentage of worms alive at each time point are shown in Figure 5.1. *In vitro* survival of *G. bullatarudis* differed significantly between temperatures (Kruskal-Wallis, $H = 60.17$, $df = 2$, $p < 0.001$), and was lowest at 25°C, intermediate at 4°C and longest at 15°C (Fig. 5.2). Probit analysis indicated that the LT_{50}'s were 27.2h (SE = 0.55); 35.0h (SE = 0.46) and 15.2h (SE = 0.37), for the 4, 15 and 25°C treatments, respectively. At the highest temperature, the greatest percentage of *in vitro* births/abortions occurred (33.3%), more than twice that at 15°C or 4°C (13.3% and 11.9%, respectively).

5.4.2 Parasite behaviour

Overall, significantly more *G. bullatarudis* individuals were observed attached to substrate or host than floating in the water column at all temperatures tested (Kruskal-Wallis, $H = 41.61$, $df = 1$, $p < 0.001$ for all comparisons). There was a significant difference between the number of individuals in the water column and those attached to the substrate at 4, 15 and 25°C (Kruskal-Wallis tests, $H = 90.30$, 85.58 and 41.61, $df = 1$, $p < 0.001$, respectively; Fig. 5.3). The relative proportion of worms present in the water column compared to worms attached to substrate or

host increased significantly with temperature (Kruskal-Wallis, $H = 32.81$, $df = 2$, $p < 0.001$; Fig. 5.4).

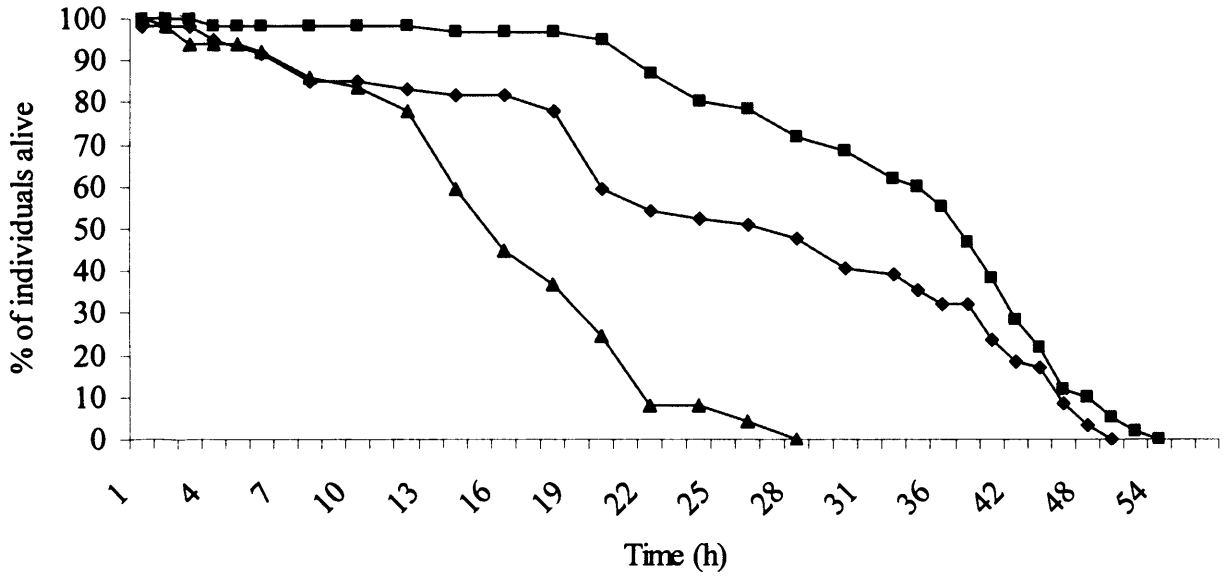

Fig. 5.1: Survival curves representing the percentage of individual *Gyrodactylus bullatarudis* worms alive at each temperature over time: 4°C (♦); 15°C (■) and 25°C (▲). Interpolation has been used to correct for time differences in screening

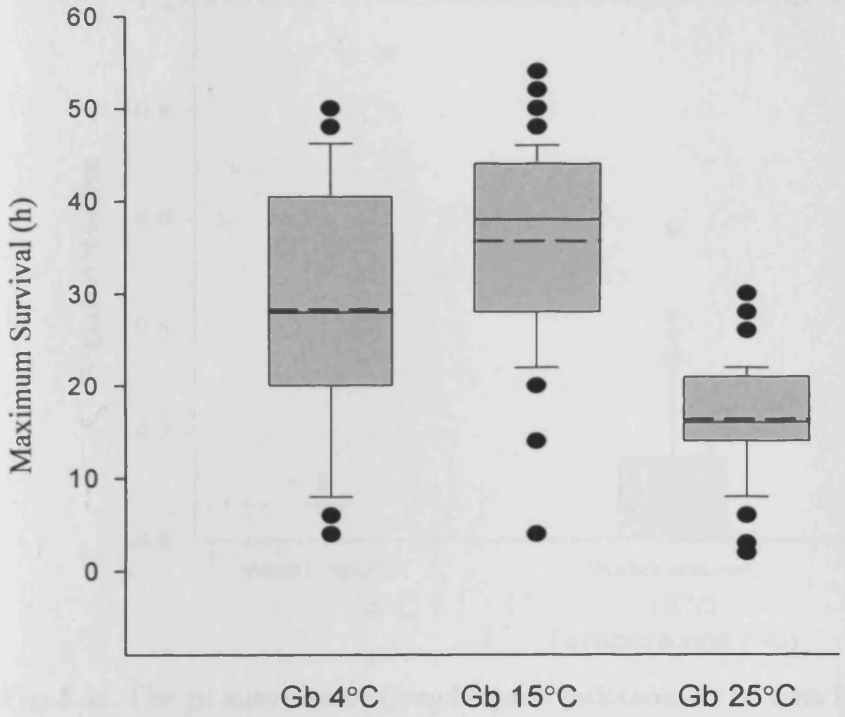

Fig. 5.2: Boxplots indicating the maximum survival time *in vitro* (h) of *Gyrodactylus bullatarudis* (Gb) individuals at 4, 15 and 25°C. Boxplots show the median (unbroken line), first and third quartiles with dots representing outlier values. The mean is indicated by the dotted line

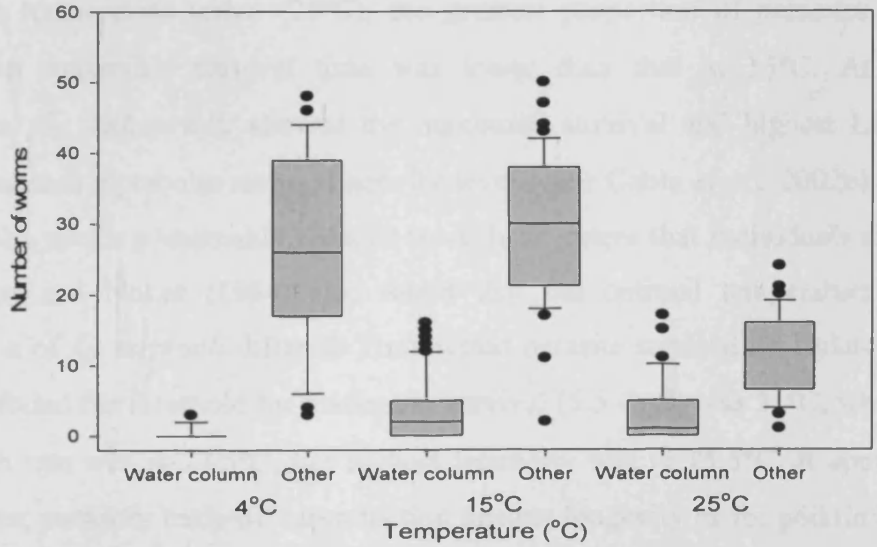

Fig. 5.3: Number of *Gyrodactylus bullatarudis* worms in the water column and attached to substrate (Other) at 4, 15 and 25°C. Boxplots show the median (unbroken line), first and third quartiles with dots representing the outlier values

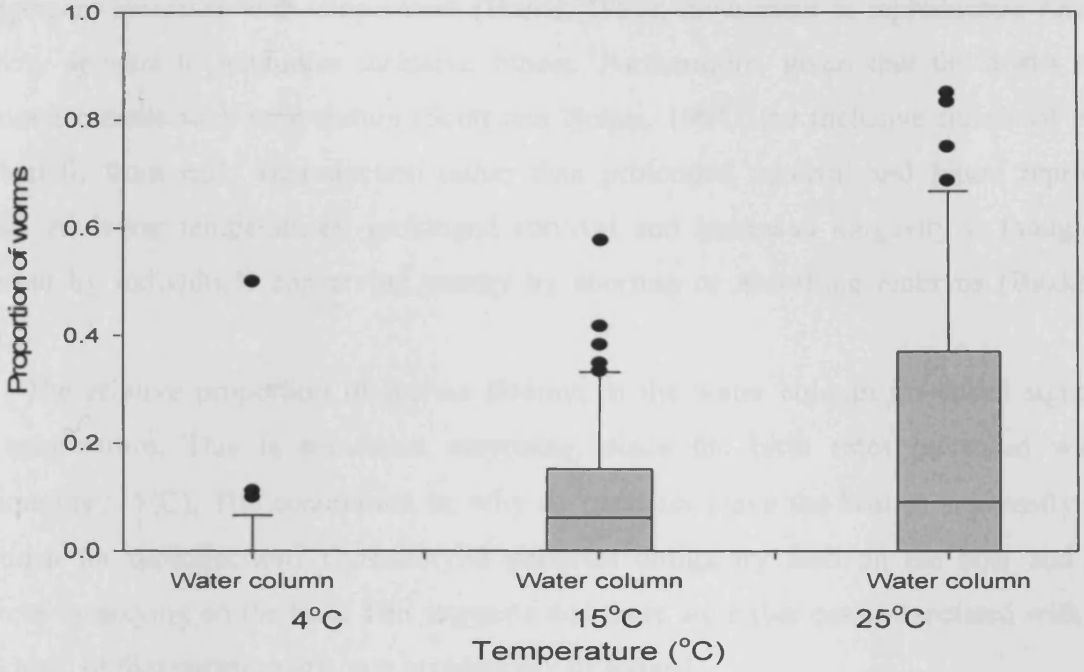

Fig. 5.4: The proportion of *Gyrodactylus bullatarudis* worms in the water column at 4, 15 and 25°C. Boxplots show the median (unbroken line), first and third quartiles with dots representing the outlier values

5.5 DISCUSSION

Survival time of *G. bullatarudis* was not inversely related to temperature, a finding discordant with studies on *G. gasterostei* (see Cable *et al.*, 2002a) and *G. salaris* (see Olstad *et al.*, 2006). At the highest temperature tested (25°C), the greatest proportion of parasites gave birth and/or aborted, but maximum survival time was lower than that at 15°C. At this intermediate temperature, *G. bullatarudis* showed the maximum survival and highest LT_{50}. This probably reflected reduced metabolic rate and activity levels (see Cable *et al.*, 2002b). However, at 4°C, the metabolic rate is presumably reduced to such an extent that individuals die. In their *in vivo* study, Scott and Nokes (1984) also found that the optimal temperature for survival and reproduction of *G. turnbulli* differed. They tested parasite survival on isolated guppies at 17 to 30°C, and found the threshold for maximum survival (5.5 days) was 21°C, whilst the highest *per capita* birth rate was at 27.5°C, but highest fecundity was at 25.5°C. It appears that at higher temperatures, parasites trade off reproduction against longevity as for poikilothermic organisms, the general trend is that reduced temperature results in increased longevity as demonstrated for organisms, such as the fish *Nothobranchius furzeri* (see Valenzano *et al.*, 2006) and the

nematode, *Caenorhabditis elegans* (see Van Voorhies, 2002). Indeed, given that accidental dislodgement increases with temperature (Harris, 1980), investment in reproduction rather than longevity appears to maximise inclusive fitness. Furthermore, given that the host's immune response increases with temperature (Scott and Nokes, 1984), the inclusive fitness of parasites may benefit from early reproduction rather than prolonged survival and future reproductive success. At lower temperatures, prolonged survival and increased longevity is thought to be facilitated by individuals conserving energy by aborting or absorbing embryos (Bakke *et al.*, 2007).

The relative proportion of worms floating in the water column increased significantly with temperature. This is somewhat surprising, since the birth rates increased with high temperatures (25°C). The conundrum is: why do parasites leave the host at apparently optimal conditions for reproduction? Gyrodactylid parasites obligatory feed on the host and acquire resources by staying on the host. This suggests that there are either costs associated with staying on the host, or that parasites are only accidentally dislodged.

Parasites may be accidentally dislodged particularly at higher temperatures as this increases the behavioural activity of hosts (Scott and Nokes, 1984). Evidence supporting increased costs comes from immunological studies showing that the host's immune response is enhanced with increasing temperature (see Scott and Nokes, 1984; Bakke *et al.*, 2007). Indeed, the present study shows that the parasites biological half-life reduces by more than 21% with a 10°C increase in temperature (from 37.1 to 29.2h at 15 and 25°C, respectively). Possibly, parasites make an actual decision to leave the host rather than accidentally being dislodged. This would suggest that the parasites trade off the increased damage to themselves by the host immune response against the risk of not finding a novel host once floating in the water column. Such active migration behaviour would be facilitated if the feeding rate of worms is significantly faster than ontogenetic development, and that worms can store food reserves which can be utilised ex situ to successfully complete gestation. This prediction can be simply tested empirically, comparing the birth rates of worms that were allowed to feed on the host for different time periods.

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CHAPTER 6: *GYRODACTYLUS ZEBRAE N. SP.* AND *GYRODACTYLUS DANIO N. SP.*, GYRODACTYLID MONOGENEANS FROM LABORATORY AND WILD CAUGHT ZEBRAFISH (*DANIO RERIO*)[1]

6.1 ABSTRACT

Gyrodactylus danio n. sp. and *G. zebrae* n. sp. are described from the skin and fins of the zebrafish (*Danio rerio*) a small freshwater fish native to south Asia and a common aquarium fish worldwide. Only a small number of *G. danio* specimens were recovered from wild (Bangladesh) fish, but for several months *G. zebrae,* isolated from a petshop stock of zebrafish imported into the UK, was maintained in culture allowing the course of infection to be followed for up to 18 days. Sequencing of the Internal Transcribed Spacers of *G. zebrae* generated an amplicon of 1194 bp. All analyses based on the 5.8S gene and ITS-2 consistently group *G. zebrae* within the *Paranephrotus-Neonephrotus-Limnonephrotus* group. Both species from zebrafish were morphologically similar to species of the *G. wageneri* group, and molecular analysis of *G. zebrae* links this species with such '*G. wageneri*' species as *G. kobayashii* and *G. gurleyi.* Discovery of these new parasite species offers the opportunity to assess gyrodactylid epidemiology on the fish equivalent of the 'laboratory rat'.

6.2 INTRODUCTION

Monogeneans of the genus *Gyrodactylus* von Nordmann, 1832 are almost ubiquitous ectoparasites of bony fishes (Harris *et al.*, 2004), and although only 400 + species have been described, the genus potentially contains several thousand species. An increasing number of gyrodactylids are known as significant pathogens of farmed and wild fish populations. The most important currently is *Gyrodactylus salaris*, a major pathogen of wild Atlantic salmon in Norway, elsewhere in Scandinavia and the Russian Republic (Bakke *et al.*, 2007), but pathogenic species have also been reported from wolffish (Mo and Lile, 1998), Atlantic Cod (Appleby, 1994), bronze bream (Turgut *et al.,* 1999) and cichlids (García-Vásquez *et al.,* 2007). The most recent reports of newly emerging potentially pathogenic gyrodactylids are those of *G. brachymystacis* infecting rainbow trout in China (You *et al.*, 2006) and an unidentified gyrodactylid infecting farmed sea bream in the Mediterranean (Paladini *et al.*, in preparation). Gyrodactylid infections are controlled by a sophisticated host immune response, and this response in part controls host

[1] The author contributed to data collection for this chapter by performing the experimental infections of *Gyrodactylus zebrae* n. sp. and conducting some of the microscopy. Other contributors to this chapter were: Dr Jo Cable (Cardiff University); Dr Phil Harris (University of Nottingham); Dr Carl Smith (University of Leicester) and Martina Ondračková (Czech Republic).

specificity and the potential for host shifts to occur (reviewed by Bakke *et al.*, 2007). Most is known about this system in commercially important salmonid hosts, such as rainbow trout (e.g. Buchmann and Bresciani, 1997) and Atlantic salmon (e.g. Cunningham, 1997; Collins *et al.*, 2000, 2004). However, these host species suffer from the drawback that their genomes are not fully characterised, relatively few genetic variants are available, they are large and require considerable resources to maintain in the laboratory, while long generation times make experimental studies of the inheritance of resistance and susceptibility difficult.

The ideal experimental host for experimental studies of gyrodactylid specificity and pathogenicity would be the zebrafish, *D. rerio*. This small fish is easy to breed and maintain in the laboratory. Most importantly, the genome of this cyprinid is available (www.ncbi.nlm.nih.gov/genome/guide/zebrafish/), thousands of genetic variants are known and knockout technology has been developed (Nasevicius and Ekker, 2000). Until recently the ecology of this fish had been neglected and perhaps it was not surprising that no gyrodactylids had previously been reported from this host. However, after extensive searches we obtained just 5 specimens from a wild population from Bangladesh and subsequently discovered an infection in a commercial shipment of *D. rerio* from South East Asia. Both gyrodactylids represented previously undescribed species, and the latter was maintained in the laboratory for a short period, providing the bulk of the data for this account.

6.3 MATERIALS AND METHODS

6.3.1 Host origin and maintenance

In January 2005, wild caught *Danio rerio* were collected in Bangladesh and screened for parasites. In 2006, *D. rerio* were obtained from two commercial aquarium fish suppliers (based in Somerset and Cardiff) having originally been imported from South East Asia. On arrival to the laboratory, fish obtained from Somerset were treated with Binox® for an unknown bacterial infection and shortly afterwards, all fish were screened for the presence of parasites under 0.02% MS222 (tricaine methane sulphonate) using a stereo-microscope with fibre optic illumination. *D. rerio* obtained from Somerset (but not those from Cardiff) were infected with *Gyrodactylus* n. sp. and were thereafter isolated in 11 jars of dechlorinated water. All fish were maintained at 25±0.5°C, with a photoperiod of 12L:12D and fed daily with Aquarian® flakes.

6.3.2 *Parasite origins and experimental infections*

Heavily infected *D. rerio* were euthanised and fixed in 95% ethanol for morphological and molecular analysis of their associated gyrodactylids (see below). For experimental infections, three replicates of four naïve *D. rerio* (petshop fish that had previously been maintained in the laboratory for one month) were maintained in 10l aquaria and an infected donor added to each replicate (n = 12). The fish were then screened every 24h and any recipient found to be infected was removed and isolated in a 1l jar of dechlorinated water and replaced with a naïve fish. If recipient fish had acquired more than 2 worms, the excess were removed and killed using a pair of watchmaker's forceps. These infected fish with either a single (n=17) or two worms (n=15) on Day 1 were monitored every 48h with the number and location of worms monitored until they had screened clear three consecutive times.

6.3.3 *Statistical analysis*

The Gyro-Scope software (van Oosterhout *et al.*, 2008) predicts no difference in outcome of infection of hosts infected with either one or two parasites, but this was tested empirically in the current study by performing a Fisher's Exact test on establishment success of *G. zebrae* n. sp. on *D. rerio* (i.e. whether the parasite was still present at the next screening (Day 2). In addition, Day of maximum parasite load, maximum parasite load, duration of infection and maximum R (*i.e.* parasite reproductive growth rate) were compared between the two groups using the non-parametric Mann-Whitney test. To determine whether *G. zebrae* had a site preference, the percentage of worms that were on the body or fins on the day of an individual's maximum parasite load when initially infected with either one or two parasites was compared using Mann-Whitney tests in Minitab vs.13. Fisher's Exact Tests were performed using a web-based programme available at http://bardeen.physics.csbsju.edu/stats/exact.html.

6.3.4 *Morphological analysis*

Parasites preserved in 95% ethanol were collected from the surface of the fish using insect pins and rehydrated briefly in distilled water. They were then placed on a slide in a drop of 1% Sodium Dodecyl Sulphate (SDS) in distilled water, and a cover slip mounted on the preparation. The specimen was monitored microscopically until the marginal hooks and hamulus roots had straightened, and then excess SDS was withdrawn from under the cover slip using filter paper. As the SDS was removed, ammonium picrate glycerine (Malmberg, 1970) was added at the other side of the cover slip and allowed to infiltrate the specimen. Measurements and photographs were

prepared using a BX61 Olympus microscope with Differential Interference Contrast. The measurement system of Shinn *et al.* (2004; Garcia-Vasquez *et al.*, 2007) was used for this work. Until holotypes are identified, measurements are given as the mean with the range in parentheses.

6.3.5 Molecular analysis

DNA was extracted as described by Harris *et al.* (1999) from four individual worms collected from the petshop strain of *Danio rerio*. Polymerase Chain Reaction (PCR) amplification of ribosomal DNA was undertaken using gyrodactylid specific primers (P3b, R1, F3 P4) spanning the internal Transcribed Spacers (ITS) and the 5.8S RNA (Cable *et al.*, 1999). Each 10 µl PCR product was cleaned by the addition of 1 µl 1:1 ratio of Exonuclease I (10 units/µl) and Shrimp Alkaline Phosphatase (1 unit/µl) (Hanke and Wink, 1994), and incubated at 37°C for 1 h followed by 80°C for 15 min. Sequencing PCR was performed using ABI Big Dye Terminator vs. 1 (Applied Biosystems) following the manufacturer's guidelines. Each PCR product was sequenced in both directions. PCR products were precipitated using isopropanol. Sequencing was performed in an ABI3100 automated sequencer.

6.3.6 Phylogenetic analyses

The 5.8S rDNA gene as defined by Ziętara *et al.* (2002) was aligned with sequences from this gene from 43 species of *Gyrodactylus*, and the genus *Gyrodactyloides*, taken from GenBank using the ClustalX editor in Mega vs. 3.1 (Kumar *et al.*, 2004), with final editing by hand. The 156 bp alignment was analysed using Minimum Evolution and Maximum Parsimony algorithms within Mega, each based upon 1000 bootstrapped replicates. This analysis placed *G. zebrae* within the *Limnonephrotus/Paranephrotus* part of the genus, and grouped it particularly with *G. wageneri* species (see below). Accordingly, a second alignment was produced, consisting of 445 bp of ITS-2 from *G. zebrae* and 84 other species of the *G. wageneri* group, taken from GenBank. This alignment was cropped to 366 bases by excluding all regions of ambiguity and indels of greater than a single base. This alignment was again analysed using Minimum Evolution and Maximum Parsimony algorithms within Mega, but was also subject to Bayesian posterior probability methods using MrBayes (Huelsenbeck and Ronquist, 2001). Bayesian analysis was undertaken for 2 x 10^6 iterations using the GTR model of DNA substitution, gamma shaped rate variation with a proportion of invariant sites and default prior probabilities. Trees were analysed and visualised in Mega (ME and MP models) or in Treeview (Page, 1996).

6.4 RESULTS

Gyrodactylus danio n. sp. (5 specimens measured)

Type Host: *Danio rerio* (Hamilton, 1822)

Site of Infection: Fins and skin surface

Type locality: Bangladesh. Remote pond in a village called Sutiakhali, about 10 km outside Mymensingh. GPS: Lat 24.41.516, Long 90.26.591. Collected Jan 2005.

Etymology: Named after the genus of the host, *Danio*.

Type Material: to be deposited in the Natural History Museum, London.

Description: Total hamulus length 46.3 (46-48) μm, shafts 36 (35.4-36.3) μm, diverging outwards from hamulus roots, which are 15.6 (14.7-16.2) μm long, at angle of approximately 20°. Hamulus points arise at an internal angle of 48° from the hamulus shafts, giving an aperture between the hamulus point and the shaft of ca. 18.4 μm. The hamulus points are 19.5 μm (18.9-20.1) long. Dorsal bar without notch or other ornamentation, straight or slightly curved. Ventral bar membrane shield like, with a blunt free end. Ventral bar ca. 19.2 μm long, width at lateral edge of the bar ca. 7 μm, ventral bar processes small, strongly triangular in outline. Marginal hooks 21.0 (19.8-22.3) μm total length, shafts 16.0 (15.5-16.6) μm in length, sickles 6.2 (6.1 - 6.4) μm in length (see Fig. 6.1).

Comments

G. danio has very similar overall dimensions to *G. zebrae*, but can be distinguished by a number of characters. The most distinctive difference lies in the relative proportion of the marginal hooks, and in the shape of the marginal hook sickles. The sickles of *G. danio* are almost half as long again as those of *G. zebrae*, and have a very elongate shape, with a narrow toe. Correspondingly, the marginal hook shafts of *G. danio* are somewhat shorter than those of *G. zebrae*. The hamuli of *G. zebrae* and *G. danio* are of a fundamentally different shape. In *G. zebrae*, the hamulus shafts and roots are straight, and form a straight line from the tip of the root to the point where the hamulus shaft starts to curve into the point. In *G. danio,* on the other hand, the shaft of the hamulus curves smoothly outwards almost from the point where the shaft emerges from the root. The hamulus shaft diverges from the hamulus root at an angle of almost 20°. The point of the hamulus of *G. danio* also diverges from the hamulus shaft at a much greater angle (the aperture distance) than in *G. zebrae*.

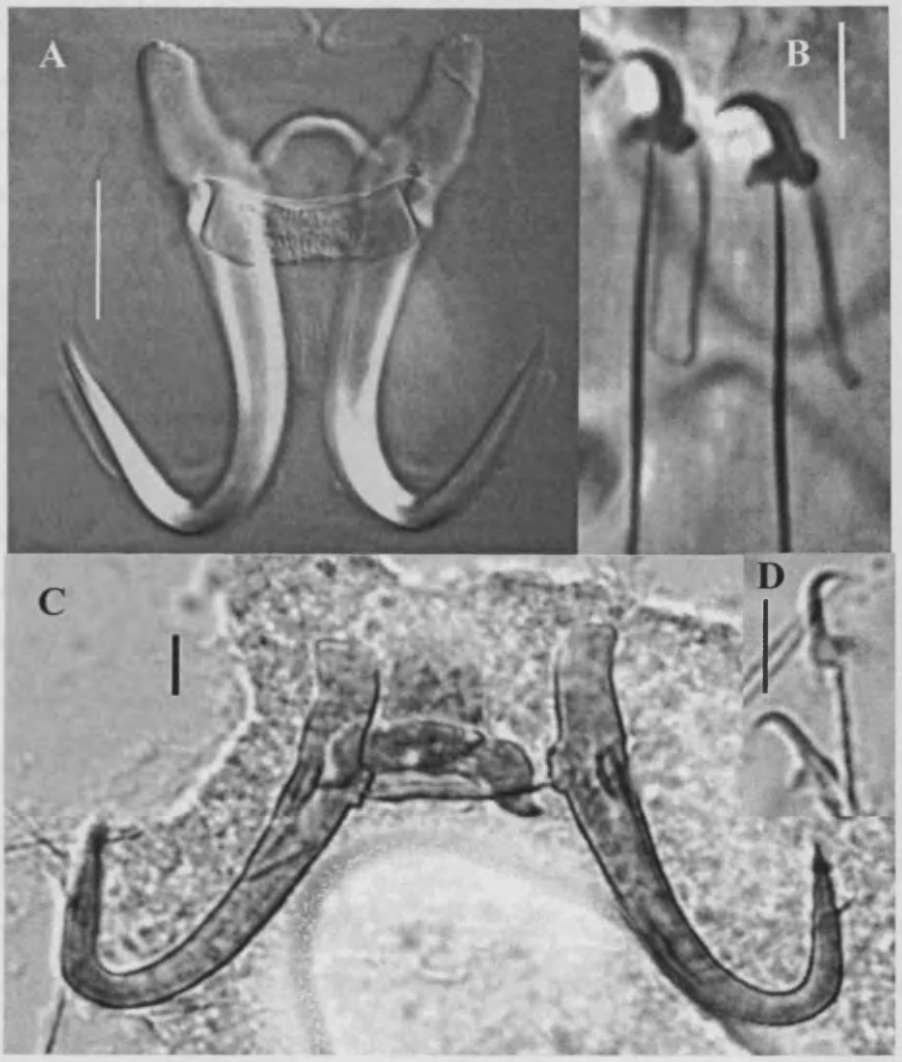

Fig. 6.1: A) *Gyrodactylus zebrae* n. sp. hamuli and bars, scale bar = 12µm, B) Marginal hooks, scale bar = 6µm, C) *G. danio* n. sp. hamuli and bars, scale bar = 6µm and D) Marginal hooks, scale bar = 6µm

Three live zebrafish infected with this gyrodactylid species were imported to the UK. The infected fish were housed with five domestic (spotted) zebrafish in an isolated aquarium but the parasites went extinct within a few days of collection. No specimens of *G. danio* were available for sequencing during this study.

Gyrodactylus zebrae n. sp. (10 specimens measured)

Type Host: *Danio rerio* (Hamilton, 1822)

Site of Infection: Fins and skin surface

Type locality: Not precisely known. Specimens collected from fishes imported to the UK through the aquarium trade, sourced to 'South East Asia'. Laboratory culture maintained from December 2006 to February 2007.

Etymology: Named after the common name of the host, zebrafish.

Type Material: Holotype and paratypes: to be deposited in the Natural History Museum, London.

Description: Total length of hamulus 48.4 (47.0-51.3) μm. Hamulus shafts lie in a straight line with the roots, with no angle between them. Hamulus roots 16.3 (14.4-17.7) μm. Hamulus points arise at an internal angle of ca. 40° (38 ± 3.5 °) from the hamulus shafts. The points of the hamuli are 23.7 (21.3-25.3) μm, and give the hamuli an aperture distance of 15.5 (14.4-16.5) μm. Dorsal bar strongly curved, without a notch, lugs or other ornamentation. Ventral bar 23μm (19.3-22.) width, 7.7 (7- 8.8) μm lateral length, with small triangular processes, 2.4 μm process length. Ventral bar membrane trapezoid tending to triangular, 10.3 (9.3 -11.8) μm long. Ventral bar weakly sculpted, but membrane lacks striae. Marginal hooks 24.0 (23.5 - 24.8) μm total length, shafts 20.0 (19.1 - 22.5) μm in length, sickles 4.3 (4.0 - 4.6) μm in length (see Fig. 6.1).

Molecular Diagnosis: A fragment of 1194 bp was amplified, spanning the Internal Transcribed Spacer 1 (ITS-1), 5.8S rDNA gene and ITS-2 region between the 18S and 28S ribosomal genes (this will be submitted to GenBank; Fig. 6.2). The 5.8S gene sequence (156 bp in length) shows greatest homology with the 5.8S gene of *Gyrodactylus* species of the *Paranephrotus-Limnonephrotus* group (see Fig. 6.3) first identified by Ziętara *et al.* (2002). The sequence is 100% homologous with that of *G. kobayashii*, a *G. wageneri*-type gyrodactylid infecting goldfish. It differs from that of *G. fossilis* only at two unique insertions (a G at position 26 and an A at position 68 of the 5.8S gene), which may represent sequencing errors in the latter species. All analyses based on the 5.8S gene unequivocally cluster *G. zebrae* within the *Paranephrotus-Limnonephrotus* cluster of the genus. The 5.8S sequence of *G. zebrae* and *G. kobayashii* differs from that of the *G. wageneri* group *sensu stricto* (represented by *G. gasterostei*, *G. salaris* and *G. leucisci*) at a single base position (base position 34 G→A substitution) and with low bootstrap support, this species is associated with the *G. wageneri* group of species. Further analysis using the longer (366 bp after exclusion of ambiguities and indels of longer than 1 base) and more

variable ITS-2 sequence with a subset of *Gyrodactylus* species drawn from the *G. wageneri* group (see Malmberg, 1970; Matějusová *et al.*, 2003; Ziętara & Lumme, 2003, 2004) confirms the placement of this new species. Both Neighbour Joining and Minimum Evolution trees based on ITS-2 are poorly resolved, with low bootstrap support, but are consistent in grouping a series of species from North West European freshwater fish (Fig. 6.4) as a single clade. All other *G. wageneri*-type species, including the new species and several species from eastern Asia, are grouped loosely around this single clade (Fig. 6.4), with bootstrap support too low to draw conclusions about relationship. The most distant of these *G. wageneri*-like forms are *G. rhodei* and a *G. rhodei*-like form, from bitterlings (Ziętara & Lumme, 2004).

ATTAACATAGTTTCCACTTTGTGTGTGTTTCAGTTGTGATTGTTGAGCTCAAGAGCTCTGAATTGAATATTATATATA
ATGAATTAAGTAGCAATTAAAGTTATGGTTATAAATGATTAAATAGTGGTGCATGAAATTAGAAGGTATCATTTCACG
CGACACATATGATACCTTCTACGTCTGGCTGACTTCTGTAGCCAGACTTTATTGAAAATTATTACGATCAAGATACAT
GTAATACCGAACGAGATACCATCGGCTATGGCCAGGAACAATCACACTGATTCATTCGCATTCAATCTGCCCTAAAAT
ATAAGGGTGAACTAAGAAGTAACCCTTCGCTGTCGTTTAGATGGTTGAATTTTTTAATACTCATTGGAGTGAACTGGT
AATCTTCCGTGCTAAAATGGTAATGGCTAGCATCGGTAAGGTCTGATTATCGGTTCGGCTACGGCCAGCTCAATGTAG
TATCCGCTATTACCAAAACATTATCCACAGTGGTTCGTTAGAGTTCCACACTCACTGCCTTGGTCCCTTCGGGTGTAC
TGATCGTAGTGCTTAGCGCCCCGTAAAAAGGGAAGAAGCTTCGCTTAATTACAACTCCATGTGGTGGATCACTCGGCT
CACGTAACGATGAAGAGTGCAGCAAACTGTGTTAACCAATGTGAAACGCAAACTGCTTCGATCATCGGTCTCTCGAAC
GCAAATGGCGGCTAAGGGCTTGCTCTTAGCCACGTTCGATCGAGTGTCGGCTTTTACCTATCGTAACGTTTAATTAGT
TACGGATTGGGAAGTTTACCATGGCTATGCGATTAACTTGTTGTTGAAAATTGGGACACTAGGTATTACACGGACTTG
ACGGTTTGCCTGGTGGTGTTCGGAATTTGGTATTACACGGTCTTTACGGTTTGCCTTATGATGTTCACATCCCGATTA
GTAAGCAGCTTCAGAGTACTACACGGACTTGACGGTTTGCTCTGAAGTAAAGACCTTTTCATCCTACACGACTTTTAC
GGTTTGATGAAAAGTGAATTAGCTCTAGTGGTTCTTCCTTAATTACTTGGGTAGTATTGTTATGTACTTAATGGTCTG
CTCTGCACAGGGTGCGTGGCTTAGTTCGCTTTGTAACGCTGTACTGATGTAGTGAGATTTGTATGTAATATACCCAGT
GAAATTTAGTCCTGACCTCGATCG

Fig. 6.2: *Gyrodactylus zebrae* n. sp. rDNA sequence to be submitted to GenBank. Partial Internal Transcribed spacer 1(1-597b), 5.8S gene (598-753 bp; highlighted), Internal Transcribed Spacer 2 (754-1180 bp) and partial 28S gene (1181-1194 bp; highlighted)

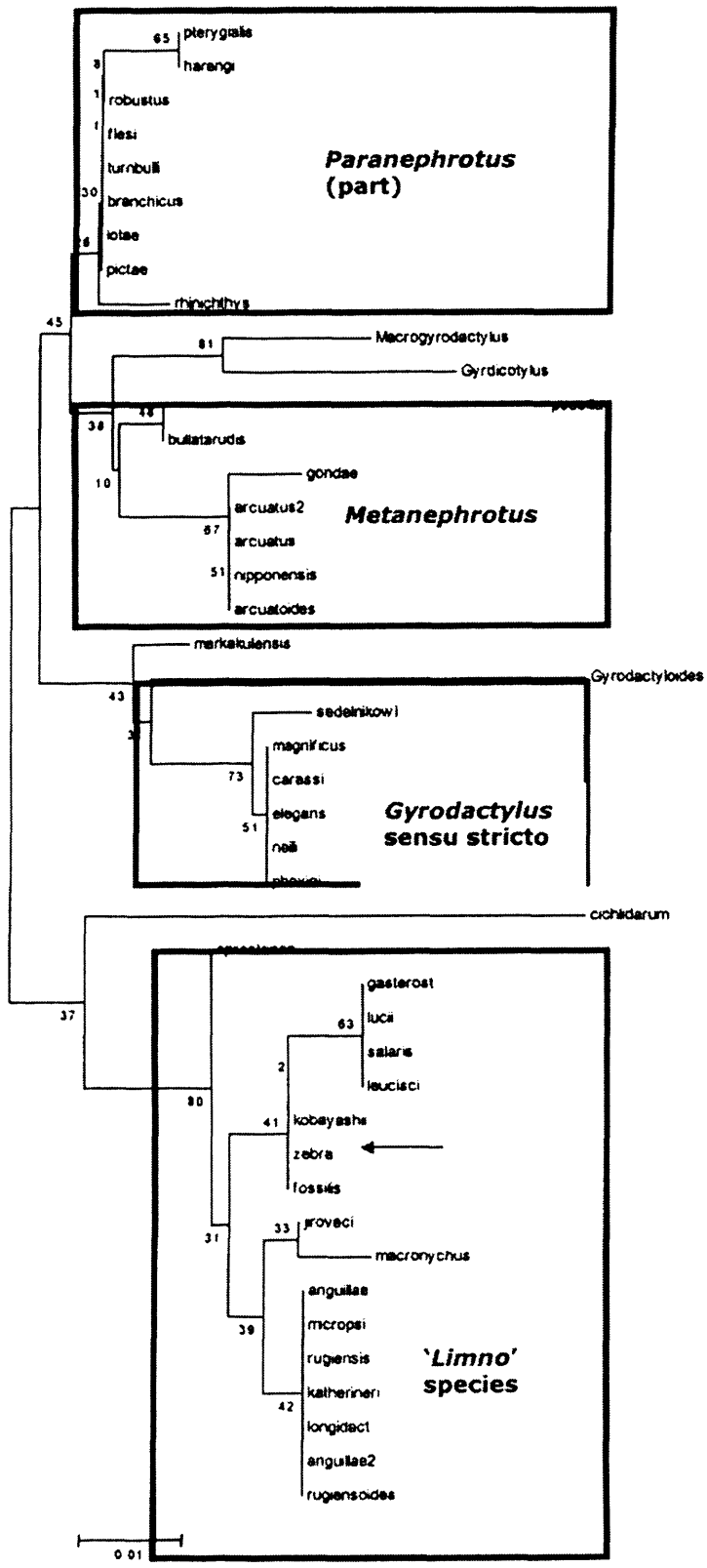

Fig. 6.3: Minimum evolution model for phylogeny of *Gyrodactylus* based on 5.8S rDNA sequences. *Gyrodactylus zebrae* clusters within the 'Paranephrotus - Limnonephrotus' grouping

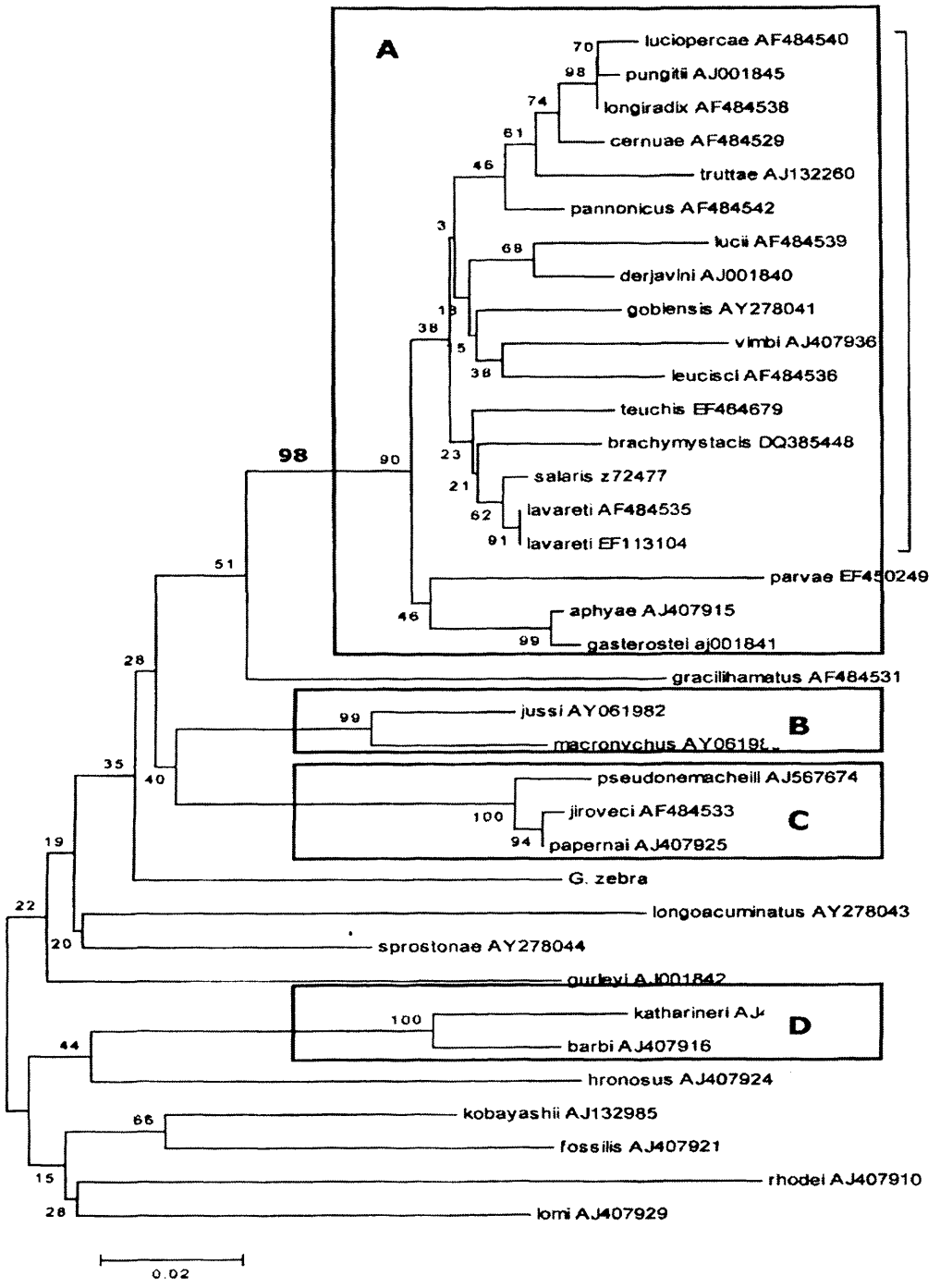

Fig 6.4: Cladogram of ITS-2 of species with "*Gyrodactylus wageneri*" type morphology and/or ITS-2 sequences, analysed using Minimum Evolution (ME) model in Mega 3.1. The block of 18 species, many found on non-cyprinid hosts (Block A), is supported with high bootstrap values and is also supported by Bayesian analysis. Three groups of species with distinct morphology cluster within the wider "*G. wageneri*" grouping. These are: Block B, the *G. katharineri* grouping, including *G. katharineri*, *G. barbi* and *G. hronosus*; C, *G. macronychus*-like forms from minnows (*Phoxinus*) and; D, the *G. pavlovskyi*-like forms from loaches. These three groups are supported with high confidence in ME, MP and Bayesian analyses. The remaining species, all with typical "*G. wageneri*" morphology, cluster basally with poor support. These species, including *G. zebrae* n. sp. from *D. rerio*, are, with the exception of *G. lomi*, all parasites of cyprinids from southern and Eastern Asia

Experimental infection: There were no significant differences for the variables tested in terms of infection trajectories ($p>0.05$) between those fish initially infected with either one or two parasites. Therefore, the data sets were pooled giving a mean maximum parasite load of 3.8 parasites per fish and a mean duration of infection of 5.1 d. The maximum parasite load attained for an individual fish was 10 parasites on Day 4, whilst maximum duration of infection for an individual fish was 18 days (Fig. 6.5).

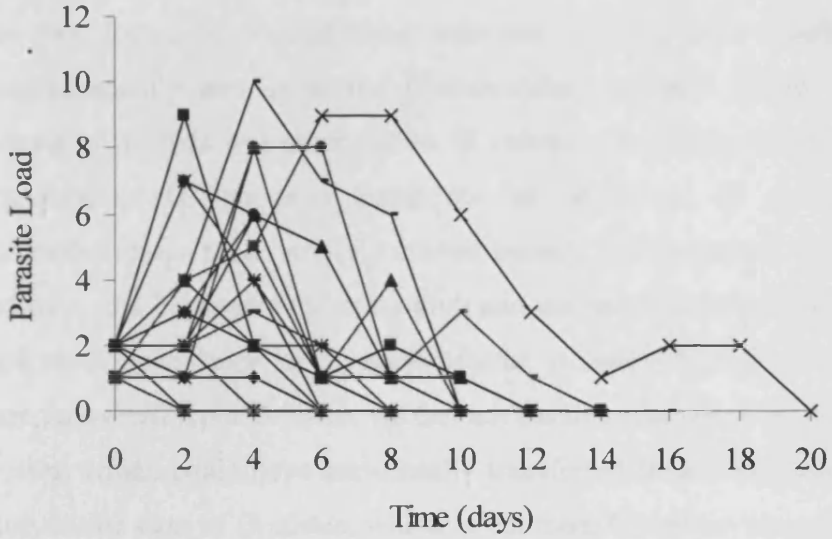

Fig. 6.5: Individual trajectories of *Gyrodactylus zebrae* n. sp. over time (initial infections with either one or two gyrodactylids, n = 32)

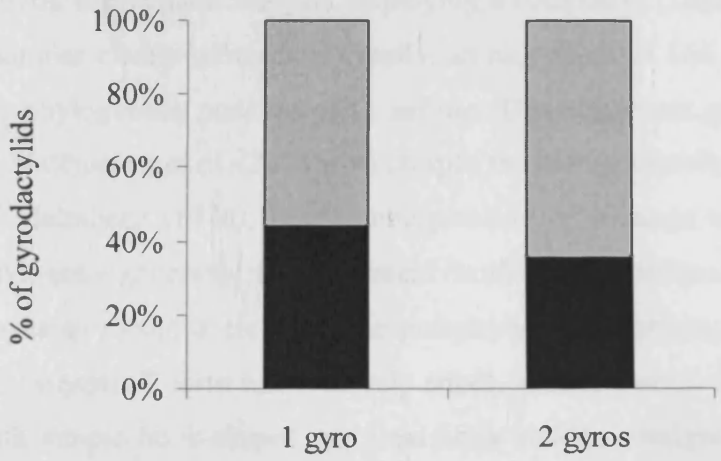

Fig. 6.6: Site preference of *Gyrodactylus zebrae* n. sp. on host (black = body, grey = fins) on day of maximum parasite load for fish initially infected with either one or two gyrodactylids

On the day of maximum parasite load, *G. zebrae* did not have a site preference between the body and fins (Mann-Whitney, $p > 0.05$) for those fish initially infected with one parasite. However, for those fish that were initially infected with two parasites, *G. zebrae* significantly more parasites were found on the fins (64.4%) compared to the body (35.7%) (Mann-Whitney, $W = 106.0$, $p = 0.0115$, see Fig. 6.6).

6.5 DISCUSSION

The two forms of *Gyrodactylus* collected in the present work from *Danio rerio* both are morphologically similar to the *Gyrodactylus wageneri* group of species, well known from Eurasian cyprinids and other fishes. *G. zebrae*, from farmed fish in Singapore, is typical of the skin-parasitic *G. wageneri* group species. However, *G. danio* from wild *Danio rerio* in Bangladesh have more strongly curved hamuli, with a greater angle between the hamulus shaft and root, and between shaft and point, and are reminiscent of gill-parasitic *G. wageneri* species, such as *G. sprostonae* or *G. markewitschi*. It should be pointed out that although both species were recovered from *D. rerio*, we can not assume that this is the typical or optimal host for these species, which could have accidentally transferred from other fish species. This is perhaps most likely in the case of *G. danio*, whilst in contrast, *G. zebrae* was maintained on a laboratory stock of *D. rerio* for over three months.

Molecular analyses using the 5.8S rDNA locus group *G. zebrae* clearly within the cluster of species identified by Ziętara *et al.* (2002) as comprising the subgenera *Limnonephrotus* and *Neonephrotus*, and part of the subgenus *Paranephrotus* as originally defined by Malmberg (1970). Subsequent analysis employing a composite ITS-1/5.8S rDNA/ITS-2 fragment identified a similar cluster of species. Finally, an alignment of 366 bp of ITS-2 was used to further refine the phylogenetic position of *G. zebrae*. This alignment generated phylogenies resembling those of Matějusová *et al.* (2003), which split the conventionally recognised *G. wageneri* species group of Malmberg (1970), by the interpolation of a range of morphologically distinct types. The phylogeny generated in the present work using minimum evolution, maximum parsimony and Bayesian methods confirms the paraphyly of morphological "*G. wageneri*" forms. The typical "*G. wageneri*" form has relatively small, gracile hamuli (typically less than 80 μm total length), with simple hook-shaped marginal hook sickles, straight hamulus roots in line with the shafts, and with small but distinct ventral bar processes. The new species *G. zebrae* is typical of the morphology of this type of gyrodactylid. Following Malmberg (1970) this has been seen as a natural group within the genus, clustering within Malmberg's (1970) subgenus *Limnonephrotus*.

Other morphological types which belong to *Limnonephrotus* but which are morphologically distinct are the *G. katharineri* group, with large (100 μm) hamuli and long, anterior-facing ventral bar processes, and the *G. pavlovskyi* types (Harris, 1985) from loaches, which have hamulus roots turned in to the midline of the haptoral apparatus. Also morphologically distinct, but less differentiated from typical *G. wageneri* forms are the *G. macronychus* types, in which the marginal hook sickles are large, and of a distinctive shape with the point of the sickle overhanging the toe (Malmberg, 1970).

The molecular analysis conducted here shows that the *G. pavlovskyi*, *G. macronychus* and *G. katharineri* forms all arise from within the more generalised *G. wageneri* morphology characterised by *G. zebrae*. The trees obtained by all methods are polytomous, and do not clarify the origins of these "*G. wageneri*" forms. However, they all distinguish a large group of northern European forms (*G. pungitii*, *G. gasterostei*, *G. gracilihamatus*, *G. salaris*, *G. derjavini*, *G. truttae* etc.) as a single clade (see Fig 6.3, Block A). These species include a small number from cyprinids (*G. aphyae*, *G. leucisci*, *G. vimbi*, *G. pannonicus* and *G. gobiensis*), but the remainder are from non-cyprinid hosts, and are assumed to have radiated via host shifts (Ziętara & Lumme, 2002). This group of species loosely corresponds to the "trout stream assemblage" of Cable *et al.* (1999), and may be considered a recent radiation of the *G. wageneri* types following glaciations. Interestingly, this group clusters with *G. parvae*, recently described from an Eastern Asian cyprinid, *Pseudorasbora parva*, by You *et al.* (2008). Apart from this group of northern Eurasian species, all other '*G. wageneri*' group species (based on morphology), including the new species *G. zebrae* from zebrafish, are loosely associated, and lack bootstrap support. Basal in these trees are the cyprinid-infecting *G. rhodei* (a northern European isolate of a widespread, possibly pan-eurasian, species), *G. gurleyi* and *G. kobayashii*, all of which originate in South East Asia, south of the zone of glaciation. The new species *G. zebrae* also clusters in this part of the tree. Originally, we suspected that *G. zebrae* might have infected *D. rerio* via a host shift within fish farms in Singapore, where these fish are bred in large numbers for the aquarium trade. However, the occurrence of a closely similar form from wild fishes in Bangladesh makes it clear that *G. wageneri* forms infect natural populations of *Danio*, and possibly other small cyprinids in SE Asia, and that these forms are basal in current phylogenies of the *G. wageneri* group. We infer from this that the *G. wageneri* group probably originated on cyprinids in SE Asia, extending north and west to infect other fishes following the large scale redistributions of freshwater fishes associated with the ice ages.

Unfortunately, the culture of *G. zebrae* was lost shortly after discovery, largely due to the immediate lack of available naïve hosts, and we were unable to establish a culture of *G. danio*. Clearly gyrodactylids on *D. rerio* are relatively rare. Despite considerable searching in the wild (Martina Ondračková and Carl Smith, personal communication), and of imported danios (Jo Cable, personal communication), only these two collections have been made, and it is then questionable whether *D. rerio* is the true host for these, or any, gyrodactylids. However, in general it is the case that many gyrodactylids are rare in nature, and it is sometimes difficult to see how their populations can persist on particular host species. In this case, it may be that the parasite preferentially infects a particular life history stage (e.g. fry or juvenile fish), rather than the adults sampled here. They may also be more abundant on breeding danios, when some element of immunosuppression may occur. However rare *G. zebrae* turns out to be, the opportunity to study a gyrodactylid with a long evolutionary history shared with *D. rerio*, a fish for which complete sequence data and a very large number of genetically characterised variants, including knockouts, is available, makes an intensive search to rediscover this gyrodactylid highly desirable.

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CHAPTER 7: LONG-TERM *GYRODACTYLUS LOMI* INFECTION ON ISOLATED JUVENILE CHUB, *LEUCISCUS CEPHALUS*

7.1 ABSTRACT

Previous studies on long-term gyrodactylid infections on isolated fish have shown that for the majority of *Gyrodactylus*-fish interactions, infections intensity peaks and then declines as the host mounts an effective immune response. In the current study, juvenile chub collected from the wild still harbored *Gyrodactylus lomi* infections after 6-10 months in the laboratory despite being individually maintained at 12°C.

7.2 INTRODUCTION

Gyrodactylids (Gyrodactylidae, Monogenea) are known for their pathogenicity to teleost hosts; the most intensively studied gyrodactylids, *G. salaris* on wild salmon in Norway and *G. turnbulli* as a model organism infecting guppies in the laboratory, are both highly pathogenic, and the parasites are notorious for causing losses in aquaculture (reviewed in Bakke *et al.*, 2007). The paradigm of gyrodactylid infection, dating back to the work of Lester and Adams (1974) and Scott and co-workers (Scott, 1982; Scott and Anderson, 1984), is of transient infections lasting weeks to days (depending on environmental temperature), after which the host eliminates the infection or dies. The basis of this rejection response remains unknown, although non-specific and specific immune responses probably play a part (Buchmann and Lindenstrøm, 2002). More recent work on salmon has implicated a variety of immune-response genes (Matějusová *et al.*, 2006; Collins *et al.*, 2007). Nevertheless, although most experimental studies have shown this pattern of infection in natural host populations, stable co-existence between gyrodactylids and hosts seems to be normal with little evidence of host mortality (e.g., MacKenzie, 1970; Harris, 1982; Appleby, 1996; Dávidová *et al.*, 2005; but see van Oosterhout *et al.* 2007). However, this is a very large taxon, with 400+ described species, and different patterns of population growth, ranging from pathogenicity to stable co-existence, might be expected. Some of these patterns were described by Harris (1993) with reference to representatives of a different gyrodactylid genera. Here, we describe the behaviour of infrapopulations of *Gyrodactylus lomi* Ergens & Gelnar, 1988, which appear able to persist as long-term stable infections on their host, *Leuciscus cephalus*.

7.3 MATERIAL AND METHODS

The fish (juvenile 0+ *Leuciscus cephalus*) used in this study were collected from the wild as part of other research dealing with gyrodactylids; and were maintained individually to mount an effective immune response and clear any natural pre-existing gyrodactylid infections. We have found this to be an effective and non-invasive method of eliminating infections from both sticklebacks (*Gasterosteus aculeatus* and *Pungitius pungitus*) and minnows (*Phoxinus phoxinus*). However, after 6 months in the laboratory, juvenile chub were infected with gyrodactylids. This study reports the identity of the parasites, and how long individual fish could sustain these infections.

Fish were collected in January 2007 from Roath Brook, Cardiff, Wales (water temperature 10°C), maintained in individual 1-L aquaria at 12°C, fed bloodworm daily with aquaria given daily water changes with dechlorinated water and maintained at a photoperiod of 12L:12D. The fish were not screened or handled further after arrival in the laboratory, but after 6 mo, they were anaethetised with 0.02% MS222 (tricaine methane sulphonate) and examined for gyrodactylids using standard techniques (e.g., King and Cable, 2007). Of the 33 fish individually maintained, 27 were infected (mean intensity = 2; infection range of 1-35 parasites). Henceforth, fish were screened fortnightly until they had either cleared their infections or died.

Individual parasites were examined microscopically using the measurement protocols of Shinn *et al.* (2004) and Olstad *et al.* (2007), and molecular analysis carried out (Cable et al., 1999).

7.4 RESULTS

A partial ITS2 sequence (375 bp) gave a 100% match with the ITS2 sequence of *G. lomi* (GenBank Accession Number AJ407929), and specimens agreed morphologically (Fig. 6.1) with the original description of this species (Ergens and Gelnar, 1988). This represents the first report of this species from the UK. Representative images of these specimens have been deposited in GyroDb (www.gyrodb.net; Harris *et al.*, 2008).

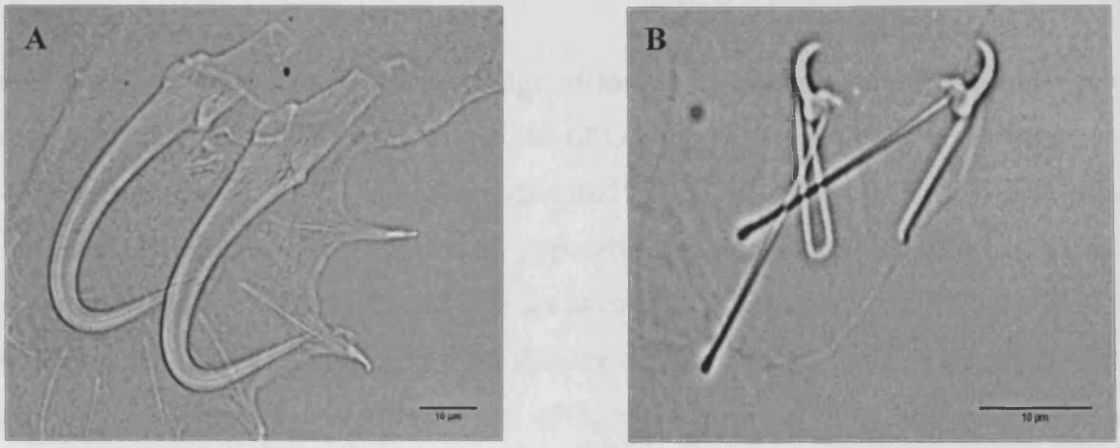

Fig. 7.1. Phase contrast light micrographs of (A) hamulus and (B) marginal hooks of *Gyrodactylus lomi*. Scale bars = 10 μm

Despite being individually maintained, these juvenile chub apparently sustained *G. lomi* infections for 24-41 weeks (24 weeks being the initial screen at 6 months). During this period, 3 of the 33 fish originally caught died of unknown causes, and the maximum infection intensity recorded for an individual fish was 35 at week 41 (this fish died shortly afterwards). Although several individuals maintained infections longer, most had lost their worms by week 31. Figure 6.2 shows individual trajectories of infection, and there was a highly significant difference between individual fish in their burden of *G. lomi* over time (Friedman's test, $P < 0.005$).

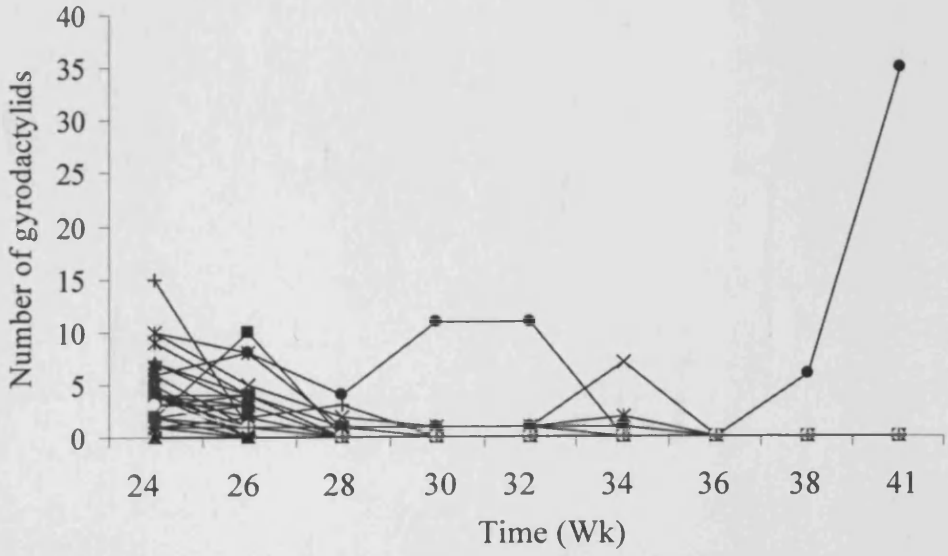

Fig. 7.2: Individual trajectories of *Gyrodactylus lomi* infection on isolated juvenile chub over time (wk) from initial screening carried out at 6 months (wk 24) until last recorded infection (wk 41)

7.5 DISCUSSION

The only comparable study to our knowledge of long-term persistence of gyrodactylids was that of Bakke *et al.* (1996), dealing with infections of *G. salaris* on Arctic charr (*Salvelinus alpinus*), some of which persisted for 150 days on individual fishes (cf. 280 days in the present study of *G. lomi* on chub). Persistence of *G. salaris* in populations of charr was similarly extended, and this host is suspected of being able to provide an asymptomatic reservoir of *G. salaris* in the field (Robertsen *et al.*, 2007). It might be that the current observations were due to the chub being immunocompromised under the conditions of culture; however, it is also possible that this is related to a specific aspect of the *G. lomi*-chub interaction. Chub are solitary fish as adults and are broadcast-spawners (Maitland and Campbell, 1992), breaking the potential link between parents and fry for gyrodactylid transmission. In these circumstances, long-term persistence of individual infections without excessive pathogenicity may be a necessary strategy for the gyrodactylid. Whatever the ultimate cause, our observations are the first to demonstrate that, when isolated, some fish species can maintain a persistent, low-level gyrodactylid infection for a long period.

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CHAPTER 8: LOCAL DIFFERENTIATION OF ISLAND AND MAINLAND UK THREE-SPINED STICKLEBACK (*GASTEROSTEUS ACULEATUS*) POPULATIONS IN RESPONSE TO INFECTION WITH THE MONOGENEAN, *GYRODACTYLUS GASTEROSTEI*

8.1 ABSTRACT

The three-spined stickleback (*Gasterosteus aculeatus*) displays significant phenotypic variation between freshwater populations. They are naturally infected with a range of *Gyrodactylus* spp. including *G. gasterostei* whose range, in the UK, is restricted to the mainland and has never been recorded in the Hebridean Islands. In the current study, we tested whether Hebridean sticklebacks show elevated susceptibility to *G. gasterostei* compared to mainland host populations and if there is evidence of local adaptation of this parasite to its mainland host. Our results demonstrate that Hebridean sticklebacks do not show elevated susceptibility when infected with this novel parasite, in fact their infection trajectories were similar to those of mainland populations. Furthermore, there was no evidence of local adaptation but rather of local differentiation of both hosts and parasites, with a patchwork of susceptibility and resistance. The current study is the first to repeatedly assay the same host-parasite combination from the natural environment and showed marked temporal variation between control populations (Cardiff) collected at different times, which we suggest overrides local selective effects leading to the development of local adaptation.

8.2 INTRODUCTION

Host-parasite systems reportedly are ideal for the study of local adaptation, which in theory should occur widely (Kawecki and Ebert, 2004). There have been a number of empirical studies testing this theory as a means of improving our understanding of host-parasite co-evolution. The most common fitness measures used to detect local adaptation are parasite life history traits, such as infectivity, whereby sympatric parasites are predicted to infect sympatric hosts more frequently than allopatric ones. The other commonly used measure is population growth rate, particularly suitable for organisms which show exponential growth (Kawecki and Ebert, 2004). However, to date, most empirical studies of local adaptation in host-parasite systems have involved invertebrates, the most well known being that of Lively and Dybdahl (2000) who demonstrated local adaptation in a snail-digenean system in two lake populations. In contrast, there have been relatively few such studies involving vertebrates (but see Jackson and Tinsley, 2005) with the only fish host studies being restricted to endoparasites with indirect life cycles.

Ballabeni and Ward (1993) demonstrated local adaptation of the digenean, *Diplostomum phoxini* to its second intermediate host, the European minnow (*Phoxinus phoxinus*). Whilst Kalbe and Kurtz (2006) looked at differences in immunocompetence between three-spined stickleback (*Gasterosteus aculeatus*) populations occurring in lake and river habitats to infection with *D. pseudospathaceum.*

The three-spined stickleback, a widely distributed teleost with a Holarctic range (Boughman, 2007), is an ideal host model for studying local adaptation. It displays high phenotypic divergence of freshwater populations from a marine ancestor, caused by post-glacial adaptive radiation (Bell and Foster, 1994). Since its synonymisation as a single species, *G. aculeatus* is considered a species complex (Foster *et al.*, 2003) with three morphs: complete, partial and low plated (Mattern, 2007). The most widely described and studied phenotypes are those found in British Columbia, where distinct benthic-limnetic species pairs occur in the Strait of Georgia (McPhail, 1994). Recently, there have been calls for legislative protection for such phenotypically unique populations, threatened mainly by anthropogenic pressures, such as habitat destruction and the introduction of predators and pathogens (Foster *et al.*, 2003). Indeed, there is clear evidence that certain benthic-limnetic species pairs in British Colombia are becoming extinct or collapsing due to introduced exotic species (Taylor *et al.*, 2006). In the United Kingdom, unique stickleback phenotypes are also found, particularly in island populations such as the Hebrides. In North Uist, an island in the Outer Hebrides, four populations of spine-deficient sticklebacks occur (Campbell, 1985; Maitland and Lyle, 1996). Loss of spines has been attributed to reduced predation pressure (Moodie and Reimchen, 1973) or a lack of environmental calcium (Giles, 1983). However, as those in North Uist are known to co-exist with a range of predators (Wright and Wright, 1991), their loss of defensive spines is probably due to calcium deficiency (Giles, 1983), particularly as these spine-deficient populations occur in peatland lochs (Maitland and Lyle, 1996).

As *G. aculeatus* populations may vary in their immune strategies, for example based on habitat differences (Scharsack *et al.* 2007), then it may follow that their associated parasites have coevolved to adapt to a particular genotype, with sympatric parasites performing better on sympatric hosts (i.e. local adaptation as described by Williams, 1966 cited by Kawecki and Ebert, 2004). In addition, sympatric and allopatric hosts may differ in their susceptibility to infection with novel parasites, as the traditional view has been that allopatric parasites may be more virulent than those that are sympatric (Ebert, 1994). One parasite group, typically found as part of the stickleback's natural parasite fauna, the monogenean ectoparasites, *Gyrodactylus* spp.,

appears ideal for testing local adaptation theory due to their direct life cycle, viviparous reproductive mode and assumed narrow host specificity. The most well known and intensively studied species is *G. salaris* due to its catastrophic effects on East Atlantic salmon stocks in Norway, since its accidental introduction in the 1970s. Previous studies on gyrodactylid epidemiology on Atlantic salmon alluded to local adaptation of the Lierelva strain of *G. salaris* to its sympatric host (e.g. Bakke *et al.*, 1990, 2002; Cable *et al.*, 2000). However, adaptation was not detected for *G. bullatarudis* and *G. turnbulli* from the Lower Aripo River, Trinidad to their sympatric guppy hosts (Cable and van Oosterhout, 2007).

Throughout its range in the wild, the three-spined stickleback is infected with 7 *Gyrodactylus* species, although normally never more than 2 or 3 species in any one location (www.gyrodb.net; see Harris *et al.*, 2008), the two most prevalent in Northwestern Europe being *G. arcuatus* and *G. gasterostei*. In the UK, the former is the more widespread and can occur in sympatry with *G. gasterostei*, when it is usually found on the gills. *G. gasterostei* is found throughout England and Wales but has never been recorded in NW Scotland including the Hebridean Islands. The distribution of this parasite is considered to be related to the post-glacial recolonisation of sticklebacks from SE England via the Channel River drainage basin (Harris *et al.*, unpublished observations). As sticklebacks moved northwards from the southern England glacial refugia, rapid adaptive radiation could have led to the parasite becoming adapted to local populations. Currently, the most northern limit of this parasite's range is the central lowlands of Scotland (Stirling area) (Harris *et al.*, unpublished observations). Although *G. gasterostei* reportedly occurs on several host species in Europe (Matějusová et al. 2000), this parasite is considered to be a specialist (Harris, 1998). This discrepancy either arose by *G. gasterostei* having been recorded from hosts on to which it accidentally transferred (Harris *et al.*, 2004) or it being confused with its sister species (see Bakke *et al.*, 2007). Based on the original species description of *G. gasterostei* as a specialist (Gläser, 1974 cited in Harris *et al.*, 2004) it is therefore more likely to demonstrate local adaptation than parasites with a broader host range (Lajeunesse and Forbes, 2002). Furthermore, *G. gasterostei* may have become adapted to local stickleback populations due to its hosts rapid adaptive radiation. It is unknown whether *G. gasterostei* could be pathogenic in a manner similar to *G. salaris* infecting East Atlantic salmon in Norway, if it were ever accidentally translocated to potentially vulnerable populations which do not encounter this parasite, such as isolated populations on the Hebridean Islands.

Using the *Gasterosteus aculeatus/Gyrodactylus gasterostei* system, the aims of the current study were twofold. Firstly, to ascertain whether Hebridean populations of *Gasterosteus aculeatus* which are naïve to *Gyrodactylus gasterostei*, are more susceptible to infection than their mainland counterparts, which have a history of infection with this parasite. Secondly, to ascertain via reciprocal cross infection experiments whether there is any evidence to suggest that *G. gasterostei* is locally adapted to its sympatric mainland hosts.

8.3 MATERIAL AND METHODS

8.3.1 Host origins and maintenance

The origins of the host populations used in the current study are given in Table 8.1. All were caught from the wild by hand netting apart from the Fada fish which were the F1 offspring of populations maintained at the University of Glasgow field station at Rowardennen. All fish were transported to Cardiff during 2004-2005. On arrival, they were transferred to 20l aquaria with approximately 20 fish per aquarium and maintained at 12.5°C ±0.5°C in a 12h L:D photoperiod while being acclimatized to Cardiff water conditions over several weeks. All fish were fed on a mixture of live *Daphnia*, *Artemia* and frozen bloodworm (*Chironomus* spp.).

8.3.2 Experimental design

Experiments were designed to test the susceptibility of stickleback populations that were outside the natural range of *Gyrodactylus gasterostei* (Hebridean Islands) and which encompassed the three recognized lateral plate morphs: low (Fada); partial (Grogary) and complete (Mull). In addition, comparisons were made between mainland populations that were within the natural range of *G. gasterostei*: Cardiff (Control), two populations from Nottingham (Syston and Clifton, 30 km apart) which are located in the same watershed, and Stirling which is at the northernmost limit of the range of this parasite. Due to the complexity of the experimental design, it was not possible to test all host-parasite combinations simultaneously and therefore three experiments were carried out. Experiment One (conducted January - March 2005) used host populations from Mull (Inner Hebrides), Cardiff and Stirling (mainland UK); Experiment Two (November 2005 - January 2006) used host populations from Fada and Grogary (Outer Hebrides) and Cardiff; whilst Experiment Three (January - March 2006) used host populations from Nottingham (Syston and Clifton) and Cardiff. Each experiment included a Cardiff host-Cardiff parasite combination as a control group and to account for inherent parasite variation, parasites from individual donor hosts were used to infect individuals from all host populations being

tested. Table 8.2 summarizes the three experiments. Where possible for Experiments One and Three, a fully reciprocal cross infection was performed. For Experiment One, Mull fish were not infected with the Stirling parasite strain as insufficient parasites were available.

Host Population collection date	Origin (Latitude/Longitude)	Host standard length, mm & weight (g)	Natural prevalence (%) of infection
Experiment 1 (Inner Hebrides) conducted January - March 2005			
Cardiff August 2004	Roath Brook, Cardiff (51°29.9'N/3°10.2'W)	21.5- 44.5 (0.105 - 1.160)	*Gyrodactylus* 33.3%; *Trichodina* 41.7%
Stirling October 2004	Commercial trout farm pond, Howietown, Stirling (56°4.3'N/3°57.0'W)	24.5 - 54.0 (0.151 - 1.871)	*Gyrodactylus* 93.4%; *Trichodina* 50.9%; fungal 19.2%; *Vorticella* 8.5%; unidentified digenean cyst 1.8%; unidentified tapeworm 0.6%
Mull October 2004	Calgary Bay, Isle of Mull, Inner Hebrides (56°34.8'N/6°16.7'W)	24.5 - 45.5 (0.231 - 1.013)	*Gyrodactylus* 45.1%; *Trichodina* 95.1%; fungal 11.8%; unidentified digenean cyst 1%
Experiment 2 (Outer Hebrides) conducted November 2005 - January 2006			
Cardiff September 2005	Roath Brook, Cardiff	24.0 - 43.5 (0.252 - 1.200)	*Gyrodactylus* 64.7%; *Trichodina* 73.5%; fungal 41.2%; *Vorticella* 29.4%; unidentified digenean cyst 8.8%; *Diplostomum* 50%; *Argulus* 2.9%
Grogary March 2005	Loch Grogary, North Uist, Outer Hebrides (57°36.7'N/7°30.0'W)	25.0 - 33.0 (0.301 - 0.504)	*Gyrodactylus* 100%; *Trichodina* 98%; fungal 2%; *Epistylus* 38%; *Thersitina gasterostei* 38%; *Apiosoma* 28%
Fada March 2005	Loch Fada, North Uist, Outer Hebrides (57°26.3'N/7°12.6'W)	26.0 - 34.0 (0.232 - 0.617)	All naïve
Experiment 3 (Mainland UK) conducted January - March 2006			
Cardiff November 2005	Roath Brook, Cardiff	31.0 - 40.5 (0.485 - 0.662)	*Gyrodactylus* 93.9%; *Trichodina* 100%; fungal 54.5%; *Vorticella* 12.1%
Syston October 2005	Tributary of Trent and Soar Rivers, Nottingham (52°42.1'N/1°05.7'W)	27.0 - 43.0 (0.297 - 1.356)	*Gyrodactylus* 93.3%; *Trichodina* 20%; fungal 93.3%; unidentified digenean cyst 3.3%
Clifton October 2005	River. Trent, Nottingham (52°55.3'N/1°09.9'W)	27.0 - 40.5 (0.415 - 0.927)	*Gyrodactylus* 100%; *Trichodina* 40%; fungal 46.7%; *Vorticella* 3.3%; unidentified digenean cyst 6.7%; *Glugea* 16.7%;unidentified copepod 3.3%

Table 8.1: Origin and size of three-spined stickleback populations used in the current study and their natural parasite infections

Parasite Strain	Host Stock		
Experiment One	Cardiff	Stirling	Mull
Cardiff	30	10	30
Stirling	30	10	Not tested
Experiment Two	Cardiff	Grogary	Fada
Cardiff	16	21	17
Experiment Three	Cardiff	Nottingham (Syston)	Nottingham (Clifton)
Cardiff	20	20	20
Nottingham (Syston)	20	20	20
Nottingham (Clifton)	20	20	18

Table 8.2: Cross infection experimental design with sample sizes for each host-parasite (*Gasterosteus aculeatus-Gyrodactylus gasterostei*) combination

8.3.3 Screening and treatment of natural parasite infections

Within a week of their arrival in Cardiff, all sticklebacks were screened for parasites using a stereo-microscope with fibre-optic illumination. With the exception of the laboratory bred Fada population, all were naturally infected with gyrodactylids (see Table 8.1). *Gyrodactylus arcuatus* was identified from sticklebacks from the Isle of Mull and Grogary while *G. gasterostei* was recovered from Cardiff, Stirling and Syston (Nottingham) sticklebacks. Sticklebacks from Clifton (Nottingham) carried mixed infections of *G. arcuatus* and *G. gasterostei.*

For use as recipient hosts in Experiments One (Inner Hebrides) and Three (Mainland UK), ectoparasites were removed by treating the sticklebacks with 0.004% formaldehyde solution for 1h (Lester and Adams, 1974a). This treatment can inflict severe damage to the epidermis and fins, and may result high fish mortality in some populations (personal observation). In order to control for the possibility of formalin-induced mortality (Smith and Price, 1972; Buchmann *et al.*, 2004), the fish were monitored daily for several weeks after treatment and then screened for any remaining gyrodactylids, any found being removed using watchmaker's forceps. On screening clear of gyrodactylids for three consecutive occasions, all fish were then given a two to four month recovery period before being used in any experimental procedure. For Experiment Two (Outer Hebrides), the fish had a marked fungal infection on arrival to the laboratory and therefore formalin treatment was not used. Instead experimental hosts were isolated and maintained individually in 1.1l jars of dechlorinated water, with regular water changes every other day, which successfully eliminated the fungal infection within a month.

8.3.4 Parasite cultures

For Experiment One, the two parasite strains were isolated from their sympatric hosts and then maintained on Cardiff sticklebacks, known to be free of any other ectoparasitic infection. The fish were maintained in 10 l containers of dechlorinated water and monitored daily with the addition of clean fish twice weekly to maintain parasite numbers.

For Experiments Two and Three, a different methodology had to be utilized due to the lack of available Cardiff sticklebacks that could maintain a culture. Parasites were removed directly from heavily infected wild caught fish, once their identity as *G. gasterostei* had been confirmed.

8.3.5 Experimental infections

Heavily infected sticklebacks were used as donors for all experiments. Having been euthanized, the donor was rinsed with dechlorinated water to remove excess body fluids and then transferred to a Petri dish of clean, dechlorinated water and left for 1h to allow gyrodactylids to detach.

Each recipient was infected with two gyrodactylids, to allow for stochastic mortality of individual parasites. A recipient host was manually restrained in dechlorinated water and its caudal fin swept along the Petri dish in order to allow parasites to attach to it. This process was observed using a stereo microscope with fibre-optic illumination, with the time recorded for each gyrodactylid to attach. The host was then transferred to an individual 1.1l jar of dechlorinated water, once a final visual check had been made to ensure that the gyrodactylids had attached. To control for potential host size differences (see van Oosterhout *et al.*, 2007), all experimental fish were size matched. Each host population included a sham infected group to control for effects of the infection protocol on host mortality. Host standard length and weight were recorded.

The day of infection was defined as Day 0. All fish were examined 24h after infection (Day 1) using light (0.05% MS222) anesthesia. Any fish found to be parasite-free were re-infected to determine whether they were totally resistant to infection or whether their gyrodactylids had been lost through stochastic mortality. Based on the known reproductive rate of *Gyrodactylus gasterostei* at 13°C (Harris, 1985), all fish were then screened every 4 days thereafter, i.e. Day 5, Day 9 etc., with the number and position of all gyrodactylids recorded. Monitoring was continued until the fish had lost all parasites and were recorded free of gyrodactylids for three consecutive screenings.

8.3.6 Statistical analyses

Bartlett's Test and the Anderson-Darling test indicated heterogeneity of variance and non-normal distribution of the data which could not be rectified by transformation, therefore non-parametric analyses were carried out using Minitab vs. 12 and the web-based programme http://bardeen.physics.csbsju.edu/stats/exact.html for Fisher's Exact Tests. Abundance and prevalence of gyrodactylid infection (as defined by Bush *et al.*, 1997) were calculated for host populations within each experiment (fish that died during the experiment being treated as missing data). Using the nomenclature of Bakke *et al.* (2002), based on the outcome of an individual's infection, fish were classified as susceptible, responding or innately resistant. The time taken for the 1st and 2nd parasite to attach was compared between host stocks in each experiment using either a Kruskal-Wallis (for three groups) or Mann-Whitney (for two groups) test. The time to loss of infection (the last day that a fish was recorded as infected) was recorded for each individual and plotted as Kaplan-Meier survival curves, which were then compared for each host population using a Wilcoxon test. Contingency tests (Chi-square or Fisher's Exact Test where sample sizes were <5) were used to test for differences between host populations in mortality (Experimental vs. Control fish), prevalence of infection at Days 5, 13 and 21 and the numbers of fish that were innately resistant, responded to infection, were susceptible or died. Day of maximum parasite load and maximum parasite growth rate (defined as the highest parasite growth rate between two consecutive screenings) were compared between groups for each experimental dataset using either Kruskal-Wallis or Mann-Whitney tests. Any tests resulting in a significant difference were then followed by *post hoc* tests i.e. Mann-Whitney with subsequent Bonferroni correction to adjust the level of significance according to the number of pairwise comparisons (i.e. 0.05/3 = 0.017).

8.4 RESULTS

All fish were successfully infected on Day 0, but the numbers that remained infected on Day 1 varied across experiments. There were no significant differences in the time taken for the 1st parasite to attach to different host stocks in all experiments, but in Experiment One there was a significant difference for the time taken for the 2nd parasite to attach between Mull, Cardiff and Stirling fish infected with the Cardiff parasite strain (Kruskal-Wallis, $H = 6.47$, $df = 2$, $p = 0.039$). Subsequent *post hoc* tests identified that the difference lay between Mull and Cardiff sticklebacks (Mann-Whitney, $W = 1078.0$, $p = 0.0158$).

There was no significant difference in mortality between experimental and control fish in any experiment (Fisher's Exact Tests, $p > 0.05$), but high mortality (27.5%) inexplicably occurred in Experiment Three, affecting both control and experimental fishes. The time when 50% of fish lost their parasite infections was similar for all experimental fish ranging between Day 18 to 26, with the exception of the Syston stock in Experiment Three where 50% of fish shed their parasites at Day 8. Comparison of Kaplan-Meier survival curves showed there were significant differences in time to loss of infection in Experiments One and Three, but not in Experiment Two. For the outcome of infections, (whether fish were innately resistant, responders, susceptible or died; see Fig. 8.1), the only significant differences were for Experiment One (Mull, Cardiff and Stirling fish infected with the Cardiff parasite strain); Experiment Three (Clifton, Syston and Cardiff fish also infected with the Cardiff parasite strain) and between the Cardiff control groups (Fisher's Exact Tests, $p = 0.005$; 0.029 and 0.006, respectively).

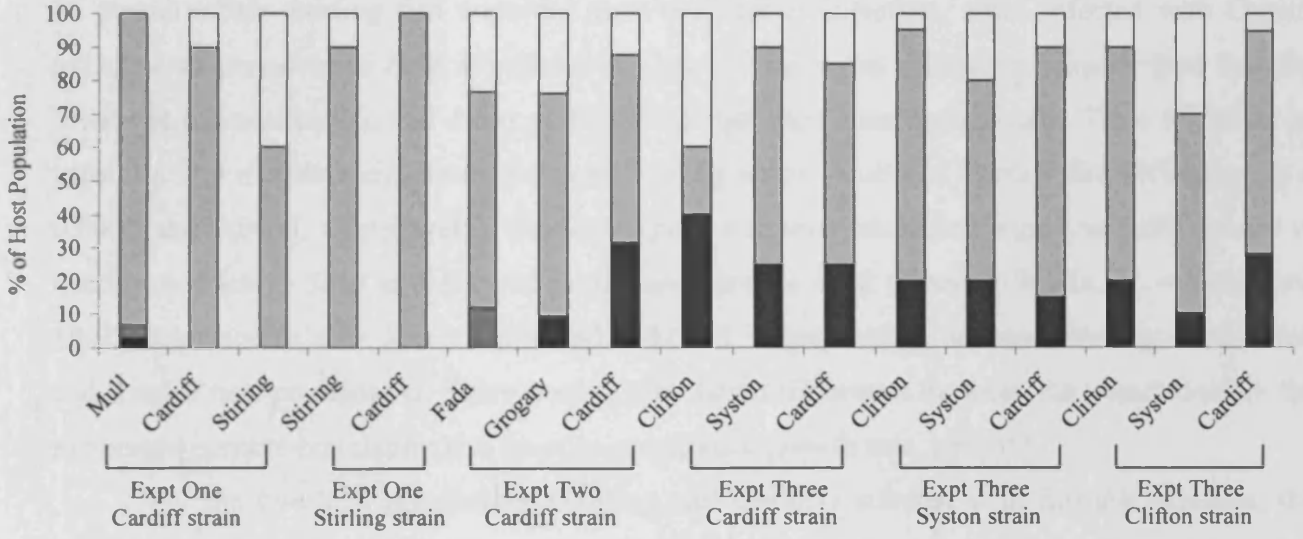

Fig. 8.1: Percentage of fish in each host population that were innately resistant (white); responders (light grey); susceptible (dark grey) and died (black). Experiment number and parasite strain are shown below the X axis

Experiment one

This first experiment compared fish from Mull (Outer Hebrides) naïve to *G. gasterostei*, with those from Stirling (central lowlands of Scotland) and Cardiff (control population) that were both naturally infected with *G. gasterostei*). Reciprocal cross infections were carried out between Cardiff and Stirling fish, using Cardiff and Stirling parasite strains.

All three host populations were successfully infected on Day 0 and infections increased on all five host-parasite combinations tested. The majority of infections followed a similar dynamic pattern, whereby the parasite load increased initially before peaking and then declining to zero, as the fish mounted an immune response (Fig. 8.2). Based on the nomenclature of Bakke *et al.* (2002), these fish were classified as responders. One Mull fish was classed as susceptible as it maintained an infection for 132 days, although it did eventually lose its infection (Fig. 8.2A). The majority of Mull fish cleared their infections by Day 29, which was not significantly different to the Cardiff fish which cleared their infections by Day 33 (Wilcoxon, $p > 0.05$). None of the Mull fish were innately resistant, although 3 out of 30 (8%) Cardiff fish infected with Cardiff parasite strain were innately resistant to infection.

Mull fish had the highest parasite load overall, with a maximum load on one individual of 74 worms at Day 21, the mean maximum parasite load being 18 parasites per fish. Cardiff fish infected with their sympatric parasite had a maximum parasite load of 43 worms, with a mean of 16 parasites/fish. Stirling fish were the most resistant to infection, when infected with Cardiff parasites as they cleared their infections by Day 29, the mean maximum parasite load was the lowest at 6 parasites/fish and 4 out of 10 (40%) fish were innately resistant. Time to failure of infection was therefore significant between Stirling versus Mull and Cardiff fish (Wilcoxon, $p = 0.0002$ and 0.0004, respectively). Similarly, *post hoc* tests identified significant differences in maximum parasite load and Day of maximum parasite load (Kruskal-Wallis, $H = 14.64$ and 18.65, respectively, $df = 2$, $p = 0.001$ and < 0.0001, respectively), between Stirling versus Mull and Cardiff host populations. There was no significant difference between the populations in the maximum parasite population size (maximum parasite growth rate, $p>0.05$).

For the two host populations (Stirling and Cardiff) infected with Stirling parasites, the maximum burden was obtained on a Cardiff (26 worms) rather than a Stirling fish (14 worms). Most fish had responded to infection by Day 25 with the exception of a single Cardiff fish which maintained a low level infection until Day 65. There was no significant difference in any of the variables tested with the exception of Day of maximum parasite load (Mann-Whitney, $W = 706.0$, $p = 0.0029$). For the two host populations subjected to a reciprocal cross infection (Stirling and Cardiff), for Stirling fish there were no significant differences in any of the variables tested between those infected with an allopatric parasite (Cardiff) and those with their sympatric parasite (Stirling). For the Cardiff fish, there was a significant difference between those infected with Cardiff and Stirling parasite strains in terms of the Day of maximum parasite load (Mann-Whitney, $W = 1106.0$, $p = 0.0032$).

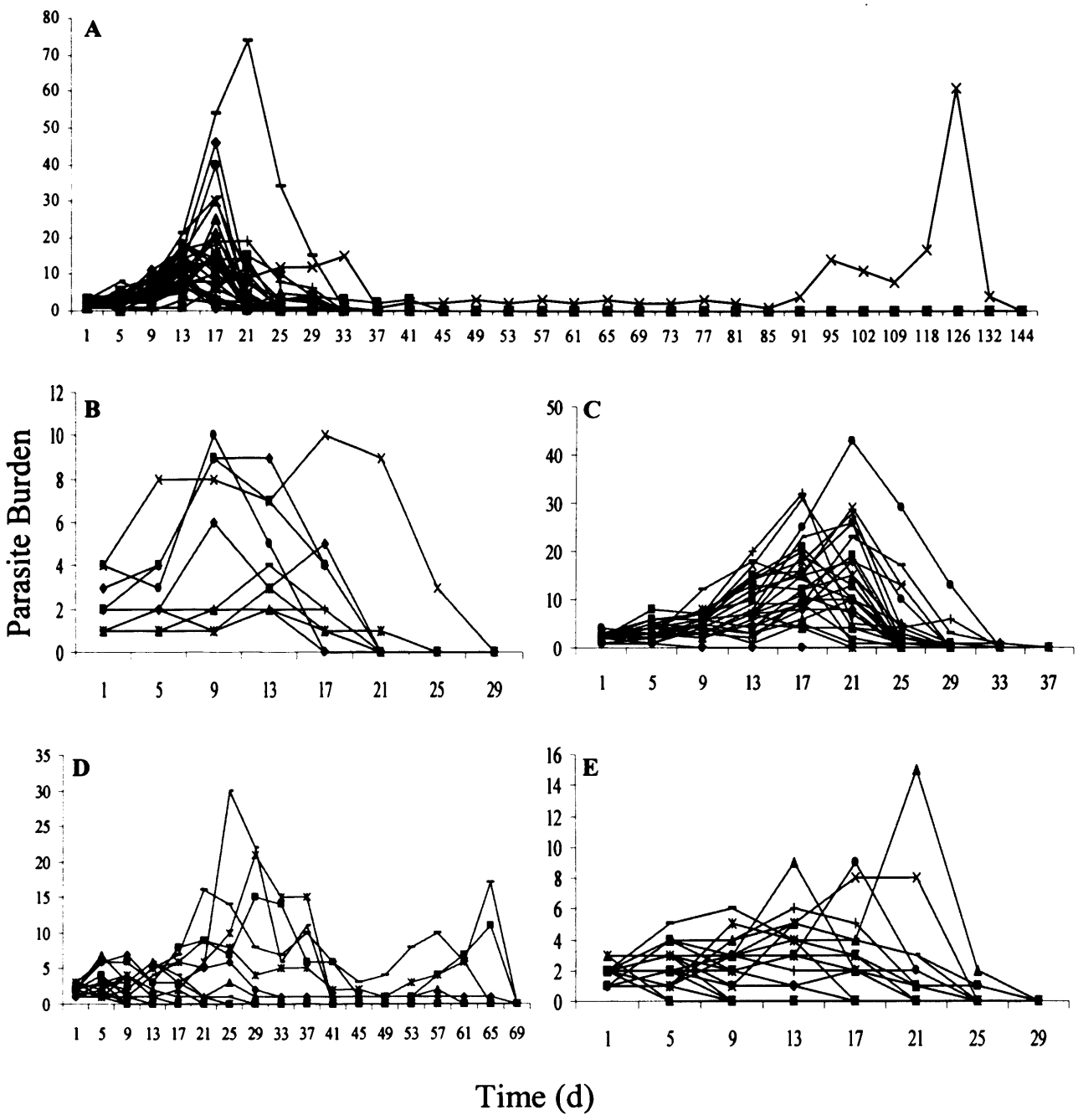

Fig. 8.2: Individual trajectories of infection for (A) Mull, (B) Stirling and (C) Cardiff fish (Experiment One), and (D) Fada and (E) Grogary fish (Experiment Two) infected with Cardiff strains of *Gyrodactylus gasterostei*

Experiment two

The second experiment compared fish from Fada (spine-deficient, Outer Hebrides, F1 lab bred), Grogary (Outer Hebrides) (Figs. 8.2D & E) and Cardiff (control population). Again, all fish were successfully infected (with Cardiff parasites) and infections grew on all populations. Fada was the only host population tested that, based on previous exposure, was completely naïve to any gyrodactylid infection and was also the only family group (full siblings). Surprisingly, the response of this group of fish to infection was also variable (see Fig. 8.2D). While the majority of fish responded to infection, 2 out of 17 fish (11.8%) were classed as susceptible, maintaining infections until Day 65, whilst 4 out of 17 fish (23.5%) were innately resistant. Overall, the highest parasite load was achieved for one individual Fada fish of 21 worms, with a peak mean abundance of 4.6 parasites/fish. Grogary fish were the most resistant to infection, all fish having cleared their infections by Day 25. A similar percentage to Fada (23.8%, 5 out of 21 fish) was innately resistant to infection. This strain supported the lowest peak load for an individual in this experiment (9 parasites), and the lowest mean peak abundance (2.5 parasites/fish). The control population, Cardiff, was intermediate between the two Hebridean populations, although they had the lowest percentage of innately resistant fish (12.5%, 2 out of 16 fish). The peak load for an individual was 13 worms, with a mean peak abundance of 5.7 parasites/fish. However, for all variables tested, there were no significant differences between the three populations.

Experiment three

In this experiment, all host populations tested were from within the natural range of *G. gasterostei* (UK Mainland). Fish from Clifton and Syston (Nottingham), which occur in the same watershed, were compared with a control population (Cardiff) with a total of nine host-parasite combinations tested. However, the highest host mortality, 49 fish (experimental and control) out of 178 (27.5%) occurred during this experiment, together with the highest infection failure rate, 27 fish out of 178 (15.2%). Therefore, although this experiment showed the greatest variation between host populations, the results should be treated with caution, as there was no *a priori* reason, based on our experimental design, for this high mortality and number of infection failures.

For those populations infected with the Cardiff parasite strain, there was a significant difference in maximum parasite growth rate (Kruskal-Wallis, $H = 9.73$, $df = 2$, $p = 0.008$), but no significant difference in the time to failure of infection ($p > 0.05$). The highest peak parasite load occurred on an individual Clifton fish (137 parasites), mean peak load being 11.2 parasites/fish,

whilst the lowest peak load occurred on the sympatric hosts (26 worms), mean peak load 5.4 parasites/fish. Syston fish had the lowest mean peak load of 3.8 parasites/fish, although the highest peak load was an individual with a peak of 55 worms. The maximum parasite load and day of maximum load were significantly different between the populations (Kruskal-Wallis, H = 7.84 and 7.35, df = 2, p = 0.020 and 0.025 respectively). Subsequent *post hoc* tests identified that the differences lay between Cardiff and Syston fish and not between the two sympatric host populations (Clifton and Syston). The highest number of innately resistant fish were found within this cross host-parasite combination, with 13 out of 60 fish, the majority being Clifton fish (8 out of the 13), and subsequently the numbers of fish that were classed as resistant, responders, susceptible and those that died was significantly different between the three host populations (Fisher's Exact Test, p = 0.029).

When infected with their sympatric parasite strain, Syston fish had the highest peak parasite load, 150 parasites for one individual, mean peak abundance of 8.9 parasites/fish. Clifton fish had an individual peak load that was close to Syston at 136 parasites for one individual, mean peak abundance being 5.2 parasites/fish. Cardiff fish had an intermediate mean peak abundance of 5.2 parasites, but the lowest peak load for one individual of 43 parasites. Although the majority of fish had cleared their infections by Day 29, one individual from Clifton maintained an infection until Day 73 after which time it died (due to unknown cause) and one individual from Cardiff maintained its infection until Day 49. Maximum parasite load and day of maximum parasite load were significantly different between populations (Kruskal-Wallis, H = 10.36 and 12.88, df = 2, p = 0.006 and 0.002; see Figs. 8.3, 8.4 and 8.5). Clifton and Syston fish were identified by *post hoc* testing as being significantly different in terms of maximum parasite load, whilst for day of maximum parasite load, the differences lay between Clifton versus Syston and Cardiff fish.

Again, Syston fish had the highest burden when infected with Clifton parasite strain (one individual with 144 parasites, mean peak abundance of 7.7 parasites per fish), one individual Clifton fish peaked at 108 worms, but overall Clifton fish had the highest mean peak abundance when infected with their sympatric parasite at 11.2 parasites/fish. Clifton parasite growth was lowest on Cardiff fish, with a peak load of 47 parasites for one individual, and a mean peak abundance of 6.8 parasites/fish. However, Syston fish responded fastest, clearing their infection by Day 21, while Cardiff fish took until Day 33 to clear their infections. The majority of Clifton fish cleared infection by Day 29 although one individual maintained an infection until Day 57. Again, maximum parasite load and day of maximum parasite load were significant (Kruskal-

Wallis, H = 7.95 and 13.17, df = 2, p = 0.019 and 0.001). *Post hoc* testing identified Clifton and Syston fish being significantly different for maximum parasite load, whilst differences lay between Syston versus Clifton and Cardiff fish for day of maximum parasite load.

Comparisons between Clifton and Syston fish which received reciprocal infections indicated that there were no significant difference in the variables tested, except for Syston fish where there was a significant difference in maximum parasite growth rate (Kruskal-Wallis, H = 7.68, df = 2, p = 0.022) between the three parasite strains. *Post hoc* test identified the difference as being between fish infected with the Cardiff and Syston parasite strains (Mann-Whitney, W = 316.5, p = 0.0100).

Temporal variation in Cardiff fish

There was considerable variation in the response of the three Cardiff control populations towards their sympatric parasite (Fig. 8.6). Time to failure of infection was significantly different between fish infected in January (Experiment One) and November 2005 (Experiment Two) (Wilcoxon, p = 0.0008). Maximum parasite load between the three control groups was significantly different (Kruskal-Wallis, H = 25.08, df = 2, p < 0.0001) with *post hoc* tests identifying the differences as being between Experiment one fish versus Experiments two and three. Day of maximum parasite load was also significant (Kruskal-Wallis, H = 20.35, df = 2, p < 0.0001) with *post hoc* tests identifying the difference lying between fish from Experiment one and three. The proportion of fish that were innately resistant, responders, susceptible and died was also significant (Fisher's Exact Test, p = 0.006; see Fig. 8.7), however, Maximum parasite growth rate between the three control groups was not significant.

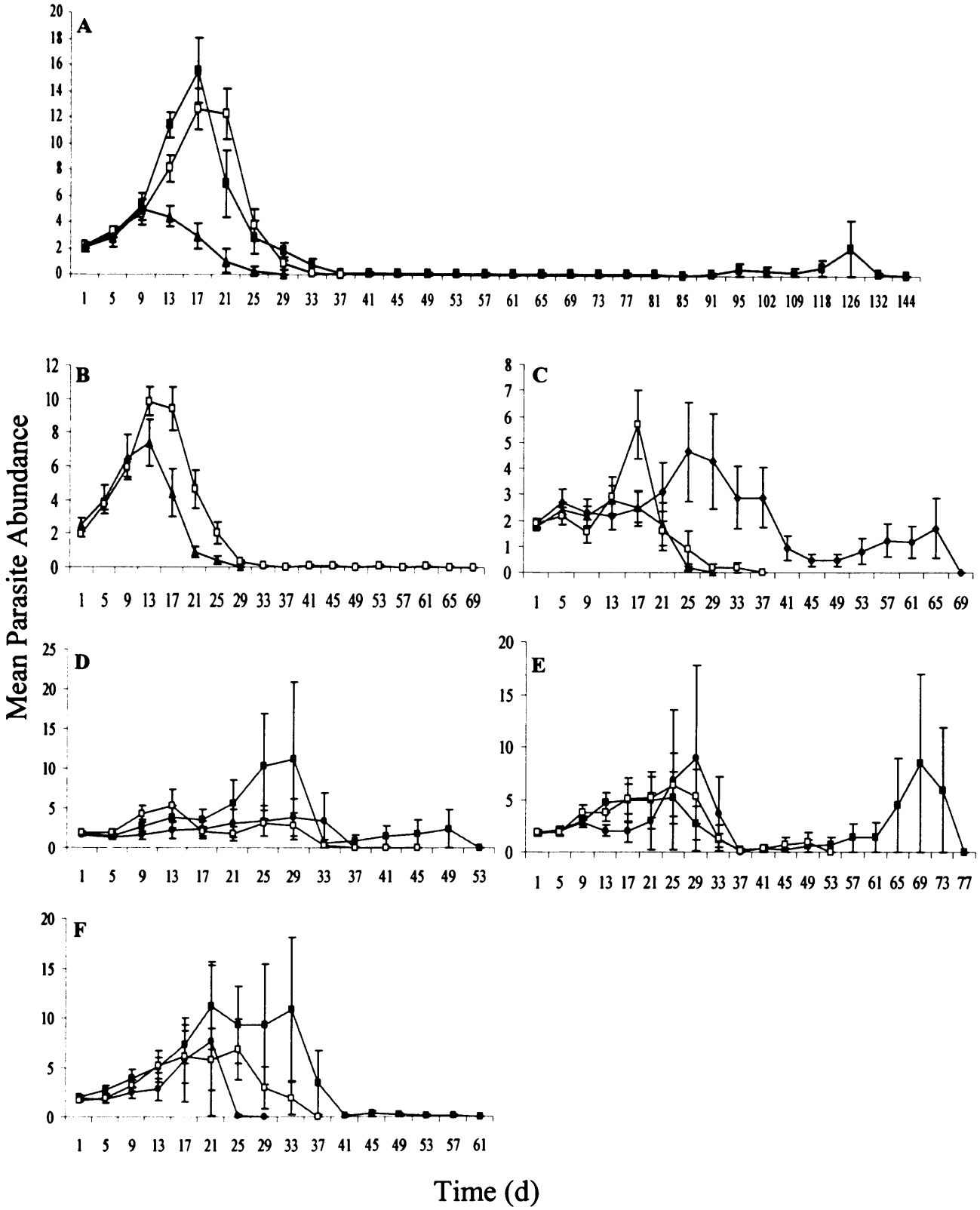

Fig. 8.3: Mean abundance of infection for: Experiment One (A) = infections with Cardiff parasite on Mull (closed square), Cardiff (open square) and Stirling (closed triangle) fish, and (B) = infections with Stirling parasite on Cardiff (open square) and Stirling (closed triangle) fish. Experiment Two (C) = infections with Cardiff parasite on Fada (closed diamond), Grogary (closed triangle) and Cardiff (open square) fish. Experiment Three (D) = infections with Cardiff parasite on Clifton (closed square), Syston (closed circle), and on Cardiff (open square) fish with (E) = Syston parasite and F) = Clifton parasite

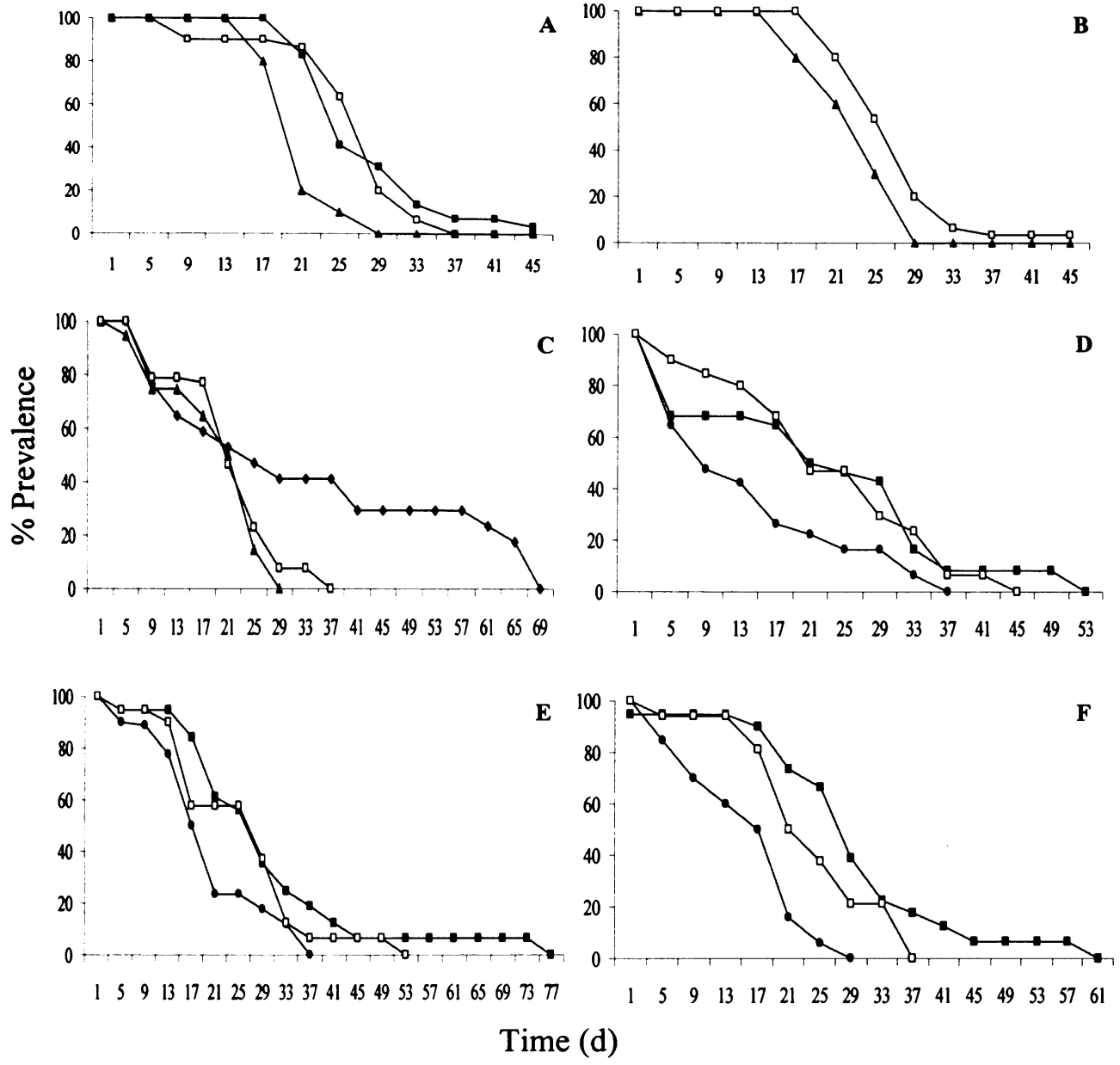

Fig. 8.4: Prevalence of infection for: Experiment One (A) = infections with Cardiff parasite on Mull (closed square), Cardiff (open square), and Stirling (closed triangle) fish, and (B) = infections with Stirling parasite on Cardiff (open square) and Stirling (closed triangle) fish. Experiment Two (C) = infections with Cardiff parasite on Fada (closed diamond), Grogary (closed triangle) and Cardiff (open square) fish. Experiment Three infections with (D) Cardiff, (E) Syston or (F) Clifton parasites on Clifton (closed square), Syston (closed circle) and Cardiff (open square) fish

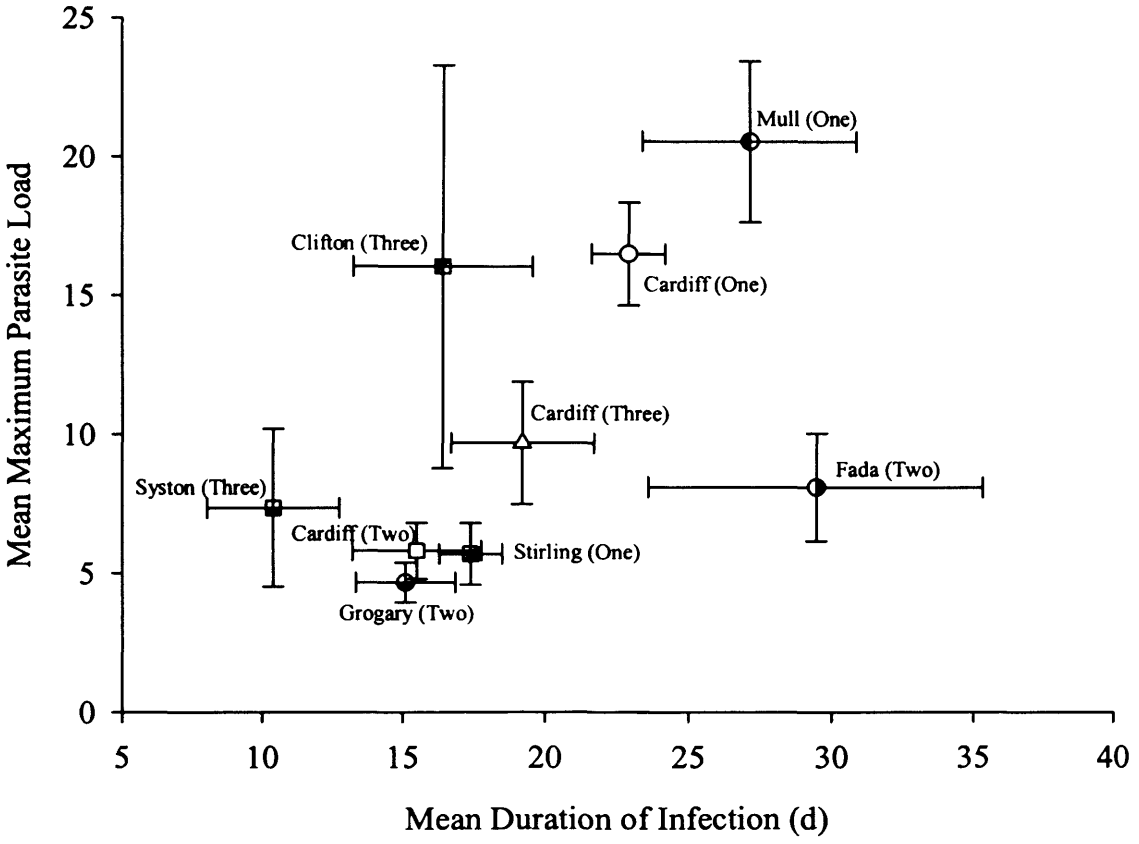

Fig. 8.5: Scatter bi-plots of mean maximum parasite load (x-axis) against mean duration of infection (y-axis) with ±SE for nine experimental host populations all infected with Cardiff *Gyrodactylus gasterostei*. Host population name is followed by the experiment number in parentheses

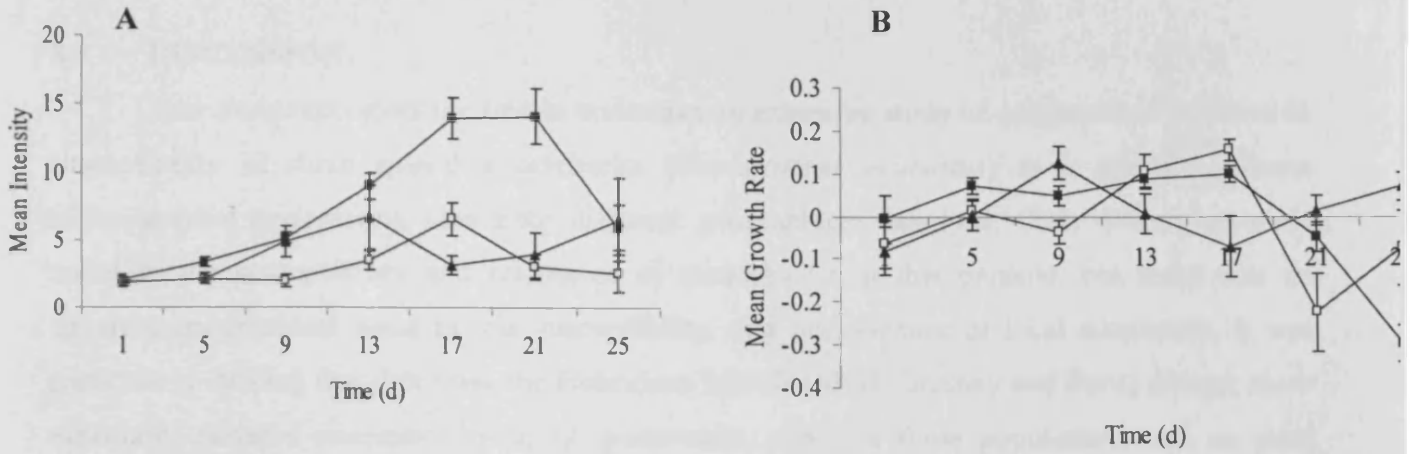

Fig. 8.6: (A) Mean intensity of infection and (B) parasite population growth rate for the control group (Cardiff) sticklebacks infected with Cardiff strain of *Gyrodactylus gasterostei*. Experiment One (2005) = closed square; Experiment Two (2005) = open square and Experiment Three (2006) = closed triangle

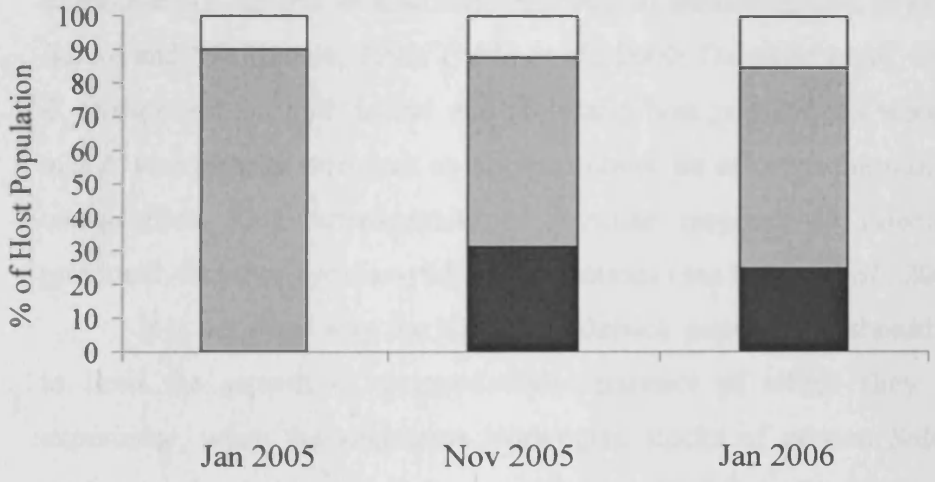

Fig. 8.7: Experimental outcome for the three control groups (Cardiff) of sticklebacks infected with Cardiff strain of *Gyrodactylus gasterostei* on three separate occasions: Experiment One = Jan 2005; Experiment Two = Nov 2005 and Experiment Three = Jan 2006. White = innately resistant fish; light grey = responding fish, infection peaks followed by sharp decline; black = fish that died during the experiment

8.5 DISCUSSION

This study represents the first to undertake an extensive study of geographical variation in susceptibility of three spined sticklebacks (*Gasterosteus aculeatus*) to a specific parasite (*Gyrodactylus gasterostei*), also from different geographical locations. Clear differences were noted in the susceptibility and resistance of sticklebacks to this parasite, but there was no apparent geographical trend in this susceptibility, and no evidence of local adaptation. It was particularly striking that fish from the Hebridean Islands (Mull, Grogary and Fada) did not show especially elevated susceptibility to *G. gasterostei*, although these populations had no prior immunological or evolutionary experience of this parasite, as they lie to the North and West of the natural range of the parasite. Although Mull fish supported the longest persistence and largest populations of *G. gasterostei* of all the stocks used during these experiments, and Mull and Fada were the only populations containing susceptible (unable to mount a host response) fish, these results were not dramatic, and certainly we did not see the extensive susceptibility noted to introduced *G. salaris* in allopatric Norwegian salmon strains (Bakke *et al.*, 1990, 2004, 2007; Bakke and MacKenzie, 1993; Cable *et al.*, 2000; Dalgaard *et al.*, 2003). Infection trajectories of *G. gasterostei* on both island and mainland host populations were similar and therefore these island sticklebacks were just as able to mount an effective immune response as their mainland conspecifics. This heterogeneity of immune response to infection has been demonstrated previously in other gyrodactylid-host infections (see Bakke *et al.*, 2007).

It is not clear why the island stickleback populations should be able to react successfully to limit the growth of a gyrodactylid parasite of which they have no prior evolutionary experience, when the analogous Norwegian stocks of salmon *Salmo salar* (also without prior evolutionary experience of *G. salaris*) entirely fail to limit parasite population growth. The response may have been due to a pre-existing immune response in the case of Mull and Grogary fish (which had experience of *G. arcuatus*), but this could not be the case in Fada fish, which had no prior experience of *Gyrodactylus* before infection and were immunologically naïve. This may be in part related to the observation that Hebridean populations of *Gasterosteus aculeatus* do have experience of *Gyrodactylus arcuatus*, and there may therefore be some selection for generic resistance to gyrodactylids, as suggested in the case of joint infections of guppies with *Gyrodactylus turnbulli* and *G. bullatarudis* (see Richards and Chubb, 1996). Norwegian salmon have no experience of gyrodactylid infection (in the absence of the introduced *G. salaris*) until after they have migrated into the marine environment, when they acquire light infections of *Gyrodactyloides*. This may represent a fundamentally different immune challenge, which fails to

confer any immunity to *G. salaris*, but it could represent a source of genetic resistance. The other difference may relate to the different responses of these two fish species to stress.

Amongst the stickleback populations from within the natural range of *G. gasterostei* (Stirling, Cardiff, Syston and Clifton), there was no clear pattern of local adaptation, but rather a patchwork of susceptibilities and resistance, with local differentiation of both parasites and hosts. This concurs with Kalbe and Kurtz's (2006) data, on sticklebacks infected with *Diplostomum pseudospathaceum*. They found no evidence of *D. pseudospathaceum* being locally adapted to its sympatric (lake) hosts, but rather local differences in immunocompetence between river and lake sticklebacks. Kalbe and Kurtz (2006) argued that lake populations of *Gasterosteus aculeatus* had greater immunocompetence against *D. pseudospathaceum* than did river populations that do not naturally encounter this particular parasite. However, lake populations were more immunocompetent due to exposure to a wider diversity of stickleback parasites (Kalbe and Kurtz, 2006), which may have influenced their response to *D. pseudospathaceum*, and it should be noted that, contrary to the situation with *D. pseudospathaceum*, *G. gasterostei* is narrowly specific to the three-spined stickleback, and (as far as we know) does not use other hosts (cf. Matějusová *et al.*, 2000). This may also have an impact upon the potential for local adaptation of gyrodactylids relative to other parasite groups.

The present study is the first to repeatedly assay the same host-parasite combination using fish and gyrodactylid parasites from the natural environment. Three replicates of the Cardiff *Gasterosteus aculeatus* – Cardiff *Gyrodactylus gasterostei* combination were undertaken, with radically different results in each. The findings of these three trials reflect the highly dynamic nature and complexity of this host-parasite interaction, showing as much variation within same-site trials as between allopatric combinations. The reasons for this are not clear. The fish used for these experiments were collected and infected at different times; for Experiment 1, using Mull and Stirling fish, control fish were collected in August and used the following January. For Experiment 2, using Hebridean fishes, Cardiff fish were collected from the wild in September for use in November, while for Experiment 3, using Trent Valley fishes, Cardiff fish and parasites were collected from the wild in November for use in January. These long time delays in the use of fish were designed to minimize the effects of the host immune response, as all had previously been exposed to *Gyrodactylus*. Originally (Lester and Adams, 1974b), it was felt that 6 weeks would be sufficient for any pre-existing immune response to be lost, and although Richards and Chubb (1996) felt that longer was required in the case of *G. turnbulli* on guppies, a minimum period of 8 weeks free of infection in the present work should have been enough to dampen the

immune response to the point where these fish could be considered naïve. Known aspects of the immune response to *Gyrodactylus* (see Harris *et al.*, 1998; Buchmann, 1998; Moore *et al.*, 1994; Bakke *et al.*, 2007) point to a non-specific complement mediated response, with no involvement of antibodies, but the inducibility of the response and memory (Scott and Robinson, 1984; Richards and Chubb, 1996) suggests an antibody mediated mechanism. Modern views of the persistence of the immune memory in fishes suggest that after 6-12 weeks memory would be retained, although the response itself would be damped, and this may have impacted upon the behaviour of controls between trials was due to an insufficiently damped immune response. A more likely reason for the difference relates to the response of *G. aculeatus* to day length changes. Each experiment was carried out in 12:12h L:D photoperiods, so in Experiments 1 and 2, the Cardiff fish were moved from a longer to a shorter photoperiod. In Experiment 3, they were moved from a shorter to a longer photoperiod. Such effects on photoperiod may have impacted upon immune function, via the action of prolactin (Bly *et al.*, 1997; Ángeles Esteban *et al*, 2006). Despite the variations between experiments, we believe that the results within experiments, between the allopatric combinations and their controls, are valid in demonstrating a patchwork of susceptibilities and resistance. However, the variation within the same host-parasite pair at different times of year does bring into question the validity of local adaptation in the case of the stickleback-*Gyrodactylus* combination, where infections are persistent and chronic throughout the year.

A further explanation of the lack of overt pathogenicity to the Hebridean fishes concerns the level of virulence of the parasite stock. In gyrodactylids, virulence is directly related to reproductive rate, because the pathogenic effects of this parasite are directly proportional to the number present. In the case of *G. salaris* on salmon, the reproductive rate of salmon-infecting clades on salmon is substantially higher than the reproductive rate of grayling-infecting clades (synonym *G. thymalli*) on grayling (Soleng and Bakke, 2001), and when tested experimentally (Cable *et al.*, 2000), populations on susceptible salmon had higher fecundity and lower mortality schedules than those on resistant fishes. In the present experiments, population growth rates on different strains of hosts within each experiment did not vary significantly, suggesting that the virulence of the parasite on different hosts did not vary.

The question of establishing local adaptation in the case of a *Gyrodactylus* infection of a fish host is problematic. Experiments in local adaptation normally establish parameters such as rate of infection (percentage of hosts infected). In the case of *Gyrodactylus* infections, characterizing the success or otherwise of an infection is much more complex, and must be

understood before a decision can be taken about the occurrence of local adaptation. In gyrodactylid infections, a founder individual will give birth to young which in turn reproduce *in situ* until after a certain time period the host reacts against the presence of the parasites and the parasite population begins to decline. It declines through the loss of parasites into the water column, from which they may infect other fishes. Eventually the infection on an isolated host may disappear entirely, but in natural populations most fish are infected, with a balance between immigration, population growth and emigration continuing in the face of an ongoing host response. Characterising the features which optimally co-adapt parasite and host in these circumstances is challenging. It can be assumed that in the case of *Gyrodactylus* infections, local adaptation must involve some local optimal trade-off between Fisher's basic reproductive rate R (the number of secondary infections produced from a primary infection, see Anderson and May, 1979), and parasite-induced host mortality, which in the case of *Gyrodactylus*, can be severe (Cable and van Oosterhout, 2007). Furthermore, it is likely that the host-parasite combinations which are observed in nature represent the most persistent, rather than those with necessarily the highest parasite population growth rates. It is not clear, for example, whether gyrodactylids should optimally be selected for long-lived individual infections, or for maximum peak population size; however, experimental studies suggest that maximal persistence is best achieved when individual infections are long-lived, and where there is heterogeneity in host response, as in the *Gyrodactylus salaris*-Arctic charr infections carried out by Bakke *et al.* (1996). Scott and Anderson (1984) demonstrated that high reproductive rate per se, coupled with high maximum population size, led to rapid extinction of *G. turnbulli* unless the host population was both large and subject to a high rate of recruitment. Indeed, the best example of local adaptation in a gyrodactylid is the relative reduction in maximum population size attained by *G. turnbulli* strain *Gt3*, which is doubtless a response to many years selection for persistence in relatively small host populations (Cable and van Oosterhout, 2007). In the light of these considerations, it is very difficult to speculate on the extent to which the *G. gasterostei* populations studied here were locally adapted. However, the high individual population sizes attained on the Mull and Fada fish by Cardiff parasites, and the presence of susceptible (no host response) fish within these populations, suggest that the Cardiff parasites are not well adapted to these fish strains. On the other hand, the relatively poor growth of Cardiff *G. gasterostei* on Stirling sticklebacks also suggests a lack of adaptation to these fish. In all of the other combinations, the relative performance of different *Gyrodactylus* on the different host strains was very similar, and it is

likely that a more refined analysis of local adaptation in this system would be impossible without a detailed understanding of the demography of the individual stickleback populations concerned.

Finally, it is worth noting that the present study has highlighted the temporal variation in response of one stickleback population to its local *G. gasterostei* strain. To date, we are aware of only one other study where local adaptation in the same vertebrate host-parasite combination was repeated at different time points. McCoy *et al.* (2002) found that in a year where greater resources where available, the ectoparasite *Ixodes uriae* was locally adapted to one of its hosts, the black-legged kittiwake (*Rissa tridactyla*). However, in the previous year, when fewer resources were available, the parasite was not locally adapted on this host. *I. uriae* is, however, a generalist and as it can complete its life cycle on any seabird so this would greatly influence any patterns of local adaptation. With regard to the current study, it is not clear how representative this temporal variation is, but where there is continuous infection with non-sterile immunity of the host (as is presumably the case with many metazoan parasites such as small monogeneans), then temporal variation, due to environmental modulation of parasite recruitment or host immunity, must be widespread. In such circumstances, sexual selection of a proxy character presumably gives a better estimate of host fitness during reproduction than does direct natural selection via parasite-induced host mortality. This would be particularly the case where parasite-induced host mortality is related to a whole community of parasites (sticklebacks for example are normally infected by two species of *Gyrodactylus* simultaneously), and individual species undergo different cycles of abundance. This role for sexual selection has been highlighted by Hamilton and Zuk (1982), but why sexual selection should be relatively more important than direct natural selection through mortality has not been clear. If variability in the outcome of infections of the sort observed here is normal, then the integrative effect of sexual selection for a proxy character could give a substantial advantage in reducing the tracking of individual parasite genotypes through episodes of parasite induced host mortality.

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CHAPTER 9: DISCUSSION

9.1 General summary

This thesis has focused on two hitherto neglected research areas of the genus *Gyrodactylus*, host specificity and local adaptation. Experimental tests of host specificity centered on two congeneric species infecting the guppy and show that previous assumptions of their host range were incorrect. *Gyrodactylus turnbulli* is not strictly host specific, although it does has a clear predilection for poeciliids (see Chapter 2). In contrast, *G. bullatarudis* is able to infect a range of fish species and can even reproduce on the three-spined stickleback *(Gasterosteus aculeatus)*, (see Chapter 3). As noted by Poulin and Keeney (2008), empirical tests of novel host-parasite combinations under laboratory conditions promote the breakdown of host specificity. Furthermore, laboratory cultures of gyrodactylids may be less host selective and thus more likely to infect atypical hosts (Bakke *et al.*, 1991, 1992; Olstad *et al.*, 2006). It was therefore a prerogative of this thesis to ascertain whether these results were a laboratory artifact by carrying out further experiments on suitable atypical hosts under semi-natural (pooled) conditions. These, pooled infections demonstrated that both parasite species were able to infect atypical hosts under their own violition. Furthermore, *G. bullatarudis*, a tropical parasite, is capable of infecting a temperate host, the three-spined stickleback within the optimal temperature range for this host (15°C). Thus, this thesis represents a comprehensive analysis of host specificity. These findings have significant implications for the aquarium trade in that gyrodactylids of aquarium fish are capable of infecting atypical hosts and currently the ornamental fish industry is less regulated than the commercial food fish industry. In addition, it has been demonstrated that *G. turnbulli* is capable of reaching lethal burdens on *Poecilia sphenops*, which has implications for the wild, where this species occurs in sympatry with *P. reticulata*. There are also important implications in view of climate change and global warming which may cause an alteration in host-parasite assemblages (Brooks and Hoberg, 2008) resulting in novel host-parasite combinations. Such combinations could have deleterious effects on novel hosts, as illustrated by the potential threat that the tropical species, *G. bullatarudis* could have on the susceptible populations of the temperate *Gasterosteus aculeatus* (see Chapter 3).

The current study also questioned whether *P. reticulata* is actually the optimal host for *Gyrodactylus turnbulli* (see Chapter 2). It is counterintuitive for a parasite to kill its host, as it depends on such a host for its very survival. Low level infections were able to persist on other poeciliids, principally *P. sphenops* and *Xiphophorus hellerii*. Both of these hosts are considerably

larger than *P. reticulata* and therefore would represent a more reliable resource (as host size is known to influence infection dynamics; Cable and van Oosterhout, 2007). Possibly *G. turnbulli* is not well adapted to *P. reticulata* even though it appears that *G. turnbulli* does a long evolutionary history with *P. reticulata* (see Harris and Lyles, 1992). It would be interesting to determine if *G. turnbulli* occurs on other poeciliids in the wild.

Field records alone can not predict the host range of parasites and this work highlights the importance of empirical data in evaluating atypical and transport hosts which should be included in any index of host specificity. It also draws attention to the need for accurate parasite identification. Based on the description that Hedrick *et al.* (2001) gave on the gyrodactylid used in their study, describing it as a gill species, it is strongly suspected that the parasite they used (and possibly Leberg and Vrijenhoek, 1994) was actually *G. bullatarudis* rather than *G. turnbulli*. However, neither study explains the methods used to identify the parasites used and no specimens were deposited. Furthermore, the assumption by Harris (1986) that studies carried out by Scott and colleagues during the 1980s, had misidentified the parasite used, could be partially incorrect. Personal observations of *G. bullatarudis* during experimental work carried out for this PhD indicate that some of the experiments by Scott and colleagues (Scott, 1982; 1985a, 1985b, 1987; Scott and Anderson, 1984; Scott and Nokes, 1984; Scott and Robinson, 1984) may have utilised *G. bullatarudis* (see Chapter 4) while others used *G. turnbulli* (see Harris, 1986).

Local adaptation in this thesis was studied using *Gyrodactylus gasterostei* which demonstrated local differentiation in susceptibility and resistance rather than local adaptation in a *G. gasterostei*-three-spined stickleback model system (Chapter 8). However, the host specificity of this gyrodactylid is still is an area of considerable contention. In the UK, this species would be regarded as a specialist (Harris, 1998) whilst in Central Europe it would be regarded as a generalist (Matějosová *et al.*, 2000). As noted by Bakke *et al.* (2007), the findings of Matějosová *et al.* (2000) that *G. gasterostei* occurs on cyprinids, may be actually a mis-identification with the sister species of this parasite, *G. aphyae*, which occurs on minnows.

G. salaris has previously been considered to be an aberrant species due to its catholic host range, however, broad host ranges could actually be the norm for this genus, as assumptions of strict host specificity are proved incorrect (Moen and Stockwell, 2006; current study). The actual host range (including atypical and transport hosts) for many gyrodactylids could be a gross underestimate. Their potential as major fish pathogens may yet be fully realised, particularly in the face of climate change and global warming which could alter host-parasite assemblages, leading to new host-parasite combinations (Brooks and Hoberg, 2008). This increases the

potential for host switching, which is the primary mode of speciation for this genus and as such, the threat posed by this taxon to vulnerable fish stocks may increase further with time.

Gyrodactylus species are considered to be amongst the most successful of invasive parasites (Kennedy, 1994). This is in part due to the fact that this genus lacks a specialised transmission stage and thus are capable of transmission to new hosts at any time during their life cycle (Boeger *et al.*, 2005). Previously, Cable *et al.* (2002) demonstrated a unique transmission strategy for *G. turnbulli* in that it migrates into the water film, and the current study has shown that this strategy appears to be density dependent (see Chapter Four). However, nothing was previously known of the transmission of *G. bullatarudis*. The findings that *G. bullatarudis* stays with a dead host regardless of parasite density (Chapter 4) is all the more remarkable given that this strategy does not appear to prolong its survival in contrast to *G. salaris* (see Olstad *et al.*, 2006). A number of hypotheses are proposed in this thesis as to the mechanisms that could promote such differing transmission strategies.

Survival of *G. bullatarudis in vitro*, does not appear to be inversely related to temperature, as maximum survival occurred at the intermediate temperature range tested (Chapter 5). Scott and Nokes (1984) carried out a similar study on *G. turnbulli* and found that optimum temperatures for fecundity, birth rate and survival differed. A similar pattern has now been demonstrated for *G. bullatarudis*, together with transmission behaviour being affected by temperature, with more worms migrating into the water film at higher temperatures.

With regard to the incidental infections that arose out of the host specificity studies, again these have generated interesting findings. Gyrodactylids observed on the zebrafish (*Danio rerio*) were on first observations, thought to be an accidental aquarium transfer, however subsequent ecological, morphological and molecular work have confirmed the existence of two new species (see Chapter 6). Although temperature was likely to be the major contributing factor to the persistence of long-term infections of *G. lomi* (its first recorded incidence in the UK) in isolated chub (*Leuciscus cephalus*), this phenomenon had not been previously observed in other temperate species (see Chapter 7). Mechanisms by which this parasite, avoids being eliminated by the host's immune response for such a considerable amount of time needs to be elucidated.

The findings of the second central theme of this thesis, local adaptation, question the validity of local adaptation theory. In contrast to the situation in Norway where Atlantic salmon stocks have proved to be highly susceptible to infection with a novel parasite, *G. salaris*, such a scenario is unlikely to occur with sticklebacks. Hebridean fish stocks were able to mount as effective an immune response as their mainland counterparts to infection with *G. gasterostei* and

importantly, that sticklebacks have a much longer immunological memory of infection than previously considered. Although, no evidence of local adaptation was found occurring in this system, but rather a pattern of local differentiation in susceptibility and resistance, that is not to say local adaptation does not occur for gyrodactylids. However, this thesis highlights that establishing this empirically for *Gyrodactylus* is problematic due to the highly dynamic nature and complexity of host-gyrodactylid interactions. This has been graphically demonstrated in this work by the over-riding effect of temporal variation detected in the control population (Cardiff). Therefore, local adaptation is unlikely to be detected in this particular host-gyrodactylid combination as it would appear that any such findings would be masked by temporal variation. Furthermore, it questions the definition of local adaptation; typically it is defined as a product of the environment and genotype. However, most studies are carried out in the laboratory and thus the environment is immediately homogenised, so can such results actually be described as local adaptation? In addition, there is also the confounding factor caused by the effect of parasite culturing. It is therefore proposed that local adaptation theory is best suited to simple organisms and may have no context in regard of vertebrate host-parasite systems due to the increasing complexity of such interactions.

9.2 Proposals for future work

This thesis has generated a wealth of data on gyrodactylids and has highlighted a range of areas for further research. In the first instance, there are 2 appendix chapters within this thesis that describe incomplete data sets which can be built upon. Studies on pooled infections of *G. turnbulli* on atypical hosts indicate that it can infect and reproduce on such hosts, particularly in the absence of its optimal host, the guppy. The aim of further studies would be to ascertain whether *G. turnbulli* can host switch to atypical hosts. This could be achieved by hybridising current cultures of *G. turnbulli* to increase genetic variation and then culturing this hybrid strain on stocks of *Poecilia sphenops* and *Xiphophorus hellerii*. Samples of worms could be removed periodically for morphological and molecular analysis to detect changes in adaptation to a new host and reciprocal cross infections could be carried out. The results of these experiments would hopefully shed light as to whether *G. turnbulli* can host switch (strains cultured on *P. sphenops* and *X. hellerii* would perform less well on *P. reticulata*) or whether its ability to infect and reproduce on new hosts is a plastic response (no differences in infectivity or population growth). With respect to transmission differences between *G. turnbulli* and *G. bullatarudis*, initial data from live host experiments could not detect any significant differences between these parasite

species. However, the time interval between observations was probably too long. Further work would aim to increase sample sizes and to carry out further experiments on interactions between parasitised guppy cadavers and transmission to naïve live hosts. The aim of such work would be to assess whether differences in virulence between these two parasite species can be explained by their transmission modes.

Typically, studies on host-gyrodactylid infections are carried out using a single host-single parasite model system but of course, co-infections commonly occur in the wild. Previously, Rohde (1977, 1979) and Morand *et al.* (2002) suggested that there was no evidence of intra- and interspecific competition in terms of niche width for monogeneans. However, by shedding light on the differences in biology of *G. bullatarudis* and *G. turnbulli,* the current study suggests competition may occur between these two species. This could be empirically tested by undertaking co-infections to determine whether one species does control the population dynamics of the other.

Research is warranted to ascertain the presence of *G. turnbulli* on the most suitable surrogate hosts identified by this thesis, namely *Poecilia sphenops* and *Xiphophorus hellerii* in the wild. The parasite strain used for the host specificity studies has been in serial passage for the last 10 years and relaxed selection pressures for this strain have probably resulted in increased virulence (Cable and van Oosterhout, 2007). Therefore, fieldwork is needed to ascertain the occurrence of *G. turnbulli* in the wild on atypical hosts and ideally reciprocal cross infection experiments should be undertaken with wild strains.

With regards to local adaptation theory, this thesis has highlighted that demonstrating its occurrence for *Gyrodactylus* is problematic. Furthermore, *Gasterosteus aculeatus* is not the most suitable host for testing this theory given the problems of interconnecting waterways that occur in the UK (see Chapter 8).

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