The biological and behavioural basis of host selection in the transmission of *Gyrodactylus* (Monogenea)

A thesis presented for the degree of
Doctor of Philosophy

By

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A mis Papas

Por darme siempre una actitud postiva y constructiva en mi vida, y todo su amor

To my Parents

for giving me a continuous constructive attitude on my life and their love

A mi hermana y hermano

Cuya existencia ha sido mi mayor soporte y alegria

To my sister and my brother,

who their existence has been my major support and happiness

To Christos

With all my innermost love and gratitude,

for his encouragement and support during this *wee* Scottish adventure.

στον Χρήστο

Με όλη την αγάπη και την ευγνωμοσύνη για την συμπαράσταση και την υποστήριξή του κατά την διάρκεια της μίκρης μου περιπέτειας στην Σκωτία,

Σ' αγαπώ πολύ!

"Try not to become a man of success but rather to become a man of value."
Albert Einstein

"In any situation, the best thing you can do is the right thing; the next best thing you can do is the wrong thing; the worst thing you can do is nothing"
Theodore Rossevelt

"Judge your success by what you had to give up in order to get it."
Dalai Lama XIV

"I have not failed. I've just found 10,000 ways that won't work"
Thomas Edison

9 In his heart a man plans his course,
but the Lord determines his steps.

Proverbs 16

10 I rejoiced greatly in the Lord that at last you renewed your concern for me.
Indeed, you were concerned, but you had no opportunity to show it.
11 I am not saying this because I am in need, for I have learned to be content whatever the circumstances.
12 I know what it is to be in need, and I know what it is to have plenty. I have learned the secret of being content in any and every situation, whether well fed or hungry, whether living in plenty or in want.
13 ***I can do all this through him who gives me strength.***

Philippians 4

1 The Lord is my shepherd, I shall not be in want.
2 He makes me lie down in green pastures, he leads me beside quiet waters,
3 He restores my soul. He guides me in paths of righteousness for his name's sake.
4 Even though I walk through the valley of the shadow of death, I will fear no evil,
for you are with me; your rod and your staff, they comfort me.
5 You prepare a table before me in the presence of my enemies.
You anoint my head with oil; my cup overflows.
6 Surely goodness and love will follow me all the days of my life,
and I will dwell in the house of the Lord forever.

Psalm 23

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All my friends and family in *mi Mexico lindo y querido*,
And to my grandparents who passed away during my PhD.

~ . ~

My heart is filled with thankfulness
To Him who bore my pain
Who plumbed the depths of my disgrace
And gave me life again
Who crushed my curse of sinfulness
And clothed me with His light
And wrote His law of righteousness
With power upon my heart

My heart is filled with thankfulness
To Him who walks beside
Who floods my weaknesses with strength
And causes fears to fly
Whose every promise is enough
For every step I take
Sustaining me with arms of love
And crowning me with grace

My heart is filled with thankfulness
To Him who reigns above
Whose wisdom is my perfect peace
Whose every thought is love
For every day I have on earth
Is given by the King
So I will give my life my all
To love and follow Him

Keith Getty & Stuart Townend

Declaration

I hereby declare that this thesis has been composed by myself and is the result of my own investigations. It has neither been accepted, nor submitted for any other degree. All sources of information have been duly acknowledged.

Signed:

Date:

Abstract

The ectoparasitic monogenean fluke, *Gyrodactylus salaris*, is a parasite known to be highly pathogenic to Atlantic salmon (*Salmo salar*). Although present in the environment of several neighbouring European countries, the UK is thought to be *G. salaris*-free, but, if national contingency plans to control this parasite are to be effective, it is vital that we understand the factors underlying its transmission from host to host. This study demonstrates that the majority of parasites transferring to new hosts are mature parasites that have reproduced at least once. Since, exploration and host transfer strategies pose a risk to survival; the parasite will endeavour to pass on its genes before attempting to transfer from one host to another. This study has also shown that when pregnant parasites are forced to leave their hosts, their offspring are aborted prematurely to ensure the survival of the mature parasite.

Gyrodactylids do not possess a free-swimming stage in their life cycle, which allows for their migration between hosts. In spite of this, they are able to rapidly colonise naïve hosts, even in non-shoaling populations of fish. This study investigates the transmission strategies employed by detached parasites in the colonisation of new hosts. Observations of gyrodactylids collected from 3-spine sticklebacks, *Gasterosteus acuelatus*, suggest that their activity increases as a stickleback approaches, alerting the host to its presence. The parasite is then ingested directly by the prospective host. A time series of experimental exposures and specimens prepared for Scanning Electron Microscopy (SEM) suggest that once ingested, the parasites attach to the lining of the buccal cavity and then migrate out to their preferred colonisation site on the outer surface of the fish. It is proposed that this may be an alternative route for host infection. Similarly, direct ingestion by the scavenging on

infected hosts by 3-spine sticklebacks suggests another route of infection of new hosts. Although these routes of transmission may be of lesser significance, infections in the buccal cavity may be an important indicator for detection of infection and those personnel involved in screening fish for gyrodactylids should be aware that this is an area in which infections can occur. This study also demonstrated that the use of the anaesthetic 2-phenoxyethanol does not affect the number of gyrodactylids which leave the host to colonise a new host.

Additionally, observations of the transmission process suggest that turbulence produced by the movement of the fish's fins may facilitate the transfer of detached parasites from the substrate. While this hypothesis appears to be supported by video evidence and photographic stills gathered throughout the duration of this study, further work should be conducted using particle tracking techniques to determine the efficacy of using a vortex effect as a means of colonising new hosts.

Field sampling processes may have an effect on this type of research, giving rise to problems with the accurate diagnosis, management and control of gyrodactylids in a variety of fish. *Gyrodactylus* infected specimens of 3-spine stickleback (*Gasterosteus aculeatus* L.), minnows (*Phoxinus phoxinus* L.) and stone loach (*Barbatula barbatula* L.) from one Scottish river were cohabited. The study found that small numbers of *Gyrodactylus* do transfer to atypical hosts. This study highlights that personnel involved in fish disease surveillance programmes should be aware of the consequences of transporting multiple species in the same transport vessel as gyrodactylids may infect species previously thought to be resistant. Equally, diagnosticians should be aware of the fact that atypical species may act as temporary hosts and that their gyrodactylid fauna should not be assumed.

Non-feeding life-cycle stages, such as the dispersal stages of parasites, are dependant for survival upon finite energy reserves gathered during feeding phases. Thus, those individuals with more limited reserves will die sooner and consequently have less time available to find a new host once detached. At this stage, the principal energy reserves in gyrodactylids are stored as large lipids droplets.

Confocal laser scanning microscopy (CLSM) has been used to investigate the distribution of lipid droplets in *Gyrodactylus*, which have migrated off their fish host, testing the hypothesis that these droplets function as a proxy for the nutritional state. This study, demonstrated that the lipid droplets were particularly associated with the gut and that there is a significant variability in the volume of stored lipid carried out by each individual. Transmission Electron Microscopy (TEM) showed that gyrodactylids carry lipid droplets at all stages of their life cycle, including at release from the birth pore. It is likely that transferring worms require stored energy reserves to survive in the event of failure to establish contact with a new host. These reserves could allow the parasite to survive without a host for several days.

As gyrodactylids appear to respond to a range of stimuli including vibration and chemicals released from the host, the presence or absence of such cues may have consequences on the rates of *Gyrodactylus* transmission. If these chemical stimuli can be identified and then mimicked or blocked, then this may offer potential opportunities for the control of gyrodactylid behaviour and for disrupting their transmission to new hosts. Baseline gyrodactylid behaviour, in the absence of a host, was determined under white light and infrared. This was achieved using a specially constructed arena and purpose written image analysis software to analyse parasite movement under different lighting conditions. The study found that gyrodactylids

were more active in the dark than in light conditions, typically displaying longer, more sinuous tracks under red light than under white light.

To begin investigating the effect of chemical presence on gyrodactylid behaviour, the activity of octopaminergic agonists and antagonist which bind to muscle receptors and alter muscle activity, were assessed. The impact of octopamine hydrochloride, clonidine hydrochloride, amitraz and, a toxic reference, chlordimeform, over a range of concentrations (0.2 to 3.2µM/L) were assessed on gyrodactylid behaviour. All of the four chemicals affected *Gyrodactylus* and produced muscle tetanus, causing muscle spasms when extension was attempted. Prolonged exposure resulted in death. Only the highest concentration of chlordimeform, the toxic reference, affected 100% of *Gyrodactylus* after 24 hours. After 48 hours, all of the *Gyrodactylus* treated with chlordimeform were either affected, moribund or dead.

Amitraz was more toxic than chlordimeform with 80% of *Gyrodactylus* being dead after 24 hours at the highest concentration. After 48 hours 100% of *Gyrodactylus* exposed to 3.2 µm/L amitraz were dead, and up to 80% were dead in those exposed to lower concentrations; with no parasites being left unaffected. Although these particular compounds are toxic to fish, the effect of these agonistic chemicals on *Gyrodactylus* behaviour and survival is interesting and suggests that a closely related compound that is safe for use against fish may offer a potential treatment for the control of *G. salaris* infections in rivers.

An ultrastructure study was undertaken to contribute to the current understanding of gyrodactylid ultrastructure. The findings of this research require broad understanding of gyrodactylid behaviour for their interpretation. Photographic evidence was gathered using transmission and electron microscopy. From these results, it is clear that *Gyrodactylus gasterostei* on a three-spine stickleback host will

respond to a range of stimuli (*i.e.* vibration or chemical cues released from the host) in their assessment of host suitability. This study illustrated for the first time a chemical sensory structure found in *Gyrodactylus gasterostei*, located close to the cephalic lobe. It also identified apparently ciliated photoreceptors; as well mechanoreceptors in this species.

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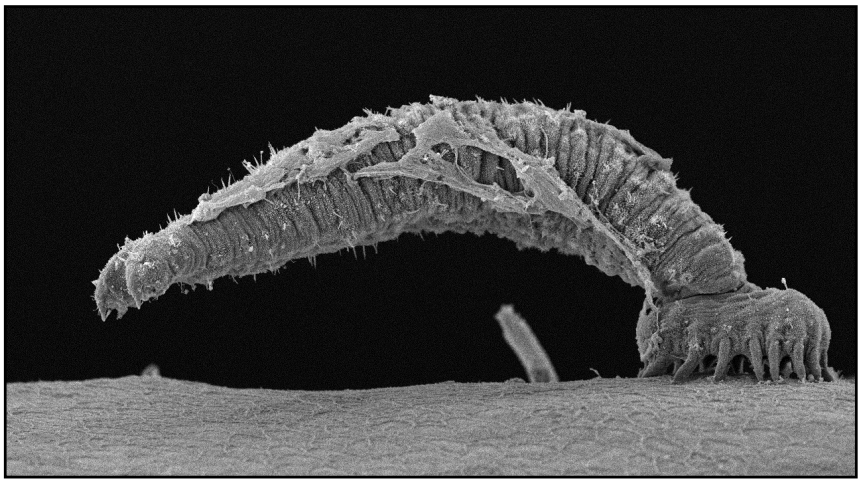
Chapter 1

General introduction



Gyrodactylus gasterostei Gläser, 1974 on its host a three-spine stickleback *Gasterosteus aculeatus* L.

1.0 General introduction

This introduction provides an overview of the current knowledge on the genus *Gyrodactylus* von Nordmann, 1832 focusing on the host-parasite system *Gyrodactylus gasterostei* Gläser, 1974 on the three-spine stickleback *Gasterosteus aculeatus* L. as a model for transmission and host-parasite interaction in this genus.

The most well characterised gyrodactylid in the last few decades has been without a doubt *Gyrodactylus salaris* Malmberg, 1957 which to a great extent has been a result of its impact on commercially important European stocks of salmon. Massive mortalities attributed to *G. salaris* are described in wild Norwegian Atlantic salmon, *Salmo salar* L., stocks with accompanying economic losses (Johnsen & Jensen 1986; Johnsen, 2006; Bakke *et al.,* 2007).

The following introduction starts with an examination of a range of different aspects of this gyrodactylid species, whose biology has been extensively described in the scientific literature since the early 1970s. The following sections will provide a general overview of current knowledge regarding transmission and host-interactions in *Gyrodactylus* spp. (Monogenea) and their devastating effects in aquaculture. Worldwide, with intensifying aquacultural activities, places such as fish farms, allow the unique colonisation abilities and pathogenicity of gyrodactylids to produce major disease epidemics since the high density of fish in such environments makes conditions for spreading pathological agents ideal (Kearn, 2004). Here, parasites can rapidly reproduce and reach high numbers and, if left untreated, they can lead to severe mortalities. Fish farming in Scotland is a major industry bringing in millions of pounds to the economy each year. In an article published by Fish News it was mentioned that "*Scotland seeks a share of the expanding global seafood market*" explaining that **"***Scottish seafood exports are estimated to be worth around £500*

million, representing more than 62% by value of total Scottish food exports (£805 million)" (see www.fishnewseu.com, Tuesday 27th April 2010).

Scotland is the world's second biggest producer of farmed salmon, exporting to more than 60 international markets (www.fishnewseu.com, Tuesday 27th April, 2010). Global seafood consumption grew from 137 million metric tons in 2006 to 140 million metric tons in 2007 and 143 million metric tons in 2008, according to a report from the United Nations' Food and Agriculture Organisation (FAO) published (www.fishnewseu.com, Tuesday 27th April, 2010).

The Scottish Salmon Producers' Organisation (SSPO) said: "*The seafood sector is Scotland's largest food export and a major supplier to global markets. Scotland is the only significant producer of farmed salmon within the European Union where it holds a reputation for high quality*".

In the UK, to date, *G. salaris* has not been detected, however, it is important that monitoring and prevention policies are in place, especially with fish movements and commercial trading all over the world. The potential introduction of this species could cause havoc to both the U.K's wild and farmed salmon, which are known to be susceptible to *G. salaris* (Bakke & MacKenzie, 1993) and to rainbow trout (*Oncorhynchus mykiss* Walbaum) which are also susceptible to a different species, *Gyrodactylus derjavini* Mikailov, 1975 [*sic G. derjavinoides* Malmberg, Collins, Cunningham *et* Jalali, 2007] (Buchmann & Uldal, 1997). However, Bakke *et al.* (2002) noted that European movements of *O. mykiss* should be more carefully monitored as it is a possible host for *G. salaris*.

1.1 Aquaculture overview

Aquaculture has been defined in a number of different of ways *e.g.* the Food and Agriculture Organisation of the United Nations (FAO) defined it as "*activity which comprises diverse systems for farming plants and animals in inland, coastal and marine areas, using and producing a wide variety of animal and plant species*" (FAO, 2009).

New sources of nutrition are needed to satisfy increasing demand. Fish meat which has a high protein content and is considered one of the most important farming products, providing a high-quality meat resource with good flavour, leading to high consumption throughout the world (Castillo, 2003). Many developing countries export fishery products such that aquaculture provides an important economic contribution. Aquaculture activities are growing so rapidly, more than any other animal food-producing sector. This fact may be demonstrated by comparing the latest data published by FAO (2009) which shows that in the 1950s, aquaculture was less than 1 million tonnes, however, production in 2006 was reported to have increased to 51.7 million tonnes, with a value of US $ 78.8 billion.

It is suggested that aquaculture also plays a major role in terms of food self-support *e.g.* in some Asian countries, where the major proportion of production especially freshwater species, are destined for domestic consumption. Aquaculture production and practice has extended globally, involving more marine and freshwater species, generally in more recent years with species which are easier to handle and are of high-value. The more fish are reared in tanks, ponds, or land-based tanks the more likely it is that such systems provide an opportunity for diseases or chronic infections associated with different pathogens to develop (Kearn, 2004).

1.2 Monogenea

Members of the Class Monogenea or monogeneans are ectoparasites of aquatic vertebrates, generally fish. They inhabit any external surface, such as the skin or gills of fishes (hence the colloquial term; skin or gill flukes) which affects their growth and may cause mortalities which lead to economic losses.

The life-cycle of monogeneans is direct with no intermediate host necessary for their transmission. These parasites can be a serious problem when there is a high population density of the host (*e.g.* in culture systems or some wild stocks *i.e. G. salaris* on Norwegian wild salmon; see Bakke & Harris, 1998). The most characteristic structure of the Monogenea is a major attachment organ called the opisthaptor. The morphology and armature of this organ is also important in the taxonomy of the class. Many monogeneans are responsible for serious impairment of fish health and welfare. Parasitic Monogenea are very successful in the colonisation of new hosts even under conditions when direct host-to-host contact is not possible. There is, however, a lack of basic understanding of the mechanisms of transmission, host finding behaviour and of the cues prompting dispersal and host seeking in monogenean groups. Gyrodactylids for example, which infect a wide range of aquaculture species, causing significant mortalities in hatcheries and in the wild.

Helminthological studies in wild animals are important because they lead to a better understanding of the behavioural links between the host and parasite in nature. This knowledge may help in avoiding epizootic diseases in other ecosystems. When wild animals are introduced for aquaculture practices, it is necessary to consider a range of strategies for reducing parasites. When looking at transmission strategies, for example, it is important to consider the life-cycle and to recognise periods where

prompt intervention may allow for some control of the infection and a reduction in parasite numbers (Lamothe, 1967).

Movements of fish from region to region for fish culture may accidentally introduce new parasites to local populations with possible disastrous results, as occurred in Norway (Bakke *et al.,* 2002).

1.3 Importance of salmonids to world aquaculture

Salmonids, particularly *Oncorhynchus* species and *S. salar* have a high market value, because of their high quality meat, which displays good texture and flavour. The FAO (2009) positioned Norway and Chile as the world's two leading producers of cultured salmonids although more recent figures now places Chile in third position behind Scotland following a series of disease outbeaks throughout the Chilean industry (www.fishnewseu.com, Tuesday 27th April 2010). World trade in cultured salmonids has increased strongly, the reason being the intensification in salmon and trout aquaculture in Northern Europe and in North and South America. Demand for farmed salmon is increasing gradually year by year, with new markets opening up in both developed, and in developing countries (www.fao.org, April 2010).

1.4 Aquaculture production in the United Kingdom

The United Kingdom provides an excellent example of the wider global aquaculture expansion. According to data provided by FAO (2009), UK fish production consists mainly of the marine culture of *S. salar* and the blue mussel *Mytilus edulis* L. The British production of salmon in 2006 was 0.137 million tonnes with an estimated value of £450 million, which represented 22% of total UK fish production.

According to a recent survey published by the Scottish Salmon Producers Organisation (SSPO) (www.scottishsalmon.co.uk, June 2009), Scotland's salmon farmers contributed £500 million to the economy in 2008. The same organisation in April 2009, suggested that Scotland was categorised as the second largest salmon producer in the world with a worldwide retail value of Scottish farmed salmon being over £1 billion. This data is supported by the Review of Current Trends in the Scottish Salmon Farming Industry', Highlands & Islands Enterprise (www.fishnewseu.com, 27th April 2010).

1.5 Aquaculture worries regarding monogenean flukes

Kearn (2004) remarked that teleost fish are the most abundant of the vertebrates and most aquatic habitats on the planet have been colonised by them. Because of this, their abundance and diversity may be matched by the parasites present on them through a process of co-evolution. According to Whittington (1998), most monogeneans are host specific with around 4000 species of Monogenea having been described to date.

The skin, gills and oral cavity (Fig. 1.1) are the principal surfaces of fish that are in contact with water, offering particularly favourable conditions for the establishment and survival of parasitic animals *i.e* epithelial cells, as food, is much more accessible.

There is no doubt, that infections by parasites have major consequences for species of fish in culture and must therefore be considered as a fundamental factor within any system of aquaculture. Bakke *et al.* (2002) remarked that salmonid gyrodactylids appear to infect more hosts than those associated with cyprinids. Some of these gyrodactylids infect two or more closely related hosts *e.g.* i) *G. salaris*

Malmberg, 1957 is recorded from 12 hosts; ii) *G. alviga* Dmitreva *et* Gerasev 2000 reputedly infects 15 hosts, and, iii) *G. arcuatus* Bychowsky 1933 infects 6 hosts (data obtained from www.gyrodb.net, 2010). Also some fish species are host to numerous *Gyrodactylus* species. The minnow, *Phoxinus phoxinus* (L.), for example plays host to 12 species (www.gyrodb.net, accessed May 2010; see also Chapter 5).

At least 12 different fish are known to act as a reservoir host for *G. salaris*. These hosts include *Coregonus lavaretus* (L.)*, Gasterosteus aculeatus* L.*, Phoxinus phoxinus, Pungitius pungitius* (L.)*, O. mykiss, Salmo salar* L.*, Salmo trutta* L.*, Salmothymus obtusirostris* (Heckel)*, Salvelinus alpinus* (L.), *Salvelinus fontinalis* (Mitchill)*, Salvelinus namaycush* (Walbaum) and *Thymallus thymallus* (L.) (www.gyrodb.net, accessed May 2010).

The introduction of *G. salaris* into Norway is believed to have been via the importation of infected juveniles from Swedish hatcheries (Johnsen, 2006; Bakke e*t al.,* 2007). There is, therefore, an emphasis on regulating the commercial movement of salmonids between countries to ensure that the likelihood of moving *G. salaris*-infected stock is minimised and that there is no further spread of *G. salaris* throughout Europe.

1.6 The biology of species belonging to the genus *Gyrodactylus*

The genus *Gyrodactylus* von Nordmann, 1832 includes in excess of 410 species, with these parasites being found on the skin and gills of their hosts (Figures 1.1-1.2). *Gyrodactylus* species are principally parasites of brackish, marine and freshwater fish worldwide (Bakke *et al*., 2007) but a small number of species are also known to parasitise amphibians (Harris *et al.,* 2004). These parasites are among the smallest monogeneans, ranging in size from 0.4 to 0.8 mm in total body length (Kearn, 2004).

The key feature of this genus is the viviparous mode of reproduction used by species, which allows for the exponential growth in population numbers. Both new born daughters and mature adults are capable of sexual reproduction. Bakke *et al*. (2007) in their review of the genus describes the viviparous capabilities of this parasite in detail and describes the invasive nature of this group to this characteristic.

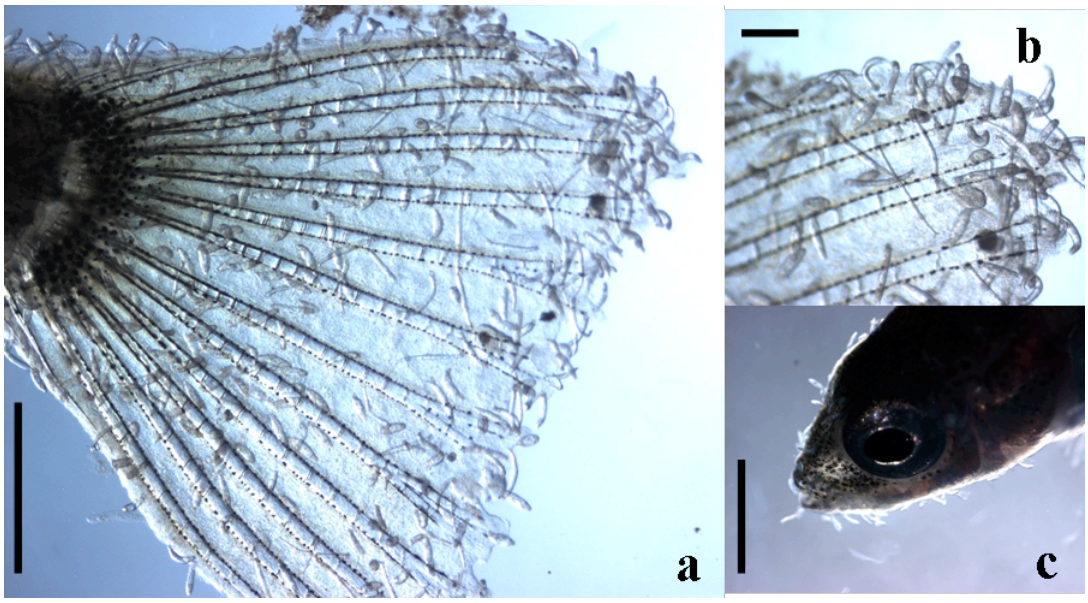


Figure 1.1. Light micrograph illustrating a massive infection of *Gyrodactylus* on the caudal fin of *Gasterosteus aculeatus* L. a) Caudal fin. Scale bar: 2 mm; b) Part of the caudal fin. Scale bar: 5 mm. c) Head of an infected *G. aculeatus*. Scale bar 5 mm.

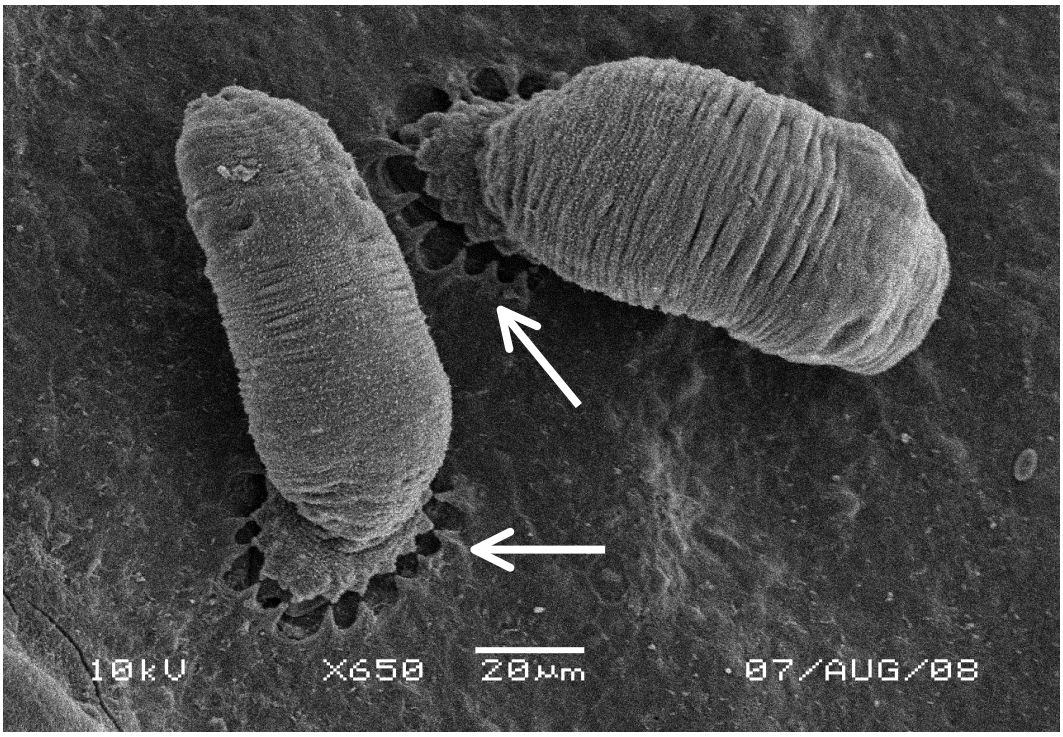


Figure 1.2. Scanning electron micrograph of two *Gyrodactylus gasterostei* Gläser, 1974 attached to the skin of their *Gasterosteus aculeatus* L. host. Note the tissue which has been pulled up by the contraction of the marginal hooks (arrowed).

Infection of new hosts can occur through direct or indirect transmission. Direct transmission occurs when an infected host makes direct skin to skin contact with another possible fish host *i.e.* fin touching *etc*. Four basic transmission profiles are described by Bakke *et al.* (2002), these being (i) contact with live hosts, (ii) contact with a dead host, (iii) by detached parasites drifting in the water column, and, (iv) by parasites attached to the substrate. In addition to these, Olstad *et al*. (2006) suggested cannibalism as another possible route of infection. El-Naggar *et al.* (2004) suggests that transmission can occur by species of *Gyrodactylus* that are able to directly swim towards and infect a host although there is some disagreement as to the validity of this observation (A. Shinn personal communication).

1.6.1 Attachment and feeding

Gyrodactylus attach to fish by means of a terminal specialised attachment organ, the opisthaptor which is equipped with two sharp, centrally positioned hooks called hamuli and an array of 16 peripherally distributed hooks (Figure 1.3).

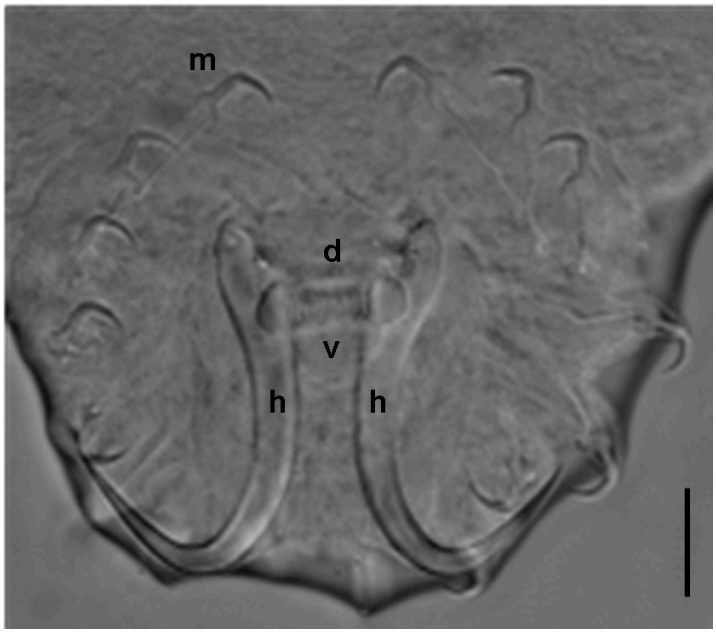


Figure 1.3. The opisthaptor of *Gyrodactylus anguillae* Ergens, 1960 parasitising the European eel, *Anguilla anguilla* L. The opisthaptor consists of two centrally positioned large hooks or hamuli (h) joined by two connecting bars, a simple dorsal bar (d) and an approximately triangular shaped ventral bar (v). There are 16 marginal hooks (m) positioned around the periphery of the opisthaptor. Scale bar = 10 μm.

The differentiation of *Gyrodactylus* species has traditionally been based on morphological studies using subtle but consistent differences in the shape of the attachment hooks (hamuli, marginal hooks, ventral and dorsal bars) to separate species (Shinn *et al.,* 1993). The parasite can also temporarily attach to its host by fixing its anterior extremity, the prohaptor, which consists primarily of two cephalic lobes, which produce a sticky secretion and the pharynx.

When *Gyrodactylus* feeds, it inverts its pharynx through its mouth and releases a digestive solution containing proteolytic enzymes *i.e* proteases and lysozyme (Buchmann & Bresciani, 1998) which act to break down the fish skin. Mucus and dissolved skin are then sucked into the gut. This feeding activity can result in small lesions in the fish skin (Cable & Harris, 2002; Bakke *et al*., 2007). A few parasites do not represent any problem for an otherwise healthy fish, but the presence of large numbers of parasites *e.g*. up to 10,000 *G. salaris* which were found on a single *S. salar* parr (Jensen & Johnsen, 1992) may cause death through disruption of normal osmoregulatory function. Extensive damage is usually only seen in higher intensity infections. The osmotic stress (*i.e.* the loss of body fluid and electrolytes) created by the perforation of the epidermis by parasite feeding is likely to be a direct cause of death, even in aquaria (Mo, 1991; Cunningham, 2002). The lesions generated by feeding activity which can extend to the dermis and the penetration of the marginal hooks, allow potential secondary pathogens *e.g.* various fungal and bacterial agents, to invade and cause infection that may compromise the health of the fish.

The prohaptor (anterior zone) consists of two prominent cephalic lobes (Figures 1.4, 8.1, 8.2) including attaching adhesive glands and sensory structures, such as the prominent spike sensilla. Lyons (1969) undertook a detailed investigation of these describing some of the fine structure and function of these sense organs which included tango- / rheoreceptors. A detailed examination of the distribution of the sensory organs might provide useful information and further insights into the stimuli and information processed by parasites prior to their transmission to a new host.

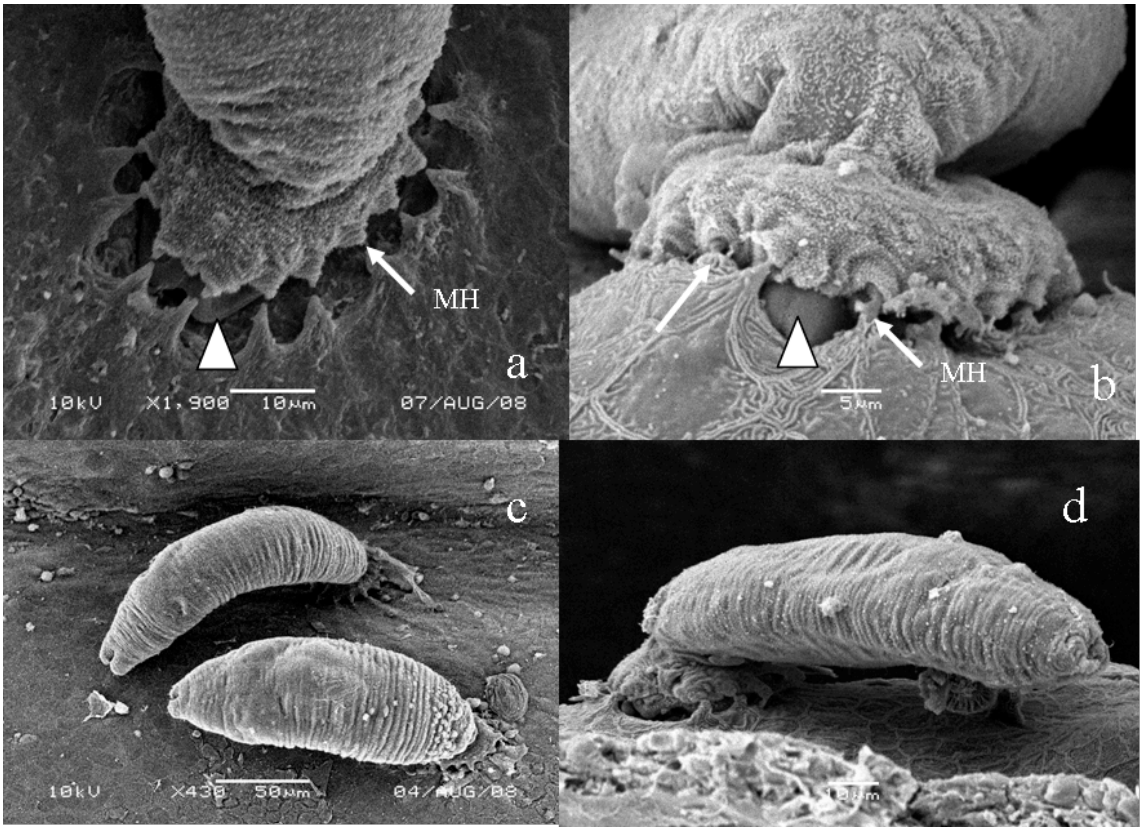


Figure 1.4. Scanning electron micrographs of *Gyrodactylus gasterostei* Gläser, 1974 attached to the skin of its *Gasterosteus aculeatus* L. host. a-d) The marginal hooks are the principal means of attachment. Their action pulls the epithelium up (arrowed) whilst the centrally positioned hamuli clasp the epithelium by clamping a piece of epithelium between them (arrowhead).

The shape of the skeletal elements of the opisthaptor, the marginal hooks, the hamuli and the dorsal and ventral bars are important in the taxonomic identification of species. Of these, the marginal hooks are the principal means of attachment whilst the hamuli provide a system preventing accidental dislodgement and assist the action of the marginal hooks by elevating the roof of the opisthaptor transferring tension through the marginal hooks (Shinn *et al.,* 2003). Two accessory bars, the ventral and dorsal bars, function to stabilise and coordinate the movements of the hamuli (Shinn *et al.,* 2003).

1.6.2 Viviparity

Gyrodactylids as monogenean flukes have direct life-cycles, are viviparous and are capable of rapid increase in numbers. Gyrodactylids possess no specific transmission stages as do the other monogeneans and do not lay eggs but give birth to full sized living individuals. Harris (1993) described this mode of reproduction as being fairly unique, allowing rapid population growth on their host and conferring an ability to transfer to a new host at all times during their life-cycle. Aphids (Hemiptera) like gyrodactylids reproduce parthenogenetically, being able to produce their young at a rapid rate (McGavin, 1993).

Gyrodactylids lack a free swimming larval stage or oncomiracidium which is present in egg-laying monogeneans, but instead have developed other highly successful reproductive strategies.

Gyrodactylids have responded to the pressure for increased reproductive output in unique ways. Noticeable studies regarding gyrodactylid reproduction have been undertaken by Cable & Harris (2002), Bakke *et al.* (2002) and reviewed in Bakke *et al.* (2007). These authors indicate that before the daughter is born, a second embryo begins to develop inside the daughter and in some species a third embryo appears within the second. Thus one individual may "encapsulate" two or three further generations and when the first of these generations is born, the second generation within it has already begun to develop. For this reason these parasites have been likened by some authors (Bakke *et al.,* 2007) to "Russian dolls" (Figure 1.5).

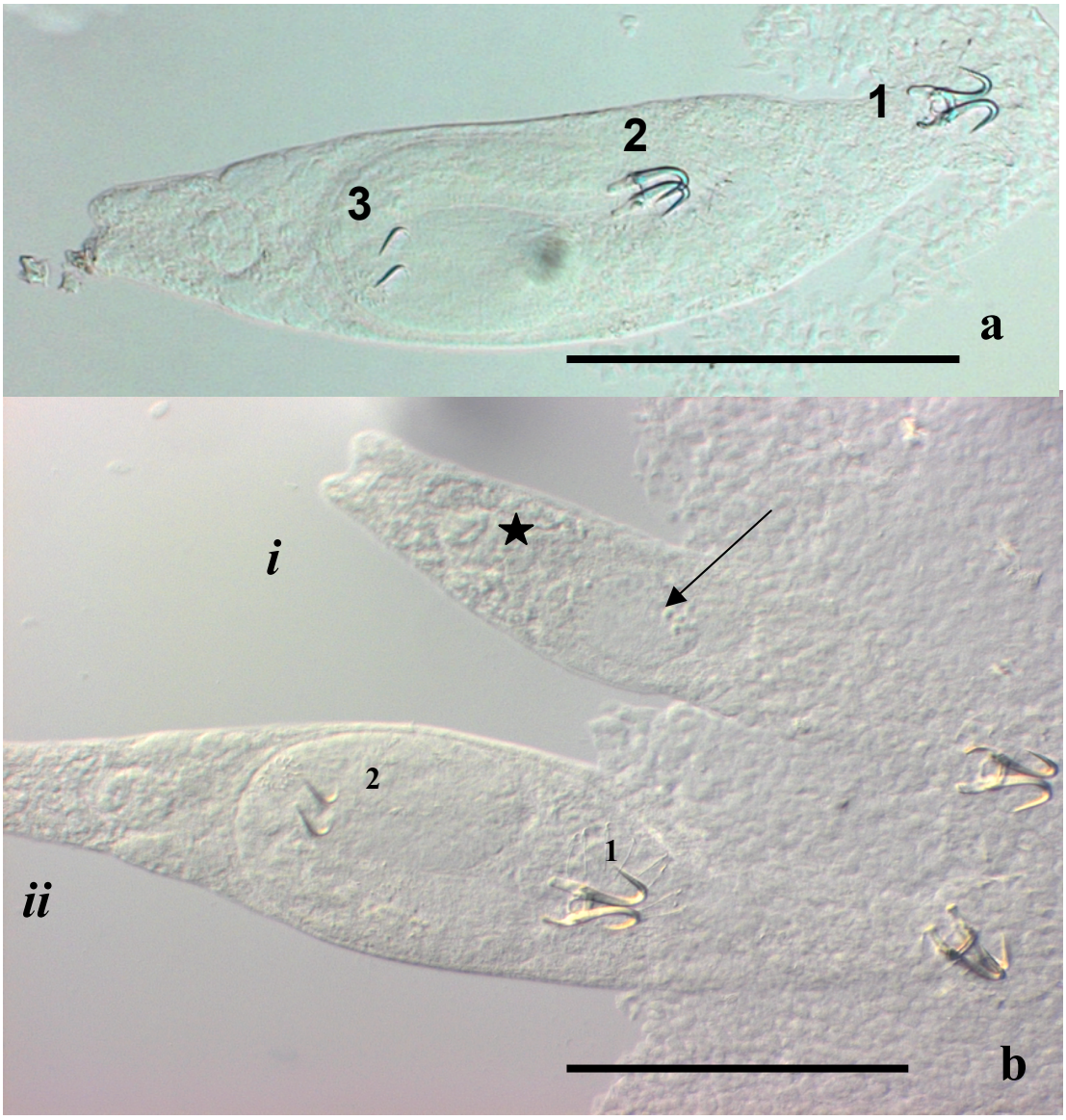


Figure 1.5. Light micrographs of *Gyrodactylus gasterostei* Gläser, 1974. a) An individual (1) containing a fully grown daughter *in utero* (2) with another daughter within her (3) - nested like "Russian dolls"; b) An individual (i) showing an empty uterus (arrowed) and pharynx (star); ii) individual showing the presence of a daughter and granddaughter (1 and 2) *in utero*. Scale bar = 0.5 mm.

The first born daughter develops from a cluster of cells within the uterus of the parent (asexual reproduction). Once this daughter *in utero* is fully-developed and ready to be born, the birth pore opens allowing the worm to escape, this worm having

the same dimensions and form as the mother. After having given birth, the mother displays a characteristic body shape which is compressed or pinched in the middle (Figure 1.6). The following daughter generation will develop from oocytes in days (Harris, 1985; Cable & Harris, 2002). This mode of reproduction (hyperviviparity) found in gyrodactylids separates these platyhelminths from other oviparous taxa and allows for "exponential" growth in number following infection. Thus, when a host is infected with one or a few parasites, numbers can subsequently increase rapidly (Harris, 1980, 1993).

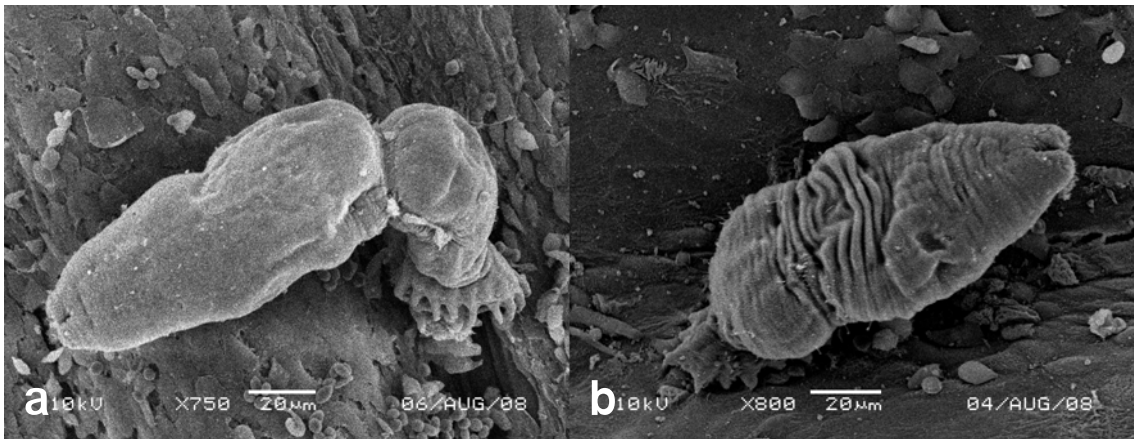


Figure 1.6. Scanning electron micrographs of *Gyrodactylus gasterostei* Gläser, 1974 attached to the skin of its *Gasterosteus aculeatus* L. host. a) and b) a mother worm having recently given birth and showing a typical compressed middle body region and or wrinkling of the tegument.

Bakke *et al*. (2007) described the "female" and "male" reproductive system of gyrodactylids. Gyrodactylids are suggested to divert resources from their own reproductive system into the development of their embryos, delaying the appearance of the male reproductive system until after the first birth daughter and the commencement of development of the second daughter (Harris, 1985). The development of the second daughter occurs by parthenogenesis. In the next

generation, development may be by sexual reproduction (after the appearance of the male copulatory organ MCO) or parthenogenetically. In gyrodactylids, the female reproductive system develops first, this being is known as protogyny, and differing from the protandry found in other hermaphrodite monogeneans where the male system is the first to develop (Kearn, 1994). There are no vitellaria present because gyrodactylids do not produce eggs, but the lipid reserves are retained sources from those of the mother.

After the development of the daughter and its birth, the MCO appears and is functional for this purpose (Figure 1.7). The MCO is a spherical and muscular organ with a series of spines which vary in size and number and are important for the taxonomic differentiation of species (Harris, 1985).

1.6.3 Transmission

As parasites, gyrodactylids are forced to employ a range of "successful" strategies in order to reach their target, a new fish. During this process of transmission or colonisation, gyrodactylids appear to display and adapt to the behaviour of the hosts using a wide range of behaviours in their transmission (Bakke *et al.,* 2002). One of the first descriptions of a specific migratory behaviour that facilitates transmission of a gyrodactylid from dead hosts was detailed by Cable *et al*. (2002). These latter authors described the method by which *Gyrodactylus turnbulli* Harris, 1986 and its hosts, guppies *Poecilia reticulata* (Peters), come into close contact. After death, guppies float at the water's surface, its burden of *G. turnbulli* parasites move off these fish into the water film, hanging motionless with the haptor held by surface tension. Since guppies are surface feeders, detached parasites in the water film are using this host's behaviour to increase the likelihood of contacting a new host. However, the majority

of gyrodactylid species, if they are dislodged from the host's skin host, they sink until they reach a solid surface (Cable *et al*., 2002).

Another interesting case of gyrodactylid behaviour was reported by El-Naggar *et al.* (2004). These authors suggested that *Gyrodactylus rysavyi* Ergens, 1973 was capable of directional swimming by flexing its body, this involving ~4-8 per sec looping contractions in any direction with a speed of ~1.7-5mm/sec and a range of ~15cm distance. This motility was suggested to be an exceptionally efficient infection mechanism with respect to the Nile catfish *Clarias gariepinus* (Burchell) host. By comparison, these authors reported that the closely related *Macrogyrodactylus congolensis* (Prudhoe, 1957) Yamaguti 1963 and *M. clarii* Gusev 1961 does not possess the ability to swim, even though they parasitise the same Nile catfish host.

Behavioural flexibility in gyrodactylids may be important in the transmission process. Parasites that had not yet given birth for the first time were suggested to be less likely to transfer to a new host than worms that had already given birth at least once (Harris, 1993). In dead hosts, however, the behaviour of gyrodactylids changes according to the observations of Bakke *et al.* (1992). Transmission from a dead host appears to be more efficient than from living ones. Olstad *et al* (2006) concluded that parasites that remained on a dead host survived and maintained their infectivity for longer periods than detached worms. According to the same authors, detached *G. salaris* uses a strategy whereby it sits stationary on the substrate at an angle waiting for a potential host and only making occasional circular exploratory movements. It has been suggested that as *G. salaris* uses a variety of transmission strategies, this makes it, potentially, one of the most infectious species of *Gyrodactylus*.

Remaining with a dead host may be a specialised behaviour attributed to a combination of the high risk related to transmission in running water and the

increased likelihood of contacting a new host due to probably feeding from a dead fish carcass as suggested by Olstad *et al.* (2006).

Gyrodactylus species usually have preferred sites on their host which, depending on the transmission strategy they use, facilitate their transmission to new host. Gyrodactylids disperse effectively using a variety of mechanisms, but the most common is most likely through contact between living hosts. The transmission could be by direct host contact, via dead fish (scavenging or food items), transmission also occurs by contact with dead hosts, parasites attached to the substratum and worms drifting in the water column (Bakke *et al*., 1992; Soleng *et al.*, 1999, Cable *et al.,* 2002). In benthic species, such as the 3-spine stickleback, transmission via the substrate is noticeable and can be one of the most important routes of transmission.

Some gyrodactylids are capable of reproducing on several hosts, whilst on others they are unable to reproduce. Nevertheless, the colonisation of a host on which reproduction cannot occur may still play a role in the transmission of the parasite towards its final host (Bakke *et al.,* 1992).

Water temperature, according to Soleng *et al.* (1999), also appears to be an important factor in the transmission rate of *G. salaris.* The transmission rate of *G. salaris* after direct host to host contact was positively correlated with water temperature. This parasite cannot reproduce at salinities higher that 7.5 ‰ and survives only a few days at salinities up to 20‰ but can survive up 5 to 6 days off the fish in a temperature range between 6 and 12 º C (Soleng & Bakke, 1997).

Olstad *et al*. (2006) focused on the transmission strategies of *G. salaris* on dead hosts, as significant reservoirs of infection. Olstad and co-workers assessed the survival and infectivity of detached worms and those removed from dead hosts. In the same study, it was revealed that the transmission was influenced by life span. The

survival rates off the host are typically 1 day at 18^{o}C and 4 days at 3^{o}C for *G. salaris.* Olstad *et al.* (2006) suggested that for parasites on a dead host, the life span is doubled compared with individuals maintained *in vitro* probably as a result of being sustained by feeding on the dead host.

1.6.4 Transmission triggers in *Gyrodactylus*

Little is known regarding the biological basis of gyrodactylid host selection and the factors underlying transmission, particularly with respect to the maturity and nutritional status of gyrodactylids moving to new hosts. Regarding this, Dmitrieva (2003) working on *Gyrodactylus sphinx* Dmitrieva et Gerasev, 2000 and its fish host the Black Sea blenny, *Blennius sphinx* Valenciennes suggested that the attainment of sexual maturity may the trigger for *G. sphinx* to begin migrating off its host and to begin seeking for a new host.

1.6.5 *Gyrodactylus salaris*

In the last few decades, *G. salaris* has gathered considerable attention with respect to the many other known species of *Gyrodactylus*. Although first described in 1957 by Dr Göran Malmberg (Malmberg, 1970), the translocation of salmonid stocks infected with *G. salaris* was not considered to be a risky endeavour. Subsequently, however, the importation of *G. salaris*-infected salmon parr of the Baltic strain of Atlantic salmon were introduced to Norway, which resulted in catastrophic fish mortalities when the parasite transferred to the Atlantic strain of Atlantic salmon (Johnsen, 1978; Johnsen & Jensen 1986; Johnsen, 2006). Unlike the Norwegian Atlantic salmon strains, Baltic salmon strains have not displayed the same high mortalities when exposed to *G. salaris*. Meinilä *et al.* (2004) considers *G. salaris* to be native to the

Baltic area, a theory that is shared by other authors (see Bakke *et al*., 2004; Johnsen, 2006). While, the appearance of *G. salaris* in Norway and elsewhere in Europe is suggested to be through anthropogenic movement (*i.e.* the fish trade) of salmon and rainbow trout populations (Johnsen & Jensen, 1986).

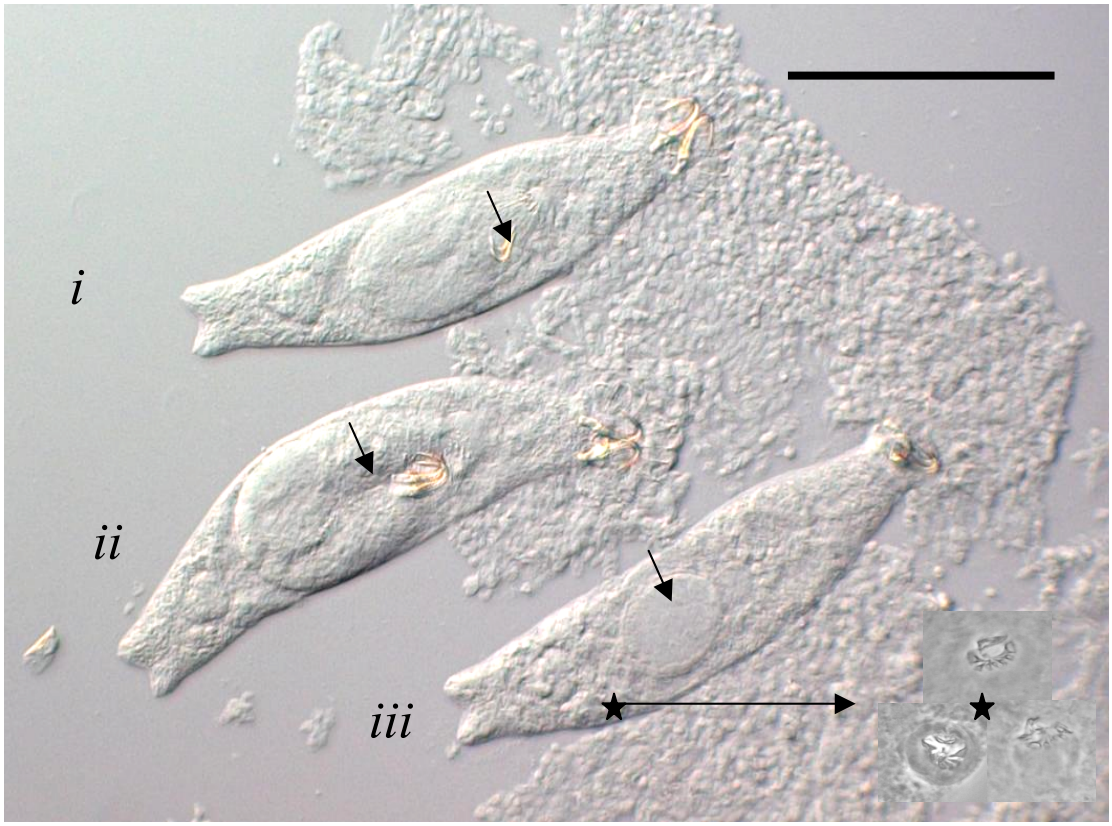


Figure 1.7. Light micrograph of *Gyrodactyus gasterostei* Gläser, 1974. *i* and *ii*) worm containing a fully grown daughter *in utero* (arrowed); *iii*) worm showing an empty uterus (arrowed) and the location of the male copulatory organ (starred; three examples shown). Scale bar = 0.5 mm.

Bakke *et al*. (2009) estimated the economic cost of *G. salaris* to the Norwegian salmon industry at around 500 million euros. Also, Winger (2009) reports that the Norwegian Directorate for Nature Management have estimated that the continued

decimation of wild salmon stocks amount to an annual economic loss of between 200 and 250 million NKR (~27,634,600 GBP).

Gyrodactylus are common parasites of both marine and freshwater teleost fish hosts with a worldwide distribution. Currently, over 420 species of *Gyrodactylus* have been described, of which approximately 29 species are recorded on salmonids (Bakke *et al.,* 2002; Harris *et al*., 2004). The number of new species recorded each year, however, continues to rise and contributing to our knowledge regarding the genus (A. Shinn personal communication).

Table 1.1. Records of *Gyrodactylus salaris* Malmberg, 1957 in Norway: its first appearance and the number of hatcheries and rivers infected at key time points through recent history.

Year	No. hatcheries infected / location	No. of rivers infected / location	Reference
1975	Sunndalsora hatchery	On the R. Lakselva	Johnsen, 1978
1975		R. Lakselva	Johnsen, 1978
1980	4	19	Heggberget & Johnsen, 1982
2007	41	41	Bakke *et al.,* 2007
2009	41	46	Winger *et al.,* 2009

Gyrodactylus salaris is highly pathogenic to Atlantic salmon, having been demonstrated as the parasite responsible for the catastrophic decline of salmon stocks in more than 40 Norwegian rivers (Mo, 1994). *Gyrodactylus salaris* was found for the first time in Norway on juvenile salmon at the Sunndalsöra hatchery in 1975 and later in the River Lakselva on which the hatchery was situated (Jonnsen & Jensen, 1991).

Gyrodactylus salaris spread rapidly infecting almost 90 percent of the parr population within the river in two months. This infection resulted in a marked decline in the salmon population - 50% in the first two years of its introduction and 2-4% of former levels five to seven years after its introduction (Johnsen & Jensen, 1986; Mo, 1994).

To date, *G. salaris* has been introduced into 46 rivers (Winger *et al.,* 2009) and has been estimated to cause a 98% reduction in affected Atlantic salmon populations over a period of 5 years (Johnsen & Jensen, 1991; Mo, 1994).

1.6.6 Movements of *Gyrodactylus salaris*

Norwegian surveillance programmes suggest that while most strains of Atlantic salmon appear to be susceptible to infection, grayling are innately resistant (Soleng & Bakke, 2001). *Gyrodactylus salaris*, however, can survive and reproduce on grayling for up to 140 days. Arctic charr, *Salvelinus alpinus*, respond in a manner similar to grayling and thus provides a good natural reservoir for *G. salaris* (Winger *et al*., 2008).

Preventing the spread of *G. salaris* to Scotland and to other uninfected countries with large natural populations of Atlantic salmon has been seen as a priority for monitoring and regulatory agencies in such regions. Most *Gyrodactylus* research in European countries has focused on three main species: *G. salaris* on *S. salar*, the morphologically similar non-pathogenic *G. thymalli* on grayling, and *G. derjavini* (sic *G. derjavinoides*) on brown trout, *Salmo trutta*. *Gyrodactylus salaris* reproduces and shows long-term survival on Atlantic salmon, rainbow trout *Oncorhynchus mykiss* and grayling (Bakke *et al.,* 1991; Soleng & Bakke, 2001). Jansen & Bakke (1995), however, demonstrated experimentally, that parasite metapopulations will not survive on brown trout past 50 days. Nevertheless, brown trout may still play a role in the

dispersal of *G. salaris* within rivers as may brook trout *Salvelinus fontinalis* Mitchill, (Sterud *et al.,* 1998) and Arctic charr *Salvelinus alpinus* L.

In Norway, research conducted by Winger (2008) gives another perspective on the role of transmission of *G. salaris*. Her studies focused on the annual role of Arctic charr as an adequate and long-term host for *G. salaris* on Atlantic salmon (Winger *et al.,* 2008, 2009). Her studies which were based in the northern-most rivers of Norway, have been treated twice with rotenone, a plant-derived poison. Her conclusion was that Arctic charr and the *G. salaris* infection they carried, escaped rotenone applied to rivers by staying in ponds and marshes connected to the river.

Winger *et al.* (2008) showed an evident seasonal dynamic in *G. salaris* infection in the charr in the two rivers of study and suggested that the reservoir infection on the charr could allow this parasite to return despite two rotenone treatments.

All of these salmonid species are therefore potentially important for the dissemination and persistence of *G. salaris* populations (Bakke & Jansen, 1991: Winger *et al.,* 2008, 2009).

Laboratory experiments carried out by Soleng & Bakke (1998) examined the susceptibility and resistance of non-salmonids to *G. salaris*. Specifically, the susceptibility of 3-spine sticklebacks, *Gasterosteus aculeatus,* and 9-spine sticklebacks, *Gasterosteus pungitius* L., and flounder, *Platichthys flesus* (L.), all fish species that are able to move between and tolerate fresh, brackish or marine waters, while they are largely resistant to *G. salaris,* they may at the same time function as reservoir or temporary hosts and therefore aid in the dispersion of this parasite.

Bakke *et al.* (1990) examined additional freshwater non-salmonid hosts including the brook lamprey, *Lampetra planeri* (Bloch), perch *Perca fluviatilis* L. and

roach *Rutilus rutilus* (L.), all of which were determined to be resistant to *G. salaris* infection. In each case, the parasites were observed to attach to these species but did not reproduce.

Bakke & Sharp (1990) demonstrated low transference rates of *G. salaris* to minnows, with no reproduction on this host, although they do survive on this host for two to four days. In common with the other species listed above, this host represents little risk as a suitable host on which numbers can increase but this host may assist in the dissemination of the parasite.

Bakke *et al.* (1991) evaluated the importance of transfer of infection to eels as a dispersal mechanism of *G. salaris* since they are transmissible from salmon to eels and *vice versa*, and also from eels to eels, both at 4 and 13°C. The study found that the infection of fish appeared to have occurred from the bottom of the tank. The transmission rate was positively correlated with water temperature and transmission was found to be more frequent from dead infected salmon rather than from living infected salmon. The maximum duration of infection on eels was 8 days.

1.6.7 Status of *Gyrodactylus salaris* in Europe and UK

According to recent records (Bakke *et. al.,* 2007), *G. salaris* has been recorded from at least 11 European member states, although records from wild fish in France and the Iberian Peninsula may be confused with *G. teuchis* (Latraite, Blanc, Thiery, Daniel *et* Vigneulle, 1999). The most recent paper by Dzika *et al.* (2009) did not provide enough conclusive evidence of the presence of *G. salaris*. In most European states, the primary host of *G. salaris* is rainbow trout. Translocations of this host, either deliberate or as escapees, presents the greatest threat of dissemination of *G. salaris.* The risk of accidental introduction of *G. salaris* is of concern as its presence is now

recorded from 11 neighbouring European countries. The most recent new records of occurrence come from Poland (Rokicka *et al*., 2007) and Italy (Paladini *et al.*, 2009). The distribution of *G. salaris* in other European countries is unknown (Bakke *et al.,* 2007). The UK was recognised as officially free of this parasite following a large survey of sites by Shinn *et al.* (1995) and on-going UK government programmes of surveillance. *Gyrodactylus salaris* is among the most significant fish disease threats to the UK, which also includes pathogens such as viral haemorrhagic septicaemia virus (VHSV), infectious salmon anaemia virus (ISAV) and infectious haematopoietic necrosis (IHN) which have been reported from the UK.

To date, *G. salaris* has not been detected within the UK (Shinn *et al.,* 1995); however it is important that monitoring and prevention policies are in place. The potential introduction could cause havoc among both natural (native) and farmed species of salmon which are known to be susceptible to this parasite. *Gyrodactylus salaris* is a notifiable disease in the UK and is known to be highly pathogenic to stocks of Atlantic salmon.

1.6.8 Distribution and importance of *Gyrodactylus salaris*

Gyrodactylus salaris is a freshwater ectoparasite whose natural host is the Baltic strains of *S. salar*, although infections in these strains rarely cause clinical disease (Johnsen, 2006).

Government-based surveillance programmes show that the UK is currently free of *G. salaris*. Nevertheless, there is considerable concern about the accidental introduction of this species, since experimental exposure of native British salmon stocks to *G. salaris* in Norway by Bakke & MacKenzie (1993) demonstrated that UK stocks are highly susceptible. The risk of natural transfer to the UK is thought to be

insignificant as the parasite is unable to tolerate full strength sea water (33.0‰) (Soleng & Bakke, 1997). It is important that policies are therefore maintained to ensure that the potential recipients of fish do not import from infected areas. It is also important import licences are obtained, screened and records of imports are kept up to date and regularly checked by the appropriate authorities. There are many potential ways that *G. salaris* could enter the UK, therefore, it is of extreme importance that the possibility of infection is well known and that protective measures are maintained. Therefore, it is vital that anglers who have been fishing abroad are aware of the dangers of possibly introducing *G. salaris* into the UK for National contingency plans for preventing and dealing with *G. salaris* in England; see www.defra.gov.uk which was updated in April 2008)

1.6.9 Treatment and control

While much has been done to ensure that the movement of live salmonids and eggs is strictly regulated between countries, arguably little has been done to research alternative gyrodactylid treatments especially those for use in river systems. Recently however, Schelkle *et al.* (2009) reviewed a wide range of treatments that have been used for the control of infections on wild and cultured fish species. The review concluded that no treatment was 100% effective and that more research is needed to find alternatives, preferably an organic biodegradable substance.

An alternative option may lie in the use of organic or natural treatments. Of these, an essential oil extracted from the Australian tea tree has been shown to have a parasiticidal effect (Steverding *et al.,* 2005). The results came out of a study which examined the effects of tea tree oil (TTA) upon *G. aculeatus* infected with *Gyrodactylus* (Steverding *et al.,* 2005). These authors findings, suggest that tea tree

oil can reduce the level of *Gyrodactylus* and reduce parasite burden on naturally infected fish.

Schelkle *et al.* (2009) in her review of treatments tested on *Gyrodactylus* commented that aqueous aluminium can act as an effective paraciticide in the control of *G. salaris* infections. The use of aqueous aluminium has been shown to effect a noticeable reduction in *G. salaris* numbers on salmon parr in resultant acidified waters (pH~5.0; Soleng *et al*. 1999, 2005). These authors underline the importance of water quality in reducing parasite infection and the value of future work which should evaluate the resistance of the parasite to aluminum and / or their reproduction after long periods of exposure.

In Norway, *G. salaris* infected rivers are treated using a poison called "rotenone" which kills all aquatic animals. Rotenone is classified by the World Health Organisation as moderately hazardous, is mildly toxic to humans and other mammals, but extremely toxic to insects and aquatic life including fish (www.wikipedia.com revised May 2011). It is estimated that the time taken to declare a river free of *G. salaris* is around 5 years, an extremely long period of time for the salmon fishing / salmon aquaculture and tourism industry. This process of eradication is severely damaging to the environment yet it is an accepted cost given the low diversity of organisms within Norwegian river ecosystems. The use of rotenone, however, in some of the larger water bodies is not possible, not only because of the large volumes of rotenone that would be required to treat them but also because larger waterbodies tend to have more complicated ecosystems. In these latter situations, there are no known measures to control the spread of *G. salaris* once it has been introduced. While the treatment of infections in rivers / wild populations is generally accepted to be more difficult, in this situation the only demonstrable method was the removal of all fish

species. Rotenone has been used for this purpose in Norway, where 46 rivers have been infected with *G. salaris*, 35 of these have been treated with rotenone with the result that 18 are now free from infection with *G. salaris* (Peeler *et al*., 2004, 2006; Winger, 2009),

1.7 The *Gyrodactylus gasterostei* and its host three-spine stickleback system

Research on monogeneans, particularly *Gyrodactylus,* have been important for understanding some of the basic interactions between hosts and their parasites and the factors modulating parasite dynamics.

Several gyrodactylid parasites and their respective hosts have been used and proven to be successful laboratory models for studying host-parasite interactions (Ikezaki & Hoffman, 1957; Lester & Adams, 1974a,b; Scott & Anderson, 1984; Harrris, 1988, 1989, 1993; Cable *et al*., 2001), with notable recent studies including those on poeciliid fishes (Harris & Cable, 2000; Cable *et al.,* 2002 , 2005).

1.8 Three-spine stickleback biology

The three-spine stickleback, *Gasterosteus aculeatus*, is a ubiquitous, small, (usually 2-5cm in total body length) sized fish commonly found in freshwater environments throughout the UK. Easily identifiable by its three dorsal, prominent spines, populations are easily mainatined in the captivity. It is for this reason, that this fish which is commonly parasitised by two species of *Gyrodactylus*, *G. arcuatus* Bychowsky, 1933 and *G. gasterostei* Gläser, 1974, makes an ideal model for studying gyrodactylid transmission strategies.

1.9 The current study

Studies on the biology of gyrodactylids have contributed to our understanding of the life-cycle and the intricacies of the biology of these parasites. The purpose of this study was to consider the mechanisms and behavioural aspects of transmission of *Gyrodactylus* under natural conditions. This has necessitated the use of a range of experimental techniques that are described in this thesis.

A series of trials were designed to examine a number of key transmission factors in gyrodactylids including: (i) the effects of maturity and reproductive status on the colonisation of new hosts; (ii) the effect of generally used fish anaesthetics on gyrodactylid transmission; (iii) the transmission routes, including initial contact sites, used by gyrodactylids in transmission and the practical application of these to surveillance and biosecurity protocols; iv) the importance of scavanging as a source of infection; (v) the effects of cohabitation of different fish species in terms of host-switching during their transportation over short time periods; (vi) the nutritional status of attached, detached and new born gyrodactylids and the distribution of lipids within each and how these are used; (vii) the behaviour of detached parasites and their response to octopamine treatment; and finally, (viii) an ultrastructural study of some of the sensory structures in *Gyrodactylus gasterostei.*

This thesis examines the transmission mechanisms used by *Gyrodactylus gasterostei,* focusing on the role played by detached parasites, the transmission of parasites that occurs during the cohabitation of infected hosts with other host species, and upon the role of scavanging in transmission. This study aimed at providing video evidence, where possible, of some of the key transmission strategies. In addition, the study aimed at establishing the distribution of lipids in terms of an energy storage reservoir in detached gyrodactylids. Prospects for future work to are also discussed.

1.10 Justification

This project set out to develop a better understanding of the behavioural research and exploratory techniques involved in the transmission of *Gyrodactylus*.

The study of fish health which includes parasitology is vital to safeguarding the UK salmon industry. Without continuing research into those parasites and pathogens which are known to infect economical farmed species like salmonids and pose a threat, then the safety of UK stocks cannot be guaranteed.

Many monogeneans or the so called “skin and gill flukes” are responsible for serious impairment of fish health and welfare which if untreated can lead to mortalities. Parasitic Monogenea are very successful in the colonisation of new hosts even under conditions when direct host-to-host contact is not possible, there is, however, a lack of basic understanding of the mechanisms of transmission, host finding behaviour and of the cues prompting dispersal and host seeking in important monogenean groups like gyrodactylids which infect a wide range of aquaculture species, which if untreated can cause significant mortalities in hatcheries.

Despite all the information regarding the biology of gyrodactylids, little is known regarding the biological basis of gyrodactylid host selection and the factors underlying transmission, particularly with respect to the maturity and nutritional status of gyrodactylids moving to new hosts.

1.11 Project objectives

Objective 1. To gain an understanding of the biological basis for, and the environmental conditions facilitating, host selection and transmission (maturity state, presence / absence of an MCO / embryo, nutritional status) in a single species of *Gyrodactylus.*

Objective 2. To investigate the effect of the anaesthetic 2-phenoxyethanol on the transmission behaviour of gyrodactylids.

Objective 3. To investigate the factors affecting transmission strategies used by gyrodactylids.

Objective 4. To determine the effects of cohabitation of different fish species in terms of host-switching during their transportation over short time periods.

Objective 5. To investigate the behaviour of detached parasites and their response to novel octopamine-like chemical treatments.

Objective 6. To establish the nutritional status of attached and detached gyrodactylids and the distribution of lipids in the body.

Objective 7. To investigate the sensory sensilla ultrastructure used by gyrodactylids and their response to external factors such as turbulence as a factor for transmission.

Chapter 2

General materials and methods

Howietoun brown trout fishery

This chapter provides a brief overview of the general materials and methods employed in the experimental work throughout this study including fish husbandry, infection protocols, microscopy and other observational techniques. Particular materials and methods will be incorporated in the related chapters.

2.1 Source of hosts and parasites

The *Gyrodactylus gasterostei* Gläser, 1974 – *Gasterosteus aculeatus* L. model was used for the purposes of this study. *Gasterosteus aculeatus* specimens were collected from a settlement pond, feeding a commercial fish farm, situated on a branch of the River Allan near Stirling, Stirlingshire, Scotland (56º 06' 37.77" N, 3º 58' 25.25" W). Fish were transferred to an aquarium facility at the Institute of Aquaculture, University of Stirling where they were held in 25 l black plastic tanks containing 15 ± 1°C, aerated "home" stream water. Fish were fed *ad libitum* on a diet of frozen bloodworms (Gamma, Chorleywood, UK). The fish were allowed to settle for a minimum of 24 h following capture before experimentation. Feeding was stopped on the day prior to the start of all experiments to maintain water quality and reduce fish stress.

Figure 2.1 Sampling for *Gasterosteus aculeatus* L. in the River Allan at Howietoun.

2.2 Fish and gyrodactylid collection in the River Endrick

Stone loach, *Barbatula barbatula* L., 3-spine sticklebacks, *Gasterosteus aculeatus* L. and minnows, *Phoxinus phoxinus* L., were hand-netted from the River Endrick near Loch Lomond, West Dunbartonshire, Scotland (56º 03' 20" N, 4º 24' 00'' W) and transferred in separate buckets to the parasitology aquarium facility at the Institute of Aquaculture.

Figure 2.2. The River Endrick near Loch Lomond, West Dunbartonshire, Scotland.

2.3 Parasite free-hosts

Parasite-free *G. aculeatus* were obtained by treating fish with low parasite burdens or fish with no visible parasites with 300 ppm formaldehyde for 60 mins under constant aeration. Following treatment, fish were transferred to a recovery tank containing clean, aerated, dechlorinated water. The fish were maintained for 10 days following treatment and examined again prior to use to ensure that they were parasite free. If fish were still infected, they were given a second formalin treatment.

2.4 Parasite migration experiments

Experiments designed to examine worms moving off dead hosts at 10°C were conducted as follows. Individual sticklebacks were euthanased with an overdose of

anaesthetic 0.01 M 2-phenoxyethanol (MERCK-Germany) and were placed in individual Petri dishes containing clean water. Dead hosts were observed under an Olympus SZ30 stereo microscope at different magnifications, with migrating gyrodactylids having their departure time recorded and being fixed and mounted for subsequent analysis.

2.5 Worm fixation and mounting

Worms required for further analyses were carefully removed from the Petri dish with a 200 µl pipette and transferred to slides. Worms were mounted on standard glass slides (25.4 × 76.2 mm, Solmedia Laboratory Supplies, UK) under 18 × 18 mm coverslips, which served to flatten the specimens. A drop of saturated ammonium picrate glycerine (Malmberg's fixative; Malmberg, 1970) was added to the edge of the coverslip and allowed to penetrate underneath in order to fix the worm. Further parasite observations were carried out using an Olympus BX51 compound microscope with specimens being observed under ×20 - ×100 objectives.

2.6 Taxonomic description

For the morphological description (×100 / oil immersion magnification), worms were identified from the ammonium picrate glycerine mounted preparations using light microscopy. Where necessary, specimens were compared with photographs from *Gyrodactylus* specimens (www.gyrodb.com).

2.7 Video recording

To investigate the gyrodactylid infection process a 16 cm (l) × 15 cm (h) × 8.5 cm (b) experimental chamber with a viewing platform was constructed from Perspex and

positioned within an 80 cm square translucent photo tent (Figure 2.3). A Veho Discovery Deluxe USB microscope with ×400 magnification (VMS-004D) with a close-up facility was focused on the platform and used to record the transmission process and relay it directly to a computer (Figure 2.3).

The video editing was performed on a Windows PC using the Microsoft Windows XP and Adobe Premiere Pro 1.5 video editing software for creating the graphics and Windows Movie Maker 5.1 for editing the original footage to create the video clips. A PowerPoint presentation was created as part of the final production and has been placed on a CD attached at the back of this thesis. The video recording was edited in Windows Movie Maker 5.1 and Adobe Premiere Pro 1.5. (Assistance in using the software programmes and editing the video was provided by Fred Phillips, Audio Visual Services, University of Stirling).

Figure 2.3. The experimental viewing chamber made of Perspex and designed so that the movement of gyrodactylids attaching to and detaching from experimental hosts could be filmed using a USB digital microscope.

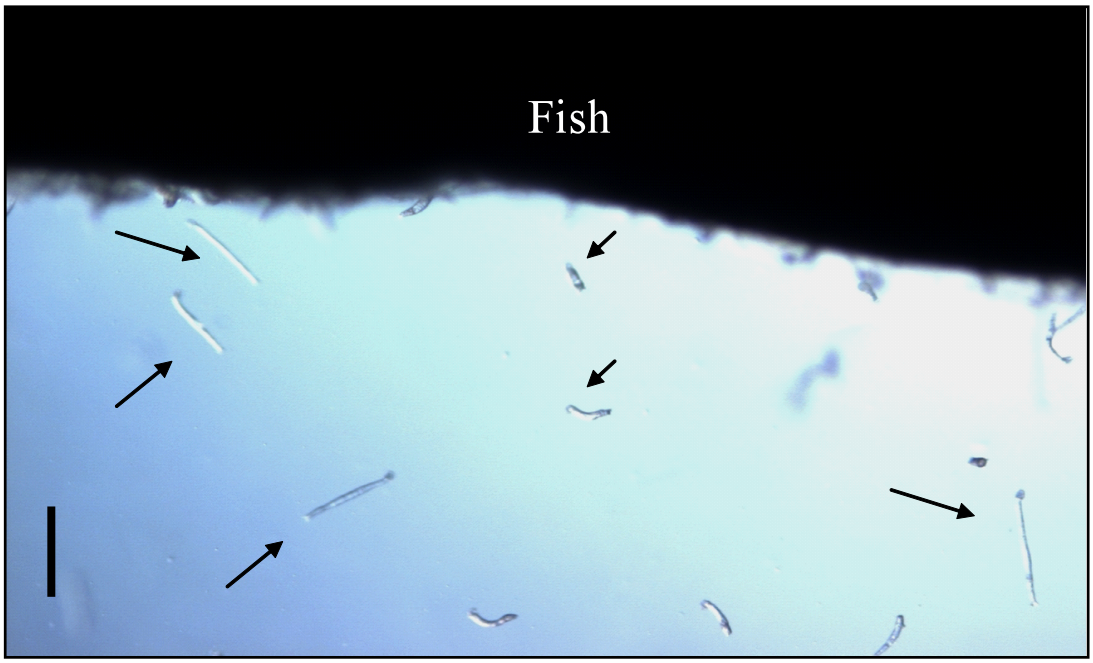


Figure 2.4. Gyrodactylids moving on and off experimental hosts could be monitored and recorded using a digital microscope linked to a laptop. Scale bar = 1 mm.

2.8 Preparation of specimens for Scanning Electron Microscopy

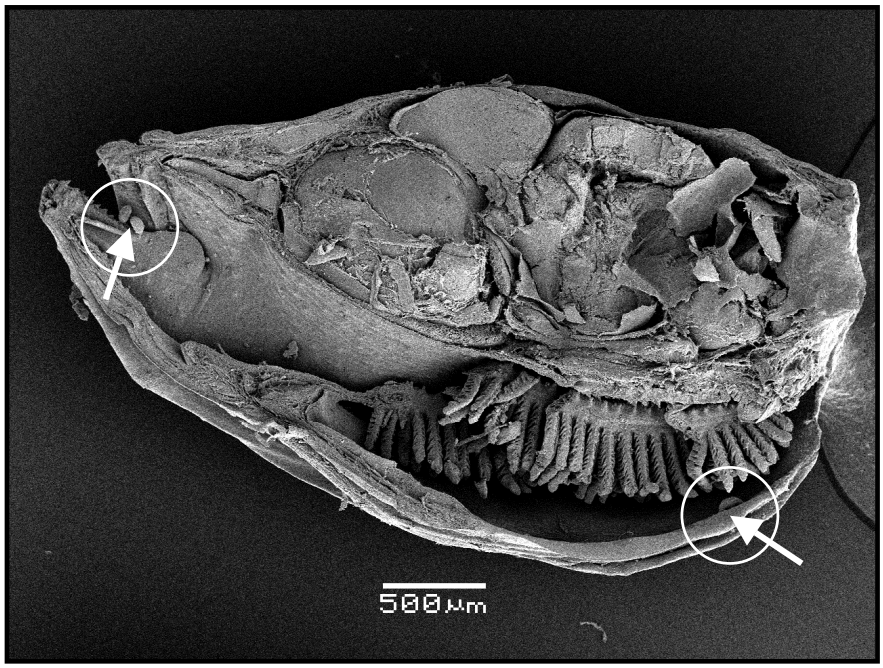
Infected hosts were examined using a scanning electron microscopy (SEM). The fish were euthanised using an overdose of 2-phenoxyethanol before the head was removed, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 10 min, and then bisected along the medial saggital line before returning them to the fixative for a further 2 days at 4^{o}C. The samples were then post-fixed in 1% osmium tetroxide, dehydrated through an ethanol series, critical point dried, mounted on aluminium stubs, sputter-coated with gold and then viewed on a Jeol JSM 6460LV scanning electron microscope at an accelerating voltage of 7-10KeV.

2.9 Preparation of specimens for transmission electron microscopy (TEM)

Individual worms were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer and then post-fixed in 1% osmium tetroxide. Specimens were then en-bloc stained in 2% uranyl acetate and 30% acetone before dehydrating them through a 60 to 100% acetone series. Specimens were then embedded in Spurr resin and cut at 50-70 nm. TEM sections were first stained with 4% uranyl acetate in 50% ethanol and then Reynolds lead citrate. Sections were viewed on a Tecnai G2 Spirit Biotwin transmission electron microscope.

Chapter 3

An oral route for infection of 3-spine sticklebacks, *Gasterosteus aculeatus* L., by *Gyrodactylus gasterostei* Gläser, 1974.



A SEM of a dissected 3-spine stickleback head showing the presence of gyrodactylids within the buccal and gill chambers.

Aspects of this study were presented at:

Stirling University SGRS student conference, April, 2008 (poster; 2nd prize)

Xth European Multicolloquium of Parasitology, Paris, 24th-28th August (poster).

Institute of Aquaculture, PhD Research Conference, 29th October 2008 (poster; 2nd prize)

6th International Symposium on Monogenea, 3rd-7th August, Cape Town, South Africa (oral and poster)

3.1 Introduction

Pathogenic monogenean parasites belonging to the family Gyrodactylidae have had significant economic impacts on populations of wild and cultured fish (Bakke *et al.,* 2007). *Gyrodactylus salaris* Malmberg 1957, for instance, is a major pathogen of wild Norwegian salmon (Johnsen & Jensen, 1986, 1991; Mo, 1994; Bakke & Harris, 1998). Like other gyrodactylids, it has a short generation time and progenetic mode of reproduction which can lead to a rapid build up of parasites on a naïve host. This in turn can lead to host death as a result of large numbers of parasites on the host through osmotic shock and secondary infections (Cable *et al*., 1996; Cable & Harris, 2002).

Bakke *et al.* (1992) suggested four routes by which gyrodactylids could transfer to a new host: (i) via contact with live hosts; (ii) via dead hosts; (iii) by detached parasites drifting in the water column; and, (iv) by parasites transferring to hosts from a non-fish substrate. The ability to employ multiple transmission strategies, coupled with their high fecundity, allows gyrodactylids to rapidly colonise new river systems (Bakke *et al.*, 1992; Johnsen *et al.*, 1999). Although the major routes of gyrodactylid transmission have been extensively studied, relatively few studies have examined the behaviour of individual gyrodactylids in the transmission process. Specifically, the factors underlying and contributing to transmission of gyrodactylids between hosts and in particular why gyrodactylids may abandon a suitable host and transfer to the water column or substrate are as yet unknown.

Studies of *G. turnbulli* Harris, 1986 occurring on the guppy *Poecilia reticulata* Peters suggested specific migratory behaviour that facilitated migration from dead hosts. In particular, it was suggested that individual gyrodactylids moved to the surface film of the water and that therefore, as guppies are surface feeders, detached parasites were more likely to contact a new host (Cable *et al.,* 2002).

The hypothesis that transmission occasionally follows accidental detachment of gyrodactylids or occurs through accidental contact of hosts alone, ignores the possibilities of behavioural mechanisms, either parasite or host that may act to improve the probability of transmission. Whilst most gyrodactylids do not display swimming abilities, *Gyrodactylus rysavyi* Ergens 1973 from the Nile catfish *Clarias gariepinus* (Burchell) is suggested to be capable of swimming when detached from the host and released into the water column (El-Naggar *et al.,* 2004).

Gyrodactylids use a variety of different strategies to infect new hosts, but robust experimentation to test these possible strategies is lacking. One theory suggests gyrodactylids can transmit from dead hosts when live hosts cannibalise the carcass of an infected host (Olstad *et al.,* 2006). This behaviour would increase the chances of parasites contacting a new host, particularly if potential hosts are either scavengers or benthophagous feeders. Three-spine stickleback feeding behaviour has been extensively studied and, whilst it is generally considered to be a benthic feeder (Hart, 2003), limnetic forms in British Columbia are known to feed preferentially on planktonic prey. Furthermore, Dukowska *et al.* (2009) demonstrated that the diet choices of sticklebacks was determined by the available food resources and readily switched from feeding on planktonic *Daphnia* to epiphytic species on macrophytes. Thus, sticklebacks appear to be somewhat generalist and opportunistic feeders. To test the hypothesis that gyrodactylids may transmit to a new host when hosts scavenge on the infected carcass of another dead infected fish, the transmission of parasites between live and dead fish was observed in order to ascertain first, whether host scavenging is a potential route of gyrodactylid transmission and second, if so, what the routes of transmission might be. In adition, consideration was given to the role of sexual maturity and gyrodactylid behaviour in enhancing transmission to new hosts.

This study employed the *Gyrodactylus gasterostei* Gläser, 1974 / *Gasterosteus aculeatus* L. infection model to examine these questions.

3.2 Materials and methods

3.2.1 Source of hosts and parasites

A *Gyrodactylus gasterostei* / 3-spine stickleback model was used for the purposes of this study as described in section 2.1. *Gasterosteus aculeatus* specimens were collected from a settlement pond, feeding a commercial fish farm, situated on a branch of the River Allan near Stirling, Stirlingshire, Scotland (56º 06' 37.77" N, 3º 58' 25.25" W). Fish were transferred to an aquarium facility at the Institute of Aquaculture, University of Stirling where they were held in 25 l black plastic tanks containing 15 ± 1^{o}C, aerated "home" stream water.

3.2.2 Parasite free-hosts

Parasite-free hosts were required for a number of experiments as described in section 2.3. Individual fish with low or no visible parasites were treated with 300 ppm formaldehyde for 60 mins under constant aeration. Following treatment, fish were transferred to a recovery tank containing clean, aerated, dechlorinated water. The fish were maintained for 10 days following treatment and re-examined under a stereomicroscope to ensure that they were parasite free during the quarantine period and at the end of the 10 day period.

3.2.3 Transmission from dead to live hosts through scavenging

To investigate the gyrodactylid infection process a 16 cm (length) × 15 cm (height) × 8.5 cm (depth) experimental chamber with a viewing platform was constructed from Perspex and positioned within an 80 cm square translucent photo tent. A Veho Discovery Deluxe USB microscope with ×400 magnification (VMS-004D) and with a close-up facility was focused on the dead fish and used to record the transmission process. Sticklebacks were obtained from a branch of the River Allen and visually screened for the presence of gyrodactylids. Heavily-infected animals (containing more than 100 parasites on the surface) were euthanised with an overdose of the anaesthetic 0.01M 2-phenoxyethanol (MERCK-Germany) and rinsed carefully in tank water to remove residue of the anaesthetic. Animals were then placed, individually, on the gravel substrate located into the chamber which contained filtered pond water having passed through a 20 μm mesh. Ten replicates of the trial were used. A live, uninfected stickleback, which had been previously starved for 3 days was then introduced into the experimental system containing the dead infected host. After three hours of cohabitation in the chamber and at the end of filming, live fish were transferred to a clean beaker and euthanised as described before and immediately fixed in 80% ethanol. The skin, gills, nostrils and the mouth cavity of each fish were examined for ectoparasites under an Olympus SZ30 stereomicroscope at ×4 magnification and the water in the beaker checked for the presence of gyrodactylids that may have been dislodged during euthanasia. Recordings of the behaviour of the live fish in proximity to the dead fish were downloaded to a MacIntosh iMovie 08 program (Apple).

The water in the observation chamber was passed through a 20 μm mesh filter to recover any dislodged parasites. The chamber was then rinsed with 100 ml 80%

ethanol and the liquid passed through the filter. The mesh was then back-washed into a separate 20 ml vial to release any gyrodactylid specimens.

The position of each gyrodactylid on each fish was carefully removed using mounted triangular surgical needles (size 16, Barber of Sheffield, UK). Each specimen was then mounted on a glass slide in a drop of distilled water ensuring that the haptoral hooks were flat. The specimens were then stained and fixed *in situ* by the addition of a drop (~3 µl) of Malmberg's fixative (ammonium picrate glycerine, APG; saturated picric acid and 100% glycerin) to the edge of the coverslip which was drawn under the coverslip by capillary action. The coverslip was then sealed with transparent nail vanish. The maturity and reproductive status of worms were recorded using a compound microscope (Olympus BX51) at 100× / oil immersion magnification. Additionally, parasites were identified to species through morphological and morphometric analysis of the opisthaptoral hard parts.

3.2.4 Scanning electron microscopy investigation of oral transmission

Fish were euthanised as above and the head was removed, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 10 min, and then bisected along the medial saggital line before returning them to the fixative for a further 2 days at 4^{o}C. The samples were then washed in 0.1M cacodylate buffer, post-fixed in 1% osmium tetroxide, dehydrated through an ethanol series, critical point dried, mounted on stubs, sputter-coated with gold and then viewed on a Jeol JSM 6460LV scanning electron microscope at an accelerating voltage of 7-10KeV. Mounted specimens were observed under SEM in order to detect gyrodactylids present in the oral and gill cavity.

3.2.5 Statistical analysis

A logistic regression was employed in order to compare the proportion of parasites transferring from dead hosts compared to the proportions remaining, thus accounting for differences in the starting number between fish. A Generalised Linear Model was used to determine whether there was an association between the number of times a fish was observed biting an infected, dead host in a 3 hour period and the number of parasites that were found to have transferred to the feeding fish. As the outcome variable was a count, a Poisson distribution was assumed. To account for differences in the starting number of parasites inhabiting the dead host, the total starting number of parasites was included in the model as a covariate. Logistic regression was used to determine if parasites of different maturity status were equally likely to transfer from a dead host to a live host. All analysis was conducted in R2.10.1 (R development Core Team, 2009).

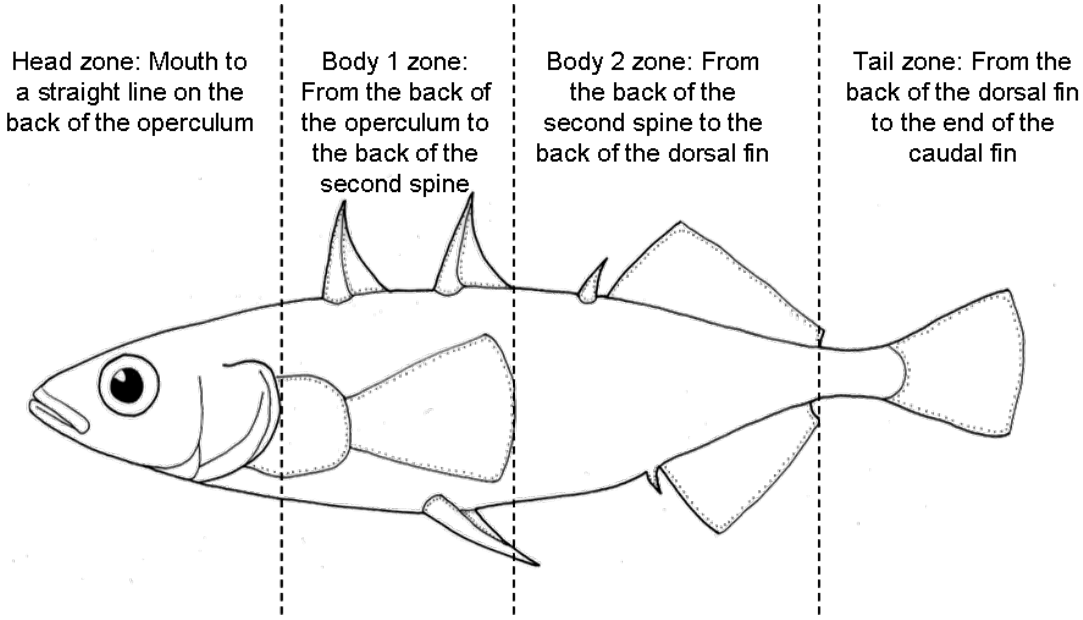


Figure 3.1. Body surface of stickleback, divided into 4 different regions to record the number and distribution of *Gyrodactylus gasterostei* infecting during the colonisation of uninfected hosts by detached gyrodactylids (n = 96).

3.3 Results

3.3.1 Transmission experiment

These experiments were carried out with a view to gaining some insight into the transmission strategies of gyrodactylids and their behaviour during scavenging activities of the fish host. During 30 h of recorded video, 72 direct contacts were recorded between the live uninfected fish and the dead parasitised fish, these contacts being described in this study in terms of bite-rate number. The average number of bites made by a live fish at the infected carcass were calculated (10.8 ± 8 (1 S.D.); n = 10) and the average contact time (*i.e.* the time spent by the live fish in direct contact with the infected carcass) was determined as 57 ± 8.3 min over the 30 hour observation period.

A total of 523 parasites were counted during these experiments with 49.22% (n = 284) being found on live hosts after scavenging interactions *i.e.* whilst live hosts took bites out of the dead fish carcass. The maturity and reproductive status of parasites that transferred to live hosts was recorded and it was found that 65.6% (193) of the parasites had a MCO present whilst 54.2% (154) had a daughter *in utero*. Also, 34.4% (101) of the parasites had no MCO present and 45.8% (130) had no daughter present.

The number of parasites remaining on the dead fish was 219 (37.95%). The maturity and reproductive status of the parasites showed a preponderance of those having no MCO present 68.0% (n = 123), with 81.4 % (n = 180) having a daughter *in utero.* Also 32.0% (n = 58) had a MCO present and 18.6% (n = 41) had no daughter present.

For the 12.82% (n = 74) of parasites collected from the mesh, 64.86% (n = 48) had a MCO and 62.16% (n = 46) showed no embryo *in utero*. Those having a daughter *in utero* were 37.83% (n = 28) and those having no MCO 35.13% (n = 26).

3.3.2 Scanning electron microscopy

SEM observations of bisected heads (Fig. 3.1a, b) demonstrated the presence of parasites within the buccal cavity. Gyrodactylids were found attached to all parts of the mouth, including the teeth, the tongue and the roof of the mouth (Fig. 3.1a, b). In a number of cases, gyrodactylids were found attached deep within the pharynx (Fig. 3.1b) and close to the mouth (Fig. 3.2).

3.3.3 Maturity status

Statistical analysis of the transmission data revealed that adding maturity and reproductive status into the model explained a significant amount of the variability observed in the proportion of parasites transferring to live hosts from dead hosts. Analysis showed a significantly higher probability of parasites transferring in the groups with a MCO that the N MCO/D group. Predicted proportion of parasites from each group transferring to the live fish: MCO (stage) was *0.313* (N MCO/D); *0.405* (N MCO/ND); *0.635* (MCO/D); *0.653* (MCO/ND) resulting that parasites with MCO presence ($P<0.05$) showed a significantly higher probability of transmission (Fig. 3.3).

Table 3.1. The number and percentage of *Gyrodactylus* found in each body zone on the sticklebacks exposed to detached gyrodactylids for 2 h and 5 h.

Time (h)	Head		Body 1		Body 2		Tail		Mouth		
	N	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**Total**
2	13	48.15	3	11.11	1	3.70	5	18.52	5	18.52	27
5	18	35.29	17	33.33	12	23.53	3	5.88	1	1.96	51
Total	**31**	39.74	**20**	25.64	**13**	16.67	**8**	10.26	**6**	7.69	78

Table 3.2. Maturity and reproductive status of the 553 *Gyrodactylus gasterostei* that were either transmitted to a new host (mouth or body), failed to transmit and were subsequently found on the mesh or remained on the original infected host. Abbreviations: the maturity and reproductive status comprise four developmental states: 1) no daughter and no MCO (ND/N MCO); 2) no MCO and daughter present (N MCO /D); 3) MCO present but no daughter (MCO /ND); and, 4) MCO present and a daughter present *in utero* (MCO /D). Values are means ± 1 S.D.

Stage	**No. of parasites found in the mouth of "clean hosts"**		**No. of parasites transmitting to the "clean" host - mouth**		**No. of parasites failing to transmit and found on the mesh**		**No. of parasites remaining on the original host**	
	No.	Mean ± S.D.	No.	Mean ± S.D.	No.	Mean ± S.D.	No.	Mean ± S.D.
N MCO /ND	1	0.1 ± 0.35	14	1.4 ± 1.58	14	1.4 ± 0.89	15	1.5 ± 1.25
N MCO /D	4	0.4 ± 0.53	54	5.4 ± 5.63	12	1.2 ± 0.93	66	6.6 ± 14.03
MCO /ND	7	0.7 ± 0.83	79	7.9 ± 7.72	33	3.3 ± 5.77	75	7.5 ± 3.06
MCO /D	3	0.3 ± 0.52	68	6.8 ± 5.71	17	1.7 ± 2.71	61	6.1 ± 2.38
	15		**215**		**76**		**217**	

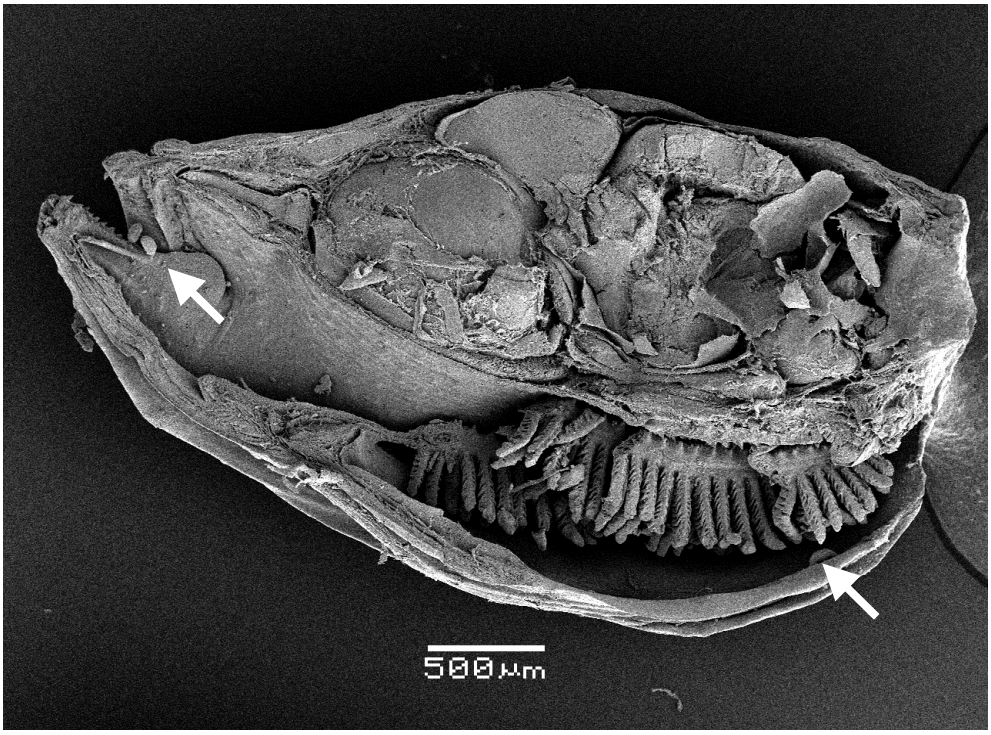


Figure 3.2. Scanning electron micrograph of *Gyrodactylus gasterostei* inside the mouth and pharynx of its stickleback host. Longitudinal section through the head showing a parasite inside the mouth and the opercular cavity (arrowhead) several hours after naïve hosts were exposed to detached parasites.

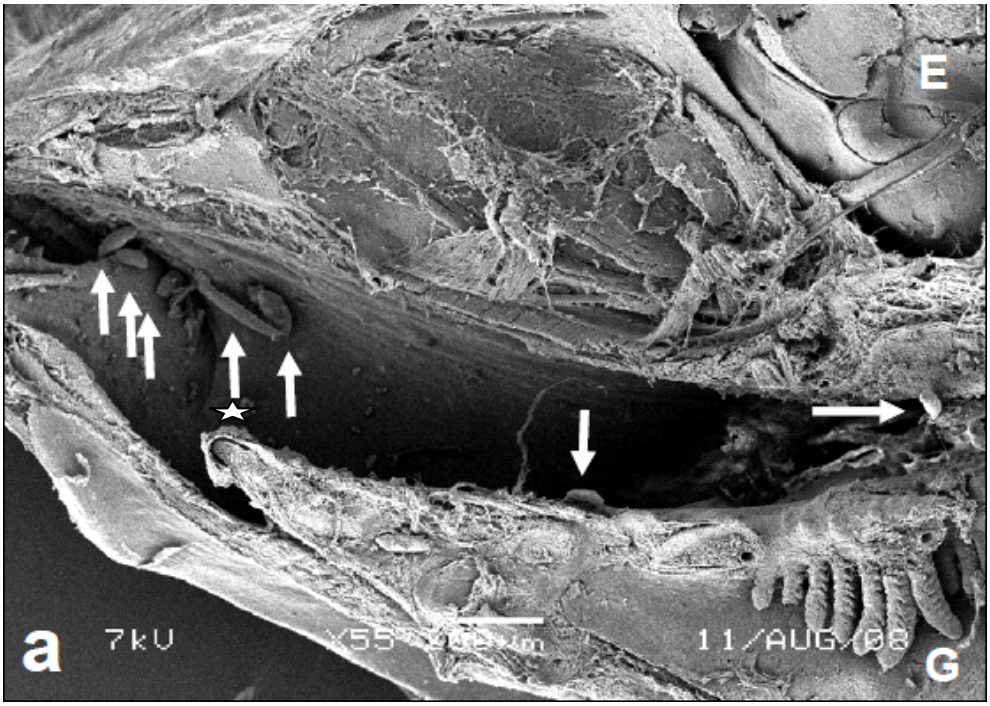


Figure 3.3. Scanning electron micrograph of *Gyrodactylus gasterostei* inside the mouth and pharynx of its stickleback host. Several parasites in close proximity to the teeth, attached to the tongue and on the roof of the mouth. E (eye); G (gill). The star shows a gyrodactylid extending its body.

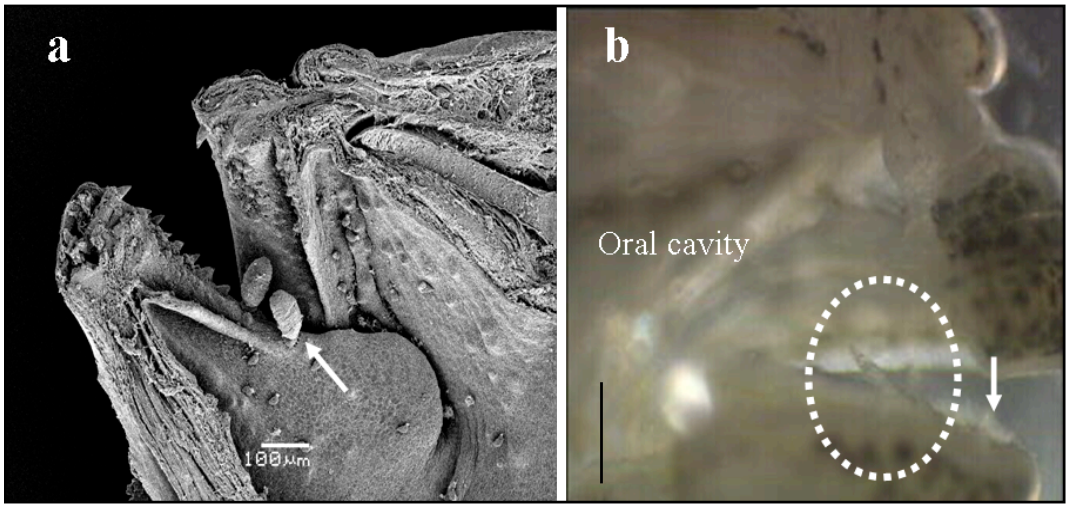


Figure 3.4. Gyrodactylids in the oral cavity a) two worms in the proximity of the teeth (arrowed); b) single worm *in vivo* (circled) inside the mouth, teeth (arrowed) using the oral route as a possible means of transmitting to a new host. Scale bar: 1 mm.

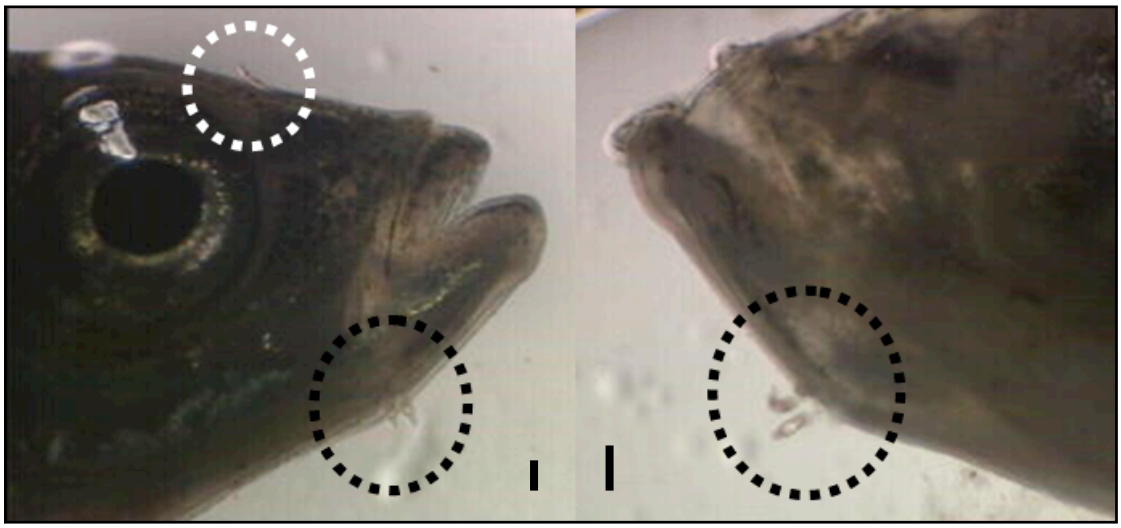


Figure 3.5. Gyrodactylids (circled) present on the chin and head area of clean *Gasterosteus aculeatus* L. after scavenging, feeding on an infected, dead carcass. Scale bar: 1 mm.

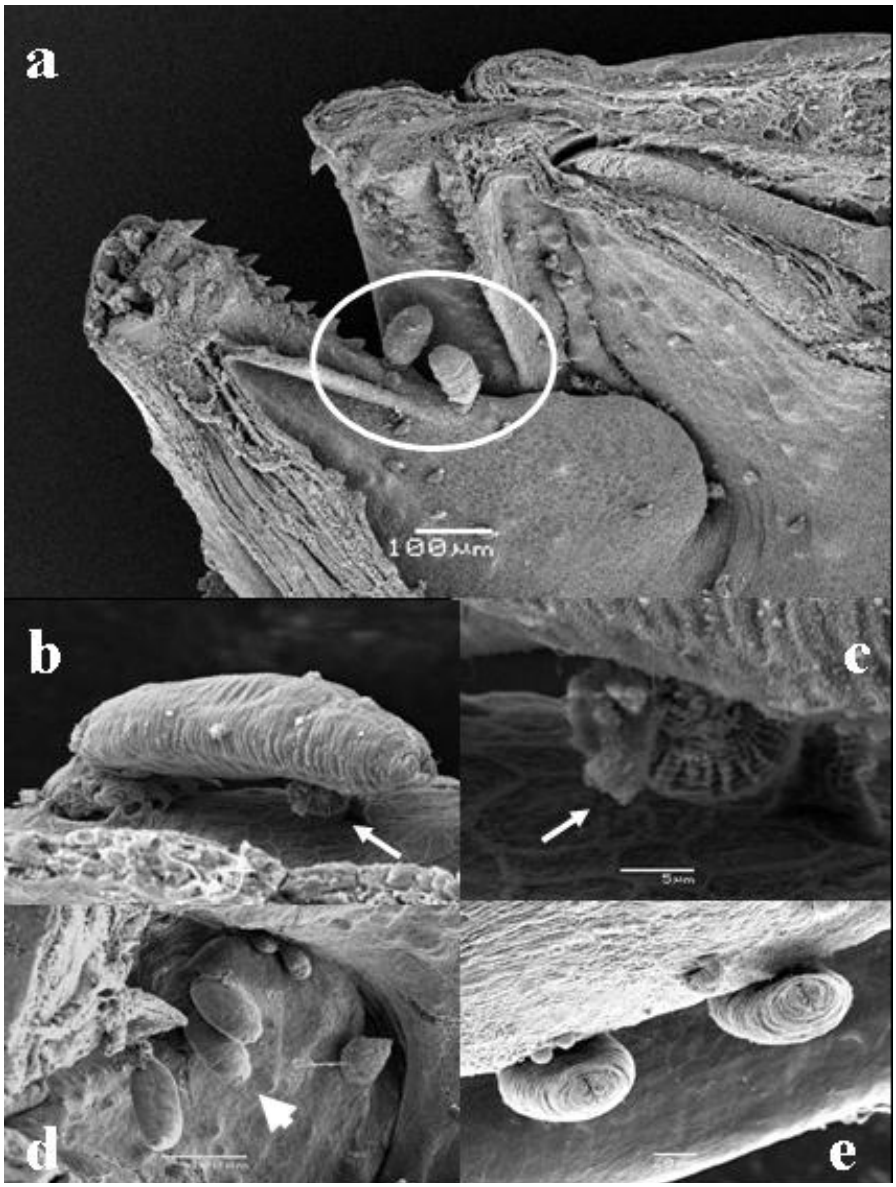


Figure 3.6 Scanning electron micrographs of *Gyrodactylus* inside the mouth of its stickleback host. a) Two parasites in close proximity to the teeth (circle). b) A single parasite attached to the tongue of its host with its pharynx everted (arrow) ready to ingest host tissue. c) Pharynx everted from the parasite. d) Three parasites situated on the roof of the mouth. The head of the parasite (arrowhead) possesses two cephalic lobes, each bearing a spike sensillum. e) Two parasites on the roof of the mouth.

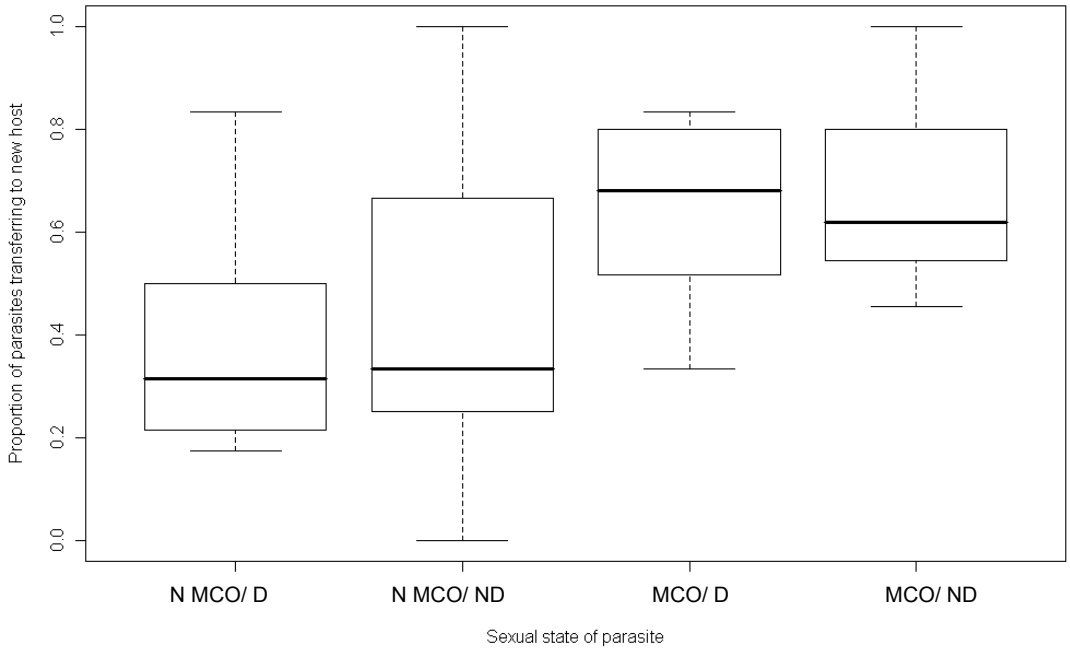


Figure 3.7. Box plot of the proportion of gyrodactylids transferring to live fish during scavengering arranged according to development and maturity status. Abbreviations: 1) no daughter and no MCO (ND/N MCO); 2) no MCO and daughter present (N MCO /D); 3) MCO present but no daughter (MCO /ND); and, 4) MCO present and a daughter present *in utero* (MCO /D).

3.5 Discussion

The experiments described in this study sought to explore the role of gyrodactylid behaviour and maturity status in transmission to new hosts and describes for the first time, transmission routes of gyrodactylids through the use of experimental and observational studies as well as scanning electron microscopy to localise parasites after transfer to new hosts that were engaged in scavenging feeding behaviours.

From the current study, a total of 72 direct contacts (*i.e.* bites) were made at dead fish across a period of 30 h video recording. This finding suggests that scavenging (see slide 11 of the Powerpoint presentation provided on the CD at the

back of this thesis) or feeding on dead animals is not a major route of infection but nonetheless fish can become infected through this activity. As the infected carcasses in this study lay on the gravel, it is easy for the parasites to move from the carcass onto the gravel-susbtrate and then infect new hosts from the substratum. This is important given that both Bakke *et al.* (1992) and Soleng *et al*. (1999) also suggested that the indirect transmission of gyrodactylids from the substratum is likely to be especially important route of infection (Figure 2.4). An interesting observation was the finding of gyrodactylids in the mouth of the clean fish after they were observed scavenging on the infected carcasses of others which may suggest another transmission route.

Harris (1988, 1989) demonstrated that *Gyrodactylus turnbulli* preferentially infected the posterior part of the host body. In these studies, he recorded the following distribution on the fish: caudal peduncle 42%, caudal fin 40%, pectoral fin 8%, dorsal fin 5%, pelvic fin 2% and the anal fin 1%. In the case of newly acquired infections, these were found predominantly upon the fins, which may suggest that these were the principal sites of initial contact with transmitting parasites (Harris & Lyles, 1992). This was supported by the studies of Harris (1988, 1989) which clearly showed that *G. turnbulli* does not infect the gill chamber. In the current study, *G. gasterostei* was, in addition to attachment via body contact, observed to be ingested directly by the prospective host. Experimental exposures for 3 h and SEM observations indicate that once ingested, the parasites can attach to the lining of the buccal cavity. Such a risky transmission strategy would not be unique amongst gyrodactylids more generally. *Gyrdicotylus gallieni,* a parasite of the toad *Xenopus laevis* (Harris & Tinsley, 1987) gains access to the host's mouth via the nostrils after migrating over the skin surface. It has been suggested that gyrodactylids may also enter the mouth attached to food

items or sloughed skin (Harris & Tinsley, 1987). The current study suggests that worms may migrate from the buccal cavity through the branchial chamber out to preferred sites on the body surface of the fish. However, the evidence for this is tentative. The observation of *G. gasterostei* within the buccal cavity of the host has important implications for detection and control of the parasite and for modelling transmission. This work suggests that movements of salmonids or other fish species conducted without specific screening of the buccal cavity for *Gyrodactylus* (and indeed for other ectoparasites) carry a risk of introducing devastating parasites to new areas (Bakke *et al.,* 2002).

It is impossible to determine from the current study whether the increased activity of the worm in the presence of hosts serves to increase visibility or attractiveness to the stickleback, which is a largely visual feeder or whether it is simply indicative of greater efforts to attach. There is evidence that in addition to fish to fish transfer, fish can acquire infections by contacting gyrodactylids that have become separated from their host and are either attached to the substrate or drifting in the water column (Bakke *et al.,* 1992). Rarely, although not the case for *G. gasterostei, Gyrodactylus* species may infect a host by swimming in the water column *e.g. G. rysavyi,* which has the ability to "propel" itself through the water. *Gyrodactylus gasterostei,* like *Macrogyrodactylus congolensis* and *M. clarii* studied by El-Naggar *et al.* (2004), failed to swim when detached experimentally from the bottom, lacking the morphological and behavioural traits possessed by *G. rysavyi*. These authors similarly indicated that there was a potential for infection of the gills should be the fish ingest the parasite specifically or should it be drawn into the inhalant current. Although it is possible that parasites may initially establish themselves on the gills and then migrate to the skin, this strategy confers the inherent

risk that the parasite may be eaten (Grano-Maldonado *et al.,* 2007*)*. The image capture speeds employed in the present study did not allow the moment of ingestion to be captured in detail, so more work needs to be carried out both to describe this event and to examine the factors that affect it.

This study provides evidence for fish to fish transfer during scavanging due to contact with dead fish infected with gyrodactylids. Olstad *et al.* (2006) examined transmission strategies of *G. salaris* from dead hosts, suggesting that they might provide a significant reservoir of infection. According to these authors the parasite can survive for up to 6 days at 12ºC on a dead host and at least 72 h post host death, their life span being doubled compared with individuals maintained *in vitro,* probably being sustained by feeding on the dead host.

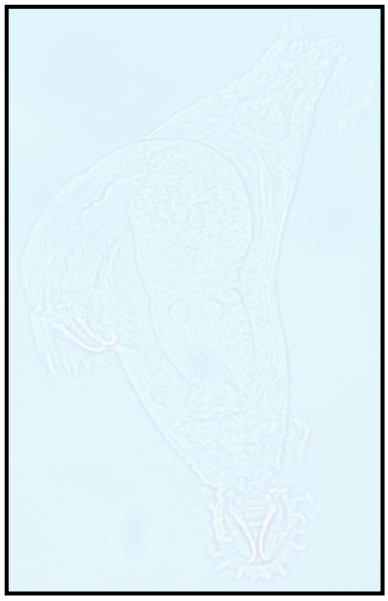
Although visual studies clearly demonstrated that *G. gasterostei* can reside in the mouth of its host, feeding upon dead parasitised hosts did not contribute significantly to the number of parasites transferring to the live feeding host in 3 hours. This suggests that transmission occurs predominantly via other pathways which involve time spent in close proximity to a dead infected host or time spent resting on the bottom near an infected host where migrating parasites may be concentrated. Analysis shows a significantly higher probability of parasites transferring in the groups with a penis than those lacking a penis (Fig. 3.7). This suggests that colonisation of new hosts is more commonly achieved by mature parasites.

The present study contributes to basic knowledge the biology of gyrodactylids, describing for the first time an oral transmission route for infection of sticklebacks by *G. gasterostei* and demonstrates that scavanging can be a route of gyrodactylid transmission. The importance of screening fish for buccal infections is highlighted

also, diagnosticians and staff involved in fish disease surveillance programmes should be aware of the consequences that oral cavity may act as temporary microhabitat.

Chapter 4

Factors affecting transmission strategies used by the monogenean ectoparasite *Gyrodactylus gasterostei* Gläser, 1974.



Gyrodactylus gasterostei giving birth (premature; haptor first).

Aspects of this study were presented at:

Xth European Multicolloquium of Parasitology. Paris. 24th-28th August, 2008 (poster).
6th International Symposium on Monogenea, Cape Town, South Africa. 3rd-7th August, 2009 (poster).

And published in:

Grano-Maldonado, M., Bron, J.E., Duguid, A., Irving, S., Longshaw, M., Turnbull, J.F. & Shinn, A.P. (2007) Factors affecting transmission strategies used by the monogenean *Gyrodactylus gasterostei* Gläser, 1974. *Parassitologia*, **49** (2), 88.

4.1 Introduction

Gyrodactylids are monogenean flukes with a direct life-cycle, they are viviparous and are capable of rapid multiplication, possessing no specific transmission stage (*i.e.* oncomiracidium) as other monogeneans, they do not lay eggs and do not possess the ability to swim to infect new hosts but reproduce progenetically to give birth to live, functional "adults". With the intensification of aquacultural practices, the unique colonisation abilities and pathogenicity of gyrodactylids has resulted in major disease epidemics. *Gyrodactylus salaris* Malmberg, 1957 is one of the most intensively studied monogeneans of recent years, having reduced salmon populations in Norwegian rivers and caused significant epidemic disease in Norwegian salmon (Johnsen & Jensen, 1986; Johnsen, 2006). Viviparity, progenesis, polyembryony and the alternation between sexual and asexual cycles of reproduction contributes to the ability of some *Gyrodactylus* species to rapidly increase their numbers on individual fish under particular environmental conditions (Harris, 1989; Cable *et al.,* 2002a; Bakke *et al*., 2007).

Gyrodactylus salaris is listed by OIE (Office International des Epizooties – World Organisation for Animal Health) as a notifiable disease (OIE, 2009). Atlantic salmon stocks vary in their susceptibility to *G. salaris*, however, it is clear that both Norwegian and Scottish stocks are susceptible (Bakke & MacKenzie, 1993). The latter authors demonstrated the possible catastrophic consequences of introducing *G. salaris* into the UK, this prompting investigation of possible measures to prevent introduction. Although known from several neighbouring European countries including the recent records from Poland (Rokicka *et al*., 2007), Germany (Dzika *et al*., 2009) and Italy (Paladini *et al*., 2009), the UK is thought to be *G. salaris*-free but contingency plans need to be in place to either minimise the likelihood of its

introduction or to control the impact of the parasite in the event of its introduction. In order to achieve this, it is vital that the life-cycle is comprehensively understood and factors underlying its transmission to new hosts.

Studies on the biology of gyrodactylids have provided a range of life-cycle observations relevant to the development of control strategies. Several gyrodactylid parasite species have successfully been used as laboratory models of micro-parasite host systems (Ikezaki & Hoffman, 1957; Lester & Adams, 1974; Scott & Anderson, 1984; Harris & Tinsley, 1987; Harris, 1993; Cable *et al*., 2002a; El-Naggar *et al.,* 2004).

Bakke *et al.* (1992) suggested four routes by which gyrodactylids could transfer to a new host: (i) via contact with live hosts; (ii) via contact with dead hosts; (iii) chance contact with detached parasites drifting / carried in the water column; and, (iv) chance contact with detached parasites attached to the substrate. The employment of multiple transmission strategies (Soleng *et al.,* 1999), coupled with their high fecundity allows gyrodactylids to rapidly colonise new host and environments (Bakke *et al.*, 2002). Although the major routes of gyrodactylid transmission have been extensively studied, relatively few studies have examined the behaviour of individual gyrodactylids in the transmission process. Specifically, the factors underlying and contributing to the transmission of gyrodactylids and, particularly, to the abandonment of a suitable host are as yet unknown.

In addition to the routes of infection proposed by Bakke *et al.* (1992), observations presented here of gyrodactylids attaching to their respective fish hosts suggested that parasites may use water turbulence and vortex formation derived from water and fish movements. It is hypothesised that by exploiting water turbulence

detached gyrodactylids may successfully recolonise prospective hosts. The importance of this, as an alternative transmission strategy, is explored.

4.2 Material and methods

Three different experiments were performed to study aspects of transmission in *G. gasterostei*. Experiments were conducted to assess the behaviour of the parasites under two different scenarios: i) transmission from dead hosts; and, ii) cohabitation of infected live fish, uninfected live fish and uninfected dead fish.

4.2.1 Source of hosts and parasites

A *G. gasterostei* / stickleback model was used for the purposes of this study as described in section 2.1.

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4.2.2 Parasite free-hosts

Parasite-free hosts were required for a number of experiments as described in section 2.3. Prior to use in experiments, treated fish were checked under an Olympus SZ30 stereo microscope to ensure that all parasites had been successfully removed. Following treatment, the fish were maintained for a further 3 days before commencing experiments. Feeding was stopped on the day prior to the start of experimentation.

4.2.3 Dead host parasite migration experiment

4.2.3.1 Choice of anaesthetic and its effect on gyrodactylids transmission

This experiment was designed to examine the maturity status of worms moving off dead hosts at 10°C. For this experiment it was hypothesised that one or more of the following conditions might be over-represented in transferring worms: i) worms that

had already given birth at least once and had a male copulatory organ (MCO) present; ii) worms having empty uterus and therefore not burdened by the extra load of carrying a daughter. To investigate this, 20 individual sticklebacks were euthanised with 0.01 M 2-phenoxyethanol (MERCK-Germany) and another 20 fish were euthanised by pithing (piercing the head and destroying the brain) as a control and placed in individual Petri dishes containing clean water. Dead hosts were observed under an Olympus SZ30 stereomicroscope and the time at which each gyrodactylid looped off the fish recorded over 100 minutes. All the gyrodactylids migrating off the fish were collected with a 200 µl pipette, transferred to slides and were mounted under a coverslip with a drop of Malmberg's fixative (ammonium picrate glycerine). After 100 min, the experiment was terminated and the population of gyrodactylids leaving the fish were staged. The maturity status of worms was recorded using a compound microscope (Olympus BX51) under a ×100 / oil immersion objective. The fish carcasses containing non-migrated parasites were fixed in 80% ethanol for assessment of the maturity status of immigrated individuals to establish the overall population structure. Several features of the recovered parasites were assessed: i) an identification of species using the hard parts of the opisthaptor; ii) four developmental states were recognised to describe the stage of maturation of each parasite – 1) no daughter and no MCO (ND/N MCO); 2) no MCO and daughter present (N MCO /D); 3) MCO present but no daughter (MCO /ND); and, 4) MCO present and a daughter present *in utero* (MCO /D). It is important to note in this context that a daughter/embryo was considered to be present if the rudimentary hard parts of the opisthaptor or attachment hooks were evident. In the case of the MCO, this was considered to be present only if the spines were clearly visible. The presence or absence of a MCO was used to evaluate the maturity of the gyrodactylids, whereas, to

evaluate the reproductive state, the feature analysed was the presence or absence of a daughter *in utero*.

4.2.4 Fish cohabitation experiment

This second experiment set out to look at the types of transmission occurring between live and / or dead hosts, and to determine whether transfer is random when given the choice, or targeted towards a certain type of host. Here, gyrodactylid-infected sticklebacks were co-habited with a live uninfected fish and an uninfected dead fish. For both experiments sticklebacks were kept in a 200 ml chamber for 3 hours at 15ºC under ambient light conditions (2800 lux) which were measured with handheld light meter. After the period of cohabitation, live fish were euthanised and both live and dead fish were preserved in 80% ethanol to fix all the gyrodactylids on each fish. The water from each experiment was also mesh-filtered (20 μm) and all parasites collected so that the maturity status of each gyrodactylid could be determined. Twelve replicate trials were carried out to assess transmission between cohabiting fish.

4.2.5 one-one Fish cohabitation experiment

On a second attempt, this cohabitation experiment was replicated increasing the number of fish hosts. Twenty replicate trials were carried out to assess the rates of transmission between cohabiting fish. In each replicate, one live parasitised fish was cohabited with one live clean fish and also one live parasitised fish was cohabited with a dead clean, fish. A live parasitised fish was used as a control. This experiment follows the same protocol that is used and detailed in section 4.2.4.

4.3 Results

4.3.1 Dead host migration experiment

From 14 replicate trials, a total of 241 worms looped of the dead host; 88.66% (213) of these within the first 70 minutes following the death of the host. Of these migrating worms, 39.62% gave birth, accounting for 95.23% of all births observed during the experiments. Currently it is unknown whether giving birth prematurely statistically affects the subsequent survival of the mother worms. From the current data, however, 58.50% of migrating worms survived 24 h off the host, 32.07% 48 h and 9.43% up to 72 h while 67% of daughters survived 24 h after birth and 33% up to 48 h. Within each of these categories, the maturity and reproductive stage of gyrodactylids was assessed to determine if these may be cues prompting their dispersal and their seeking out of a new host.

The majority of parasites transferring off dead hosts held in 10^{o}C water were mature *i.e.* 70.83% had a MCO present and 64.58% had a daughter *in utero*. The remaining population on the host was not mature (Table 4.1).

Table 4.1. Maturity and reproductive status of worms migrating off dead hosts, the majority (~70%) are mature worms *i.e.* those that have given birth at least once and bear a MCO.

Reproductive status	**% non-migrating parasites** n = 241	**% total**	**% migrating** n = 213	**% total**
N MCO / ND	15.09 %		4.17%	
N MCO / D	56.6 %	71.69%	25%	64.58%
MCO / ND	10.37 %		31.25%	
MCO / D	17.92 %	28.29%	39.58%	70.83%

Abbreviations: the maturity and reproductive status is recognized by four developmental states 1) no daughter and no MCO (ND/N MCO); 2) no MCO and daughter present (N MCO /D); 3) MCO present but no daughter (MCO /ND); and, 4) MCO present and a daughter present *in utero* (MCO /D).

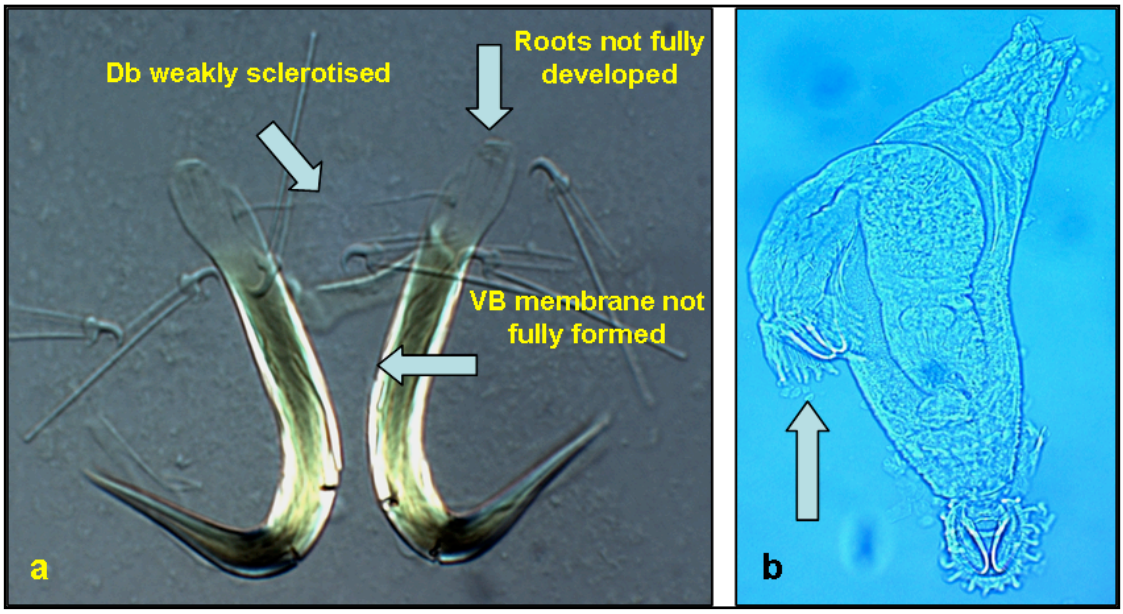


Figure 4.1. a) A premature birth – the haptoral hooks are not fully developed. b) Breach birth (haptor first).

Table 4.2. The developmental status of gyrodactylids transferring to either a dead or a live host and the dislodged worms collected in the mesh. 1) no daughter and no MCO (ND/N MCO); 2) no MCO and daughter present (N MCO /D); 3) MCO present but no daughter (MCO /ND); and, 4) MCO present and a daughter present *in utero* (MCO /D).

Fish category	Maturity status of the gyrodactylid				
	N MCO / ND	N MCO / D	MCO / ND	MCO / D	Total
Dead clean	4	1	8	1	14
Live clean	1	6	11	5	23
Live parasitised	79	510	346	274	1209
Mesh	12	29	17	12	79
Total	**96**	**546**	**382**	**292**	**1325**

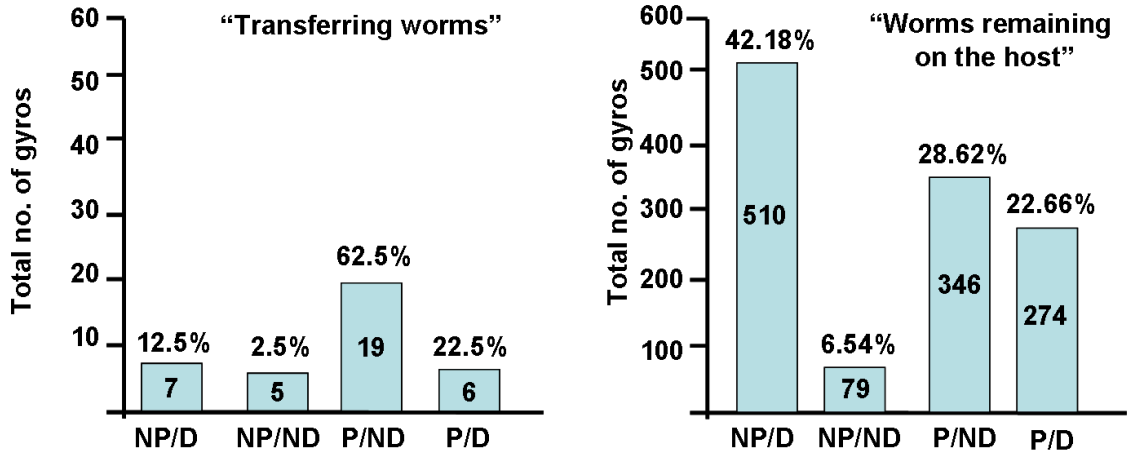


Figure 4.2. The developmental status of gyrodactylids transferring to either a dead or a live host (n = 37; left) and those remaining on the host after the 3 hour cohabitation period (n = 1209; right). Abbreviations: 1) no daughter and no MCO (ND/N MCO); 2) no MCO and daughter present (N MCO /D); 3) MCO present but no daughter (MCO /ND); and, 4) MCO present and a daughter present *in utero* (MCO /D).

4.3.2 Choice of anaesthetic and its effect on gyrodactylid transmission

From 40 trials, a total of 544 gyrodactylids were recorded and staged. The results showed that when hosts were euthanised using the anaesthetic 2-phenoxyethanol, then a total of 47 parasites were found to have transferred to the bottom of the Petri dish. Of these, 8 ± 0.82 (*i.e.* 17 %) parasites had an MCO and a daughter present; 14 ± 0.73 (*i.e.* 30 %) had no MCO but a daughter present; 23 ± 1.26 (*i.e.* 49%) had an MCO and daughter absent, and finally, 2 ± 0.30 (*i.e.* 4%) had no MCO and no daughter present.

Examination of the 198 worms that remained on the parasitised fish host were made up of 42 ± 1.65 (*i.e.* 21 %) parasites that had an MCO and a daughter present; 108 ± 3.0 (*i.e.* 54%) parasites that had no MCO but a daughter present; 36 ± 2.01 (*i.e.* 18%) that had an MCO but no daughter, and finally, 12 ± 6.06 (*i.e.* 6%) parasites that had neither an MCO or a daughter present.

Table 4.3. The developmental status of gyrodactylids transferring from the anaesthetic euthanised fish (n = 20). Abbreviations: 1) no daughter and no MCO (ND / N MCO); 2) no MCO but a daughter present (N MCO / D); 3) MCO present but no daughter present (MCO / ND); and, 4) an MCO present and a daughter present *in utero* (MCO / D).

	MCO/D	**N MCO/D**	**MCO/ND**	**N MCO/ND**	**TOTAL**
	8	**14**	**23**	**2**	**47**
SD	0.82	0.73	1.26	0.30	
%	17.02	29.78	48.93	4.25	

Table 4.4. The developmental status of gyrodactylids remaining on the anaesthetic euthanised fish (n = 20). Abbreviations: 1) no daughter and no MCO present (ND / N MCO); 2) no MCO but a daughter present (N MCO / D); 3) MCO present but no daughter (MCO / ND); and, 4) both an MCO and a daughter present *in utero* (MCO / D).

	MCO /D	**N MCO/D**	**MCO/ND**	**N MCO/ND**	**TOTAL**
	42	**108**	**36**	**12**	**198**
SD	1.65	3.01	2.015	0.68	
%	21.21	54.54	18.18	6.06	

4.3.3 Pithing hosts and its effect on gyrodactylid transmission

The results showed when hosts were pithed a total of 61 parasites transferred from the hosts to the bottom of the Petri dish. Of these, 15 ± 1.20 (*i.e.* 25 %) had an MCO and a daughter present; 12 ± 0.82 (*i.e.* 20 %) lacked a MCO but had a daughter present; 22 ± 1.29 (*i.e.* 36%) had an MCO but a daughter absent, and finally, 12 ± 0.68 (*i.e.* 20%) parasites had neither an MCO or a daughter present.

Table 4.5. The developmental status of gyrodactylids transferring from hosts that were killed by pithing (n = 20). Abbreviations: 1) no daughter and no MCO (ND / N MCO); 2) no MCO and daughter present (N MCO / D); 3) MCO present but no daughter (MCO / ND); and, 4) MCO present and a daughter present *in utero* (MCO / D).

	MCO/D	**NMCO/D**	**MCO/ND**	**NMCO/ND**	**TOTAL**
	15	**12**	**22**	**12**	**61**
SD	1.2	0.8	1.29	0.68	
%	24.59	19.67	36.06	19.67	

Of the 238 worms that remained on the host killed by pithing 64 ± 3.57 (*i.e.* 27%) parasites had an MCO and a daughter present; 115 ± 5.0 (*i.e.* 48%) parasites had no MCO but a daughter present; 36 ± 2.04 (*i.e.* 15%) had an MCO but no daughter, and finally, 23 ± 1.46 (*i.e.* 10%) parasites lacked both an MCO and a daughter.

Table 4.6. The developmental status of gyrodactylids that remained on hosts that were killed by pithing (n = 20). Abbreviations: 1) no daughter and no MCO (ND / N MCO); 2) no MCO and daughter present (N MCO / D); 3) MCO present but no daughter (MCO / ND); and, 4) MCO present and a daughter present *in utero* (MCO / D).

	MCO/D	**NMCO/D**	**MCO/ND**	**NMCO/ND**	**TOTAL**
	64	**115**	**36**	**23**	**238**
SD	3.57	5.01	2.04	1.46	
%	26.89	48.31	15.12	9.66	

4.3.3 Statistical analysis of the methods used to kill fish

A Wilcoxon (non-parametric) test was used to test whether there was a difference in the number of parasites transferring to new hosts from infected hosts killed by one of two methods (*i.e.* pithing *versus* anaesthetic) (W = 157; p = 0.245); the results were not significant. The mean number of parasites transferring from hosts killed with

anaesthetic were 2.35 ± 2.23 (mean ± S.D.) whilst those from hosts killed by pithing were 3.05 ± 2.21. These results demonstrate that the use of the anaesthetic 2-phenoxyethanol does not affect the population of gyrodactylids which transmit off the host (Figures 4.3 and 4.4).

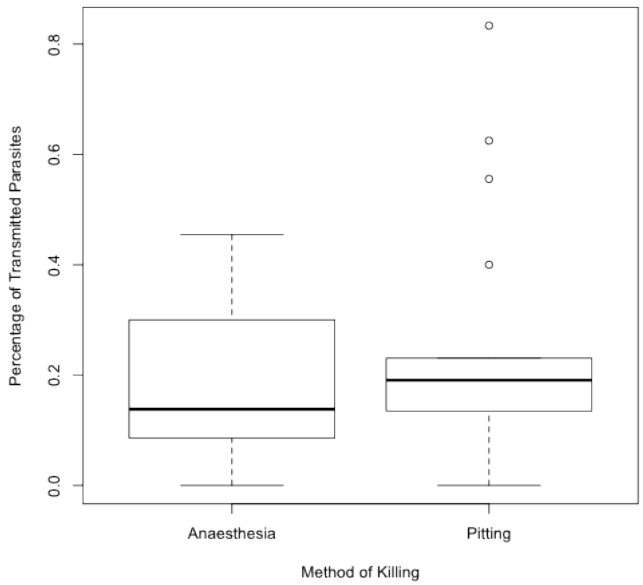


Figure 4.3. Box plot of the proportion of gyrodactylids transferring from fish killed by an overdose of anaesthetic and from those killed by pithing.

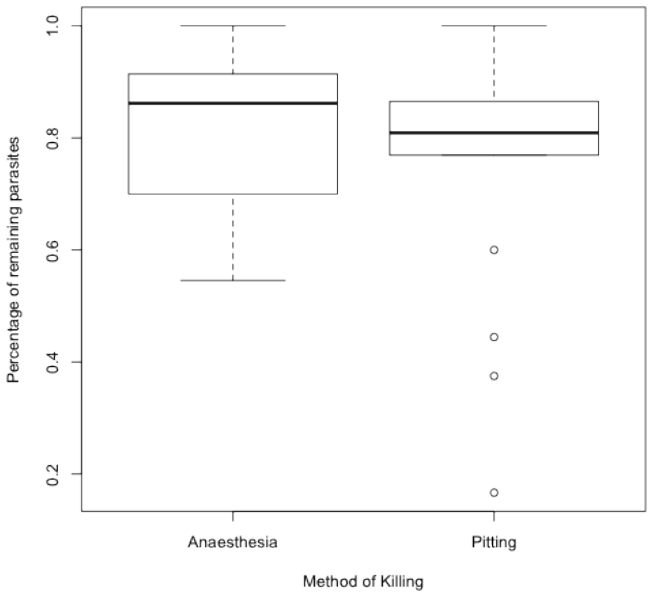


Figure 4.4. Box plot of the proportion of gyrodactylids remaining on fish having been killed by an overdose of anaesthetic and on those killed by pithing.

4.4.2 Fish cohabitation experiment

Overall, from 12 replicate trials, a total of 1325 gyrodactylids were recorded. The results showed that 23 (1.73%) parasites transferred to the live/clean fish, 14 (1.05%) parasites transferred to the dead/clean fish, 79 (5.96%) parasites failed to re-attach and 1209 (91.25%) parasites remained on the original parasitised host.

Determining the reproductive maturity status of each worm revealed that the largest group of worms transferring to live hosts consisted of two overlapping categories of gyrodactylids: those with an MCO (85%) and those lacking a daughter *in utero* (65%). Of the worms remaining on the hosts, however, the largest single class was the most immature class *i.e.* those with no MCO (48.72%).

Regarding the influence of water flow and turbulence as a possible transmission factor, a series of video recordings (see Figure 4.5 and slides 4 and 5 of the PowerPoint presentation on the attached CD) which show the movement of three gyrodactylids which were placed on the viewing platform and their subsequent transmission onto a new host was followed and recorded. The movement of the parasite through the water column suggests that the parasite exploits water vortices generated by the movement of the host's fins to assist its transmission onto a potential host (see slides 6-9 of the PowerPoint presentation on the attached CD).

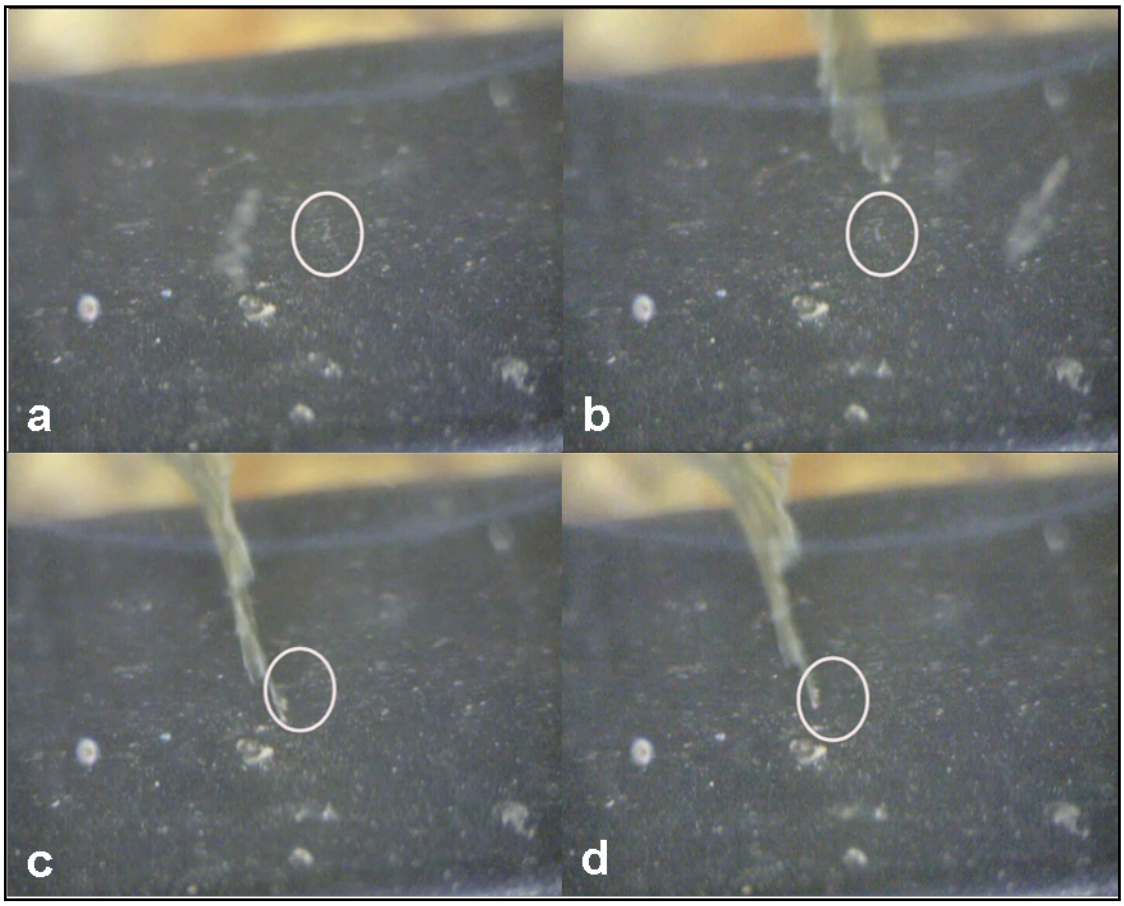


Figure 4.5. (a-d) A sequence of stills taken from video footage showing the attachment of a detached gyrodactylid (circled) attaching to the caudal fin of a 3-spine stickleback.

4.4.3 Fish (live infected *versus* dead uninfected) cohabitation experiment

A total of 3877 parasites were collected from all the replicate trials conducted for the 3 h fish cohabitation experiment. Of these, a total of 290 (22%) parasites transmitted from the live infected fish on to the dead uninfected fish (Table 4.7). Of these parasites that transferred, 104 (36%) were mature in that they had an MCO present and a daughter present *in utero*. A Kruskal-Wallis (non-parametric) test applied to the data suggests that there are significant differences ($K = 18.66$, $p<0.001$) between the parasites remaining on live parasitised fish exposed individually, and live clean fish, those on dead clean fish and those on the control fish (Table 4.7 and Figure 4.6).

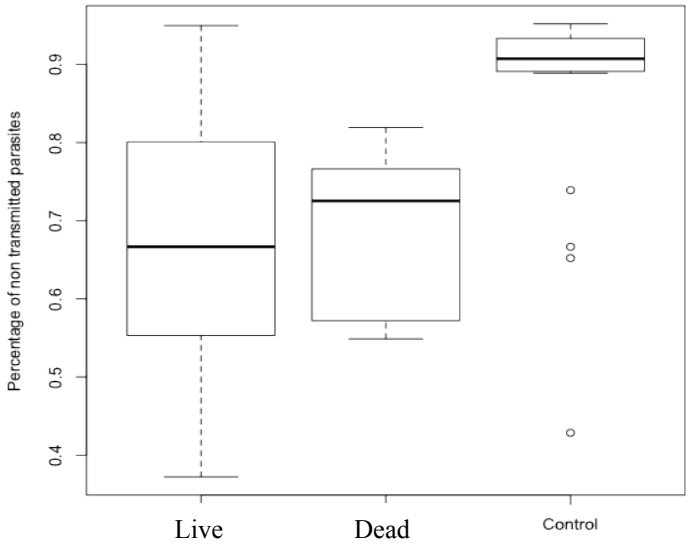


Figure 4.6. Box plot of the proportion of gyrodactylids remaining on the live parasitised fish and on the live clean fish and the dead clean fish which they were exposed to following a 3 hour period of cohabitation. In addition, the numbers of parasites on the control fish are also considered.

The use of the non-parametric Wilcoxon test suggests significant differences (W = 100; p = 0.007), concerning the percentage of parasites transmitting to either a clean dead or live fish (Figure 4.7).

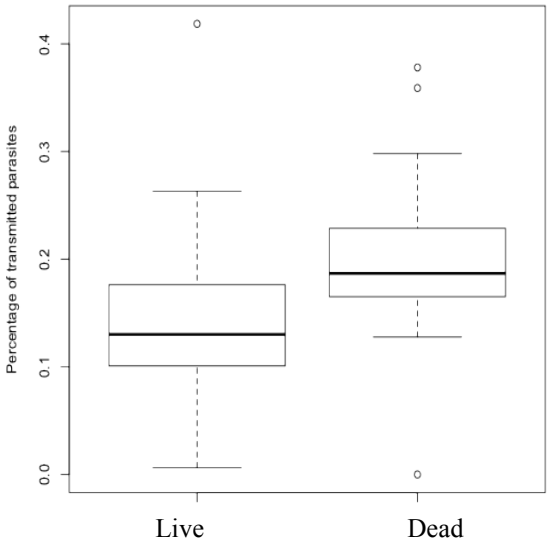


Figure 4.7. Box plot of the proportion of gyrodactylids transmitting to either a clean dead or live host during a 3 h period of cohabitation with an infected live host.

Table 4.7. The cohabitation experiment one-one parasite transmission showing the developmental status of gyrodactylids during 3 h cohabitation using fish (n = 20) for each category: live clean, live parasitised and dead clean. Abbreviations: 1) no daughter and no MCO (ND / N-MCO); 2) no MCO and daughter present (N-MCO / D); 3) MCO present but no daughter (MCO / ND); and, 4) MCO present and a daughter present *in utero* (MCO / D).

Fish category	**MCO/D**	**%**	**NMCO/D**	**%**	**MCO/ND**	**%**	**NMCO/ND**	**%**	**TOTAL developmental stages**	**Total parasites in the system**	**%**
live clean	46	33.33	21	15.21	49	35.50	22	15.94	**138**	**1315**	**10.49**
live paras	291	27.58	447	42.36	184	17.44	133	12.60	**1055**		**80.22**
mesh	18	14.75	13	10.65	66	54.09	25	20.49	**122**		**9.27**
dead clean	104	35.86	56	19.3	85	29.31	45	15.51	**290**	**1320**	**21.96**
live paras	283	30.49	399	42.99	162	17.45	84	9.05	**928**		**70.30**
mesh	25	24.50	9	8.82	53	51.96	15	14.70	**102**		**7.72**
control	314	27.86	430	38.15	243	21.56	140	12.42	**1127**	**1242**	**90.74**
mesh	28	24.34	25	21.73	47	40.86	15	13.04	**115**		**9.25**
TOTAL	**1109**		**1400**		**889**		**479**		**3877**	**3877**	
Average	7.701		9.72		6.17		3.32				
SD	11.136		17.54		8.00		5.70				
%	28.60		36.11		22.93		12.35				

4.5 Discussion

This study describes aspects of the transmission of *G. gasterostei* to its *Gasterosteus aculeatus* host, including the role of maturity and reproductive status in migration. In the two experiments that were performed, the maturity state of each parasite was evaluated by observing the presence or absence of an MCO, which appears after gyrodactylids have given birth for the first time (at an age of 24-30 h at 13ºC for *G. gasterostei*; see Harris, 1985). Regarding their reproductive status, the presence or absence of a daughter was also recorded.

In the dead host migration experiments, the results suggested that worms with the presence of a developed MCO were more likely to leave the host (Table 4.2). The population remaining on the host was largely immature. This suggests that gyrodactylids that have given birth at least once are more likely to leave the host following host death in order to colonise new hosts. Young flukes, which have not given birth, have high life expectancy, and may remain on the fish until after they have given birth in order to maximise population numbers. The decision to leave a fish may also reflect the nutritional status of the worm, these perhaps requiring a filled gut or high stored reserves before leaving the host. In this regard, it is possible that the large size of *Gyrodactylus* embryos *in utero* physically constrains feeding during the latter stages of development by blocking food entry into the intestinal crura. This suggestion could be further investigated by future studies utilising confocal microscopy. Cable *et al.* (2002) noted that detached, starved parasites can abort their offspring (embryos) and that an interruption in nutrient flow to the embryo might have a significant impact on reproductive rate.

For *Gyrodactylus* species which have been studied, it is suggested that individuals give birth to less than four daughters in their life time *e.g. Gyrodactylus*

alexanderi Mizelle et Kritsky 1967 on 3-spine sticklebacks (Lester & Adams, 1974) and *Gyrodactylus bullatarudis* Turnbull, 1956 on guppies (*Poecilia reticulata* Peters) (see Scott, 1982). However, Harris (1985) established that in wild or laboratory populations of *G. gasterostei,* less than 5% of all individuals survive to give birth twice. The survival observed in the present study (~3 days at 10 °C) is comparable with the life-span of detached *G. gasterostei* described by Cable *et al*. (2002); the same authors reported the maximum survival times for *G. gaserostei* as being 103 h at 4°C, 89 h at 10°C, and 66 h at 15°C.

In the cohabitation experiment, transferring worms showed the same behaviour as the previous experiment involving the dead host. Mature parasites with an MCO present are the first to leave the live host. Those lacking an MCO and having a daughter present made up the majority of those remaining on the fish (n = 510, 42.18%).

Of the worms which had failed to re-attach to hosts and were recovered from a mesh to catch them (n = 79), 51.9% lacked an MCO, representing either newborn worms or young mothers. Of the worms found on the mesh, only a small percentage was recovered still alive (7.59%).

It is suggested that these recovered worms represented either failed transfers by healthy worms, accidental dislodgements, mothers surviving from a birth event or full-term daughters. Given the large size of the worms being born, it is not surprising that a significant proportion of births result in either death (29.11%) or damage (41.77%) to the mother.

A significant percentage of worms failing to attach during a transfer opportunity or accidentally dislodged, appear to lose their embryos subsequently. Of the daughters that were lost, some were quite advanced in their development *i.e.* had

fully developed marginal hooks and near complete hamuli but had under-developed hamuli roots and dorsal / ventral bars. This represents an interesting observation because if worms can be forced to transfer off their hosts prematurely and this results in an increased chance of embryo loss, then this could have a significant effect on the number of worms surviving and managing to reattach.

In the first trial, the cohabitation experiment was performed using hosts in three different states in the same container (*i.e.* live and parasitised, live and clean and dead and clean), however, the limitation of this study was that all the three types of host were cohabited together at the same time and therefore this misses some of the subtleties of paired experiments (*i.e.* using only two types of host at a time). However, in the second experiment, the one-to-one fish cohabitation trial, it was clear that the condition of dead fish was "favourable" for the transmission of gyrodactylids on to it.

A chance observation made in the course of this study was that when fish were infected with the common skin parasite "white spot" *Ichthyophthirius multifiliis* (Figure 4.8), gyrodactylid infections were unsuccessful (100% failed). It is possible that this may be evidence of one parasite making the host unsuitable for the colonisation of the second. This phenomenon has been previously described for *Ichthyophthirius multifiliis* (Hoffman & Putz, 1964; Buchmann, 1999; Buchmann *et al.,* 1999) due to a response to parasites invading the epidermis. Gyrodactylids might also influence one another's host range *e.g. G. bullatarudis* and *G. turnbulli* on guppies interfere with each other's population growth via the host response (Richards & Chubb, 1996). This study reports for the first time a possible unfavourable condition generated by 3-spine sticklebacks infected with *Ichthyophthirius multifiliis* and *G. gasterostei / G. arcuatus,* however, as mixed infections do occur (see Figure

4.8) further experiments are required to determine under what conditions "unsuitability for infection" might occur.

Figure 4.8. The caudal fin of *Gasterosteus aculeatus* showing a co-infection of *Ichthyophthirius multifiliis* and *Gyrodactylus gasterostei.* Scale bar: 5 mm

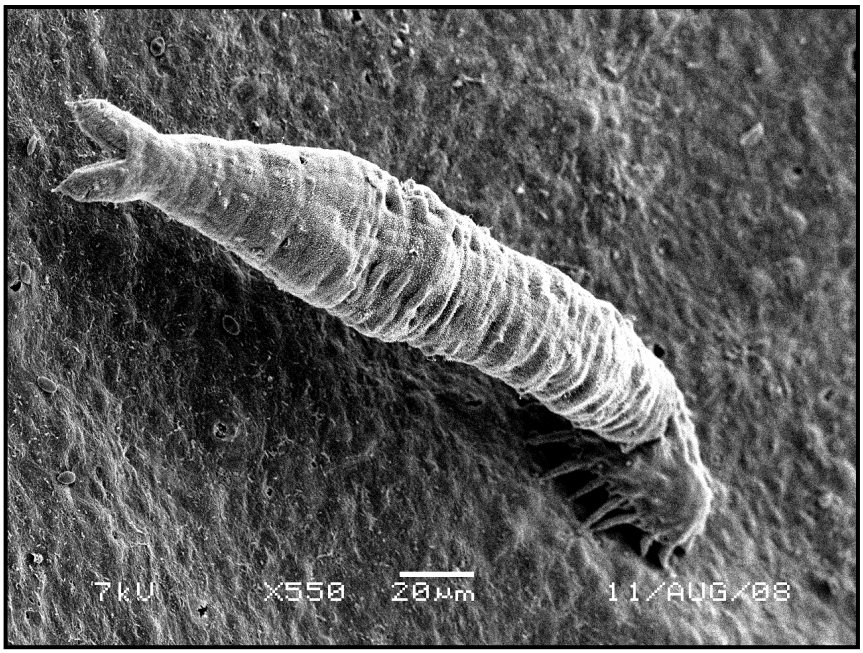
Gyrodactylus gasterostei is not capable of directional swimming as has been reported for *G. rysavyi* by El-Naggar *et al*. (2004) who suggests that this parasite uses this ability to increase its chances of transmitting to a new host. In the present study, based on video observation, it is suggested that detached worms may employ turbulence to assist their transfer onto a new host; this strategy might possibly be advantageous for those parasites attached to the substratum. It is possible that parasites may initially detect water movements associated with an approaching host and then prepare themselves for attachment. The turbulence could considerably affect the parasites movement (*i.e.* transmission pathway) showing that in complex flows, *e.g.* the pectoral-fin wake, flows can have dramatic effects on the parasite

transmission performance. Turbulence has been used by a number of authors (Tytell, 2006, Bozkurttas *et al*., 2006). Drucker & Lauder (2003) describing rainbow trout locomotion, focused on the functioning of the pectoral fin, and found that the pectoral fins produced vortices that could be used by the parasite in the same way that is described in this study. Future experiments should be conducted to examine these suggestions.

Finally, the present study shows that the *G. gasterostei* / *Gasterosteus* model is a simple and successful system to examine aspects of transmission of parasites from live and dead fish. These experiments include determining the maturity and reproductive status of transmitting worms, in order to consider the effects of factors that influence parasite choice, upon the likelihood of migration to a new fish host. This study demonstrates that parasites with an MCO present are more likely to abandon the host and attempt a host tranfer than those parasites that have a daughter *in utero*.

Chapter 5

The accidental transfer of *Gyrodactylus* (Monogenea) during short duration fish transportation



SEM of *Gyrodactylus gasterostei* attached to the epithelium of *Gasterosteus aculeatus*

Aspects of this chapter have been accepted for publication in Fish Pathology

5.1 Introduction

The viviparous ectoparasitic monogenean *Gyrodactylus salaris* Malmberg, 1957 is a pathogen of notable significance, with infections of Atlantic salmon *Salmo salar* in Norwegian rivers having historically resulted in heavy losses (Johnsen and Jensen, 1986, 1992; Johnsen *et al.,* 1999). *Gyrodactylus salaris* is a notifiable disease in the UK and is known to be highly pathogenic to British Atlantic salmon (Bakke and MacKenzie, 1993). Statutory national surveillance programmes throughout the UK, therefore sample rivers and fish farms on a regular basis for the purposes of screening for pathogens that may impact on the health and sustainability of fish stocks. These diseases are detailed by Defra (DOF 21) and include *G. salaris* as a List III pathogen.

The present study focuses on the potential of gyrodactylids to switch hosts and the frequency with which could occur under normal field sampling. One of the principal modes of host-infection within the gyrodactylids is direct transfer from host to host, with movement from substrate to host also recognised as an important route. Both these routes of infection are likely to be further facilitated in the context of field sampling, with dislodgement of parasites and crowding of fish encouraging transfer. The importance of inter-species transfer for this group of parasites is discussed by Harris (1993), Bakke *et al.* (2002) and Bakke *et al.* (2007), who suggested host-switching to be the predominant mode of spreading within the gyrodactylid group. This study looks at the manner in which field samples are collected and whether practices have a bearing on the accurate allocation of gyrodactylid species to hosts. Specifically, there are concerns that holding two different fish species in the same transportation vessel may either cause gyrodactylids to detach from their respective hosts, causing parasite burdens to be under-reported or allowing gyrodactylids to

transfer to new hosts providing specificity estimates that are lower than expected. With these considerations in mind, current routine collection and screening procedures may lead to a proportion of worms going undetected or misidentified. Previous studies that have considered host to host transfer have tended to do so over longer time periods of several days. For example, Moen and Stockwell (2006) exposed *Cyprinodon tularosa* and *C. variegatus* to the *C. tularosa*-specific gyrodactylid *Gyrodactylus tularosae* Kritsky et Stockwell, 2005 for up to 4 days. Whilst the parasite was able to use *C. variegatus* as a transient host, its preference was for its typical host. No data was obtained by the authors on the number of host transfers that occurred over the experimental period. In a similar study, Blazek *et al.* (2008) held ruffe, *Gymnocephalus cernuus,* and perch, *Perca fluviatilis,* together for around two weeks and minnows, *Phoxinus phoxinus,* and roach, *Rutilus rutilus,* together for up to 3 weeks. *Gyrodactylus macronychus* Malmberg, 1957, a parasite of *P. phoxinus*, transferred to the atypical host *R. rutilus* only once. Under natural conditions where gyrodactylid numbers are high, the *P. phoxinus* parasites *G. macronychus* and *Gyrodactylus aphyae* Malmberg, 1970 have similarly been shown to undergo limited transfer to brown trout, *Salmo trutta* (Mo, 1997). It has been suggested, however, that under experimental conditions, *Gyrodactylus* spp. appear to transfer to atypical hosts, so the conclusions that may be drawn from inter-species transfers under such conditions are uncertain (*e.g.* Bakke and Sharp, 1990; Bakke *et al*., 1990, 1991; King and Cable, 2007).

The current study looks at the level of gyrodactylid transfer over a short time span between cohabited hosts (three-spine sticklebacks *Gasterosteus aculeatus*; *P. phoxinus*; and stone loach *Barbatula barbatula*) from one Scottish river.

5.2 Materials and methods

5.2.1 Host and gyrodactylid collection

Specimens of *B. barbatula*, *G. aculeatus* and *P. phoxinus* were hand-netted as described in section 2.2.

5.2.2 Fish cohabitation experiment

Upon arrival at the research aquarium, *Gyrodactylus*-infected fish were randomly assigned to 1 L beakers each containing 900 mL, 20 μm filtered water from the River Endrick which was taken at the point of fish capture. The fish were distributed in three replicate vessels each contained three *B. barbatula* and three *G. aculeatus*, another three replicate vessels each contained three *P. phoxinus* and three *G. aculeatus*. Each series of control vessels were set up so that three replicate vessels each contained three *B. barbatula* only (*B. barbatula* control), three *G. aculeatus* only (*G. aculeatus* control) or *P. phoxinus* only (*P. phoxinus* control). The experimental vessels were maintained for 3 h under ambient light conditions (2800 lux) and at the same temperature as the river water at the point of capture (15ºC).

After 3 h, each fish from each beaker was euthanised in accordance with UK Home Office regulations and subsequently fixed, individually, in 80% alcohol. The water from each beaker was then passed through a 20 μm mesh filter to recover any dislodged parasites. The beaker was then rinsed with 100 mL 80% ethanol and the liquid passed through the filter. The mesh was then back-washed into a separate 20 mL vial to release any gyrodactylid specimens. The skin, gills, nostrils and the mouth cavity of each fish were examined for ectoparasites under an Olympus SZ30 stereomicroscope at ×4 magnification. The position of each gyrodactylid on each fish was noted before it was carefully removed using mounted triangular surgical needles

(size 16, Barber of Sheffield, UK). Each specimen was then mounted on a glass slide with a drop of distilled water ensuring that the haptoral hooks were flat. The specimens were then stained and fixed *in situ* by the addition of a drop (~3 μL) of Malmberg's fixative (ammonium picrate glycerine, APG; saturated picric acid and 100% glycerine) to the edge of the coverslip, which was drawn under the coverslip by capillary action. The coverslip was then sealed with transparent nail varnish. Each specimen of *Gyrodactylus* was identified following examination and measurement of the hard parts of the haptor using a compound microscope (Olympus BX51) at ×100 / oil immersion magnification. The maturity status of each worm was also determined as either 1) lacking both a male copulatory organ (MCO) and an embryo *in utero*, 2) lacking an MCO but with an embryo *in utero*, 3) possessing an MCO but lacking an embryo *in utero*, or 4) possessing both an MCO and an embryo *in utero*.

5.2.3 Taxonomic identification

Hook morphology, particularly that of the marginal hook sickle, and morphometry was used to identify each gyrodactylid. The total length of the hamulus and a marginal hook on each specimen was recorded from images captured using a JVC KY-F30B 3CCD video camera, mounted on an Olympus BH2 microscope and using a 2.5 interfacing lens at ×100 oil immersion and KS300 (ver.3.0) (Carl Zeiss Vision GmbH, 1997) image analysis software. For specimens of *Gyrodactylus gasterostei* Gläser, 1974 and *G. aphyae* which have similar hook morphologies, the length of the ventral bar was also taken but for these two species only. In addition, the armature of the MCO, where present, was used to facilitate specimen identification. Images of the MCO and hooks were captured using a Zeiss AxioCam MRc digital camera interfacing with an Olympus BH2 compound microscope using a ×0.75 lens and

MRGrab 1.0.0.4 (Carl Zeiss Vision GmbH, 2001) software. The morphometric measurements made on the attachment hooks and ventral bar of each specimen follow those provided in Shinn *et al.* (2004) and are expressed in micrometers as the mean ± standard deviation followed by the range in parentheses and are shown in Table 5.1.

5.3 Results

5.3.1 Morphometric identification

Identification of the gyrodactylids (n = 567) was based on attachment hook morphology and facilitated by measuring the length of the hamuli and the marginal hooks. The discrimination of *G. gasterostei* (n = 406) from *G. aphyae* (n = 42) was more difficult but was made possible by measuring the length of the ventral bar in addition to the previous measurements. The ventral bar of *G. aphyae* is shorter (*i.e.* 26.9 ± 6.1; 20.1 - 35.5) than that of *G. gasterostei* (*i.e.* 31.3 ± 2.7; 28.1 - 36.7) and this assists their discrimination from one another (Figure 5.1; Table 5.1). The MCO, when present, was also used to support species identification (Figure 5.1). The MCO of *G. gasterostei* is presented for the first time, as are the tissue-free haptoral hooks of *G. aphyae* and *G. gasterostei* (Figure 5.1).

5.3.2 The *Gyrodactylus* population on experimental fish

Eleven species of *Gyrodactylus* were recovered from the three fish species sampled (Table 5.1). Over all states and replicates (3 replicates of *B. barbatula* + *G. aculeatus*; 3 replicates of *P. phoxinus* + *G. aculeatus*; 3 replicates of *B. barbatula* only; 3 replicates of *P. phoxinus* only; 3 replicates of *G. aculeatus* only), the results showed that the single-host controls had no apparent accidental infections (n = 303 gyrodactylids), all gyrodactylids recovered being normally associated with that host

(Table 5.2). However, 8 individual gyrodactylid specimens were observed to transfer to an atypical host (Table 5.2) and 9 specimens of *G. gasterostei*, which failed to attach, were recovered from the bottom of the experimental vessels. In the *G. aculeatus – P. phoxinus* trials (18 hosts; 191 gyrodactylids), one specimen of the *G. aculeatus* specific *Gyrodactylus arcuatus* Bychowsky, 1933 and one specimen of *G. gasterostei* were recorded on the *P. phoxinus,* whilst a single specimen of the *P. phoxinus* parasite *Gyrodactylus limneus* Malmberg, 1964 and 2 specimens of the *P. phoxinus*–specific *G. macronychus* (11.8% of the *G. macronychus* population) transferred to *G. aculeatus* (Table 5.2). Similarly, in the *B. barbatula – G. aculeatus* experiment (18 hosts; 82 gyrodactylids), a total of 2 specimens of *G. gasterostei* (4.7% of the *G. gasterostei* population), a species usually restricted to *G. aculeatus*, were found on two *B. barbatula* and a single specimen of the *B. barbatula* specific *Gyrodactylus pavlovskyi* Ergens et Bychowsky, 1964 was noted on a *G. aculeatus*. All of these transfers, with the exception of *G. gasterostei* transferring to *P. phoxinus* (see Dorovskikh, 1997), are recorded for the first time (see Table 5.3). The maturity status of each gyrodactylid transferring to a new host was determined and the details are given in Table 5.2. Of the 8 specimens transferring, 2 had a visible, well developed MCO, while 6 specimens did not. Four had an embryo *in utero* while the remaining four did not. Parasites were recorded on fins, skin and gills of all hosts. All of those gyrodactylids found on "atypical" hosts were recorded from the fins and skin.

Four unattached specimens of *G. gasterostei* were also recovered from the bottom of the experimental vessels in the *B. barbatula – G. aculeatus* trials and 5 from the *G. aculeatus – P. phoxinus* trials representing 9.3% and 4.4% of the *G. gasterostei* population in each set of experiments. The maturity status of each detached specimen was also determined, 4 specimens possessed an MCO, 5 did not

whilst 6 of the 9 specimens had an embryo *in utero*. Given the low number of specimens failing to attach or transferring to an atypical host, it is not possible to identify underlying drivers. Possession of an MCO, detachment during the process of giving birth or encumbrance by a large embryo *in utero* may all make a contribution to the behaviour / status of these individuals.

5.4 Discussion

This study provides evidence for the accidental host transfer of *Gyrodactylus* species maintained in artificially cohabiting host communities over a short time (3 h) period. Under normal conditions, the ability to transfer between hosts, even for short periods, can extend the effective host population available for colonisation, which may be important for survival of individuals and species, particularly under conditions of stress (*e.g*. low recruitment success of the usual host). The ability of *Gyrodactylus* species to transfer between hosts during the sampling process or through mixed-species transportation in the same container, raises important questions over the potential accuracy and reliability of assessments of parasite fauna made following such transport. In this study, the gyrodactylid fauna of artificially cohabiting *G. aculeatus*, *B. barbatula* and *P. phoxinus* following a series of 3 h cohabitation experiments was assessed. The results showed that a number of worms were found to have transferred to an atypical host, with each of these transfers, being recorded for the first time (Table 5.3). Except for the transfer of a single specimen of *G. gasterostei* onto *P. phoxinus*, No accidental infections were found among the 303 gyrodactylids recovered from the control fish. An ongoing gyrodactylid surveillance programme in the River Endrick (>10 years), undertaken by some of the current authors, has similarly documented no accidental infections within these hosts. Whilst

it is possible that the observed accidental transfers may have occurred prior to collection of fish from the wild, the lack of accidental transfers in control groups suggest that this is not the case. A refinement of this study could be to treat all fish with a general anthelminthic to remove all gyrodactylids and then experimentally expose fish to a known number of gyrodactylids. Subsequent cohabitation with mixed naïve species could then lead to parasite transfer. However, this was outside the remit of the current study, which was concerned with transfer under transport conditions. This study has revealed that the opportunities provided for transfer of gyrodactylids between host species arising from a relatively standard sampling routine may affect the correct allocation of parasites to hosts, and the diagnosis, management and control of gyrodactylosis in a variety of fish.

Robertsen *et al.* (2008) suggested that host transfer under natural conditions could cause significant expansion of the geographical range of pathogenic variants of *G. salaris,* allowing it to spread. Gyrodactylids can rapidly colonise an entire river system (Bakke *et al*. 1992), such colonisation being presumably largely dependent upon host densities / availability, such that an expanded host range might be expected to contribute to the speed of colonisation. Colonisation ability also depends on survival time off hosts and since gyrodactylid survival time depends particularly upon water temperature (Soleng and Bakke, 1997), this will also affect colonisation. *Gyrodactylus salaris* can survive for 4 days at 3°C (Olstad *et al*. 2006); the survival of detached *G. gasterostei* is similarly temperature dependent, being 103 h at 4°C (Cable *et al*. 2002). Some host species are clearly more suitable for infection by a wide number of *Gyrodactylus* species. *Phoxinus phoxinus*, for instance has been reported to be associated with a total of 14 gyrodactylid species belonging to seven species groups, more than any other single host (Bakke *et al*. 2002). The presence, in sampled

fish fauna, of hosts with a broad range of parasites or indeed the presence of *Gyrodactylus* species showing low host specificities in such samples might be considered to exacerbate problems of inter-species transfers. This said, in the present study only three gyrodactylids for whom *P. phoxinus* is the primary (typical) host (*Gyrodactylus macronychus, G. limneus* and *G. pavlovskyi)* were found to have erroneously transferred to *G. aculeatus* (atypical host). This fact may be related to the short period of exposure provided (3 h), although this time period reflects an assumed typical transfer time of fish from field sample sites to a laboratory. During a longer term study, Blazek *et al.* (2008) demonstrated that a single specimen of *G. macronychus* transmitted from *P. phoxinus* to *R. rutilus*. Although not expressly measured or observed, it is interesting to speculate on the mode of gyrodactylid transfer between hosts.

Whilst accidental host transfer has been demonstrated to occur following artificial cohabitation of hosts, it is difficult to assess the relevance of such accidental transfer abilities to wild populations. The fact that control hosts and the historical fish-parasite record for this particular river showed no evidence for presence of "foreign" gyrodactylid species may be indicative of the fact that in the capture environment different host species are isolated from one another by a number of factors. Differing environmental preferences (*e.g.* benthic / open water habits) or behavioural isolating mechanisms may isolate host species which effectively decrease probabilities of accidental transfer between host species. Furthermore, host specificity has been used previously as a diagnostic criterion for some species, although this is variable. Many *Gyrodactylus* species are considered host specific, at least to genus or host family. For example, whilst *G. salaris* is considered as a salmonid parasite, it has been found to occur on *P. phoxinus*, *Platichthys flesus* and several genera of salmonid. Of those

species that were shown to transfer to an atypical host during the current study, the two *G. aculeatus* gyrodactylids, *G. arcuatus* and *G. gasterostei* have been recorded on at least 9 and 12 different hosts respectively under normal conditions (Table 5.3). In contrast, of the two *P. phoxinus* parasites, *G. limneus* and *G. macronychus*, only the former has been found on one additional host (Table 5.3). Similarly, *G. pavlovskyi* has only been recorded on another host *Silurus asota* (Table 5.3). Thus, for *P. phoxinus* and *B. barbatula* gyrodactylids, host specificity seems to be normal. The wide host specificity of the *G. aculeatus* gyrodactylids may be either due to previous misidentifications providing an overestimate of the number of true hosts or may be due to a biological transmission strategy on the part of those parasites. *Gasterosteus aculeatus* occurs in a wide range of lentic and lotic water conditions ranging from freshwater to fully marine. Gyrodactylid parasites of *G. aculeatus* may therefore show a measure of the same plasticity with respect to host and environment.

This study has demonstrated that the accidental transfer of gyrodactylids may occur during artificial cohabitation following field sampling of host fish species. Hence standard field sampling practices involving transportation of multiple host species in the same container may affect the correct allocation of parasites to hosts, and the diagnosis, management and control of gyrodactylosis in a variety of fish and it may thus be prudent to reconsider sampling protocols in the light of these findings.

Table 5.1. A summary of the total length of the hamulus and marginal hook (mean ± standard deviation) in micrometers followed by the range in parentheses for each species of *Gyrodactylus* parasitising stone loach *Barbatula barbatula*, three-spine sticklebacks *Gasterosteus aculeatus* and minnows *Phoxinus phoxinus* from the River Endrick, Scotland. The total width of the ventral bar was measured on *G. aphyae* and *G. gasterostei*, which are morphologically similar species, to facilitate their discrimination from one another.

Species	**N**	**Hamulus total length**	**Marginal hook total length**	**Ventral bar total width**	**Usual fish host in the UK**
G. aphyae Malmberg, 1970	42	61.7 ± 5.0 (47.8-71.1)	31.8 ± 2.2 (26.3-34.8)	26.9 ± 6.1 (20.1-35.5)	*P. phoxinus*
G. arcuatus Bychowsky, 1933	18	41.7 ± 4.4 (32.9-53.5)	22.8 ± 3.5 (19.4-27.6)		*G. aculeatus*
G. barbatuli Achmerov, 1952	10	38.8 ± 1.2 (37.5-40.8)	19.6 ± 1.3 (17.9-21.3)		*B. barbatula*
G. gasterostei Gläser, 1974	406	61.0 ± 2.1 (49.4-65.8)	32.5 ± 1.8 (25.9-36.8)	31.3 ± 2.7 (28.1-36.7)	*G. aculeatus*
G. jiroveci Ergens et Bychowsky, 1967	1	55.7	27.1		*B. barbatula*
G. laevis Malmberg, 1957	5	41.4 ± 1.6 (38.5-42.3)	17.2 ± 1.7 (15.3-19.9)		*P. phoxinus*
G. limneus Malmberg, 1964	15	55.1 ± 3.7 (47.1-62.9)	26.6 ± 3.4 (21.0-32.2)		*P. phoxinus*
G. macronychus Malmberg, 1957	33	72.9 ± 2.9 (65.6-78.7)	32.9 ± 2.1 (28.6-37.0)		*P. phoxinus*
G. pannonicus Molnar, 1968	8	51.1 ± 1.1 (50.1-52.7)	26.9 ± 1.5 (23.5-28.2)		*P. phoxinus*
G. pavlovskyi Ergens et Bychowsky, 1967	14	49.7 ± 2.8 (42.9-52.7)	28.4 ± 2.0 (24.6-32.6)		*B. barbatula*
G. sedelnikowi Gvosdev, 1950	24	37.26 ± 1.26 (34.9-39.7)	20.2 ± 1.9 (17.6-23.7)		*B. barbatula*

Table 5.2. The species of *Gyrodactylus* and the number of specimens recorded on each fish host at the end of a 3 h period of cohabitation. The figures marked with an asterisk represent species of *Gyrodactylus* that were deemed to have transferred onto another host during the 3 h period of cohabitation.

Species	***B. barbatula* cohabited with *G. aculeatus***		***P. phoxinus* cohabited with *G. aculeatus***		***B. barbatula* controls (n = 9)**	***G. aculeatus* controls (n = 9)**	***P. phoxinus* controls (n = 9)**	**Total**
	***B. barbatula* (n = 9)**	***G. aculeatus* (n = 9)**	***P. phoxinus* (n = 9)**	***G. aculeatus* (n = 9)**				
G. aphyae			30[m]				12[y]	42
G. arcuatus		1[k]	**1*[c]**	9[r]		7[w]		18
G. barbatuli	9[g]				1[t]			10
G. gasterostei	**2*[a]**	41†[l]	**1*[d]**	112‡[s]		250[x]		406
G. jiroveci	1[h]							1
G. laevis			5[n]					5
G. limneus			7[o]	**1*[e]**			7[z]	15
G. macronychus			16[p]	**2*[f]**			15[aa]	33
G. pannonicus			7[q]				1[ab]	8
G. pavlovskyi	12[i]	**1*[b]**			1[u]			14
G. sedelnikowi	15[j]				9[v]			24
Total	39	43	67	119	11	257	35	567

Abbreviations: †4 of these specimens failed to attach and were recovered from the bottom of the experimental vessels (one specimen lacked an MCO but had a embryo *in utero* (NP/D), one specimen had an MCO and embryo *in utero* (P/D), two specimens had an MCO but no embryo *in utero* (P/ND)). ‡ 5 of these specimens failed to attach (one specimen lacked both an MCO and embryo *in utero* (NP/ND), 1P/D, 3NP/D), **a-f**, the maturity status of each gyrodactylid transferring to an atypical host; **a**, 2P/ND; **b**, 1NP/D; **c**, 1NP/ND; **d**, 1NP/D; **e**, 1NP/ND; **f**, 2NP/D. **g-ab**, the maturity status of each gyrodactylid remaining on its primary host; **g**, 1NP/ND, 1NP/D, 4P/ND, 3P/D; **h**, 1P/D; **i**, 1NP/ND, 4NP/D, 5P/ND, 1P/D; **j**, 1NP/ND, 7NP/D, 1P/ND, 6P/D; **k**, 1P/ND; **l**, 13NP/ND, 24NP/D, 3P/ND, 1P/D; **m**, 2NP/ND, 20 NP/D, 3P/ND, 5P/D; **n**, 1NP/ND, 1NP/D, 1P/ND; **o**, 1NP/ND, 2NP/D, 4P/D; **p**, 1NP/ND, 5NP/D, 3P/ND,7P/D; **q**, 2NP/ND, 4NP/D, 1P/ND; **r**, 4NP/D, 3P/ND, 2P/D; **s**, 2NP/ND, 45NP/D, 25P/ND, 30P/D; **t**, 1P/D, **u**, 1P/ND; **v**, 1NP/ND, 5NP/D, 1P/ND, 2P/D; **w**, 6NP/D, 1P/ND; **x**, 22NP/ND, 130NP/D, 45P/ND, 53P/D; **y**, 9NP/D, 2P/ND, 1P/D; **z**, 1NP/ND, 2NP/D, 4P/D; **aa**, 5NP/D, 3P/ND, 7P/D; **ab**, 1P/D.

Table 5.3. Hosts records for the species of *Gyrodactylus* von Nordmann, 1832 considered in the current study. † Denotes the primary host for each species.

***Gyrodactylus* species**	**Host**	**References**
G. aphyae	*Leuciscus leuciscus*	Dorovskikh (1997), Ivashevsky (1999)
	Phoxinus phoxinus†	Malmberg (1970), Pugachev *et al.* (2010)
	Rhynchocypris czekanowskii (syn. *Phoxinus czekanowskii czerskii)*	Pugachev *et al.* (2010)
	Rhynchocypris percnurus (syn. *Phoxinus percnurus*)	Dorovskikh (1997)
	Rutilus rutilus	Dorovskikh (1997)
	Salmo trutta	Kiskaroly (1988), Mo (1997)
G. arcuatus	*Gadus morhua*	Hemmingsen & MacKenzie (2001)
	Gasterosteus aculeatus†	Bychowsky (1933), Pugachev *et al.* (2010)
	Gobiusculus flavescens	Longshaw *et al.* (2003)
	Pomatoschistus lozanoi	Geet *et al.* (1999)
	Pomatoschistus minutus	Geet *et al.* (1999)
	Pomatoschistus pictus	Geet *et al.* (1999)
	Pungitius pungitius	Domnich & Sarabeev (2000), Sterud (1999)
	Salmo salar	Shinn *et al.* (1996), Sterud (1999)
	Salvelinus alpinus	Sterud (1999)
G. barbatuli	*Barbatula barbatula*†	Achmerov (1952), Pugachev *et al.* (2010)
	Barbatula toni	Pugachev *et al.* (2010)
G. gasterostei	*Alburnus alburnus*	Dorovskikh (1997)
	Blicca bjoerkna	Gussev (1985)
	Gasterosteus aculeatus†	Gläser (1974), Pugachev *et al.* (2010)
	Gobio gobio	Dorovskikh (1997)
	Leuciscus leuciscus	Dorovskikh (1997)
	Perca fluviatilis	Valtonen *et al.* (1997, 2003), Nedeva & Babacheva, (1999)
	Phoxinus phoxinus	Dorovskikh (1997)
	Pungitius pungitius	Pugachev *et al.* (2010)
	Rutilus aula	Galli *et al.* (2002)
	Rutilus rutilus	Dorovskikh (1997)
	Squalius cephalus (syn. *Leuciscus cephalus)*	Dusek *et al*. (1998), Nedeva & Babacheva (1999), Gelnar *et al*. (1997)
G. jiroveci	*Barbatula barbatula*†	Ergens & Bychowsky (1967), Pugachev *et al.* (2010)
	Barbatula toni	Pugachev *et al*. (2010)
G. laevis	*Leuciscus idus*	Pugachev *et al*. (2010)
	Leuciscus baicalensis (syn. *Leuciscus leuciscus baicalensis*)	Pugachev *et al.* (2010)
	Phoxinus phoxinus†	Malmberg (1957), Pugachev *et al.* (2010)
	Rhynchocypris lagowskii (syn. *Phoxinus lagowski*)	Pugachev *et al.* (2010
	Rhynchocypris percnurus	Pugachev *et al.* (2010)
G. limneus	*Phoxinus phoxinus*†	Malmberg (1964), Pugachev *et al.* (2010)
	Rhynchocypris percnurus	Pugachev *et al.* (2010)
G. macronychus	*Phoxinus phoxinus*†	Malmberg (1957), Pugachev *et al.* (2010)
	Rhynchocypris percnurus	Pugachev *et al.* (2010)
	Rutilus rutilus	Blazek *et al.* (2008), Pugachev *et al.* (2010)
	Salmo trutta	Mo (1997)
	Squalius cephalus (syn. *Leuciscus cephalus cabeda*)	Pugachev *et al.* (2010)
G. pavlovskyi	*Barbatula barbatula*†	Gvosdev (1950), Pugachev *et al.* (2010)
	Silurus asota	Pugachev *et al*. (2010)
G. sedelnikowi	*Barbatula barbatula*†	Gvosdev (1950), Pugachev *et al.* (2010)
	Barbatula toni	Pugachev *et al*. (2010)
	Silurus asota	Pugachev *et al*. (2010)

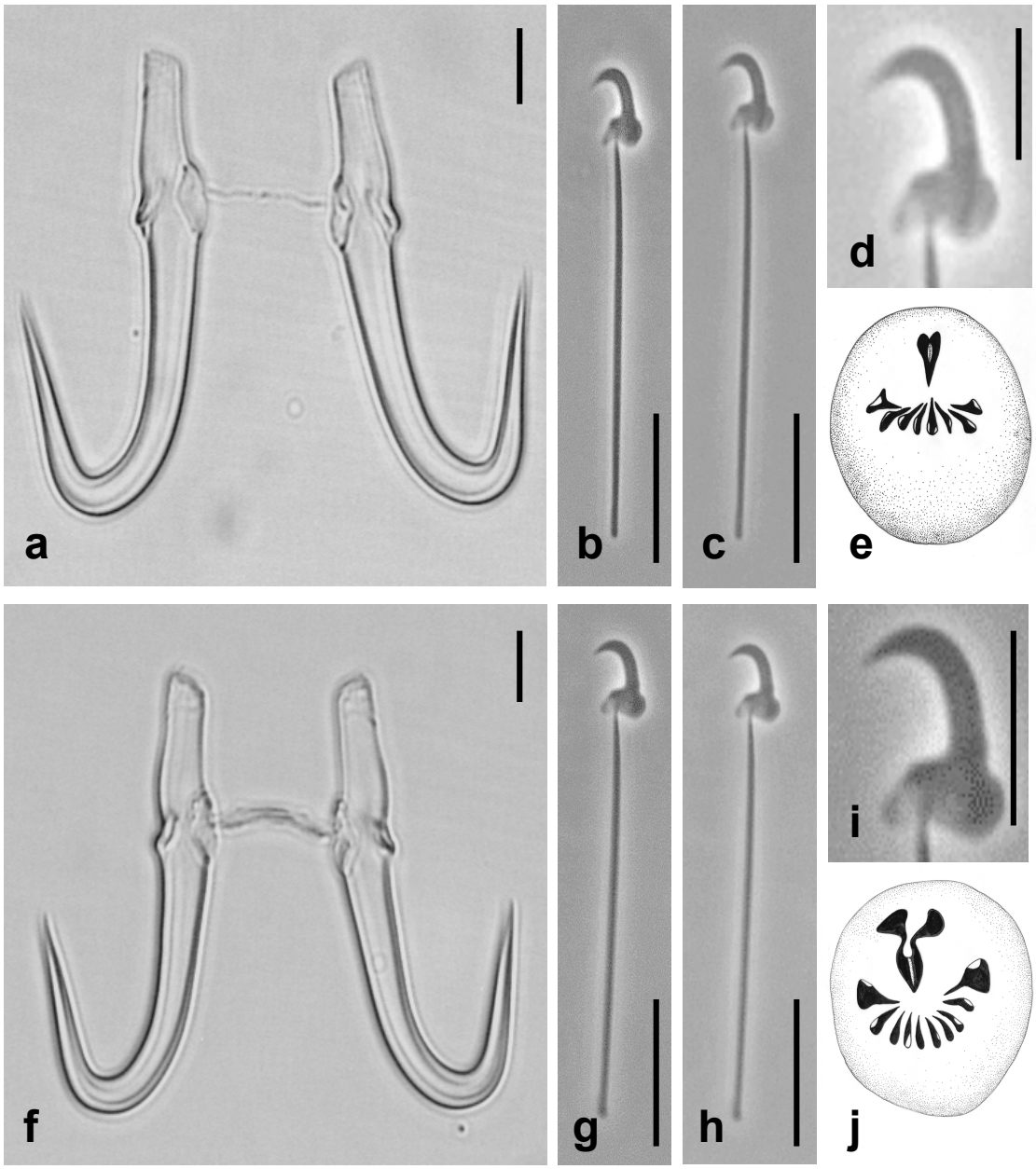
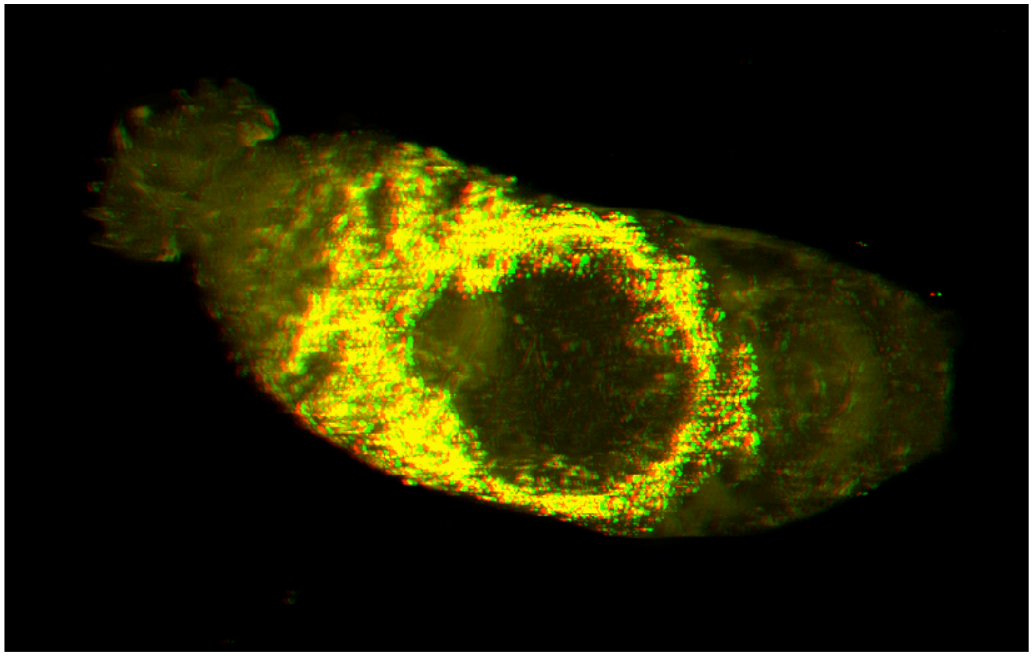


Figure 5.1. The hamuli, marginal hooks and male copulatory organ of *Gyrodactylus aphyae* Malmberg, 1964 (**a-e**) and *G. gasterostei* Gläser, 1974 (**f-j**). **a**, *G. aphyae* hamuli and dorsal bar, **b-c**, marginal hooks, **d**, marginal hook sickle, **e**, male copulatory organ armed with 1 large spine facing a single row of 8 approximately equal sized spines. **f**, *G. gasterostei* hamuli and dorsal bar, **g-h**, marginal hooks, **i**, marginal hook sickle, **j**, male copulatory organ bearing 1 large spine facing a row of smaller spines consisting of 2 medium sized terminal spines and 8 small central spines. Scale bars a-c, f-h = 10 µm, d, i = 5 µm.

Chapter 6

Lipid reserves in *Gyrodactylus gasterostei* Gläser, 1974 migrating from their 3-spine stickleback (*Gasterosteus aculeatus* L.) hosts.



A 3D anaglyph confocal laser scanning micrograph of a high lipid specimen of *Gyrodactylus* sp. showing the presence of lipid rich material within its gut

Aspects of this study were presented at:

Scottish Aquaculture: A Sustainable Future. International Conference, Heriot-Watt, Edinburgh. 21-22nd April, 2009 ,p. 23 (poster; 2nd prize).

6th International Symposium on Monogenea, 3rd-7th August, Cape Town, South Africa. (poster)

European Association of Fish Pathologist (EAFP). Prague. 14th-19th September, 2009 (poster)

6.1 Introduction

Gyrodactylus salaris is a freshwater, monogenean ectoparasite of Baltic strains of Atlantic salmon *Salmo salar* L. on which it generally causes no clinical disease. Infection of other strains of Atlantic salmon in Norway has resulted in high levels of juvenile salmon mortality and highly significant reductions in the population causing significant epidemic disease in Norwegian salmon (Johnsen & Jensen, 1986, 1992; Johnsen *et al.,* 1999; Johnsen, 2006). Work in Norway involving infection of UK Atlantic salmon stocks demonstrated that this species can also be highly pathogenic to stocks UK salmon (Bakke & MacKenzie, 1993) although to date there have been no records of infection.

The decision to leave a dead fish may reflect a given worm's status in two ways. First, the worm's reproductive and developmental status may inform the decision to abandon the host (see Chapters 3 and 4), Second, the nutritional status of the worm, may either prompt or allow migration from the host. It is hypothesised here that the existence of a full gut or high stored reserves might favour decisions to leave the host. Cable *et al.* (2002) noted that detached starved parasites can abort their offspring (embryos) and that an interruption in nutrient flow to the embryo might have a significant impact on reproductive rate. Recently, the study elaborated by Cook *et al.* (2010) study has provided in the copepodid *Lepeophtheirus salmonis* (Krøyer, 1837), a novel technique allowing measurement lipid reserves in individuals and, by extension chronological changes in lipid levels in these small aquatic organisms. Another study by Cooper *et al*. (2010) elaborated on an initial study of cellular lipid content in a flagellated microalga *Chrysochromulina* sp. using fluorescent dye and confocal microscopy.

This study examines the distribution and depletion of stored lipids in *G. gasterostei* Gläser, 1974 migrating off its stickleback host *Gasterosteus aculeatus* L., with the prospect that it might prove informative for interpreting the biology of other gyrodactylids species more generally.

In this study, laser scanning confocal microscope has been employed to quantify the number, size and distribution of lipid droplets in each worm and their depletion with time. Transmission electron microscopy was employed to localise the intestinal wall showing the presence lipid droplets storage vesicles in the underlying gut epithelium.

6.3 Materials and methods

6.3.1 Source of hosts and parasites

A *Gyrodactylus gasterostei* / *Gasterosteus aculeatus* model was used as described in section 2.1 in Chapter 2.

6.3.2 Lipid in migrating worms

This experiment was designed to examine the lipid characteristics of worms moving off dead hosts at 10°C. For this experiment we hypothesised that host transfer might be more favoured in those parasites having higher energy/lipid reserves than non-transferring individuals. Individual sticklebacks were euthanised with an overdose ($0.01/L^{-1}$) of anaesthetic 2-phenoxyethanol (Merck-Germany) and were placed in individual Petri dishes containing clean water at 10 ± 1º C. Dead hosts were observed under an Olympus SZ30 stereomicroscope at different magnifications, with the time at which each gyrodactylid looped off the fish during 60 minutes being recorded. Worms detaching naturally from the host tissue within 60 minutes were then used for analysis. Worms were carefully removed with a 200 µl pipette and were placed individually into

3 cm Petri dishes containing 5ml of filtered (0.45 µm Minisart Sartorius Stedim, Biotech) water taken from the same source as that used for fish maintenance and incubated al 10°C for 24, 48, and 72 hours.

Following incubation for the appropriate period of time, worms were fixed in 10% neutral buffered formalin (NBF) at least 48 hours prior to staining. The worms remaining on the fish after 60 minutes were fixed in 10% NBF an employed as a control.

6.3.3 Microscopy and analysis

Following fixation in 10% NBF, the worms were stained with a lipid specific stain green-fluorescent BODIPY/FL® 505/513 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene; Molecular Probes, Eugene, OR, USA). Worms were incubated in the dark for one hour in 0.1% v/v solution of BODIPY in filtered water. Thereafter, worms were rinsed three times with filtered water, and were then transferred to slides and mounted under a coverslip with a drop of water and sealed with transparent nail varnish. Worms were viewed using a Leica SP2 AOBS Confocal Laser Scanning Microscope (CLSM) (Leica Microsystems AG, Wetzlar, Germany) coupled to a Leica DM IRE2 inverted microscope employing a 20× glycerol-immersion lens to observe the lipid drops stained. Unstained, worms were used as a negative control to assess autofluorescence. Confocal protocol set up is described as follows:

SECTIONS	50
µc	1.0201
GAIN	504.1
OFFSET	0.4
GREEN	494-601 nm
ZOOM	1.5
IMAGE	1024 × 1024

6.3.4 Statistical analysis

Normality and homogeneity tests were employed.

6.3.5 Image analysis software

The image analysis was performed on serial images using Fiji-Win 32 (ver. 2011) software which permits images taken with CLSM to to be processed and analysed as follows:

Step	Operation performed in the main window	Function	Parameters used in this study	Observation
1	Image Sequence	this command opens the image sequence		
2	Image properties	Calibrate image measurements		
3	Analyse 3D objects counter	Threshold	Intensity threshold: 24	Threshold lipid *vs* non-lipid
	"3D OC "	3D select measurements	a. Volume b. Nb. of obj voxel c. Integrated density d. Std dev gray value e. Minimum gray value f. Median gray value g. Maximum gray value	
	"3D viewer"	Reconstruct 3D image		

6.3.6 Transmission electron microscopy (TEM)

Individual worms were fixed as described in Chapter 2 (see Section 2.9).

6.4 Results

Lipid droplets in unstained (control) worms did not fluoresce (Figure 6.1), but once stained a number of nutritional states could be recognised (Figure 6.4), with significant differences (Kruskal-Wallis Test = 9.8287, df = 3, *p*-value = 0.02008) in size, distribution and number of lipid droplets evident (Figure 6.5). The levels of lipid in worms that had abandoned dead hosts were followed over a period of time. Observations during the trial indicate that embryos have a maternally derived lipid store but that the majority of newborn daughters (66%) die within 24 hours if not fed.

Serial confocal images taken through the worm indicate that the highest lipid staining was largely confined to vesicles located within cells positioned in the intestinal wall of the worm rather than residing in food items within the gut, although fluorescent lipid could also be observed in other tissues (Figures 6.2 and 6.3) .

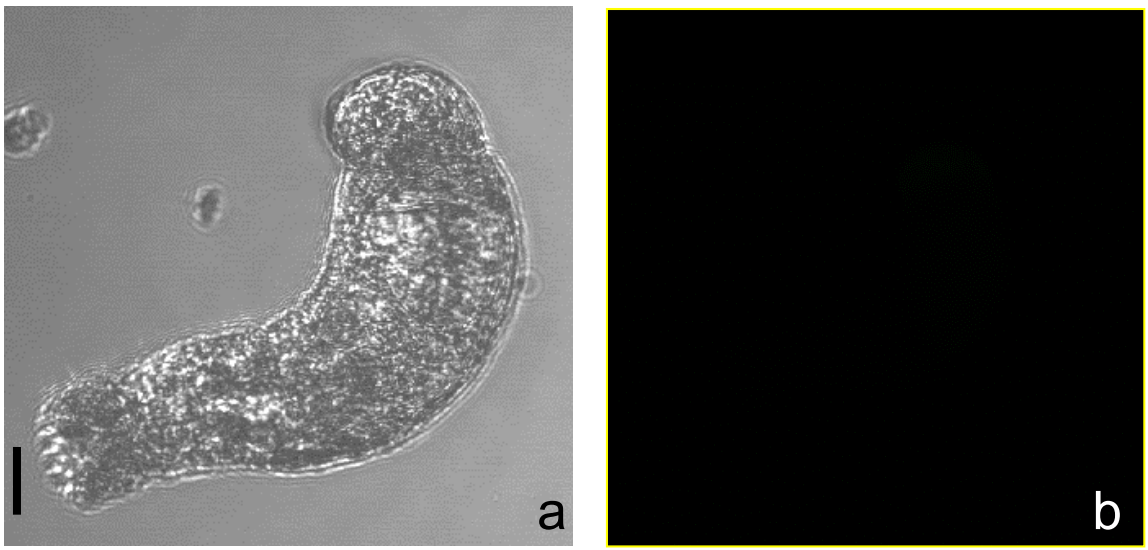


Figure 6.1. Worms were picked off a host, stained with a lipid specific stain and viewed using a confocal laser scanning microscope (CLSM). a) Gyrodactylid in transmitted light and b) negative control (no stain; lipids are not autofluorescent). Scale bar = 0.16 mm.

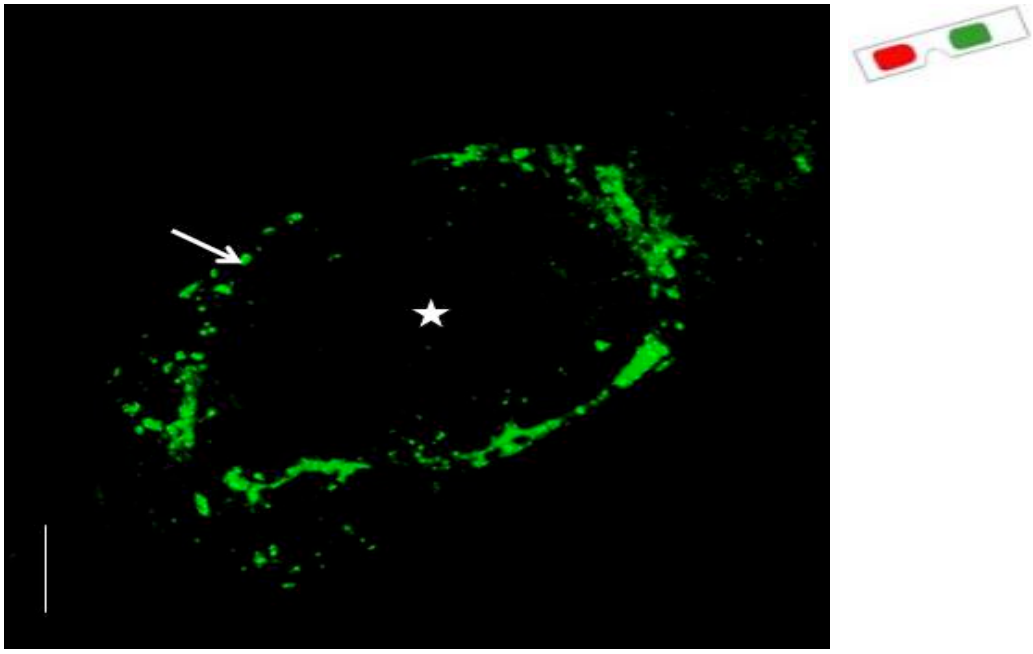


Figure 6.2. Confocal laser scanning micrograph of lipid droplets within *Gyrodactylus* seen as bright green spots when stained with the fluorescent dye BODIPY. Lipid droplets were observed distributed around the intestinal caeca. Serial images taken through a worm starved for 48 hours confirming that lipid staining is of material within cells positioned in the intestinal wall of the worm (arrowed) rather than of lipid in food items within the gut. This worm presents an empty uterus (star) (scale bar= 50µm).

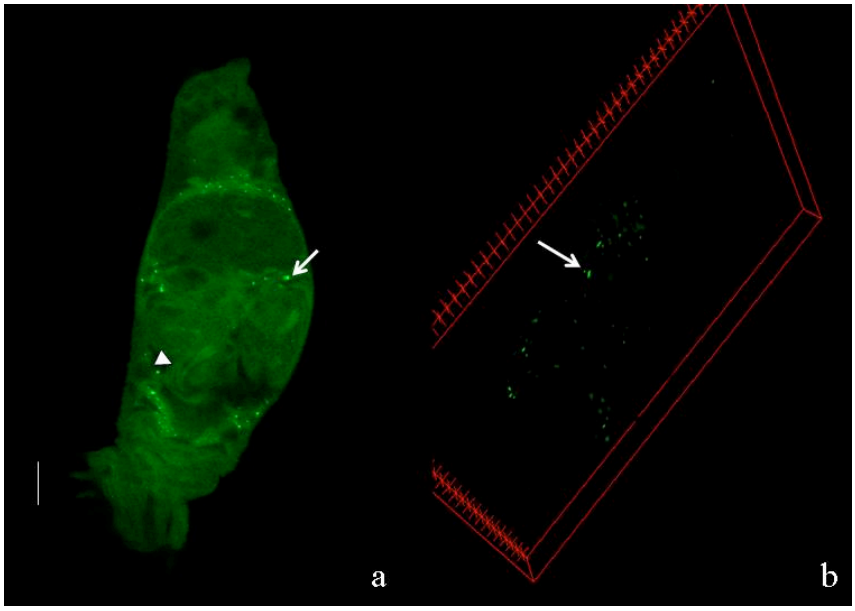
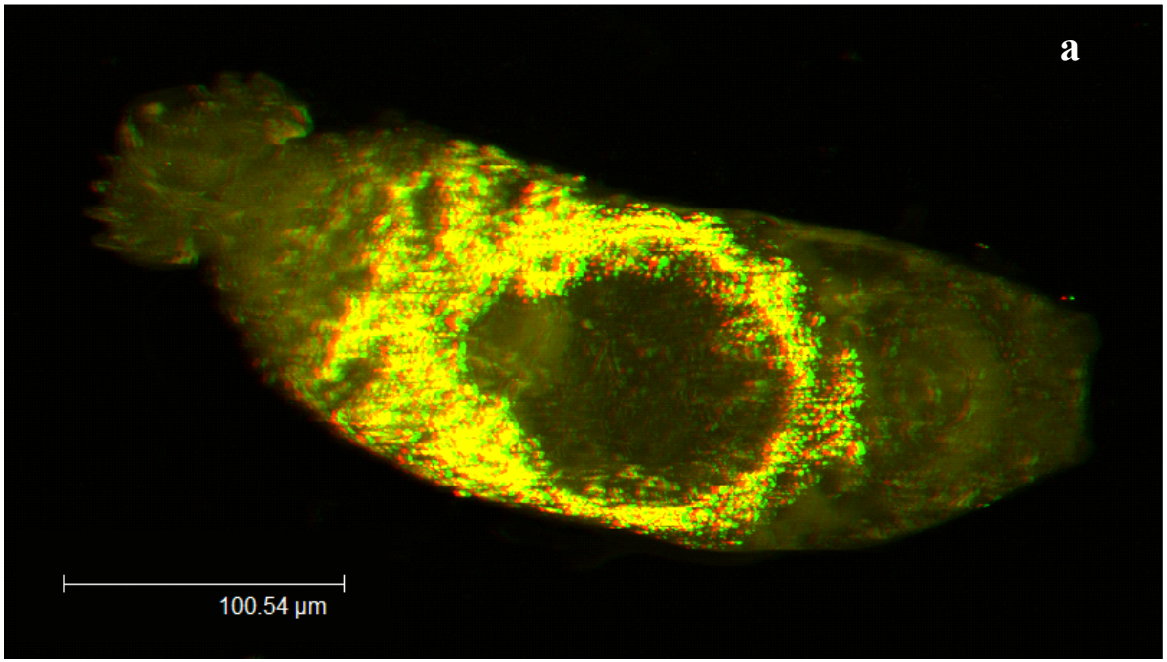
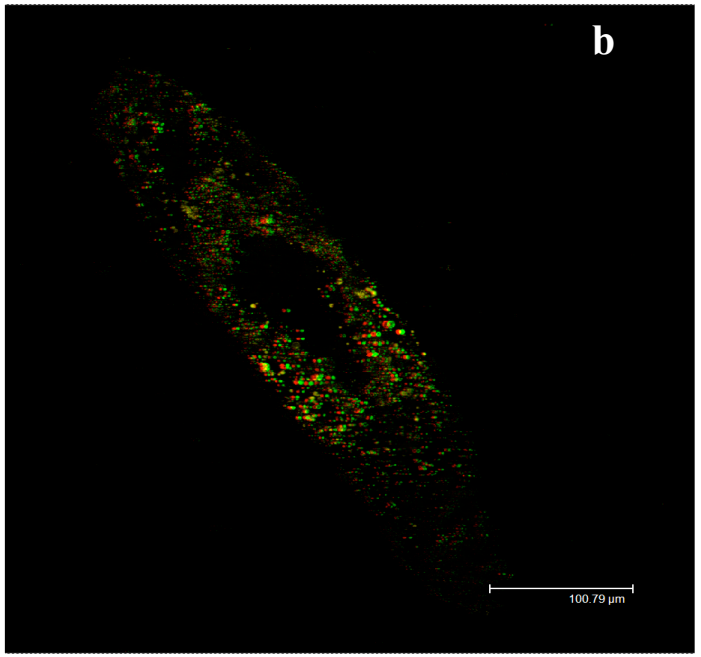


Figure 6.3. Confocal laser scanning photography of lipid droplets within *Gyrodactylus* seen as bright green spots (arrows) when stained with the fluorescent dye BODIPY. Image taken from a worm starved for 72 hours confirming that lipid staining is of material within cells positioned in the intestinal wall of the worm rather than of lipid in food items within the gut (scale bar= 50µm) b) 3D reconstruction image of lipid droplets (arrow) were observed distributed around the intestinal caeca.

Figure 6.4. 3D anaglyph confocal micrographs showing a) a fed, and b) a starved *Gyrodactylus.* The lipid is stained with BODIPY and shows as yellow bodies within the gut sides. The differences in lipid levels, in terms of fluorescence intensity and vesicle number, can be observed clearly. In each case, intestinal lipid droplets are distributed around the vicinity of the paired intestinal caecae. The worms are not bearing a daughter *in utero.*





3D image

6.4.1 Image analysis

6.4.1.1 Statistical analysis

The data did not support the assumptions of ANOVA, therefore a Kruskal-Wallis (non-parametric) test was applied to the data suggesting significant differences (Kruskal-Wallis Test= 9.8287, df = 3, *p*-value = 0.02008) between the content lipid droplets in parasites starved at different times (Figure 6.5).

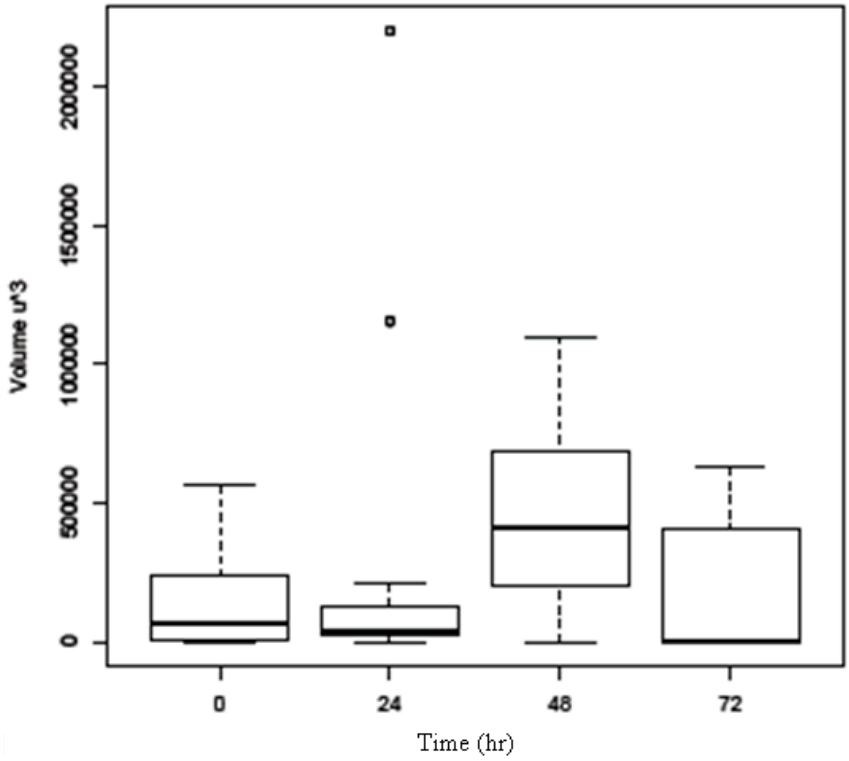


Figure 6.5. Box plot of the volume of lipid droplets found in gyrodactylids after 72 hours starvation. In addition, the volume of the droplets found in the control parasites (0 hour) are also considered.

6.4.2 Transmission electron microscopy (TEM)

To confirm the presence of lipid droplets, individual worms were fixed and studied using TEM. The luminal surface of the gut was observed to be highly microvillar, maximising surface area for absorptive functions (Figure 6.6). Although small

amounts of lipid were observed in the gut lumen (Figure 6.6) these were not substantial in studied specimens. Clear evidence for the presence of lipid storage vesicles in the epithelium of the gut, however, was provided by these studies, confirming storage of lipid in the areas previously suggested by the CLSM study (Figures 6.2 and 6.4).

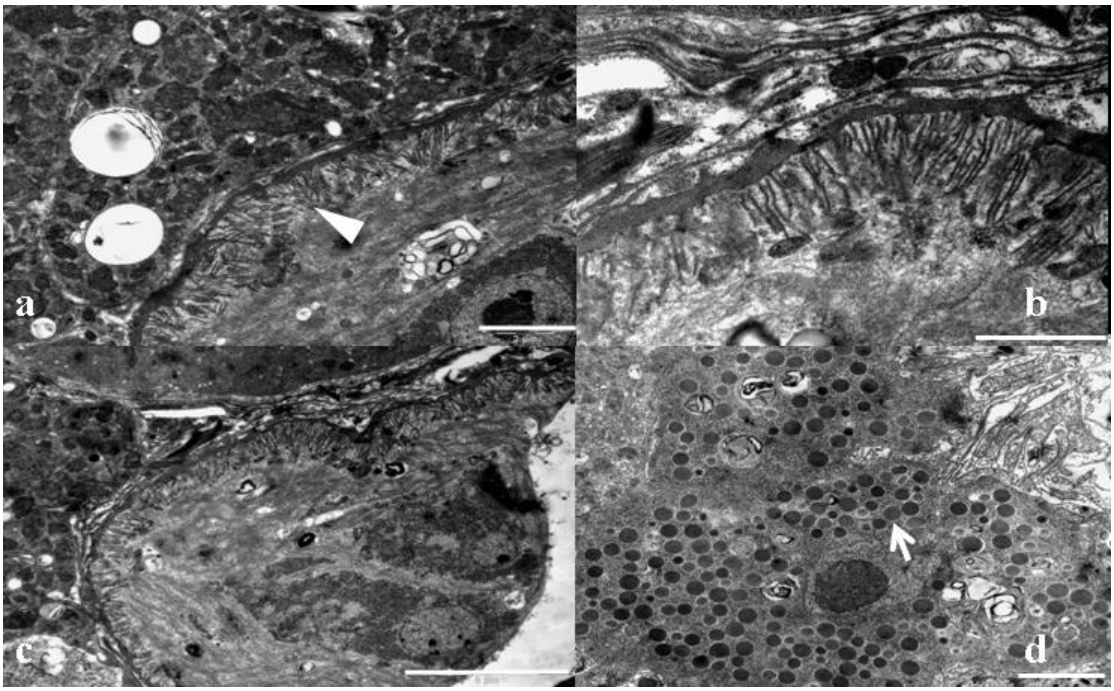


Figure 6.6. TEM micrographs of the intestine and surrounding tissue of *Gyrodactylus gasterostei*. **a**, intestinal wall showing the presence of absorptive microvilli (arrowhead), scale bar = 2 μm; **b**, high magnification of the microvillous border, scale bar = 1 μm; **c**, presence of lipid droplets (arrow) in the intestinal lumen, scale bar = 5 μm; **d**, lipid storage vesicles in the underlying gut epithelium (arrow), scale bar = 2 μm.

6.5 Discussion

Non-feeding life-cycle stages, such as dispersal stages of parasites, are dependant for survival upon finite energy reserves gathered during previous feeding phases. Thus, those individuals with more limited reserves will die sooner and consequently have less time available to find a new host once detached. In many such stages, lipids represent the principal form of stored energy reserves, these often being stored as large droplets. Lipid studies in parasites are scarce; however confocal laser scanning microscopy in gyrodactylids has been successfully used previously by El-Naggar *et al.* (2004) to reveal the neuromusculature of *Macrogyrodactylus clarii*, a gill parasite of the Nile catfish *Clarias gariepinus*. In the present study, this microscopical tool was used to investigate and characterise the distribution of lipid droplets in *Gyrodactylus gasterostei* which have migrated off their fish host, using a working hypothesis that these droplets function as a proxy for nutritional state. The apparent changes in the lipid content and distribution during time in free-living aquatic organisms such as copepods were studied by Cook *et al.* (2010) and marine microalgae (Cooper *et al.,* 2010). The current research which focuses on gyrodactylids, provides information on the localisation of lipid droplets and how the number and volume of these change over increasing periods of starvation.

The work presented here has demonstrated that the majority of droplets were located within vesicles in the gut wall and that individuals were variable in the amount of stored lipid that they carry. It is likely that transferring worms require a buffer of stored reserves to protect them against failure, this allowing survival off a host for several days. Clear observations in this study suggest that part of the stored lipid is derived from maternal reserves; this is also reported to occur in copepodids (Cook *et al.,* 2010). In the current study, it is suggested that the lipid reserves passed

from the mother to the embryo, increase with the increasing developmental state of the daughter *in uterus*. The worms that were starved for 48 hours contained more stored lipid than the control group (Figure 6.5), thus it may be the case that 87.5% (*i.e.* 14 worms) hold a daughter *in uterus,* this fact might increase the amount of lipids during the image analysis. However, the lipid reserves of individuals are exclusive and might differ between organisms of the same species. Another factor to consider is that cells in the tissue of moribund worms lyse, releasing lipid consequentially causing a brighter general lipid distribution but a poorer intensity of localised staining.

For the gyrodactylids infecting 3-spine-sticklebacks, the importance of parasites remaining on the host and attaining an optimum nutritional status is crucial. While lipid consumption may be related with temperature and survival, detached *Gyrodactylus alexanderi* (see Lester & Adams, 1974) kept at 15ºC, had a mean survival of ~ 1.8 days. In a second example, Cable *et al.* (2002) showed that the survival of detached *G. gasterostei* depended on temperature and found that they could survive for a maximum of 101 hours at 4ºC but only 67 hours at 15ºC. As mortality was continuous during the first 60 hours off the host at 10ºC, these authors suggested worms could survive until the extinction of energy reserves. However, in the present study, the worms kept at 10ºC progressively decreased their lipid content over the 72 hour experimental period. Olstad *et al.* (2006) looking at the maximum survival of *Gyrodactylus salaris* remaining on a dead salmon host, concluded that at 18ºC, worms moving off the dead host survived for up to 27 hours whilst those remaining on the dead host benefited and survived for up to 72 hours.

This technique using image analysis of laser scanning confocal microscope images (3D) has been used to assess the distribution of lipid droplets in other aquatic organisms (Cooper *et al.*, 2010; Cook *et al.,* 2010). The techniques used here for lipid

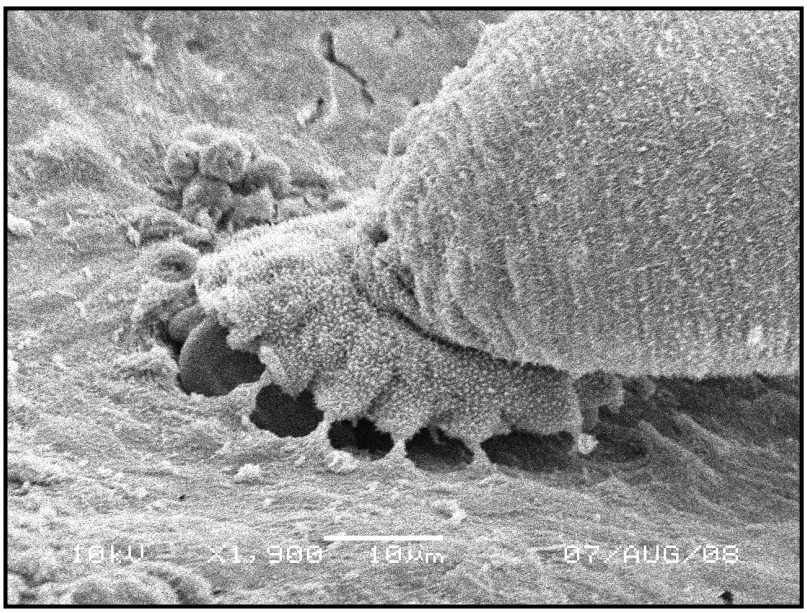
measurement and evaluation in *Gyrodactylus gasterostei* can also be applied to other parasitic organisms, having the advantage of rapid preparation and observation of specimens and the production of lipid distribution map. The CLSM is used to detect and image structures stained using target specific dyes. The approximate size and distribution of these structures, *e.g.* lipid droplets, can then be determined from composite images reconstrcuted through multiple scans through the specimen. The use of the Fiji-image analysis software then permits the size and volume of each droplet to be calcuated and the data exported in a format that permits subsequent statistical analysis.

In the current study, the high variability in the lipid reserves between individuals means that a larger number of gyrodactylids in each nutritional state need to be examined to determine the distribution and use of lipids by each. Nevertheless, this study does suggest that the presence of high lipid reserves may encourage or facilitate the early migration / transmission of gyrodactylids.

The present study describes the lipid measurement in *Gyrodactylus gasterostei* where 3D reconstruction of lipid vesicles from confocal image stacks showed lipid vesicles distributed around the intestinal caecae. Serial images taken through the worm confirm that lipid staining is localised within cells positioned in the intestinal wall of the worm rather than localising to lipid in food items within the gut. The number and volume of all lipid vesicles in each specimen were determined from stacked serial images using the Fiji-Win 32 image analysis programme. This technique has the advantage of allowing rapid preparation and observation of specimens.

Chapter 7

The effect of octopaminergic compounds on the behaviour and transmission of *Gyrodactylus* spp.



The opisthaptor of *Gyrodactylus gasterostei*

Aspects of this study were presented at:

6th International Symposium on Monogenea, 3rd-7th August, Cape Town, South Africa. (oral)

Participation in the study:

Grano-Maldonado assisted in the study design, in the collection of specimens and in the preparation of specimens for tracking and chemotherapy

Aspects of this chapter have been accepted for publication in Parasites & Vectors

7.1 Introduction

As *Gyrodactylus* von Nordmann, 1832 (Monogenea) has no specific transmission stage in its life-cycle, movement between hosts must be achieved by strategies employed by the adult. Bakke *et al.* (1992) suggested four routes by which gyrodactylids could transfer to a new host: (i) via contact with live hosts, (ii) via dead hosts, (iii) by detached parasites drifting in the water column, and (iv) by parasites attached to the substrate. This transmission potential, coupled with their high fecundity allows gyrodactylids to rapidly colonise new river systems (Bakke *et al.* 1992; Johnsen *et al.* 1999). Although transmission routes in gyrodactylids have been studied extensively, few workers have investigated the behaviour of individual gyrodactylids.

Gyrodactylus salaris Malmberg, 1957 has devastated Atlantic salmon (*Salmo salar* L.) populations where it is present in North European rivers (Hansen *et al.* 2003) and currently the only method of eradicating *G. salaris* from river systems is by using biocides, such as rotenone. However, this is devastating for the river habitat and, once it has recovered, *G. salaris* can re-colonise the river if measures are not taken to prevent its re-introduction Bakke *et al.* (1992). Consequently, the focus of research is moving towards finding alternative methods to control *G. salaris*, which target the pathogen without seriously affecting the river ecosystem. This requires an increased understanding of gyrodactylid biology and behaviour (Olstad *et al.* 2006).

In the control of other pathogens, chemicals treatments often target specific stages of the life cycle, which can be exploited to reduce the survival or infectivity of the parasites. Teflubenzuron, for example, is used to disrupt the moult of sea lice (*Lepeoptheirus salmonis* Krøyer, 1837 and *Caligus elongatus* Nordmann, 1832) (Branson *et al.* 2000). Agonists and antagonists are compounds that elicit a response

by binding to a receptor (*e.g.* muscle) and mimic the natural transmitter. In this study, the effect of octopaminergic receptor agonists / antagonists on gyrodactylids was investigated. It is suggested that exposing gyrodactylids to these compounds may affect their ability to attach to a host using their haptor, rendering them immobile and unable to infect a host.

Four octopaminergic compounds ((±)-octopamine hydrochloride ($C_8H_{11}NO_2$.ClH; O0250 Sigma), clonidine hydrochloride ($C_9H_9Cl_2N_3$.ClH; C7897 Sigma), amitraz (N-methylbis-(2,4-xylyl iminomethyl) amine, $C_{19}H_{23}N_3$; 45323 Riedel-de Haën / Sigma) and chlordimeform ($C_{10}H_{13}ClN_2$; 35913 Riedel-de Haën / Sigma)) were tested in this trial. Chlordimeform was selected as a toxic reference as it is known to be extremely toxic to aquatic life (Sigma-Aldrich, 2010). Octopamine is a biogenic monoamine found in both vertebrates and invertebrates and modulates physiological activity by binding to adrenoceptors. In invertebrates it acts as a neurohormone, a neuromodulator or as a neurotransmitter and modulates almost every physiological process (Roeder, 1999). Octopamine is homologous to noradrenaline in vertebrates and is found at concentrations less than 1% of noradrenaline, with its physiological activity being only 1 – 2% of noradrenaline (Williams *et al.* 1987). Clonidine is a centrally-acting α-adrenergic receptor agonist and is prescribed as an anti-hypertensive agent in humans (Kolb *et al.* 1984; Kinzie and Leung, 1989). It is used several conditions such as insomnia, migraines and attention deficit hyperactivity disorder (ADHD), and alleviate the withdrawal symptoms associated with the use of narcotics, alcohol and nicotine. Clonidine is also known to reduce involuntary muscle contractions, or tics, in humans by binding to α2-adrenergic receptors (Cohen *et al.* 1979). Its mode of action is inhibition of adrenergic receptors, which results in reduced motor activity (Altobelli *et al.* 2001). Amitraz and chlordimeform belong to a

group of insecticides / acaricides whose mode of action is by interaction with octopamine receptors (Altobelli *et al.* 2001). They work by mimicking the action of octopamine at the neuromuscular junction in invertebrates (Evans and Gee, 1980). Amitraz acts as a receptor agonist, whereas chlordimeform has an antagonistic effect (Matsumura and Beeman, 1976; Altobelli *et al.* 2001). Although certain groups of invertebrates have been shown to be particularly sensitive to formamidic compounds (Acarines, Lepidoptera and Hemiptera), vertebrates in general are relatively insensitive (Hollingworth, 1976). Both chemicals have antihelminthic properties (Benkó *et al.,* 1968) and have been shown to induce hyperexcitation and detachment of feeding ticks (Gladney *et al.* 1974; Stone, *et al.* 1974). Products containing amitraz were banned in 2010 for pesticidal uses in agriculture due to concerns of human exposure and risks to the environment (Rotterdam Convention on the Prior Informed Consent Procedure for Certain Hazardous Chemicals and Pesticides for International Trade, 2011). Chlordimeform is banned for use as an agricultural pesticide due to concerns that it is carcinogenic to humans and is toxic to aquatic life (Joint FAO / UNEP Programme for the Operation of Prior Informed Consent, 1911).

In order to investigate the effect of these octopaminergic chemicals on the behaviour of gyrodactylids, it was necessary to develop a bioassay to observe their behaviour. Therefore, the objectives of the study were to: 1) develop a system for recording and observing the movements of gyrodactylids under different lighting conditions; 2) determine optimum lighting conditions for observing the behaviour of gyrodactylids, by comparing their movements under white light, red light and in dark conditions; and 3) determine the efficacy of the four octopaminergic compounds on detached gyrodactylid behaviour.

7.2 Materials and methods

As *Gyrodactylus salaris* is a notifiable pathogen in the UK, it was not possible to acquire them for use in this study and therefore gyrodactylids from three spine sticklebacks, which are easily obtainable, were used as a gyrodactylid model.

Three spine sticklebacks (*Gasterosteus aculeatus* L.) were netted from a tributary of the River Forth, Stirlingshire (56º 06' 37.77" N, 3º 58' 25.25" W) and maintained at 10ºC in 30 litre, static tanks in an aquarium facility at the Institute of Aquaculture, University of Stirling. A 50% water change was carried out daily, using water collected from Loch Airthrey (56º 08' 39.53" N, 3º 53' 51.20" W) and the sticklebacks were fed *ad libitum* with frozen bloodworm (Gamma, Chorleywood, UK). *Gyrodactylus* for use in the behaviour experiments were removed from the sticklebacks using triangular mounted surgical needles (size 16, Barber of Sheffield, UK). Parasites were identified to species level using standard descriptions. Once the behaviour of each gyrodactylid had been determined, it was fixed and mounted in ammonium picrate glycerine according to the method detailed by Malmberg (1970), speciated and its maturity status determined (*i.e.* presence or absence of a male copulatory organ and / or an embryo *in utero*).

7.2.1 Investigation of lighting conditions

Initially, a simple experiment was undertaken to determine the activity of gyrodactylids under light and dark conditions. A mark was made on the underside of a 9 cm diameter Petri dish using a permanent marker and a single *Gyrodactylus* spp. was placed onto the mark in the Petri dish and filled with stream water at 10ºC. Twenty replicates of each were maintained in either ambient light (2800 lux) or dark conditions (0 lux). The replicates in ambient light were placed inside a cotton light

diffusing box to scatter the light and eliminate any direction cues. After three hours the straight line distance between the final position of the *Gyrodactylus* spp. and the initial mark was measured.

7.2.2 *Gyrodactylus* tracking

An experimental system was constructed to record the behaviour of individual *Gyrodactylus* spp. (Figure 7.1). This consisted of a 110 mm section of PVC pipe with a circular divider inserted inside the pipe. A circular hole 52 mm in diameter was cut in the divider and a mirror was placed underneath the divider at an angle of 45º. A 5 cm diameter Petri dish with a painted matt black base was placed onto supports surrounding the circular hole. Light was provided by a Carousel S 150W slide projector, which was directed onto the mirror, deflecting the light up through the divider and around the Petri dish. A foil cone set at an angle of ~30^{o} directed light back into the centre of the Petri dish, forming a ring of incident light. This allowed the gyrodactylid to be detected in the arena and eliminated any directional light cue as the light level was consistent around the whole dish (Figure 7.1). A Canon MiniDV MD205 video camera was mounted on a stand above the arena to record the movements of the *Gyrodactylus* spp. Inflated circular rubber inner tubes measuring 20 and 50 cm in diameter were placed underneath the projector and the tray containing the light chamber to dampen vibrations from the projector.

For each replicate a new 5 cm diameter painted Petri dish was filled with 10 ml of 0.2µm filtered stream water at 10ºC and a single *Gyrodactylus* spp. was placed into the centre of the arena using a Gilson pipette. It was then placed into the light chamber and left to settle for 20 minutes. The subsequent behaviour of the parasite was then recorded ($T_{20\text{-}50}$ mins) onto MiniDV cassettes, using the video camera, for 30

minutes, before being fixed and mounted onto a glass slide. Ten replicates were recorded in white light (~2800 lux) and ten in red light, using a Hoya 600 nm (590-2750 nm) red photographic filter placed over the projector lens.

The 30 minute videos were converted to digitial video files in .avi format using Windows Moviemaker software (version 2.1.4028.0, Microsoft Corporation, 2007). Individual frames in bitmap format were extracted using Bink and Smacker software (Bink version 1.9L, Smacker version 4.2d, RAD Game Tools Inc., 2009) at a frame rate of 1 frame per 5 seconds. Shade correction and segment analysis of the image set was performed in KS300 software (version 3.0 Carl Zeiss Vision GmbH, 1997) to facilitate the tracking of the parasite. Paratrack software (version 2.4, A. Brooker, University of Stirling, 2007) was used to track the movements of the parasite in each frame, creating an image of the gyrodactylids' movements and a text file containing a list of co-ordinates of the parasite's location in each frame. Once the parasites had been tracked the lists of co-ordinates were time averaged over three steps (15 seconds) to smooth the data. This removes any bias in the calculated behaviour parameters caused by exploratory extensions by the gyrodactylids whilst their haptors are stationary. The resultant co-ordinates were then used to calculate behavioural information including the mean and maximum velocity of each parasite, the distance travelled, turn rate, meander and heading. Fractal dimensions, which are a measure of track complexity, were also calculated for the parasite tracks using the 'box counting' method (Seuront *et al.* 2004; Uttieri *et al.* 2005). These operations were all undertaken using the Paratrack software. Principal Component Analysis (Statistica 6.1 software, 2004, Statsoft Inc., USA) was used to investigate differences between gyrodactylid movements in white and red light.

7.3 Chemical efficacy

As the efficacy of the four octopaminergic compounds (octopamine, clonidine, amitraz and chlordimeform) on *Gyrodactylus* spp. was unknown, a simple dose ranging exposure experiment was carried out using serial dilutions of each chemical with distilled water prepared in concentrations of 32, 16, 8, 4 and 2 µM plus a control consisting of distilled water only. One ml of each of these dilutions was pipetted into 5 cm diameter Petri dishes containing 9 ml of filtered stream water at 10 ºC to give final concentrations of 3.2, 1.6, 0.8, 0.4 and 0.2 µM. A single *Gyrodactylus* spp. specimen was introduced into each Petri dish, which were then kept in an incubator at 10 ºC. Each chemical concentration was replicated 15 times. The parasites were checked after 24 and 48 h and recorded as alive, affected (*i.e.* not attached and showing muscular spasms), moribund (*i.e.* not attached, curled up and showing minute muscular contractions) or dead (*i.e.* no response to physical stimulus). After 48 h the gyrodactylids were preserved in ethanol for future identification and maturity assessment. Probit analysis (Minitab 13.1 Software, 2000, Minitab Inc., USA) was used to calculate 24 h and 48 h 50% effective concentration (EC50) values for each of the octopaminergic compounds. Where EC50 values are given, figures in parentheses are fiducial limits.

7.4 Results

Two species of *Gyrodactylus* were identified from sticklebacks, *G. gasterostei* Gläser, 1974 and *G. arcuatus* Bychowsky, 1933, although the former were in the majority (60% and 40%, respectively). Both species were used in the behaviour experiments.

7.4.1 Lighting conditions

As there was no significant difference between the distances travelled by each species of *Gyrodactylus* the data was combined. The investigation showed that *Gyrodactylus* spp. are more active in dark than in light conditions (P = <0.001, one-way ANOVA) (Figure 7.2). After three hours, parasites in dark conditions moved a mean distance of 28.37 ± 10.18 mm from their starting point, whereas those in white light conditions moved only 11.8 ± 10.13 mm.

7.4.2 Tracking

Observation of the 30 minute tracks of individual *Gyrodactylus* spp. shows several different behaviour patterns that were common to both species of *Gyrodactylus* tested. The most common behaviour involved moving in one direction with little deviation from the chosen heading (Figure 7.3a). The movements of some individuals were confined to a very small area around the starting point (Figure 7.3b). The final behaviour pattern can be described as extensive sinuous movements, with several path crossovers (Figure 7.3c). Individuals recorded in white light conditions appeared to display the first and second behaviour patterns, whereas individuals recorded under red light appeared to have longer, more sinuous tracks.

Analysis of the tracks revealed that gyrodactylids in red light (n = 10) had a higher mean velocity (0.18 ± 0.17 mm / sec) and maximum velocity (0.78 ± 0.35 mm / sec), travelled further (6.32 ± 5.81 cm) and had a higher turn rate (± 26.6 degrees / sec) compared to those in white light (n = 10), which had a mean velocity of 0.11 ± 0.10 mm / sec, maximum velocity of 0.51 ± 0.28 mm / sec, travelling distance of 4.04 ± 3.35 cm and turn rate of 20.35 ± 6.79 degrees / sec (Figure 7.4). However, none of these values were significantly different (one-way ANOVA). Fractal dimensions and

meander were lower for gyrodactylids in red light (0.69 ± 0.2 and 856 ± 397 degrees / mm) than for those in white light (0.85 ± 0.2 and 1195 ± 373 degrees / mm), indicating less complex tracks for those in red light, although again none of these values were significantly different (one-way ANOVA).

The behaviour data was subjected to Principal Component Analysis (PCA) to reveal differences between gyrodactylid behaviour in white light and red light. The behaviour parameters that showed the greatest differences between white light and red light (one-way ANOVA) were chosen (*i.e.* maximum velocity, meander and fractal dimension) and checked for normality (the remaining parameters were found to be too variable to show any patterns in behaviour). The maximum velocity data was found to be skewed, so was log transformed to normalise it. Eigen values for Factors 1 and 2 were 66.3% and 25.8%, respectively, describing a total of 92.1% of the variation in the data. The PCA plot shows two distinct groups according to behaviour in white light and red light, although some individuals in white light were grouped with those in red light (Figure 7.5). Examination of the individual tracks comfirmed that those individuals in white light that were grouped with those in red light exhibited behaviour typical of those in red light (*i.e.* long, sinuous tracks).

7.4.3 Chemical efficacy

All of the four compounds affected *Gyrodactylus* spp. and produced involuntary muscular contractions (spasms) when normal body extension was attempted. 10% mortality was seen in the control group after 48 hours, although no muscle spasms were observed. As the toxic reference, the highest concentration of 3.2 µM of chlordimeform affected 87% of gyrodactylids after 24 h as denoted by limited movements (Figure 7.6a). However, after 48 h 27% of gyrodactylids were unaffected

(Figure 7.6b) suggesting that (i) the muscular spasms may only be temporary at that concentration; (ii) the gyrodactylids needed to be at a particular physiological state before they became susceptible; (iii) the persistence of the compound affects its efficacy. As there was no clear trend in the numbers of dead, moribund and affected gyrodactylids (Figure 7a,b), it was not possible to accurately calculate EC50 values for chlordimeform.

Octopamine had a dose dependent response after 24 h, with 73% of gyrodactylids being either affected, moribund or dead at the highest concentration of 3.2 µM, compared to 27% at the lowest concentration of 0.2 µM (EC50 = 0.631 µM (0.109 – 1.703 µM)) (Figure 7.6c). After 48 h the majority (67%) of the gyrodactylids were dead at 3.2µM (Figure 7.6d). Numbers of affected and moribund gyrodactylids were low for all concentrations (7% – 27%) after 48 h suggesting that the optimum exposure time for octopamine is between 24 and 48 h. The 48 h EC50 for octopamine was 0.14 µM (0 – 0.41 µM).

Clonidine was effective after 24 h with 60% of gyrodactylids being either affected, moribund or dead at both 3.2 µM and 0.2 µM (Figure 7.6e). After 48 h this figure had increased to 87% at 3.2 µM and 80% at 0.2 µM (Figure 7.6f). As there was little difference in the number of affected gyrodactylids between the highest and lowest doses, it is possible that either the concentration range selected was too narrow to determine the effective range or there are other factors affecting the efficacy of the compound. Therefore it was not possible to accurately calculate EC50 values for clonidine. However, as the number of affected and moribund gyrodactylids was low after 48 h (7% – 27%), it is suggested that, similar to octopamine, the optimum exposure time for clonidine is between 24 and 48 h.

Amitraz was the most effective of the compounds tested with 100% of gyrodactylids being either affected, moribund or dead after 24 h at the highest concentration of 3.2 µM (53% dead) (Figure 7.6g). At 0.2 µM 66% remained unaffected with 20% being either affected or moribund. The 24 h EC50 for amitraz was 0.29 µM (0.15 – 0.41 µM). After 48 h 60% were dead at 3.2 µM and 27% were dead at 0.2 µM (Figure 7.6h). As there were a considerable number of gyrodactylids either affected or moribund after 48 h (33 – 47%), and the numbers either affected, moribund or dead after 48 h were similar to those after 24 h, it is likely that the optimum exposure time for amitraz is longer than 48 h. The 48 h EC50 value for amitraz was 0.155 µM (0.024 – 0.248 µM).

7. 5. Discussion

These results suggest that gyrodactylids are more active in the dark than in light and therefore imply that they possess some form of photoreceptor. Watson and Rohde (1994) found sensory receptors in *Gyrodactylus* sp., which closely resemble photoreceptors found in other platyhelminths (Rohde & Watson, 1990; Sopott-Ehlers 1991). The light / dark experiment shows a significant difference in the distance travelled between those gyrodactylids in the dark and those exposed to light. However, as this experiment only records the start and end position of the parasite, the trial assumes that parasites have travelled in a straight line and, therefore, it is impossible to quantify their movements during the period of the experiment *i.e.* whether they follow a straight or sinuous path. This does, however, suggest that there may be differences in the distance travelled by gyrodactylids under different lighting conditions.

Although parasite tracks cannot be determined in the "dark", they can be measured under red and infrared light. By recording and tracking all the movements of individual *Gyrodactylus* it is possible to quantify their movements. While most of the measured movement parameters (velocity, distance travelled, turn rate) were higher for those gyrodactylids in red light than those in white light, none of the differences were significant. This is an indication of the wide variation in behaviours, resulting in large deviations from the mean. Conversely, meander and fractal dimensions were lower for gyrodactylids in red light than those in white light, indicating less complex tracks than those in white light. By using the movement parameters showing the greatest differences between white and red light it was possible to discriminate between the two lighting conditions using PCA. This suggests that the different conditions do result in different behaviours, although more replicates would be required to state categorically whether there are significant differences in their movements.

Observations of the tracks showed that gyrodactylids in white light often had unidirectional tracks, whereas those in red light were generally more sinuous. However, in several individuals the converse was true. Therefore, it appears that exposure to a specific cue (*e.g.* red or white light) does not always elicit a behavioural response typical of the majority of individuals exposed to the cue.

The difference in behaviours in red and white light may relate to their natural behaviour *in situ*. The long sinuous tracks of the gyrodactylids in red light, which had lower complexity and meander than those in white light, may indicate a host-seeking behaviour. Covering a large surface area as quickly as possible may allow them to identify chemical or physical cues used in host location. For example, ciliary structures likely to be photoreceptors found in *Gyrodactylus* sp. Watson & Rohde

(1994) may be involved in a shadow response (Lyons, 1973), allowing gyrodactylids to detect a potential host moving overhead whilst attached to the substrate. In comparison, the behaviour exhibited by the gyrodactylids in white light (uni-directional tracks or limited movements) may indicate a response to either seek shade or conserve energy in anticipation of darkness. This implies that host-seeking behaviour is more likely to occur in dull or dark conditions. Host transmission may be more favourable at night depending on host behaviour *e.g.* if they are less active at night and aggregate with other hosts. Transmission during darkness may also minimise the chances of being eaten by hosts that forage during the day. Photoreceptors require pigmentation in order to detect directional light and as pigmented photoreceptors are usually absent in adult Monogenea (Lyons, 1973) and the sensory receptors found in *Gyrodactylus* sp. (Watson & Rohde, 1994) were unpigmented it is likely that gyrodactylids cannot detect directional light. This suggests that directional choices made by individual gyrodactylids are random and not related to directional light cues.

The distances travelled by gyrodactylids in this study gives an indication of the transmission potential via the substrate. In the tracking experiment gyrodactylids in red light travelled a mean distance of 6.32 cm, which equates to 3.03m over a 24 h period and in white light travelled a mean distance of 4.04 cm, equating to 1.94m over 24 h. Transmission rates are temperature dependent and activity may increase at higher temperatures (Bakke *et al.,* 1991), indicating the dispersal and transmission potential via the substrate for detached gyrodactylids. Comparing the distances travelled in the tracking experiment with those in the experiment investigating lighting conditions, gyrodactylids travelled significantly further in the dark than in

white light, suggesting that distances travelled by gyrodactylids in the dark may be even greater.

Of the four octopaminergic compounds tested, all had an effect on gyrodactylids. The initial effect was to induce muscular spasms as the parasites attempted to extend their bodies. Prolonged exposure resulted in death. It is not known if this response reflects an interaction at the peripheral or central nervous system, but does imply the presence of octopaminergic receptors. Although chlordimeform severely affected the parasites, amitraz had an even stronger effect, even at low concentrations down to 0.2µM. Only chlordimeform at higher concentrations and amitraz significantly affected the parasites after 24 h. With octopamine and clonidine the full effect was not seen until after 48 h. This has implications for use of this type of treatment in the field, as prolonged exposure (24+ h) would be required to have any significant effect on gyrodactylids. As octopamine is a natural biogenic amine, it will be subject to metabolism and uptake by the gyrodactylids so its effect will be affected by other physiological processes. This may also be the case for clonidine. As chlordimeform and amitraz are synthetic compounds, they are less likely to be affected by uptake and metabolism. In addition, it should be noted that the bioassay used in this study is relatively crude. The complex behaviours of sensory host detection followed by co-ordinated tactic motor activity involve considerable complexity and it is probable that the small behavioural effects found at very low concentrations can confer considerable efficacy.

As the survival rates of gyrodactylids off the host are 1 day at 18^{o}C and 4 days at 3^{o}C for *G. salaris* (Olstad *et al.,* 2006) and 2.7 days at 15^{o}C and 4.2 days at 4^{o}C for *G. gasterostei* (Cable *et al*., 2002), this type of experiment is prone to error as a result

of natural mortalities. Although mortalities in the control were only 10% it is important to bear in mind the survival rates off the host when interpreting the results.

Before any chemical treatment against *G. salaris* can be used for entire river habitats, the toxicity of the compound to human operators and to other flora and fauna must be established. An effective treatment should affect the target organism, without having adverse effects on other aquatic life. However, as the desired mode of action of any octopaminergic treatment is to interfere with the behaviour of gyrodactylids by inducing muscle spasms, the concentrations of compound required will be considerably lower than those required to kill the parasites. As octopamine modulates virtually all physiological processes in invertebrates, but shows very little activity in vertebrates, being homologous to noradrenaline in vertebrates (Roeder, 1999), it is likely that it will have minimal effects on vertebrates at the concentrations required to disrupt physiological processes in invertebrates. No information is available on the toxicity of octopamine in fish, although results have shown that it is non-toxic to mammals (Sigma-Aldrich, 2002). However, it is likely that the toxicity of octopamine in other aquatic invertebrates is similar to that of gyrodactylids. Although it was not possible to calculate EC50 values for Chlordimeform in this study, 73% of gyrodactylids were affected or dead after 48 h at the lowest concentration of 0.2 µM (0.04 mg/L), which is considerably lower than the 96 h LC50 for rainbow trout (*Oncorhynchus mykiss*) at 13.2 mg/L (Sigma-Aldrich, 2010). Similarly, it was not possible to calculate EC50 values for clonidine. However, at 0.8 µM (0.21 mg/L) 93% of gyrodactylids were affected or dead after 48 h. Considering that the 96 h LC50 for clonidine in ide (*Leuciscus idus*) is 87 mg/L (Fisher Scientific AB, 2006), it is likely that the EC50 in gyrodactylids is significantly lower. In addition, 80% of gyrodactylids were affected by clonidine after 48 h at 0.2 µM (0.053 mg/L), which is

a concentration significantly lower than the 48 h EC50 for *Daphnia* of 182 mg/L Fisher Scientific AB, 2006). Amitraz has a 24 h EC50 of 0.29 μM (8.5 mg/L) for gyrodactylids, which is higher than the 24 h LC50 in rainbow trout of 2.7 - 4.0 mg/L (Intervet Australia Pty Ltd, 2006). The 48 h EC50 for amitraz in gyrodactylids is 0.16 μM (4.6 mg/L), whereas in *Daphnia magna* it has been calculated as 3.4 mg/L (Sigma-Aldrich, 2010). Although the EC50 values for amitraz are of the same magnitude as the LC50 and EC50 values for trout and *Daphnia*, it is anticipated that the concentrations required to disrupt the host seeking and attachment behaviour of gyrodactylids will be considerably lower. However, this requires further investigation.

The economic cost of any new potential treatments must also be considered. For any treatment, once it is scaled up to treat whole river systems, the costs can be massive. The cost of surveillance and eradication programmes in Norway was estimated to be around USD 23 million (Bakke *et al.,* 2007). If new treatments are to be found, they must be inexpensive, or required in low concentrations, otherwise their cost may be prohibitive.

Both amitraz and chlordimeform have been banned in the EU for use as agricultural pesiticides (Rotterdam Convention on the Prior Informed Consent Procedure for Certain Hazardous Chemicals and Pesticides for International Trade, 2011). Chlordimeform has a carcinogenic risk to humans and is toxic to wildlife, especially aquatic fauna. However, as the half life of chlordimeform is <60 days and it is relatively immobile in soils (Joint FAO / UNEP Programme for the Operation of Prior Informed Consent, 1991), the risk to the wider environment is limited. Amitraz has been banned due to concerns that it may enter the human food chain. However, it is still used on mammalian domestic pets for the control of ticks, lice and mites, *etc.* (Rotterdam Convention on the Prior Informed Consent Procedure for Certain

Hazardous Chemicals and Pesticides for International Trade, 2011). Although it is relatively toxic to wildlife it is rapidly broken down, having a half-life of less than one day in soil (Intervet Australia Pty Ltd, 2006), and therefore has a low long term environmental risk. Consequently, it is considered that amitraz would have limited effects on river fauna and virtually no impact on the wider environment, if used at the low concentrations required to disrupt the behaviour of gyrodactylids.

7.6 Comments

This work has made a significant step forward in the observation of gyrodactylid behaviour and is the first time that movements / activity have been studied in detail, suggesting that gyrodactylids are more active in dark than light conditions. Now that the experimental procedures have been developed to observe and record gyrodactylid movements, this system can be used for a wide variety of gyrodactylid behaviour experiments. Further work is required to confirm that gyrodactylid behaviour is affected by light conditions, specifically their behaviours in white light, red light, infrared light and dark conditions. The efficacy experiments have shown that octopaminergic receptors exist in gyrodactylids as the octopaminergic compounds tested have an effect on gyrodactylids resulting in muscular spasms and eventually death. The next logical step is to investigate the ability of affected gyrodactylids to reattach to a fish host once they have been exposed to low doses of octopaminergic compounds and whether the effect is permanent or temporary, once they have been removed from the compounds.

These initial results observing gyrodactylid behaviour and the effect of octopaminergic compounds are promising and indicate that there might be potential use of compounds affecting octopamine receptors to control gyrodactylid infections.

With the constant threat of *G. salaris* entering UK waterways and the lack of any effective treatment, other than the total eradication of all river fauna using rotenone, it important that investment is made now to develop new chemical treatments that will specifically target *Gyrodactylus* infections, without seriously affecting whole river ecosystems.

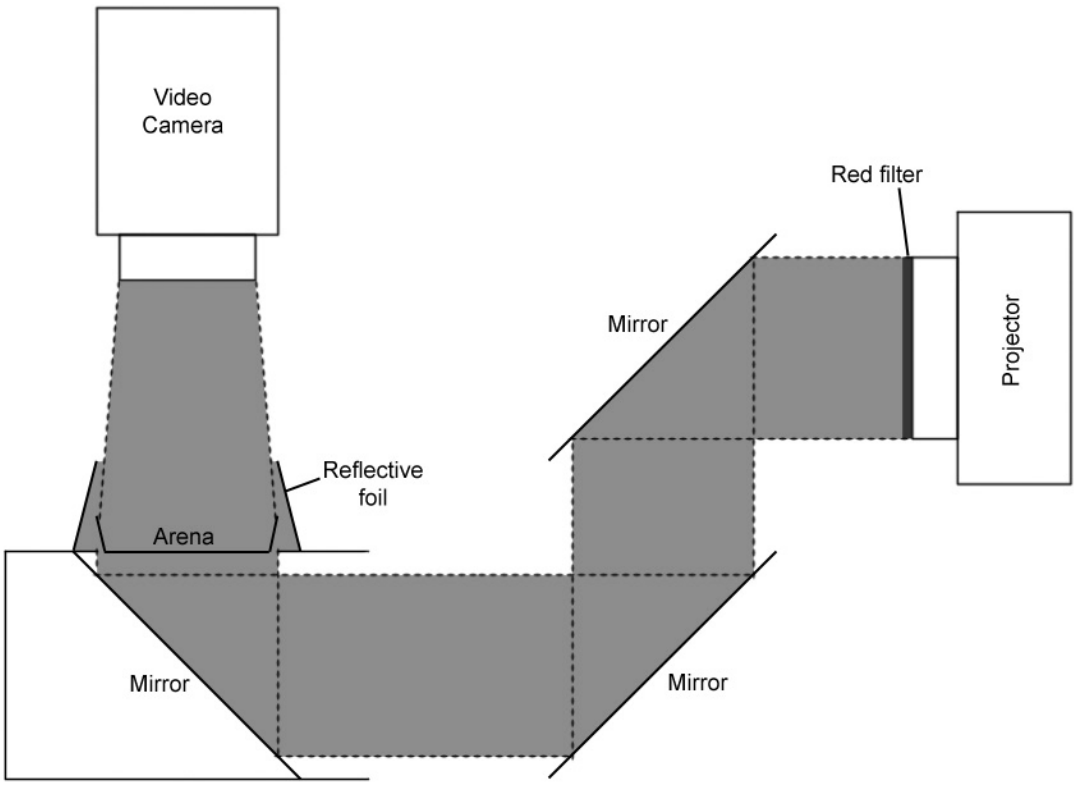


Figure 7.1**.** Diagram of the experimental setup used to record the behaviour of detached gyrodactylids under various lighting conditions or when exposed to a range of muscle agonists.

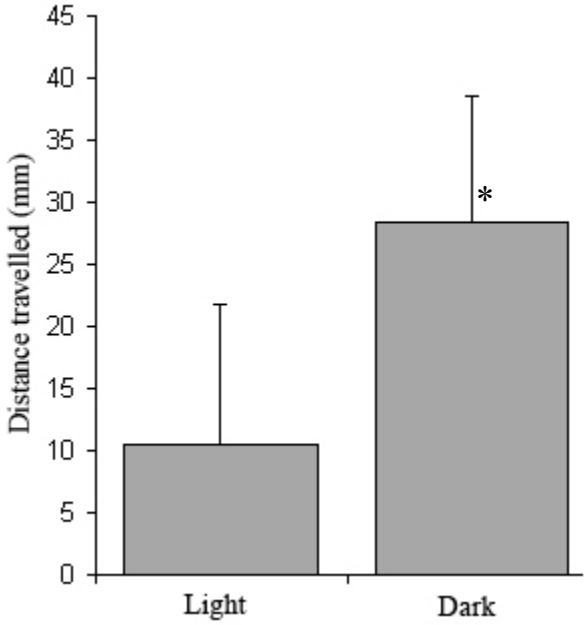


Figure 7.2**.** Distance travelled by *Gyrodactylus* spp. after 3 h in light (n = 19) and dark (n = 19) conditions. Bars = 1 S.D., * = significant difference from white light response ($p < 0.001$).

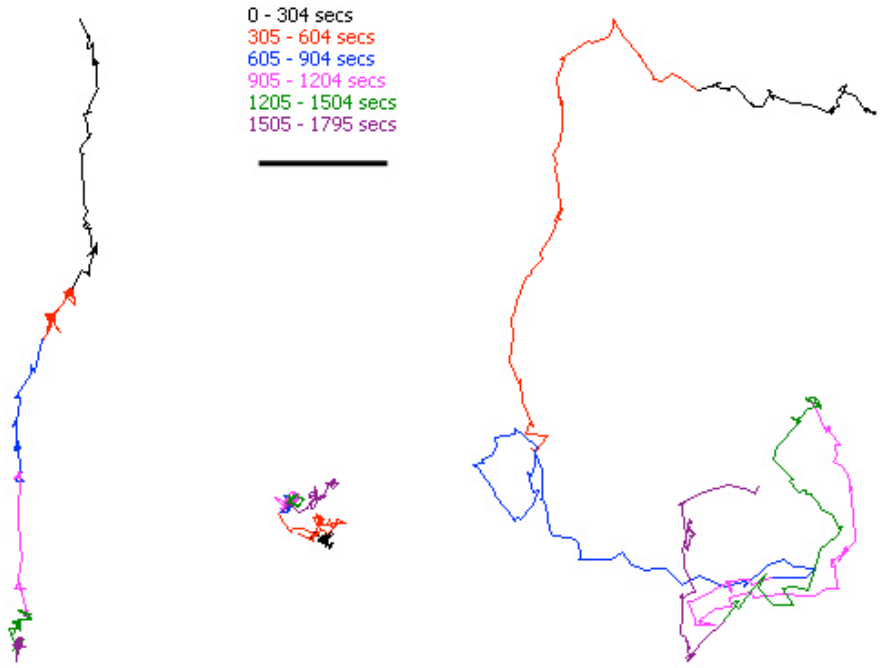


Figure 7.3**.** Thirty minute *Gyrodactylus* spp. tracks, showing different types of behaviour. (a) Linear movement, (b) limited movement and (c) long sinuous movements with track crossovers. Bar = 5 mm.

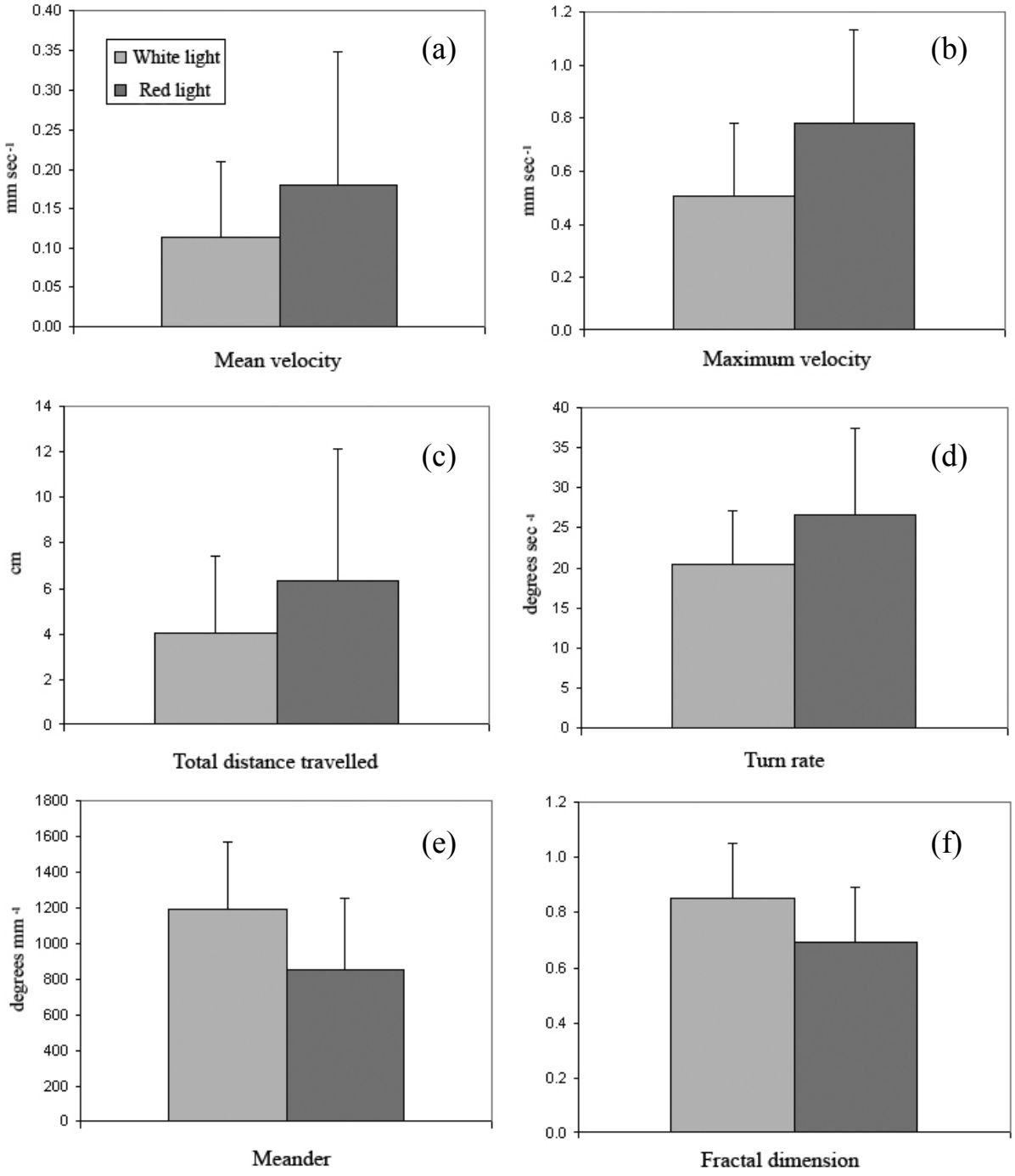


Figure 7.4**.** Behaviour parameters for *Gyrodactylus* spp. recorded in white and red light conditions (n = 10). (a) mean velocity; (b) maximum velocity; (c) distance travelled; (c) turn rate; (e) meander and (f) fractal dimension. Bars = 1 S.D.

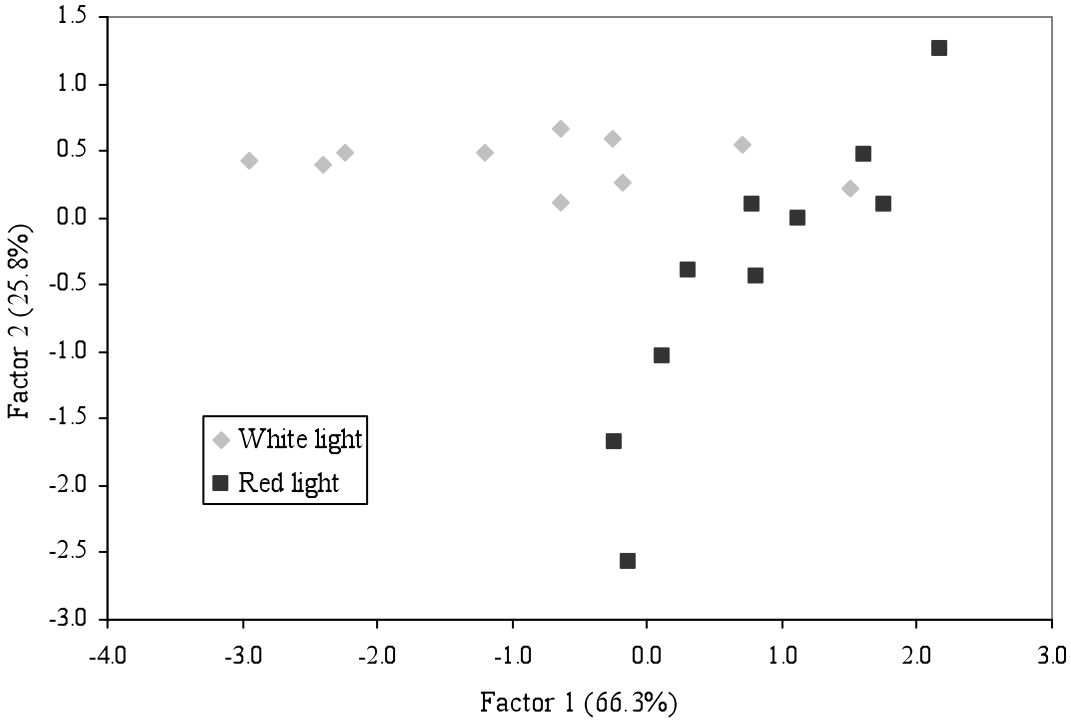


Figure 7.5. Principal Component Analysis of maximum velocity, meander and fractal dimension for gyrodactylids exposed to white light (n = 10) and red light (n = 10).

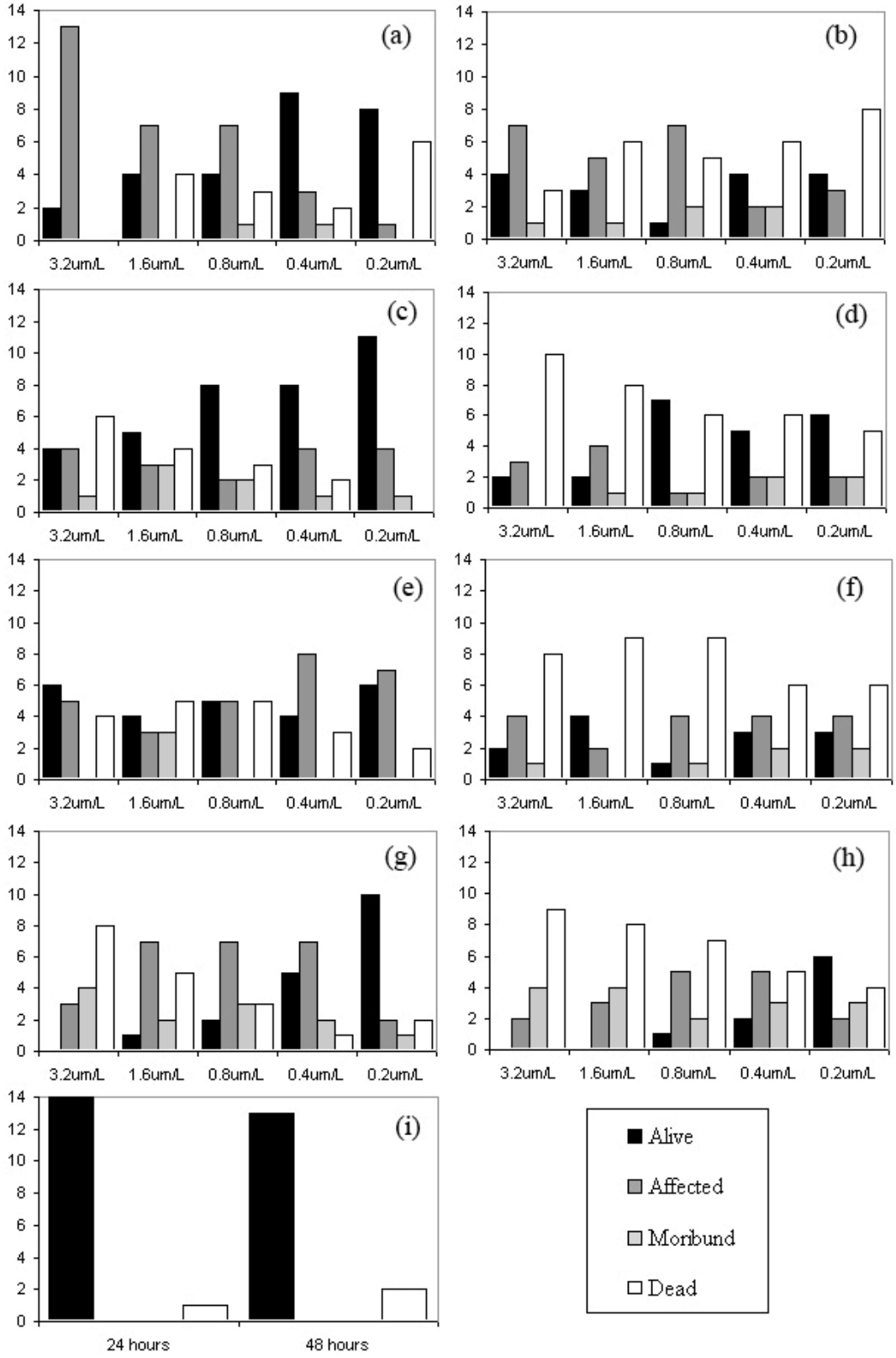
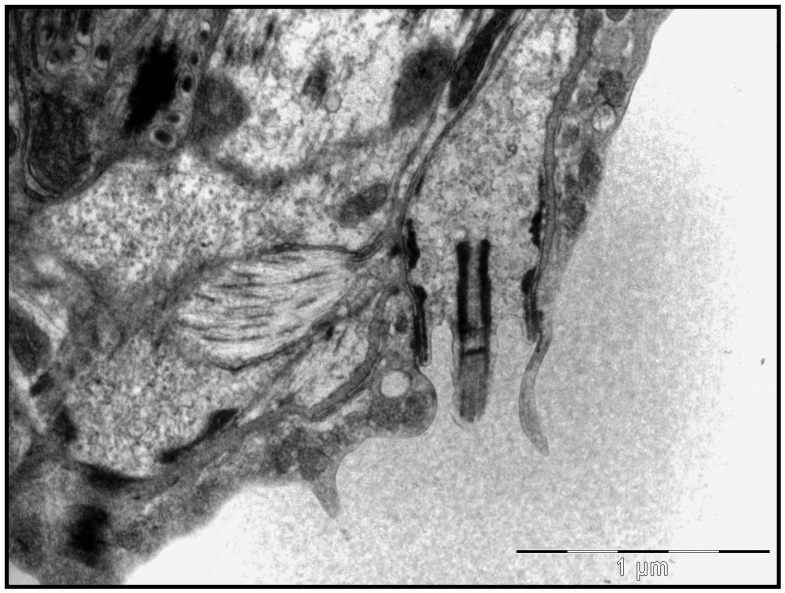


Figure 7.6. Effect on *Gyrodactylus* of (a) chlordimeform after 24 h, (b) chlordimeform after 48 h, (c) octopamine after 24 h, (d) octopamine after 48 h, (e) clonidine after 24 h, (f) clonidine after 48 h, (g) amitraz after 24 h, (h) amitraz after 48 h and (i) control. (y axes = number of individual *Gyrodactylus* spp., n = 15 for each compound tested).

Chapter 8

Ultrastructure of the external sensory apparatus of *Gyrodactylus gasterostei* Gläser, 1974



Transmission scanning micrograph of an unidentified receptor close to the cephalic lobe region on a specimen of *Gyrodactylus* sp.

8.1 Introduction

Infection by the *Gyrodactylus* parasites relies on the use of an array of sensory receptor and motor abilities which activate and modify gyrodactylid behaviour patterns, allowing transmission to a new host as part of the parasite's survival and reproductive strategy. Bakke *et al.* (1992) suggested four routes by which gyrodactylids could transfer to a new host: (i) via contact with live hosts, (ii) via dead hosts, (iii) by detached parasites drifting in the water column, and, (iv) by parasites attached to the substrate. Although these transmission routes have been studied extensively, few workers have investigated the ultrastructure of these sense organs and their relationship with specific behaviours displayed by individual gyrodactylids.

As gyrodactylids appear to respond to a range of stimuli in close proximity to the host, including vibrations *etc*, water currents and the turbulence created by approaching / passing hosts may be key factors in the transmission of *Gyrodactylus gasterostei* Gläser, 1974. Other stimuli include chemical (Neill, 1990) and physical responses *i.e.* light, gravity and vibration (Pike, 1990), which have been documented in other organisms such as copepods (Poulin *et al.,* 1990). This assumption may have consequences in the rates of *Gyrodactylus* transmission which need to be studied. For this reason, it is vital to understand the factors underlying transmission to a new host, and a detailed, ultrastructural examination of the sensory structures that are used may improve current understanding of the receptors that *Gyrodactylus* species employ to interpret both their host and ambient environments. Such information may assist in the interpretation of transmission behaviours, particularly their responses to chemical or

physical cues which gyrodactylids employ in host location during the transmission process.

Classically, the term *sensillum* and *sensilla*, is applied extensively to describe sensory structures in arthropods. This term has deliberately been misrepresented in numerous previous platyhelminth-based studies and that their use here follows that of the earlier works for consistency in terminology (A. Shinn personal communication), see for example the monogenean-based works of Lyons (1969 a,b, 1972, 1973), Shinn *et al.* (1997, 1998) and Bakke *et al.* (2007). In this study, the term *sensillum* or *sensilla* will be used to describe the prominent hair-like structures which project perpendicularly from the tegument of the parasite and are direct contact with the external aquatic environment.

Earlier research on monogenean sense organs was conducted on the works of Lyons (1969 a,b, 1972, 1973) and Watson & Rohde (1994). Lyons (1973) examination of certain structures on *Gyrodactylus* suggested their function as photoreceptors; her suggestions were based on gyrodactylids reacting to a shadow response, representing a host moving overhead, whilst attached to the substrate. Soleng *et al*. (1999) agreed with Lyons (1973) suggesting that the indirect transmission of parasites from the substratum is likely to be an important route of infection by detached gyrodactylids.

While the arrangement and distribution of surface sensilla on several species of *Gyrodactylus* has been reported (Shinn *et al.,* 1997, 1998; Bakke, Nilsen & Shinn, 2004), the use of the scanning and transmission electron microscopy will assist in determining the ultrastructure of each type of sensory structure (Watson & Rohde, 1994). Little is known about the ultrastructure of the sensilla monogeneans.

The aim of this study, therefore, was to investigate the ultrastructure of gyrodactylid sensilla and to ascertain how these may be employed in the colonisation of new hosts.

8.2 Material and methods

8.2.1 Source of hosts and parasites

Specimens of *G. gasterostei* from 3-spine sticklebacks were collected from a settlement pond on a commercial farm site (see Section 2.1 of Chapter 2).

8.2.2. Preparation of specimens for scanning electron microscopy (SEM)

The preparation of individual gyrodactylids for SEM follows the methods detailed in Section 2.8 of Chapter 2.

8.2.3. Preparation of specimens for transmission electron microscopy (TEM)

Specimens of *G. gasterostei* were prepared for TEM following the methodologies detailed in Section 2.9 of Chapter 2. The criteria to identify the photoreceptors follow those detailed in Watson & Rohde (1994).

8.3 Results

Scanning and transmission electron microscopy revealed two types of external receptor. One of these structures is suggested to serve as a mechano-receptor (Figure 8.1-8.3) and, the other as a chemoreceptor (Figure 8.4-8.5). In addition, two internal structures were found that may represent possible photoreceptors (Figures 8.6 and 8.7).

8.3.1 Observations with the scanning electron microscopy

The use of a scanning electron microscopy in this study provided a three- dimensional resolution of the surface sensory structures on the specimens of *G. gasterostei* infecting 3-spine sticklebacks. Figures 8.1 and 8.2 show an array of sensory structures on the tegument of *G. gasterostei*. Of particular interest, are the prominent arrangements of six sensilla on the dorsal surface, close to the cephalic lobes (Figure 8.3). The identification of each gyrodactylid specimen was based on hook morphology, particularly that of the marginal hook sickle. In addition, the armature of the MCO, where present, was used to facilitate specimen identification as described in Chapter 5 (section 5.2.3, Table 5.1 and Figure 5.1).

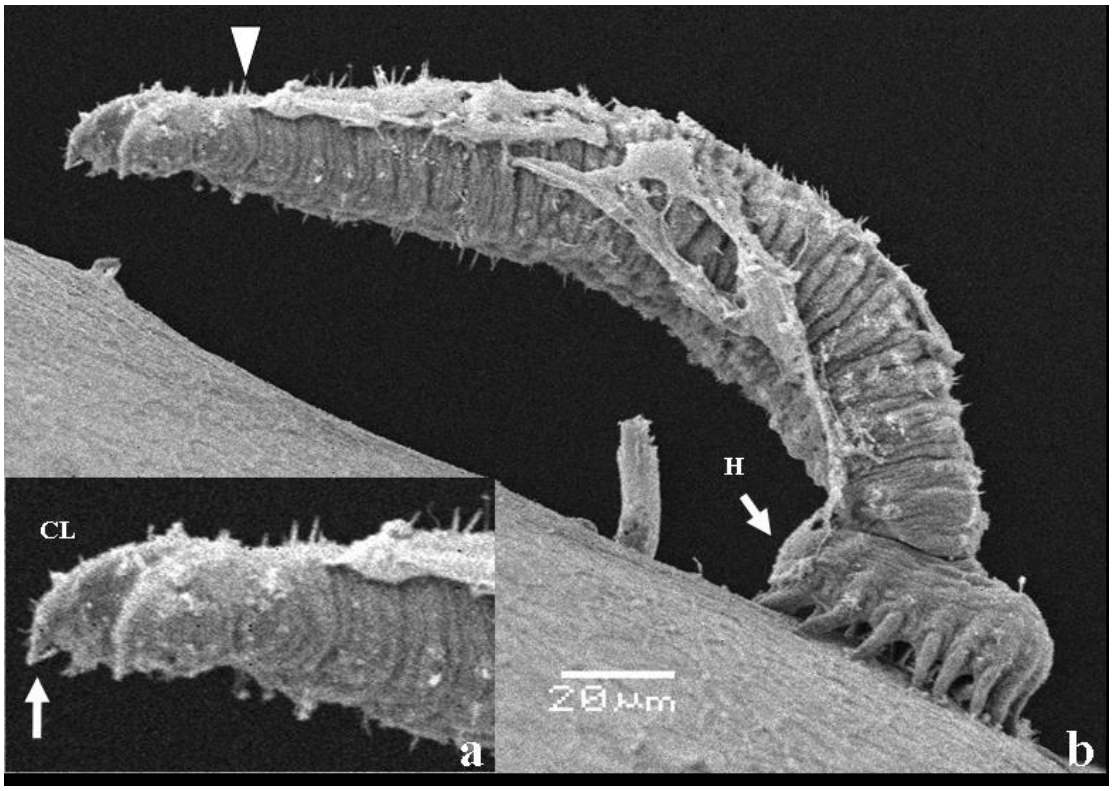


Figure 8.1 Scanning electron micrographs of *Gyrodactylus*. a) The anterior end of the parasite possesses two prominent cephalic lobes (CL), each bearing a spike sensillum as first described by Lyons (1969). This compound receptor consists of a number of associated sensilla, each ending in a single cilium" (Lyons, 1969). This concentration of sensory sensilla (arrow) has been compared with that in the ciliary endings of other invertebrates where mechano- and chemoreceptors are used to process information regarding the environment (Lyons, 1969b; Bakke *et al.* 2007) and in the potential recognition of suitable hosts (Whittington *et al.,* 2000). b) A single parasite attached to its fish host by means of its haptor (H). Note the numerous sensory sensilla covering the body. The arrowhead highlights, the prominent hair-like sensilla which project perpendicularly from the tegument.

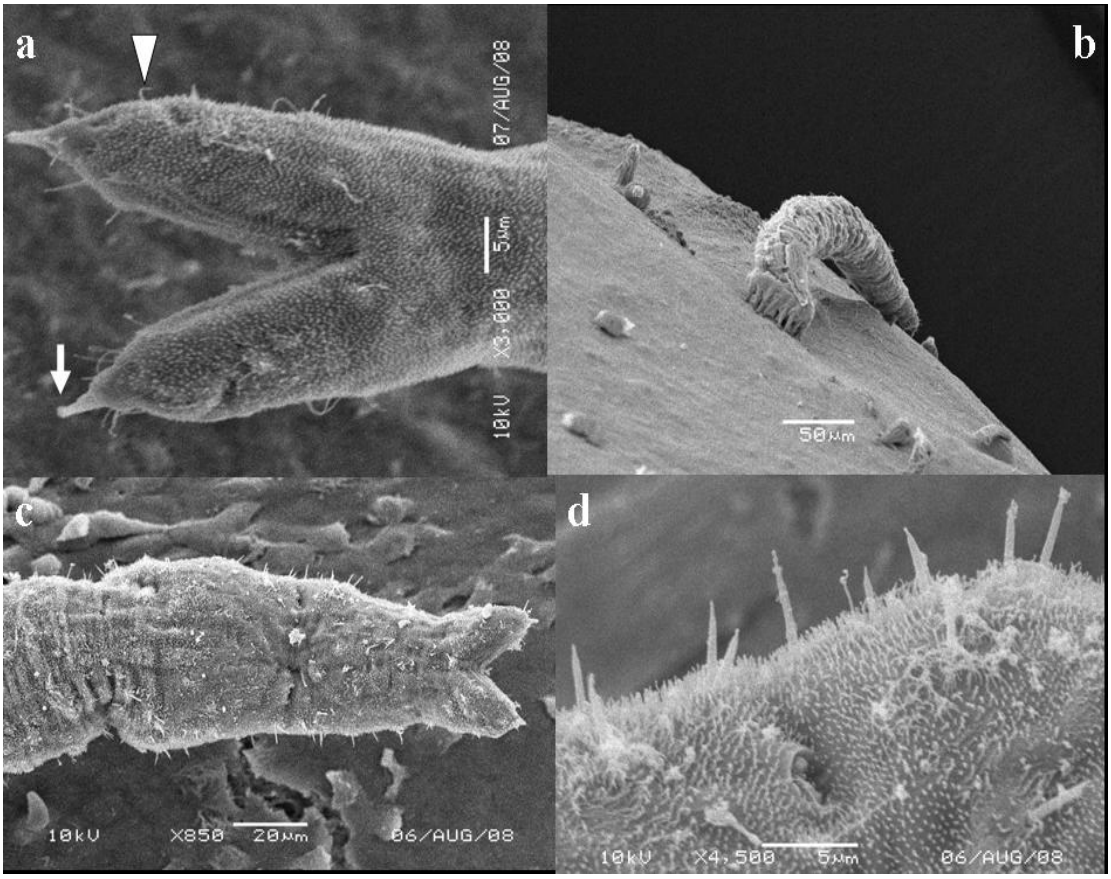


Figure 8.2 Scanning electron micrographs of *Gyrodactylus* spp. infecting 3-three spine sticklebacks, *G. aculeatus*. a) The head of the parasite possesses two cephalic lobes, each bearing a spike sensillum (arrow). Notice the structural difference of these structures to the finer sensilla (arrowhead) that are also present on the cephalic lobes. b) A single parasite in the process of traversing the skin of its host. Note the position of the haptor to the orientation of the body. c-d) the body of the parasite is covered with numerous sensory structures. These may serve as possible mechano-receptors which are able to detect vibrations or turbulence in water currents generated by a host in relative close proximity. These observable facts were shown step by step on the slow-motion videos (see attached CD).

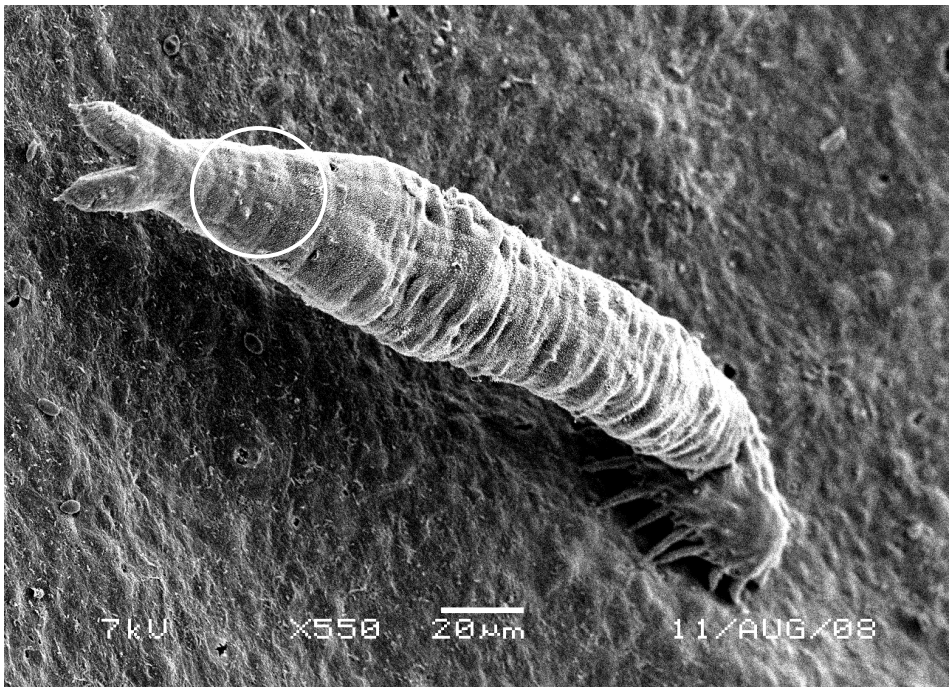


Figure 8.3. SEM of a specimen of *G. gasterostei* attached to the epithelium of its 3-spine stickleback (*Gasterosteus aculeatus*) host. Note the circular arrangement of six sensilla close to the cephalic lobes (circled). This study set out to ascertain whether these six sensilla serve as photoreceptors. Harris (1983) suggested similar structures may exist lining the pharynx region, close to the cerebral organ, in fixed and stained specimens of *Macrogyrodactylus polypteri.* These structures, however, have not been investigated further.

8.3.2 Transmission electron microscopy

The use of a transmission electron microscope permitted the ultrastructure of the sensory structures to be investigated contributing to our current understanding of gyrodactylid sensilla. The following section details the internal structure of different sensilla found on *G. gasterostei*.

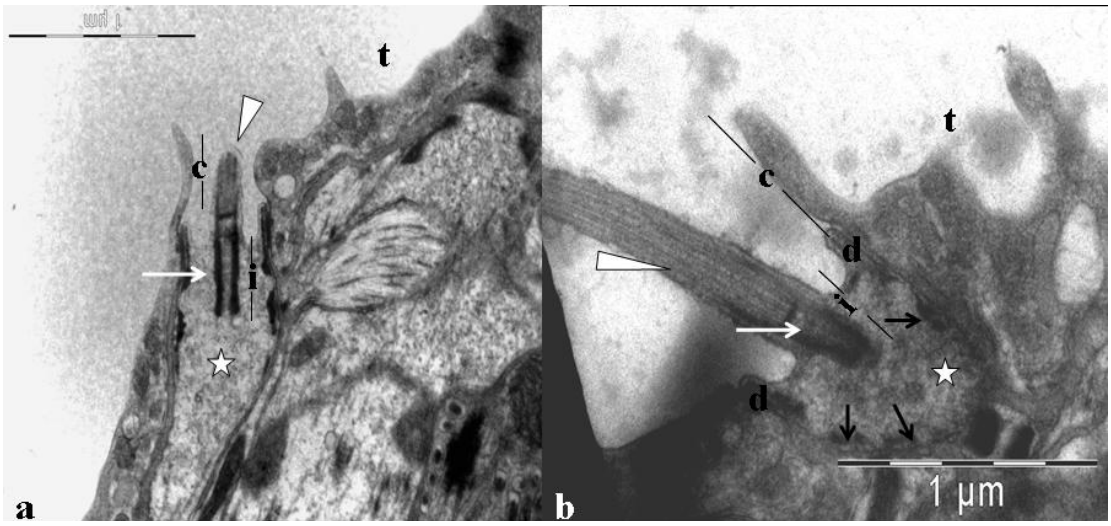


Figure 8.4. Transmission electron micrographs of two types of gyrodactylid sensory structure. a) A chemo-receptor. Note the short cilia based structure (arrowhead) and long basal body (white arrow) providing stability to the cilium (c) enclosed within a lumen, that has a large opening on the tegument (t), and deep basal invagination (star). b) A mechano-receptor on the anterior zone of the body. Note the cavity that it occupies within the tegument (c), the insertion of the cilium (i), the electron-dense collar of dendrite components (black arrows), and, the presence of desmosomes (d).

8.3.2.1 Presumable photoreceptors on *Gyrodactylus gasterostei*

8.3.2.2 Type I ciliated photoreceptor (anterior)

Type I ciliated photoreceptor are sub-surface ciliary receptors, localised in close proximity to the spike sensilla as reported by Watson & Rohde (1994)

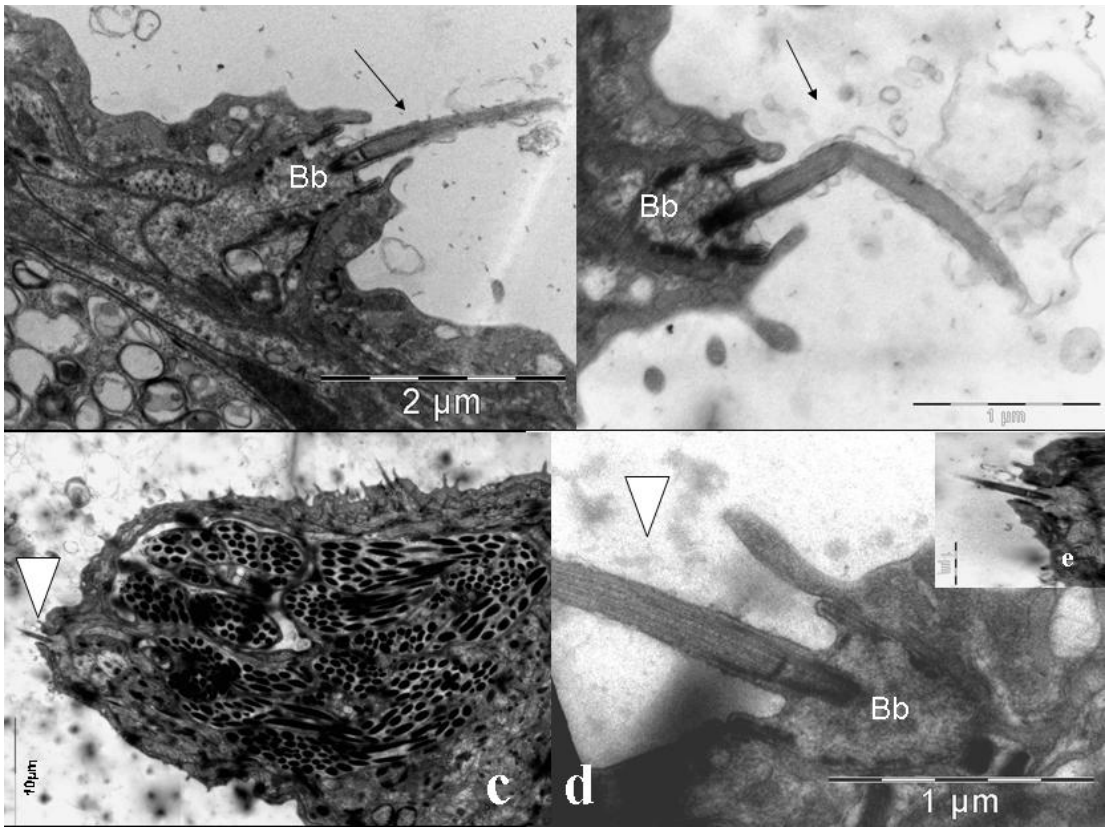


Figure 8.5. Transmission electron micrographs of a variety of sensory structures. a-b) The parasite's tegument is covered with numerous sensory sensilla. The sensillum based structure (arrow) and basal body (Bb). c) Shows a sensillum localised on the worm's anterior zone (arrowhead). Notice the compact shape and its projection. d) The parasite's tegument is covered with numerous sensory sensilla; the sensillum (arrowhead) and associated basal body (Bb) of each are clear. The function of each type remains to be established but these may serve as "mechano-receptors" presumably to detect changes in water currents or turbulence on *Gyrodactylus*.

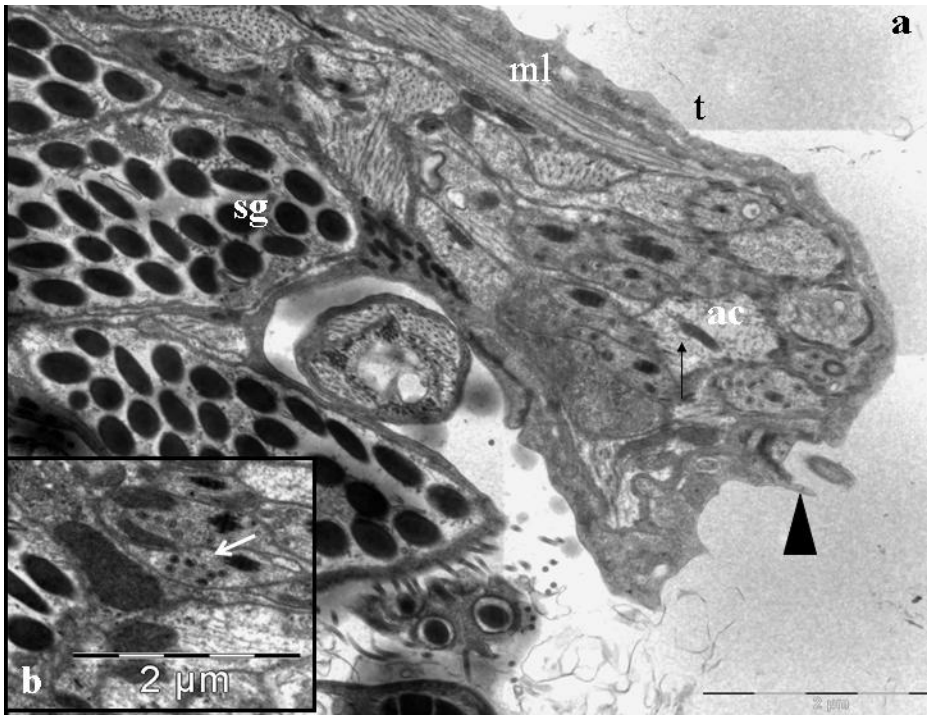


Figure 8.6. Transmission electron micrograph through a cephalic lobe of *Gyrodactylus* showing a presumed photoreceptor type I. **a**) A spike sensilla (arrowhead); presumed cilia (arrow) localised on anterior cavity (ac). Note its relative close proximity to the spike sensilla; muscle layer (ml), secretion glands (sg); tegument (t). **b**) Cilia microtubules (arrow).

8.3.2.3 Type II ciliated photoreceptor

This photoreceptor is localised in close proximity to type 1 ciliated photoreceptor of the type reported by Watson & Rohde (1994). This structure is found immediately underneath the tegument of *G gasterostei,* close to its surface.

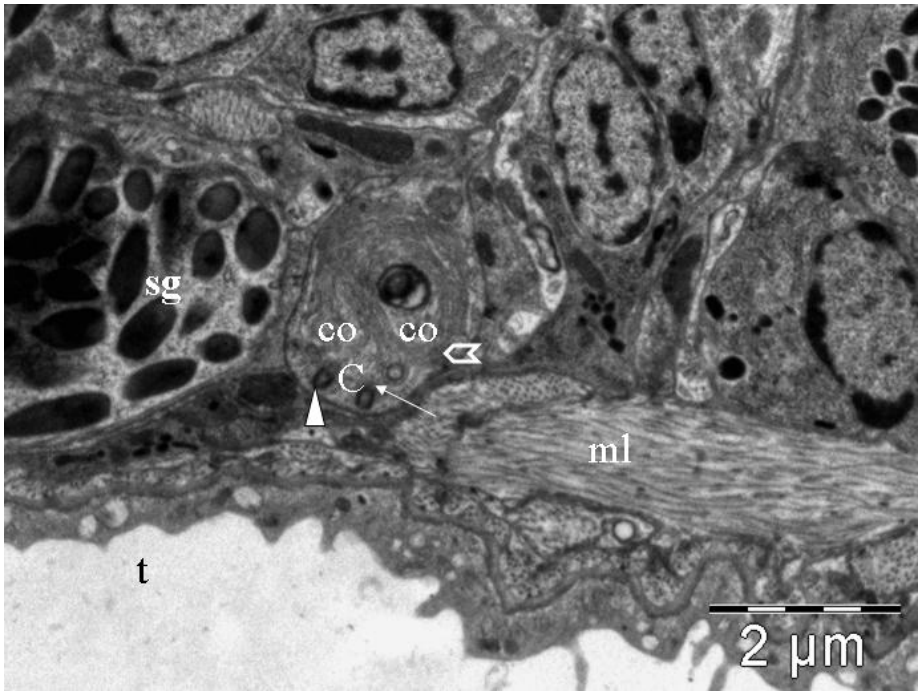


Figure 8.7. Transmission electron micrograph of *Gyrodactylus.* Presumed photoreceptor compressed in a single defined structure. Basal body (arrowhead) providing the structural base of the cilium (arrow); cilia (c); ciliary membranes compress into whirls (open arrow); cytoplasmic foundation (co); muscle layer (ml), secretion glands (sg), tegument (t).

8.4 Discussion

The results of this study provide photographic data concerning the sensory structures that are found on the tegument and in the sub-tegumental zone of *G. gasterostei* that infect 3-spine sticklebacks. This investigation complements preceding sensilla work on gyrodactylids (Lyons, 1969a, b, 1972, 1973; Watson & Rohde, 1994; Shinn *et al.,* 1997, 1998) and offers additional information on tegmental external and internal structures.

The findings in the current study are compared to those of Kearn (1984), who described a ciliary photoreceptor in the tegument of the monogenean *Sphyranura* sp.,

and Watson & Rohde (1994) who described a structure in a species of *Gyrodactylus*. The general arrangement of the structures and the proximity to the tegument and microtubules, entering lamellae and the position of the cilium in both studies were similar to those found in the current study. Some of the sensory structures described here follow those observed by Cribb *et al.* (2003) namely, the cavity formed by the epidermis, the invagination of the cilium, the presence and location of an electron-dense collar of dendrite components, the presence of desmosomes that attach to the tegument surface, and, the saccular shaped cavity containing the sensillum.

Adams *et al.* (2008) defined the role of cilia, as having contact with the exterior and providing chemo, thermo and mechano-sensation of the environment establishing, "a sensory role mediating specific signalling cues, including soluble factors in the external environment". In gyrodactylids, mechano and chemical receptors play a vital role providing accurate information on the surroundings. This includes: *i.e.* identifying, or targeting a possible host (Lyons, 1969a, b, 1972, 1973), exploring the surroundings and proximity of other parasites; detection of water turbulence and vortices used as an outer force to make contact with a host. In addition, the presence of some form of photoreceptor may increase the chances of transmitting to a new host if the parasite responds to a shadow response.

The concept of a photoreceptor existing in gyrodactylids was proposed by Lyons (1973) suggesting that gyrodactylids behaviour may be triggered by a shadow response allowing gyrodactylids to detect a potential host moving overhead whilst attached to the substrate. In the present study, it is hypothesised that during the parasite's substrate-attached phase, the parasite may use turbulence generated by the movement of fins to assist infection (Drucker & Lauder, 2003).

Video footage collected during the current study clearly shows the attractive behaviour of a gyrodactylid towards a host as a fish approaches the substrate to which the parasite is attached (see enclosed CD). The water turbulence generated on the bottom by the movements of the fins produces a vertical uplift movement, dislodging the parasite and moving towards the fish. This transfer may be facilitated by the response of mechanoreceptors present on the tegument, detecting the aquatic turbulence. Additionally, the presence of a photoreceptor, which register the shadow produced by a passing fish could increase the chances of a successful transmission.

Previous research on sense organs in *Gyrodactylus* species infecting green swordtails, *Xiphophorus helleri* Heckel, 1848, were conducted by Watson & Rohde (1994). This work identified ciliary structures, identified as possible photoreceptors, on the antero-dorsal section of the cephalic lobe just below the spike sensilla. These findings also support the results found in Chapter (7) where gyrodactylid activity is increased in the dark when compared to light conditions, strongly suggesting that some type of photo stimulus is important in the host-finding system.

It is known, that many mechanoreceptors enable humans to detect touch or to monitor their position (Johansson & Flanagan, 2009). In humans, these cutaneous receptors are found next to hair follicles, so even if the skin is not touched directly, movement of the hair is detected or felt. In the same way, water movements or the presence of a host can be detected using an array of "hair-like" sensilla as found on gyrodactylids. The slow-motion videos suggest that the parasites response to the presence of an approaching host is triggered by detection of water turbulence / vibrations.

Neuromasts, which are mechanoreceptive hair cells (Pitcher *et al.,* 1976) exist on the tegument of many aquatic organisms; these, however, were not studied in the

present study. Neuromasts, however, typically possess bundles of 40-50 microvilli or "hairs" which function as mechanoreceptors in fish (Pitcher *et al.,* 1976; Peach & Rouse, 2000).

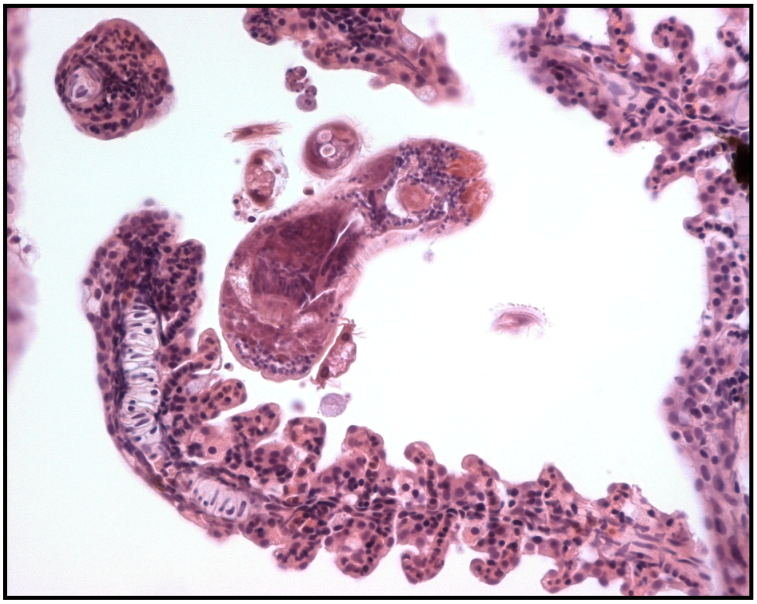
Several studies have described the arrangement of the sensilla and their pattern on the body (Shinn *et al.,* 1997, 1998); however, these studies did not identify the existence or verify their function as mechano and / or chemical receptors. Lyons (1969a, b, 1972, 1973) undertook the first intensive investigation regarding the functionality of monogenean sense organs including a detailed description of their structure. According to Lyons (1969a), chemosensory function in monogenean could be associated with structures that bear a short cilium ending in a lamellar process, which does not extend beyond the bulbus depression. On the other hand, mechanoreceptors have been described by Lambert *et al.* (1981), who performed an ultrastructure investigation studying uncilliated sensilla functioning as mechanoreceptors. Likewise, Cribb *et al.* (2003) described three different types of sensilla in monogeneans. Other authors such as Justine *et al.* (1994) described tegumentary receptors of the uniciliary type in monogeneans. The present study attempted to describe mechano receptors which were located on the anterior end of the worm *(i.e.* the cephalic lobes) and have noticeable, long cilia. This type of sensillum displays a modest invagination where the cilium can be observed at its distal end to connect to the gyrodactylids rudimentary nervous system.

In this study, it is clear that *G. gasterostei* on 3-spine sticklebacks respond to a range of stimuli (*i.e.* vibration or chemical cues released from the host, see CD Power Point presentation) as part of their assessment of host suitability. This study suggests that certain sensilla, presented for the first time, may serve as chemoreceptors.

An alternative explanation was provided by Welsh & Storch (1969; quoted in Bourne & Danielli, 1980) where they state that "candidates for chemoreceptors may possess an apical process that reaches the surface of the epithelium or are restricted to the basal portion of the epithelium". In the gastropod genus *Aplysia*, the presence of centrally located primary chemosensory neurons was assumed in electrophysiology studies in non-arthropods (Jahan-Parwar, 1975 quoted in Bourne & Danielli, 1980). In 1977, the same author confirmed them in the posterior region by identifying long distal processes filled with microtubules. Further research with supportive electrophysiology should be considered in gyrodactylids. Immunohistochemical studies, using antibodies raised against opsin or some other light sensitive pigment may also prove to be useful. Future studies, could verify or disprove this hypothesis.

Chapter 9

General discussion and conclusions



Haematoxylin and eosin stained section of the gills from an infected glass eel showing the presence of *Gyrodactylus* and *Trichodina* sp.

9.1 General discussion

In the introduction to this thesis it was stated that the aim of this research was to obtain an improved understanding of the behavioural aspects of transmission / host transfer in gyrodactylid parasites and to determine how transmission relates to individual parasites in terms of their reproductive maturity, developmental stage and nutritional status. To date, the number of gyrodactylid studies published, which specifically address behavioural aspects of transmission, has been limited. A few notable exceptions, such as Soleng & Bakke (1999), Cable *et al.* (2002) and Olstad *et al.* (2006) have described factors relevant to the transmission of *G. turnbulli* and *G. salaris*. Such studies and the understanding they bring are important in preventing the spread of pathogenic parasites and in improving management and control, thereby reducing the impact and the costs for wild and cultured fisheries. The current research focused on the model fish host 3-spine stickleback, *Gasterosteus aculeatus*, and the gyrodactylid ectoparasite *Gyrodactylus gasterostei* to answer some of these questions (for supporting film footage, please see slides 1-16 of the PowerPoint presentation on the attached CD).

The transmission of *Gyrodactylus* between live and / or dead hosts was examined through the use of digital recording equipment to have a better understanding of the biological factors underlying the aspects of transmission; assisting in the prediction, reduction and the elimination of certain pathogenic species like *G. salaris*. Whilst many previous studies of host transfer have been largely descriptive *e.g.* Cable *et al*. (2002), few have examined the role of specific host behaviour. In this respect, Olstad *et al.* (2006) suggested that cannibalism may be a source of transmission in *G. salaris.* The transmission routes used by *G. gasterostei* in

the colonisation of new hosts, therefore, was investigated in Chapter 3 which included observations on the transfer of parasites between live hosts, transfer from dead to live hosts, transfer through the water column and transfer from substrates on to live hosts. The study also looked at the transmission of gyrodactylids from infected dead hosts to live hosts that were scavenging and / or feeding on the dead carcass of an infected fish. Fish displayed clear scavenger activity towards dead conspecifics (see slides 10-11 of the PowerPoint presentation on the attached CD). Despite recognition of a number of different scavenging activities, *e.g.* taking bites out of dead carcasses; carrying dead carcasses in the mouth etc, the number of parasites passed by the oral route did not correlate with the number of bites observed. Whilst host scavenge feeding may represent an additional route for gyrodactylid transmission, its relative importance, with respect to other recognised transmission routes, appears to be minimal (Chapter 3).

In addition, it is suggested that the movements associated with vigorous searching behaviour of gyrodactylids on a dead host may attract the attention of predatory sticklebacks and may then elicit a feeding response which could allow transfer to a new host as it approaches to feed. Video recordings and a subsequent scanning electron microscopy study conducted in Chapter 3 suggested that once parasites transfer to the mouth, during feeding, they can attach to the lining of the buccal cavity and then migrate to their preferred colonisation site on the outer surface of the fish. This interesting finding should be used to inform diagnosticians of the fact that the oral cavity may play a role in transmission as a temporary colonisation site through which the worms move to get to more conventional sites of colonisation. The importance of this has been highlighted in different related studies (Grano-Maldonado *et.al* 2011 a, b).

The examination of worms transferring to a new host during scavenging activities found that there is a significantly higher proportion of parasites with a MCO transferring than those having no MCO or daughter present. This suggests that colonisation of new hosts is more commonly achieved by mature parasites (see Chapters 3, 4 and 5). The results also showed, that this mode of transmission, was relatively minor when compared to the rates of transmission that occur a result of direct contact between live hosts, as described by Bakke *et al.* (2002), or through detached gyrodactylids carried through the water current. This may be due to the fast approach of scavenging fish providing a low contact time and therefore a small window of opportunity for parasite transmission. Given the evidence for transmission through scavenging seen in the stickleback model, it will be important to consider other host-parasite pairs, *e.g. Salmo salar* / *G. salaris,* and to establish the importance and the contribution of this behaviour in gyrodactylid transmission dynamics.

During the transfer process, it was observed that a small percentage of worms failed to re-attach to new hosts, irrespective of whether the host was dead or alive. These worms were caught on a mesh placed in the bottom of the experimental tanks and subsequently staged. Of these, 51.9% of the worms lacked an MCO, representing either newborn worms or those having recently given brith for the first time. Of the worms that were recovered, only a small percentage (*i.e.* 7.59%) was still alive. It is suggested that these recovered worms represented either failed transfers by healthy worms, accidental dislodgements from mothers surviving a birth event or full-term daughters that failed to attach following birth. Given the large size of the worms being born, it is not surprising that a significant proportion of the births result in either death (29.11%) or damage (41.77%) to the mother. The most significant finding of the experiment, however, was that 21.52% of the worms that were recovered appeared to

be premature births. The term "premature birth" here was used to describe those worms showing poorly developed haptoral structures *i.e.* attachment hooks. This suggests that pregnant worms either failed to attach during a potential transfer opportunity or were dislodged and subsequently lost their embryos, which may act to extend their longevity off the host.

Given that the anaesthetic 2-phenoxyethanol does not affect the population of gyrodactylids which transmit off the host, the proportion of "premature births" that were observed could be important because if worms are forced to leave their hosts prematurely, this could result in an increased chance of embryo loss, assuming that the loss of an embryo means that the paraent worms would then have an increased likelihood of surviving.

Cable, Tinsley & Harris (2002) noted that detached starved parasites can abort their offspring (embryos) and that an interruption in nutrient flow to the embryo might have a significant impact on reproductive rate. These authors did not refer to any other factors related to this phenomenon, however, from the research described in this thesis it is apparent that gyrodactylids are subject to premature births under stress conditions including low temperatures (<3ºC), and during physical interference such as 'picking-off' activities. The consequence of nutritional status was also considered during the course of this thesis and is discussed below and in Chapter 6.

The experimental approach in Chapter 4 continued to examine aspects of the transmission of parasites from live and dead fish. The maturity and reproductive status of transmitting gyrodactylids was also examined in order to consider the influence of parasite status upon the likelihood of migration and searching for a new fish host. The results showed that a higher than expected proportion of individuals showing full maturity and having a male copulatory organ present, transmitted from dead and live

hosts in order to reach a new host. Non-migrating parasites showed a higher than expected representation of parasites with a daughter *in utero* which can be suggested as a possible biological strategy to increase the worm population *in situ*.

One small but important study that was conducted prior to all these experiments being undertaken, set out to assess the effect of the anaesthetic 2-phenoxyethanol on the transmission of *Gyrodactylus* to or from euthanased hosts. The study concluded that there was no effect on the rates of transmission when using anaesthetic (see Chapter 4) and that this could be used in subsequent experimentation.

The ability of parasites to rapidly transfer from one host to the next was explored in Chapter 5 whereby the transfer rates of different *Gyrodactylus* species and different fish species, when cohabited for short periods of time (~3 h), was explored. This study was based on a concern that when multiple fish species are sampled in the field and placed in a communal container, or subsequently transported together in the same vessel, that certain parasites may transfer to other hosts. The concern is therefore that this may affect the correct allocation of parasites to hosts, and the diagnosis, management and control of gyrodactylosis in a variety of fish. To investigate this, *Gyrodactylus* infected specimens of three-spine sticklebacks, minnows *Phoxinus phoxinus* and stone loach *Barbatula barbatula* from one Scottish river were cohabited with one another in small volumes of water for 3 h, a time period representing the average transfer time from the field to the laboratory. The study found that small numbers of *Gyrodactylus* transfer to atypical hosts. This study indicates that personnel involved in fish disease surveillance programmes should be aware of the possible consequences, in terms of inter-host transfer, of transporting multiple species in the same transport vessel. Diagnosticians should be aware of the fact that fish may act as

temporary or paratenic hosts and that the apparent gyrodactylid fauna present following transport may not reflect that encountered under normal circumstances.

Bakke *et al.* (2007) suggested that fish movements due to commercial trading may increase the potential to disperse gyrodactylids. The results obtained in Chapter 5 highlight the risks of parasite transfer during fish cohabitation such as that which occurs when sampling or moving fish. Such transfers affect the accurate assessment of native parasite fauna and increase the possibility of introducing new parasites into a different environment.

To explain some aspects of transmission it was hypothesised that the nutritional status of the worms might play an important role in their decision as to whether or not migrate, from the fish. Nutritional status could therefore represent a key to the interpretation of movement and migration behaviour. Non-feeding life-cycle stages, such as dispersal stages of parasites *i.e.* mature *Gyrodactylus*, are dependant upon finite energy reserves, gathered during previous feeding phases, for their survival. Thus, those individuals with more limited reserves will have shorter survival times and consequently have less time available to find a new host, once detached. At this stage, energy reserves are most commonly stored as lipids, these often being stored as large droplets. In Chapter 6, confocal laser scanning microscopy was used to investigate the distribution of lipid droplets in *Gyrodactylus*, which have migrated from their fish host, testing the hypothesis that these droplets function as a proxy for nutritional state. The study found that the majority of droplets were located in the gut wall, that individuals stored variable volumes of lipid and that there was a difference in the volume of lipid in worms starved for different periods of time (Kruskal-Wallis, $p = 0.02$). All stages, including individuals just released from the birth pore, carried lipid droplets, this being confirmed by TEM imaging. It is likely

that transferring worms require an energy store to protect them against failure, to attach to a host. This could allow survival off a host for several days. The study that was used describes a technique for lipid measurement in *Gyrodactylus* and other parasitic worms, which has the advantage of allowing rapid preparation and observation of specimens.

The high transmission potential of *Gyrodactylus* species, coupled with their high fecundity, allows them to rapidly colonise new hosts and to increase in number. *Gyrodactylus salaris* has been responsible for the devastation of Altantic salmon populations in a number of Norwegian rivers. Current methods of eradicating *G. salaris* from river systems centre on the use of non-specific biocides, such as rotenone and aluminium sulphate. Although transmission routes in gyrodactylids have been studied extensively (Cable *et al*., 2002; Bakke *et al*., 2002, 2007; Olstad *et al*., 2006), characterisation of the behaviour of the parasite has received relatively little attention. In Chapter 7, the behaviour of individual gyrodactylids was observed and the effect of selected octopaminergic compounds on gyrodactylids was investigated. It is hypothesised that exposing gyrodactylids to octopaminergic receptor agonists and antagonists would affect their ability to attach to a host using their haptor, rendering them immobile and unable to infect a host. Initial experiments that were conducted suggested that gyrodactylids were more active in dark than in light conditions. An experimental system was constructed to observe the behaviour of individual gyrodactylids in an arena and record their movements using a video camera, with video files being digitised and analysed for behavioural patterns using specialised tracking software (Paratrack). A simple dose ranging experiment was used to assess the efficacy of four octopaminergic compounds. Results showed that all of the compounds inhibited movement and ultimately led to the death of gyrodactylids at

low concentrations (0.2 μM), although prolonged exposure (48 h) was necessary in some instances. Although the particular compounds tested are also toxic to fish and other aquatic life in varying degrees, the effect of these chemicals on *Gyrodactylus* behaviour and survival is informative and suggests that these, or closely related compounds might provide alternative or supplementary treatments for the control of *G. salaris* infections in rivers. With more research there is potential for octopaminergic compounds to be used as parasite-specific treatments against *G. salaris* infections, which have minimal effects on the host or its environment.

The initial experiments conducted in Chapter 7, suggested that gyrodactylids are most active in dark conditions but are also more active in red light conditions than in white light. These results suggest that perhaps some type of photo stimulus or photoreceptor is present in *G. gasterostei* and that they play a role in its transmission to new hosts (see Chapter 8).

The transmission strategies employed by gyrodactylids are influenced by the accurate and rapid response to chemical and mechanic stimuli. Chapter 8 hypothesised that some of the numerous surface sensory structures observed over the body of each gyrodactylid might serve as either mechano-sensory or and chemo-sensory structures providing information regarding the worm's environment. In addition, sub-tegumental photoreceptors have been observed in other monogeneans (*e.g.* see the study of Watson & Rohde, 1994) prompting a study to determine whether similar structures also existed in *Gyrodactylus*. While a scanning electron microscope (SEM) study revealed numerous sensilla over the surface of the worm, many concentrated around the cephalic lobes, a transmission electron microscope (TEM) study was used to determine the possible function of selected groups of sensilla. One interesting group of six sensilla on the dorsal surface just posterior to the cephalic

lobes, for example, were of particular interest. TEM sections taken through various sensilla revealed the presence of both chemo- (short sensilla in pits) and mechano- (long sensilla standing perpendicular to the tegument) receptors. In addition, the TEM study suggested the presence of two types of photoreceptor. The first type is localised within a chamber posterior to the spike sensilla (Type I), while the proposed Type II was observed as a small sub-tegumental structure occurring in the anterior third of the body between the pharynx and the spike sensilla.

If the mechano-receptors are considered, it is suggested that gyrodactylids respond to host-generated water currents through the dense array of sensilla that cover their bodies (Chapter 8). These are in addition to the other types of receptor (*i.e.* chemo and photo) that serve to process information regarding the parasite's environment. These perceptive mechanisms may allow gyrodactylids to transfer between moving hosts by sensing changes in water movement or turbulence and then detaching and using the water vortices generated by an approaching host to assist their transfer on the new host. The CD enclosed at the back of this thesis provides a video record of parasites attached to the substrate using the water turbulence caused by approaching hosts to facilitate their attachment to that host. The video footage of several parasites using water turbulence to transmit to new hosts is very interesting and represents a mechanism not previously considered. This makes it worthy of further study.

In summary, this thesis has investigated a range of physical and behavioural mechanisms used in the transmission of *Gyrodactylus gasterostei* to its host the 3-spine stickleback. It has considered the maturity (Chapters 3 and 4) and nutritional status (Chapter 6) of worms transferring between alive and / or dead hosts. It has considered alternative routes of transmission including scavenging (Chapter 3) and

water turbulence (Chapter 4), both of which are presented and discussed for the first time. This thesis has also looked at the interaction between different parasite and host species when held together and what the consequences might be (Chapter 5). Chapter 8 of this thesis has briefly investigated what sensilla might be involved in assisting their transmission and demonstrated that structures approximating photoreceptors are present. Chapter 7 has looked at the parasite's searching behaviour while unattachment to a host under different lighting conditions and their response when exposed to different octopamines.

Future work may wish to investigate the super extension capability of the parasite when attaching from either a host passing in close proximity or from the substrate. The presence of coiled muscle (elastic), has been observed in a number of other invertebrates (see Kritsky, 1971), which increases the parasite's probability of transferring to a new host. Future studies should investigate the ultrastructure of the muscles in *Gyrodactylus* and how they function in the transmission process.

Chapter 7 of this study set out to consider alternative compounds for the control of *Gyrodactylus*. If chemicals affecting their release from the host or causing the ejection of their embryos could be identified, then this may lead to strategies for the control of pathogenic species of *Gyrodactylus*, like *G. salaris*, in the future.

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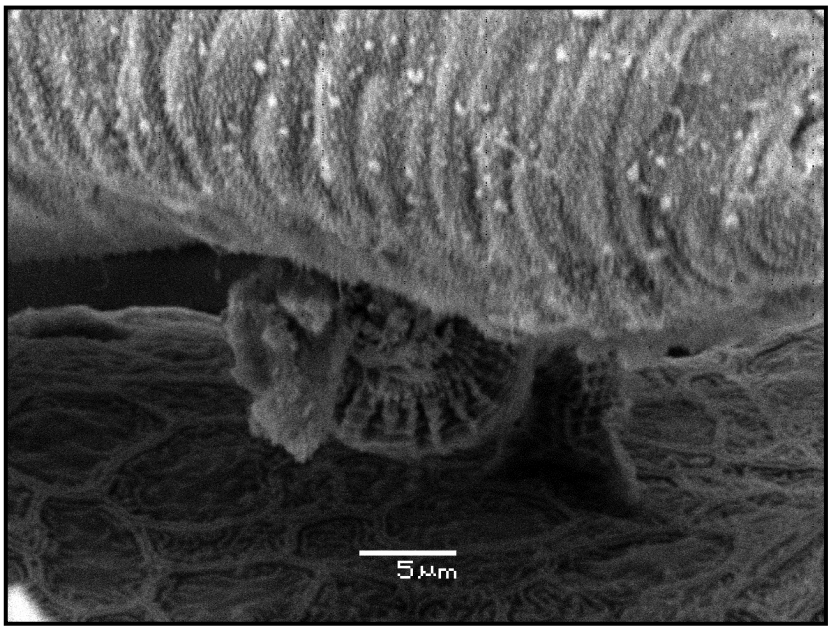

Scanning electron microscopy photography showing the protruded pharynx of a gyrodactylid.

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Appendix

Published papers

魚病研究 **Fish Pathology, 46 (3), 71–79, 2011. 9**

The Accidental Transfer of *Gyrodactylus* (Monogenea) during Short Duration Fish Transportation

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ABSTRACT—The potential of parasite transfer to an alternative host during short periods of cohabitation was explored. The work described in this paper suggests that either the field sampling process itself or the subsequent transportation of multiple fish species in the same container, may affect the correct allocation of parasites to hosts, and the diagnosis, management and control of gyrodactylosis in a variety of fish. *Gyrodactylus* infected specimens of three-spine sticklebacks *Gasterosteus aculeatus*, minnows *Phoxinus phoxinus* and stone loach *Barbatula barbatula* from one Scottish river were cohabited with one another in small volumes of water for 3 h. The study found that a small number of *Gyrodactylus* spp. transfer to atypical hosts. This study indicates that personnel involved in fish disease surveillance programmes should be aware of the possible consequences, in terms of inter-host transfer, of transporting multiple species in the same transport vessel. Diagnosticians should be aware of the facts that fish may act as temporary/paratenic hosts and that the apparent gyrodactylid fauna present following transport may not reflect that encountered under normal circumstances.

Key words: *Gyrodactylus*, Monogenea, accidental infection, surveillance, health check

The viviparous ectoparasitic monogenean *Gyrodactylus salaris* Malmberg, 1957 is a pathogen of notable significance, with infections of Atlantic salmon *Salmo salar* in Norwegian rivers having historically resulted in heavy losses (Johnsen and Jensen, 1986, 1992; Johnsen *et al.,* 1999). *Gyrodactylus salaris* is a notifiable pathogen in the UK and is known to be highly pathogenic to British Atlantic salmon (Bakke and MacKenzie, 1993). Statutory national surveillance programmes throughout the UK, therefore sample rivers and fish farms on a regular basis for the purposes of screening for pathogens that may impact on the health and sustainability of fish stocks. These pathogens are detailed by Defra (DOF 21) and include *G. salaris* as a List III pathogen.

The present study focuses on the potential of gyrodactylids to transfer between hosts and the frequency with which this could occur under normal field sampling. One of the principal modes of host-infection within the gyrodactylids is direct transfer from host to host (Bychowsky, 1961), with movement from substrate to host also recognised as an important route (Bakke *et al*., 2007). Both these routes of infection are likely to be further facilitated in the context of field sampling, with dislodgement of parasites and crowding of fish encouraging transfer. The importance of inter-species transfer for this group of parasites is discussed by Harris (1993), Bakke *et al.* (2002) and Bakke *et al.* (2007), who suggested host-switching to be the predominant mode of radiation within the gyrodactylid group. This study looks at the manner in which field samples are collected and whether practices have a bearing on the accurate allocation of gyrodactylid species to hosts. Specifically, there are concerns that holding different fish species in the same transportation vessel may either cause gyrodactylids to detach from their respective hosts causing parasite burdens to be under-reported or allow gyrodactylids to transfer to new hosts that are encountered in transit providing specificity estimates that are lower than expected. With these considerations in mind, current routine collection and screening procedures may lead to a proportion of worms going undetected or misidentified. Previous studies that have considered host to host transfer have tended to do so over longer time periods of several days. For example, Moen and Stockwell (2006) exposed *Cyprinodon tularosa* and *C. variegatus* to the *C. tularosa*-specific gyrodactylid *Gyrodactylus tularosae* Kritsky et Stockwell, 2005 for up to 4 days. Whilst the parasite was able to use *C. variegatus* as a transient host, its preference was for its typical host. No data was obtained by the authors on the number of host transfers that occurred over the experimental period. In a similar study, Blazek *et al.* (2008) held

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ruffe, *Gymnocephalus cernuus,* and perch, *Perca fluviatilis,* together for around two weeks and minnows, *Phoxinus phoxinus,* and roach, *Rutilus rutilus,* together for up to 3 weeks. *Gyrodactylus macronychus* Malmberg, 1957, a parasite of minnow, transferred to the atypical host *R. rutilus* only once. Under natural conditions where gyrodactylid numbers are high, the minnow parasites *G. macronychus* and *Gyrodactylus aphyae* Malmberg, 1970 have similarly been shown to undergo limited transfer to brown trout, *Salmo trutta* (Mo, 1997). It has been suggested, however, that under experimental conditions, *Gyrodactylus* spp. appear to more readily transfer to atypical hosts, so that the conclusions that may be drawn from inter-species transfers under such conditions are uncertain (*e.g.* Bakke and Sharp, 1990; Bakke *et al*., 1990, 1991; King and Cable, 2007).

The current study looks at the level of gyrodactylid transfer over a short time span between cohabited hosts (three-spine sticklebacks *Gasterosteus aculeatus*, minnow and stone loach *Barbatula barbatula*) from one Scottish river.

Materials and Methods

Host and gyrodactylid collection

Specimens of stone loach (n = 18; 1.03 ± 0.20 g; 48.2 ± 4.47 mm), three-spine sticklebacks (n = 18; 0.61 ± 0.12 g; 35.5 ± 7.18 mm) and minnows (n = 18; 0.25 ± 0.05 g; 31.3 ± 2.91 mm) were hand-netted from the River Endrick near Loch Lomond, West Dunbartonshire, Scotland (56°03′20″N, 4°24′00″W). Fish were transferred in separate 10 L vessels, each containing 8 L of fresh river water, one host species per bucket, to the parasitology aquarium facility at the Institute of Aquaculture, University of Stirling, a journey which took 40 mins.

Fish cohabitation experiment

Upon arrival at the research aquarium, *Gyrodactylus*-infected fish were randomly assigned to 1 L beakers each containing 900 mL, 20 μm filtered, fresh, aerated water from the River Endrick which was taken at the point of fish capture. The fish were distributed such that three replicate vessels each contained three stone loach and three sticklebacks, another three replicate vessels each contained three minnows and three sticklebacks. A series of control vessels were set up so that three replicate vessels each contained three stone loach only (stone loach control), another three replicates contained three sticklebacks only (stickleback control) and a further three replicate vessels contained three minnows only (minnow control). The experimental vessels were maintained for 3 h under ambient light conditions (2800 lux) and at the same temperature as the river water at the point of capture (15°C).

After 3 h, each fish from each beaker was euthanised in accordance with UK Home Office regulations and subsequently fixed, individually, in 80% alcohol. The water from each beaker was then passed through a 20 μm mesh filter to recover any dislodged parasites. The beaker was then rinsed with 100 mL 80% ethanol and the liquid passed through the filter. The mesh was then back-washed into a separate 20 mL vial to release any gyrodactylid specimens. The skin, gills, nostrils and the mouth cavity of each fish were examined for ectoparasites under an Olympus SZ30 stereomicroscope at ×4 magnification. The position of each gyrodactylid on each fish was noted before it was carefully

Table 1. A summary of the total length of the hamulus and marginal hook (mean ± standard deviation) in micrometers followed by the range in parentheses for each species of *Gyrodactylus* parasitising stone loach *Barbatula barbatula*, three-spine sticklebacks *Gasterosteus aculeatus* and minnows *Phoxinus phoxinus* from the River Endrick, Scotland. The total width of the ventral bar was measured on *G. aphyae* and *G. gasterostei*, which are morphologically similar species, to facilitate their discrimination from one another

Species	N	Hamulus total length	Marginal hook total length	Ventral bar total width	Usual fish host in the UK
G. aphyae Malmberg, 1970	42	61.7 ± 5.0 (47.8–71.1)	31.8 ± 2.2 (26.3–34.8)	26.9 ± 6.1 (20.1–35.5)	*P. phoxinus*
G. arcuatus Bychowsky, 1933	18	41.7 ± 4.4 (32.9–53.5)	22.8 ± 3.5 (19.4–27.6)		*G. aculeatus*
G. barbatuli Achmerov, 1952	10	38.8 ± 1.2 (37.5–40.8)	19.6 ± 1.3 (17.9–21.3)		*B. barbatula*
G. gasterostei Gläser, 1974	406	61.0 ± 2.1 (49.4–65.8)	32.5 ± 1.8 (25.9–36.8)	31.3 ± 2.7 (28.1–36.7)	*G. aculeatus*
G. jiroveci Ergens et Bychowsky, 1967	1	55.7	27.1		*B. barbatula*
G. laevis Malmberg, 1957	5	41.4 ± 1.6 (38.5–42.3)	17.2 ± 1.7 (15.3–19.9)		*P. phoxinus*
G. limneus Malmberg, 1964	15	55.1 ± 3.7 (47.1–62.9)	26.6 ± 3.4 (21.0–32.2)		*P. phoxinus*
G. macronychus Malmberg, 1957	33	72.9 ± 2.9 (65.6–78.7)	32.9 ± 2.1 (28.6–37.0)		*P. phoxinus*
G. pannonicus Molnár, 1968	8	51.1 ± 1.1 (50.1–52.7)	26.9 ± 1.5 (23.5–28.2)		*P. phoxinus*
G. pavlovskyi Ergens et Bychowsky, 1967	14	49.7 ± 2.8 (42.9–52.7)	28.4 ± 2.0 (24.6–32.6)		*B. barbatula*
G. sedelnikowi Gvosdev, 1950	24	37.3 ± 1.26 (34.9–39.7)	20.2 ± 1.9 (17.6–23.7)		*B. barbatula*

removed using mounted triangular surgical needles (size 16, Barber of Sheffield, UK). Each specimen was then mounted on a glass slide in a drop of distilled water ensuring that the haptoral hooks were flat. The specimens were then stained and fixed *in situ* by the addition of a drop (~3 μL) of Malmberg's fixative (ammonium picrate glycerine, APG; saturated picric acid and 100% glycerine) to the edge of the coverslip which was drawn under the coverslip by capillary action. The coverslip was then sealed with transparent nail varnish. Each specimen of *Gyrodactylus* was identified following examination and measurement of the hard parts of the haptor using a compound microscope (Olympus BX51) at × 100 oil immersion magnification. The maturity status of each worm was also determined and for this study is given as either Stage 1) lacking an MCO but with an embryo *in utero*, Stage 2) lacking both a male copulatory organ (MCO) and an embryo *in utero*, Stage 3) possessing both an MCO and an embryo *in utero*, or Stage 4) possessing an MCO but lacking an embryo *in utero*.

Taxonomic identification

Hook morphology, particularly that of the marginal hook sickle, and morphometry was used to identify each gyrodactylid. The total length of the hamulus and a marginal hook on each specimen was recorded from images captured using a JVC KY-F30B 3CCD video camera mounted on an Olympus BH2 microscope using a 2.5 interfacing lens at ×100 oil immersion and KS300 (ver. 3.0) (Carl Zeiss Vision GmbH, 1997) image analysis software. For specimens of *Gyrodactylus gasterostei* Gläser, 1974 and *G. aphyae* which have similar hook morphologies, the length of the ventral bar was also taken but for these two species only. In addition, the armature of the MCO, where present, was used to facilitate specimen identification. Images of the MCO and hooks were captured using a Zeiss

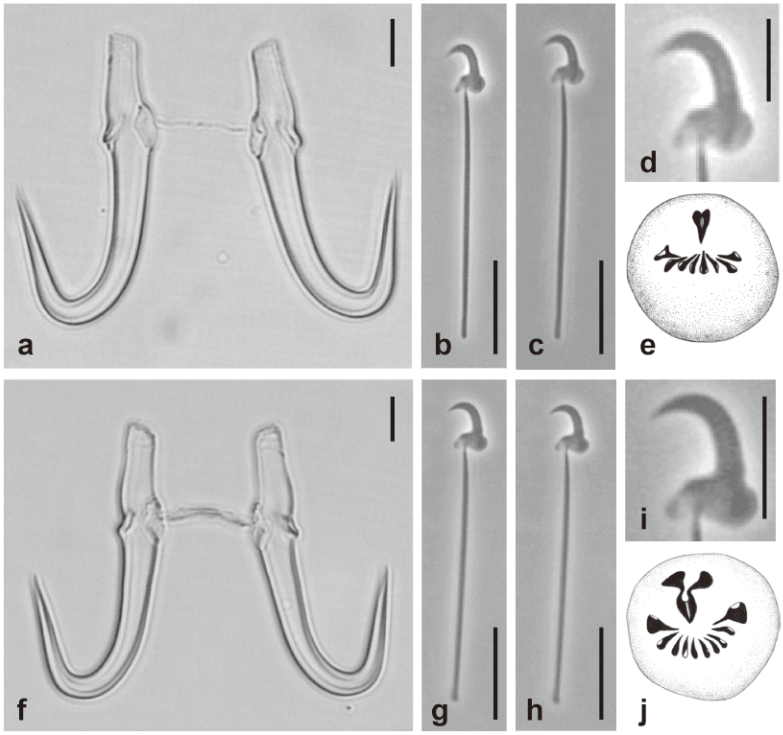

Fig. 1. The hamuli, marginal hooks and male copulatory organ of *Gyrodactylus aphyae* Malmberg, 1964 (a–e) and *G. gasterostei* Gläser, 1974 (f–j). a, *G. aphyae* hamuli and dorsal bar, b–c, marginal hooks, d, marginal hook sickle, e, male copulatory organ armed with 1 large spine facing a single row of 8 approximately equal sized spines. f, *G. gasterostei* hamuli and dorsal bar, g–h, marginal hooks, i, marginal hook sickle, j, male copulatory organ bearing 1 large spine facing a row of smaller spines consisting of 2 medium sized terminal spines and 8 small central spines. Scale bars a–c, f–h = 10 μm, d, i = 5 μm.

AxioCam MRc digital camera interfacing with an Olympus BH2 compound microscope using a × 0.75 lens and MRGrab 1.0.0.4 (Carl Zeiss Vision GmbH, 2001) software. The morphometric measurements made on the attachment hooks and ventral bar of each specimen follow those provided in Shinn *et al.* (2004) and are expressed in micrometers as the mean ± 1 standard deviation followed by the range in parentheses and are shown in Table 1.

Results

Morphometric identification

Identification of the gyrodactylids (n = 567) was based on attachment hook morphology and facilitated by measuring the length of the hamuli and the marginal hooks. The discrimination of *G. gasterostei* (n = 406) from *G. aphyae* (n = 42) was more difficult but was made possible by measuring the length of the ventral bar in addition to the previous measurements. The ventral bar of *G. aphyae* is shorter (*i.e.* 26.9 ± 6.1; 20.1–35.5) than that of *G. gasterostei* (*i.e.* 31.3 ± 2.7; 28.1–36.7) and this assists their discrimination from one another (Fig. 1; Table 1). The MCO, when present, was also used to support species identification (Fig. 1). The MCO of *G. gasterostei* is presented for the first time, as are the tissue-free haptoral hooks of *G. aphyae* and *G. gasterostei* (Fig. 1).

The Gyrodactylus *population on experimental fish*

Eleven species of *Gyrodactylus* were recovered from the three fish species sampled (Table 1). Over all states and replicates (three replicates of stone loach + sticklebacks; three replicates of minnows + sticklebacks; three replicates of stone loach only; three replicates of minnows only; three replicates of sticklebacks only), the results showed that the single-host controls had no apparent accidental infections (n = 303 gyrodactylids), all gyrodactylids recovered being normally associated with that host (Table 2). Two specimens of *G. gasterostei* in a stickleback control and a single specimen of *G. aphyae* in a minnow control, however, were recovered from the bottom of their respective vessels. Eight individual gyrodactylid specimens, however, were observed to transfer to an atypical host (Table 2) and nine specimens of *G. gasterostei*, which failed to attach, were recovered from the bottom of the experimental vessels. In the stickleback–minnow trials (18 hosts; 191 gyrodactylids), one specimen of *Gyrodactylus arcuatus* Bychowsky, 1933 and one specimen of *G. gasterostei*, species usually restricted to sticklebacks in the UK, were recorded on minnows whilst a single specimen of the minnow parasite *Gyrodactylus limneus* Malmberg, 1964 and 2 specimens of *G. macronychus*

Table 2. The species of *Gyrodactylus* and the number of specimens recorded on each fish host at the end of a 3 h period of cohabitation. The figures marked with an asterisk represent species of *Gyrodactylus* that were deemed to have transferred onto another host during the 3 h period of cohabitation

Species	*B. barbatula* cohabited with *G. aculeatus*		*P. phoxinus* cohabited with *G. aculeatus*		*B. barbatula* controls (n = 9)	*G. aculeatus* controls (n = 9)	*P. phoxinus* controls (n = 9)	Total
	B. barbatula (n = 9)	*G. aculeatus* (n = 9)	*P. phoxinus* (n = 9)	*G. aculeatus* (n = 9)				
G. aphyae			30[m]				12[y]	42
G. arcuatus		1[k]	1[*c]	9[r]		7[w]		18
G. barbatuli	9[g]				1[t]			10
G. gasterostei	2[*a]	41[†l]	1[*d]	112[‡s]		250[x]		406
G. jiroveci	1[h]							1
G. laevis			5[n]					5
G. limneus			7[o]	1[*e]			7[z]	15
G. macronychus			16[p]	2[*f]			15[aa]	33
G. pannonicus			7[q]				1[ab]	8
G. pavlovskyi	12[i]	1[*b]			1[u]			14
G. sedelnikowi	15[j]				9[v]			24
Total	39	43	67	119	11	257	35	567

Abbreviations: [†]4 of these specimens failed to attach and were recovered from the bottom of the experimental vessels (one specimen lacked an MCO but had a embryo *in utero* (Stage 1 or S1), one specimen had an MCO and embryo *in utero* (Stage 3 or S3), two specimens had an MCO but no embryo *in utero* (Stage 4 or S4)). [‡] 5 of these specimens failed to attach (one specimen lacked both an MCO and embryo *in utero* (Stage 2 or S2), 1 S3, 3 S1), a-f, the maturity status of each gyrodactylid transferring to an atypical host; a, 2 S4; b, 1 S1; c, 1 S2; d, 1 S1; e, 1 S2; f, 2 S1. g-ab, the maturity status of each gyrodactylid remaining on its primary host; g, 1 S1, 1 S2, 3 S3, 4 S4; h, 1 S3; i, 4 S1, 1 S2, 1 S3, 5 S4; j, 7 S1, 1 S2, 6 S3, 1 S4; k, 1 S4; l, 24 S1, 13 S2, 1 S3, 3 S4; m, 20 S1, 2 S2, 5 S3, 3 S4; n, 1 S1, 1 S2, 1 S4; o, 2 S1, 1 S2, 4 S3; p, 5 S1, 1 S2, 7 S3, 3 S4; q, 4 S1, 2 S2, 1 S4; r, 4 S1, 2 S3, 3 S4; s, 45 S1, 2 S2, 30 S3, 25 S4; t, 1 S3, u, 1 S4; v, 5 S1, 1 S2, 2 S3, 1 S4; w, 6 S1, 1 S4; x, 130 S1, 22 S2 (of which 1 was dislodged), 53 S3 (of which 1 was dislodged), 45 S4; y, 9 S1 (of which 1 was dislodged), 1 S3, 2 S4; z, 2 S1, 1 S2, 4 S3; aa, 5 S1, 7 S3, 3 S4; ab, 1 S3.

Table 3. Hosts records for the species of *Gyrodactylus* von Nordmann, 1832 including accidental transfers observed in the current study

Gyrodactylus species	Host	References
G. aphyae	*Leuciscus leuciscus*	Dorovskikh (1997), Ivashevsky (1999)
	Phoxinus phoxinus†	Malmberg (1970), Pugachev *et al.* (2010)
	Rhynchocypris czekanowskii (syn. *Phoxinus czekanowskii czerskii*)	Pugachev *et al.* (2010)
	Rhynchocypris percnurus (syn. *Phoxinus percnurus*)	Dorovskikh (1997)
	Rutilus rutilus	Dorovskikh (1997)
	Salmo trutta	Kiskaroly (1988), Mo (1997)
G. arcuatus	*Gadus morhua*	Hemmingsen and MacKenzie (2001)
	Gasterosteus aculeatus†	Bychowsky (1933), Pugachev *et al.* (2010)
	Gobiusculus flavescens	Longshaw *et al.* (2003)
	Phoxinus phoxinus	Current study
	Pomatoschistus lozanoi	Geet *et al.* (1999)
	Pomatoschistus minutus	Geet *et al.* (1999)
	Pomatoschistus pictus	Geet *et al.* (1999)
	Pungitius pungitius	Domnich and Sarabeev (2000), Sterud (1999)
	Salmo salar	Shinn *et al.* (1996), Sterud (1999)
	Salvelinus alpinus	Sterud (1999)
G. barbatuli	*Barbatula barbatula*†	Achmerov (1952), Pugachev *et al.* (2010)
	Barbatula toni	Pugachev *et al.* (2010)
G. gasterostei	*Alburnus alburnus*	Dorovskikh (1997)
	Barbatula barbatula	Current study
	Blicca bjoerkna	Gussev (1985)
	Gasterosteus aculeatus†	Gläser (1974), Pugachev *et al.* (2010)
	Gobio gobio	Dorovskikh (1997)
	Leuciscus leuciscus	Dorovskikh (1997)
	Perca fluviatilis	Valtonen *et al.* (1997, 2003), Nedeva and Babacheva (1999)
	Phoxinus phoxinus	Dorovskikh (1997); current study
	Pungitius pungitius	Pugachev *et al.* (2010)
	Rutilus aula	Galli *et al.* (2002)
	Rutilus rutilus	Dorovskikh (1997)
	Squalius cephalus (syn. *Leuciscus cephalus*)	Dusek *et al.* (1998), Nedeva and Babacheva (1999), Gelnar *et al.* (1997)
G. jiroveci	*Barbatula barbatula*†	Ergens and Bychowsky (1967), Pugachev *et al.* (2010)
	Barbatula toni	Pugachev *et al.* (2010)
G. laevis	*Leuciscus idus*	Pugachev *et al.* (2010)
	Leuciscus baicalensis (syn. *Leuciscus leuciscus baicalensis*)	Pugachev *et al.* (2010)
	Phoxinus phoxinus†	Malmberg (1957), Pugachev *et al.* (2010)
	Rhynchocypris lagowskii (syn. *Phoxinus lagowski*)	Pugachev *et al.* (2010
	Rhynchocypris percnurus	Pugachev *et al.* (2010)
G. limneus	*Gasterosteus aculeatus*	Current study
	Phoxinus phoxinus†	Malmberg (1964), Pugachev *et al.* (2010)
	Rhynchocypris percnurus	Pugachev *et al.* (2010)
G. macronychus	*Gasterosteus aculeatus*	Current study
	Phoxinus phoxinus†	Malmberg (1957), Pugachev *et al.* (2010)
	Rhynchocypris percnurus	Pugachev *et al.* (2010)
	Rutilus rutilus	Blazek *et al.* (2008), Pugachev *et al.* (2010)
	Salmo trutta	Mo (1997)
	Squalius cephalus (syn. *Leuciscus cephalus cabeda*)	Pugachev *et al.* (2010)
G. pavlovskyi	*Barbatula barbatula*†	Gvosdev (1950), Pugachev *et al.* (2010)
	Gasterosteus aculeatus	Current study
	Silurus asota	Pugachev *et al.* (2010)
G. sedelnikowi	*Barbatula barbatula*†	Gvosdev (1950), Pugachev *et al.* (2010)
	Barbatula toni	Pugachev *et al.* (2010)
	Silurus asota	Pugachev *et al.* (2010)

†Denotes the primary host for each species.

(11.8% of the *G. macronychus* population), species restricted to minnows in the UK, were found to have transferred to sticklebacks (Table 2). Similarly, in the stone loach–stickleback experiment (18 hosts; 82 gyrodactylids), a total of two specimens of *G. gasterostei* (4.7% of the *G. gasterostei* population), a species usually restricted to sticklebacks in the UK, were found on two stone loach and a single specimen of *Gyrodactylus pavlovskyi* Ergens et Bychowsky, 1964, which only infects stone loach in the UK, was recovered from a stickleback. All of these transfers, with the exception of a specimen of *G. gasterostei* transferring to a minnow (see Dorovskikh, 1997), are recorded for the first time (see Table 3). The maturity status of each gyrodactylid transferring to a new host was determined and the details are given in Table 2. Of the eight specimens transferring, two had a visible, well developed MCO while six specimens did not, four had an embryo *in utero* while the remaining four did not. Parasites were recorded on fins, skin and gills of all hosts. All of those gyrodactylids found on "atypical" hosts were recorded from the fins and skin.

Two unattached specimens of *G. gasterostei* (Stage 2 and Stage 3) and a single unattached specimen of *G. aphyae* (Stage 1) were recovered from the bottom of the stickleback and minnow control vessels respectively. In addition, four unattached specimens of *G. gasterostei* were also recovered from the bottom of the experimental vessels in the stone loach–stickleback trials and five from the stickleback–minnow trials representing 9.3% and 4.4% of the *G. gasterostei* population in each set of experiments. The maturity status of each detached specimen was also determined, four specimens possessed an MCO, five did not whilst six of the nine specimens had an embryo *in utero*. Given the low number of specimens failing to attach or transferring to an atypical host, it is not possible to identify underlying drivers (*e.g.* possession of an MCO, detachment during the process of giving birth or encumbrance by a large embryo *in utero*) for the behaviour/status of these individuals.

Discussion

This study provides evidence for the accidental host transfer of *Gyrodactylus* species maintained in artificially cohabiting host communities over a short time (3 h) period. Under normal conditions, the ability to transfer between hosts, even for short periods, can extend the effective host population available for colonisation, which may be important for survival of individuals and species, particularly when access to optimal hosts is limited (*e.g.* low recruitment success of the usual host). The ability of *Gyrodactylus* species to transfer between hosts during the sampling process or through mixed-species transportation in the same container, raises important questions over the potential accuracy and reliability of assessments of parasite fauna made following such transport. In this study, the gyrodactylid fauna of artificially cohabiting sticklebacks, stone loach and minnows following a series of 3 h cohabitation experiments was assessed. The results showed that a number of worms were found to have transferred to an atypical host, with each of these transfers, except for the transfer of a single specimen of *G. gasterostei* onto minnow, being recorded for the first time (Table 3). No accidental infections were found among the 303 gyrodactylids recovered from the control fish. An ongoing gyrodactylid surveillance programme in the River Endrick (> 10 years), undertaken by some of the current authors, has similarly documented no accidental infections within these hosts. Whilst it is possible that the observed accidental transfers may have occurred prior to collection of fish from the wild, the lack of accidental transfers in control groups suggest that this is not the case. A refinement of this study could be to treat all fish with a general anthelminthic to remove all gyrodactylids then experimentally expose fish to a known number of gyrodactylids. Subsequent cohabitation with mixed naïve species could then lead to parasite transfer. However, this was outside the remit of the current study, which was concerned with transfer under transport conditions. This study has revealed that the opportunities provided for transfer of gyrodactylids between host species arising from a relatively standard sampling routine may affect the correct allocation of parasites to hosts, and the diagnosis, management and control of gyrodactylosis in a variety of fish.

Robertsen *et al.* (2008) suggested that host transfer under natural conditions could cause significant expansion of the geographical range of pathogenic variants of *G. salaris,* allowing it to spread. Gyrodactylids can rapidly colonise an entire river system (Bakke *et al*., 1992), such colonisation being presumably largely dependent upon host densities and / or availability, such that an expanded host range might be expected to contribute to the speed of colonisation. Colonisation ability also depends on survival time off hosts and gyrodactylid survival time depends particularly upon water temperature (Soleng and Bakke, 1997). *Gyrodactylus salaris* can survive for 4 days at 3°C (Olstad *et al*., 2006) and survival of detached *G. gasterostei* was similarly temperature dependent, being 103 h at 4°C (Cable *et al*. 2002). Some host species are clearly more susceptible to inpection by a wide number of *Gyrodactylus* species. Minnows, for instance have been reported to be associated with a total of 14 gyrodactylid species belonging to seven species groups, more than any other single host (Bakke *et al*., 2002). The presence, in sampled fish fauna, of hosts with a broad range of parasites or indeed the presence of *Gyrodactylus* species showing low host specificities in such samples

might be considered to exacerbate problems of interspecies transfers. This said, in the present study only three gyrodactylids for whom minnows (*G. macronychus, G. limneus*) and stone loach (*G. pavlovskyi*) are the primary (typical) host were found to have erroneously transferred to a stickleback (atypical host). This fact may be related to the short period of exposure provided (3 h), although this time period reflects an assumed typical transfer time of fish from field sample sites to a laboratory. During a longer term study, Blazek *et al.* (2008) demonstrated that a single specimen of *G. macronychus* transmitted from a minnow to a roach. Although not expressly measured or observed, it is interesting to speculate on the mode of gyrodactylid transfer between hosts.

Whilst accidental host transfer has been demonstrated to occur following artificial cohabitation of hosts, it is difficult to assess the relevance of such accidental transfer abilities to wild populations. Both the control hosts in the present study and the historical fish-parasite record for this stretch of river showed no evidence of *Gyrodactylus* cross-infection for the hosts examined. This may be indicative of the fact that in the capture environment different host species are isolated from one another by differing environmental preferences (*e.g.* benthic / open water habits) or behavioural isolation mechanisms, which effectively decrease probabilities of accidental transfer between host species. Furthermore, host specificity has been used previously as a diagnostic criterion for some species, although this is variable. Many *Gyrodactylus* species are considered host specific, at least to genus or host family. For example, whilst *G. salaris* is considered as a salmonid parasite, it has been found to occur on minnows, *Platichthys flesus* and several genera of salmonid. This, however, is an oversimplification with different haplotypes potentially occurring on different hosts. Of those species that were shown to transfer to an atypical host during the current study, the two stickleback gyrodactylids, *G. arcuatus* and *G. gasterostei* have been recorded on at least 9 and 12 different hosts respectively under normal conditions (Table 3). In contrast, the number of fish species serving as hosts, typical or accidental, for *G. limneus, G. macronychus* and *G. pavlovskyi* is lower (see Table 3). The wide host specificity of the stickleback gyrodactylids may be either due to previous misidentifications providing an overestimate of the number of true hosts or may be due to a biological transmission strategy on the part of those parasites. Three-spine sticklebacks occur in a wide range of lentic and lotic water conditions ranging from freshwater to fully marine. Gyrodactylid parasites of sticklebacks may therefore show a measure of the same plasticity with respect to host and environment.

This study has demonstrated that accidental transfer of gyrodactylids may occur during artificial cohabitation following field sampling of host fish species. Hence standard field sampling practices involving transportation of multiple host species in the same container may affect the correct allocation of parasites to hosts, and the diagnosis, management and control of gyrodactylosis in a variety of fish and it may thus be prudent to reconsider sampling protocols in the light of these findings.

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RESEARCH Open Access

The effect of octopaminergic compounds on the behaviour and transmission of *Gyrodactylus*

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Abstract

Background: The high transmission potential of species belonging to the monogenean parasite genus *Gyrodactylus*, coupled with their high fecundity, allows them to rapidly colonise new hosts and to increase in number. One gyrodactylid, *Gyrodactylus salaris* Malmberg, 1957, has been responsible for devastation of Altantic salmon (*Salmo salar* L.) populations in a number of Norwegian rivers. Current methods of eradicating *G. salaris* from river systems centre around the use of non-specific biocides, such as rotenone and aluminium sulphate. Although transmission routes in gyrodactylids have been studied extensively, the behaviour of individual parasites has received little attention. Specimens of *Gyrodactylus gasterostei* Gläser, 1974 and *G. arcuatus* Bychowsky, 1933, were collected from the skin of their host, the three-spined stickleback (*Gasterosteus aculeatus* L.), and permitted to attach to the substrate. The movements of individual parasites were recorded and analysed.

Results: The behaviour patterns of the two species were similar and parasites were more active in red light and darkness than in white light. Four octopaminergic compounds were tested and all four inhibited the movements of parasites. Treatment ultimately led to death at low concentrations (0.2 μM), although prolonged exposure was necessary in some instances.

Conclusions: Octopaminergic compounds may affect the parasite's ability to locate and remain on its host and these or related compounds might provide alternative or supplementary treatments for the control of *G. salaris* infections. With more research there is potential for use of octopaminergic compounds, which have minimal effects on the host or its environment, as parasite-specific treatments against *G. salaris* infections.

Keywords: *Gyrodactylus*, octopamine, behaviour, toxicology

Background

As *Gyrodactylus* von Nordmann, 1832 (Monogenea) has no specific transmission stage in its life-cycle, movement between hosts must be achieved by strategies employed by the adult. Bakke *et al.* [1] suggested four routes by which gyrodactylids could transfer to a new host: (i) via contact with live hosts, (ii) via dead hosts, (iii) by detached parasites drifting in the water column, and (iv) by parasites attached to the substrate. This transmission potential, coupled with their high fecundity allows gyrodactylids to rapidly colonise new river systems [1,2]. Although transmission routes in gyrodactylids have been studied extensively, few workers have investigated the behaviour of individual gyrodactylids.

Gyrodactylus salaris Malmberg, 1957 has devastated Atlantic salmon (*Salmo salar* L.) populations where it is present in North European rivers [3] and currently the only method of eradicating *G. salaris* from river systems is by using biocides, such as rotenone. However, this is devastating for the river habitat and, once it has recovered, *G. salaris* can re-colonise the river if measures are not taken to prevent its re-introduction [2]. Consequently, the focus of research is moving towards finding alternative methods to control *G. salaris*, which target the pathogen without seriously affecting the river ecosystem. This requires an increased understanding of gyrodactylid biology and behaviour [4].

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In the control of other pathogens, chemical treatments often target specific stages of the life-cycle, which can be exploited to reduce the survival or infectivity of the parasites *e.g.* teflubenzuron is used to disrupt the moult of sea lice (*Lepeophtheirus salmonis* Krøyer, 1837 and *Caligus elongatus* Nordmann, 1832) [5]. Neurotransmitter receptor agonists/antagonists are compounds that elicit a response by binding to a postsynaptic receptor (*e.g.* on muscle or nerve) and mimicking or blocking the natural transmitter. In this study, the effect of octopaminergic receptor agonists/antagonists on gyrodactylids was investigated. It is suggested that exposing gyrodactylids to these compounds may affect their ability to attach to a host, rendering them immobile and unable to infect a host.

In order to investigate the effect of octopaminergic chemicals on the behaviour of gyrodactylids, it was necessary to develop a bioassay to observe their behaviour. Therefore, the objectives of the study were to: 1) develop a system for recording and observing the movements of gyrodactylids under different lighting conditions; 2) determine optimum lighting conditions for observing the behaviour of gyrodactylids, by comparing their movements under white light, red light and in dark conditions; and 3) determine the efficacy of the four octopaminergic compounds on detached gyrodactylid behaviour.

Materials and methods

Source of parasites

As *Gyrodactylus salaris* is a notifiable pathogen in the UK, it was not possible to acquire them for use in this study and therefore gyrodactylids from three-spined sticklebacks (*Gasterosteus aculeatus* L.), which are easily obtainable, were used as a gyrodactylid model. Two species of *Gyrodactylus* were identified from sticklebacks, *G. gasterostei* Gläser, 1974 and *G. arcuatus* Bychowsky, 1933, although the former were in the majority (80% and 20%, respectively). Both species were used in the behaviour experiments.

Three-spined sticklebacks were netted from a tributary of the River Forth, Stirlingshire (56° 06' 37.77" N, 3° 58' 25.25" W) and maintained at 10°C in 30 litre, static tanks in an aquarium facility at the Institute of Aquaculture, University of Stirling. A 50% water change was carried out daily, using water collected from Loch Airthrey (56° 08' 39.53" N, 3° 53' 51.20" W) and the sticklebacks were fed *ad libitum* with frozen bloodworm (Gamma, Chorleywood, UK). *Gyrodactylus* for use in the behaviour experiments were removed from the sticklebacks using triangular mounted surgical needles (size 16, Barber of Sheffield, UK). Parasites were identified to species level using standard descriptions. Once the behaviour of each gyrodactylid had been determined, it was fixed and mounted in ammonium picrate glycerine according to the method detailed by Malmberg [6], identified and its maturity status determined (*i.e.* presence or absence of a male copulatory organ and/or an embryo *in utero*).

Investigation of lighting conditions

Initially, a simple experiment was undertaken to determine the activity of gyrodactylids under light and dark conditions. A mark was made on the underside of a 9 cm diameter Petri dish using a permanent marker and a single *Gyrodactylus* was placed onto the mark in the Petri dish filled with stream water at 10°C. Parasites attached themselves by the haptor and twenty replicates of each were maintained in either ambient light (2800 lux) or dark conditions (0 lux). The replicates in ambient light were placed inside a cotton light-diffusing box to scatter the light and eliminate any directional cues. After three hours the straight line distance between the final position of the *Gyrodactylus* and the initial mark was measured.

Gyrodactylus tracking

An experimental system was constructed to record the behaviour of individual *Gyrodactylus* (Figure 1). This consisted of a 110 mm section of PVC pipe with a divider inserted inside the pipe. A circular hole 52 mm in diameter was cut in the divider and a mirror was placed underneath the divider at an angle of 45°. A 5 cm diameter Petri dish with a painted matt black base was placed onto supports surrounding the circular hole. Light was provided by a Carousel S 150W slide projector, which was directed onto the mirror, deflecting the light up through the divider and around the Petri dish. A foil

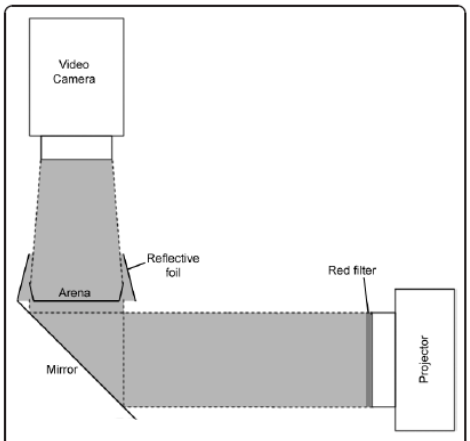

Figure 1 Diagram of the experimental setup used to record the behaviour of detached specimens of *Gyrodactylus* under various lighting conditions or when exposed to a range of muscle agonists.

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cone set at an angle of ~30° directed light back into the centre of the Petri dish, forming a ring of incident light. This allowed the gyrodactylid to be detected in the arena and eliminated any directional light cue as the light level was consistent around the whole dish (Figure 1). A Canon MiniDV MD205 video camera was mounted on a stand above the arena to record the movements of the *Gyrodactylus*. Inflated circular rubber inner tubes measuring 20 and 50 cm in diameter were placed underneath the projector and the tray containing the light chamber to dampen vibrations from the projector.

For each replicate a new 5 cm diameter painted Petri dish was filled with 10 ml of 0.2 μm filtered stream water at 10°C and a single *Gyrodactylus* was placed into the centre of the arena using a Gilson pipette. It was then placed into the light chamber and left to settle for 20 minutes. The subsequent behaviour of the parasite was then recorded ($T_{20\text{-}50}$ mins) onto MiniDV cassettes, using the video camera, for 30 minutes, before being fixed and mounted onto a glass slide. Ten replicates were recorded in white light (~2800 lux) and ten in red light, using a Hoya 600 nm (590-2750 nm) red photographic filter placed over the projector lens.

The 30 minute videos were converted to digital video files in.avi format using Windows Moviemaker software (version 2.1.4028.0, Microsoft Corporation, 2007). Individual frames in bitmap format were extracted using Bink and Smacker software (Bink version 1.9L, Smacker version 4.2d, RAD Game Tools Inc., 2009) at a frame rate of 1 frame per 5 seconds. Shade correction and segment analysis of the image set was performed in KS300 software (version 3.0 Carl Zeiss Vision GmbH, 1997) to facilitate the tracking of the parasite. Paratrack software (version 2.4, A. Brooker, University of Stirling, 2007) was used to track the movements of the parasite in each frame, creating an image of the gyrodactylid's movements and a text file containing a list of co-ordinates of the parasite's location in each frame. Once the parasites had been tracked the lists of co-ordinates were time averaged over three steps (15 seconds) to smooth the data, removing any bias in the calculated behaviour parameters as a result of exploratory extensions by the gyrodactylids whilst their haptors are stationary. The resultant co-ordinates were then used to calculate behavioural information including the mean and maximum velocity of each parasite, the distance travelled, turn rate, meander and heading. Fractal dimensions, which are a measure of track complexity, were also calculated for the parasite tracks using the 'box counting' method [7,8]. These operations were all undertaken using the Paratrack software. Principal Component Analysis (PCA -Statistica 6.1 software, 2004, Statsoft Inc., USA) was used to investigate differences between gyrodactylid movements in white and red light.

Chemical efficacy

The following four octopaminergic compounds were tested in this trial: (±)-octopamine hydrochloride ($C_8H_{11}NO_2$.ClH; O0250 Sigma), clonidine hydrochloride ($C_9H_9Cl_2N_3$.ClH; C7897 Sigma), amitraz (N-methylbis-(2,4-xylyl iminomethyl) amine, $C_{19}H_{23}N_3$; 45323 Riedel-de Haën/Sigma) and chlordimeform ($C_{10}H_{13}ClN_2$; 35913 Riedel-de Haën/Sigma). Chlordimeform was selected as a positive reference as it is known to be effective in the control of invertebrates and is toxic to aquatic life [9]. Octopamine is a biogenic monoamine found predominantly in invertebrates and modulates physiological activity by binding to adrenoceptors. In invertebrates it acts as a neurohormone, a neuromodulator or a neurotransmitter and modulates almost every physiological process [10]. In vertebrates noradrenaline is homologous to octopamine in invertebrates. Octopamine is found at concentrations less than 1% of noradrenaline in vertebrates, with its physiological activity being only 1-2% of noradrenaline [11]. Clonidine is a centrally-acting α-adrenergic receptor agonist and is known to reduce involuntary muscle contractions, or tics, in humans by binding to α2-adrenergic receptors [12]. Its mode of action is inhibition of adrenergic receptors, which results in reduced motor activity [13]. Amitraz and chlordimeform belong to a group of insecticides/acaricides (formamidines) whose mode of action is by interaction with octopamine receptors [13]. They work by mimicking the action of octopamine (centrally and at the neuromuscular junction) in invertebrates [14]. Amitraz acts as a receptor agonist, whereas chlordimeform has an antagonistic effect [15,13]. Although certain groups of invertebrates have been shown to be particularly sensitive to formamidine compounds (Acarines, Lepidoptera and Hemiptera), vertebrates in general are relatively insensitive [16]. Both chemicals have anthelmintic properties [17] and have been shown to induce hyperexcitation and detachment of feeding ticks [18,19].

As the efficacy of the four octopaminergic compounds on *Gyrodactylus* was unknown, a simple dose ranging exposure experiment was carried out using serial dilutions of each chemical with distilled water prepared in concentrations of 32, 16, 8, 4 and 2 μM plus a control consisting of distilled water only. One ml of each of these dilutions was pipetted into 5 cm diameter Petri dishes containing 9 ml of filtered stream water at 10°C to give final concentrations of 3.2, 1.6, 0.8, 0.4 and 0.2 μM. A single *Gyrodactylus* specimen was introduced into each Petri dish, which were then kept in an incubator at 10°C. Each chemical concentration was replicated 15 times. The parasites were checked after 24 and 48 h and recorded as alive, affected (*i.e.* not attached and showing muscular spasms), moribund (*i.e.* not attached, curled up and showing minute muscular contractions) or dead (*i.e.* no response to physical stimulus). After 48 h the gyrodactylids were preserved

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in ethanol for future identification and maturity assessment. After applying Abbot's correction factor [20] to account for control mortality, probit analysis (Minitab 13.1 Software, 2000, Minitab Inc., USA) was used to calculate 24 h and 48 h 50% effective concentration (EC50) values for each of the octopaminergic compounds. Where EC50 values are given, figures in parentheses are fiducial limits.

Results

Lighting conditions

As there was no significant difference between the distances travelled by each species of *Gyrodactylus* the data were combined. The investigation suggested that *Gyrodactylus* are more active in dark than in light conditions ($P = < 0.001$, one-way ANOVA) (Figure 2). After three hours, parasites in dark conditions moved a mean distance of 28.37 ± 10.18 mm from their starting point, whereas those in white light conditions moved only 11.8 ± 10.13 mm.

Tracking

Observation of the 30 minute tracks of individual *Gyrodactylus* showed several different behaviour patterns that were common to both species of *Gyrodactylus* tested: The most common behaviour involved moving in one direction with little deviation from the chosen heading (Figure 3a); the movements of some individuals were confined to a very small area around the starting point

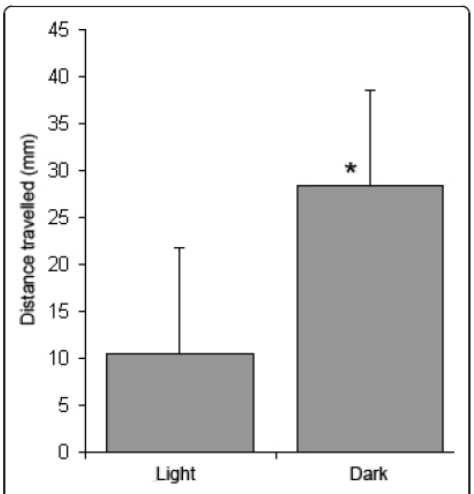

Figure 2 Distance travelled by *Gyrodactylus* after 3 h in light (n = 19) and dark (n = 19) conditions. Bars = 1 S.D., * = significant difference from white light response ($p < 0.001$).

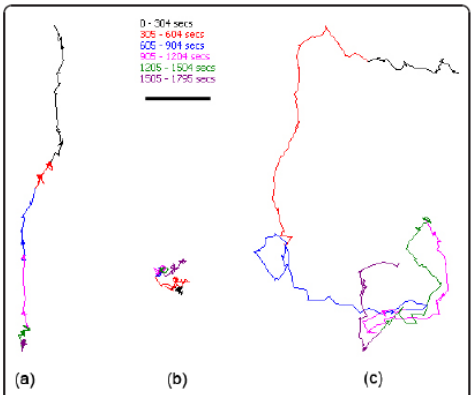

Figure 3 Thirty minute *Gyrodactylus* tracks, showing different types of behaviour. (a) Linear movement, (b) limited movement and (c) long sinuous movements with track crossovers. Bar = 5 mm.

(Figure 3b); the final behaviour pattern can be described as extensive sinuous movements, with several path crossovers (Figure 3c). Individuals recorded in white light conditions appeared to display the first and second behaviour patterns, whereas individuals recorded under red light appeared to have longer, more sinuous tracks. No correlation was found between the different behaviour types and the species or maturity status of the individual gyrodactylids.

Analysis of the tracks revealed that gyrodactylids in red light (n = 10) had a higher mean velocity (0.18 ± 0.17 mm/sec) and maximum velocity (0.78 ± 0.35 mm/sec), travelled further (6.32 ± 5.81 cm) and had a higher turn rate (± 26.6 degrees/sec) compared to those in white light (n = 10), which had a mean velocity of 0.11 ± 0.10 mm/sec, maximum velocity of 0.51 ± 0.28 mm/sec, travelling distance of 4.04 ± 3.35 cm and turn rate of 20.35 ± 6.79 degrees/sec (Figure 4). However, none of these values were significantly different (one-way ANOVA). Fractal dimensions and meander were lower for gyrodactylids in red light (0.69 ± 0.2 and 856 ± 397 degrees/mm) than for those in white light (0.85 ± 0.2 and 1195 ± 373 degrees/mm), indicating less complex tracks for those in red light, although again none of these values were significantly different (one-way ANOVA). The behavioural data was analysed (one-way ANOVA) for differences according to species of *Gyrodactylus* and maturity status, but none were found and no patterns in the data were apparent, suggesting that the different behaviours observed in this study are unrelated to the species and/or age of the gyrodactylids.

The behaviour data was subjected to Principal Component Analysis (PCA) to reveal differences between

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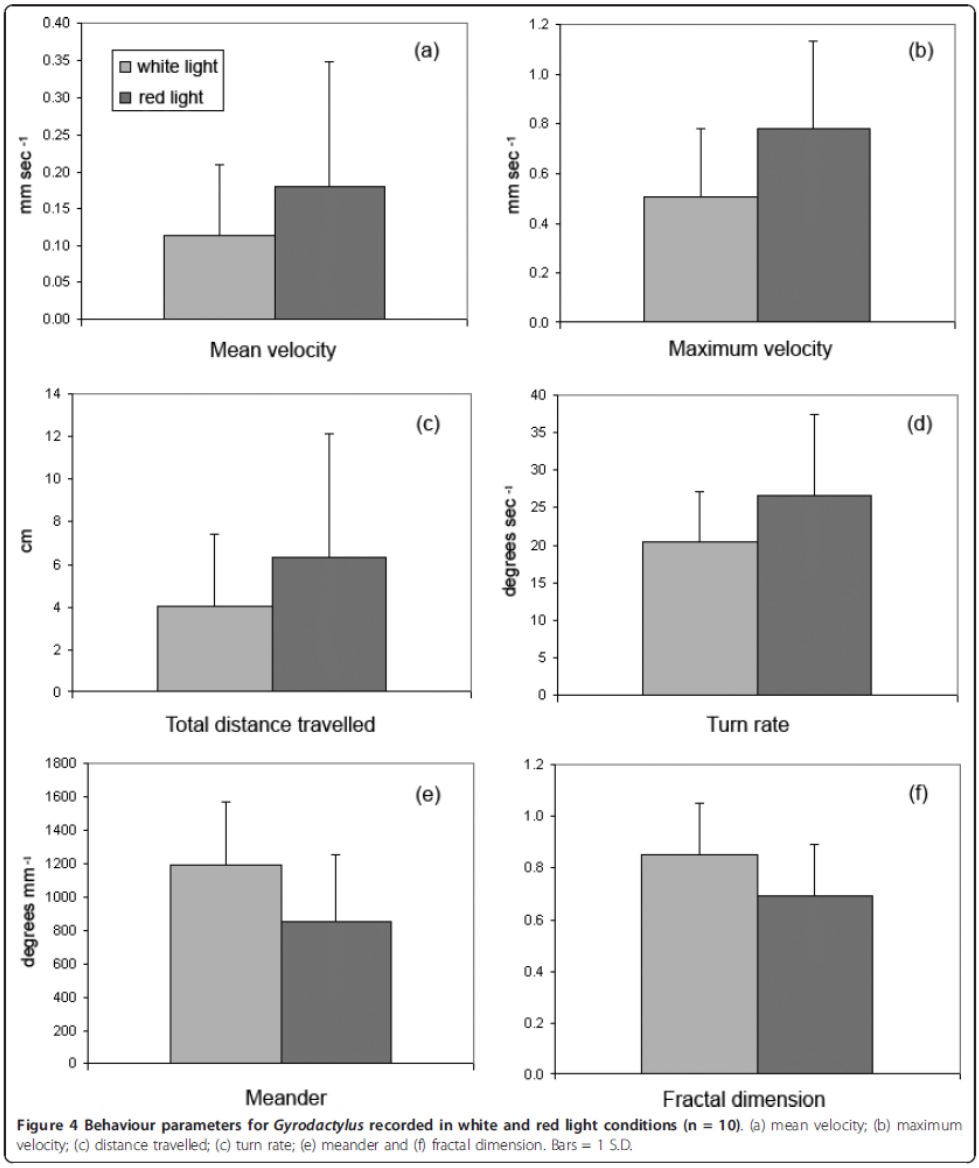

Figure 4 Behaviour parameters for *Gyrodactylus* recorded in white and red light conditions (n = 10). (a) mean velocity; (b) maximum velocity; (c) distance travelled; (c) turn rate; (e) meander and (f) fractal dimension. Bars = 1 S.D.

gyrodactylid behaviour in white light and red light. The behaviour parameters that showed the greatest differences between white light and red light (one-way ANOVA) were chosen (*i.e.* maximum velocity, meander and fractal dimension) and checked for normality (the remaining parameters were found to be too variable to show any patterns in behaviour). The maximum velocity data was found to be skewed, so was log transformed to

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normalise it. Eigen values for Factors 1 and 2 were 66.3% and 25.8%, respectively, describing a total of 92.1% of the variation in the data. The PCA plot shows two distinct groups according to behaviour in white light and red light, although some individuals in white light were grouped with those in red light (Figure 5). Examination of the individual tracks confirmed that those individuals in white light that were grouped with those in red light exhibited behaviour typical of those in red light (*i.e.* long, sinuous tracks).

Chemical efficacy

All of the four compounds affected *Gyrodactylus* and produced involuntary muscular contractions (spasms) when normal body extension was attempted. A mortality of 10% was seen in the control group after 48 hours, although no muscle spasms were observed. As the positive reference, the highest concentration of 3.2 μM of chlordimeform affected 87% of gyrodactylids after 24 h as denoted by limited movements (Figure 6a). However, after 48 h 27% of gyrodactylids were unaffected (Figure 6b) suggesting that (i) the muscular spasms may only be temporary at that concentration; (ii) the gyrodactylids needed to be at a particular physiological state before they became susceptible; (iii) the persistence of the compound affects its efficacy. As there was no clear trend in the numbers of dead, moribund and affected gyrodactylids (Figure 6a, b), it was not possible to accurately calculate EC50 values for chlordimeform.

Octopamine had a dose dependent response after 24 h, with 73% of gyrodactylids being either affected, moribund or dead at the highest concentration of 3.2 μM, compared to 27% at the lowest concentration of 0.2 μM (EC50 = 0.89 μM (0.46-1.94 μM)) (Figure 6c). After 48 h the majority (67%) of the gyrodactylids were dead at 3.2 μM (Figure 6d). Numbers of affected and moribund gyrodactylids were low for all concentrations (7%-27%) after 48 h suggesting that the optimum exposure time for octopamine is between 24 and 48 h. The 48 h EC50 for octopamine was 0.25 μM (0-0.54 μM).

Clonidine was effective after 24 h with 60% of gyrodactylids being either affected, moribund or dead at both 3.2 μM and 0.2 μM (Figure 6e). After 48 h this figure had increased to 87% at 3.2 μM and 80% at 0.2 μM (Figure 6f). As there was little difference in the number of affected gyrodactylids between the highest and lowest doses, it is possible that either the concentration range selected was too narrow to determine the effective range or there are other factors affecting the efficacy of the compound. Therefore, it was not possible to accurately calculate EC50 values for clonidine. However, as the number of affected and moribund gyrodactylids was low after 48 h (7%-27%), it is suggested that, similar to octopamine, the optimum exposure time for clonidine is between 24 and 48 h.

Amitraz was the most effective of the compounds tested with 100% of gyrodactylids being either affected, moribund or dead after 24 h at the highest concentration of 3.2 μM (53% dead) (Figure 6g). At 0.2 μM 66% remained unaffected with 20% being either affected or moribund. The 24 h EC50 for amitraz was 0.31 μM (0.18-0.44 μM). After 48 h 60% were dead at 3.2 μM and 27% were dead at 0.2 μM (Figure 6h). As there were a considerable number of gyrodactylids either affected or moribund after 48 h (33-47%), and the numbers either affected, moribund or dead after 48 h were similar to those after 24 h, it is likely that the optimum exposure time for amitraz is longer than 48 h. The 48 h EC50 value for amitraz was 0.18 μM (0.05-0.27 μM).

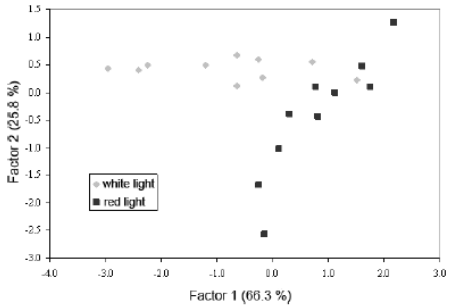

Figure 5 Principal Component Analysis of maximum velocity, meander and fractal dimension for gyrodactylids exposed to white light (n = 10) and red light (n = 10).

Discussion

These results suggest that gyrodactylids are more active in the dark than in light and therefore imply that they possess some form of photoreceptor. Watson and Rohde [21] found sensory receptors in *Gyrodactylus* sp., which closely resemble photoreceptors found in other platyhelminths [22,23]. The light/dark experiment shows a significant difference in the distance travelled between those gyrodactylids in the dark and those exposed to light. However, as this experiment only records the start and end position of the parasite, the trial assumes that parasites have travelled in a straight line and, therefore, it is impossible to quantify their movements during the period of the experiment *i.e.* whether they follow a straight or sinuous path. This does, however, suggest that there may be differences in the distance travelled by gyrodactylids under different lighting conditions.

Although parasite tracks cannot be determined in the "dark", they can be measured under red and infrared light. By recording and tracking all the movements of

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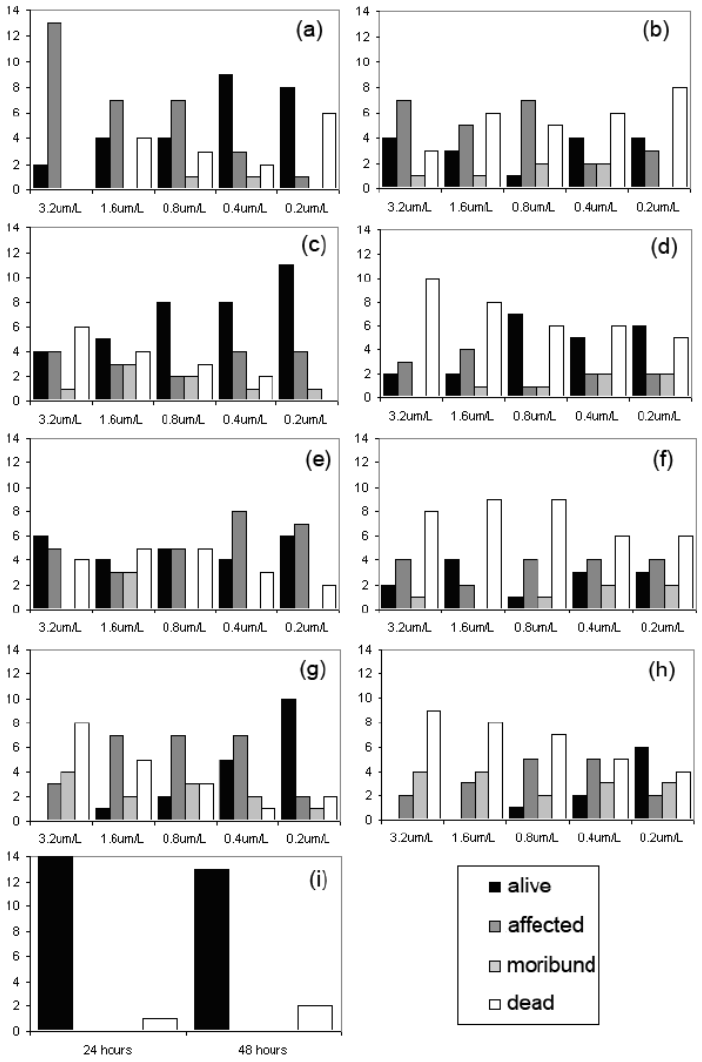

Figure 6 Effect on *Gyrodactylus* of (a) chlordimeform after 24 h, (b) chlordimeform after 48 h, (c) octopamine after 24 h, (d) octopamine after 48 h, (e) clonidine after 24 h, (f) clonidine after 48 h, (g) amitraz after 24 h, (h) amitraz after 48 h and (i) control. (y axes = number of individual gyrodactylids, n = 15 for each compound tested).

individual *Gyrodactylus* it is possible to quantify their movements. While most of the measured movement parameters (velocity, distance travelled, turn rate) were higher for those gyrodactylids in red light than those in white light, none of the differences were significant. This is an indication of the wide variation in behaviours, resulting in large deviations from the mean. Conversely, meander and fractal dimensions were lower for gyrodactylids in red light than those in white light, indicating less complex tracks than those in white light. By using the

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movement parameters showing the greatest differences between white and red light it was possible to discriminate between the two lighting conditions using PCA. This suggests that the different conditions do result in different behaviours, although more replicates would be required to state categorically whether there are significant differences in their movements.

Observations of the tracks showed that gyrodactylids in white light often had unidirectional tracks, whereas those in red light were generally more sinuous. However, in several individuals the converse was true. Therefore, it appears that exposure to a specific cue (*e.g.* red or white light) does not always elicit a behavioural response typical of the majority of individuals exposed to the cue.

The difference in behaviour in red and white light may relate to their natural behaviour *in situ*. The long sinuous tracks of the gyrodactylids in red light, which had lower complexity and meander than those in white light, may indicate a host-seeking behaviour. Covering a large surface area as quickly as possible may allow them to identify chemical or physical cues used in host location. For example, ciliary structures likely to be photoreceptors found in *Gyrodactylus* sp. [21] may be involved in a shadow response [24], allowing gyrodactylids to detect a potential host moving overhead whilst attached to the substrate. In comparison, the behaviour exhibited by the gyrodactylids in white light (uni-directional tracks or limited movements) may indicate a response to either seek shade or conserve energy in anticipation of darkness. This implies that host-seeking behaviour is more likely to occur in dull or dark conditions. Host transmission may be more favourable at night depending on host behaviour *e.g.* if hosts are less active at night and aggregate with other hosts. Transmission during darkness may also minimise the chances of being eaten by hosts that forage during the day.

Orientation with respect to directional light requires photoreceptors with pigment shields. Although structures assumed to be light receptors have been found in gyrodactylids [21] they have no associated pigment shields. Therefore, it is likely that directional choices made by individual gyrodactylids are random and not related to directional light cues.

Host transmission may be associated with particular maturity stages of individual gyrodactylids, *e.g.* when newborn or after giving birth. However, no correlation was found between the behaviour patterns of individual *Gyrodactylus* and their maturity status. Although this does not necessarily indicate that maturity status is not linked to tranmission, (as the gyrodactylids had already been physically removed from their hosts) it does suggest that light may provide a stronger behavioural cue than maturity status, once they are detached.

The distances travelled by gyrodactylids in this study give an indication of the transmission potential via the substrate. In the tracking experiment, gyrodactylids in red light travelled a mean distance of 6.32 cm, which equates to 3.03 m over a 24 h period and in white light travelled a mean distance of 4.04 cm, equating to 1.94 m over 24 h. Transmission rates are temperature dependent and activity may increase at higher temperatures [25], indicating the dispersal and transmission potential via the substrate for detached gyrodactylids. Comparing the distances travelled in the tracking experiment with those in the experiment investigating lighting conditions, gyrodactylids travelled significantly further in the dark than in white light, suggesting that distances travelled by gyrodactylids in the dark may be even greater.

Of the four octopaminergic compounds tested, all had an effect on gyrodactylids. The initial effect was to induce muscular spasms as the parasites attempted to extend their bodies. Prolonged exposure resulted in death. It is not known if this response reflects an interaction at the peripheral or central nervous system, but does imply the presence of octopaminergic receptors. Although chlordimeform severely affected the parasites, amitraz had an even stronger effect, even at low concentrations down to 0.2 μM. Only chlordimeform at higher concentrations and amitraz significantly affected the parasites after 24 h. With octopamine and clonidine the full effect was not seen until after 48 h. This has implications for use of this type of treatment in the field, as prolonged exposure (24+ h) may be required to have any significant effect on gyrodactylids, although this delay between application and effect may be shortened with another compound due to pharmacokinetic considerations. As octopamine is a natural biogenic amine, it will be subject to metabolism and uptake by the gyrodactylids so its effect will be affected by physiological processes. This may also be the case for clonidine. As chlordimeform and amitraz are synthetic compounds, they are less likely to be affected by uptake and metabolism. In addition, it should be noted that the bioassay used in this study is relatively crude. The complex behaviours of sensory host detection followed by co-ordinated tactic motor activity involve considerable complexity and it is probable that the small behavioural effects found at very low concentrations can confer considerable efficacy for control in the field.

As the survival rates of gyrodactylids off the host are 1 day at 18°C and 4 days at 3°C for *G. salaris* [4] and 2.7 days at 15°C and 4.2 days at 4°C for *G. gasterostei* [26], this type of experiment is prone to error as a result of natural mortalities. Although mortalities in the control were only 10% it is important to bear in mind the survival rates off the host when interpreting the results. To account for

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control mortality an appropriate correction factor must be used, such as Abbots or Schneider-Orelli.

Before any chemical treatment against *G. salaris* can be used for entire river habitats, the toxicity of the compound to human operators and to other flora and fauna must be established. An ideal effective treatment should affect the target organism, without having adverse effects on other aquatic life. However, as the desired mode of action of any octopaminergic treatment is to interfere with the subtle behaviour of gyrodactylids, the concentrations of compound required will be considerably lower than those required to kill the parasites. As octopamine modulates virtually all physiological processes in invertebrates, but shows very little activity in vertebrates, being homologous to noradrenaline in vertebrates [7], it is likely that it will have minimal effects on vertebrates at the concentrations required to disrupt physiological processes in invertebrates. No information is available on the toxicity of octopamine in fish, although results have shown that it is non-toxic to mammals [27]. However, it is likely that the toxicity of octopamine in other aquatic invertebrates is similar to that of gyrodactylids. Although it was not possible to calculate EC50 values for chlordimeform in this study, 73% of gyrodactylids were affected or dead after 48 h at the lowest concentration of 0.2 μM (0.04 mg/L), which is considerably lower than the 96 h LC50 for rainbow trout (*Oncorhynchus mykiss* Walbaum) at 13.2 mg/L [6]. Similarly, it was not possible to calculate EC50 values for clonidine. However, at 0.8 μM (0.21 mg/L) 93% of gyrodactylids were affected or dead after 48 h. Considering that the 96 h LC50 for clonidine in ide (*Leuciscus idus* (L.)) is 87 mg/L [28], it is likely that the EC50 in gyrodactylids is significantly lower. In addition, 80% of gyrodactylids were affected by clonidine after 48 h at 0.2 μM (0.053 mg/L), which is a concentration significantly lower than the 48 h EC50 for *Daphnia* of 182 mg/L [28]. Amitraz has a 24 h EC50 of 0.29 μM (8.5 mg/L) for gyrodactylids, which is higher than the 24 h LC50 in rainbow trout of 2.7-4.0 mg/L [29]. The 48 h EC50 for amitraz in gyrodactylids is 0.16 μM (4.6 mg/L), whereas in *Daphnia magna* Straus, 1820 it has been calculated as 3.4 mg/L [30]. Although the EC50 values for amitraz are of the same magnitude as the LC50 and EC50 values for trout and *Daphnia*, it is anticipated that the concentrations required to disrupt the host seeking and attachment behaviour of gyrodactylids will be considerably lower. However, this requires further investigation.

Products containing amitraz were banned in 2010 for pesticidal uses in agriculture due to concerns of human exposure and risks to the environment [31]. Chlordimeform is banned for use as an agricultural pesticide due to concerns that it is carcinogenic to humans and is toxic to aquatic life [32]. Despite these concerns, the compounds can be used to illustrate the presence of key octopaminergic pathways in gyrodactylids.

Conclusions

This work has made a significant step forward in the observation of gyrodactylid behaviour and is the first time that movements/activity have been studied in detail, suggesting that gyrodactylids are more active in dark than light conditions. Now that the experimental procedures have been developed to observe and record gyrodactylid movements, this system can be used for a wide variety of gyrodactylid behaviour experiments. Further work is required to confirm that gyrodactylid behaviour is affected by light conditions, specifically their behaviours in white light, red light, infrared light and dark conditions. The efficacy experiments have shown that octopaminergic receptors exist in gyrodactylids, as the octopaminergic compounds tested have an effect on gyrodactylids resulting in neuromuscular disturbance and eventually death. The next logical step is to investigate the ability of affected gyrodactylids to reattach to a fish host once they have been exposed to low doses of octopaminergic compounds and whether the effect is permanent or temporary, once they have been removed from the compounds.

These initial results observing gyrodactylid behaviour and the effect of octopaminergic compounds are promising and indicate that there might be potential use of compounds affecting octopamine receptors to control gyrodactylid infections. With the constant threat of *G. salaris* entering UK waterways and the lack of any effective treatment, other than the total eradication of all river fauna using rotenone, it is important that investment is made now to develop new chemical treatments that will specifically target *Gyrodactylus* infections.

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Authors' contributions
AJB made significant contributions to the conception and design of the study, constructed experimental equipment, carried out data acquisition, data analysis and interpretation, and drafted the manuscript. MIGM contributed to the design of the study and data acquisition. JEB, SI and ML contributed to the study concept and were involved in critically revising the manuscript. APS supervised the study, contributed to the conception and design of the study, interpretation of results and critically revising the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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