Aspects of systematics and host specificity for *Gyrodactylus* species in aquaculture

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"*There are only two ways to live your life.*
One is as though nothing is a miracle.
The other is as though <u>everything</u> is a miracle."
(Albert Einstein, German physicist, 1879-1955)

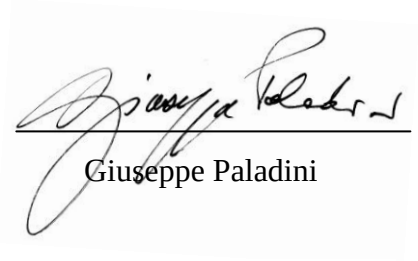
Alla mia famiglia,
presente, passata e futura.

Declaration

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree. All images presented in this thesis are original, unless otherwise stated.

All experimental procedures and husbandry practices involving animals were conducted in compliance with the Animals Scientific Procedures Act 1986 (Home Office Code of Practice. HMSO: London, January 1997), in accordance with EU regulation (EC Directive 86/609/EEC), and approved by the Animal Ethics and Welfare Committee of the University of Stirling.

Signature of candidate:



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Abstract

Of the 430+ extant species of *Gyrodactylus*, ectoparasitic monogenetic flukes of aquatic vertebrates, *Gyrodactylus salaris* Malmberg, 1957 is arguably the most well-known. Following the introduction of this species into Norway in the 1970s with consignments of infected Atlantic salmon smolts, *Salmo salar* L., this species has had a devastating impact on the Norwegian Atlantic salmon population, decimating wild stocks in over 40 rivers. *Gyrodactylus salaris* is the only OIE (Office International des Epizooties) listed parasitic pathogen of fish and has been reported from 19 countries across Europe, though many of these records require confirmation. The UK, Ireland and some selected watersheds in Finland are currently recognised as *G. salaris*-free states; however, the threat that this notifiable parasite poses to the salmon industry in the UK and Ireland is of national concern. Current British contingency plans are based on the assumption that if *G. salaris* were to be introduced, the parasite would follow similar dynamics to those on salmonid stocks from across Scandinavia, *i.e.* that Atlantic strains of Atlantic salmon would be highly susceptible to infection, with mortalities resulting; that brown trout, *Salmo trutta fario* L., would be resistant and would lose their infection in a relatively short period of time; and that grayling, *Thymallus thymallus* (L.), would also be resistant to infection, but would carry parasites, at a low level, for up to 143 days.

Two of the objectives of this study were to confirm the current distribution of *G. salaris* across Europe, and then, to investigate the relative susceptibility of British salmonids to *G. salaris*, to determine whether they would follow a similar pattern of infection to their Scandinavian counterparts or whether, given their isolation since the last glaciation and potential genetic differences, they would exhibit different responses.

It has been almost six years since the distribution of *G. salaris* across Europe was last evaluated. Some of the European states identified as being *G. salaris*-positive, however, are ascribed this status based on misidentifications, on partial data resulting from either morphological or molecular tests, or according to records that have not been revisited. Additional *Gyrodactylus* material from selected salmonids was obtained from several countries to contribute to current understanding regarding the distribution of *G. salaris* across Europe. From the work conducted in the study, *G. salaris* is reported from Italy for the first time, alongside three other species, and appears to occur extensively throughout the central region without causing significant mortalities to their rainbow trout, *Oncorhynchus mykiss* (Walbaum), hosts. The analysis of archive material from *G. salaris*-positive farms would suggest that *G. salaris* has been in the country for at least 12 years. Material obtained from rainbow trout from Finland and Germany was confirmed as *G. salaris* supporting existing data for these countries. No specimens of *G. salaris*, however, were found in the additional *Gyrodactylus* material obtained from Portuguese and Spanish rainbow trout, only *Gyrodactylus teuchis* Lautraite, Blanc, Thiery, Daniel *et* Vigneulle, 1999, a morphologically similar species was found. *Gyrodactylus salaris* is now reported from 23 out of ~50 recognised states throughout Europe, only 17 of these however, have been confirmed by either morphology or by an appropriate molecular test, and only ten of these records have been confirmed by a combination of both methods.

To assess the susceptibility of English and Welsh salmonids to *G. salaris*, a number of salmonid stocks of wild origin, were flown to the Norwegian Veterinary Institute (NVI) in Oslo, where they were experimentally challenged with *G. salaris*. Atlantic salmon from the Welsh River Dee, *S. trutta fario* from the English River Tyne and *T. thymallus* from the English River Nidd, raised from wild stock in government hatcheries, were flown out and subsequently challenged with *G. salaris* haplotype A. After acclimation, each fish was infected with ~50–70 *G. salaris* and marked, so that parasite numbers on individual fish

could be followed. The dynamics on individual fish were followed against a control (Lierelva Atlantic salmon). The experiment found that the number of *G. salaris* on *S. salar* from the River Dee continued to rise exponentially to a mean intensity (m.i.) of ~3851 *G. salaris* fish^{-1} (day 40 post-infection). These salmon were highly susceptible, more so than the Norwegian salmon control (m.i. ~1989 *G. salaris* fish^{-1} d40 post-infection) and were unable to regulate parasite numbers. The *S. trutta fario* and *T. thymallus* populations, although initially susceptible, were able to control and reduce parasite burdens after 12 (m.i. ~146 *G. salaris* fish^{-1}) and 19 (m.i. ~253 *G. salaris* fish^{-1}) days, respectively when peak infections were seen. Although the latter two hosts were able to limit their *G. salaris* numbers, both hosts carried infections for up to 110 days (*i.e.* when the experiment was terminated). The ability of *S. trutta fario* and *T. thymallus* to carry an infection for long periods increases the window of exposure and the potential transfer of *G. salaris* to other susceptible hosts. The potential role that brown trout may play in the transmission and spread of *G. salaris* in the event of an outbreak, needs to be considered carefully, as well as the interpretation of the term "resistant" which is commonly used when referring to brown trout's susceptibility to *G. salaris*. The current British surveillance programmes for *G. salaris* are focused on the screening of Atlantic salmon and on the monitoring of the rainbow trout movements. The findings from this study demonstrate that *G. salaris* can persist on brown trout for long periods, and suggest that brown trout sites which overlap with Atlantic salmon or rainbow trout sites are also included within surveillance programmes and that the role that brown trout could play in disseminating infections needs to be factored into contingency/management plans.

Throughout the course of the study, a number of parasite samples were sent to the Aquatic Parasitology Laboratory at Stirling for evaluation. Some of these samples represented *Gyrodactylus* material that were associated with fish mortalities, but the species of *Gyrodactylus* responsible appeared to be new to science. A further aspect of this

study was, therefore, to investigate these *Gyrodactylus* related mortalities in aquaculture stock and to describe the species found in each case, which may represent emerging pathogens. The two new species, *Gyrodactylus orecchiae* Paladini, Cable, Fioravanti, Faria, Di Cave *et* Shinn, 2009 and *Gyrodactylus longipes* Paladini, Hansen, Fioravanti *et* Shinn, 2011 on farmed gilthead seabream, *Sparus aurata* L., were collected from several Mediterranean farms. The finding of *G. orecchiae* in Albania and Croatia was associated with 2–10% mortality of juvenile stock and represents the first species of *Gyrodactylus* to be formally described from *S. aurata*. Subsequently, *G. longipes* was found in Bosnia-Herzegovina and Italy, and at the Italian farm site, it occurred as a mixed infection with *G. orecchiae*, but these infections did not appear to result in any loss of stock. Unconfirmed farm reports from this latter site, however, suggest that a 5–10% mortality of juvenile *S. aurata* was also caused by an infection of *Gyrodactylus*, which is suspected to be *G. longipes*. Additional samples of *Gyrodactylus* from a gilthead seabream farm located in the north of France have been morphologically identified as *G. longipes*, extending the geographical distribution of this potentially pathogenic species to three countries and three different coasts.

In addition to these samples, some specimens of *Gyrodactylus* from a Mexican population of rainbow trout were sent for evaluation. These latter specimens were later determined to be a new morphological isolate/strain of *Gyrodactylus salmonis* (Yin *et* Sproston, 1948), a notable pathogen of salmonids throughout North America. The current material was of particular interest as it extends the current geographic range of this parasite from Canada and the USA to the south-eastern region of Mexico. This new Mexican isolate was genetically identical with *G. salmonis* from Canada and USA, although small morphological differences were evident in the marginal hook sickle shape, which allows to discriminate between the two strains. The results from this study are important as they reflect a similar situation in Europe with *G. salaris* and *Gyrodactylus thymalli* Žitňan,

1960, two morphological different but genetically similar species. Discriminating *G. salaris* from other species of *Gyrodactylus* infecting salmonids is difficult and, according to OIE, the identification should be based on a combination of data resulting from morphological and molecular approaches.

The impact of *Gyrodactylus salaris* in Norway currently costs £38 million p.a., including loss of revenue from tourism and angling restrictions, and also the cost of on-going surveillance programmes and river treatments. The infection in certain rivers is removed through the addition of either 100 ppb biocide rotenone, which kills all the fish that are host to the parasite, or by a 10–14 day-treatment with 100 µg L^{-1} aluminium sulfate, which removes the parasite but does not kill its salmon host. If *G. salaris* were to enter the UK, it is unlikely that either of these compounds would be used because of the human health concerns (*i.e.* potential links to Parkinson's and Alzheimer's diseases) linked to their use. There are, however, very few compounds that could be used as alternatives for the control of wild infections, and there is little research investigating possible replacements. To begin exploring alternatives, a minor component of the study was to explore the effectiveness of two compounds: bronopol (2-bromo-2-nitropropane-1,3-diol) - a broad spectrum disinfectant - and tannic acid - a natural polyphenol that is released from the breakdown of plant material. The evaluation of bronopol was conducted against two strains of *G. salaris* from Atlantic salmon and on a single population of *Gyrodactylus arcuatus* Bychowsky, 1933 from three-spined sticklebacks, *Gasterosteus aculeatus aculeatus* L., as a continuous exposure and for 1 hour only. The results showed that there was a significant increase in the mortality rate of *G. salaris* as the dose of bronopol increased, but as time progressed, the influence of dose on mortality decreased. Bronopol had a statistically significant ($p<0.001$) greater effect on *G. salaris* than it did on *G. arcuatus*. The analysis suggested that the 1 hour-LC50 for *G. salaris* was ~384 ppm bronopol, while that needed to kill 50% of *G. arcuatus* within a 1 hour window of

exposure was ~810 ppm bronopol. The trial with tannic acid represented a preliminary assessment and its effects as a continuous exposure and as a 10 minute treatment on *G. salaris* only were determined. The effect of tannic acid caused the tegument of *G. salaris* to lift away and the 1 hour-LC50 for tannic acid was <100 ppm although lower doses administered over long periods of time (*i.e.* 10–14 days as is currently used for aluminium sulfate) may have greater impacts on the survival of the parasite population. While these results demonstrate that bronopol could be used to control infections of *G. salaris* in confined aquaria, this does not mean that this advocates its use in river systems, as there are a plethora of logistic, economic and environmental considerations to take into account. The study does, however, take important steps towards investigating alternative control agents for use in the event of an outbreak, and both these products are worthy of further evaluation.

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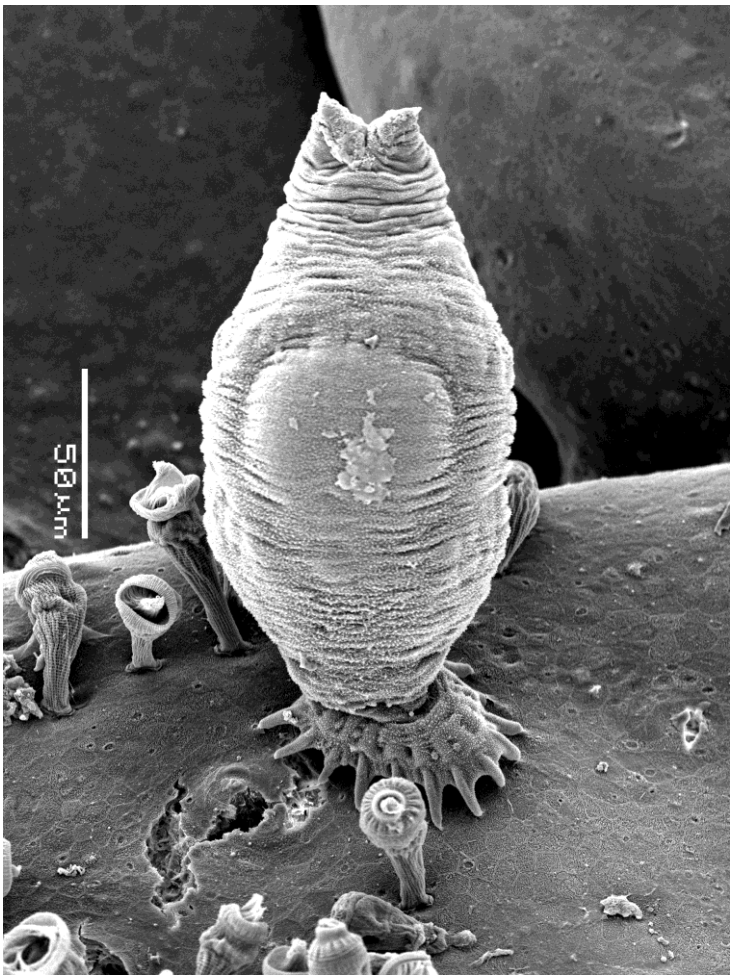
Chapter 1

General introduction



Gyrodactylus arcuatus Bychowsky, 1933 and *Apiosoma* sp. on the skin of a three-spined stickleback, *Gasterosteus aculeatus aculeatus* L. [original image].

1.1. Genus *Gyrodactylus* von Nordmann, 1832

1.1.1. Biology and reproduction

Monogeneans of the genus *Gyrodactylus* von Nordmann, 1832 are small (<1mm), viviparous, polyembryonic ectoparasites. The latter feature has earned them the colloquial label "Matryoshka dolls" or "Russian dolls" (dolls of decreasing sizes placed one inside the other) for their singular method of reproduction (Bakke *et al*., 2007). The first embryo contains in its uterus another embryo, which includes a third embryo, sometimes reaching up to 4 generations, one inside the other (see Fig. 1.1). This rare hyperviviparity and the ability to alternate between sexual and asexual modes of reproduction, allow for exponential increases in the size of the parasite population in relatively short periods of time (Cable & Harris, 2002).

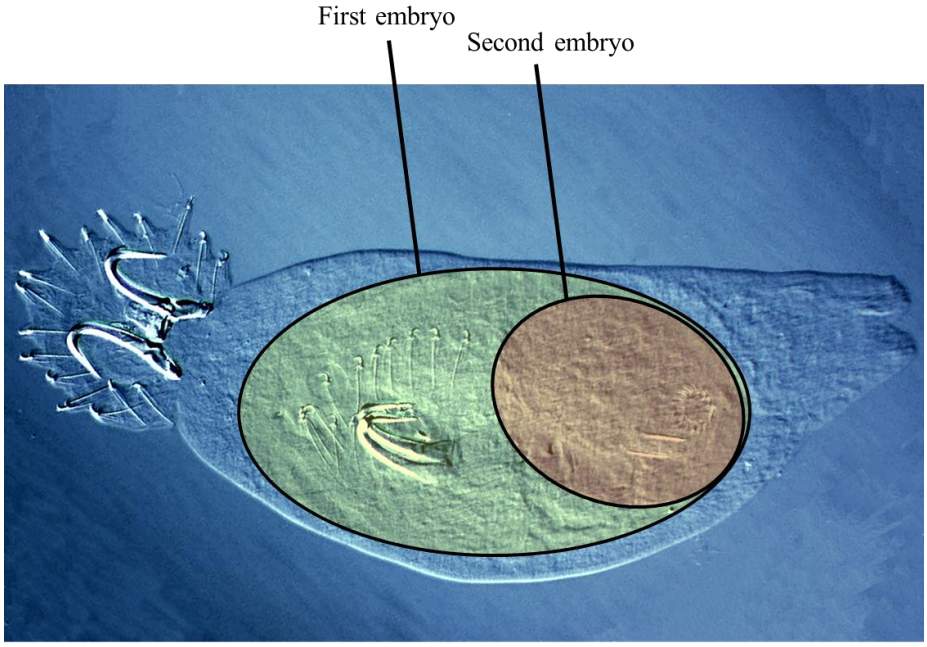


Figure 1.1. Light micrograph of *Gyrodactylus salaris* Malmberg, 1957 collected from a rainbow trout *Oncorhynchus mykiss* (Walbaum) reared in Italy, showing the hooks of the first and second embryos, one inside the other [original image].

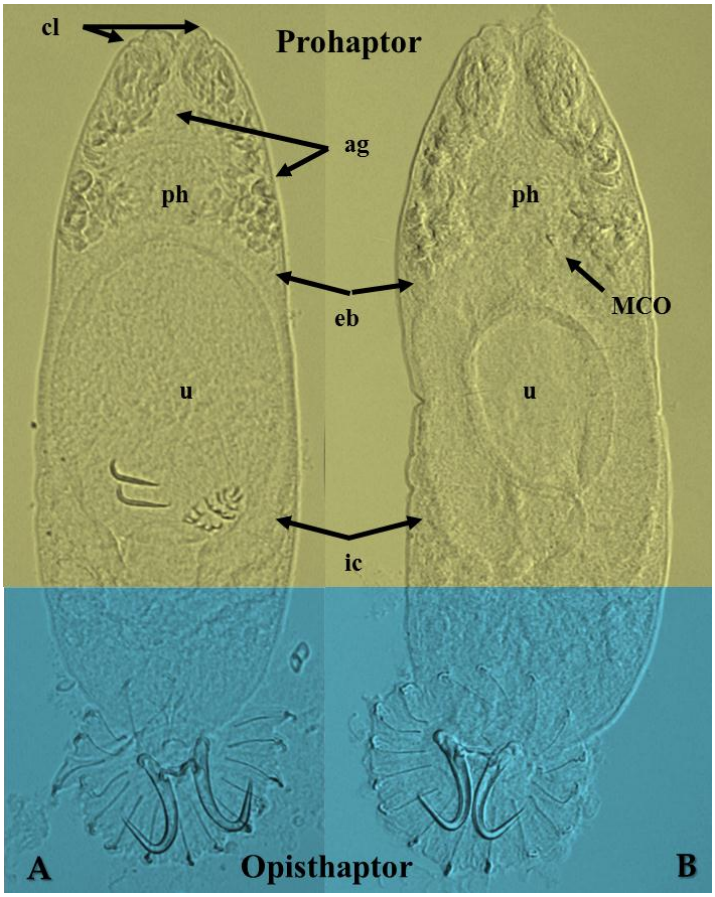


Figure 1.2. Light micrographs of *Gyrodactylus longipes* Paladini, Hansen, Fioravanti *et* Shinn, 2011, illustrating the division between the anterior part of the body (light yellow), which includes the prohaptor, and the posterior part of the body (light blue), which includes the opisthaptor. **A**: Pregnant *G. longipes* with daughter's hooks visible in the uterus and no MCO; **B**: A specimen of *G. longipes* having recently given birth with an empty uterus and an MCO, which appears after the first birth, visible. Abbreviations: *ag*: anterior glands; *cl*: cephalic lobes; *eb*: excretory bladders; *ic*: intestinal crura; *MCO*: male copulatory organ; *ph*: pharynx; *u*: uterus [original images].

The first-born offspring develops at the centre of an immature embryo cluster in the parent's uterus, which suggests that the first born daughter arises asexually (Cable & Harris, 2002). The second-born daughter develops from oocytes by parthenogenesis, whilst subsequent daughters develop either sexually or parthenogenetically (Harris, 1993), all of which are morphologically indistinguishable from their parent, both in size and in shape (Cable & Harris, 2002). Gyrodactylids are considered protogynous hermaphrodites, *i.e.* they are born "female" and following the first birth event develop visible external features

associated with the male reproductive system. The female reproductive system is a relatively simple one, in which gyrodactylids do not possess vaginae but in which cross-fertilisation occurs through tegumental impregnation of sperm (Cable & Harris, 2002).

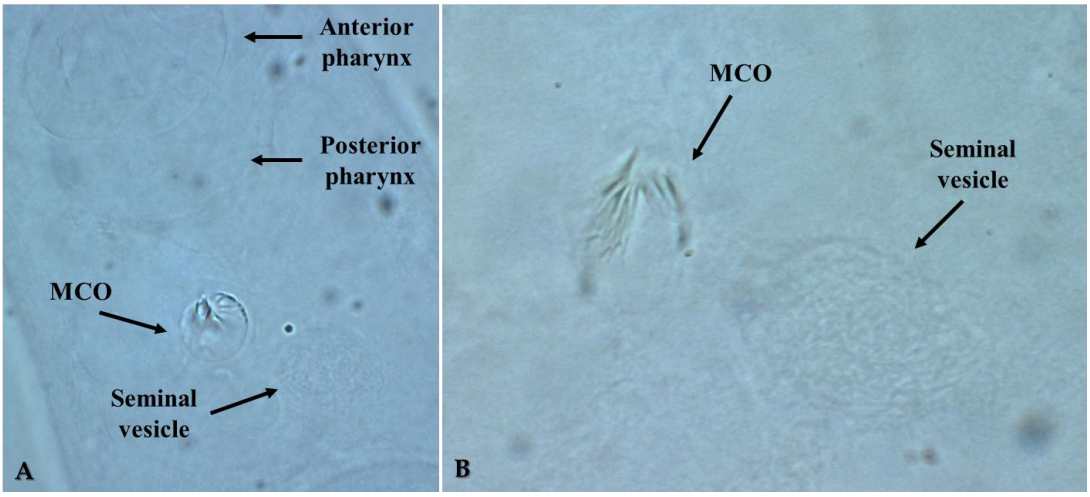


Figure 1.3. Light micrographs of *Ieredactylus rivuli* Schelkle, Paladini, Shinn, King, Johnson, van Oosterhout, Mohammed *et* Cable, 2011 (Monogenea, Gyrodactylidae), a genus of viviparous flukes closely related to *Gyrodactylus*, showing (**A**) the anterior and posterior bulbs of the pharynx, and the male copulatory organ (MCO) connected to the seminal vesicle. Image (**B**) shows the MCO and associated seminal vesicle at higher magnification [original images].

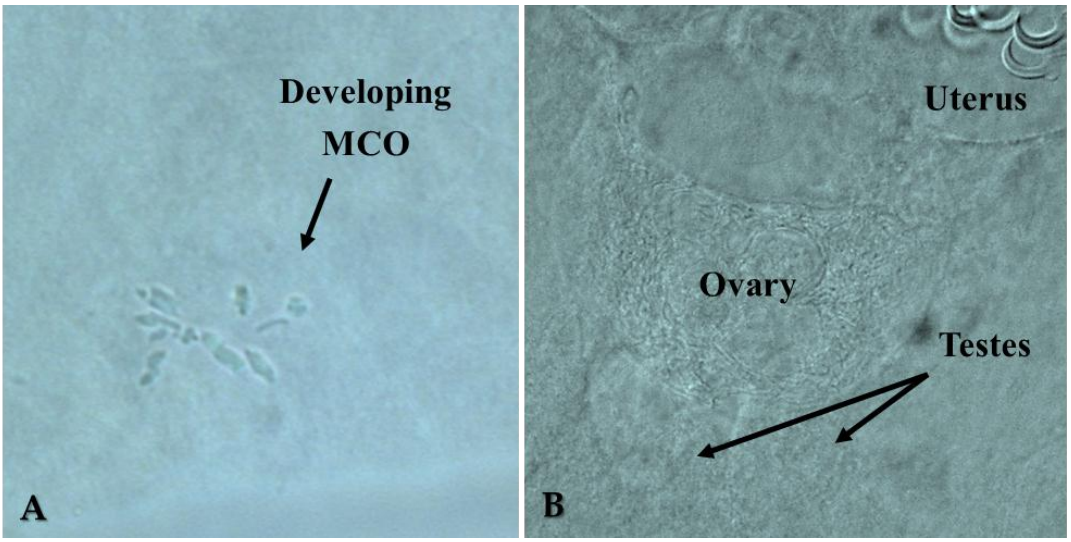


Figure 1.4. Light micrographs of (**A**) *Ieredactylus rivuli* Schelkle, Paladini, Shinn, King, Johnson, van Oosterhout, Mohammed *et* Cable, 2011 (Monogenea, Gyrodactylidae) showing the male copulatory organ (MCO) in formation, and (**B**) *Gyrodactylus salaris* Malmberg, 1957 illustrating the presence of more than one testis [original images].

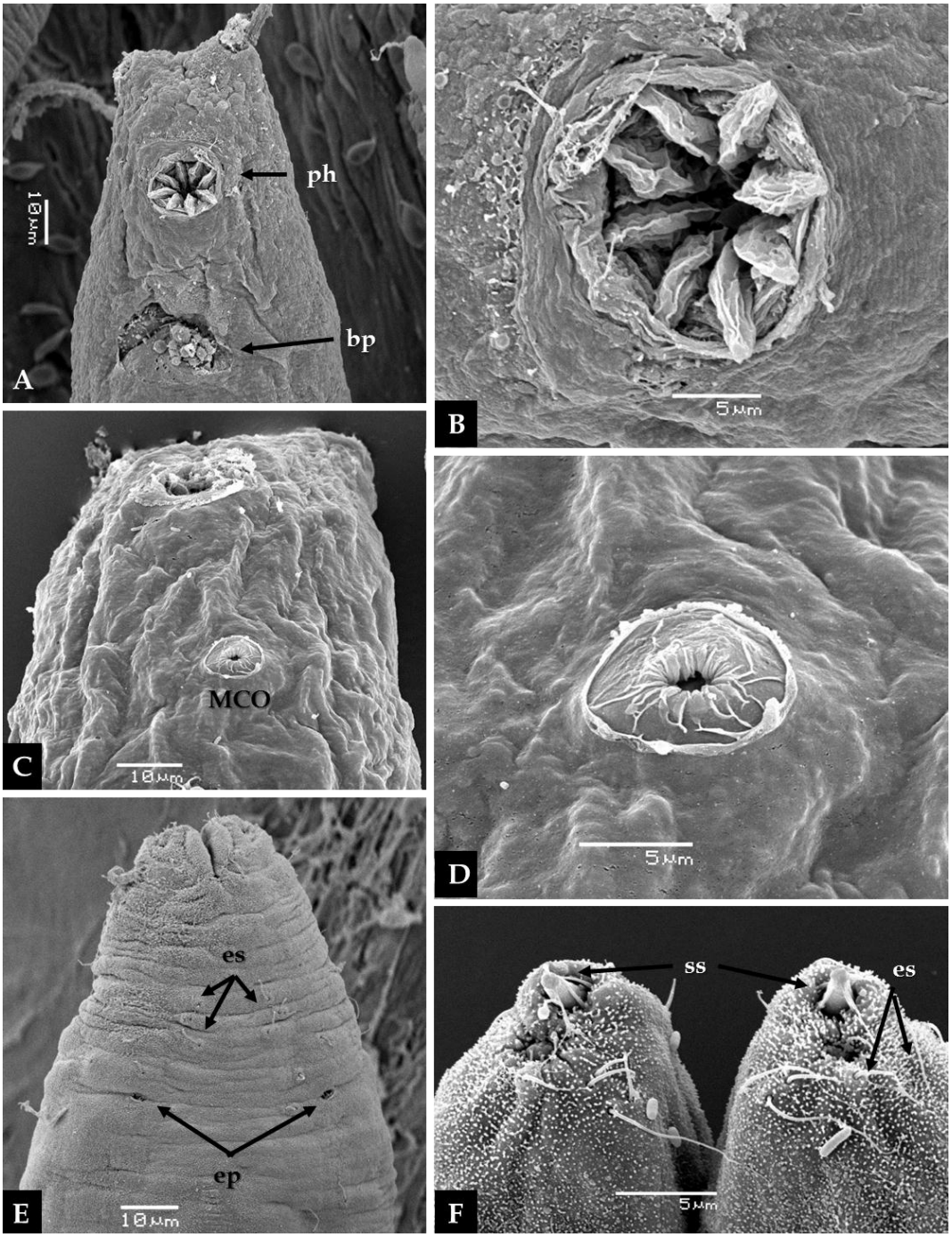


Figure 1.5. Scanning electron micrographs (SEM) of the anterior region of *Gyrodactylus salaris* Malmberg, 1957. **A**-**B**: details of the protruded pharynx (*ph*), possessing 8 processes, and the birth pore (*bp*); **C**-**D**: the male copulatory organ (*MCO*) bulb showing its aperture and a raised rim marking its periphery; this is the first time that SEMs of the MCO have been presented in the scientific literature; **E:** *Gyrodactylus* possesses a branched excretory system of ducts and flame cells that, in some species, terminate in bladders, but in all species empty onto the dorsal body surface *via* two excretory pores (*ep*); **F**: the prohaptor consists of two cephalic lobes, each equipped with a spike sensillum (*ss*) and a large number of elongated sensilla (*es*) and sensory pits constituting the sensory apparatus [original images].

The male reproductive system is located in the anterior portion of the body (Fig. 1.2) and consists of an anterior seminal vesicle connected to the male copulatory organ (MCO) (Fig. 1.3), and of one or more posteriorly positioned testes (Fig. 1.4; Malmberg, 1957; Kritsky, 1971). The MCO is not present on new-born parasites, but is evident after the first birth event and becomes fully functional when the second embryo begins to develop (Harris, 1985). The MCO is a spherical muscular bulb armed with one principal hook used to penetrate the tegument of the partner, and several smaller surrounding spines, which serve to hold the MCO bulb in position during the process of fertilisation (Harris, 1993). A rim marking the external periphery of the MCO (Fig. 1.5C, D) may ensure a tight seal between mating partners and promote the efficient transfer of sperm.

1.1.2. Life-cycle and transmission

Species of *Gyrodactylus* are known to colonise a vast array of marine, brackish and freshwater hosts, making this genus one of the most commonly encountered groups of parasites (Williams & Jones, 1994).

The life-cycle of *Gyrodactylus* is simple and direct (see Fig. 1.6): there is no specific transmission stage and the infection of new hosts occurs through a variety of different mechanisms. Once the parasite gives birth, the new-born attaches directly to the same host. After a period of feeding and/or subsequent birth events, the parasite may then transfer, by direct skin-to-skin (or fin) contact, to a new host. According to Bakke *et al*. (2002), transmission does not only occur between living hosts, but also by transfer from dead hosts and of those parasites attached to inorganic substrates. The transmission of *Gyrodactylus salaris* Malmberg, 1957 to Atlantic salmon, *Salmo salar* L., by detached parasites drifting in the water column has been demonstrated by Soleng *et al*. (1999), while the transfer between hosts by cannibalism and predation has been also suggested (Malmberg, 1973; Harris & Tinsley, 1987; El-Naggar *et al*., 2006). The re-attachment of detached parasites

from the substrate by water currents generated by the movement of the fish's fin has also been demonstrated (Grano-Maldonado, 2012).

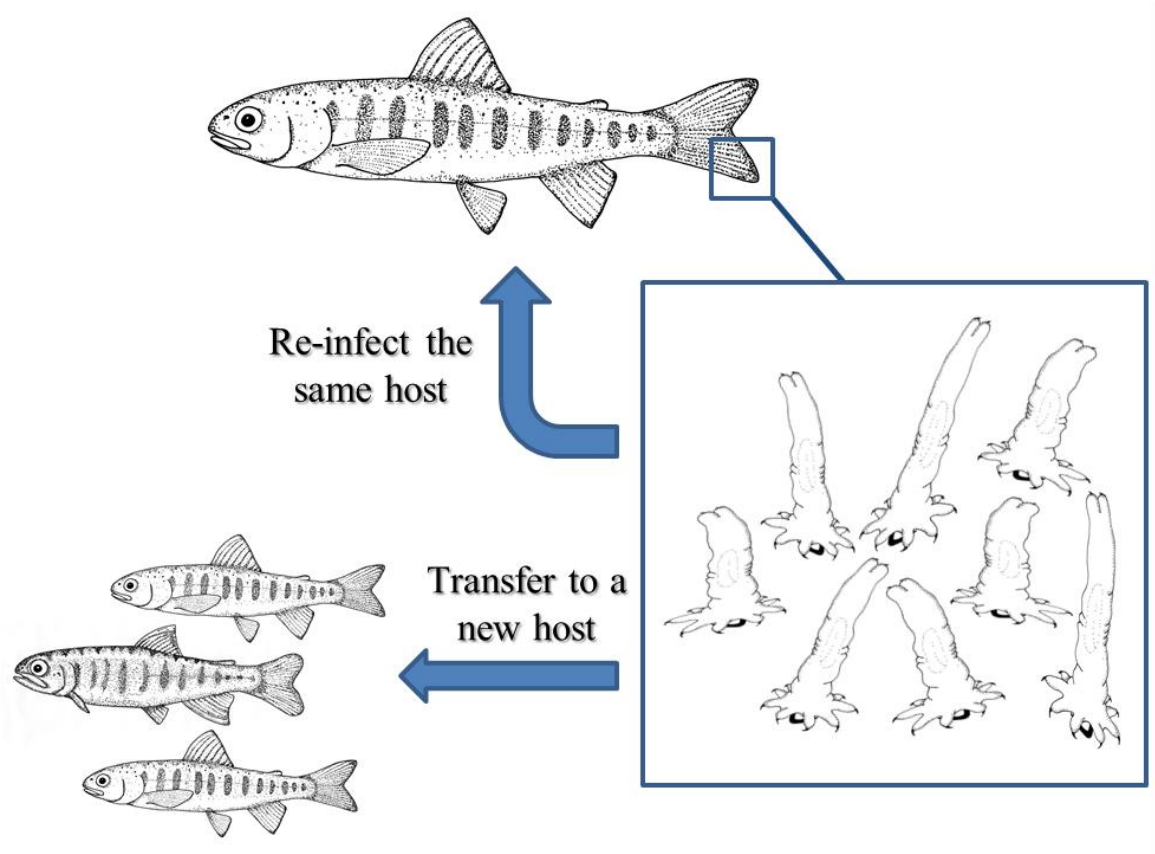


Figure 1.6. Schematic diagram of the life-cycle of *Gyrodactylus* von Nordmann, 1832 [original drawings].

1.1.3. Host specificity

The term "host specificity" in parasitology should be considered with some caution and perhaps replaced with a more suitable term, such as "host preference" or "host range", given that not all fish species have been tested against each and every parasite species. A parasite record typically indicates the preference for a host within a community. The current usage of the term "host specificity" does not, unfortunately, consider the infection potential of a parasite under all environmental conditions that it can be found and the hosts it could potentially encounter. The term "host specificity", however, will be used in the

current thesis until a time when further studies are carried out to support the proposed change in terminology.

Since the description of the first species (*i.e. Gyrodactylus elegans* von Nordmann, 1832) and the erection of the genus, over 400 species have been subsequently described. Although the last review of species by Harris *et al*. (2004) listed 409 species, more than 50 new species have since been discovered, making *Gyrodactylus* one of the largest genera within the class Monogenea Carus, 1863. The description of new species has been facilitated by a number of new methodologies; notably advancements in molecular technologies have helped in the characterisation of new strains (*e.g.* the haplotypes of *G. salaris* detailed by Ziętara & Lumme, 2002 and Hansen *et al*., 2003), but also in the discovery of cryptic species (*i.e.* distinct species but morphologically almost identical) such as *Gyrodactylus ulinganisus* García-Vásquez, Hansen, Christison, Bron *et* Shinn, 2011 (see García-Vásquez *et al*., 2011).

The genus *Gyrodactylus* is largely host specific, with the hosts listed in Harris *et al.* (2004) infecting ~200 teleosts (Bakke *et al*., 2002) and a small number of amphibian hosts, *i.e. Gyrodactylus ambystomae* Mizelle, Kritsky *et* McDougal, 1969 from *Ambystoma macrodactylum* Baird (see Mizelle *et al*., 1969); *Gyrodactylus arcuatus* Bychowsky, 1933 from *Hyla arborea* L. (see Volgar-Pastukhova, 1959; Vojtkova, 1989), which probably represents an accidental host transfer (Prudhoe & Bray, 1982); *Gyrodactylus aurorae* Mizelle, Kritsky *et* McDougal, 1969 from *Rana aurora aurora* Baird *et* Girard (see Mizelle *et al*., 1969); *Gyrodactylus catesbeianae* Wootton, Ryan, Demaree *et* Critchfield, 1993 and *Gyrodactylus jennyae* Paetow, Cone, Huyse, McLaughlin *et* Marcogliese, 2009, both from *Rana catesbeiana* Shaw (see Wootton *et al*., 1993; Paetow *et al*., 2009); and *Gyrodactylus ensatus* Mizelle, Kritsky *et* Bury, 1968 from *Dicamptodon ensatus* Eschscholtz (see Mizelle *et al*., 1968); plus a number of unidentified species (Paetow *et al*., 2009).

Given that there are an estimated ~24,000 teleost species, it has been suggested by Bakke *et al*. (2007) that there could be as many as ~20,000 species of *Gyrodactylus* in the world. The mechanisms underlying host specificity, and therefore their ability to exploit different host species, are not completely clear, and could include several processes, *e.g.* survival instinct (when the parasite is "forced" to infect the first available host in order to survive when detached and subsequently adapting to this "new" host), parasite behavioural (host preference), host behavioural (parasites of demersal fish are more likely to transfer to other benthic hosts rather than pelagic fish), physiological (*e.g. G. salaris* experimentally infecting European eel, *Anguilla anguilla* (L.), fails to feed and reproduce, maybe due to non-specific mechanisms, *e.g.* thickness of mucus layer, or toxic components on eel skin), immunological (the host responds in some way), phylogenetic (closeness to other hosts), geographical (parasites exposed to different communities can transfer to new hosts from one area to another), and/or ecological (host-parasite interaction) (Harris, 1980; Madhavi & Anderson, 1985; Jansen *et al*., 1991; Bakke *et al*., 1992a; Poulin *et al*., 2011). For *Gyrodactylus* host specificity is very variable, with some species apparently showing strict host specificity and known to infect only a single host (*e.g. Gyrodactylus margaritae* Putz *et* Hoffman, 1963; *Gyrodactylus imperialis* Mizelle *et* Kritsky, 1967; *Gyrodactylus neretum* Paladini, Cable, Fioravanti, Faria *et* Shinn, 2010), whilst other species appear to be cosmopolitan (*e.g. Gyrodactylus alviga* Dmitrieva *et* Gerasev, 2000, recorded from at least 15 hosts; *Gyrodactylus arcuatus*, recorded from at least 12 hosts; *G. salaris* documented to be able to reproduce on at least eight salmonid hosts) (see Bychowsky, 1933; Putz & Hoffman, 1963; Mizelle & Kritsky, 1967; Dmitrieva & Gerasev, 2000; Harris *et al*., 2004; Paladini *et al*., 2010a). Bychowsky (1957) considered *Gyrodactylus* the least-specific genus within the class Monogenea. Despite numerous species descriptions of *Gyrodactylus* having been associated with a single host, giving the impression that *Gyrodactylus* is narrowly host specific (Malmberg, 1970), this could represent a sampling

artefact and Bakke *et al*. (1992a) suggested that gyrodactylids are less host specific than generally believed.

1.1.4. Diagnosis of Gyrodactylus *species: taxonomic tools*

The identification and discrimination of *Gyrodactylus* species have, until recently, been based only on the morphological comparison of the opisthaptoral hard parts using light microscopy (*e.g.* Mizelle & Kritsky, 1967; Malmberg, 1970; Ogawa & Egusa, 1978; Ergens, 1980; Mo & Lile, 1998; Nack *et al*., 2005). With the advent of new techniques, such as the employment of molecular tools used in conjunction with morphological methods, the standards of the gyrodactylid species descriptions have improved considerably (*e.g.* see Christison *et al*., 2005; Le Blanc *et al*., 2006; Přikrylová *et al*., 2012). The morphological studies of monogeneans supplemented by relatively new techniques, such as proteolytic digestion methods in order to release the hooks from the surrounding tissue (Harris & Cable, 2000; Paladini *et al*., 2009a), have led to a better examination of the opisthaptoral hard parts, supported also by phase-contrast microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and in some cases also by confocal laser scanning microscopy (CLSM) (see *e.g.* Shinn *et al*., 1993, 2003; Huyse *et al*., 2004; Galli *et al*., 2007; King *et al*., 2009; Paladini *et al*., 2011a, b; Ziętara *et al*., 2012; García-Vásquez *et al*., 2012).

Morphological studies are made principally on the opisthaptoral hard parts, which consist of a pair of centrally positioned anchors or hamuli, single ventral and dorsal bars, and 16 marginal hooks distributed round the periphery of the posterior haptor or "opisthaptor", which is the principal attachment organ (Fig. 1.7; Shinn *et al*., 2004). The shape and configuration of the MCO spines (Figs. 1.4 and 1.8) are also used, but to a lesser degree, as these are present only in parasites having given birth at least once and, given their small size, are not always evident (Paladini *et al*., 2010a).

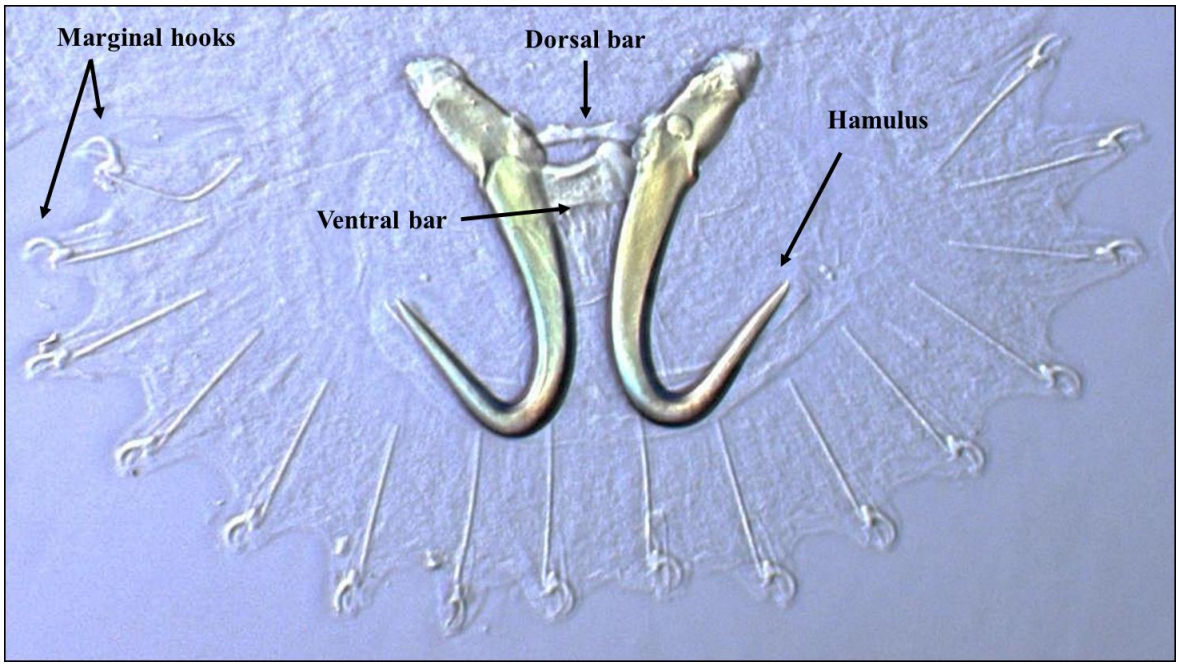


Figure 1.7. Light micrograph of *Gyrodactylus salaris* Malmberg, 1957 collected from farmed Italian rainbow trout, *Oncorhynchus mykiss* (Walbaum), showing the attachment structures of the opisthaptor, formed by 16 peripherally distributed marginal hooks, two centrally positioned anchors or hamuli, one dorsal bar connecting the two hamuli together and one ventral bar over which the two hamuli pivot during attachment [original image].

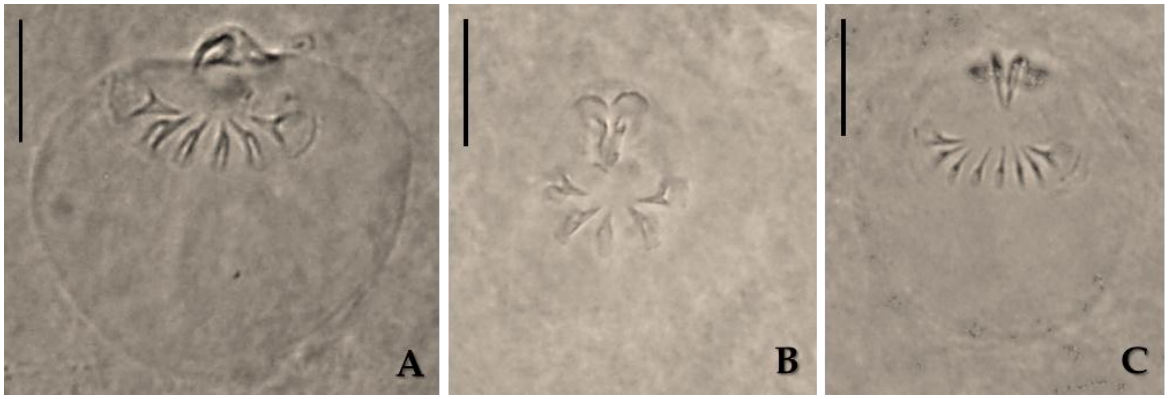


Figure 1.8. Light micrographs of the male copulatory organs (MCOs) of (**A**) *Gyrodactylus salaris* Malmberg, 1957, (**B**) *Gyrodactylus teuchis* Latraite, Blanc, Thiery, Daniel *et* Vigneulle, 1999, and (**C**) *Gyrodactylus derjavinoides* Malmberg, Collins, Cunningham *et* Jalali, 2007 collected from farmed Italian *Oncorhynchus mykiss* (Walbaum). The figure shows the different spine arrangements of the MCO of the three species, used as a supporting morphological feature in the discrimination of species. Scale bars: 10 μm [images modified from Paladini *et al*. (2009a)].

The taxonomic classification and identification of *Gyrodactylus* species is assisted by the differing morphologies of the marginal hook sickles, which are used as one of the key diagnostic criteria for discriminating species (see Fig. 1.9; Malmberg, 1970; Cunningham, 2002; Shinn *et al*., 2004; Rubio-Godoy *et al*., 2010).

For the morphological identification, Malmberg (1970) suggested a series of point-to-point morphometric measurements (18 in total: four for the hamulus, six for the ventral bar, two for the dorsal bar and six for the marginal hooks) to be taken from the opisthaptoral sclerites. These were subsequently modified by Shinn *et al*. (2004), who added 10 new descriptors and removed three from the previous set of measurements to give a total of 25 point-to-point morphometric characters (11 for the hamulus, six for the ventral bar and eight for the marginal hooks; Figs. 1.10–1.13).

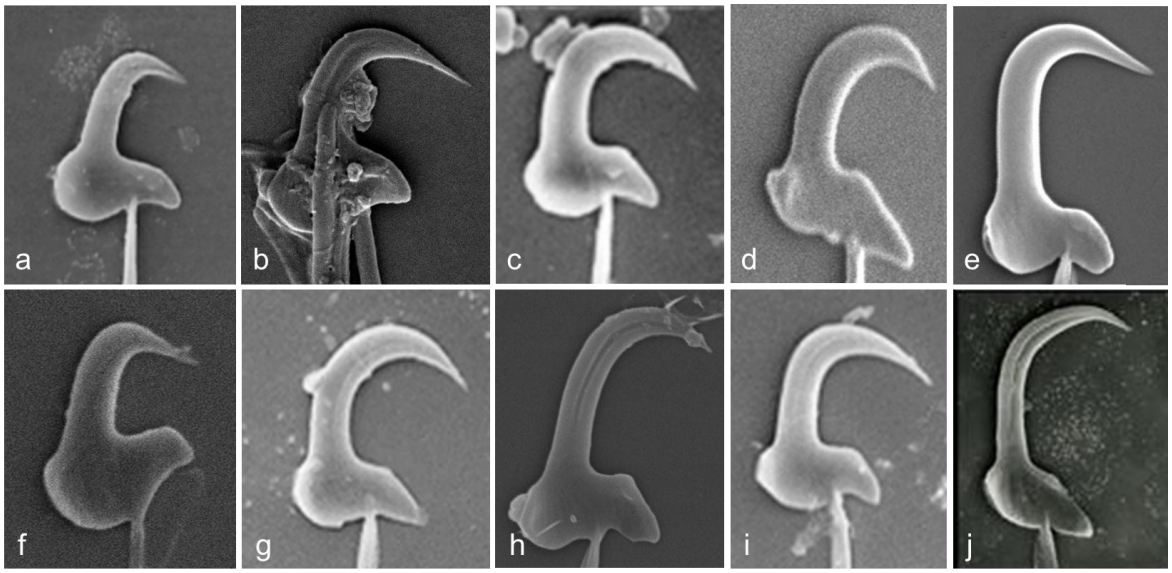


Figure 1.9. Different morphologies of the marginal hook sickles of 10 species of *Gyrodactylus* von Nordmann, 1832 (**a**) *G. arcuatus* Bychowsky, 1933; (**b**) *G. corleonis* Paladini, Cable, Fioravanti, Faria *et* Shinn, 2010; (**c**) *G. derjavinoides* Malmberg, Collins, Cunningham *et* Jalali, 2007; (**d**) *G. longipes* Paladini, Hansen, Fioravanti *et* Shinn, 2011; (**e**) *G. notatae* King, Forest *et* Cone, 2009; (**f**) *G. orecchiae* Paladini, Cable, Fioravanti, Faria, Di Cave *et* Shinn, 2009; (**g**) *G. salaris* Malmberg, 1957; (**h**) *G. salinae* Paladini, Huyse *et* Shinn, 2011; (**i**) *G. truttae* Gläser, 1974; (**j**) *G. turnbulli* Harris, 1986 [images **a**, **c**, **g**, **i** and **j** courtesy of Dr A.P. Shinn; image **e** courtesy of Dr S. King; all other images are original].

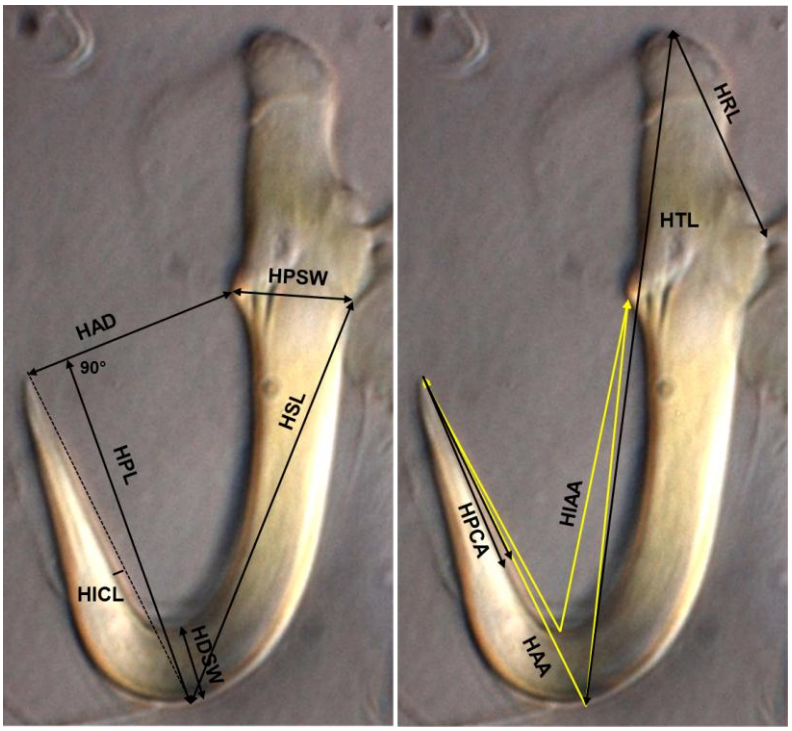


Figure 1.10. Light micrograph of the hamulus of *Gyrodactylus teuchis* Latraite, Blanc, Thiery, Daniel *et* Vigneulle, 1999 from *Oncorhynchus mykiss* (Walbaum), illustrating the point-to-point measurements that are typically obtained for a specimen of *Gyrodactylus* (modified from Shinn *et al*., 2004). HAA: hamulus aperture angle; HAD: hamulus aperture distance; HDSW: hamulus distal shaft width; HIAA: hamulus inner aperture angle; HICL: hamulus inner curve length; HPCA: hamulus point curve angle; HPL: hamulus point length; HPSW: hamulus proximal shaft width; HRL: hamulus root length; HSL: hamulus shaft length; HTL: hamulus total length [original images].

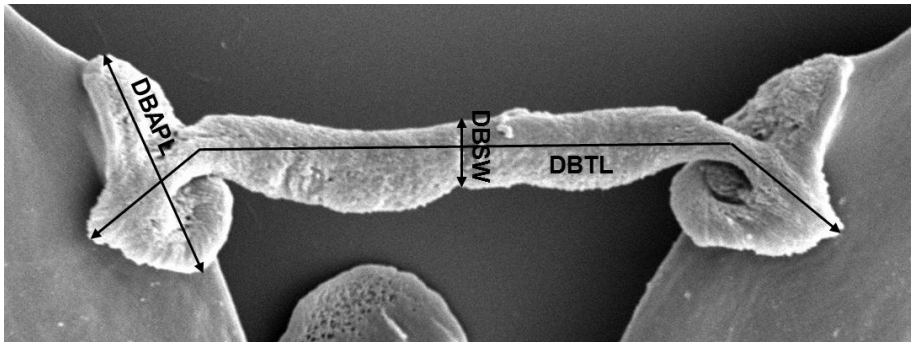


Figure 1.11. Scanning electron micrograph of the dorsal bar of *Gyrodactylus longipes* Paladini, Hansen, Fioravanti *et* Shinn, 2011 from *Sparus aurata* L., illustrating the point-to-point measurements that are taken. DBAPL: dorsal bar attachment point length; DBSW: dorsal bar shaft width, taken at the middle point of the dorsal bar; DBTL: dorsal bar total length [original image].

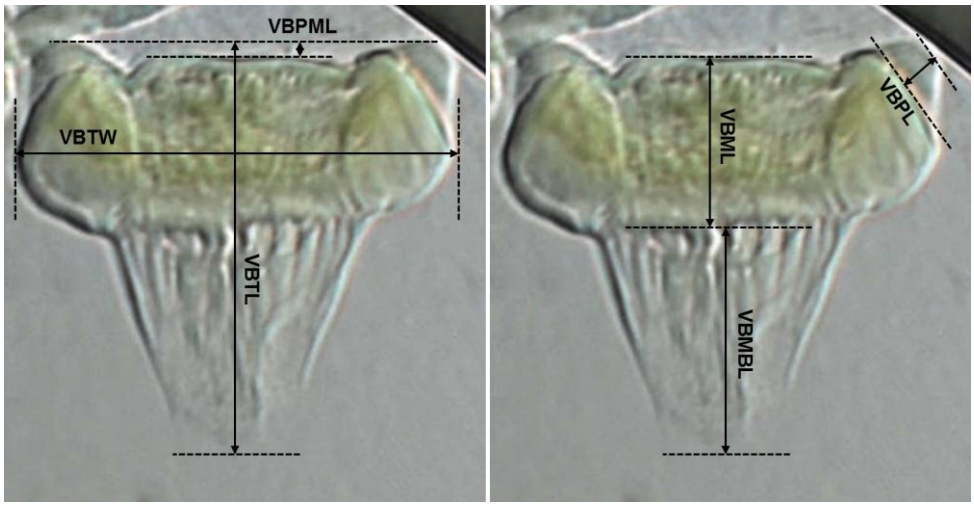


Figure 1.12. Light micrograph of the ventral bar of *Gyrodactylus teuchis* Latraite, Blanc, Thiery, Daniel *et* Vigneulle, 1999 from *Oncorhynchus mykiss* Walbaum, illustrating the point-to-point measurements that are typically taken (modified from Shinn *et al*., 2004). VBMBL: ventral bar membrane length; VBML: ventral bar median length; VBPL: ventral bar process length; VBPML: ventral bar process-to-mid length; VBTL: ventral bar total length; VBTW: ventral bar total width [original images].

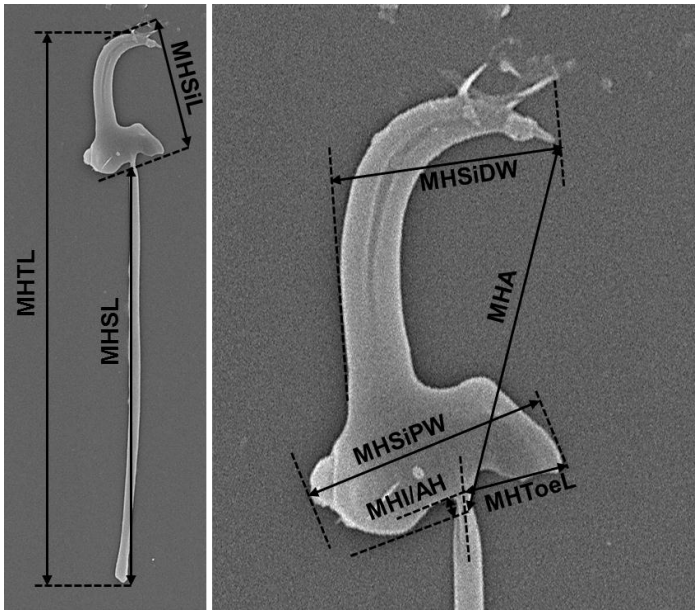


Figure 1.13. Scanning electron micrograph of the marginal hook of *Gyrodactylus salinae* Paladini, Huyse *et* Shinn, 2011 from *Aphanius fasciatus* (Valenciennes), illustrating the point-to-point measurements that are usually obtained from specimens (modified from Shinn *et al*., 2004). MHA: marginal hook aperture; MHI/AH: marginal hook instep/arch height; MHSiDW: marginal hook sickle distal width; MHSiL: marginal hook sickle length; MHSiPW: marginal hook sickle proximal width; MHSL: marginal hook shaft length; MHTL: marginal hook total length; MHToeL: marginal hook toe length [original images].

The shape of the hamuli and the ventral bar are used to a lesser degree, whilst the shape of the dorsal bar generally makes a weak contribution to the separation of species. This is not always the case, however, as the shape of the dorsal bar is useful in separating some species of *Gyrodactylus* infecting poeciliids (*e.g. Gyrodactylus bullatarudis* Turnbull, 1956; *Gyrodactylus costaricensis* Kritsky *et* Fritts, 1970; *Gyrodactylus xalapensis* Rubio-Godoy, Paladini, García-Vásquez *et* Shinn, 2010; see review of species infecting poecilids in Rubio-Godoy *et al*., 2010). The marked peculiarities in the dorsal bar morphology of these latter species, for example, warranted the re-inclusion of two of the features originally proposed by Malmberg (1970), *i.e.* the total length (DBTL) and the dorsal bar shaft width (DBSW), and the proposal of one new feature, the length of the dorsal bar attachment point (DBAPL; Fig. 1.11), for the description of certain new species (Paladini *et al*., 2011b; Schelkle *et al*., 2011).

In order to support morphological identification of existing species or new descriptions, the use of molecular tools has been stressed by many authors (Harris *et al*., 1999; Cunningham *et al*., 2003; Huyse *et al*., 2004; Bakke *et al*., 2007; Paladini *et al*., 2009b; Přikrylová *et al*., 2012; Ziętara *et al*., 2012), who use sequences from the rDNA spanning internal transcribed spacer ITS1, 5.8S and ITS2 regions, and where possible also the rRNA intergenic spacer (IGS) and the mitochondrial DNA cytochrome *c* oxidase subunit I (COI) gene to discriminate species.

The ITS regions have been sequenced for the majority of the *Gyrodactylus* species existing in GenBank but are not entirely useful as molecular markers (www.ncbi.nlm.nih.gov). These markers successfully separate a large number of sibling species (*e.g.* Ziętara & Lumme, 2002, 2003), but in some cases, *i.e.* the discrimination of *G. salaris* and *Gyrodactylus thymalli* Žitňan, 1960 is not possible as their ITS regions are identical (see Cunningham, 1997; Meinilä *et al*., 2002; Ziętara & Lumme, 2002; Kuusela *et al*., 2005). To avoid this confusion in identification and the consequences of

misidentifications, a more sensitive DNA marker has been developed (Meinilä *et al*., 2002). The circular mitochondrial genome is a good molecular marker for maternal patterns of inheritance as it is inherited *via* the oocyte cytoplasm and lacks recombination. COI was first sequenced for *Gyrodactylus salaris* by Meinilä *et al*. (2002) and since then it has been used to identify evolutionary patterns within the genus and to study mitochondrial haplotype diversity, which is a result of heteroplasmy (presence of more than one mitochondrial DNA due to mutation) (Hansen *et al*., 2003; Kuusela *et al*., 2005).

1.1.5. Factors influencing hook morphology

The chemical composition of the attachment hooks of *Gyrodactylus* have been suggested to consist of keratin-like and chitin-like proteins, depending on which haptoral structure is being considered (Kayton, 1983; Shinn *et al*., 1995). The hamuli and marginal hook elements are high in sulphur, whilst the ventral bar possesses a higher amount of calcium (Shinn *et al*., 1995). The significant presence of sulphur as a structural component in the hamuli and marginal hooks suggests a keratin-like component and gives strength to these structures, whilst the higher presence of calcium, rather than sulphur, in the ventral bar, which serves as an anchoring plate for many of the muscles within the opisthaptor, has been associated with a chitin-like substance, consisting in a long-chain polymer of a *N*-acetylglucosamine, a derivative of glucose (Neville, 1975; Shinn *et al*., 1995). While certain environmental factors, such as temperature and host adaptation can influence the phenotypic plasticity of these hooks (Malmberg, 1970; Ergens, 1976; Solomatova & Luzin, 1977; Ergens & Gelnar, 1985; Mo, 1991a, b; Shinn *et al*., 1995), much of the variation is linked to changes in size rather than shape (Mo, 1991a). Specifically, it has been demonstrated that the size of the haptoral hard parts increases with decreasing temperature, and *vice versa* (Malmberg, 1970; Mo, 1991a). This is explained by increases in water temperature accelerating embryonic development, reducing the lifespan of *Gyrodactylus*,

resulting in smaller-sized individuals with smaller-sized attachment hooks than their counterparts growing in colder waters (Ergens, 1976; Ergens, 1981; Kulemina, 1977; Jansen, 1989; Mo, 1991a). The hook plasticity is a key factor to consider when identifying economically important and pathogenic *Gyrodactylus* species, such as *G. salaris*. If species identifications are based on size alone, then specimens of the same species collected at different times of the year could appear as two different species which, in turn, could lead to misidentifications being made. Collecting gyrodactylid specimens throughout the year at different temperatures, is therefore highly recommended, when possible, in order to provide a better set of information, which includes the size range for certain structures, especially when new species are being described. Dmitrieva and Dimitrov (2002) also demonstrated the effect of temperature, alongside the host and salinity, on hamulus and marginal hook size of four *Gyrodactylus* species from the Black Sea, *i.e. G. alviga* Dmitrieva *et* Gerasev, 2000; *G. crenilabri* Zaika, 1966; *G. flesi* Malmberg, 1957; and *G. sphinx* Dmitrieva *et* Gerasev, 2000. According to Dmitrieva and Dimitrov (2002), freshwater *Gyrodactylus* species show larger hook sizes when the salinity is lower, while marine species have larger hook dimensions when the salinity is higher. As a general statement, the size of the opisthaptoral hard parts appear to increase when the parasite lives in favourable environmental conditions, as hostile environmental situations reduce the time of embryogenesis, which increases the reproduction rate, but at the same time, decreases the time for hook development (Dmitrieva & Dimitrov, 2002). Finally, the morphology of the opisthaptoral attachment hooks, it is suggested, can also be influenced by the host and by the site of attachment on the host (Huyse & Volckaert, 2002; Robertsen *et al*., 2007).

1.1.6. Influence of salinity and temperature on parasite biology

Salinity might also influence the site preference on the host. *Gyrodactylus callariatis* appears to prefer the body of Atlantic cod, when it is found in the marine environment,

however, when this host inhabits brackish waters, the parasite appears to principally infect the gills (Malmberg, 1970; Appleby, 1996). The same situation has been observed in *G. arcuatus*, with marine populations found on the skin and freshwater populations on the gills (Harris, 1993; Bakke *et al*., 2007). Temperature does not affect only the size of the opisthaptoral hard parts, but also the reproduction and survival of the parasite. It is known, for example, that the mean life-span of *G. salaris* is negatively correlated with water temperature, *i.e.* 33.7 days at 2.5°C and 4.5 days at 19°C (Jansen & Bakke, 1991). There is not, however, a standard temperature for the genus *Gyrodactylus*, as each species requires a different temperature range depending on the host and its geographical distribution. It is possible that certain species of *Gyrodactylus* can tolerate and "adapt" to a wide range of environmental conditions (*e.g.* salinity and temperature). This is the case for *Gyrodactylus arcuatus* from the three-spined stickleback *Gasterosteus aculeatus aculeatus* L. (see Harris, 1982); *Gyrodactylus callariatis* Malmberg, 1957 from Atlantic cod, *Gadus morhua* L.; and *Gyrodactylus salinae* Paladini, Huyse *et* Shinn, 2011 from the south European toothcarp *Aphanius fasciatus* (Valenciennes), amongst other species, which remain on their respective hosts even when the environmental conditions change drastically. *Gyrodactylus callariatis* for example, tolerates salinities from 5–35‰ (Malmberg, 1970), and, *G. salinae* survives on its host at temperatures ranging from 5–30°C and salinities ranging from 0–65‰ (Paladini *et al*., 2011b).

1.2. Impact of *Gyrodactylus salaris* and other emerging pathogenic species

Over 430 species of *Gyrodactylus* have been described, excluding synonyms and erroneous reports (Harris *et al*., 2004; www.gyrodb.net; www.monodb.org); some species are recognised as being highly pathogenic. Their pathogenicity has been linked to feeding activity and to the pathology of parasite attachment, which creates micro-wounds that destroy the osmotic integrity of the epidermis and, consequentially, facilitate the entry of

secondary infections, *i.e.* viral, bacterial and fungal agents (Snieszko & Bullock, 1968; Cone & Odense, 1984; Bakke *et al.*, 2006). Whilst species of *Gyrodactylus* have been reported from marine, brackish and freshwater environments in cold and warm latitudes, only a few species have gained notoriety and attention because of their pathogenicity to hosts. The most well-known pathogenic species is *G. salaris*, which is an OIE (Office International des Epizooties) listed pathogen, and a notifiable parasite in many European states, and which principally infects freshwater populations of Atlantic salmon (OIE, 2012). *Gyrodactylus salaris* has had devastating impact on the juvenile Atlantic salmon populations in 46 Norwegian rivers, and it represents the most significant threat to the existence of natural Atlantic salmon populations (Johnsen *et al*., 1999; Bakke *et al*., 2007). Given the reported decrease in wild parr populations, which has been up to 86% in some infected rivers, the annual loss caused by *G. salaris* has been estimated to be between 250–500 metric tonnes. The total cost of this parasite for the Norwegian government is now in excess of £330 million (Bakke *et al*., 2004). Although *G. salaris* has had a catastrophic impact in Norway, it has also been reported to have had a pathogenic effect on salmon populations elsewhere in Scandinavia and in Russia (Rintamäki, 1989; Ieshko *et al*., 1995; Alenäs, 1998; Alenäs *et al*., 1998). *Gyrodactylus salaris* has also been reported from rainbow trout, *Oncorhynchus mykiss* (Walbaum), from many European countries, where it is generally non-pathogenic (see Chapters 2 and 3). Some European states, including the UK, which is currently recognised as a *G. salaris*-free zone, now have mandatory surveillance programmes screening wild salmonid populations (*e.g.* brown trout *Salmo trutta fario* L., Arctic charr *Salvelinus alpinus alpinus* (L.), grayling *Thymallus thymallus* (L.), Atlantic salmon) for the presence of this notifiable pathogen.

Gyrodactylus salaris, however, is not the only pathogenic species within the genus *Gyrodactylus*. Many other species have been reported to cause mortality to their hosts. For example, *Gyrodactylus anarhichatis* Mo *et* Lile, 1998 was found to be highly pathogenic

on farmed Atlantic wolffish, *Anarhichas lupus* L., and spotted wolffish, *Anarhichas minor* Olafsen, causing heavy infections, notably on adult specimens weighing up to 10 kg (Mo & Lile, 1998). *Gyrodactylus callariatis* has been responsible for heavy mortalities of farmed Atlantic cod juveniles in Norway (Appleby, 1994, 1996). Likewise, *Gyrodactylus anguillae* Ergens, 1960 was found to be one of the contributory factors resulting in the heavy mortality of the glass stage of the European eel collected from Spain (Grano-Maldonado *et al*., 2011). Infections by *Gyrodactylus salmonis* (Yin *et* Sproston, 1948) are also worthy of note. This species has a low host specificity, has been widely recorded from several salmonid hosts (Rubio-Godoy *et al*., 2012; see Chapter 5 of the current thesis), and it is specifically highly pathogenic for brook trout, *Salvelinus fontinalis* (Mitchill), causing extensive damage of the fins as a consequence of parasite feeding and attachment activities: the marginal hooks penetrate deep into the host's epidermis (Cone & Odense, 1984; Cusack & Cone, 1986). Given the pathogenic potential this species poses to North American salmonid species, it has received almost as much attention as its European counterpart *G. salaris.* Buchmann and Uldal (1997) reported *Gyrodactylus derjavinoides* Malmberg, Collins, Cunningham *et* Jalali, 2007 (referred to as *Gyrodactylus derjavini* Mikhailov, 1975 before its later reclassification) causing a 10% mortality in brown trout fry even at low intensities of infection (*i.e.* 10 parasites fish^{-1}), while on rainbow trout fry, losses of up to 22% were seen when the mean intensities of the parasite were ~26 parasites fish^{-1} (Busch *et al*., 2003). *Gyrodactylus brachymystacis* Ergens, 1978 on rainbow trout reared in China has been reported to be highly pathogenic, resulting in extensive caudal fin erosion (You *et al*., 2006). Given the potential damage that this parasite can cause, *G. brachymystacis* needs close monitoring as it may become a significant pest in aquaculture (You *et al*., 2006). *Gyrodactylus cichlidarum* Paperna, 1968 has been reported to be the cause of several mass mortalities of juvenile Nile tilapia, *Oreochromis niloticus niloticus*

(L.) (see Fryer & Iles, 1972; Roberts & Sommerville, 1982; García-Vásquez *et al*., 2007, 2011).

Recently, two outbreaks of gyrodactylosis on juvenile gilthead seabream, *Sparus aurata* L., in Albania and Croatia were of interest because of the reported losses attributed to this parasite and this may represent an emerging disease (Paladini *et al*., 2009b; see Chapter 4). The species of *Gyrodactylus* collected from *S. aurata* were previously unknown and the causal factors responsible for the outbreak can only be theorised upon, *i.e.* local climatic changes and/or the migration of fish species into the area from which the parasite transferred to the more susceptible host, *S. aurata*. The investigation of this mortality event led to the subsequent description of two new species (see Chapter 4). Of these two, *Gyrodactylus orecchiae* Paladini, Cable, Fioravanti, Faria, Di Cave *et* Shinn, 2009 was found as a heavy infection (*i.e.* 1000+ parasites fish^{-1}) resulting in a 2–10% loss of farm stock. The other new species that was found, *Gyrodactylus longipes* Paladini, Hansen, Fioravanti *et* Shinn, 2011, has been recorded from two sites located in Italy and Bosnia-Herzegovina, and most recently, from a further site in Northern France. The samples of *S. aurata* collected from the Italian site were found as a co-infection with *G. orecchiae* (see Paladini *et al*., 2011a; see Chapter 4). High numbers of *G. orecchiae* associated with the mortality of juvenile *S. aurata* raise concerns regarding the pathogenic potential of this species and the consequences of finding it as a co-infection with *G. longipes*.

1.3. Global aquaculture production

The Food and Agriculture Organization of the United Nations (FAO) describe aquaculture as follows: "…*the farming of aquatic organisms: fish, molluscs, crustaceans and aquatic plants. Farming implies some form of intervention in the rearing process to*

enhance production, such as regular stocking, feeding and protection from predators…" (see FAO, 2012).

Globally, the largest source of aquaculture production is associated with the farming of fish. The culture of microalgae, also known as phytoplankton or microphytes, represents the second largest sector (see Fig. 1.14). The complete history of aquaculture is not known, but it is generally believed that the first practices of aquaculture concerned the raising of eels around 6000 B.C. by the indigenous Gunditjmara in Victoria, Australia, and then later, the production of common carp, *Cyprinus carpio carpio* L., in China around 2500 B.C. (Rabanal, 1988).

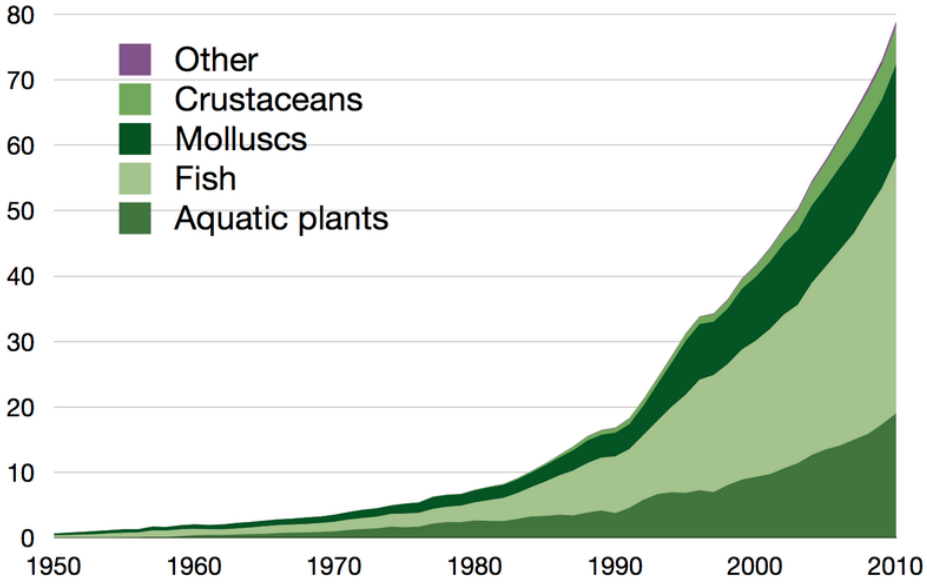


Figure 1.14. Global aquaculture production during 1950-2010 expressed in million tonnes [image from http://en.wikipedia.org/wiki/Aquaculture, which is based from FAO data].

Aquaculture represents the fastest growing animal food-producing sector and the *per capita* consumption from aquaculture products has increased from 0.7 kg in 1970 to 7.8 kg in 2006, with a mean annual growth rate of 6.9% (FAO, 2012). The drivers for the development of aquaculture are various. For example, the indigenous Gunditjmara of Australia used to catch eels, and then keep them so that they could be eaten all year round. Today, some of the underlying reasons for the expansion in certain aquaculture production

systems are driven by the need to protect wild stocks from extinction, *e.g.* Atlantic bluefin tuna, *Thunnus thynnus* L. (see Safina & Kinlger, 2008), to alleviate pressure on wild fisheries stocks, and also to feed the increasing demand for fish and aquatic products (Naylor *et al*., 2000). This demand has increased rapidly and, in 2006, the total production from aquaculture was reported to be 51.7 million tonnes, worth an estimated £52 billion, representing a marked increase when compared with <1 million tonnes per year produced in the early 1950s (FAO, 2012).

The intensification and expansion of aquaculture practices has, however, raised problems concerning the health status of the fish that are being grown. These include stocks being reared at higher densities and the stress caused by these intensive aquaculture systems, which can facilitate the establishment and manifestation of previously undetected pathogens (Smith, 1998; Kearn, 2004; *pers. obs*.).

1.4. The role of salmonids in the world aquaculture

The family Salmonidae is composed of three subfamilies: the Coregoninae, the Salmoninae and the Thymallinae, which collectively encompass ten genera. Of these, *Oncorhyncus mykiss* and *Salmo salar* represent the two species commanding the highest market value given the quality of their flesh (Farmer *et al*., 2000; Bugeon *et al*., 2010). Atlantic salmon production in freshwater began in the 19th Century in the UK in order to stock local waters with parr for recreational purposes. The subsequent stages of Atlantic salmon, *i.e.* smolts to adults, were reared in sea cages in the 1960s in Norway to raise the fish to a commercial size. The successful production of salmon by the Norwegians drove the development of Atlantic salmon culture not only in Norway but also in Scotland, followed by Ireland, the Faroe Islands, Canada, USA, Chile and Australia. The current worldwide annual production of farmed Atlantic salmon (Figs. 1.15-1.16) now exceeds 1 million tonnes and represents more than 50% of the total global salmon market (FAO,

2012). The main producer of Atlantic salmon is Norway, which produced 900,000 tonnes in 2010, followed by Chile with 288,000 tonnes and Scotland with 154,000 tonnes (sources: www.marineharvest.com, www.scottishsalmon.co.uk, last access November 2012). Norway is by far the largest producer, but Chile has rapidly increased its production since Atlantic salmon were introduced from Norway and Scotland in the early 1980s. Chile benefits from low costs for labour and raw materials, and can therefore efficiently enter distant markets and compete with traditional producing countries.

Figure 1.15. Countries, shown in orange, producing Atlantic salmon, *Salmo salar* L., through aquaculture [image from FAO, 2012].

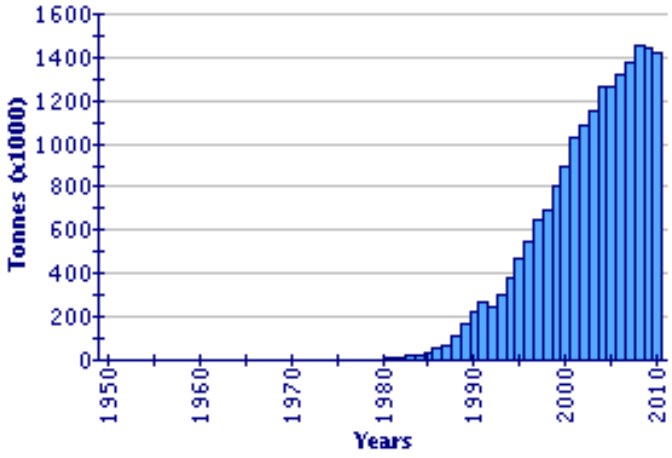


Figure 1.16. The increasing global aquaculture production of Atlantic salmon, *Salmo salar* L., during the period 1950-2010 [graph from FAO, 2012].

1.5. UK aquaculture industry and *G. salaris* national contingency plans

Scotland represents the third largest producer of farmed salmon in the world and it is the major producer within the United Kingdom. The Scottish Salmon Producers Organisation (SSPO) (www.scottishsalmon.co.uk) reported that Scotland's salmon farmers contributed about £500 million to the economy in 2009, and that the worldwide retail value of Scottish farmed salmon is over £1 billion, with more than 60 countries importing fresh Scottish salmon in 2011. While the production of Atlantic salmon in 2010 in the UK was 154,625 tonnes (of which 154,164 tonnes was produced in Scotland), it is important to consider other salmonid species cultured in the UK, such as rainbow trout and brown trout. In 2010, for example, the UK produced 11,988 tonnes of freshwater rainbow trout, 1,606 tonnes from marine rainbow trout production and 574 tonnes of freshwater brown trout (listed as inland sea trout production; see FAO, 2012). Given the impact that *G. salaris* has on Atlantic salmon in Scandinavia and Russia (Rintamäki, 1989; Johnsen & Jensen, 1991; Ieshko *et al*., 1995; Alenäs, 1998; Alenäs *et al*., 1998), and the risk that other salmonid species pose as potential carrier hosts in the dispersal of *G. salaris* within and between countries, the OIE has listed this parasite as a notifiable disease (OIE, 2012). As the UK is currently recognised *G. salaris*-free (Platten *et al*., 1994; Shinn *et al*., 1995; European Commission Decision 2004/453/EC; http://eur-lex.europa.eu), and given the importance of wild Atlantic salmon stocks, the fish inspectorates throughout the UK now have mandatory surveillance programmes, which include the screening of wild salmonid populations, such as brown trout, Arctic charr, grayling and Atlantic salmon. The presence of this notifiable pathogen has not been recorded in the UK but the susceptibility of Scottish populations of Atlantic salmon to *G. salaris* has been experimentally tested, demonstrating high susceptibility of the fish (Bakke & MacKenzie, 1993). Given the value of the UK salmonid industry and the relevance of its wild stocks, it is important the UK's *G. salaris*-free status is upheld. Current UK dispersion models and contingency plans for its control (see

www.defra.gov.uk) are based on the assumption that British stocks of Atlantic salmon would be highly susceptible to *G. salaris* if exposed, that brown trout would be unaffected by the parasite, and that grayling would be moderately resistant. Brown trout and grayling have been demonstrated to harbour low-level infections for a few weeks, without showing the pathogenic effects that *G. salaris* has on Atlantic salmon (see review in Bakke *et al.*, 2007). Whether native UK stocks of brown trout and grayling would respond in the same way as their Scandinavian counterparts is unknown, as they have been separated from mainland Europe since the last period of glaciation (Halvorsen & Hartvigsen, 1989). Should differences in susceptibility be demonstrated, then this could necessitate a revision of current contingency plans and a redrafting and analysis of current dispersion models (see Chapter 6).

1.6. Previous studies on salmonid susceptibility to *G. salaris*

A number of experimental studies have been carried out testing the susceptibility to *G. salaris* of different Atlantic salmon strains collected from Canada, Denmark, Norway, Russia, Scotland and Sweden (see Chapter 6). The majority of these studies have used *G. salaris* haplotype F, a strain that normally infects rainbow trout, but also Atlantic salmon, whilst only a few studies have used *G. salaris* haplotype A (see Chapter 6), a strain that typically infects Atlantic salmon in Norway and Sweden (Hansen *et al*., 2003). The susceptibility to *G. salaris* has been tested for several salmonid species (*e.g.* Bakke *et al*., 1990; Bakke *et al*., 1991a; Bakke & Jansen, 1991a, b; Bakke *et al*., 1992a, b, c; Jansen & Bakke, 1995; Bakke *et al*., 1996; Lindenstrøm *et al*., 2000; Soleng & Bakke, 2001a, b; Dalgaard *et al*., 2004; Robertsen *et al*., 2007; Winger *et al*., 2008) and also for a number of non-salmonid hosts (Mo, 1987; Bakke *et al*., 1990; Bakke & Sharp, 1990; Bakke *et al*., 1991b; Soleng & Bakke, 1998), which may represent suitable and undetected carriers

contributing to the dispersal of *G. salaris* (see Chapter 6). The results of these studies will be briefly considered below but also examined in greater detail in Chapter 6 of this thesis.

1.6.1. Haplotypes and pathogenicity of G. salaris

A total of 18 *G. salaris* mitochondrial haplotypes (Table 1.1.) have been identified by cytochrome oxidase I (COI) analysis (Hansen *et al.*, 2003, 2006, 2007a, b; Meinilä *et al*., 2004; Kuusela *et al*., 2005, 2007; Robertsen *et al*., 2007; Paladini *et al*., 2009a). Hansen *et al.* (2003) was the pioneer in characterising the first six haplotypes from Atlantic salmon from Latvia, Norway and Sweden, identified as haplotypes "A-F", one of which, haplotype "F", is also commonly encountered on rainbow trout from Sweden and Italy (Hansen *et al*., 2003; Paladini *et al*., 2009a), and on Arctic charr from Norway (see Hansen *et al*., 2007a; Robertsen *et al*., 2007). All haplotypes were well supported and linked with their respective host and locality, with the exception of haplotype F (Hansen *et al*., 2003).

Meinilä *et al*. (2004) subsequently described a further five haplotypes from Atlantic salmon, *i.e.* "*Sal* Keret1", "*Sal* Keret2", "*Sal* Lagan", "*Sal* Tornio" and "*Sal* Vefsna" from Sweden, Finland and Russia, in addition to the previously known haplotype F from rainbow trout, recorded for the first time from Denmark. Furthermore, Meinilä *et al*. (2004) commented on the finding of haplotype F from Atlantic salmon in the River Pistojoki, in Russia, suggesting that this may have been introduced *via* rainbow trout farms.

One year later, Kuusela *et al*. (2005) discovered two further haplotypes from Lake Onega (Russia), namely "*Sal* Lizhma" and "*Sal* Kumsha", but the latter has been indicated by Kuusela *et al*. (2007) as a synonym of the haplotype "*Sal* Keret1", previously described by Meinilä *et al*. (2004).

In 2007, Kuusela *et al*. (2007) commented on other five new *G. salaris* haplotypes: "RBT2" (named accordingly to the GenBank accession number EF570120 provided in the

paper, otherwise named "specific" in the same article) infecting Ohrid trout, *Salmo letnica* (Karaman), from a fish farm in Macedonia; "*Sal* Ba06" from Finnish Atlantic salmon; "*Sal* Ba09" and "*Sal* Ba10" from Swedish Atlantic salmon; and "*Sal* Ba11" from Russian Atlantic salmon, together with haplotype F from a new locality in Russia.

Table 1.1. *Gyrodactylus salaris* Malmberg, 1957 mitochondrial haplotypes listed in alphabetical order.

No.	Haplotype name	Hosts	Locality	GenBank COI accession numbers	Reference
1	A	*Ss*	Norway	AF542161, 6; AY146597-9; AY146606-7; AY258336, 38-42, 45-57; AY486488–96; AY486527-30, 33-38, 42-43	Hansen *et al*. (2003, 2006)
		Ss	Sweden	AY258337, 43-44, 48-49; AY486500, 08-09, 11, 17-18, 21-22	Hansen *et al*. (2003, 2006)
2	B	*Sa*	Norway	AY486497; AY486525	Robertsen *et al*. (2007)
		Ss	Norway	AY146600-5; AY486497; AY486525-6; AF542162-5	Hansen *et al*. (2003, 2006)
		Ss	Sweden	AY258367-70; AY486499	Hansen *et al*. (2003, 2006)
3	C	*Ss*	Sweden	AY258358-66[1]; AY486501-02, 04-06, 10, 13-16, 23-24, 31-32	Hansen *et al*. (2003, 2006)
4	D	*Ss*	Latvia	AY146593-4; AY486507	Hansen *et al*. (2003, 2006)
5	E	*Ss*	Sweden	AY258373-4; AY486512	Hansen *et al*. (2003, 2006)
6	F	*Om*	Denmark	AF479750[2]	Meinilä *et al*. (2004)
		Om	Finland	AF479750[2]	Meinilä *et al*. (2004)
		Om	Italy	none[3]	Paladini *et al*. (2009a)
		Om	Sweden	AF479750[2]; AY146589-90; AY486503	Hansen *et al*. (2003, 2006); Meinilä *et al*. (2004)
		Sa	Norway	DQ923578	Robertsen *et al*. (2007)
		Ss	Norway	AY146591-2, 95-96; AY146614; AY258370-2; AY486498; AY486519-20, 39-41	Hansen *et al*. (2003, 2006)
		Ss	Russia	AF479750; DQ517533; DQ778628[2]	Meinilä *et al*. (2004); Kuusela *et al*. (2007)
7	RBT2	*Sl*	Macedonia	EF570120	Kuusela *et al*. (2007)
8	*Sal* Ba06	*Ss*	Finland	DQ993189	Kuusela *et al*. (2007)
9	*Sal* Ba09	*Ss*	Sweden	DQ993193	Kuusela *et al*. (2007)
10	*Sal* Ba10	*Ss*	Sweden	DQ993194	Kuusela *et al*. (2007)
11	*Sal* Ba11	*Ss*	Russia	EF117889	Kuusela *et al*. (2007)
12	*Sal* Keret1	*Ss*	Russia	AF540891; AY840223[4]	Meinilä *et al*. (2004)
13	*Sal* Keret2	*Ss*	Russia	AF540892[5]	Meinilä *et al*. (2004)
14	*Sal* Lagan	*Ss*	Sweden	AF540904[6]	Meinilä *et al*. (2004)
15	*Sal* Lizhma	*Ss*	Russia	AY840222[7]	Kuusela *et al*. (2005)
16	*Sal* Nera	*Om*	Italy	GQ370816	Paladini *et al*. (2009a)
17	*Sal* Tornio	*Ss*	Finland	AF540905[8]	Meinilä *et al*. (2004)
18	*Sal* Vefsna	*Ss*	Norway	AF540906[9]	Meinilä *et al*. (2004)

Footnotes: [1]Synonym: "*Sal*Ba08" (Kuusela *et al*., 2007); [2]Synonyms: "*Onc*FI-S-DK" (Meinilä *et al*., 2004) and "RBT" (Ziętara *et al*., 2006; Kuusela *et al*., 2007); [3]Haplotype F from Italian rainbow trout has 100% COI identity with haplotype F from Swedish rainbow trout described by Hansen *et al*. (2003). Sequence not deposited in GenBank; [4]Synonyms: "KA" (Kuusela *et al*., 2005), "*Sal*Ba01" (Kuusela *et al*., 2007) and "*Sal*Kumsha" (Hansen *et al*., 2007b); [5]Synonyms: "KB" (Kuusela *et al*., 2005) and "*Sal*Ba02" (Kuusela *et al*., 2007); [6]Synonym: "*Sal*Ba08" from Smedjeån (Kuusela *et al*., 2007); [7]Synonym: "*Sal*Ba03" (Kuusela *et al*., 2007); [8]Synonyms: "*Sal*Ba04" and "*Sal*Ba05" (Kuusela *et al*., 2007); [9]Synonym: "*Sal*Ba07" (Kuusela *et al*., 2007). Abbreviations: *Om*: *Oncorhynchus mykiss*; *Sa: Salvelinus alpinus*; *Sl: Salmo letnica*; *Ss*: *Salmo salar*.

Robertsen *et al*. (2007) reported for the first time two *G. salaris* haplotypes from Arctic char, *Salvelinus alpinus alpinus* (L.), identical to haplotypes B and F described by Hansen *et al*. (2003).

More recently, Paladini *et al*. (2009a) found a further haplotype, "*Sal* Nera", on an Italian population of farmed rainbow trout from the River Nera, together with haplotype F. Although the differentiation of *G. salaris* strains through characterisation of their COI represents a useful molecular tool, it does not, however, provide information concerning their potential pathogenicity (Bakke *et al*., 2004; Hansen *et al*., 2007b), such that their potential virulence, with respect to each population of fish, needs to be investigated. Using the haplotype classification of *G. salaris* strains provided by Hansen *et al*. (2003), it is most likely that the strain of *G. salaris* collected originally from the River Lierelva and used by Bakke and co-workers in their salmonid susceptibility trials was haplotype F (see Chapter 6).

1.6.2. Baltic strain of Salmo salar

It is believed that the Baltic strain of Atlantic salmon, particularly the populations from the River Neva in Russia and the River Tornio between Finland and Sweden are more resistant to the pathogen *G. salaris* than the Atlantic strain (Bakke *et al*., 1990, 1992a; Anttila *et al*., 2008). The salmon populations from the Finnish Rivers Oulujoki, Lijoki and Kemijoki have also been shown to have a high resistance to natural infections of *G. salaris* (see Rintamäki-Kinnunen & Valtonen, 1996). This demonstrable resistance, however, is not always the case, as it has been demonstrated by several other studies with salmon populations from the Swedish Rivers Luleälven and Indalsälven (Bakke *et al*., 2002, 2004; Dalgaard *et al*., 2003) and with triploid salmon originating from the Estonian River Kunda (Ozerov *et al*., 2010).

There are at least three geographically separated groups of *S. salar*: the Western Atlantic, the Eastern Atlantic and the Baltic Sea populations (Ståhl, 1987). A comparison of the genetic distances between these three populations, however, suggests that relatively few differences have appeared among these three since the last glaciation (Ståhl, 1987). The observed different susceptibilities displayed by Atlantic and Baltic strains of *S. salar* to *G. salaris* (see Bakke *et al.*, 1990) may result from the degree of "isolation" between the strains.

1.6.3. Atlantic strain of Salmo salar

One of the first experimental infection studies conducted, investigated three strains of *S. salar* infected with *G. salaris* (see Bakke *et al*., 1990). The two Atlantic strains were collected from the Rivers Alta and Lone (Norway), whilst one Baltic strain from the River Neva (Russia) was tested with a strain of *G. salaris* originating from *S. salar* from the River Drammenselva, Norway (most likely haplotype F, according to Hansen *et al*., 2003). The hatchery-reared Baltic strain showed innate resistance to the *G. salaris* infection, managing to respond within 3 weeks and to reduce the parasite number (Fig. 1.17). The Norwegian Alta and Lone salmon populations, however, were highly susceptible to infection (Bakke *et al*., 1990). The experiment was terminated after 5 weeks after several fish mortalities, probably due to external, unknown causes (Bakke *et al*., 1990).

Similar results were also obtained by Bakke (1991) testing the susceptibility of the Atlantic Alta and Lone populations from Norway, and the Baltic Neva population from Russia plus two other Atlantic strains originating from the Norwegian Rivers Drammenselva and Lierelva. All four Norwegian strains were susceptible to *G. salaris*, whilst the Neva strain again was able to launch a good response to the infection (Bakke, 1991). Atlantic salmon parr from the River Alta, Norway, showed moderate to high susceptibility to *G. salaris* infection over the 6-week (42 days) experiment (Bakke *et al*.,

1999). Sixteen fish managed to control the infection after ~30 days, while eight fish did not manage to respond to the infection (Bakke *et al*., 1999). In both cases the experiment was terminated after 42 days and there is no evidence to suggest whether the infection in the responding fish would have ultimately crashed to extinction or not.

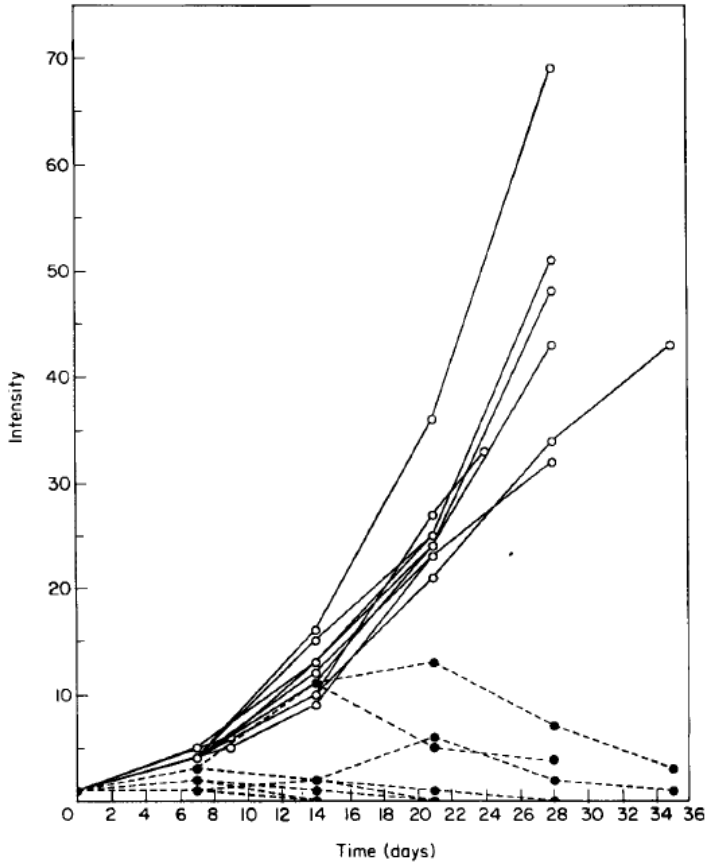


Figure 1.17. Schematic example of the course of an experimental *Gyrodactylus salaris* Malmberg, 1957 infection on two strains of Atlantic salmon, *Salmo salar* L., parr. Atlantic River Lone (Norway) stock (solid lines) and Baltic River Neva (Russia) stock (broken lines) [image from Bakke *et al.*, 1990].

1.6.4. Experimental studies with Salmo trutta fario

Brown trout is genetically the most closely related species of the family Salmonidae to the Atlantic salmon (Phillips *et al*., 1992) and could, therefore, represent a suitable host for *G. salaris*. Brown trout parr naturally infected with *G. salaris* at low intensities have been reported by several authors (Tanum, 1983; Mo, 1988; Malmberg & Malmberg, 1991; Johnsen & Jensen, 1992). The susceptibility of this host to *G. salaris* collected from

Atlantic salmon from the River Lierelva (Norway) (haplotype F, according to Hansen *et al*., 2003) has been experimentally tested by Jensen and Bakke (1995), using both anadromous and resident stocks in Norway. Twenty-one individually isolated and 22 grouped anadromous brown trout were exposed to 25 infected salmon parr for 5 days. In the isolated brown trout, the *G. salaris* population declined gradually but persisted for 7 weeks (49 days) post-infection with a mean intensity of 0.3 parasites $fish^{-1}$ (range 1-5). Similarly, in the grouped fish after 49 days, there was still one parasite remaining (mean intensity was 0.05 parasites $fish^{-1}$) (Jansen & Bakke, 1995). The same experiment was carried out with resident stocks, but the infection period was extended to 15 days. The results show that the infection persisted for 28 and 22 days, respectively (Jansen & Bakke, 1995). In a third experiment, fed fish eliminated the infection briefly within 9 days, whilst the starved fish carried on the *G. salaris* infection for longer (27 days) (Jansen & Bakke, 1995). These results open a discussion on the susceptibility of fish stocks conditioned by their general health status, which is higher if the host is stressed or starved (Gelnar, 1987). This was also demonstrated by Harris *et al*. (2000), who found that brown trout were more susceptible to *G. salaris* infection following the administration of hydrocortisone acetate. Other experimental infections of brown trout with *G. salaris* have demonstrated that the fish maintain their infection as long as they co-exist with infected Atlantic salmon in the same tank (Tanum, 1983; Mo, 1988). This was also demonstrated by Bakke *et al*. (1999), who found that brown trout from the River Fossbekk (Norway) eliminated their *G. salaris* (haplotype F) infection in less than two weeks, showing an innate resistance to this parasite (see Fig. 1.18).

The lower rate of reproduction of *G. salaris* on brown trout does not exclude the risk of its dissemination within a river and spread to connecting systems, especially considering that the parasite population can remain on this host for up to 49 days under certain conditions (Jansen & Bakke, 1995).

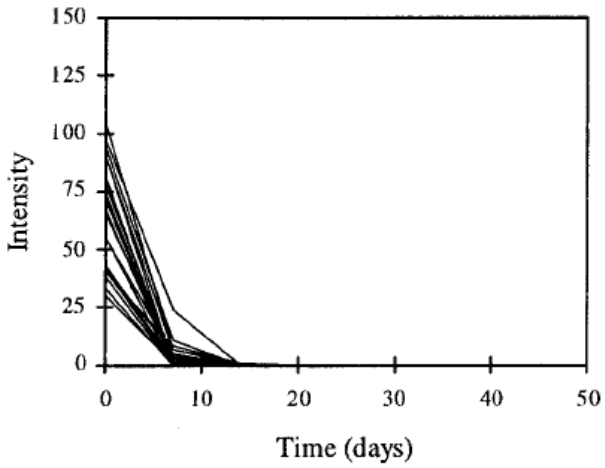


Figure 1.18. Representation of the course of *Gyrodactylus salaris* Malmberg, 1957 (haplotype F) infection on individually isolated brown trout, *Salmo trutta fario* L., from the River Fossbekk (Norway), showing innate resistance to the infection [graph from Bakke *et al*., 1999].

1.6.5. Experimental studies with Salmo salar × Salmo trutta fario *hybrids*

The susceptibility of *Salmo salar* × *Salmo trutta fario* hybrids to *G. salaris* from the River Lierelva (most likely haplotype F - see Hansen *et al*., 2003) was also investigated to determine whether there are differences in genetic resistance to gyrodactylid infection and to assess the role of interspecific salmonid hybridisation in the ecology of *G. salaris* (see Bakke *et al*., 1999; Figs. 1.19-1.20). Two experiments, one using female Atlantic salmon × male brown trout (hybrid 1), and a second using male Atlantic salmon × female brown trout (hybrid 2), were conducted by Bakke *et al*. (1999). The results showed that the first hybrid (n = 23) displayed a range of susceptibilities to *G. salaris*, with 9 fish eliminating the infection within two weeks; four fish sustaining the infection for the first 3 weeks before slowly declining over the following 2 months (one of them was still infected after 70 days when the experiment was terminated); and 10 fish being highly susceptible for the first 3 weeks, after which period they responded, reducing the infection to almost zero by the end of the experiment (Bakke *et al*., 1999). The second set of hybrids (n = 24) demonstrated resistance to the *G. salaris* infection, eliminating the parasite population within the first two weeks of the trial, with the exception of one fish which, after an initial

decline in parasite burden, started to show increasing parasite numbers again (Bakke *et al*., 1999).

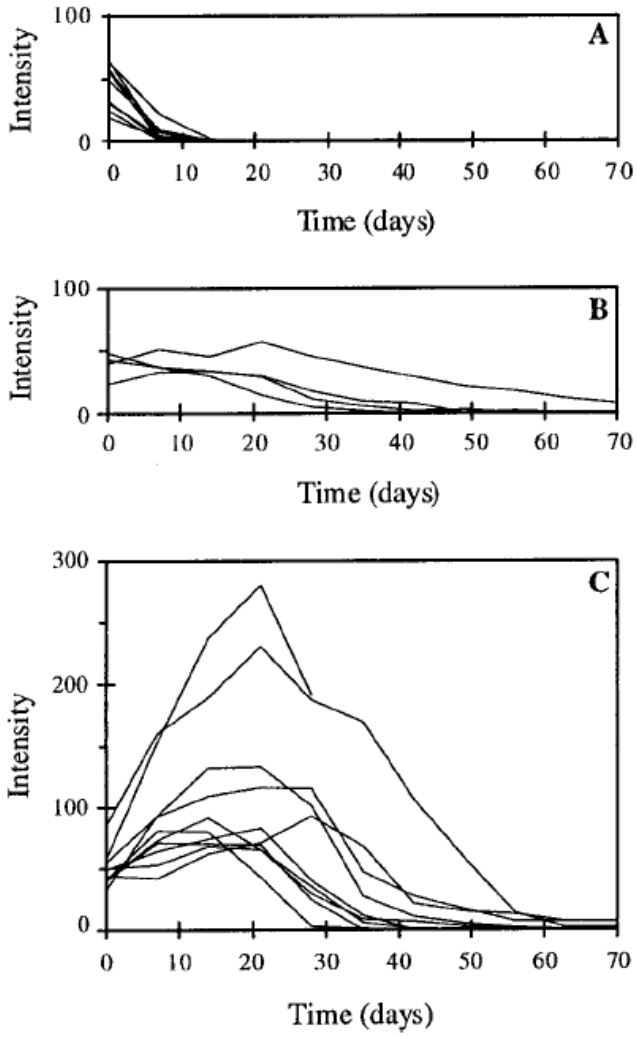


Figure 1.19. Representation of the course of *Gyrodactylus salaris* Malmberg, 1957 (haplotype F) infection on individually isolated hybrids of ♀Atlantic salmon, *Salmo salar* L., × ♂ brown trout, *Salmo trutta fario* L., showing (**A**) innately resistant specimens; (**B**) initially susceptible fish, responding and controlling the infection; (**C**) moderate to highly susceptible individuals, eliminating the infection after 1-2 months [graphs from Bakke *et al*., 1999].

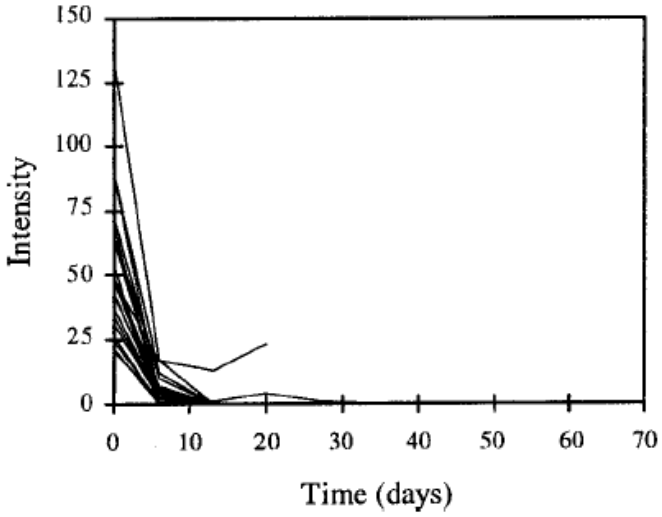


Figure 1.20. Course of *Gyrodactylus salaris* Malmberg, 1957 (haplotype F) infection on individually isolated hybrids of ♂Atlantic salmon, *Salmo salar* L., × ♀ brown trout, *Salmo trutta fario* L., demonstrating innately resistant fish eliminating the infection after 2 weeks [graph from Bakke *et al*., 1999].

1.6.6. Experimental studies with Thymallus thymallus

Grayling, *Thymallus thymallus*, within the 11 species existing in the genus, is the only species of the subfamily Thymallinae that has been tested to determine its susceptibility to *G. salaris* (see Soleng & Bakke, 2001b; Figs. 1.21–1.22). The reason why this species is considered important is because grayling is the principal host of *Gyrodactylus thymalli*, a species that is morphologically and genetically similar to *G. salaris* (see McHugh *et al*., 2000; Shinn *et al*., 2004). When Žitňan (1960) described this species from wild grayling taken from the Danubian headwaters in Moravia, he highlighted its morphological similarities to the pathogenic species *G. salaris*. Later, Cunningham (1997) found that both species had identical ITS (internal transcribed spacer) sequences, which did not permit their ready discrimination from one another. *Gyrodactylus thymalli*, however, is the only species to have been reported from wild grayling, *i.e. G. salaris* has not been recorded (Soleng & Bakke, 2001b). Nevertheless, the possibility that grayling may carry *G. salaris* remains a concern and a taxonomic challenge.

In the experiment conducted by Soleng and Bakke (2001b), the strain of *G. salaris* used was obtained from heavily infected Atlantic salmon caught in the River Lierelva, Norway (haplotype F, see Hansen *et al*., 2003). The grayling were aged 0+ (mean weight 1.0 g; mean fork length 5.4 cm) and 1+ (mean weight 3.9 g; mean fork length 8.3 cm) and were hatchery-reared, Lake Sølensjøen (Norway) stock.

The young grayling (*i.e.* 0+) were experimentally challenged as isolated individuals (n = 21 fish) and as groups of fish (n = 50 fish), by exposure to *G. salaris* for 24 hours. A second group of 1+ grayling were also tested individually (n = 20 fish) and as groups (n = 100 fish), and exposed to *G. salaris* for a period of 7 days (Soleng & Bakke, 2001b). At the beginning of the experiment, all the fish became infected with *G. salaris*, and the results were divided by fish age. From Soleng and Bakke's (2001b) study, of the 21 individually isolated 0+ fish, three were innately resistant eliminating the infection shortly within the first 3 weeks (Fig. 1.21.A), while on the remaining 18 fish, the parasite population increased slightly (Fig. 1.21.B). The infection declined after two weeks, persisting for up to 35 days, when the experiment was terminated because of host mortalities; however, three of the grayling were still infected (Soleng & Bakke, 2001b).

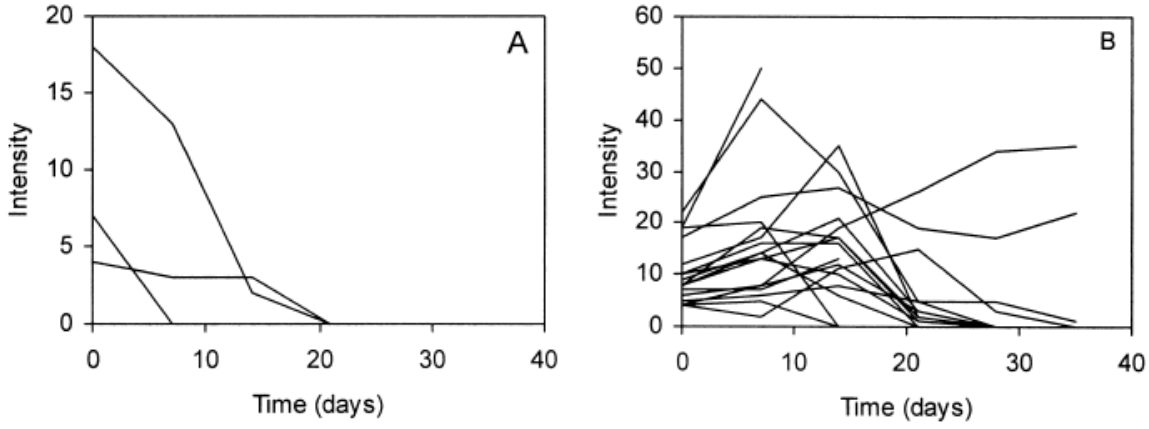


Figure 1.21. Course of infection of *G. salaris* (haplotype F) on grayling aged 0+: (**A**) individually isolated fish (n = 3) showing innate resistance; (**B**) individually isolated fish (n = 18) shown to be susceptible [graphs from Soleng & Bakke, 2001b].

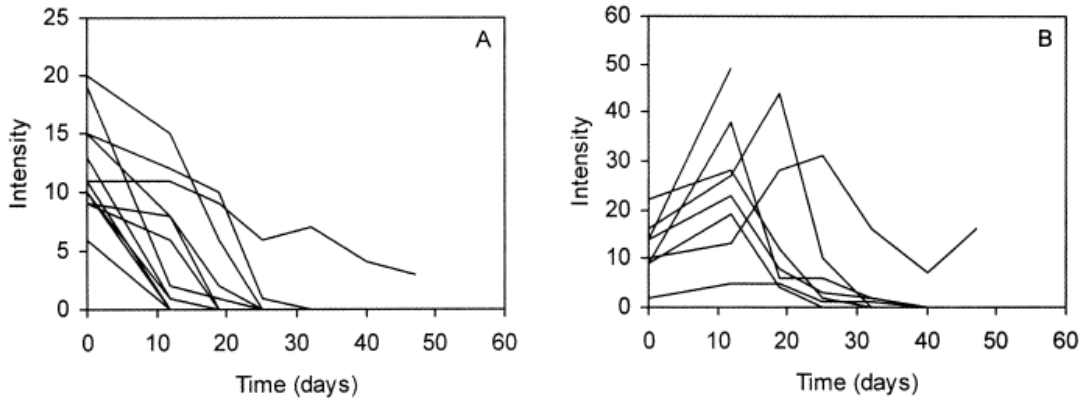


Figure 1.22. The course of *G. salaris* (haplotype F) infection on 1+ aged grayling: (**A**) individually isolated fish (n = 12) showing innate resistance; (**B**) individually isolated fish (n = 8) shown to be susceptible to infection [graphs from Soleng & Bakke, 2001b].

In the 0+ grayling infected as a group, the infection increased during the first week to its peak (mean infection 13.5 parasites fish^{-1}), and subsequently declined until elimination after 35 days (Soleng & Bakke, 2001b).

The 1+ grayling followed the same pattern of infection with twelve out of 20 individually held fish showing an innate resistance (Fig. 1.22.A), while the remaining 8 were susceptible to infection (Fig. 1.22.B). After 12 days, however, the parasite population increased and thereafter declined slowly, persisting on two fish for up to 47 days when the experiment was terminated because of host mortality (Soleng & Bakke, 2001b). The infection on the grouped fish (aged 1+) declined shortly after the first week, but in one of the two replicates the *G. salaris* population lasted for more than 50 days (Soleng & Bakke, 2001b). These results demonstrated that the pathogen *G. salaris* can easily attach to and reproduce on grayling, with some differences between the host populations, where the innate resistance increases with the age of the fish, apart for the grouped fish. When grayling were held in isolation, *i.e.* not cohabited with *S. salar,* the infection period was short (Soleng & Bakke, 2001b). This indicates that *T. thymallus* might represent a carrier for *G. salaris,* but in absence of salmon, it is unable to develop the parasite infection.

In order to discriminate between *G. salaris* and *G. thymalli*, their host specificity and pathogenicity was tested experimentally by Sterud *et al*. (2002). Atlantic salmon that were experimentally infected with *G. thymalli* managed to eliminate the infection within 42 days when held as isolated individuals and within 70 days when held in groups of fish (Sterud *et al*., 2002). Using the same approach but infecting grayling with *G. salaris* from the River Lierelva (most likely haplotype F - see Hansen *et al*., 2003), the infection of *G. salaris* partially failed to reproduce and grow, but remained on two individually isolated fish for up to 143 days (one of the two, even increased from 5 parasites to 22 in the last count), when the experiment was terminated. In the trial using groups of fish, a single fish carried the infection with four *G. salaris* specimens until the end of the experiment at 143 days (Sterud *et al*., 2002).

1.6.7. British salmonids previously tested with G. salaris

The early surveys of Platten *et al*. (1994) and Shinn *et al*. (1995) conducted throughout the UK examined more than 4000 wild and farmed salmonids and did not find *G. salaris*. On-going surveillance programmes from that time have also taken samples from key sites and identified any collected *Gyrodactylus* specimens to ensure they were not *G. salaris*. Based on the combined data from these investigations the UK is considered to be *G. salaris* free (see European Commission Decision 2004/453/EC; EC Decision 2006/272/EC; http://eurlex.europa.eu). If *G. salaris*, however, were to be introduced into the UK, then it is believed that the consequences of this could be potentially catastrophic. To determine whether UK stocks were susceptible, Atlantic salmon stocks from two Scottish rivers, the Conon and the Shin were experimentally challenged with *G. salaris* (see Bakke & MacKenzie, 1993; Dalgaard *et al*., 2003, 2004). Hatchery-reared 0+ salmon parr from both rivers were flown to Norway and exposed to a strain of *G. salaris* from the River Figga, Norway (most likely haplotype A, see Fig. 1 in Hansen *et al*., 2003). After 3

days exposure, the prevalence of infection was found to be 100% (Bakke & MacKenzie, 1993). The 50 day experiment assessed fish held in isolation and as groups. During this period, none of the fish were able to completely eliminate their infection. Peak infections *i.e.* ~1500 parasites fish^{-1} were seen on between days 22 and 36, after which some fish appeared to mount a response and were able to considerably reduce their parasite burdens. Other fish, however, failed to respond and died during the experimental period (Bakke & MacKenzie, 1993; Fig. 1.23).

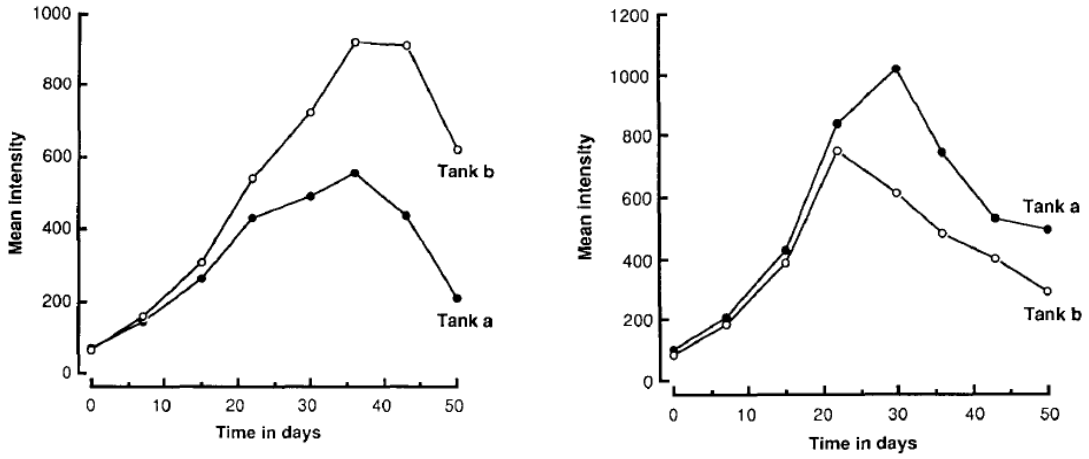


Figure 1.23. The course of *Gyrodactylus salaris* Malmberg, 1957 (haplotype A) infection on individually isolated salmon parr originating from the Scottish River Conon (left) and from the River Shin (right), expressed as the average mean intensity of two replicates groups [graphs from Bakke & MacKenzie, 1993].

Later, Dalgaard *et al*. (2003) flew a sample of *S. salar* from the River Conon to Denmark and assessed their susceptibility to a strain of *G. salaris* collected from the River Lærdaselva, Norway (most likely haplotype F, see Hansen *et al*., 2003). The 0+ fish were either infected with *G. salaris* following the normal procedures or treated with corticosteroids in order to induce a state of stress before they were exposed to the parasite. As expected, the treated salmon were more susceptible to infection and had a mean intensity of ~280 parasites fish^{-1} by the end of the experiment (8 weeks). The untreated salmon, by comparison, had 98 parasites fish^{-1}, and in both cases a 40% fish mortality

occurred (Dalgaard *et al*., 2003). A subsequent trial by Dalgaard *et al*. (2004) tested the River Conon population again alongside three other populations of *S. salar* from Canada, Denmark and Sweden, and a population of rainbow trout from Denmark, and the River Conon stock showed similar susceptibilities to *G. salaris* to those described by Dalgaard *et al*. (2003).

1.7. Existing control methods and management

The uncontrolled increases in parasite numbers on wild Atlantic salmon populations have necessitated extreme measures to manage infections, and in Norway these management measures have included the use of the plant extract rotenone, a broad spectrum piscicide, to "clean out" the entire fish population within a *G. salaris* infected river (Bakke *et al*., 2007). Rotenone has been used and reused in several Norwegian rivers with mixed success (*e.g.* River Skibotnelva was treated twice but both treatment failed to completely eliminate *G. salaris* infection; see Winger *et al*., 2012), but given environmental concerns regarding its use and its alternative "treatment", *i.e.* aluminium sulfate, there is a drive to identify alternatives for use in farms and in rivers where approval has been granted. *Gyrodactylus salaris* currently costs Norway £38 million p.a.; £23 million is linked to impacts on tourism and restrictions imposed on fishing and the associated industries, whilst the remaining £15 million results from on-going surveillance programmes and treatments. Whilst *G. salaris* infections in farmed fish populations can be readily controlled using formalin, *etc.*, infections on wild fish pose a series of logistical problems including the scale and volume of certain water systems, their complexity and species diversity, treating salmonids which are highly mobile hosts, impacts on non-target species and on the environment, and cost (Shinn & Bron, 2012). Commonly used, alternative treatments are those used elsewhere in the salmonid industry, *i.e.* salt and formalin. Both of these latter control compounds are effective (Buchmann *et al*., 2004; see

treatment review of Schelkle *et al*., 2009, 2011), but salt treatment requires use of large quantities. Its use in the UK, for example, is now regulated by the local protection agency, and it is difficult to administer, and there are human health concerns regarding the use of formalin. In some countries like Italy, the use of formalin is already restricted. The current alternative to rotenone is aluminium sulfate, $Al_2(SO_4)_3$ (see Soleng *et al*., 1999; Bakke *et al*., 2007). Acidified aluminium sulfate is being trialled as an alternative because it does not kill salmonids but does appear effective at removing *G. salaris* which is unable to survive at below pH 5. Current "treatments" are given as a 10 to 14 day regime followed by a rotenone treatment (dose ~100 μg L^{-1} for both treatments). Although trials using $Al_2(SO_4)_3$ are on-going, within the UK at least, there are concerns regarding the impact of aluminium on the environment and on human health, *e.g.* Alzheimer's (Doll, 1993). The UK's concern is well-founded in that the UK's largest poisoning incident involved undiluted aluminium sulfate entering the domestic water supply *via* the water treatment plant at Camelford, UK (www.guardian.co.uk/society/2012/mar/14/camelford-water-poisoning-inquest).

Temperature has also been explored to control *G. salaris* infections. Rintamaki (1989) demonstrated that the reproduction of *G. salaris* decreases if the water temperature exceeds 16°C and that the parasite prefers low temperatures (Malmberg, 1973). Salinity also appears to be an effective method to control the reproduction of *G. salaris*, which readily reproduces on rainbow trout at salinities of ~5‰. At higher salinities, *i.e.* 7.5‰, the population slowly declines (56 days at 6–12°C) (Soleng & Bakke, 1997). At higher salinities yet, *i.e.* 20‰, *G. salaris* can survive for a few days but can continue to reproduce if returned to freshwater after 8 hours. This tolerance to salinity supports the hypothesis that *G. salaris* may disperse through brackish water (Bakke *et al*., 2002).

Most recently the utility of octopamine-like compounds in disrupting the behaviour of gyrodactylids has been investigated (Brooker *et al*., 2011). These compounds affected

the ability of parasites to locate and remain on their hosts, inhibiting their movements and inducing death at low concentrations of 0.2 μM (Brooker *et al.*, 2011). While such compounds, which are invertebrate specific, may have value, those tested in Brooker *et al.* (2011) were not chosen for their practical applicability, *i.e.* they were assessed to determine whether such classes of compound have an effect on *Gyrodactylus* species rather than these being candidate compounds to replace the use of rotenone and aluminium sulfate for use in rivers. There is much work to be done in this area of monogenean research and the situation of *G. salaris* in Norway and the threat it poses to salmon populations elsewhere, including the UK, gives urgency to the search for an effective, safe alternative treatment.

1.8. Aims of the thesis

The overarching aim of the current thesis is to improve our understanding of the emergence and control of gyrodactylid infections linked with fish diseases. To achieve this, the current research project investigates several lines of research surrounding *Gyrodactylus salaris* and other potentially pathogenic species associated with emergent disease problems.

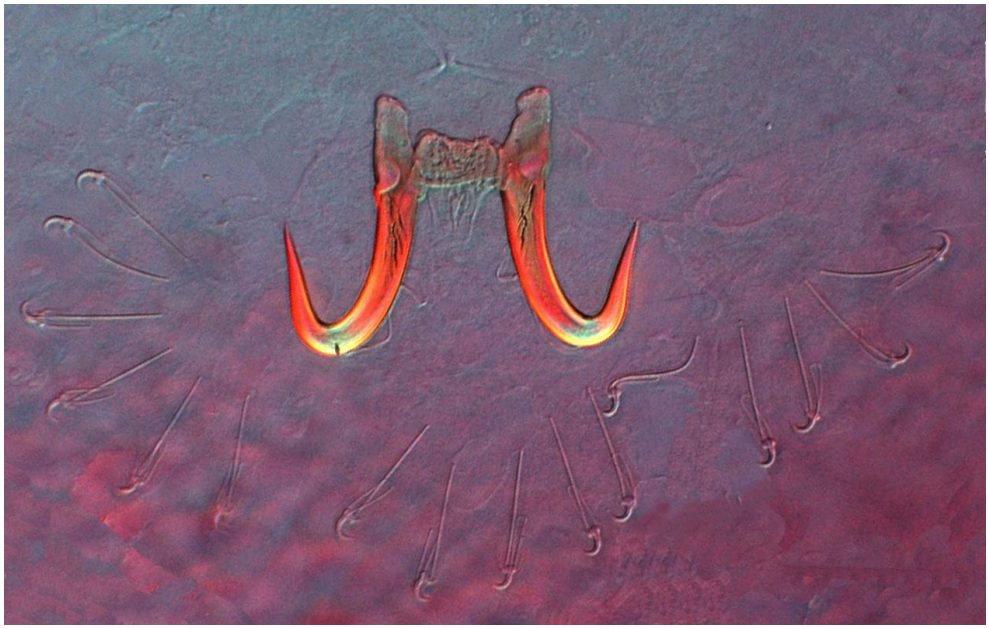
More in details, the specific aims of this thesis are as follows:

1) To re-evaluate the geographical distribution of *G. salaris* throughout Europe. The first report of *G. salaris* in Poland and then subsequently in Italian populations of farmed rainbow trout (Paladini *et al*., 2009a; Chapter 2) in the early stages of this research project highlighted a need to define the distribution of *G. salaris* within Europe. To achieve this, a review of the known geographical distribution was required, including the collection of new *Gyrodactylus* material from salmonids obtained from a number of European states. This study and its findings are reported on and discussed in Chapter 3.

2) To identify and discriminate *G. salaris* from other potentially pathogenic *Gyrodactylus* species. This study required a comprehensive understanding of gyrodactylid taxonomy. To achieve this it was necessary to be exposed to a large number of species in a range of formats. The Aquatic Parasitology Laboratory within the Institute of Aquaculture regularly receives parasite material for either evaluation as part of its diagnostic service or has material donated for research purposes. Some of the fish and parasite samples received during the tenure of this study led to the discovery and description of several new species. Some of these new species, which impact on reared aquaculture species, are presented in Chapters 4 and 5. Some of these are highlighted because they may represent potential emerging pathogens.
3) To determine the relative susceptibilities of English and Welsh populations of salmonids, *i.e.* Atlantic salmon, brown trout and grayling, to *G. salaris* for the first time. National contingency plans in the UK have been based on the assumption that British salmonids would respond in the same way as their Scandinavian counterparts. This study, detailed in Chapter 6, represents one of the central issues of this research project.
4) To investigate for alternative treatments to control and manage *Gyrodactylus* infections. While there is a general consensus that rotenone and aluminium sulfate are inappropriate options for the management of *G. salaris* infections, there has been little work to look for alternatives. Chapter 7 represents a preliminary investigation and begins by assessing the suitability of broad spectrum disinfectants such as bronopol and of natural compounds like tannic acid, determining whether they have an impact on the survival of different species of *Gyrodactylus*. The action of these two compounds is assessed against two species of *Gyrodactylus*, *i.e.* the OIE-notifiable pathogen *G. salaris* and *Gyrodactylus arcuatus*.

Chapter 2

The first report of *Gyrodactylus salaris* in Italy



Gyrodactylus salaris Malmberg, 1957 from farmed Italian rainbow trout, *Oncorhynchus mykiss* (Walbaum) [original image].

Paper I

Paladini G., Gustinelli A., Fioravanti M.L., Hansen H., Shinn A.P. (2009). The first report of *Gyrodactylus salaris* Malmberg, 1957 (Platyhelminthes, Monogenea) on Italian cultured stocks of rainbow trout (*Oncorhynchus mykiss*). *Veterinary Parasitology*, 165: 290–297.

Aspects of this paper were presented as:

Paladini G., Hansen H., Fioravanti M.L., Shinn A.P. (2009). The potential impact of monogeneans on Italian fish stocks. *Proceedings of the 6th International Symposium on Monogenea (ISM6), Cape Town, South Africa, 2nd-7th August 2009*: P15 (poster).

2.1. General introduction of Paper I

The following paper has been published in *Veterinary Parasitology* officially reporting for the first time in Italy the presence of *Gyrodactylus salaris* Malmberg, 1957, alongside three other species: *Gyrodactylus derjavinoides* Malmberg, Collins, Cunningham *et* Jalali, 2007; *Gyrodactylus teuchis* Lautraite, Blanc, Thiery, Daniel *et* Vigneulle, 1999; and *Gyrodactylus truttae* Gläser, 1974. These four species were found on Italian farmed rainbow trout, *Oncorhynchus mykiss* Walbaum, which were collected from several regions in the northern and central Italy. The report of *G. salaris* on Italian *O. mykiss* represents an important finding, given the economic impact that *G. salaris* has had on Atlantic salmon, *Salmo salar* L., in Scandinavia and the size of the Italian farmed rainbow trout industry.

2.2. Authors' contribution

For this study, I personally visited all the farm sites and collected the gyrodactylid material with my colleague Dr Andrea Gustinelli from the University of Bologna. Following collection, all the material was transported to the Institute of Aquaculture, University of Stirling and then analysed. Prior to this study, Dr Andrew P. Shinn provided training in *Gyrodactylus* taxonomy and systematics. I carried out both morphometric and morphological methodologies, therefore, all the measurements and the pictures have been taken by me. Dr Haakon Hansen, working in an OIE-reference laboratory in Norway, performed the molecular part of this study. I drafted the first version of the paper which was subsequently revised with my co-authors Dr Andrew Shinn, Professor Maria Letizia Fioravanti, Dr Andrea Gustinelli and Dr Haakon Hansen. All authors read and approved the final version of the manuscript.

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The first report of *Gyrodactylus salaris* Malmberg, 1957 (Platyhelminthes, Monogenea) on Italian cultured stocks of rainbow trout (*Oncorhynchus mykiss* Walbaum)

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ABSTRACT

The monogenean *Gyrodactylus salaris* Malmberg, 1957 is considered one of the most important parasites of wild salmonids in the European Community due to the heavy ecological and economical damage it has inflicted on Atlantic salmon (*Salmo salar*) parr populations. Rainbow trout (*Oncorhynchus mykiss*) is susceptible to *G. salaris* and can act as a suitable carrier host and, consequently, its trade in EU territory is restricted in relation to the status of "recognized free" zones. Despite the economic importance of rainbow trout farming in Italy, information on the Italian gyrodactylid fauna is lacking and prior to this study, *G. salaris* had not been officially reported. During a routine health examination of farmed rainbow trout stock throughout Central and Northern Italy in 2004–2005, five fish farms were found to be infected with *G. salaris* alongside three other gyrodactylids. Morphological and molecular characterisation confirmed the presence of *G. salaris*, *Gyrodactylus teuchis* Lautraite, Blanc, Thiery, Daniel et Vigneulle, 1999 and *Gyrodactylus derjavinoides* Malmberg, Collins, Cunningham et Jalali, 2007, while *Gyrodactylus truttae* Gläser, 1974 was identified by morphological analysis only. The findings from this study extend the distribution of *G. salaris* within Europe and highlight the importance of the rainbow trout trade in its dissemination.

1. Introduction

The importance of *Gyrodactylus salaris* Malmberg, 1957 is demonstrated by the heavy losses that this monogenean has caused over the last thirty years in parr and smolt stages of wild Atlantic salmon (*Salmo salar* L.), principally throughout Norway (Johnsen et al., 1999; Bakke et al., 2007). Apart from being a parasite of Atlantic salmon, *G. salaris* can colonise and reproduce on a wide number of salmonid species without clinical signs of disease and these hosts represent important carriers of the parasite (Bakke et al., 2002). In particular, rainbow trout (*Oncorhynchus mykiss* Walbaum) is considered as an ideal reservoir for *G. salaris*, being a very receptive and generally asymptomatic host (Bakke et al., 2002). For this reason, the movement of rainbow trout within the European Community is strictly regulated and is permitted only between regions of equivalent health status (Peeler et al., 2006).

Most species of *Gyrodactylus* can be differentiated by the morphological features of their haptoral hard parts (Malmberg, 1970) and/or by differences in their ribosomal internal transcribed spacer (ITS1 and ITS2) regions

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(Matejusová et al., 2001; Ziętara and Lumme, 2002). The discrimination of *G. salaris* from other related species is, however, not always straightforward. *Gyrodactylus salaris* and the purported non-pathogenic *Gyrodactylus thymalli* Žitňan, 1960 from grayling, *Thymallus thymallus* L., are morphologically similar, their ITS sequences are practically identical, and recent studies indicate that the two species might be conspecific (Ziętara and Lumme, 2002; Hansen et al., 2003, 2006, 2007; Meinilä et al., 2004). Analyses of the mitochondrial cytochrome oxidase I gene (COI), however, show that *G. salaris* and *G. thymalli* can be grouped in different clades and that all the gyrodactylids from rainbow trout appear to belong to the same COI haplotype (Hansen et al., 2003; Meinilä et al., 2004). Although characterisation by COI does not seem to be linked to the virulence of *G. salaris* (Hansen et al., 2007), its determination, however, does provide hints to its possible origin and relationship to other populations within the species. Similarly *Gyrodactylus teuchis* Lautraite, Blanc, Thiery, Daniel et Vigneulle, 1999 was first considered to be morphologically similar to *G. salaris* but it was later shown that it could be characterised as a separate species based on its ITS sequences (Lautraite et al., 1999; Cunningham et al., 2001).

Rainbow trout production in Italy represents a significant proportion of the nation's freshwater production with over 39,000 tons/year (API, 2008), but data on infection of *Gyrodactylus* spp. of farmed salmonids is scarce. With reference to *G. salaris*, Molnár and Ghittino (1977) reported a gyrodactylid from cultured rainbow trout and brown trout (*Salmo trutta* L.) from Italy but no further studies were carried out to confirm the identity of these or to define their distribution throughout the country. The current study, therefore, set out to establish the gyrodactylid fauna on captive held populations of rainbow trout in Northern and Central Italy.

2. Materials and methods

During the period March to May 2005, five Italian rainbow trout farms were visited throughout the Central and Northern regions of Italy and a sample of stock at each site was examined for the presence of *Gyrodactylus* spp. Ten fish, ranging in 10–40 cm total length, were sampled from each site. Fish were euthanased and a representative mucus sample was taken from the body and fins of each fish using a scalpel and then fixed immediately in 70% ethanol for analysis in the laboratory. The fish farms situated on five different water systems were positive for *Gyrodactylus*: the River Sile, Veneto (45°38′23.18″N, 12°08′14.29″E), the Avisio Torrent, Trentino Alto Adige (46°16′42.70″N, 11°26′39.52″E), the River Sérchio, Tuscany (44°02′52.00″N, 10°27′39.74″E), and two sites in the Umbria region, the Clitunno Fountain (42°44′41.95″N, 12°42′24.81″E) and the River Nera (42°51′41.38″N, 12°58′48.84″E) (Fig. 1).

Gyrodactylid parasites were isolated from the fixed mucus and prepared for morphological and molecular analyses. Individual specimens were placed on a glass slide, the haptor was removed using a scalpel and subjected to proteolytic digestion using a modification of the method given in Harris and Cable (2000), *i.e.* 3 μl of digestion solution (100 μg/ml proteinase K (Cat # 4031-1, Clontech UK Ltd., Basingstoke, UK), 75 mM Tris–HCl (Sigma–Aldrich, Poole, UK), 10 mM EDTA (Sigma–Aldrich), 5% SDS (Sigma–Aldrich)) added to each haptor. The digestion of each specimen was continuously monitored under a 4× objective on an Olympus SZ30 dissecting microscope. Tissue digestion was then arrested and mounted *in situ* by the addition of 2 μl of a 1:1 saturated ammonium picrate: 100% glycerine mix. The edges of the coverslip were then sealed with nail varnish.

The digested, ammonium picrate glycerine mounted specimens were photographed using a JVC KY-F30B 3CCD camera with an interfacing 2.5× top lens fitted to an Olympus BH2 compound fitted with phase contrast under a 100× oil immersion objective and features of the hooks measured using Zeiss KS300iC/Windows release ver 3.0 (1997) (Carl Zeiss Vision GmbH, Munchen, Germany/Imaging Associates Ltd., Thame, Oxfordshire, UK) software. For identification, a total of 25 point-to-point morphometrics (11 on the hamulus, 6 on the ventral bar and 8 on the marginal hooks) were made on each specimen (see Shinn et al., 2004) using the purpose written software PointR ver 1.0 (© Shinn and Bron, 2003, University of Stirling, UK) within KS300.

The excised body of each gyrodactylid was transferred to an individual, labelled 1.5 ml Eppendorf tube and stored in 95% ethanol until required. A limited number of specimens from each of the sampled locations were available for molecular analysis. DNA was extracted from individually isolated specimens using the DNEasyKit or Mini Kit (Qiagen) following the manufacturer's instructions. The primer pairs ITS1A (5′-GTAACAAGGTTTCCG-TAGGTG-3′) and ITS2 (5′-TCCTCCGCTTAGTGATA-3′) (Matejusová et al., 2001) were used to amplify a fragment spanning the 3′ end of the 18S subunit, ITS1-5.8S-ITS2 and the 5′ end of the 28S subunit. In instances where this full fragment did not amplify, the primers ITS4.5 and ITS2 (Matejusová et al., 2001) were used to amplify the ITS2 region separately. ITS2 alone contains less variation than ITS1, but nevertheless differs between all species studied herein.

The primer pairs ZMO1 (5′-GCGMCTAAATGCTT-TAAGGGCTTG-3′) and ZMO4 (5′-GAGGATAGCAC-TATCCCTGTCAC-3′) (Hansen et al., 2003) were used to amplify the mitochondrial COI. All PCR reactions were performed with puRe Taq Ready-to-Go PCR beads (Amersham Biosciences) in a GeneAmp PCR System 9700 (Applied Biosystems) using the following protocol: 4 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 50 °C and 2 min at 72 °C.

All PCR-products were purified using a QIAquick PCR Purification Kit (Qiagen) or Macherey-Nagel NucleoSpin® Extract II according to the manufacturer's recommendations. Both DNA strands were sequenced on a MEGABACE 1000 (GE Healthcare) using DyeET-terminator mix (GE-Healthcare) and were carried out in 10 μl reactions. The PCR primers and the internal primers ITS1R (5′-ATTTGCGTTCGAGAGACCG-3′) and ITS2F (5′-TGGTGGAT-CACTCGGCTCA-3′) (Ziętara and Lumme, 2003) were used for sequencing of the full ITS fragment. The ITS2 fragments

Fig. 1. The location of the rainbow trout farms sampled throughout Italy and found to be positive for *Gyrodactylus* von Nordmann. (1) Avisio Torrent, Trentino Alto Adige (46°16′42.70″N, 11°26′39.52″E); (2) River Sile, Veneto (45°38′23.18″N, 12°08′14.29″E); (3) River Sérchio, Tuscany (44°02′52.00″N, 10°27′39.74″E); (4) Clitunno Fountain, Umbria (42°44′41.95″N, 12°42′24.81″E); (5) River Nera, Umbria (42°51′41.38″N, 12°58′48.84″E).

were sequenced using the PCR primers only. Amplified fragments of COI were sequenced using the PCR primers in addition to ZMO2 (5′-CCAAAGAACCAAAATAAGTGTTG-3′) and ZMO3 (5′-TGTCYCTACCAGTGCTAGCCGCTGG-3′) (Hansen et al., 2003). Sequences were proofread in VectorNTI (Invitrogen) and identity established by submitting the sequences to a GenBank BlastN search (http://www.ncbi.nlm.nih.gov/) (Altschul et al., 1990; Zhang et al., 2000). Calculation of genetic distances was performed in Mega 4.0 (Tamura et al., 2007).

In addition, one formalin fixed sample of *Gyrodactylus* was found within the fish pathology archive held by the Department of Veterinary Public Health and Animal Pathology, University of Bologna. The skin scrape, which contained five gyrodactylids, was collected in April 2000 from an unspecified rainbow trout farm within the Veneto region. The gyrodactylids were rinsed in distilled water and then prepared as whole mounts in ammonium picrate glycerine and identified by morphometry and morphology.

The sampling protocols and experimentation conducted throughout the course of the investigation complied with the laws and statutes of Italy and the diagnostic approaches required by OIE (Office International des Epizooties - World Organisation for Animal Health) for the confirmation of *G. salaris* (OIE, 2006).

3. Results

Gyrodactylids were recovered from the mucus scrapes taken from the body and the fins of the fish sampled at each of the five rainbow trout farms that were visited. All fish were found to be infected (100%) with approximately 10 (range 5–16) gyrodactylids being recovered from each site. On the basis of morphological features taken from ~10 parasites per site ($n = 53$ gyrodactylids in total), the following species were identified: *Gyrodactylus derjavinoides* Malmberg, Collins, Cunningham et Jalali, 2007, *G. salaris*, *G. teuchis* and *G. truttae* Gläser, 1974 (Table 1). Not

Table 1
A summary of the methods used to identify the specimens of *Gyrodactylus* von Nordmann, 1832 collected from the five Italian rainbow trout farms visited during the current study.

Region	*G. derjavinoides*	*G. salaris*	*G. teuchis*	*G. truttae*
Trentino Alto Adige ($n = 5$)	–	4 M; 1 ITS2[a]; 1 COI[b]	–	1 M; 1 ITS[c]; 1 COI[c]
Tuscany ($n = 16$)	–	16 M; 3 ITS1-2[a]; 3 COI[d]	–	–
Umbria (R. Nera; $n = 15$)	3 M; 2 ITS1-2[e]; 1 COI[c]	2 M; 1 ITS1-2[f]; 1 COI[g]	10 M; 3 ITS1-2[h]; 1 COI[c]	–
Umbria (Clitunno Fountain; $n = 11$)	4 M	2 M	5 M	–
Veneto (R. Sile; $n = 6$)	–	6 M; 3 ITS2[a]; 2 COI[b]	–	–
Veneto (archive sample; $n = 5$)	–	5 M	–	–

In addition, one *Gyrodactylus* positive sample collected from an unspecified rainbow trout farm dated April 2000 and deposited in the University of Bologna fish tissue archive was also examined. Each specimen was identified initially by morphology and morphometry (M) and then, where possible, confirmed by comparing the base sequence of its cytochrome oxidase 1 gene (COI) and the internal transcribed spacer 1 and 2 regions (ITS1-2) with sequences held in NCBI GenBank (http://www.ncbi.nlm.nih.gov/).

[a] 100% ITS identity with NCBI acc. nos. Z72477, DQ916137, DQ919059, AF484544 and AF328871.
[b] 100% COI identity with haplotype F from rainbow trout (Hansen et al., 2003).
[c] Not amplified.
[d] Corresponds to haplotype F from rainbow trout (Hansen et al., 2003).
[e] 100% ITS identity with NCBI acc. no. AF484530.
[f] 100% ITS identity with NCBI acc. nos. DQ919059 and AF328871.
[g] New haplotype (acc. no. GQ370816).
[h] 100% ITS identity with NCBI acc. no. AJ249350.

every species was found at all farms; *G. salaris*, however, was present on the stock at all five farms, notably Tuscany and Veneto where *G. salaris* was the only gyrodactylid species found. *Gyrodactylus teuchis* was identified from the two farm sites located in the Umbria region (Clitunno Fountain and River Nera), alongside *G. salaris* and *G. derjavinoides*. *Gyrodactylus truttae* was found in Trentino Alto Adige (Avisio Torrent) together with *G. salaris*. As the morphology of the attachment hooks of *G. salaris* and *G. teuchis* are similar and photographic images of the latter have not been formerly presented elsewhere, figure plates of these alongside *G. derjavinoides* and *G. truttae* are provided to assist in their future identification and discrimination (Figs. 2 and 3).

From Figs. 2 and 3, the four species of *Gyrodactylus* can be readily discriminated from each other based on the unique shape of the marginal hook sickle. As all specimens were collected in the same season (March–May 2005) and from water bodies of a similar temperature (~11–12.5 °C), the size of the attachment hooks of the four species can be compared. *Gyrodactylus salaris* is the largest of the four species, the total length of the hamuli and the marginal hooks were ~76 μm and ~39.5 μm respectively compared to ~68 μm and ~36 μm for *G. teuchis*, ~63 μm and ~23.5 μm for *G. truttae*, and 55 μm and ~33 μm for *G. derjavinoides*.

Although frequently drawn, relatively few photographic images of the male copulatory organ (MCO) exist within the literature; those of *G. derjavinoides*, *G. salaris* and *G. teuchis* are presented for the first time. Only one specimen of *G. truttae* was found in the current study but this individual was prepared for molecular analysis and therefore there was no opportunity to look at the configuration of spines on the MCO. The armature of the MCO, however, in the former three species are different from each other. The MCO of *G. salaris* which measures approximately 30 μm in diameter, bears a single arch of 6–7 spines (2 large, terminal ~6.2 μm long and 4–5 medium-sized ~5.3 μm long central spines). In addition, 4–6 small, circular studs (~1.2 μm in diameter), the precise structure of which have not been formally described, are observed scattered around the main arch of spines but are not in any set, discernible configuration (Fig. 2f–h). The MCO of *G. teuchis*, which measures ~25 μm in diameter, bears 4–5 similar, large-sized spines (~5 μm long) arranged in a single arch with 1–2 visible small, circular studs (Fig. 3f and g). The MCO of *G. derjavinoides* measures 26 μm in diameter and bears 8 spines in a single arch (2 medium-sized, terminal spines ~4.4 μm long and 6 smaller-sized ~3.2 μm long central spines). No circular studs were observed (Fig. 3j).

Only fourteen specimens were available for molecular characterisation by sequencing of the ITS1 and ITS2 or ITS2 separately (Table 1). From these samples, *G. salaris*, *G. teuchis* and *G. derjavinoides* were confirmed. Only one specimen of *G. truttae* was available for the molecular analysis and no sensible sequence reads were obtained from it. For the seven specimens identified as *G. salaris* by morphological analysis or by sequencing of ITS, the base sequence of their cytochrome oxidase I genes were also determined. Six of the sequences corresponded to the mitochondrial F haplotype that is common in rainbow trout farms across Europe (see Meinilä et al., 2004; Hansen et al., 2003, 2006, 2007) and three of these sequences contained some ambiguities that could be the result of PCR or sequencing errors. The last sequence represents a new haplotype of *G. salaris* and is submitted under GenBank accession number GQ370816. This haplotype (774 bp) differs from haplotype F with 11 nucleotide substitutions (K2-distance: 0.0147) and is not identical to any other currently known haplotypes. The most closely related sequences in GenBank are AY225307 and AY225308 (5 nucleotide substitutions).

4. Discussion

Although gyrodactylosis represents a common and economically significant parasitic disease of rainbow trout

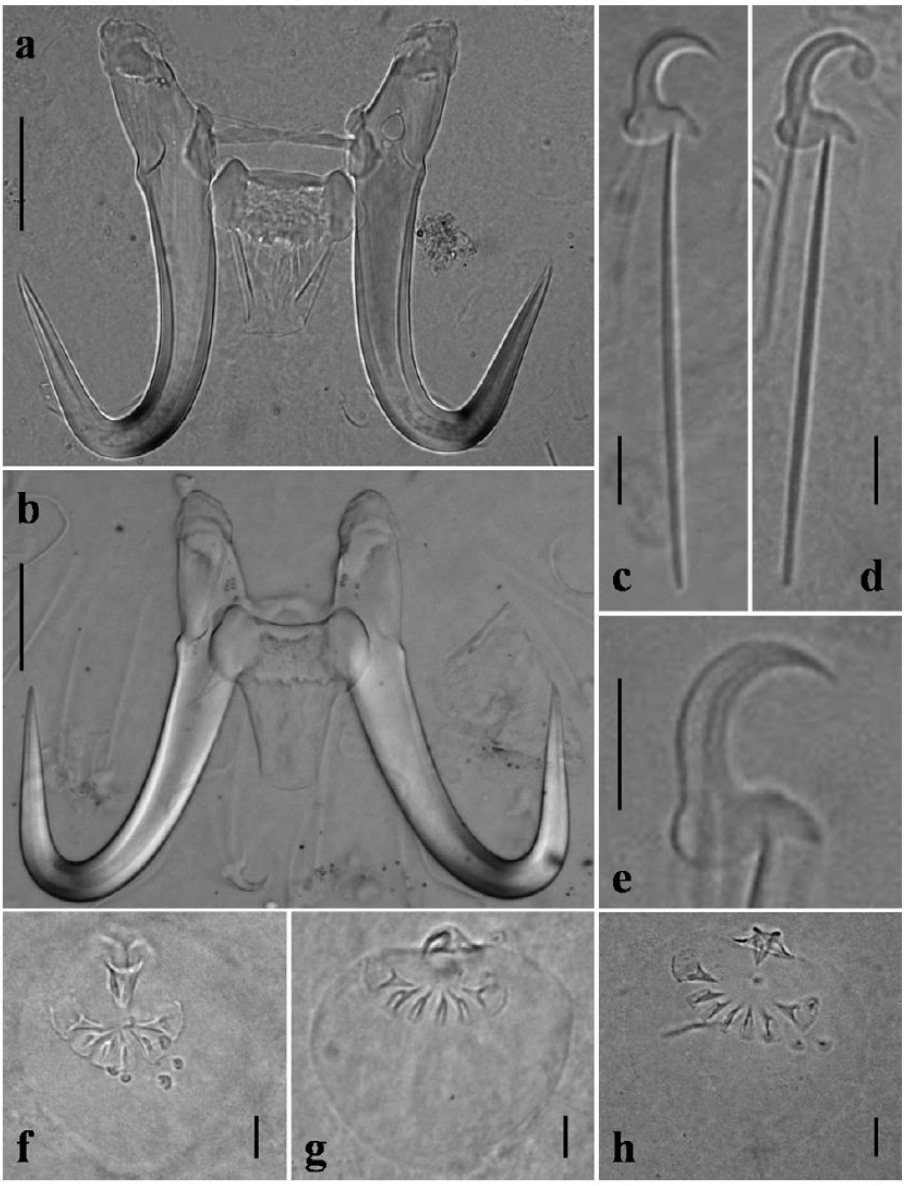

Fig. 2. Light micrographs of the haptoral hard parts and male copulatory organ of *Gyrodactylus salaris* Malmberg, 1957. (a and b) Central hook complex; (c–e) marginal hooks; (f–h) male copulatory organ. Scale bars, a and b = 20 μm; c–h = 5 μm.

farmed in Italy (Fioravanti and Caffara, 2007), studies aimed at identifying the *Gyrodactylus* species involved in its aetiology have been scarce. Molnár and Ghittino (1977) commented on the occurrence of a gyrodactylid "morphologically like *G. salaris*" on cultured rainbow trout and brown trout, but prior to the current study, this report was not confirmed and the figures that were presented in the earlier account do not permit a definitive identification. *Gyrodactylus derjavinoides* (cited as *G. derjavini* Mikhailov, 1975), however, is already known from Italy and has been reported from Italian brown and rainbow trout (Malmberg, 1993).

Malmberg and Malmberg (1993) suggested that *G. salaris* originated in the Baltic area; it is known to occur naturally at low intensities in this area including the Russian Onega and Ladoga water systems and within some Swedish and Finnish rivers that drain into the Baltic Sea (Ieshko et al., 1996; Shulman et al., 2000). Throughout Europe, *G. salaris* has also been reported from Norway (Johnsen and Jensen, 1991; Johnsen et al., 1999), from rivers on the Swedish west coast (Malmberg and Malmberg, 1993; Alenäs, 1998), Denmark (*e.g.* Buchmann and Bresciani, 1997; Buchmann et al., 2000), Finland (Rimaila-Pärnänen and Wiklund, 1987; Keränen et al., 1992; Koski

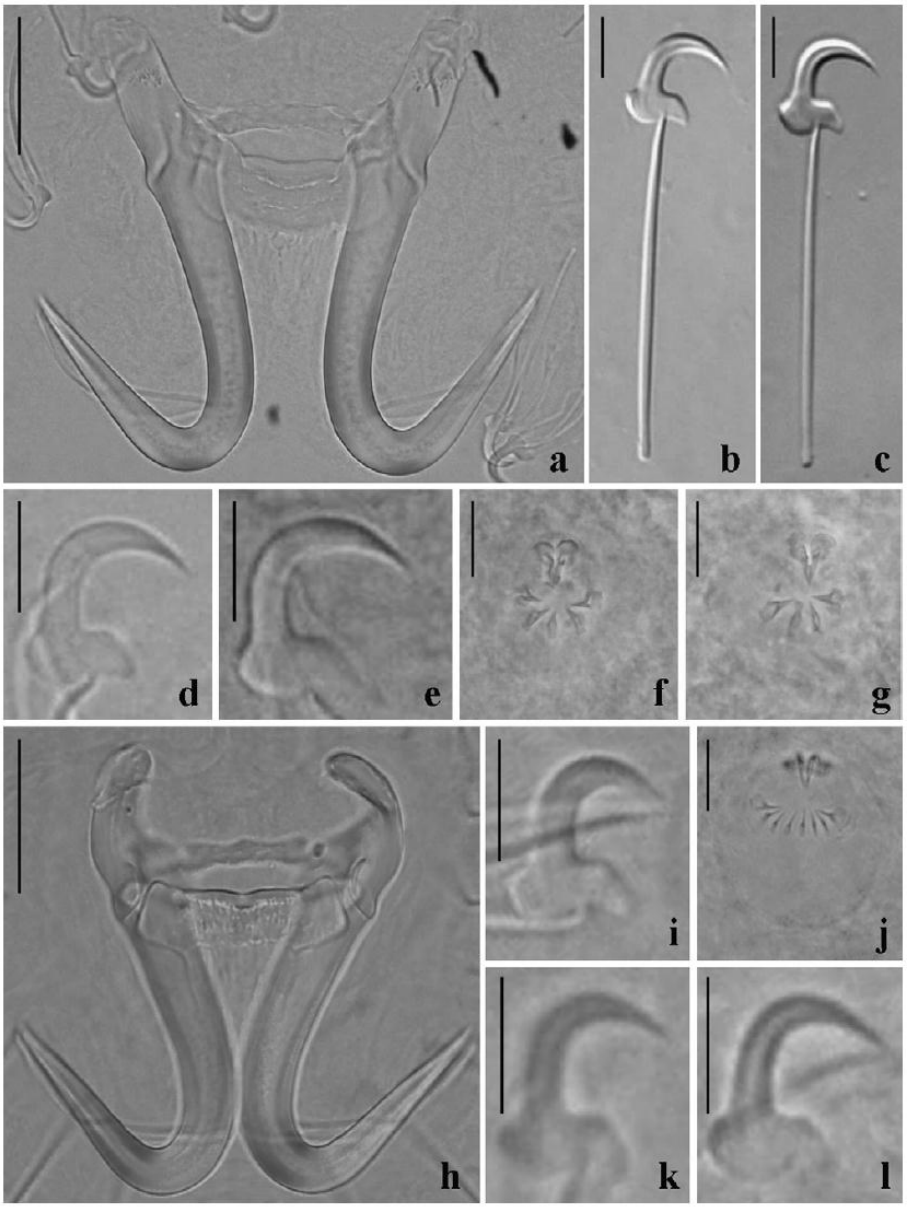

Fig. 3. Light micrographs of the haptoral hard parts of *Gyrodactylus teuchis* Lautraite, Blanc, Thiery, Daniel et Vigneulle, 1999 (a–g), *Gyrodactylus derjavinoides* Malmberg, Collins, Cunningham et Jalali, 2007 (h–j) and *Gyrodactylus truttae* Gläser, 1974 (k and l). *Gyrodactylus teuchis*: a, central hook complex; b and c, marginal hooks; d and e, marginal hook sickles; f and g, male copulatory organ; *G. derjavinoides*: h, central hook complex; i, marginal hook sickle; j, male copulatory organ; *G. truttae*: k and l, marginal hook sickles. Scale bars, a, h = 20 μm; f–g, j = 10 μm; b–e, i, k–l = 5 μm.

and Malmberg, 1995; Koski, 1996; Rintamäki-Kinnunen and Valtonen, 1996), Russia (Ergens, 1983; Ieshko et al., 1996, 1997; Meinilä et al., 2004), Germany (Lux, 1990; Dzika et al., 2009), Spain (Malmberg, 1993), France (Johnston et al., 1996) and, most recently, from Poland (Rokicka et al., 2007). While the presence of *G. salaris* has been confirmed by molecular methods for many of these, the reports from certain countries, *i.e.* France, Spain and Portugal, awaits verification.

In addition to Atlantic salmon, *G. salaris* has also been recorded in the wild from other salmonids such as Arctic charr (*Salvelinus alpinus* L.) (see Table 2 in Bakke et al., 1992; Robertsen et al., 2007) and Adriatic trout (*Salmo obtusirostris* Heckel) (see Žitňan and Cankovic, 1970). Although brown trout has a limited susceptibility to *G. salaris* (Mo, 1988; Jansen and Bakke, 1995), it has nevertheless been reported on this host in the wild on several occasions (*e.g.* Mo, 1988). It is also common in many

rainbow trout farms across Europe (Lux, 1990; Koski and Malmberg, 1995; Meinilä et al., 2004). In addition to these salmonid hosts, *G. salaris* has also experimentally been shown to attach and survive for a short period on some non-salmonid fish species such as the eel (*Anguilla anguilla* L.) and the flounder (*Platichthys flesus* L.) (see Mo, 1987), which may act as transport hosts (see Table 2 in Bakke et al., 1992).

The *G. salaris* findings from the current study, however, prompted a detailed study of preserved fish material from farm archives dating back to 2000. The formalin fixed *Gyrodactylus salaris* specimens were identified solely on hook morphology suggesting that this species may have been in the country for the past nine years. This latter finding has provided the impetus for a larger study of gyrodactylids on Italian salmonids which is currently underway.

Only one specimen of *G. truttae* from Trentino Alto Adige (Avisio Torrent) was found in the current study. Given the small number of fish that were sampled from each site and the sampling strategy that was used (*i.e.* skin scrapes), the likelihood of *G. truttae* occurring on stock held at the other farms cannot be ruled out. *Gyrodactylus teuchis* has previously been reported in France, sporadically in Denmark and the UK and appears to be common on rainbow trout in Polish fish farms (Lautraite et al., 1999; Cunningham et al., 2001; Rokicka et al., 2007). Lautraite et al. (1999) found *G. teuchis* to be widely distributed on both wild and farmed salmonids from Brittany to the Western Pyrénées, and the current survey now extends its distribution into Italy. In the current study, *G. teuchis* was found at two sites in Central Italy and, on both occasions, in association with *G. salaris*.

This study represents the first confirmed presence of *G. salaris* in Italy, which was the most commonly encountered gyrodactylid species on farmed rainbow trout and this extends the reported range of this parasite in Europe. Identification of most of the *G. salaris* specimens as haplotype F (Table 1), which are common in rainbow trout farms, provides supporting evidence to suggest that *G. salaris* has mainly been spread via the rainbow trout trade rather than from the local indigenous fish population. The finding of a new haplotype on rainbow trout is not surprising as several haplotypes have been recovered from salmon and grayling (Hansen et al., 2003, 2006, 2007; Meinilä et al., 2004). Further investigation, however, is needed to ascertain whether this infection originates from rainbow trout introduced to the farm or from wild fish in the River Nera. This survey together with recent studies on gyrodactylids in rainbow trout farms in Europe (*e.g.* Rokicka et al., 2007; Dzika et al., 2009) points to the importance of this industry for spreading of *G. salaris* in Europe. It seems more than likely that the examination of rainbow trout farms in other countries will extend the range further.

Acknowledgements

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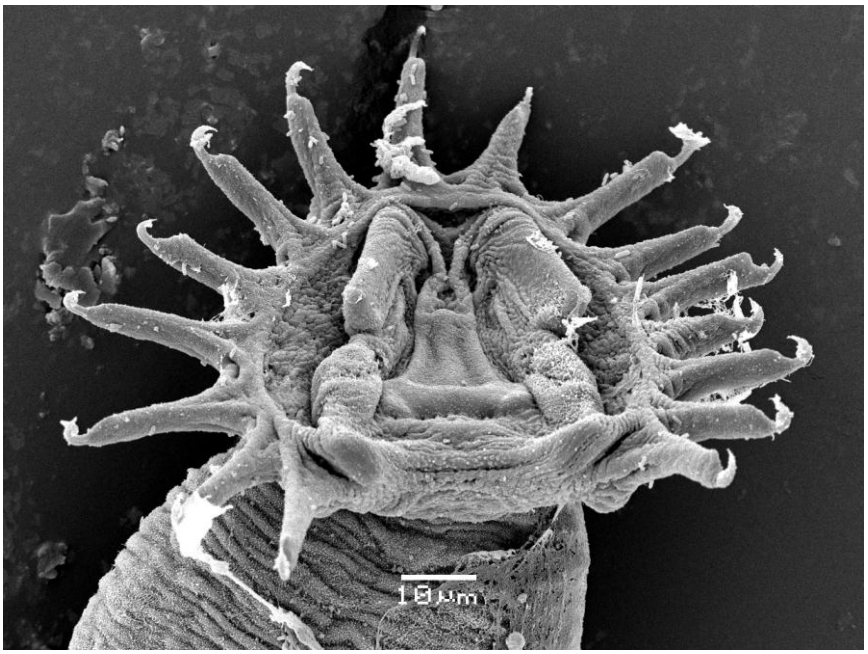
Chapter 3

Geographical distribution of *Gyrodactylus salaris* Malmberg, 1957 (Monogenea, Gyrodactylidae) throughout Europe



The haptor of *Gyrodactylus salaris* Malmberg, 1957 haplotype A [original image].

Aspects of this work were presented as:

Paladini G. (2012) *Gyrodactylus*: tales of invasion, resistance and control strategies. *Aquaculture UK 2012, Aviemore, Scotland, 23rd-24th May 2012* (talk).

Paladini G., Williams C., Hansen H., Taylor N.G.H., Rubio-Mejía O.L., Denholm S.J., Hytterød S., Bron J.E., Shinn A.P. (2012) *Gyrodactylus salaris*: the good, the bad and the ugly. *Institute of Aquaculture 3rd PhD Research Conference, Stirling, Scotland, 24th October 2012*: 16 (talk).

3.1. Introduction

Gyrodactylus salaris Malmberg, 1957 has been shown to be extremely pathogenic to the Atlantic strain of *Salmo salar* L., and to a lesser degree to the Baltic strain (Bakke *et al*., 1990; Bakke, 1991; see Chapter 6). Whilst *G. salaris* has been reported from at least nine salmonid hosts (see www.gyrodb.net), its occurrence on rainbow trout, *Oncorhynchus mykiss* (Walbaum), a species which is traded extensively across Europe, is of particular concern (Peeler & Thrush, 2004; Peeler & Oidtmann, 2008). Rainbow trout have been demonstrated to be susceptible to *G. salaris* infection, and although these infections are self-limiting, they can persist for up to 90 days or more (Bakke *et al*., 1991a). Low levels of infection and the absence of evident clinical signs means that the parasite could go undetected in a consignment of fish (Peeler & Thrush, 2004; Peeler & Oidtmann, 2008). This, coupled with the ability of hosts to carry an infection for long periods, increases the window of exposure and raises concerns regarding the movement of rainbow trout, in terms of their potential role as a carrier and source of *G. salaris* infection of other susceptible fish populations, across Europe.

3.1.1. OIE guidelines for the identification of G. salaris

In the *Manual of Diagnostic Tests for Aquatic Animals* the Office International des Epizooties (OIE) recommends that the diagnosis of *G. salaris* should be based on information resulting from a combination of both morphological and molecular analyses (OIE, 2012). This approach has not always been followed in the past and certain *G. salaris* reports have been based solely on data derived from morphological investigations (*e.g.* recording of *G. salaris* in Germany by Lux, 1990) or, in other cases based on molecular data only (*e.g.* recording of *G. salaris* in Latvia by Hansen *et al*., 2003). To help understand the existing distribution and recording of *G. salaris* across Europe, a map

grayscale coding each *G. salaris*-positive country by the diagnostic method used to characterise the record is presented in Fig. 3.2 and Table 3.1.

3.1.2. G. salaris vs G. thymalli*: a taxonomic challenge*

The difficulty in discriminating *G. salaris* from *Gyrodactylus thymalli* Žitňan, 1960 has been stressed and debated over by many authors (Malmberg, 1987; McHugh *et al*., 2000; Sterud *et al*., 2002; Meinilä *et al*., 2004; Olstad *et al*., 2007). If morphology alone is considered, then while there are some subtle differences in the marginal hook sickles to permit the discrimination of these two species (McHugh *et al*., 2000), host information is ideally required to support identification. Olstad *et al.* (2007), however, looking at a large data set of material collected from 10 populations, suggested that an *a priori* species delineation based on host alone is not possible and that more information is required to support identification. The study of Shinn *et al.* (2010), however, demonstrates that when all supporting information is removed and morphology experts are asked to make an identity based on the specimen only (*i.e.* no supporting information relating to host or location, *etc*.), then misclassification rates of between 4.88–29.27% for six *G. salaris* morphology experts and greater, *i.e.* 4.88–100%, for six *Gyrodactylus* morphology experts were seen. Although the internal transcribed spacer (ITS) region of the rRNA gene is frequently used in the description and discrimination of most *Gyrodactylus* species, this region for *G. salaris* and *G. thymalli* is nearly identical (Cunningham, 1997; Ziętara & Lumme, 2002), and so differences in the intergenic spacer (IGS) and cytochrome oxidase I (COI) are used instead to discriminate these two species (Sterud *et al*., 2002; Cunningham *et al*., 2003; Meinilä *et al*., 2004). Despite some morphological similarities, *G. thymalli* appears restricted to grayling, *Thymallus thymallus* (L.), whilst *G. salaris* has never been recorded from grayling in nature (Soleng & Bakke, 2001a). Although this study comments on whether the *G. salaris*-positive status of each country is valid, it does not necessarily

enter into debate on the validity of other species of *Gyrodactylus* parasitising salmonids, therefore, the distribution of *G. thymalli* from grayling throughout Europe is not considered.

3.1.3. Aims of the study

The aim of the present study is to provide a revised update of *G. salaris* in each European state, supplemented and supported by the analysis of additional *Gyrodactylus* specimens collected from salmonid populations from certain European states. Although the European distribution of *G. salaris* has been discussed several times in the scientific literature (Malmberg, 1993; Bakke *et al*., 2007; Paladini *et al*., 2009a), some of the countries reported as being *G. salaris* positive were based on misidentifications of morphologically similar species, whilst other *G. salaris* countries appear to have been overlooked.

3.2. Materials and methods

To further investigate the *status inquirendae* for the presence of *G. salaris* in certain European states, *e.g.* Portugal and Spain, additional salmonid samples were collected and screened. The results from each of these additional samples will be commented upon under the entry for each country. These specimens included new material from rainbow trout from Finland, Germany, Italy, Portugal and Spain between the period 2008 and 2010.

In order to provide a revised update of the *G. salaris* distribution across Europe, a literature review was necessary. This literature, however, was not always easily accessible, *e.g.* very old papers; no electronic versions; records only mentioning the presence of the parasite, but not providing any evidence of the correct identification; and, in many cases, the papers were published in their original country language; therefore, a translation to

English was necessary to extrapolate reports and data. Given the amount of work involved in hunting the literature and fill in the gaps of the countries with the unknown or unclear *G. salaris*-status, the historical records of each country where *G. salaris* has been reported, officially or unofficially, are listed below in the "Results" section, in chronological order.

3.2.1. Acquisition of Gyrodactylus *specimens from Finland, Germany, Italy, Portugal and Spain*

Ten ethanol fixed specimens of *Gyrodactylus* collected from a rainbow trout farm in the Jyväskylä region of Finland (location withheld for confidentiality) were donated to the Institute of Aquaculture (IoA), University of Stirling (UoS) by Professor E. Tellervo Valtonen. The specimens had already been removed from their hosts and no details regarding the number of hosts they were collected from, or the size of infection were available, but it is assumed they were taken from multiple hosts. The specimens were analysed following morphological and molecular methods.

Twenty ammonium picrate glycerine-mounted specimens of *Gyrodactylus* collected from a rainbow trout stock from an undisclosed fish farm in Germany were sent to the Parasitology Laboratory at IoA (UoS) by Professor Ewa Dzika, and their identity was assessed by morphology and morphometrics only.

Between the period 2008-2009, twenty-seven samples of *Gyrodactylus* were collected from twenty Italian rainbow trout farms located in seven different regions (Friuli-Venezia Giulia, Lombardy, Piedmont, Trentino-Alto Adige, Tuscany, Umbria and Veneto) throughout the central and northern regions of Italy (fish farm sites undisclosed for confidentiality). From each site, ten fish ranging from 10-40 cm total length were sampled and examined for the presence of gyrodactylids. Fish were euthanised by an overdose of 100 mg L^{-1} Finquel® (Argent Chemical Laboratories, Redmond, WA, USA) and a sample of their mucus collected and fixed in 80% ethanol by scraping the body and fins of each

fish using the back edge of a scalpel blade. The parasites were identified by both morphological and molecular approaches.

A Portuguese sample of fins removed from 10 fingerling rainbow trout (~15 cm total length), that had been fixed in 80% ethanol, were sent to IoA, UoS in December 2008 (site details withheld). A total of three specimens of *Gyrodactylus* were found. Each specimen was subjected to morphological and molecular examination.

A sample of 60 *Gyrodactylus* specimens collected from ten rainbow trout fingerlings from a farm in the Galicia region of Spain (site details withheld) in 2010 was sent by a local contact, and subsequently processed for morphological and molecular analyses. Representative specimens were prepared as whole mounts and cleared *in situ* using ammonium picrate glycerine.

3.2.2. Morphological analysis

The specimens collected were prepared for both morphological and molecular analyses following the methods detailed in Paladini *et al*. (2009a) and Shinn *et al*. (2010). When unmounted parasites were available, gyrodactylids were cleaned of extraneous mucus using mounted triangular surgical needles (size 16, Barber of Sheffield, UK) and observed under an Olympus SZ40 dissecting microscope at ×4 magnification. Each individual specimen was then transferred to a glass slide and cut in half with a scalpel blade. The anterior part was transferred to a 1.5 mL Eppendorf containing 95% ethanol for subsequent molecular characterisation (Paladini *et al*., 2009a). The posterior part of the specimen, containing the attachment organ, was subjected to proteolytic digestion, to remove tissue surrounding the attachment hooks, following the method detailed in Paladini *et al*. (2009a) which is a modification of the protocol given in Harris and Cable (2000). Tissue digestion was arrested and sclerites mounted *in situ* by the addition of 2 µl of a 1:1 saturated ammonium picrate: 100% glycerine mix solution. The edges of the coverslip

were then sealed with common nail varnish to make a semi-permanent mount. The digested specimens were then photographed using a JVC KY-F30B 3CCD camera with an interfacing ×2.5 top lens fitted to an Olympus BH2 compound microscope with phase contrast.

3.2.3. Molecular analysis

The corresponding upper parts of the parasite bodies, previously stored in 95% ethanol, were subjected to molecular characterisation, which was performed by Dr Haakon Hansen at the Norwegian Veterinary Institute of Oslo. DNA was extracted only from the specimens collected from Finland, Italy, Portugal and Spain, using DNeasy® Blood & Tissue minikit (Qiagen). To amplify (PCR) a fragment spanning the 3′ end of the 18S ribosomal RNA subunit, internal transcribed spacers 1 and 2 (ITS1 and ITS2), the 5.8S subunit and the 5′ end of the 28S subunit, the primer pair ITS1A (5′-GTAACAAGGTTTCC GTAGGTG-3′) and ITS2 (5′-TCCTCCGCTTAGTGATA-3′) (Matejusová *et al*., 2001) were used. The PCR reactions were performed with PuReTaq Ready-To-Go™ PCR beads (GE Healthcare) following the manufacturer's instructions. The PCR program was as follows: 4 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 55°C and 2 min at 72°C. PCR products were then purified using a NucleoSpin® Purification Kit (Macherey–Nagel) and sequencing reactions were carried out on a MegaBACE 1000 analysis system (GE Healthcare) using DYEnamic ET dye terminators. For sequencing, the internal primers ITS4.5 (5′-CATCGGTCTCTCGAACG-3′) (Matejusová *et al*., 2001), ITS1R (5′-ATTTGCGTTCGAGAGACCG-3′), ITS18R (5′-AAGACTACCAGTTCACT CCAA-3′), ITS2F (5′-TGGTGGATCACTCGGCTCA-3′) and ITS28F (5′-TAGCTCTAG TGGTTCTTCCT-3′) (Ziętara & Lumme, 2003) were used in addition to the PCR primers. The obtained sequences (ITS1, 5.8S and ITS2 only) were

proofread and assembled in Vector NTI 11 (Invitrogen) and subjected to a BlastN search (Zhang *et al*., 2000).

3.3. Results

A re-evaluation of the distribution of *G. salaris* across Europe showed that this species is currently known from 17 countries throughout Europe, although its presence has been reported from 23 countries (Fig. 3.2). The report from Slovakia is not considered valid, whilst the identity of the specimens recovered from some countries, *e.g.* France, Portugal and Spain, are questionable and their *G. salaris* status requires further re-examination. The specimens from these latter three countries were most likely *Gyrodactylus teuchis* Lautraite, Blanc, Thiery, Daniel *et* Vigneulle, 1999, a species bearing some morphological similarities to *G. salaris* but undescribed at the time of the "*G. salaris*" report for each country.

The *G. salaris* records for each European state are discussed below chronologically by the date of first official observation. The acquisition of new *Gyrodactylus* material from Finland, Germany, Italy, Portugal and Spain and its subsequently examination, is discussed under each European state. A summary table listing only the valid reports of *G. salaris* by country is presented in Table 3.1.

3.3.1. Chronological record of G. salaris *in each European state*

1951 - Sweden

In 1951, Dr Göran Malmberg based at the University of Gothenburg, received a sample of *Gyrodactylus* collected from Atlantic salmon held at the experimental fish farm station in Hölle (now Hölleforsens Laxodling) situated on the River Indalsälven, Sweden. The findings from this material were reported on six years later, although the description of

G. salaris was performed on one specimen only (see Malmberg, 1957). In 1954, the salmon parr held at the Hölle farm were observed to harbour a heavy *G. salaris* infection. While the salmon from the River Indalsälven responded well to treatment, the salmon parr originating from the River Gullspångsälven, emptying into the Lake Vänern in western Sweden, proved to be more difficult to treat. This was the first observation regarding the differential sensitivity and susceptibility of Atlantic salmon stocks to *G. salaris* (see Malmberg, 2004). Since then, *G. salaris* has been recorded from salmonids from 11 rivers on the Swedish west coast draining into the Kattegat and Skagerak (Malmberg & Malmberg, 1993; Koski & Malmberg, 1995; Buchmann *et al*., 2000). The parasites found in the Swedish rivers draining into the North Sea, however, are suggested to originate from the Baltic Sea (Hansen *et al*., 2003). *Gyrodactylus salaris* is believed to occur naturally in Sweden and is not considered pathogenic in the wild, as supported by several records of *G. salaris* infection in the Baltic watershed, without causing any host mortalities (Malmberg & Malmberg, 1991, 1993).

As a generalisation, whilst infections of *G. salaris* on Swedish populations of Atlantic salmon do not appear to be particularly pathogenic (Bakke *et al*., 2002; Dalgaard *et al*., 2003, 2004), not all the Baltic strains of Atlantic salmon are resistant to *G. salaris*. In 1998, Alenäs and colleagues (Alenäs, 1998; Alenäs *et al*., 1998) reported a 90% decrease in the salmon parr density from the River Säveån, a tributary to the River Göta älv, which were infected with high burdens (~1700 specimens $fish^{-1}$) of *G. salaris* (tentatively haplotype E, based on the map and tables presented in Hansen *et al*., 2003). It is important to note that this river drains into Lake Mjörn, which is closer to the Atlantic side of Sweden than to the Baltic side. This could be a possible explanation for the unexpected pathogenicity of *G. salaris* on this strain of Atlantic salmon. Another hypothesis could be that the haplotype of *G. salaris* found on this *S. salar* stock may be more pathogenic than those found on the Baltic strain of *S. salar*. The haplotype

pathogenicity between different fish populations has not been tested yet, and therefore it would be interesting to verify their potentially different virulence. *Gyrodactylus salaris* haplotype A, which is widespread throughout Norway where it commonly infects Atlantic salmon, has also been found in Sweden from the Rivers Ätran and Surtan (Alenäs *et al*., 1998; Hansen *et al*., 2003), suggesting that this pathogenic haplotype is not confined geographically and could be widespread. This is also the case for haplotype B, which has been recorded from Norway and Sweden, whilst haplotype F has also been reported from Denmark, Finland, Italy and Russia (Hansen *et al*., 2003; Meinilä *et al*., 2004; Jørgensen *et al*., 2008; Paladini *et al*., 2009a).

1960 – Ukraine

A parasitological survey on 295 fish sampled from two Ukrainian rivers, the Tisa and the Seret, found *G. salaris* on brown trout, *Salmo trutta fario* L., collected from the River Seret (Kulakovskaja, 1967). Later in 1973, Malmberg (1973) reported finding *G. salaris* on *S. trutta fario* collected from a Carpathian hatchery, and although he did not specify the exact location of the hatchery at the time, in a later account Malmberg (1993) indicated that these represented specimens originating from the River Seret that had been donated by Dr Kulakovskaja to Dr Malmberg back in 1960. Further records of *G. salaris* result from an investigation conducted by Tesarcik and Ivasik (1974) on brown trout and rainbow trout (referred to as its old name *Salmo gairdneri irideus*) sampled from a number of Carpathian ponds. The authors reported finding *G. salaris* on both hosts from ponds fed by the Rivers Dniester and Danube, within the Ukraine (Tesarcik & Ivasik, 1974).

In 1983, Ergens described *Gyrodactylus* sp. material collected from the fins of *S. trutta fario* from two localities within the Autonomous Republic of Crimea, Ukraine. The first sample was taken in 1975 from the River Salgir, whilst the second sample, collected in 1976, was from the River Angara (Ergens, 1983). Ten years later, Malmberg (1993)

suggested that *Gyrodactylus* sp. *sensu* Ergens, 1983 was a synonym of *G. salaris*. No specimens of *G. salaris* collected from Ukrainian waters, however, have been confirmed by molecular methods.

1967 – Bosnia and Herzegovina

The first two reports of *G. salaris* from *S. salar* bred in Bosnia and Herzegovina date back to 1967 (Čanković & Kiškarolj, 1967; Žitňan, 1967). Žitňan and Čanković (1970) later recorded *G. salaris* from rainbow trout and brown trout from the Rivers Buna and Pliva, which run through two fish farms sited at Blagaj and Jezero, near the towns of Jajce and Mostar, respectively, and from Adriatic trout *Salmo obtusirostris* Heckel, from the River Buna site. Ergens (1983) listed the species of *Gyrodactylus* collected from the Rivers Buna and Pliva as *Gyrodactylus truttae* Gläser, 1974, and it is not clear whether Ergens (1983) based this assumption on a re-examination of the specimens that were collected during the earlier study or on the assessment of new material that was collected. The validity of this record was also questioned by Bakke *et al.* (1992a), an opinion based on Tanum's (1983) assessment of the material, who considered the reports of *G. salaris* from *O. mykiss* and from *S. trutta fario* as misidentifications, but not the record of *G. salaris* from *S. obtusirostris*. Following Žitňan and Čanković's (1970) study, several other reports of *G. salaris* infections from the skin and fins of rainbow trout fry were recorded from three fish farms situated at Blagaj near the town Jajce, Ljuta near Konjic and Jezero near Mostar, where mortalities of 3-5% were reported, and also from the River Ribnik (Imamović, 1984, 1987). Although the report of Imamović (1987) is considered as valid, it is not possible to verify this based on the drawings of the attachment hooks that are presented in the paper.

1972 – Russia (including the Republic of Karelia but not Kaliningrad)

Specimens of *Gyrodactylus* sp. were collected by Ergens and Rumyantsev in June 1972 from *S. salar* caught in Lake Ladoga, Republic of Karelia (Ergens, 1983). A subsequent re-examination of these specimens and a comparison with the re-described type material of *G. salaris*, confirmed that the Karelian material was *G. salaris* (see Ergens, 1983). Although the first record of *G. salaris* in Russia appears to have been made by Yekimova (1976) working in the River Pechora, a subsequent re-examination of the specimens suggested that this was a misidentification (Dorovskikh, 2000; Kudersky *et al*., 2003). One year after the re-description of *G. salaris*, its occurrence on *S. salar* from River Pyalma, Lake Onega, Republic of Karelia was reported (Permyakov & Rumyantsev, 1984). *Gyrodactylus salaris* has also been recorded on salmon from the River Keret with prevalences close to 100% and mean intensities of approximately 300 parasites $fish^{-1}$, suggesting that this parasite is a likely cause for the decline of the salmon parr population in this river (Ieshko *et al*., 1995). The introduction of *G. salaris* to the River Keret in Russia was suggested to originate from Finland by anthropogenic activities, following an epidemic in the White Sea salmon stock in Russia (Malmberg, 1993; Mo, 1994; Johnsen *et al*., 1999; Bakke *et al*., 2004), although it was not clear exactly when the parasite introduction took place. The confirmation of this though was not possible until a mitochondrial DNA-based analysis was conducted (Meinilä *et al*., 2002). Following mitochondrial characterisation, the presence of *G. salaris* in the River Keret appeared to originate from the Vyg (White Sea) hatchery during the period 1986-1989, when native salmon juveniles were transported by helicopter (Kuusela *et al*., 2005). Kuusela *et al*. (2005) and Ieshko *et al*. (2008) suggested that the same canvas bag had been used to transfer fish to Lake Onega, where the parasite normally resides and does not cause any damage. The presence of *G. salaris* has also been recorded from the landlocked salmon population in the River Pistojoki, Lake Kuitozero (Meinilä *et al*., 2004), but this strain of

G. salaris is most likely to have originated from rainbow trout that were stocked into fish farms in Kuusamo, Finland, upstream of the Pistojoki (Kuusela *et al*., 2005). The molecular identification of *G. salaris* from Russian *S. salar* has been confirmed by a number of authors (Cunningham *et al*., 2003; Meinilä *et al*., 2004; Kuusela *et al*., 2007, 2009).

Given the size of the Russian landmass, it might be advisable in the future to divide the country into zones when considering the occurrence of *G. salaris*. Defining these "zones", however, is not a simple matter and may be restricted to *G. salaris*-positive watersheds, as there are no geographic features that would otherwise limit its spread across the entire country. *Gyrodactylus salaris* has not, however, been reported from the Russian exclave Kaliningrad, which is positioned between Poland and Lithuania. Although both latter states have been reported as being *G. salaris* positive, the record for Lithuania is based on a single web reference (Cefas; www.westcountry angling.com/pdf/gyrodactylus_salaris.pdf) and requires verification. *Gyrodactylus* material, therefore, from this region is required before comment on its *G. salaris* status can be made.

1974 – Czech Republic

The first report of *G. salaris* in Czech Republic results from the study conducted by Tesarcik and Ivasik (1974) which included the North-Moravian River Moravice in the Czech Republic and Carpathian ponds in the Ukraine. Specimens of *G. salaris* were collected from brown trout from the River Moravice, and although no images of the attachment hooks are presented in Tesarcik and Ivasik's (1974) account, the record is considered as valid.

The discovery of a *G. salaris*-morphologically similar species, namely *Gyrodactylus bohemicus* Ergens, 1992 from farmed *O. mykiss* and *Salvelinus fontinalis*

(Mitchill) in the Czech Republic (Ergens, 1992), raises the question of whether this is a valid species or a misidentification, also due to the absence of molecular data. Ergens (1992) commented on the morphological similarities of *G. bohemicus* with *G. thymalli* and *Gyrodactylus magnus* Konovalov, 1967, but made no reference to *G. salaris*. Although three paratypes of *G. bohemicus* are deposited in the monogenean collection maintained by the Institute of Parasitology, Czech Academy of Sciences, these represent valuable specimens and are not available for scientific loan. However, pictures of the paratypes of *G. bohemicus* were kindly taken by Dr Roman Kuchta, and their morphological examination during the course of this study suggests a very close similarity to *G. salaris* (*personal identification*; see Fig. 3.1). Lindenstrøm *et al*. (2003) also remarked on the similarities between the two species; further comments on this, however, must wait until more specimens can be collected and evaluated through a molecular comparison with congeners. It is for these latter reasons that Bakke *et al*. (2007) suggested that *G. salaris* is probably absent from the Czech Republic, but comments that a detailed study to establish its presence or otherwise would be worthwhile. In a study carried out by Matejusová and colleagues (2001), a single specimen of *Gyrodactylus* was recovered from a brown trout sampled from the River Vlára. The identity of this specimen, however, was not clearly defined and it was referred to as *G. salaris*/*G. thymalli* (see Matejusová *et al*., 2001). There are, however, other reports of "*G. salaris*" from the Czech Republic, which represent misidentifications of either *Gyrodactylus derjavinoides* Malmberg, Collins, Cunningham *et* Jalali, 2007 and/or *G. truttae*, neither of which had been discovered and described at the time the relevant "*G. salaris*" report was made. These include the record of *Gyrodactylus* specimens from brown trout from the River Osoblaha (Ergens, 1965) and from rainbow trout from a fish farm near the town Český Krumlov (Lucký, 1963). In a study by Řehulka (1973), specimens of brown trout, rainbow trout and brook trout were infected with *G. salaris sensu* Ergens, 1961, which later was determined to be a misidentification of *G.*

truttae, whose attachment hooks vary markedly in size from those of *G. salaris* (see Mo, 1983; Ergens, 1992).

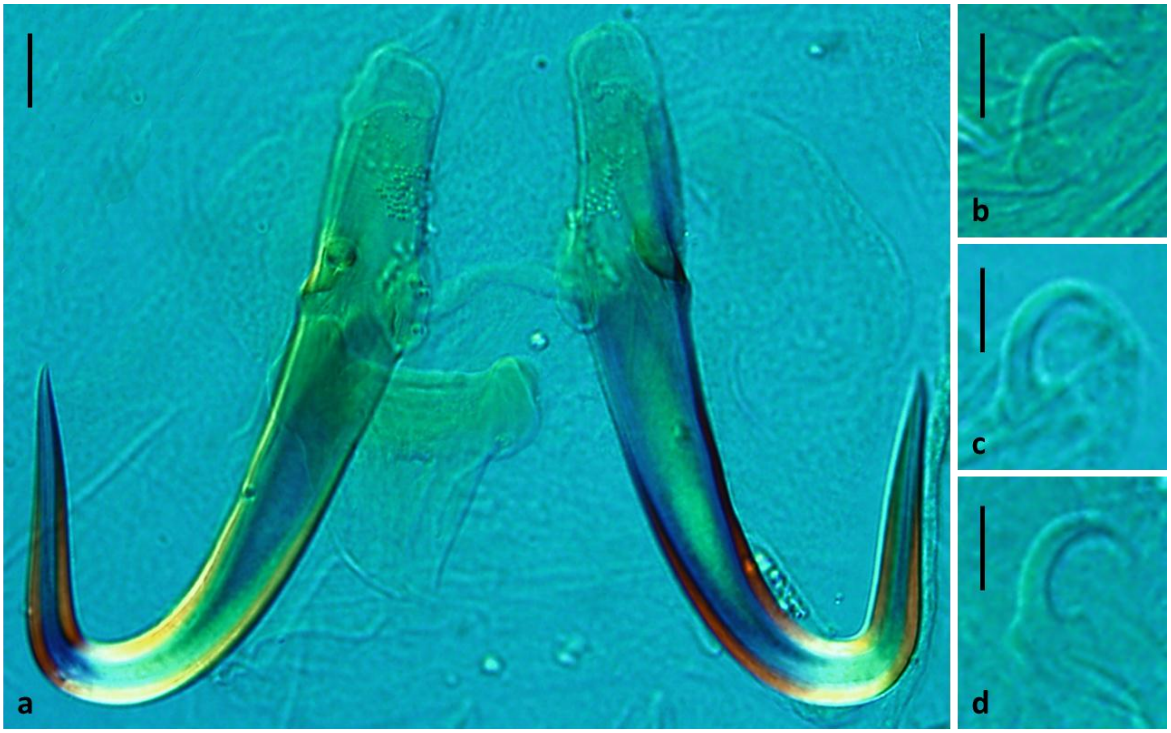


Figure 3.1. Light micrographs of the paratypes (acc. no. M-342) of *Gyrodactylus bohemicus* Ergens, 1992 from farmed *Oncorhynchus mykiss* (Walbaum) and *Salvelinus fontinalis* (Mitchill) from Czech Republic. **a**: hamulus complex; **b**–**d**: marginal hook sickles. Scale bars: a = 10 μm; b–d = 5 μm [images kindly provided by Dr R. Kuchta].

1975 – Norway

The first observation of *G. salaris* in Norway was made in 1975 at the Akvaforsk fish hatchery in Sunndalsøra, Møre and Romsdal County (Bergsjö & Vassvik, 1977), although Johnsen and Jensen (1991) indicated that the first official record was made by Tanum (1983). In the same year (1975), *G. salaris* was found in the Rivers Lakselva and Ranaelva following the heavy loss of Atlantic salmon parr stocks (Johnsen, 1978). The first assumption was that *G. salaris* occurred naturally in Norway, but later studies suggested that this parasite had been introduced, most likely from Sweden (Heggberget &

Johnsen, 1982; Johnsen & Jensen, 1986, 1992; Mo, 1994) and that the Atlantic strain of *S. salar* was more susceptible to infection than the Baltic strain (Bakke *et al*., 2004). The true story on how the parasite has been introduced, however, is still controversial. According to Johnsen and Jensen (1991), *G. salaris* was accidentally introduced with a consignment of parr from Sweden into Norway for aquaculture purposes, whilst Winger (2009) suggested that in 1978, infected salmon smolts being transported by a vehicle were accidentally dumped in the Norwegian River Skibotnelva and one year later the presence of *G. salaris* was observed in that river (Heggberget & Johnsen, 1982; Mo, 1994).

In 1983, gyrodactylosis on salmon by *G. salaris* was declared a notifiable disease in Norway and from then, the Norwegian government introduced active measures to control and eradicate the parasite from infected river systems (Mehli & Dolmen, 1986; Dolmen & Mehli, 1988). Given the high level of mortality seen in the wild, *G. salaris*-infected salmon populations, the Norwegian salmon authorities approved the treatment of the rivers with the non-selective insecticide and pesticide rotenone, which is a natural extract of the leguminous plant *Derris elliptica*. This biocide, however, kills the fish as well as the parasites by inhibiting the transfer of electrons in mitochondria (Marking & Bills, 1976). Although rotenone has been administered in several rivers to control *G. salaris* infections (Arnekleiv *et al*., 2001; Eriksen *et al*., 2009), not all treatments have proven successful, with either some infected fish avoiding treatment or other infected fish moving in from elsewhere (Mo, 1988; Winger *et al*., 2007). As such, some rivers have had to be treated several times, *e.g.* although the River Skibotnelva in northern Norway has been treated twice, it is now re-infected again (Winger *et al*., 2007). Despite these problems, the cost–benefit plan made by Krokan and Mørkved (1994) justified the use of rotenone to be deployed on a large scale, with some modification to the treatment (Haukebø *et al*., 2000) after *G. salaris* was found to re-establish in certain rivers (Bakke *et al*., 2007). The dose of rotenone used ranges from 0.5–5.0 ppm of a 5% formulation, but

typically is around 2 ppm (*i.e.* 100 ppb active ingredient). In addition to the use of rotenone, the use of acidified aluminium sulfate [$Al_2(SO_4)_3$] to eradicate the parasite infection in Norwegian rivers is now being explored, and current treatments are a 10–14 day aluminium treatment followed by a rotenone treatment (Soleng *et al*., 1999; Poléo *et al.*, 2004; Bakke *et al*., 2007).

To date, 46 salmon rivers have been infected in Norway since the first record of *G. salaris*; current infections account for an estimated annual loss of between 250 and 500 tonnes of salmon (see Table 2 in Bakke *et al*., 2007). The molecular identification of *G. salaris* throughout Norway has been confirmed by many authors (*e.g.* Cunningham *et al*., 2003) with numerous sequences deposited in GenBank. Hansen *et al.* (2003) was the first to characterise six different *G. salaris* mitochondrial haplotypes from Atlantic salmon from throughout Norway and Sweden. Although further haplotypes have since been described (Meinilä *et al*., 2004; Hansen *et al*., 2007b; Paladini *et al*., 2009a), the studies suggest that instead of a single introduction of *G. salaris* into Norway, there have been several (Bakke *et al.*, 2007; Hansen *et al*., 2007b).

1983 – Georgia

Malmberg (1993) suggested that *Gyrodactylus salaris* was also present in Georgia, given that the description of *Gyrodactylus* sp. *sensu* Ergens, 1983 was shown to be a synonym of *G. salaris*. The report of this species from *S. trutta fario* collected from Chernorechenskoye fish farm in 1978 by Ergens (1983), therefore, is considered as valid, although future collections should, additionally, be verified by molecular-based approaches.

1984 – Finland

Although Rintamäki (1989) reported the presence of *G. salaris* on Baltic salmon dating back to 1984, the first official record of this parasite from Finnish fish farms was published in 1987 by Rimaila-Pärnänen and Wiklund (1987), who reported an infection on 18 fish farms that were studied between 1986 and 1987. Rintamäki (1989) reported moderate to heavy infections of *G. salaris* on Baltic salmon from the Ossauskoski fish farm situated on the River Kemijoki, resulting in 8% mortality in the one-year-old fish stocks. The occurrence of *G. salaris* from salmon fish farms connected to the River Iijoki and the River Kemijoki and, also reported for the first time from Finland, on rainbow trout, presented no clinical signs of disease (Keränen *et al*., 1992). An additional investigation was carried out by Koski and Malmberg (1995) on a number of rainbow trout and salmon farms in northern Finland, who confirmed finding *G. salaris* on salmon and rainbow trout without linked mortality. During these surveys, they also found *Gyrodactylus lavareti* Malmberg, 1957 only on rainbow trout in a mixed infection with *G. salaris* (see Koski & Malmberg, 1995). The presence of *G. salaris* originating from Finland has also been confirmed by molecular analysis (see Cunningham *et al*., 2003; Meinilä *et al*., 2004; Kuusela *et al*., 2007). During the course of the present study, ten additional *Gyrodactylus* specimens from rainbow trout reared in the Jyväskylä region were confirmed as *G. salaris* by both morphology and molecular-based approaches (*personal identification*; part of the molecular results are also presented in Table 1 in Shinn *et al*., 2010).

Parts of Finland, however, have been declared *G. salaris*-free under EC Decision (see section below of *G. salaris*-free states).

1990 – Germany

Lux (1990) was the first to report *G. salaris* in Germany from a survey of rainbow trout farms in the Brandenburg, Saxony and Thuringia districts, but in the absence of

supporting molecular data, the validity of this particular report is doubtful (Bakke *et al*., 2007). In 2003, however, Cunningham *et al.* (2003) acquired a sample of specimens from a rainbow trout in Berlin and confirmed them as being *G. salaris* by analysis of the ribosomal RNA intergenic spacer (IGS) region. In 2005, Dzika *et al.* (2009) sampled a rainbow trout pond at Rogg in Bavaria, on a tributary of the River Danube, and reported finding *G. salaris* alongside *G. derjavinoides*, *G. truttae* and *G. teuchis*. However, the accuracy of the *G. salaris* drawings, notably those of the marginal hooks, questions the validity of the identification, given that no reference specimens were deposited in a national collection, nor was molecular analysis conducted. During the course of the present PhD project, 20 specimens of *Gyrodactylus* from *O. mykiss* reared in Germany were kindly donated by Dr Ewa Dzika. These specimens were mounted in ammonium picrate glycerine and confirmed as *G. salaris* by morphological identification only, supporting the findings of Dzika *et al*. (2009), at least for *G. salaris* (*personal identification*). Voucher specimens of *G. salaris* from this sample will be deposited in the Parasitic Worms collection in The Natural History Museum, London (UK).

1993 - Moldova (including Transnistria)

Although *G. salaris* has not been reported from Moldova *per se*, it has been reported from *S. trutta fario* from the River Seret, Ukraine (Kulakovskaja, 1967), which is a tributary of the River Dniester, which forms the eastern boundary of Moldova and the breakaway territory of Transnistria. On the NHM, London host-parasite database, *G. salaris* is reported from *S. trutta fario* in the "Ukraine, including Moldavia" with the report being accredited to Malmberg (1993). Although Moldova and Transnistria attained independence in 1991 and 1990, Malmberg (1993) referred to this record as the [former] Ukraine. Based on this information Moldova and Transnistria can be considered as *G. salaris*-positive states.

1997 – Denmark

Malmberg (1973) conducted a *Gyrodactylus* survey in three Danish rainbow trout hatcheries and reported the presence of two unidentified *Gyrodactylus* species that were very different to the highly pathogenic *G. salaris*. Although these most likely represented *G. derjavinoides* and *G. truttae*, both species were still undescribed at the time. In 1997, Buchmann and Bresciani (1997) published the first official report of *G. salaris* on Danish rainbow trout, which was found to co-occur alongside *G. derjavinoides*. Later, Nielsen and Buchmann (2001) confirmed the presence of *G. salaris*, alongside *G. derjavinoides*, from eight rainbow trout farms during an 11-month sampling, using both morphology and molecular-based approaches. Although the latter study found only *G. salaris* and *G. derjavinoides*, an earlier study on Danish brown trout and other salmonids found also other two species, *i.e. G. truttae* and *G. teuchis* (see Buchmann *et al*., 2000; Buchmann, 2005). During a survey of wild Atlantic salmon from the Fladså (River Ribe å system), only one specimen of *G. salaris* was found, which was identified by morphological and molecular analyses (Jørgensen *et al*., 2008). Three *G. salaris* variants have been reported from Denmark: two from farmed rainbow trout (see Lindenstrøm *et al*., 2003; Jørgensen *et al*., 2007) and one from wild Atlantic salmon (see Jørgensen *et al*., 2008), all of them non-pathogenic, suggesting a high rate of genetic variation within this parasite in Denmark. Bakke *et al.* (2007) suggested that there are no *G. salaris* epidemics on Danish wild salmon probably because the rainbow trout variants of *G. salaris* do not reproduce on Danish salmon, or due to the scarcity of wild salmon in Danish watersheds. There are only four Atlantic salmon rivers in Denmark, *i.e.* Rivers Guden, Haderup, Skjern and Varde (www.salmonatlas.com). A recent catch-and-release survey of 2153 Atlantic salmon represented 55% of the total number of rod-caught fish, which suggests that the total number is around 3915 salmon (ICES, 2012).

2000 – Italy

A survey of five rainbow trout farms from four different regions in central and northern Italy by Paladini *et al.* (2009a; see Chapter 2) found that fish were infected with four species of *Gyrodactylus*, including *G. salaris*. The other three species were *G. derjavinoides*, *G. teuchis* and *G. truttae*. The specimens were collected throughout 2004–2005 and the morphological identification was confirmed by molecular analysis (*personal identification*; part of the molecular results are also presented in Table 1 in Shinn *et al*., 2010). An additional archived sample of formalin-fixed rainbow trout mucus scraped from infected fish dating back to 2000 was also found to contain *G. salaris.* Although these latter specimens were identified by morphology only, this confirmed that *G. salaris* had been in the country since at least 2000 and had persisted without causing any ascribed mortality (Paladini *et al.*, 2009a). For the current study, an investigation conducted throughout 2008-2009 investigated the distribution of *G. salaris* throughout the central and northern regions of Italy, and found that 22 of the 27 (81.5%) samples collected were positive for the presence of *Gyrodactylus* spp. at low intensities of infection (4–30 parasites fish^{-1}). *Gyrodactylus salaris* and *G. derjavinoides* were found in 17 samples from all 7 regions; only two specimens of *G. truttae* were found, one in a sample from Veneto and one from Trentino-Alto Adige. *Gyrodactylus teuchis* was the predominant species found in all 22 *Gyrodactylus* positive samples from all seven regions (Paladini *et al.*, 2010b; Shinn *et al*., 2010). The origin of *G. salaris* haplotype F in Italy may be attributed to the trade in rainbow trout, given that this haplotype has also been recorded from rainbow trout in several European countries, including Denmark. Italy's recent history of importing rainbow trout from Denmark and Spain, coupled with the fact that the same four *Gyrodactylus* species, *i.e. G. derjavinoides*, *G. salaris*, *G. teuchis* and *G. truttae* (see Lindenstrøm *et al*., 2003; Paladini *et al*., 2009a), have also been found in Denmark, lends support to one possible hypothesis that *G. salaris* haplotype F may have been introduced

via rainbow trout trade from Denmark. Voucher specimens of *G. salaris* and *G. teuchis* collected from Italy will be deposited in the Parasitic Worms collection held within The Natural History Museum, London (UK).

2002 – Latvia

Specimens of *Gyrodactylus* collected from Baltic salmon from a fish farm near to the River Gauja were identified as a new haplotype of *G. salaris* (haplotype D) by molecular analysis (Hansen *et al*., 2003). This new mitochondrial haplotype, which clusters with haplotypes A and B (from Norway and Sweden) and haplotype C (Sweden only) as a single clade of *G. salaris* strains that only infects Atlantic salmon, appears to differ from the other two haplotypes (*i.e.* haplotype E on Atlantic salmon only; haplotype F on Atlantic salmon and rainbow trout), which form two separate single-haplotype clades (Hansen *et al*., 2003). Later, Hansen *et al.* (2006) added further information by analysing the nucleotide sequence of the intergenic spacer (IGS) and the mitochondrial cytochrome oxidase I (COI) of *G. salaris* haplotype D from the same Latvian fish farm, finding the same IGS arrangements that are typical of *G. salaris* from Norway.

2007 – Macedonia

Although there is no specific mention in the literature of *G. salaris* occurring in Macedonia, DNA sequences of *G. salaris* from Ohrid trout, *Salmo lectnica* (Karaman), and from rainbow trout, both collected from a fish farm located on the River Vardar in the Aegean Sea basin, Macedonia are reported in Kuusela *et al.* (2007) and in Ziętara *et al.* (2010).

2007 – Poland

The first survey of *Gyrodactylus* on Polish salmonids was made from a fish farm and from the Rivers Soła and Czarna by Prost (1991), who found two species: *G. derjavinoides* from *S. trutta fario*, *O. mykiss* and *S. fontinalis*; and *G. truttae* from *S. trutta fario.* Subsequently, Rokicka *et al*. (2007) reported finding specimens representing three molecular forms belonging to the *G. salaris*/*G. thymalli* group that were collected from Polish rainbow trout, sea trout (*Salmo trutta trutta* L.) and grayling from tributaries of the River Vistula, near Pomerania province. Identification of the forms was based on a PCR-RFLP analysis of the nuclear ITS fragment of rDNA. These three forms were represented by: 1) the standard ITS type which is found only on grayling; 2) a heterogenic *G. salaris* type previously described by Lindenstrøm *et al.* (2003) found on rainbow trout and sea trout; and 3) a form found on rainbow trout, which was a complementary homozygous clone differing by three nucleotides. The molecular identification was supported by a parallel morphometric analysis, and from the drawings presented in Rokicka *et al.* (2007), it is clearly visible that the specimens of *G. salaris* and *G. thymalli* collected represent two distinct species, although not pointed out. In Rokicka *et al.* (2007) the measurements of the two forms of *G. salaris* from rainbow trout and from grayling were grouped together, but if the two species are considered separately as *G. salaris* and *G. thymalli*, then the measurements of the hamulus total length reported in the literature are 61–69 μm for *G. salaris* and 75–84 μm for *G. thymalli* (see Ergens, 1983), which together correspond with the range reported in Rokicka *et al.* (2007), *i.e.* 69.1–87.9 μm.

2009 – Romania

Although no official report exists, the recent findings in 2009 of the OIE Reference Laboratory in Norway would suggest that *G. salaris* is also present within Romania from an unspecified farmed fish (OIE, 2012).

2010 – Estonia

Atlantic salmon populations from the Baltic basin are believed to be more resistant to *G. salaris* infection than are the salmon populations from the Atlantic and the White Sea coasts (Rintamäki-Kinnunen & Valtonen, 1996; Dalgaard *et al.*, 2003; Bakke *et al.*, 2004). A recent survey on triploid Atlantic salmon from the Baltic basin showed a high susceptibility to *G. salaris* infection (Ozerov *et al.*, 2010). This fish population originated from a hatchery in northern Estonia, situated on the Kunda River, Gulf of Finland, Baltic Sea (Ozerov *et al.*, 2010). Identification of *G. salaris* was confirmed by molecular analyses, including sequencing of the ITS rDNA and mitochondrial COI (Ozerov *et al.*, 2010). Although there is no doubt regarding the identification of these specimens, no morphological data was presented. According to the mtDNA sequences obtained by Ozerov *et al.* (2010), the closest relatives to the Estonian strain of *G. salaris* – with a single nucleotide difference in the COI region – are the strain of *G. salaris* found in Genevadsån on the Swedish west coast and those collected from the Raasakka hatchery, Iijoki, Gulf of Bothnia, Finland.

3.3.2. Countries where the G. salaris *status requires confirmation*

France

The first record of *G. salaris* in France and also in Portugal was made by Johnston *et al.* (1996) with reference to material collected from rainbow trout and identified using morphology and a DNA probe. The subsequent discovery of *G. teuchis*, a species which has morphological similarities with *G. salaris*, makes the validity of this earlier *G. salaris* finding questionable (Lautraite *et al*., 1999). This latter study and that of Cunningham *et al*. (2001) – which looked at material collected from a large scale survey of Atlantic salmon, rainbow trout and brown trout farms – did not find *G. salaris*, and therefore was

unable to support the suggestion that France is a *G. salaris*-positive state. Given this results, the earlier report was most likely the result of a misidentification between *G. salaris* and *G. teuchis*.

Kazakhstan and/or Tajikistan

The Natural History Museum (NHM), London, maintains a "host-parasite" database (www.nhm.ac.uk) which was populated with published parasite data up to and including 2002. On this database, there is a record of *G. salaris* from Aral trout, *Salmo trutta aralensis* Berg, from Kazakhstan linked to a paper by Gvozdev and Karabekova (2001). From this reference, however, Amu-Darya trout *Salmo trutta oxianus* Kessler is listed as a host for *G. salaris* from the River Kafirnigan, in Tajikistan, which could have been misidentified with *Gyrodactylus derjavini* Mikhailov, 1975, the only "other" *Gyrodactylus* species previously recorded from this host (see www.gyrodb.net; Ergens, 1983; Prof. Margaritov N.M., *pers. comm.*). An earlier, similar reference by Gvozdev and Karabekova (1990) does not mention *G. salaris* within the 43 listed species of *Gyrodactylus*, although the abstract indicates that 48 *Gyrodactylus* species are listed. The validity of the *G. salaris* report from Kazakhstan and/or Tajikistan is questionable, and although attempts have been made to contact the authors, no communication has been established. This report cannot be confirmed until further detailed information on this report is available, or specimens can be obtained and assessed.

Lithuania

The record of *G. salaris* in Lithuania is cited in an on-line publication published by Cefas (www.westcountryangling.com/pdf/gyrodactylus_salaris.pdf) and its occurrence is suggested, but not confirmed, in a second website (www.europe-aliens.org/pdf/Gyrodactylus_salaris.pdf). Cefas have been contacted and asked if an

official statement can be provided. Although Lithuania sits in the middle of the suggested natural distribution of *G. salaris*, until further information is forthcoming this record cannot be verified.

Portugal

The assessment of Johnston *et al.* (1996) of the *Gyrodactylus* specimens collected from farmed Portuguese rainbow trout was based on both morphological and on molecular data. The specimens, however, were initially fixed in buffered formalin and then rinsed in 70% ethanol before being assessed. It is likely that the formalin fixation would have prevented flat preparations of *Gyrodactylus* and, therefore, a clear view of the marginal hooks, which are considered the key morphological feature upon which to identify species. *Gyrodactylus teuchis* was an unknown species at the time of study and given the morphological similarities between this and *G. salaris*, it is possible that the subtle differences in hook shape were not recognised as deviating from those of *G. salaris*. Subsequent studies by Lautraite *et al*. (1999) and Cunningham *et al*. (2001) described *G. teuchis* and its discrimination from *G. salaris* by morphology and differences in PCR-RFLP patterns of the ITS1, 5.8S gene and ITS1 regions. A survey of salmonids throughout France by both latter studies led to the conclusion that France was most likely a *G. salaris*-free state and that the original report was a result of a misidentification. Although Eiras (1999) conducted a survey on several Portuguese rainbow trout and brown trout farms, no specimens of *G. salaris* were found. Johnston *et al*.'s (1996) identification of *G. salaris* from Portugal, therefore, remains in doubt until demonstrated otherwise.

In September 2007, three specimens of *Gyrodactylus* were recovered from a sample of 20 Portuguese rainbow trout. All three specimens were confirmed, during the current study, as *G. teuchis* by morphological and molecular examinations (*personal*

identification). Voucher specimens of *G. teuchis* from Portugal will be deposited in the Parasitic Worms collection at The Natural History Museum, London (UK).

Slovakia

Ergens (1961, 1963) recorded the presence of *G. salaris* in Slovakia (formerly Czechoslovakia) from brown trout from the River Topl'a, near the town of Bardějov. A later re-examination of this material (Ergens, 1983) found that the species in question was *G. truttae*, a species not described at the time of Ergens' original study. The identity of *G. truttae* was evident from the measurements of the haptoral hard parts (Gläser, 1974; Ergens, 1983). The record of *G. salaris* from Slovakia, therefore, is not considered valid.

Spain

Two drug trials conducted in Spain on the species of *Gyrodactylus* collected from rainbow trout from Carballo, La Coruña, were identified, on the basis of hook morphology, by Professor Göran Malmberg (University of Stockholm) as *G. salaris* (see Santamarina *et al*., 1991; Tojo *et al*., 1992). Similarly to the reports for France and Portugal, it is likely that these specimens were *G. teuchis* and were mistaken for *G. salaris*. A sample of 60 *Gyrodactylus* specimens collected for the current study from rainbow trout fingerlings from a farm in the Galicia region of Spain, were all identified as *G. teuchis* by morphological and molecular analyses (*personal identification*). Voucher specimens from this collection will be deposited in The Natural History Museum, London (UK).

3.3.3. Gyrodactylus salaris-*free states*

Finland

Parts of the Finnish territory have been declared *G. salaris*-free under EC Decision 2004/453/EC (http://eurlex.europa.eu). These regions include the water catchment areas of the Tenojoki and Näätämönjoki, whilst the water catchment areas of the Paatsjoki, Luttojoki, and Uutuanjoki are considered as buffer zones.

Republic of Ireland

The Republic of Ireland is declared *G. salaris*-free under the EC Decision 2004/453/EC (http://eurlex.europa.eu) based on evidence that its government submitted to the European Commission.

United Kingdom

Following the events in Norway, *G. salaris* was made a notifiable pathogen in the UK in 1987 under the Diseases of Fish Acts 1937 and 1983, which can impose movement restrictions on fish stocks from fish farms, rivers, or from entire catchments (OIE, 2012). Following notification, a survey of 7 rivers and 17 fish farms in Northern Ireland (Platten *et al*., 1994), and a parallel investigation of 63 fish farms and 164 wild salmonid sites throughout Great Britain by Shinn *et al.* (1995), set out to establish the *G. salaris* status of each. Neither survey found *G. salaris* or the morphologically similar *G. teuchis*, but the surveys did find *Gyrodactylus arcuatus* Bychowsky, 1933 and *Gyrodactylus caledoniensis* Shinn, Sommerville *et* Gibson, 1995 from *S. salar*; *G. derjavinoides* from *O. mykiss*, *Salvelinus alpinus alpinus* (L.), *S. salar* and *S. trutta fario*; *G. truttae* on *S. trutta fario*; and a number of unidentified *Gyrodactylus* morphotypes from *S. alpinus alpinus* and *S. salar*. Mandatory surveillance programmes by the relevant fish inspectorate authorities within

each constituent country continue to screen fish samples for *G. salaris* and other pathogens of concern. National contingency planning in the event of a *G. salaris* introduction began in 2006 in Scotland (www.scotland.gov.uk), in 2008 in England (www.oie.int) and Wales (http://wales.gov.uk), and in 2009 in Northern Ireland (www.dardni.gov.uk). Great Britain is officially a *G. salaris*-free zone under EC Decision 2004/453/EC and its subsequent amendments provided under EC Decision 2006/272/EC (http://eurlex.europa.eu).

Although the UK is *G. salaris*-free, there is a single report of *G. salaris* from *S. trutta fario* from Loch Leven, Scotland (Campbell, 1974). Malmberg (1987) considered this a misidentification of *G. derjavinoides* or *G. truttae*, species that were both still undescribed at the time of publication. Salmonids from Loch Lomond were sampled during the study of Shinn *et al*. (1995) but no specimens of *G. salaris* were found.

3.4. Discussion

In Europe, Atlantic salmon are widespread and are found along the coasts of the North Atlantic including the Baltic Sea and their range extends from the Bay of Biscay to the White Sea. Colonisation of northern Europe most likely occurred from the sea after the last glaciation event (Halvorsen & Hartvigsen, 1989). Although most species of *Gyrodactylus* are fairly host specific (www.gyrodb.net), *G. salaris* displays lower host specificity and is able to colonise and reproduce on a range of salmonid hosts.

Gyrodactylus salaris has, under natural conditions in the wild, been recorded from *S. salar* (see *e.g.* Ergens, 1983; Johnsen & Jensen, 1985), *O. mykiss* (see *e.g.* Mo, 1988), *S. trutta fario* (see *e.g.* Tanum, 1983; Malmberg & Malmberg, 1991), *Salvelinus alpinus alpinus* (see Mo, 1988), *Salmo obtusirostris* (see Žitňan & Čanković, 1970) and *Platichthys flesus* (see Mo, 1987), although the latter, as a non-salmonid species, has proven to be an unsuitable host (Bakke *et al.*, 1992a). The relative susceptibility of these

hosts to *G. salaris* varies, as does the pathology induced (see *e.g.* Bakke *et al.*, 1991b; Bakke & Jansen, 1991a, b; Bakke *et al*., 1992a, b; Soleng & Bakke, 2001a, b).

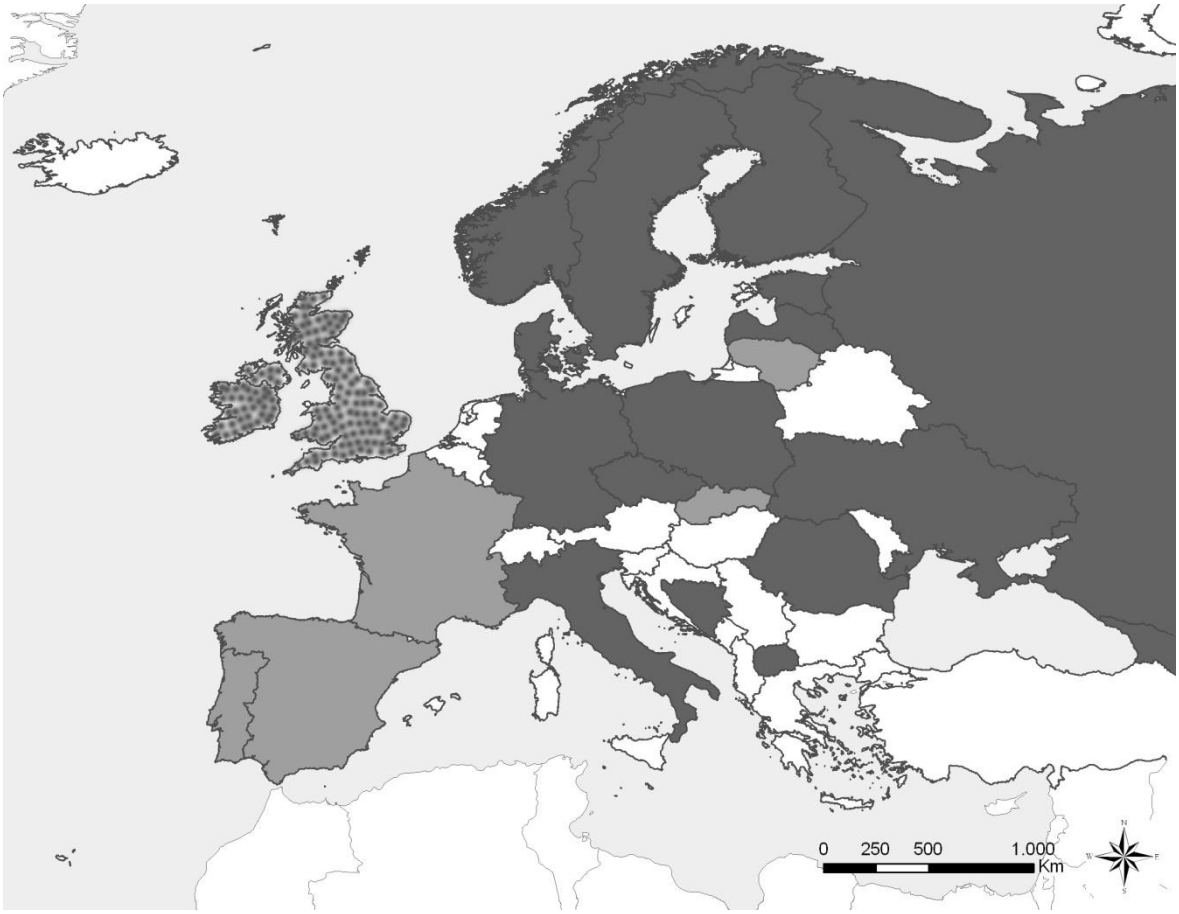


Figure 3.2. Map of Europe highlighting *Gyrodactylus salaris*-positive states (dark grey colour). For territories such as France, Italy, Spain and Portugal only the status of the mainland is considered and larger islands (*e.g.* Balearic, Canary, Corsica, Sardinia, Sicily, *etc*) under their respective sovereignty are considered as separate geographic entities. For the purposes of this study, Kaliningrad, the Russian exclave, is considered as a separate geographic zone to the main Russian state. The reports of *G. salaris* from Kazakhstan and / or Tajikistan (not shown), France, Lithuania, Portugal, Slovakia and Spain (light grey colour) are questionable and need further verifications regarding the presence or absence of *G. salaris*. Republic of Ireland and the UK (dotted grey colour) are the only two countries currently declared *G. salaris*-free. Countries where the status of *G. salaris* is unknown are left in white colour [original image].

Although *G. salaris* was initially classified as a List III pathogen under the European Council Directive 91/67/EEC regarding measures against certain diseases in

aquaculture animals, it has since been removed following EC Directive 2006/88/EC, but remains on OIE lists as a "significant disease" and "notifiable pathogen" (OIE, 2012).

Of all the European countries, considerable stocks of wild salmon populations are present only in Norway, Scotland, Faroe Islands, Ireland and Iceland (Peeler *et al*., 2006). In other states within Europe (see Table 3.2) the number of salmon populations is small (Mills, 1991; Maitland & Campbell, 1992; Hendry & Cragg-Hine, 2003). The dissemination of *G. salaris* across Europe appears mainly to be linked to movements of rainbow trout between countries (Peeler & Thrush, 2004; Peeler *et al*., 2006; Bakke *et al*., 2007). This appears to be the case for most of the *G. salaris* reports from southern Europe, *e.g.* Italy where salmon is not present, but *G. salaris* has been recorded all over the country where rainbow trout is farmed (Paladini *et al*., 2009a, 2010b). There are approximately 50 states within Europe and although most contain salmonid species, others such as Gibraltar, Malta, Monaco and Vatican City, do not and, therefore, the *G. salaris* status of these cannot be assessed (Table 3.2). Although the Republic of San Marino is considered salmonid-free by the on-line database fishbase (www.fishbase.org), Lake Faetano, a small artificial lake created in 1968 for recreational fishing, does contain rainbow trout and brown trout, and the *G. salaris* status of these stocks requires establishing. The lack of clinical signs of gyrodactylosis on species such as rainbow trout, means that *G. salaris* infections may go undetected for many years, *e.g.* Italy where *G. salaris* infections had persisted unknown for at least 9 years prior to its first official report (Paladini *et al*., 2009a; see Chapter 2). This finding is an important consideration when moving salmonid stocks, and calls for more rigorous biosecurity control measures in the trade and transfer of fish species from one country to another. A lesson to learn from the past is the spreading of *Gyrodactylus cichlidarum* Paperna, 1968 on Nile tilapia, *Oreochromis niloticus niloticus* (L.), a species which from its African origins has been exported, undetected, with its host

worldwide, and being associated with mass mortalities of Nile tilapia (García-Vásquez *et al*., 2010).

Gyrodactylus salaris has been reported from 23 out of ~50 recognised states throughout Europe (Tables 3.1-3.2). Only 17 of these records, however, are considered valid, having been identified by either morphology, molecular or a combination of both methods. Only ten of these reports though have been confirmed by a combination of both molecular and morphological approaches (Table 3.1). The records of *G. salaris* from France, Portugal, Slovakia and Spain all appear to have been based on misidentifications and although some additional specimens have been obtained from some of these countries, and found only to contain *G. teuchis*, larger numbers of samples are required before a definitive statement can be made. In the case of France, however, a large survey was conducted, but only the morphologically similar species *G. teuchis* was found. Likewise, the reports of *G. salaris* from Kazakhstan (and/or Tajikistan) and Lithuania are doubtful and further samples are required for evaluation. The records of *G. salaris* from Bosnia-Herzegovina, Georgia, Moldova and Ukraine are all based on morphology only, and ideally these reports require confirmation by an appropriate molecular test. The only two states that are currently considered *G. salaris*-free are the Republic of Ireland and the United Kingdom; on-going government-based surveillance programmes continue to screen key salmonid sites in these countries.

Table 3.1. List of European countries from which *Gyrodactylus salaris* has been reported to occur on salmonids. Some of the records are questionable and the confirmation of the presence of *G. salaris* needs further verification. For each country only the mainland is considered; larger island groups are considered separately.

Country	Host	ID status	Method of ID*	Presence in GenBank	Representative references
Bosnia-Herzegovina	*Om, Ss, Stf, So*	valid	A	no	Čanković & Kiškarolj (1967); Žitňan (1960); Žitňan & Čanković (1970)
Czech Republic	*Stf*	valid	A+B	no	Tesarcik & Ivasik (1974); Matejusová *et al*. (2001)
Denmark	*Om, Ss*	valid	A+B	yes	Buchmann & Bresciani (1997); Lindenstrøm *et al.* (2003); Jørgensen *et al*. (2008)
Estonia	*Ss*	valid	B	yes	Ozerov *et al.* (2010)
Finland	*Om, Ss*	valid	A+B	yes	Rimaila-Pärnänen & Wiklund (1987); Kuusela *et al.* (2007); current study
France[1]	*Om*	unconfirmed	A	no	Johnston *et al.* (1996)
Georgia	*Stf*	valid	A	no	Ergens (1983); Malmberg (1993)
Germany	*Om*	valid	A+B	no	Lux (1990); Dzika *et al*. (2009); Cunningham *et al.* (2003)
Kazakhstan and / or Tajikistan	*Sto*	unconfirmed	A	no	Gvozdev & Karabekova (2001)
Italy[1]	*Om*	valid	A+B	yes	Paladini *et al.* (2009a); current study
Latvia	*Ss*	valid	B	yes	Hansen *et al.* (2003)
Lithuania	unknown	unconfirmed	unknown	no	unofficial reports[2]
Macedonia	*Om, Sl*	valid	B	yes	Kuusela *et al.* (2007); Ziętara *et al.* (2010)
Moldova	*Stf*	valid	A	no	Malmberg (1993)
Norway	*Ss*	valid	A+B	yes	Johnsen (1978); Cable *et al*. (1999); Meinilä *et al.* (2004)
Poland	*Om, Stt*	valid	A+B	yes	Rokicka *et al.* (2007)
Portugal[1]	*Om*	unconfirmed	A	no	Johnston *et al.* (1996)
Romania	unknown	valid	A+B	no	OIE (2012)
Russia	*Ss*	valid	A+B	yes	Ergens (1983); Meinilä *et al.* (2004); Kuusela *et al.* (2007)
Slovakia	*Stf*	not valid	A	no	Ergens (1961, 1983)
Spain[1]	*Om*	unconfirmed	A	no	Santamarina *et al.* (1991)
Sweden	*Ss*	valid	A+B	yes	Malmberg (1957); Meinilä *et al.* (2004)
Ukraine	*Om, Stf*	valid	A	no	Kulakovskaja (1967); Tesarcik & Ivasik (1974)

Footnotes: *Method of identification: A. morphology only; B. molecular only; A+B. morphology + molecular characterisation. Abbreviations: *Om*: *Oncorhynchus mykiss*; *Sl*: *Salmo letnica*; *So*: *Salmo obtusirostris*; *Ss*: *Salmo salar*; *Stf*: *Salmo trutta fario*; *Sto*: *Salmo trutta oxianus*; *Stt*: *Salmo trutta trutta*. [1]Large islands, such as Corsica (France), Sardinia and Sicily (Italy), Balearic and Canary Islands (Spain) or Madeira (Portugal) are included, although their *G. salaris* status should be considered separately from their respective mainland territories. [2]Unofficial records of *G. salaris* from Lithuania were taken from two websites: www.westcountryangling.com/pdf/gyrodactylus_salaris.pdf and www.europe-aliens.org/pdf/Gyrodactylus_salaris.pdf.

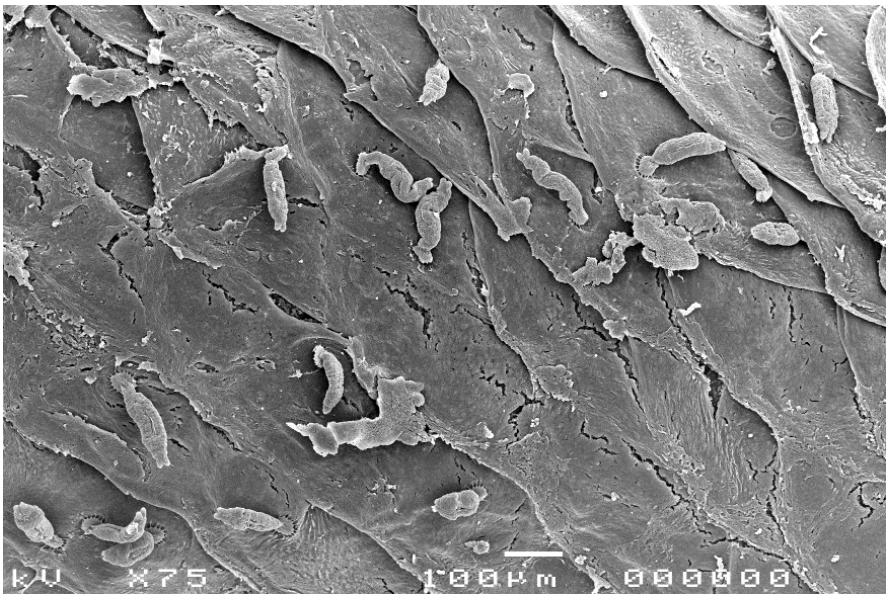
Table 3.2. A summary of the occurrence of salmonids in each European state (independent and recognised territories), excluding those listed in Table 3.1 and the two *G. salaris*-free states (Republic of Ireland and the UK), with comments on the other species of *Gyrodactylus* recorded from them.

European countries	*Gyrodactylus* species	Host	References	Presence of salmonids	Status of *G. salaris*
Albania	-	-	-	yes	unknown
Andorra	-	-	-	yes	unknown
Armenia	-	-	-	yes	unknown
Austria	*G. teuchis*	*Stf*	Hahn *et al.* (2011)	yes	unknown
	G. thymalli	*Tt*	Hahn C., *pers. comm.*[1]		
	G. truttae	*Saa*	Kadlec *et al.* (1997)		
Azerbaijan	-	-	-	yes	unknown
Belarus	-	-	-	yes	unknown
Belgium	-	-	-	yes	unknown
Bulgaria	*G. truttae*	*Om, Stf*	Kakacheva-Avramova & Menkova (1982)	yes	unknown
Croatia	*G. salmonis*	*Om*	Zrnčić & Oraić (2008)	yes	unknown
Cyprus	-	-	-	yes	unknown
Faroe Islands	-	-	-	yes	unknown
Gibraltar	-	-	-	no	-
Greece	-	-	-	yes	unknown
Greenland	-	-	-	yes	unknown
Hungary	-	-	-	yes	unknown
Iceland	-	-	-	yes	unknown
Liechtenstein	-	-	-	yes	unknown
Lithuania	*G. rarus*	*Om*	Host-parasite DB[2]	yes	unknown
Luxembourg	-	-	-	yes	unknown
Malta	-	-	-	no	-
Monaco	-	-	-	no	-
Montenegro	-	-	-	yes	unknown
Netherlands	-	-	-	yes	unknown
San Marino	-	-	-	yes	unknown
Serbia	-	-	-	yes	unknown
Slovenia	*G. thymalli*	*Tt*	Hansen *et al*. (2007a)	yes	unknown
Switzerland	-	-	-	yes	unknown
Turkey	*Gyrodactylus* spp.	*Om*	Ozkan Ozyer (2008)	yes	unknown
Vatican City	-	-	-	no	-

Footnotes: *Om*: *Oncorhynchus mykiss*; *Saa*: *Salvelinus alpinus alpinus*; *Stf*: *Salmo trutta fario*; *Tt*: *Thymallus thymallus*. [1]*G. thymalli* has been found on both fresh and preserved museum materials from the Natural History Museum, Vienna, Austria, suggesting that this parasite has been there for at least 130 years, long before its description in 1960 (Hahn C., *pers. comm.*); [2]Host-parasite database of the Natural History Museum of London (UK): www.nhm.ac.uk.

Chapter 4

Gyrodactylus species associated with emergent disease problems



Skin of gilthead seabream, *Sparus aurata* L., infected with *Gyrodactylus orecchiae* Paladini, Cable, Fioravanti, Faria, Di Cave *et* Shinn, 2009 [original image].

Paper II

Paladini G., Cable J., Fioravanti M.L., Faria P.J., Di Cave D., Shinn A.P. (2009). *Gyrodactylus orecchiae* sp. n. (Monogenea: Gyrodactylidae) from farmed population of gilthead seabream (*Sparus aurata*) in the Adriatic Sea. *Folia Parasitologica*, 56: 21–28.

Paper III

Paladini G., Hansen H., Fioravanti M.L., Shinn A.P. (2011). *Gyrodactylus longipes* n. sp. (Monogenea: Gyrodactylidae) from farmed gilthead seabream (*Sparus aurata* L.) from the Mediterranean. *Parasitology International*, 60: 410–418.

Aspects of these papers were presented as:

Paladini G. (2011). Is my PhD a fluke? An Italian's journey through the dark side of parasitology. *Lunchtime seminar, University of Stirling, Stirling, Scotland, UK, 22nd November 2011* (talk).

Paladini G., Williams C., Hansen H., Taylor N.G.H., Rubio-Mejía O.L., Denholm S.J., Hytterød S., Bron J.E., Shinn A.P. (2012). *Gyrodactylus salaris*: the good, the bad and the ugly. *Proceedings of the Institute of Aquaculture 3rd PhD Research Conference, Stirling, Scotland, 24th October 2012*: 16 (talk).

4.1. General introduction of Papers II and III

The following two papers have been published in *Folia Parasitologica* and *Parasitology International* which describe two new species of *Gyrodactylus* infecting farmed populations of gilthead seabream, *Sparus aurata* L., in the Mediterranean. From my personal experience and that of other senior colleagues working at the Department of Veterinary Medical Sciences (formerly Dept. of Veterinary Public Health and Animal Pathology) of the University of Bologna (Italy), *Gyrodactylus* was never been reported on gilthead seabream, despite several years of rigorous screening and health assessments of farmed stocks. This study highlights that emerging diseases/new parasites can appear in long-established industries. The World Health Organisation (WHO) defines an emerging disease as "*one that has appeared in a population for the first time, or that may have existed previously but is rapidly increasing in incidence or geographic range*" (www.who.int/topics/emerging_diseases/en/). It could be speculated that these two new species, *Gyrodactylus orecchiae* Paladini, Cable, Fioravanti, Faria, Di Cave *et* Shinn, 2009 and *Gyrodactylus longipes* Paladini, Hansen, Fioravanti *et* Shinn, 2011: 1) were already present in the environment around farm sites at a very low level below the limits of detection by routine sampling practices, but changes to local conditions (*e.g.* climate, farm practices) have enhanced the pathogenicity of these parasites; or 2) these parasites have been carried into the Mediterranean by another host species, which has then subsequently found an alternative susceptible host, *i.e.* gilthead seabream. The mortalities associated with the presence of these two parasites, and the recent record of *G. longipes* from northern France, highlight the potential pathogenicity of these two species and their wide geographic spread within the European area. *Gyrodactylus orecchiae* is currently known from three countries (Albania, Croatia and Italy) and *G. longipes* from other three (Italy, Bosnia-Herzegovina and France).

4.2. Authors' contribution

For both papers, I drafted the first version of each manuscript which were subsequently revised with my co-authors. All authors read and approved the final version of the two manuscripts.

4.2.1. Paper II

For the description of *G. orecchiae*, several samples of gilthead seabream from two sites in Albania were sent to me, while working at the University of Bologna with Professor Maria Letizia Fioravanti, for health assessing and parasite screening. Further samples from two sites in Croatia were sent at about the same time to an Italian colleague, Dr David Di Cave, who subsequently passed the material to me to evaluate. All samples were identified by myself as being the same species and, therefore, it was decided that the results would be prepared as a joint collaboration. All the material was transported to the Institute of Aquaculture of the University of Stirling, where I carried out the morphological and morphometric description under the guidance of Dr Andrew P. Shinn. Drawings and images of the light microscope (LM) and of the scanning electron microscope (SEM) were prepared, processed and produced by me. Technical support in using the SEM was provided by Mr Linton Brown (Stirling University), whilst assistance in reading the histopathology was provided by Professor Massimo Trentini (University of Bologna). Whilst I conducted some of my own molecular evaluation of the parasite material, the procedures and data used in this study was conducted by Dr Patricia J. Faria and Dr Joanne Cable from Cardiff University, Wales.

4.2.2. Paper III

For the description of *G. longipes*, two samples of gilthead seabream from Italy and from Bosnia-Herzegovina were sent to Professor Fioravanti and myself. As per the description of *G. orecchiae*, the two samples were identified by myself and described at the

University of Stirling, where I carried out the morphological and morphometric analyses under Dr Shinn's close supervision. All the specimens used for light and scanning electron microscope evaluation were prepared by myself with some technical SEM assistance from Mr Linton Brown. Dr Haakon Hansen, from the National Veterinary Institute, Oslo, Norway, performed the molecular component of this study.

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Gyrodactylus orecchiae sp. n. (Monogenea: Gyrodactylidae) from farmed populations of gilthead seabream (*Sparus aurata*) in the Adriatic Sea

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Abstract: *Gyrodactylus orecchiae* sp. n. (Monogenea, Gyrodactylidae) is described from the skin, fins, eyes and gills of juvenile *Sparus aurata* L. (gilthead seabream) following two outbreaks of gyrodactylosis amongst stocks held in inshore floating cages on the Adriatic coast of Albania and Croatia. Fish were heavily infected (1000+ gyrodactylids/fish) with *G. orecchiae* which reportedly resulted in ~2–10% mortality amongst the infected stock. Morphologically, the haptoral hooks of *G. orecchiae* most closely resemble those of *Gyrodactylus arcuatus* Bychowsky, 1933 in the approximate shape of the ventral bar with its pronounced ventral bar processes and marginal hook sickles which possess a square line to the inner edge of the sickle blade and large rounded heels. The marginal hooks are also morphologically similar to those of *Gyrodactylus quadratidigitus* Longshaw, Pursglove et Shinn, 2003 and *Gyrodactylus colemanensis* Mizelle et Kritsky, 1967, but *G. orecchiae* can be readily discriminated from all three species by the characteristic infolding of the hamuli roots and the shape of the marginal hook sickle. Molecular sequencing of the ITS1, 5.8S, ITS2 regions (513+157+404 bp, respectively) of *G. orecchiae* and alignment with other gyrodactylids for which these same genomic regions have been determined, suggests that this is a new species. No similarities were found when the ITS1 region of *G. orecchiae* was compared with 84 species of *Gyrodactylus* available on GenBank.

Key words: Monogenea, *Gyrodactylus orecchiae*, gilthead seabream, *Sparus aurata*, Croatia, Albania

Monogeneans, notably infections with the microcotylid *Sparicotyle chrysophrii* (van Beneden et Hesse, 1863) and the diplectanid *Furnestinia echeneis* (Wagener, 1857), are commonly encountered in both cultured and wild populations of gilthead seabream *Sparus aurata* L. (Sparidae) within the Mediterranean (Euzet 1984, Radujkovic and Euzet 1989, Di Cave et al. 1998, Varriale and Baroncelli 1998, De Liberato et al. 2000). Of these, the report by De Liberato et al. (2000) and a chemotherapy study by Santamarina et al. (1991) also refer to the presence of an unidentified *Gyrodactylus* von Nordmann, 1832 on *S. aurata*. During 2005–2006, routine diagnostic sampling of inshore floating gilthead seabream cages at Orikum, Albania and Ugljan Island, Croatia revealed heavy infections with gyrodactylids on the skin and gills of juvenile stock. Infected fish were observed to be hypermelanotic, lethargic, anorexic and displayed a progressive loss of weight. Stock mortality was determined to be 2–5% within the inland-based farm at Orikum, rising to 10% in the floating cages at the same location. Looking at the on-line database "GyroDb" (www.gyrodb.net, Harris et al. 2008), only one other gyrodactylid, *Gyrodactylus alviga* Dmitrieva et Gerasev, 2000, is known to parasitize the sparids *Diplodus annularis* (L.) and *Sarpa salpa* (L.) from the Black Sea (Dmitrieva and Gerasev 2000). Given the increasing importance of *S. aurata* in the Mediterranean as a species for aquaculture (86,700 tonnes in 2006; FAO/GLOBEFISH 2007), this study was undertaken to describe a new species of *Gyrodactylus* using molecular, light and scanning electron microscopy techniques.

MATERIALS AND METHODS

Collection of material and morphological determination. At each of the two farm sites (Fig. 1), approximately 20 juvenile *S. aurata* (weight ca. 5–10 g) were randomly sampled from several cages, killed by pithing and then fixed immediately in 70%

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Fig. 1. *Gyrodactylus orecchiae* sp. n. sample sites within the Adriatic. 1 – Ugljan Island, Croatia (44°7'45.87"N, 15°6'9.77"E); 2 – Orikum, Albania (40°18'50.92"N, 19°28'32.93"E).

ethanol. On return to the laboratory at the University of Bologna, the fish were screened using an Olympus SZ40 stereomicroscope at ×4 magnification and specimens of *Gyrodactylus* were removed using mounted triangular surgical needles (size 16, Barber of Sheffield, UK). All fish (*i.e.* 2 sites; n = 40 fish screened) were found to be infected with mean intensities in excess of 1,000 gyrodactylids per fish; no other metazoan parasites were detected. A further five fish from each site were processed for histology following standard procedures.

Parasite specimens were washed in distilled water and representatives prepared as whole mounts by clearing them in ammonium picrate glycerine following the procedure detailed by Malmberg (1970). A further 40 worms were removed, washed in distilled water and then digested on glass slides using a modification of the proteolytic method given in Harris and Cable (2000) and then mounted in ammonium picrate. The haptoral hooks of ten specimens were digested on 11 mm round glass coverslips, sputter-coated with gold and then examined using a JEOL JSM 5200 scanning electron microscope operating at an accelerating voltage of 25 kV. Five specimens were removed from their hosts, their haptors excised and prepared for proteolytic digestion and morphological study while the bodies were fixed in 95% ethanol for molecular characterisation.

For the morphological study, the haptoral hard parts were studied and drawn at magnifications of ×40 and ×100 oil immersion from images grabbed 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. A total of 27 point-to-point morphometric measurements were made on haptoral hooks of each specimen from images grabbed 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. The measurements follow those given in Shinn et al. (2004) and are expressed in micrometres as the mean ± standard deviation followed by the range in parentheses, unless otherwise stated.

Molecular characterisation. Sequencing of the ITS1, 5.8S and ITS2 regions of *G. orecchiae* was performed using primers P3b (TAGGTGAACCTGCAGAAGGATCA) and P4 (GTCCGGATCCTCCGCTTATTGAATGC) (Cable et al. 2005) which anneal to the 18S and 28S, respectively. Amplifications were carried out in a Perkin Elmer thermocycler (9700) using an initial denaturation of 95 °C, followed by 35 cycles of 94 °C 30 s, 50 °C 1 min, 72 °C 2 min and a final extension of 72 °C 10 min. PCR products were purified using Exonuclease *I* and SAP (Shrimp Alkaline Phosphatase) (Biolabs) and both strands were sequenced using BigDye (version 3.1; Applied Biosystems) on an ABI3100 sequencer. Strands were manually aligned and corrected using the program BioEdit (Hall 1999).

The consensus sequence from three individuals were aligned with EMBLALIGN: Align_000605 (Matějusová et al. 2003) using CLUSTAL X (Jeanmougin et al. 1998) following the criteria detailed by Matějusová et al. (2003) and deleting the hypervariable sections of the ITS1 and ITS2 in order to optimize the alignment without ambiguities. The following sequences from GenBank were used for the alignment analysis: *Gyrodactylus alburniensis* Prost, 1972 (AY278032); *G. alexgussevi* Ziętara and Lumme, 2003 (AY061979); *G. anguillae* Ergens, 1960 (AB063294); *G. arcuatus* Bychowsky, 1933 (AF328865); *G. branchicus* Malmberg, 1964 (AF156669); *G. bullatarudis* Turnbull, 1956 (AY692024); *G. cichlidarum* Paperna, 1968 (DQ124228); *G. elegans* von Nordmann, 1832 (AJ407870); *G. flesi* Malmberg, 1957 (AY278039); *G. lotae* Gusev, 1953 (AY061978); *G. macronychus* Malmberg, 1957 (AY061980 and AY061981); *G.* cf. *niger* Huyse, Audenaert et Volckaert, 2003 (AY338452); *G. pictae* Cable, van Oosterhout, Barson et Harris, 2005 (AY692023); *G. rarus* Wegener, 1910 (AY338445); *G. robustus* Malmberg, 1957 (AY278040); *G. rugiensis* Gläser, 1974 (AF328870); *G. rugiensoides* Huyse et Volckaert, 2002 (AJ427414); *G. salaris* Malmberg, 1957 (AF328871); and *G. turnbulli* Harris, 1986 (AJ001846). MEGA version 4.0 (Tamura et al. 2007) was used to estimate *p*-distance between species.

RESULTS

Gyrodactylus orecchiae sp. n. Figs. 2–4, Table 1

Morphological description. Coverslip-flattened specimens 275.0–455.9 (356.3) long; 62.1–92.1 (81.5) wide at level of uterus. Anterior bulb of pharynx 25.1 (22.8–28.5) long × 41.3 (36.7–46.9) wide bearing 8 processes 11.5 (8.9–15.2) long; posterior bulb 18.3 (12.8–22.6) long × 51.7 (44.8–67.2) wide. Intestinal crura, short, extend to the posterior end of uterus. Haptor, sub-ovate to spherical when attached, clearly delineated from body, 76.8 (67.6–96.1) long × 65.0 (50.0–77.7) wide (Fig. 3e, f). Male copulatory organ ventro-lateral to posterior pharyngeal bulb or posterior to it, 13.8 (11.0–15.4) long × 13.7

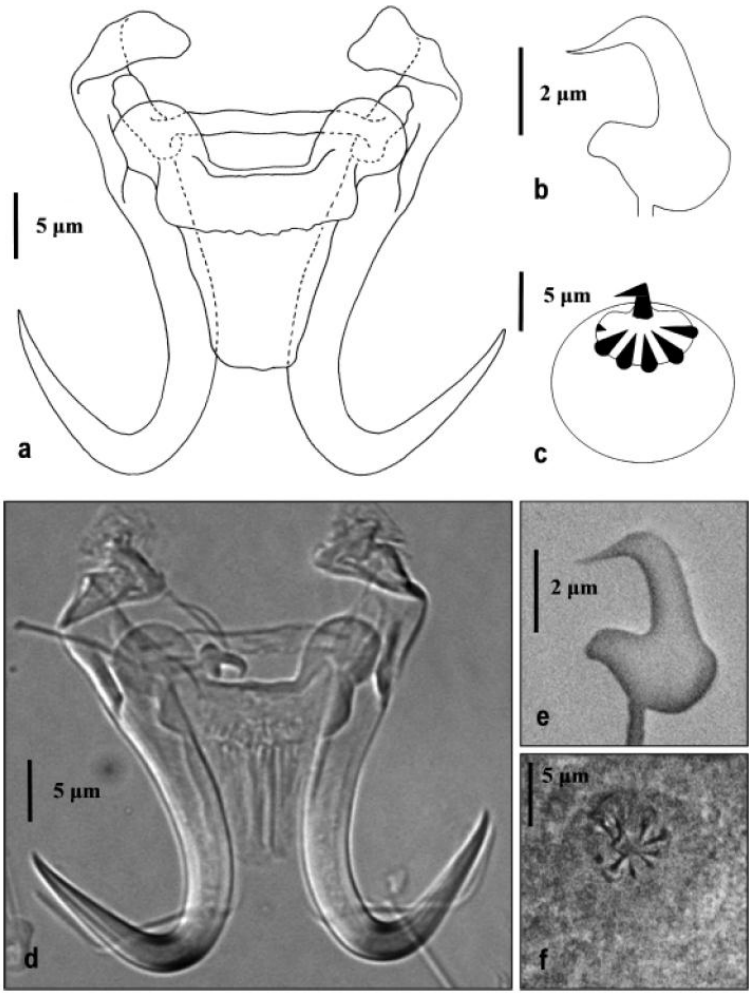

Fig. 2. *Gyrodactylus orecchiae* sp. n. **a, d** – the haptoral central hook complex of hamuli, dorsal and ventral bars; **b, e** – marginal hook sickle; **c, f** – male copulatory organ showing one large apical spine and a single row of five equal-sized small spines.

(11.1–16.6) wide, spherical, armed with large apical hook and single arch of 5 (4–6) small even-sized spines (Fig. 2c, f). Hamuli total length 34.6 (32.6–38.1); shaft length 21.1 (20.2–22.6); point 15.7 (14.6–16.2) long with a 37.3° (33.3–40.6°) aperture; inwardly directed roots 10.2 (7.7–14.2) long with central depression and thickened margins (Figs. 2a, d, 3a). Dorsal bar 16.4 (15.5–18.2) long; 2.1 (1.8–2.3) wide (Figs. 2a, d, 3a). Ventral bar 20.6 (18.6–22.0) long; 21.7 (19.0–24.0) wide; ventral bar processes prominent, rounded, 4.6 (4.0–5.6) long; ventral bar membrane lingulate, posteriorly rounded, 10.6 (9.3–11.8) long (Figs. 2a, d, 3a). Marginal hook length 18.2 (17.5–18.7); shaft length 14.7 (14.2–15.9); sickle proper length 3.3 (3.0–4.0); sickle base tangential to plane of shaft with proximal width 3.2 (2.6–3.5); rhomboid toe 1.8 (1.2–2.0) long; heel rounded; sickle shaft parallel to long axis of entire hook; sickle point perpendicular to sickle shaft, tapers to fine point with distal width 2.2 (1.9–2.5); sickle aperture 3.4 (3.1–3.8); inner curve of sickle proper approximately rhomboid (Figs. 2b, e, 3b1–b5).

Type host: *Sparus aurata* L. (gilthead seabream), Sparidae.

Site: Skin, fins, eyes and gill filaments.

Type locality: Orikum, Albania (40°18'50.92" N, 19°28'32.93" E)

Other reported localities: Ugljan Island, Croatia (44°7'45.87" N, 15°6'9.77" E)

Type material: Forty specimens were studied for light microscopy and ten digested specimens for SEM studies. Holotype (BMNH Reg. No. 2008.12.15.1) and paratype (BMNH Reg. No. 2008.12.15.2) are deposited in the parasitic worm collection at The Natural History Museum, London. Additionally, one paratype (M-475) is deposited in the gyrodactylid collection held at the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice.

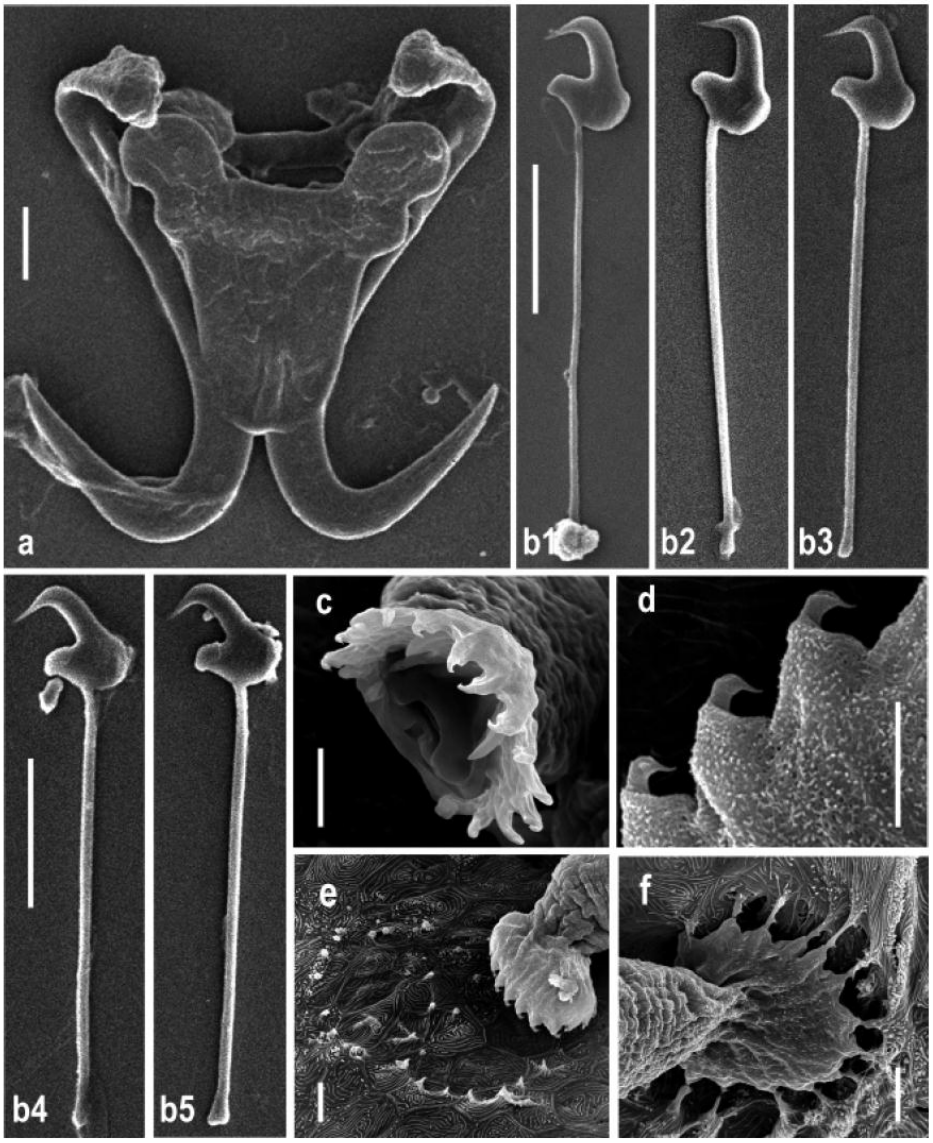

Fig. 3. Scanning electron micrographs of *Gyrodactylus orecchiae* sp. n. **a** – central hook complex showing the characteristic inwardly directed hamuli roots; **b1–b5** – marginal hooks; **c** – ventral surface of the haptor showing the position of the ventral and dorsal bar which may project into the epithelium of its host, preventing the haptor from slipping backwards, promoting the efficiency of attachment; **d** – characteristic marginal hook sickle points projecting from the haptoral tegument; **e** – attachment wound; **f** – sub-ovate haptor. Scale bars: a, b1–b5, d = 5 µm; c, e, f = 10 µm.

Molecular sequence data: The 1147 bp amplified fragment (18S (1–16) + ITS1 (17–529) + 5.8S (530–686) + ITS2 (687–1090) + 28S (1091–1147)) is deposited in GenBank under Accession Number FJ013097.

Etymology: Named in honour of Professor Paola Orecchia.

Histopathology. Acute dermatitis (hyperplasia and necrosis) was observed in the seabream with large numbers (1000+) of *Gyrodactylus* attached to the epidermis; epidermal spongiosis and some hydropic degeneration was also evident. Infections by *Gyrodactylus* on the gills showed secondary infection by bacteria with cellular exfoliation and mild haemorrhaging.

Molecular characterisation. The amplified nucleotide sequence of the rDNA cluster was 1147 bp and consisted of the 3' end of the 18S subunit (16 bp), the ITS1 (513 bp), the 5.8S gene (157 bp), the ITS2 (404 bp) and the 5' end of the 28S subunit (57 bp). Submitting the ITS1 to a BLASTN (Altschul 1991) search revealed no related sequences while a search using the 5.8S gave total ho-

Table 1. Morphological measurements (mean ± standard deviation followed by the range in parentheses; in micrometres) of *Gyrodactylus orecchiae* sp. n. from *Sparus aurata* collected from Orikum, Albania, which are respectively compared with those of *Gyrodactylus arcuatus* Bychowsky, 1933 from freshwater *Gasterosteus aculeatus*, *Gyrodactylus arcuatus* Bychowsky *sensu* Bychowsky et Poljansky, 1953 from *Gasterosteus aculeatus* from the Baltic Sea off Sweden, and *Gyrodactylus quadratidigitus* Longshaw, Pursglove et Shinn, 2003 from *Thorogobius ephippiatus*. Measurements taken from original descriptions in the literature are shown in a bold font, whilst those in regular font represent new measurements made in the current study. New measurements for *G. quadratidigitus* are provided from a syntype.

Measurement	***G. orecchiae* sp. n. (n = 40)**	***G. arcuatus* Bychowsky, 1933 (n = 24)[1]**	***G. arcuatus* Bychowsky *sensu* Bychowsky et Poljansky, 1953[2]**	***G. quadratidigitus* Longshaw, Pursglove et Shinn, 2003**
Total body length	356.3 ± 51.4 (275.0–455.9)	399.8 ± 36.3 (340.4–464.0)[4]	**336–460**	**430.0 ± 63.0 (334.0–486.0)**
Total body width	81.5 ± 7.6 (62.1–92.1)	102.1 ± 14.3 (82.4–122.6)[4]	**100–128**	**122.0 ± 19.0 (104.0–149.0)**
Haptor length × width	76.8 ± 12.1 (67.6–96.1) × 65.0 ± 12.0 (50.0–77.7)[3]	52.5 ± 9.7 (45.4–69.5) × 70.3 ± 8.8 (63.6–85.1)[3]	–	**48.9 ± 3.7 (45–54) × 81.4 ± 8.0 (70.5–88.0)**
Pharynx length × width (anterior; posterior bulb)	Ant: 25.1 ± 2.6 (22.8–28.5) × 41.3 ± 4.3 (36.7–46.9); Post: 18.3 ± 3.4 (12.8–22.6) × 51.7 ± 8.0 (44.8–67.2)[3]	Ant: 23.8 ± 5.1 (16.1–27.8) × 32.2 ± 2.8 (28.2–35.1); Post: 14.9 ± 3.8 (8.6–18.3) × 39.7 ± 2.9 (35.7–42.7)[3]	–	**30.0 ± 1.8 (28.5–32.5) × 30.3 ± 2.7 (27.0–33.5)**
Male copulatory organ length × width	13.8 ± 1.7 (11.0–15.4) × 13.7 ± 2.0 (11.1–16.6)[3]	13.9 ± 1.2 (12.1–14.9) × 13.9 ± 1.7 (11.9–16.4)[3]	–	**8.2 ± 0.5 (7.0–8.5) armed with 5–7 small spines**
Hamulus				
Ham aperture	11.5 ± 0.6 (10.5–12.4)	17.1 ± 1.0 (15.0–19.0)	–	12.0
Ham prox. shaft width	5.5 ± 0.3 (5.0–6.4)	6.7 ± 1.0 (5.0–9.3)	–	6.2
Ham point length	15.7 ± 0.4 (14.6–16.2)	18.9 ± 1.0 (17.0–20.6)	**15.2–18.5**	**14.0 ± 1.6 (12.3–15.5)**
Ham distal shaft width	3.5 ± 0.2 (3.2–3.8)	3.7 ± 0.4 (2.9–4.4)	–	2.2
Ham shaft length	21.1 ± 0.6 (20.2–22.6)	26.0 ± 1.2 (24.1–27.1)	**28.3–32.9**	**21.0 ± 3.8 (17.7–24.6)**
Ham inner curve length	2.3 ± 0.4 (1.5–2.9)	2.8 ± 0.7 (1.5–3.6)	–	1.2
Ham aperture angle (°)	37.3 ± 2.2 (33.3–40.6)	44.0 ± 4.1 (37.0–53.6)	–	44.0
Ham point curve angle (°)	14.0 ± 2.9 (9.8–20.7)	12.2 ± 2.1 (7.6–15.1)	–	20.0
Inner ham apert angle (°)	44.2 ± 3.3 (38.4–49.0)	50.9 ± 4.7 (43.1–61.7)	–	**53.9 ± 0.8 (53.2–54.6)**
Ham root length	10.2 ± 1.9 (7.7–14.2)	11.1 ± 1.2 (8.2–13.9)	**9.6–13.7**	**10.0 ± 0.9 (8.9–11.1)**
Ham total length	34.6 ± 1.9 (32.6–38.1)	40.4 ± 2.0 (35.8–43.5)	**35.9–43.1**	**28.0 ± 3.8 (24.4–31.8)**
Dorsal bar				
DB total length	16. 4 ± 1.0 (15.5–18.2)[3]	16.0 ± 0.9 (14.9–16.8)[3]	**17.4**	**12.3 ± 0.9 (11.4–13.4)**
DB width	2.1 ± 0.2 (1.8–2.3)[3]	2.0 ± 0.2 (1.7–2.2)[3]	**0.9**	**0.8 ± 0.1 (0.8–0.9)**
Ventral bar				
VB total width	21.7 ± 1.3 (19.0–24.0)	23.6 ± 2.0 (20.6–27.5)	**15.7–20.0**	**18.0 ± 1.5 (16.2–19.5)***
VB total length	20.6 ± 0.9 (18.6–22.0)	24.9 ± 2.1 (19.7–28.0)	**24.4–27.0**	**12.1 ± 0.7 (11.3–13.0)***
VB process to mid-length	5.2 ± 0.7 (4.0–7.5)	8.0 ± 0.9 (5.6–9.7)	–	3.0
VB median length	5.0 ± 0.4 (4.3–5.8)	4.9 ± 0.7 (3.6–6.0)	**3.9–5.2**	2.1
VB process length	4.6 ± 0.4 (4.0–5.6)	7.1 ± 0.9 (5.7–9.2)	–	**3.0 ± 0.0 (3.0–3.0)**
VB membrane length	10.6 ± 0.7 (9.3–11.8)	12.5 ± 1.7 (8.1–15.3)	**11.3–11.8**	**8.2 ± 0.6 (7.7–8.8)**
Marginal hook				
MH total length	18.2 ± 0.3 (17.5–18.7)	22.4 ± 1.3 (20.3–24.4)	**19.6–22.2**	**25.2 ± 1.1 (24.2–31.8)**
MH shaft length	14.7 ± 0.4 (14.2–15.9)	18.2 ± 1.2 (15.9–20.2)	**15.7–18.3**	**21.0 ± 0.9 (19.9–22.4)**
MH sickle length	3.3 ± 0.2 (3.0–4.0)	5.1 ± 0.3 (4.6–5.9)	**4.4**	**4.9 ± 0.4 (4.4–5.7)**
MH sick prox width	3.2 ± 0.2 (2.6–3.5)	3.9 ± 0.3 (3.4–4.3)	**3.5–3.9**	**3.0 ± 0.3 (2.8–3.7)**
MH toe length	1.8 ± 0.2 (1.2–2.0)	1.4 ± 0.1 (1.1–1.7)	–	1.1
MH sick dist width	2.2 ± 0.2 (1.9–2.5)	2.6 ± 0.2 (2.1–2.9)	**2.2**	**3.9 ± 0.4 (3.1–4.5)**
MH aperture	3.4 ± 0.2 (3.1–3.8)	3.9 ± 0.3 (3.4–4.3)	–	3.8
MH instep / arch height	0.4 ± 0.1 (0.3–0.5)	0.6 ± 0.1 (0.4–0.8)	–	0.2

[1]Specimens taken from a freshwater population of *Gasterosteus aculeatus* L. from Loch Airthrey, Stirlingshire, Scotland (56°8'47.6"N, 3°59'33.5"W); [2]Data taken from Malmberg (1970) represent specimens in marine environments; [3]Based on the measurement of 5 specimens; [4]Based on the measurement of 10 specimens; *The terms ventral bar length and width in this study are used in relation to longitudinal axis of the worm's body. The measurements in Longshaw et al. (2003), however, follow those of Malmberg (1970) and have been switched for direct comparison in this study.

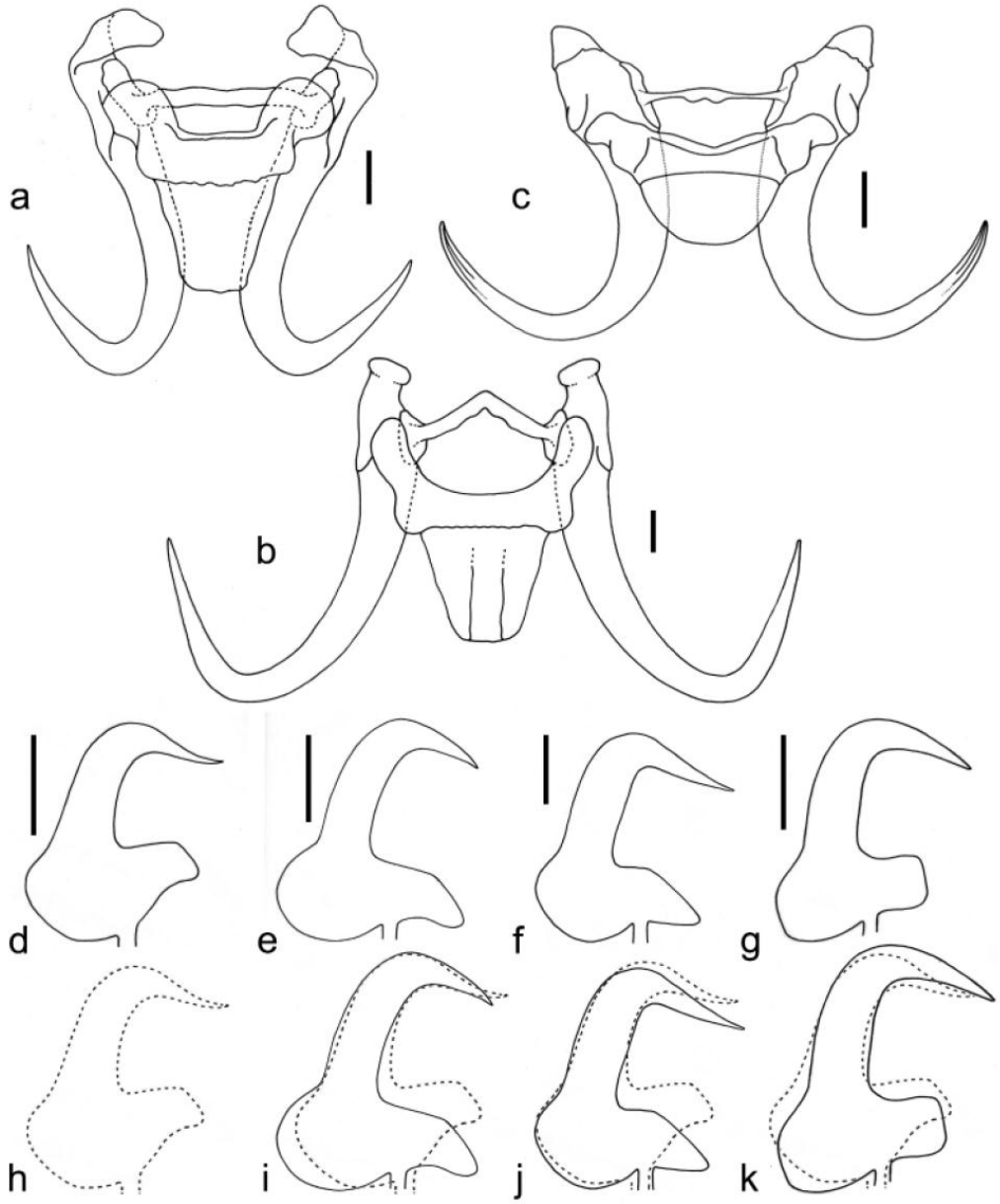

Fig. 4. A comparison of the haptoral hooks of *Gyrodactylus orecchiae* sp. n. with morphologically similar species. **a–c** – comparison of the central haptoral complex; **a** – *G. orecchiae*; **b** – *G. arcuatus* Bychowsky, 1933; **c** – *G. quadratidigitus* Longshaw, Pursglove et Shinn, 2003 (redrawn from original); **d–g** – marginal hooks of four morphologically similar species; **d** – *G. orecchiae*; **e** – *G. arcuatus*; **f** – *G. colemanensis* Mizelle et Kritsky, 1967; **g** – *G. quadratidigitus*; **h–k** – overlays of the marginal hook sickle for *G. orecchiae* with morphologically similar species (marginal hook sickles size invariant); **h** – *G. orecchiae* as a broken line; **i** – overlay of *G. orecchiae* with *G. arcuatus*; **j** – overlay of *G. orecchiae* with *G. colemanensis*; **k** – overlay of *G. orecchiae* with *G. quadratidigitus*. Scale bars: a–c = 5 μm; d–g = 2 μm.

mology (*p*-distance = 0) with *Gyrodactylus alexgussevi*, *G. branchicus*, *G. flesi*, *G. lotae*, *G. rarus*, *G. robustus* and *G. rugiensoides*. When the ITS2 was blasted separately, then the closest gyrodactylid with a homology of 78% (coverage of 97%) was the *Gyrodactylus* species parasitizing *Gobius niger* L. (see Huyse et al. 2003). Furthermore, homology of 86% on ITS2 (coverage of only 47%) was also obtained with *G. alexgussevi*, *G. branchicus*, *G. lotae* and *G. rarus* (*p*-distance = 0.101).

DISCUSSION

Gyrodactylus orecchiae is the first species of this genus to be formally described from *Sparus aurata* although one other species, *G. alviga*, is recorded from two other sparid hosts, *Diplodus annularis* and *Sarpa salpa* (Dmitrieva and Gerasev 2000). The morphology of the attachment hooks of these two gyrodactylids, however, differs markedly. Although large ventral bar processes are a characteristic feature of many gyrodactylid species, notably among the Nearctic gyrodactylid fauna, *viz*. the freshwater species *G. colemanensis* Mizelle et Kritsky, 1967 from *Salvelinus fontinalis* (Mitchill), and the brackish/marine species *viz*. *G. groenlandicus* Levinsen, 1881 from *Myoxocephalus scorpius* (L.), *G. nainum* Hanek et Threlfall, 1970 from *Triglopsis* (*Myoxocephalus*) *quadricornis* (L.), *G. pleuronecti* Cone, 1981 from *Pseudopleuronectes americanus* (Walbaum) and *G. stephanus* Mueller, 1937 from *Fundulus heteroclitus* (L.), *G. orecchiae* can be discriminated from these other species based on the morphology of its marginal hook sickle. For example, when the marginal hook sickle of *G. orecchiae* is aligned to a morphologically similar species, such as *G. colemanensis*, although the shaft and point regions are proportionally alike and describe the same rhomboid inner curve to the sickle (Fig. 4j), other marginal hook features allow their differentiation from each other. For example, the toe of *G. colemanensis* is triangular whilst that of *G. orecchiae* is square to rhomboid and is upwardly oriented in the direction of the sickle point. The sickle base of *G. orecchiae* is proportionally deep with a large rounded heel (Fig. 4d, f, j). The size of the marginal hooks of these two gyrodactylids also differ markedly: (31.1 (col) *vs*. 18.2 (orec) total length; 25.8 (col) *vs*. 14.7 (orec) shaft length; 6.0 (col) *vs*. 3.3 (orec) sickle length; 4.2 (col) *vs*. 3.2 (orec) sickle proximal width; 4.1 (col) *vs*. 2.2 (orec) sickle distal width, 1.5 (col) *vs*. 1.8 (orec) toe length; 4.6 (col) *vs*. 3.4 (orec) aperture) (data for *G. colemanensis* taken from Shinn 1993).

Two other morphologically similar species are *G. arcuatus* Bychowsky, 1933 (Fig. 4b, e) and *G. quadratidigitus* Longshaw, Pursglove et Shinn, 2003 (Fig. 4c, g). The former is known from both freshwater and marine populations of three-spined sticklebacks (*Gasterosteus aculeatus* L.) and as seen in *G. orecchiae*, it also possesses large ventral bar processes, hamuli roots that are commonly observed to turn inwards over the ventral bar processes, and marginal hooks with a square line to the inner edge of the sickle blade and large rounded heels. *Gyrodactylus arcuatus*, however, can be readily discriminated from *G. orecchiae* based on the shape of the sickle proper toe, which is long and triangular in the former (Fig. 4d, e, i). *Gyrodactylus quadratidigitus* from *Thorogobius ephippiatus* (Lowe), prior to the current study, appeared to be unique in that it possesses marginal hooks with a square toe (Fig. 4g), a male copulatory organ (*sic*. cirrus) positioned in line with or anterior to the posterior pharyngeal bulb, and unusually short intestinal crura which do not extend beyond the level of the testis. The position of the male copulatory organ in mature specimens of *G. orecchiae* appears to be variable. It has been observed in positions ranging from medial or posterior to the posterior pharyngeal bulb to lateral, the centre of the male copulatory organ level with the posterior edge of the pharyngeal bulb. The intestinal crura of *G. orecchiae* also appear to be very short in that they do not extend beyond the most posterior limit of the uterus. It is the blunt-ended toe of the marginal hook sickle, however, of both *G. orecchiae* and *G. quadratidigitus* that are characteristic but the morphology of each is not so subtle as to prevent their discrimination from one another (Fig. 4d, g, k).

The angles at which the ventral bar processes and the hamuli roots project under the haptoral tegument and their alignment to one another create a series of ridges that may serve to increase the efficiency of attachment in this species (Fig. 3c). The apparently robust processes of the ventral bar, it is hypothesized, would press into the epidermal tissues of its host at an opposing angle to the principal force of action by the marginal hooks contributing to the worm's attachment and minimising the risks of its dislodgement.

Of the 409 species of *Gyrodactylus* described so far, only around 20% have been sequenced at the ITS. In the absence of molecular data for *G. colemanensis* and *G. quadratidigitus*, both of which are nominally "similar" to *G. orecchiae*, a thorough analysis of the taxonomic affinities of these species must await a more thorough molecular coverage of the group.

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Gyrodactylus longipes n. sp. (Monogenea: Gyrodactylidae) from farmed gilthead seabream (*Sparus aurata* L.) from the Mediterranean

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ABSTRACT

Gyrodactylus longipes n. sp. (Monogenea, Gyrodactylidae) is described from the gills of farmed juvenile gilthead seabream (*Sparus aurata* L.) from two sites located in Italy and Bosnia–Herzegovina and represents the second species of *Gyrodactylus* to be described from *S. aurata*. *Gyrodactylus orecchiae* Paladini, Cable, Fioravanti, Faria, Di Cave *et* Shinn, 2009 was the first gyrodactylid to be described from *S. aurata*, from populations cultured in Albania and Croatia. In the current study, *G. longipes* was found in a mixed infection with *G. orecchiae* on fish maintained in Latina Province, Italy, thus extending the reported distribution of the latter throughout the Mediterranean. The morphology of the opisthaptoral hard parts of *G. longipes* is compared to those of *G. orecchiae*, using light and scanning electron microscopy. *Gyrodactylus longipes* is characterised by having larger, elongated ventral bar processes and long, triangular-shaped toe region to their marginal hook sickles which, by comparison, are rhomboid in *G. orecchiae*. The marginal hook sickles of *G. longipes* are almost double the size of *G. orecchiae* which allows for their rapid discrimination from each other in mixed infections. A comparison of the DNA sequence of the ribosomal internal transcribed spacer 1 and 2 regions (ITS1 and ITS2) of *G. longipes* with the corresponding sequence from *G. orecchiae* and with those available in GenBank, supports the separate species status of *G. longipes*. Part of this study necessitated an overview of the existing *Gyrodactylus* fauna from Italy and Bosnia–Herzegovina; a summary from each country is provided here to assist future investigations.

1. Introduction

Gilthead seabream (*Sparus aurata* L.) (Sparidae) is ranked among the most important fish species farmed in the Mediterranean with annual production now exceeding 125,000 tonnes. The largest producer in the Mediterranean is Greece (49%), followed by Turkey (15%), Spain (14%) and Italy (6%) [1].

As the production of *S. aurata* throughout the Mediterranean has increased, commercial enterprises have placed a greater emphasis on health management. If infections by Monogenea only are considered, then the most commonly encountered species infecting the gills of *S. aurata* are: the microcotylid *Sparicotyle chrysophrii* (van Beneden *et* Hesse, 1863) Mamaev, 1984, which can cause anaemia and high mortality at low intensities (8–10 parasites/gill arch) in fish weighing 10–300 g; and, the diplectanid *Furnestinia echeneis* (Wagener, 1857) Euzet *et* Audouin, 1959, which although common, does not represent a significant threat. Most recently, *Gyrodactylus orecchiae* Paladini, Cable, Fioravanti, Faria, Di Cave *et* Shinn, 2009, the first species of *Gyrodactylus* to be described from *S. aurata*, was found to be responsible for a ~10% mortality in juvenile stock [2]. This latter material obtained from Albania and Croatia was infected with a single species of *Gyrodactylus*, parasitising the skin, fins, eyes and gills in high numbers (1000+ parasites/fish) [2]. Additional *S. aurata* samples subsequently received from a farm site located on the Tyrrhenian coast of Italy and from a second farm site from Bosnia–Herzegovina, in addition to harbouring *G. orecchiae*, were found to have a second species of *Gyrodactylus*. This study provides a morphological description of the new species using light and scanning electron microscopy (SEM), which is supplemented with a reference DNA sequence of the internal transcribed spacer region.

2. Materials and methods

2.1. Specimens collection

Two samples of *S. aurata*, submitted as part of each farm's routine health assessment of stock, were processed by the fish health diagnostics team in the Department of Veterinary Medical Sciences, Univesity of Bologna, Italy. The first sample came from a marine cage site in Latina Province, located on the Tyrrhenian coast of Italy (41°13′

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36.45″N; 13°34′23.62″E), and the second sample from a marine hatchery located at Neum, Bosnia–Herzegovina (42°55′00.07″N; 17°36'59.91"E). A small sub-sample of both collections was fixed in 70% ethanol to preserve any ectoparasites present. The Italian sample, collected in January 2007, consisted of three fish weighing ~25 g, whilst the second sample of five fish weighing ~50 g was collected in May 2007 from Bosnia–Herzegovina.

2.2. Specimens preparation for morphological analysis

Individual gyrodactylids, principally infecting the gills, were removed from the fixed hosts and then rinsed in distilled water. Specimens were prepared either as whole mounts in ammonium picrate glycerine according to the method detailed by Malmberg [3] or had their opisthaptors excised and then subjected to proteolytic digestion. The alcohol-fixed body corresponding to each digested opisthaptor was subsequently transferred to 95% ethanol for molecular characterisation. For digestion, individual opisthaptors were placed on a glass slide and the tissues enclosing the attachment hooks were removed using 3 µl of digestion solution [4]. The digestion of each specimen was continuously monitored under a ×4 objective on an Olympus SZ30 dissecting microscope. Tissue digestion was then arrested by the addition of 2 µl of a 1:1 formaldehyde : glycerine mix. A glass coverslip (18×18 mm, "0" thickness, VWR International, Lutterworth, UK) was then placed over the hook preparation and the edges sealed with nail varnish. For specimens prepared for scanning electron microscopy (SEM), individual opisthaptors were digested on 13 mm diameter glass coverslips (Chance Propper Ltd., Warley, UK), rinsed several times with distilled water, air-dried, sputter-coated with gold and then examined using a JEOL JSM5200 scanning electron microscope operating at an accelerating voltage of 10 kV.

For the morphological study, the opisthaptoral hard parts were studied from images captured using a Zeiss AxioCam MRc digital camera mounted on top of an Olympus BH2 compound microscope using a ×0.75 interfacing lens. Images of the opisthaptoral hard parts were captured using ×40 and ×100 oil immersion objectives and MRGrab 1.0.0.4 (Carl Zeiss Vision GmbH, 2001) software. A total of 27 point-to-point morphometric measurements were made on the opisthaptoral hooks of each specimen from images captured using a JVC KY-F30B 3CCD (JVC, Yokohama, Japan) video camera mounted on

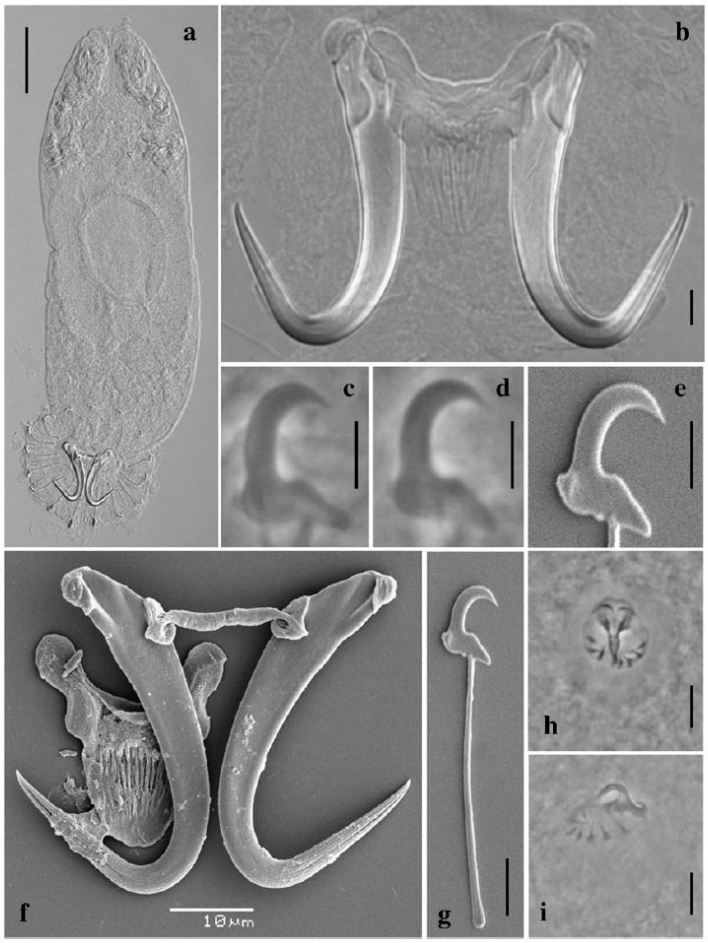

Fig. 1. *Gyrodactylus longipes* n. sp. from gilthead seabream (*Sparus aurata* L.) from Latina Province, located on the Tyrrhenian coast of Italy (type-locality). a – holotype, whole parasite in ventral view; b – light micrographs of the opisthaptoral central hook complex showing the hamuli, the dorsal and the ventral bar (ventral view); c – light micrograph of a marginal hook sickle; d – light micrograph of a marginal hook sickle from *G. longipes* from Neum, Bosnia-Herzegovina; e – scanning electron micrograph of the marginal hook sickle. f – scanning electron micrograph of the opisthaptoral central hook complex (dorsal view); g – scanning electron micrograph of a marginal hook; h – male copulatory organ (MCO); i – MCO of *G. longipes* from Neum, Bosnia-Herzegovina. Scale bars: a = 50 µm; b, g-i = 5 µm; c-e = 3 µm.

an Olympus BH2 microscope using a ×2.5 interfacing lens. Images were captured using a ×100 oil immersion objective and the KS300 (ver.3.0) (Carl Zeiss Vision GmbH, 1997) image analysis software. The measurements follow those detailed in Shinn et al. [5] and are expressed in micrometres as the mean ± 1 standard deviation followed by the range in parentheses.

2.3. Molecular analysis

DNA was extracted from four specimens, two individuals from each population, using a DNeasy® Blood & Tissue minikit (Qiagen). The primer pair ITS1A (5′-GTAACAAGGTTTCCGTAGGTG-3′) and ITS2 (5′-TCCTCCGCTTAGTGATA-3′) [6] were used to amplify (PCR) a fragment spanning the 3′ end of the 18S ribosomal RNA subunit, internal transcribed spacers 1 and 2 (ITS1 and ITS2), the 5.8S subunit and the 5′ end of the 28S subunit. The PCR reactions were performed with PuReTaq Ready-To-Go™ PCR beads (GE Healthcare) following the manufacturer's manual. The PCR program was as follows: 4 min at 95 °C followed by 35 cycles of 1 min at 95 °C, 1 min at 55 °C and 2 min at 72 °C. PCR products were subsequently purified using a NucleoSpin® Purification Kit (Macherey–Nagel) and sequencing reactions were carried out on a MegaBace 1000 analysis system (GE Healthcare) using DYEnamic ET dye terminators. In addition to the PCR primers, the internal primers ITS4.5 (5′-CATCGGTCTCTCGAACG-3′) [6], ITS1R (5′-ATTTGCGTTCGAGAGACCG-3′), ITS18R (5′-AAGACTACCAGTTCACTCCAA-3′), ITS2F (5′-TGGTGGATCACTCGGCTCA-3′) and ITS28F (5′-TAGCTCTAGTGGTTCTTCCT-3′) [7] were used for sequencing. Sequences (ITS1, 5.8S and ITS2 only) were proofread and assembled in Vector NTI 11 (Invitrogen) and submitted to a BlastN search [8] with default parameter settings to reveal possible identity with other species already in GenBank.

To place the species found in the current study within the phylogeny of *Gyrodactylus*, 32 sequences from species representing the major groupings of the genus as used by *e.g.* Ziętara & Lumme [9] and Vanhove et al. [10] were selected. In addition, the sequences that retrieved a 100% hit when performing a BlastN search with the conservative 5.8S separately were also included. *Macrogyrodactylus heterobranchii* N'Douba *et* Lambert, 1999 was used as the outgroup.

Due to the difficulty of aligning ITS1, as commented upon by other authors (see *e.g.* Ziętara & Lumme [9]), this fragment was excluded from the current analyses. The remaining fragments, consisting of 5.8S and ITS2, were aligned in MUSCLE within Mega 5 [11] using the default parameters. The 3′ ends of ITS2 were trimmed manually to remove ambiguous sites and the resulting data set was 606 bp.

The phylogenetic reconstruction was performed in Mega 5 by the Maximum Likelihood (ML) method based on the Tamura-Nei model [12]. Nodal support was assessed through 1000 bootstrap samples. All positions with less than 95% site coverage were eliminated, giving a total of 412 positions in the final dataset. In addition to the ML-method, a neighbor-joining analysis using the maximum composite likelihood method of calculating evolutionary distances was performed.

3. Results

Gyrodactylus longipes n. sp. is described from an Italian farmed stock of *S. aurata* from the Tyrrhenian coast near Latina Province. Although *G. longipes* was found to co-occur alongside *G. orecchiae* on the skin (1–2 individuals of each species per fish), *G. longipes* was the only species found on the gills and at a low intensity of infection (~20 gyrodactylids/fish). A subsequent sample of *S. aurata* received from Neum, Bosnia–Herzegovina was infected, principally on the gills, with a single species, *G. longipes*, at intensities similar to those found on the material originating from Italy.

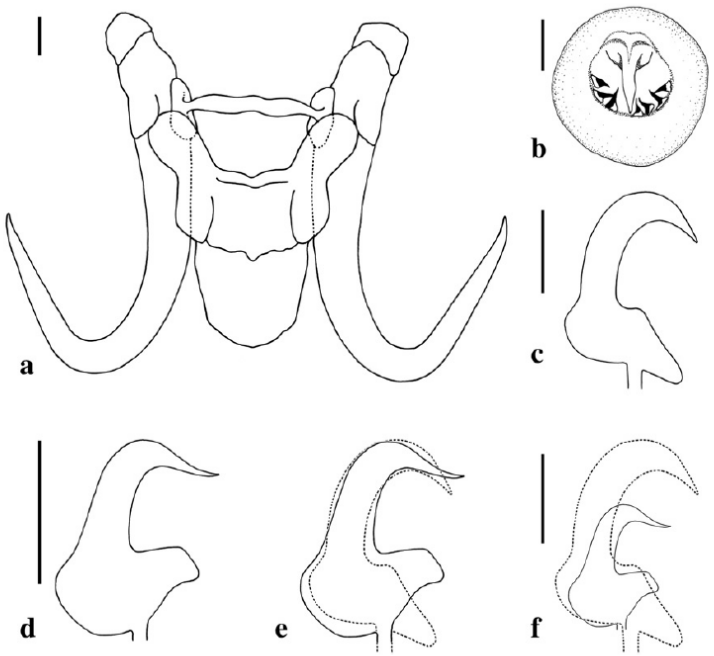

Fig. 2. Drawings of the opisthaptoral hard parts and male copulatory organ (MCO) of *Gyrodactylus longipes* n. sp. from gilthead seabream (*Sparus aurata* L.) from Latina Province, Italy (type-locality). a – opisthaptoral central hook complex; b – MCO; c – marginal hook sickle of *G. longipes*; d – marginal hook sickle of *Gyrodactylus orecchiae* Paladini, Cable, Fioravanti, Faria, Di Cave *et* Shinn, 2009 from *S. aurata*; e – a size invariant overlay of the marginal hook sickles of *G. longipes* (broken line) with *G. orecchiae* (solid line); f – a size variant overlay of the marginal hook sickles of *G. longipes* (broken line) with *G. orecchiae* (solid line) Scale bars: a, b = 5 μm; c, d, f = 3 μm.

3.1. Gyrodactylus longipes n. sp

Type host: *Sparus aurata* L. (gilthead seabream) (Perciformes: Sparidae).
Site on the host: principally on the gills; occasionally on the skin.
Type locality: Latina Province, Italy (41°13′36.45″N; 13°34′23.62″E).
Additional locality: Neum, Bosnia–Herzegovina (42°55′00.07″N; 17°36′59.91″E).
Type material: twenty-two specimens (twelve from the type-locality and ten from Neum) were studied for light microscopy. Holotype (BMNH accession no. 2009.6.2.1) and 7 paratypes (BMNH accession nos. 2009.6.2.2–8) from the type-locality, in addition with 2 paratypes from Neum (BMNH accession nos. 2009.6.2.9–10), are deposited in the parasitic worm collection at The Natural History Museum, London. Additionally, four paratypes from the type-locality (AHC accession nos. 29822–29825) and 3 paratypes from Neum (AHC accession nos. 29826–29828) are deposited in the Australian Helminthological Collection (AHC) of The South Australian Museum (SAMA), North Terrace, Adelaide. Five paratypes from Neum (accession no. USNPC 102014) are deposited in the United States National Parasite Collection, Beltsville, Maryland, USA.
DNA reference sequence: the 1064 bp ribosomal DNA sequence consisting of the 3′-end of 18S (20 bp), ITS1 (412 bp), 5.8S (157 bp), ITS2 (433 bp) and the 5′-end of 28S (42 bp) is deposited in GenBank under accession number GQ150536.
General: a species profile including taxonomic traits, host details and additional metadata is provided on the on-line databases www.gyrodb.net[13,14] and www.monodb.org[15].
Etymology: named after the long toe portion of the marginal hook sickle (from Latin: *longi* = long; *pes* = foot).

Table 1
Morphological measurements (mean ± 1 standard deviation followed by the range in parentheses, are provided in micrometres) of *Gyrodactylus longipes* n. sp. from *Sparus aurata* L. collected from Latina Province, located on the Tyrrhenian coast of Italy (type-locality), and from Neum, Bosnia-Herzegovina, which are presented alongside the previously reported species on the same host, *Gyrodactylus orecchiae* Paladini, Cable, Fioravanti, Faria, Di Cave *et* Shinn, 2009, from Albania and Croatia [2].

Measurement	*Gyrodactylus longipes* n. sp. from Italy (n = 12)	*Gyrodactylus longipes* n. sp. from Bosnia-Herzegovina (n = 10)	*Gyrodactylus orecchiae* Paladini *et al.*, 2009 (n = 40)
Total body length	390 ± 60.7 (300–490)[a]	419 ± 43.8 (350–475)[b]	356.3 ± 51.4 (275–455.9)
Total body width	136 ± 26.1 (105–175)[a]	159 ± 19.4 (125–175)[b]	81.5 ± 7.6 (62.1–92.1)
Opisthaptor length × width	89.9 ± 20.6 (50–125) ×	91.0 ± 11.2 (75–110) ×	76.8 ± 12.1 (67.6–96.1) ×
	109.5 ± 18.0 (85–150)[a]	132.3 ± 19.2 (100–150)[b]	65.0 ± 12.0 (50.0–77.7)
Anterior pharynx bulb length × width	16.5 ± 2.6 (13.4–19.7) ×	17.0 ± 2.9 (13.5–20.2) ×	25.1 ± 2.6 (22.8–28.5) ×
	28.6 ± 0.7 (27.6–29.4)	22.7 ± 5.7 (17.5–31.6)	41.3 ± 4.3 (36.7–46.9)
Posterior pharynx bulb length × width	30.3 ± 4.6 (21.7–34.4) ×	28.2 ± 2.9 (24.3–30.4) ×	18.3 ± 3.4 (12.8–22.6) ×
	44.7 ± 2.4 (41.5–47.5)[a]	45.4 ± 8.2 (35.8–53.3)[b]	51.7 ± 8.0 (44.8–67.2)
MCO length × width	15.6 ± 1.0 (14.6–16.8) ×	16.1 ± 1.7 (14.3–17.7) ×	13.8 ± 1.7 (11.0–15.4) ×
	14.7 ± 1.2 (13.1–16.3)[c]	16.2 ± 0.4 (16.0–16.7)[d]	13.7 ± 2.0 (11.1–16.6)
Hamulus (H)			
H aperture	19.4 ± 0.9 (17.6–20.6)	18.9 ± 1.3 (16.7–21.9)	11.5 ± 0.6 (10.5–12.4)
H proximal shaft width	7.7 ± 0.3 (7.4–8.2)	7.6 ± 0.4 (6.7–8.3)	5.5 ± 0.3 (5.0–6.4)
H point length	24.9 ± 0.6 (24.3–25.8)	24.5 ± 0.7 (23.3–25.5)	15.7 ± 0.4 (14.6–16.2)
H distal shaft width	4.3 ± 0.2 (4.1–4.7)	4.4 ± 0.3 (3.9–5.0)	3.5 ± 0.2 (3.2–3.8)
H shaft length	33.5 ± 2.0 (30.7–36.1)	35.1 ± 1.5 (33.0–37.6)	21.1 ± 0.6 (20.2–22.6)
H inner curve length	2.6 ± 0.3 (1.9–3.1)	2.8 ± 0.7 (1.4–3.5)	2.3 ± 0.4 (1.5–2.9)
H aperture angle (°)	40.2 ± 1.5 (37.4–42.1)	39.2 ± 2.1 (35.1–43.4)	37.3 ± 2.2 (33.3–40.6)
H point curve angle (°)	8.6 ± 1.3 (5.7–10.8)	10.0 ± 2.5 (4.4–13.2)	14.0 ± 2.9 (9.8–20.7)
Inner H aperture angle (°)	46.1 ± 1.9 (42.9–49.4)	45.4 ± 3.0 (40.8–53.1)	44.2 ± 3.3 (38.4–49.0)
H root length	13.9 ± 0.5 (13.2–14.6)	13.8 ± 0.5 (12.6–14.8)	10.2 ± 1.9 (7.7–14.2)
H total length	46.4 ± 0.7 (45.1–47.5)	46.5 ± 1.3 (44.4–48.5)	34.6 ± 1.9 (32.6–38.1)
Dorsal bar (DB)			
DB total length	19.4 ± 1.6 (16.5–22.0)	19.5 ± 1.7 (16.6–22.1)	16.4 ± 1.0 (15.5–18.2)
DB width	2.5 ± 0.3 (2.1–2.8)	2.5 ± 0.3 (2.1–2.8)	2.1 ± 0.2 (1.8–2.3)
Ventral bar (VB)			
VB total width	28.5 ± 1.5 (25.8–31.9)	29.3 ± 1.7 (26.1–31.9)	21.7 ± 1.3 (19.0–24.0)
VB total length	29.8 ± 0.8 (28.0–31.0)	30.9 ± 1.3 (29.0–31.9)	20.6 ± 0.9 (18.6–22.0)
VB process-to-mid length	7.7 ± 0.6 (6.8–8.6)	7.9 ± 0.9 (6.2–9.2)	5.2 ± 0.7 (4.0–7.5)
VB median length	8.4 ± 0.6 (6.8–9.1)	8.2 ± 0.6 (7.1–9.3)	5.0 ± 0.4 (4.3–5.8)
VB process length	8.6 ± 0.3 (8.1–9.3)	8.8 ± 0.7 (8.1–10.2)	4.6 ± 0.4 (4.0–5.6)
VB membrane length	14.0 ± 0.7 (13.0–15.4)	14.9 ± 1.0 (13.5–16.4)	10.6 ± 0.7 (9.3–11.8)
Marginal hook (MH)			
MH total length	29.8 ± 1.0 (27.9–31.2)	30.1 ± 1.0 (27.7–31.4)	18.2 ± 0.3 (17.5–18.7)
MH shaft length	24.0 ± 0.7 (22.8–25.1)	24.3 ± 1.1 (21.2–26.2)	14.7 ± 0.4 (14.2–15.9)
MH sickle length	6.5 ± 0.1 (6.3–6.7)	6.6 ± 0.2 (6.1–7.1)	3.3 ± 0.2 (3.0–4.0)
MH sickle proximal width	5.1 ± 0.2 (4.8–5.5)	5.2 ± 0.3 (4.8–5.7)	3.2 ± 0.2 (2.6–3.5)
MH toe length	2.2 ± 0.1 (2.0–2.5)	2.6 ± 0.3 (2.2–3.0)	1.8 ± 0.2 (1.2–2.0)
MH sickle distal width	4.2 ± 0.1 (4.0–4.5)	4.3 ± 0.2 (3.9–4.5)	2.2 ± 0.2 (1.9–2.5)
MH aperture	5.3 ± 0.1 (5.1–5.6)	5.3 ± 0.3 (4.9–5.9)	3.4 ± 0.2 (3.1–3.8)
MH instep / arch height	0.4 ± 0.1 (0.4–0.6)	0.4 ± 0.1 (0.3–0.6)	0.4 ± 0.1 (0.3–0.5)

[a] Based on the measurement of 11 specimens.
[b] Based on the measurement of 8 specimens.
[c] Based on the measurement of 5 specimens.
[d] Based on the measurement of 3 specimens.

3.1.1. Morphological description (Figs. 1–2 and Table 1)

Body elongate, 390 (300–490) long; 136 (105–175) wide at the level of the uterus. Prohaptor with a single pair of cephalic lobes each bearing a gland and a spike sensillum. Anterior bulb of pharynx 16.5 (13.4–19.7) long × 28.6 (27.6–29.4) wide; posterior bulb 30.3 (21.7–34.4) long × 44.7 (41.5–47.5) wide. Intestinal caeca short which extend to a point level with the posterior end of the uterus. Presence or absence of excretory bladders not discernible on whole mounts. Opisthaptor, sub-ovate to spherical, clearly delineated from the body, 80.9 (50.0–125.0) long × 109.5 (85.0–150.0) wide (Fig. 1a). Male copulatory organ (MCO) (observed in five specimens) ventro-lateral to posterior pharyngeal bulb, the anterior edge slightly overlapping the posterior pharyngeal bulb, usually on the right, 15.6 (14.6–16.8) long × 14.7 (13.1–16.3) wide, spherical, armed with one large principle hook and a single ring of 6–9 (usually 6) approximately even-sized spines (Figs. 1h-i and 2c). Hamulus total length 46.4 (45.1–47.5); shaft length 33.5 (30.7–36.1); point 24.9 (24.3–25.8) long with a 40.2° (37.4–42.1°) aperture; root 13.9 (13.2–14.6) long (Figs. 1b, f and 2a). Dorsal bar 19.4 (16.5–22.0) long, which narrows at its union with the hamuli; 2.5 (2.1–2.8) wide (Figs. 1f and 2a). Ventral bar 29.8 (28.0–31.0) long; 28.5 (25.8–31.9) wide; ventral bar processes prominent, 8.6 (8.1–9.3) long; ventral bar membrane lingulate, lateral margins not thickened, posteriorly rounded, median zone of membrane striated, 8.4 (6.8–9.1) long (Figs. 1b, f and 2a). Ventral aspect of the median portion of the ventral bar smooth. Dorsal aspect of the median portion rugose. Marginal hook 29.8 (27.9–31.2) long; shaft length 24.0 (22.8–25.1); sickle proper length 6.5 (6.3–6.7); sickle base slender; sickle proximal width 5.1 (4.8–5.5). Toe triangular, distance from tip to union with marginal hook shaft 2.2 (2.0–2.5) long. Zenith of toe bridge in line with the attachment point of marginal hook shaft. Toe:heel ratio 1:2 (underside of sickle base); toe bridge:heel ratio 2:1 (upperside of sickle base). Heel rounded but not pronounced. Sickle shaft broad; sickle tip short terminating at a point beyond the apex of the bridge and approximately in line with the mid-point of the toe; distal width 4.2 (4.0–4.5); sickle aperture 5.3 (5.1–5.6) (Figs. 1c–e, g and 2b and d).

3.1.2. Molecular characterisation and phylogenetic analyses

From all four specimens of *G. longipes* analysed, a 1002 bp fragment of ITS1 (412 bp), 5.8S (157 bp) and ITS2 (433 bp) was sequenced. No intra-specific variation was observed between the four sequences. The result from the BlastN search [8] (11.10.2010) of this 1002 bp ITS fragment confirmed the separate species status of *G. longipes* shown by the morphological analysis, as no identical or close hits were found. The hit with the highest score was *Gyrodactylus coriicepsi* Rokicka, Lumme *et* Ziętara, 2009 (FJ009451) with a homology of 92% (coverage 90%). Submitting the conservative 157 bp of 5.8S of *G. longipes* to a separate BlastN search did, as expected, show 100% homology with a number of species of *Gyrodactylus*, including *G. orecchiae*, the only other species described from *S. aurata*.

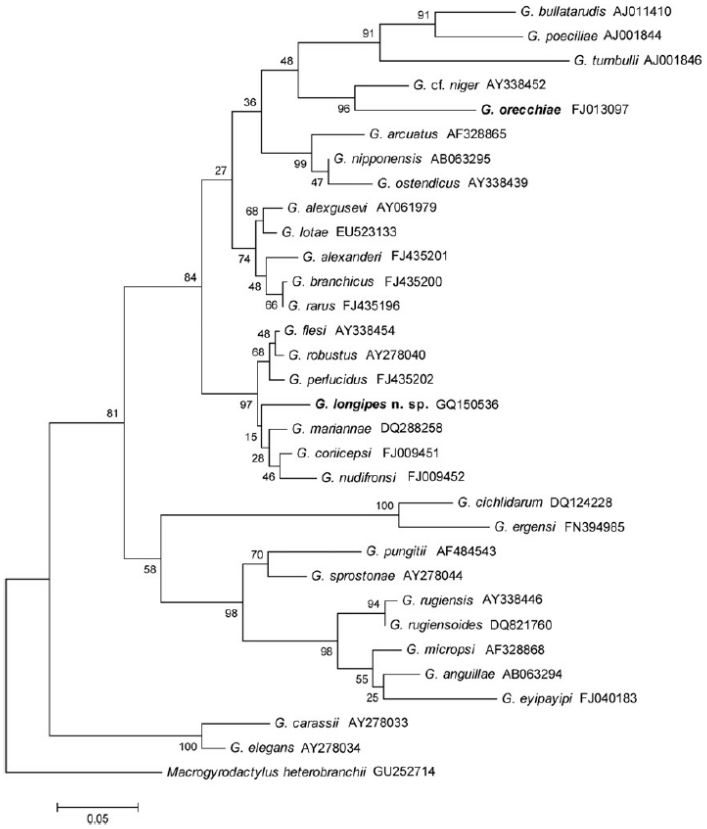

Fig. 3. Phylogenetic reconstruction by the Maximum Likelihood (ML) method based on 5.8S and ITS2 sequences for selected species of *Gyrodactylus* von Nordmann, 1832 with *Macrogyrodactylus heterobranchii* N'Douba *et* Lambert, 1999 used as the outgroup. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The tree with the highest log likelihood (−4054,7367) is shown. Nodal support (1000 replicates) is shown next to the branches. The corresponding GenBank accession numbers are shown next to each species of *Gyrodactylus* and *Macrogyrodactylus*.

Table 2
Estimates of evolutionary divergence between sequences of *Gyrodactylus* spp. The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Kimura 2-parameter model [21]. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). The analysis involved sequences from 32 species, but only the species clustering with *Gyrodactylus longipes* n. sp. are shown. The table also includes *Gyrodactylus orecchiae* Paladini, Cable, Fioravanti, Faria, Di Cave and Shinn, 2009, (see Fig. 3) which co-occurs on the same host [2] and *Gyrodactylus* cf. *niger* Huyse, Audenaert *et* Volckaert, 2003 which clusters along with it. All ambiguous positions were removed for each sequence pair. There were a total of 606 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [11].

		1	2	3	4	5	6	7	8
1	*G. longipes* n. sp.								
2	*G.* cf. *niger*	0.35							
3	*G. coriicepsi*	0.10	0.34						
4	*G. flesi*	0.10	0.30	0.10					
5	*G. mariannae*	0.10	0.31	0.08	0.08				
6	*G. nudifronsi*	0.11	0.38	0.05	0.10	0.09			
7	*G. orecchiae*	0.39	0.24	0.33	0.34	0.35	0.36		
8	*G. perlucidus*	0.09	0.31	0.30	0.06	0.04	0.06	0.08	
9	*G. robustus*	0.10	0.33	0.31	0.10	0.01	0.08	0.10	0.04

The phylogenetic reconstruction based on a maximum likelihood analysis is shown in Fig. 3. *Gyrodactylus longipes* clusters with high bootstrap support (97%) in a group consisting of five other marine species and the freshwater species *Gyrodactylus mariannae* Winger, Hansen, Bachmann *et* Bakke, 2008. All these six species share an identical 5.8S sequence. The Kimura 2-parameter (K2-parameter) distance (based on the 606 bp alignment) between these species varies between 0.01 and 0.10 (Table 2). The neighbor-joining analysis recovered the same well supported clusters as with ML (data not shown). The phylogenetic reconstruction corroborates the findings from the BlastN search in that *G. orecchiae* is distinct from *G. longipes*. The genetic distance (K2-parameter) between *G. longipes* and *G. orecchiae* is 0.39. *Gyrodactylus orecchiae* clusters with high bootstrap support (96%) with *Gyrodactylus* cf. *niger* Huyse, Audenaert *et* Volckaert, 2003 from *Gobius niger* L. The two latter species share an identical 5.8S sequence and the distance between them is 0.24.

4. Discussion

The record of *G. longipes* from Italy and Bosnia–Herzegovina adds to the known gyrodactylid fauna of each country (see Tables 3 and 4) and represents the seventh and first marine species of *Gyrodactylus* to be reported from each. The records of the fauna from Bosnia-Herzegovina are dominated by freshwater species. This is not surprising given the short length of its coastline (~26 km). Until a decade ago the *Gyrodactylus* fauna of Italy was largely unknown but now 21 named species are recorded (Table 3).

The specimens of *S. aurata* collected from Latina Province harboured a mixed infection of *G. orecchiae* and *G. longipes*. The latter species was mostly on the gills whereas *G. orecchiae* was found only on the skin. Despite some morphological similarities in the shape of the opisthaptoral hooks, *e.g.* the possession of prominent ventral bar processes, the two species can be readily discriminated on their differing marginal hook morphology (Fig. 2c–f). The toe of *G. longipes* is triangular and downwardly pointing, the point of which falls below the level of the marginal hook sickle shaft and its union with the sickle proper. The toe of *G. orecchiae*, by comparison, is rhomboid and orientated in an upwards direction. The heel of *G. orecchiae* is larger and more deeply rounded than that of *G. longipes* which is evident when the outline of the two size invariant sickles are overlaid one another (Fig. 2e). Similarly, the marginal sickle points show opposing deflections; the broader point of *G. longipes* curves down towards the toe (Fig. 2c) whilst the more slender sickle point of *G. orecchiae* is parallel with the sickle base (Fig. 2d). Given the relative shape and position of the sickle points with respect to the sickle base, the inner edge of *G. longipes* describes a smooth curve whilst that of *G. orecchiae* is approximately rhomboid. The hamuli roots also differ between

Table 3
Gyrodactylus species recorded from Italy, including the species and family details pertaining to their hosts.

Gyrodactylus species	Valid host name	Host family	Reference
G. anguillae Ergens, 1960	*Anguilla anguilla* (L.)	Anguillidae	[22]
G. arcuatus Bychowsky, 1933	*Knipowitschia panizzae* (Verga)	Gobiidae	[23]
G. branchialis Huyse, Malmberg *et* Volckaert, 2004	*Pomatoschistus marmoratus* (Risso)	Gobiidae	[23]
G. carassii Malmberg, 1957	*Telestes muticellus* (Bonaparte)	Cyprinidae	[24]
G. corleonis Paladini, Cable, Fioravanti, Faria *et* Shinn, 2010[a]	*Syngnathus typhle* L.	Syngnathidae	[25]
G. derjavini sensu Malmberg *et* Malmberg, 1987	*Oncorhynchus mykiss* (Walbaum)	Salmonidae	[26]
	Salmo trutta fario L.	Salmonidae	[26]
G. derjavinoides Malmberg, Collins, Cunningham *et* Jalali, 2007	*Oncorhynchus mykiss*	Salmonidae	[4]
G. gasterostei Gläser, 1974	*Rutilus aula* (Bonaparte)	Cyprinidae	[27]
G. katharineri Malmberg, 1964	*Cyprinus carpio carpio* L.	Cyprinidae	[27]
G. longipes n. sp.	*Sparus aurata* L.	Sparidae	current study
G. lucii Kulakowskaja, 1952	*Esox lucius* L.	Esocidae	[27]
G. neretum Paladini, Cable, Fioravanti, Faria *et* Shinn, 2010[b]	*Syngnathus scovelli* (Evermann *et* Kendall)	Syngnathidae	[25]
G. orecchiae Paladini, Cable, Fioravanti, Faria, Di Cave *et* Shinn, 2009	*Sparus aurata*	Sparidae	current study
G. ostendicus Huyse *et* Malmberg, 2004	*Pomatoschistus marmoratus*	Gobiidae	[23]
G. salaris Malmberg, 1957	*Oncorhynchus mykiss*	Salmonidae	[4]
G. salinae Paladini, Huyse *et* Shinn, 2011	*Aphanius fasciatus* (Valenciennes)	Cyprinodontidae	[28]
G. sprostonae Ling Mo-en, 1962	*Cyprinus carpio carpio*	Cyprinidae	[29]
G. teuchis Lautraite, Blanc, Thiery, Daniel *et* Vigneulle, 1999	*Oncorhynchus mykiss*	Salmonidae	[4]
G. tincae Malmberg, 1957	*Rutilus aula*	Cyprinidae	[24]
G. truttae Gläser, 1974	*Oncorhynchus mykiss*	Salmonidae	[4]
G. turnbulli Harris, 1986	*Poecilia reticulata* Peters	Poeciliidae	[30]
Gyrodactylus sp	not specified		[31]
Gyrodactylus sp.	not specified		[32]
Gyrodactylus sp.	not specified		[33]
Gyrodactylus sp.	*Sparus aurata*	Sparidae	[34]
Gyrodactylus sp.	*Carassius auratus auratus* (L.)	Cyprinidae	[35]
Gyrodactylus sp.	*Carassius auratus auratus*	Cyprinidae	[36]
Gyrodactylus sp.	*Liza ramada* (Risso)	Mugilidae	[37]
Gyrodactylus spp.	*Chondrostoma soetta* Bonaparte	Cyprinidae	[38]

[a] Specimens of *S. typhle* held in an Italian aquarium were reputedly collected off the French coast near Marseille.
[b] Aquarium specimens of *S. scovelli* purportedly originated from a site off the N. American Atlantic coast near Baltimore, Maryland.

Table 4
Gyrodactylus species recorded from Bosnia–Herzegovina, including the species and family details pertaining to their hosts. The original reports, where the host species names presented are no longer available, are shown in square parentheses.

Gyrodactylus species	Valid host name	Host family	Reference
G. albaniensis Ergens, 1960 (syn. of *G. markewitschi* Kulakowskaja, 1951)[a]	*Barbus petenyi* [=*B. meridionalis petenyi*] Heckel	Cyprinidae	[39]
G. aphyae Malmberg, 1957	*Phoxinus phoxinus* (L.)	Cyprinidae	[39]
	Salmo trutta fario L.	Salmonidae	[39]
G. barbatuli Achmerov, 1952	*Barbatula barbatula* [=*Nemacheilus barbatulus*] (L.)	Balitoridae	[40]
G. carassii Malmberg, 1957	*Alburnus alburnus* (L.)	Cyprinidae	[40]
	Scardinius erythrophthalmus (L.)	Cyprinidae	[40,41]
G. cernuae Malmberg, 1957	*Gymnocephalus* [=*Acerina*] *cernua* (L.)	Percidae	[40]
G. chondrostomatis Žitňan, 1964	*Chondrostoma nasus* [=*C. n. nasus*] (L.)	Cyprinidae	[40,41]
G. cobitis Bychowsky, 1933	*Cobitis taenia* (L.)	Cobitidae	[40]
G. cyprini Diarova, 1964	*Cyprinus carpio carpio* L.	Cyprinidae	[42–44]
G. decorus Malmberg, 1957	*Scardinius erythrophthalmus*	Cyprinidae	[40]
G. elegans Nordmann, 1832	*Abramis brama* [=*A. b. danubii*] (L.)	Cyprinidae	[40]
	Ballerus [=*Abramis*] *sapa* (Pallas)	Cyprinidae	[40]
G. fairporti Van Cleave, 1921	*Ameiurus nebulosus* (Lesueur)	Ictaluridae	[40]
G. gracilihamatus Malmberg, 1964	*Alburnus alburnus*	Cyprinidae	[40]
	Rutilus rutilus [=*R. r. carpathorossicus*] (L.)	Cyprinidae	[40]
G. hronosus Žitňan, 1964	*Alburnoides bipunctatus* (Bloch)	Cyprinidae	[40]
	Alburnus alburnus	Cyprinidae	[40,45]
G. katharineri Malmberg, 1964	*Cyprinus carpio carpio*	Cyprinidae	[42–44]
G. laevis Malmberg, 1957	*Alburnus alburnus*	Cyprinidae	[40]
	Leucaspius delineates (Heckel)	Cyprinidae	[40]
	Rutilus rutilus [=*R. r. carpathorossicus*]	Cyprinidae	[40]
G. latus Bychowsky, 1933	*Cobitis taenia*	Cobitidae	[40]
G. leucisci Žitňan, 1964	*Squalius* [=*Leuciscus*] *cephalus* (L.)	Cyprinidae	[40]
G. longipes n. sp.	*Sparus aurata* L.	Sparidae	current study
G. longiradix Malmberg, 1957	*Gymnocephalus* [=*Acerina*] *cernua*	Percidae	[45]
G. longoacuminatus Žitňan, 1964	*Carassius carassius* (L.)	Cyprinidae	[40]
G. lotae Gusev, 1953	*Lota lota* (L.)	Lotidae	[40]
G. lucii Kulakovskaya, 1952	*Esox lucius* L.	Esocidae	[46]
G. luciopercae Gusev, 1962	*Sander* [=*Stizostedion*] *lucioperca* (L.)	Percidae	[46]
G. malmbergi Ergens, 1961	*Barbus barbus* (L.)	Cyprinidae	[40]
G. markewitschi Kulakowskaja, 1951	*Barbus barbus*	Cyprinidae	[40,45]
G. matovi Ergens *et* Kakacheva-Avramova, 1966	*Cobitis taenia*	Cobitidae	[40]
G. medius Kathariner, 1893	*Carassius auratus auratus* (L.)	Cyprinidae	[40]
	Carassius gibelio [=*C. auratus gibelio*] (Bloch)	Cyprinidae	[40]
	Carassius carassius	Cyprinidae	[40]
	Cyprinus carpio carpio	Cyprinidae	[42–44]
G. minimus Malmberg, 1957	*Phoxinus phoxinus*	Cyprinidae	[39]
G. misgurni Ling Mo-en, 1962	*Cobitis taenia*	Cobitidae	[40]
	Misgurnus fossilis (L.)	Cobitidae	[40]
G. pannonicus Molnar, 1968	*Phoxinus phoxinus*	Cyprinidae	[40]
G. paralaevis Ergens, 1966	*Phoxinus phoxinus*	Cyprinidae	[39]
G. paraminimus Ergens, 1966	*Phoxinus phoxinus*	Cyprinidae	[39]
G. prostae Ergens, 1963	*Alburnus alburnus*	Cyprinidae	[40]
	Rutilus rutilus [=*R. r. carpathorossicus*]	Cyprinidae	[40]
G. rarus Wegener, 1910	*Gymnocephalus* [=*Acerina*] *cernua*	Percidae	[40]
	Perca fluviatilis L.	Percidae	[40]
G. rhodei Žitňan, 1964	*Rhodeus amarus* [=*R. sericeus amarus*] (Bloch)	Cyprinidae	[40]
G. salaris Malmberg, 1957	*Oncorhynchus mykiss* [=*Salmo gairdneri irideus*] (Walbaum)	Salmonidae	[47,48]
	Salmo obtusirostris [=*Salmothymus o. oxyrhynchus*] Heckel	Salmonidae	[44]
	Salmo trutta fario	Salmonidae	[44]
G. scardinii Malmberg, 1964	*Scardinius erythrophthalmus*	Cyprinidae	[40]
G. sedelnikowi Gvozdev, 1950	*Barbatula barbatula* [=*Nemachilus barbatulus*]	Balitoridae	[40]
G. shulmani Ling Mo-en, 1962	*Carassius gibelio* [=*C. auratus gibelio*]	Cyprinidae	[40]
	Carassius carassius	Cyprinidae	[40]
	Cyprinus carpio carpio	Cyprinidae	[42–44]
G. sprostonae Ling Mo-en, 1962	*Carassius gibelio* [=*C. auratus gibelio*]	Cyprinidae	[40]
	Cyprinus carpio carpio	Cyprinidae	[40,41,44]
G. stankovici Ergens, 1970	*Cyprinus carpio carpio*	Cyprinidae	[42–44]
G. thymalli Žitňan, 1960	*Thymallus thymallus* (L.)	Salmonidae	[39]
G. tincae Malmberg, 1957	*Tinca tinca* L.	Cyprinidae	[41,44]

[a] See Malmberg [3].

species, in *G. orecchiae* they are square-ended which curve inwards while in *G. longipes*, they are shorter, straight and rounded ended. In addition, the sickle length of the marginal hook of *G. longipes* (6.5 μm) is almost double that of *G. orecchiae* (3.3 μm), as is the proximal width of the sickle (5.1 for *G. longipes vs* 3.2 for *G. orecchiae*; Fig. 2f).

Sequences of the internal transcribed spacer region (ITS1 and 2) have been applied extensively as species specific reference sequences (barcodes) in the genus *Gyrodactylus*[7,16,17] and, to date, partial or complete ITS1 and 2 sequences representing more than 100 *Gyrodactylus* species are available in GenBank. This marker seems to correspond well to morphological markers, *i.e.* separate species as defined by morphological characters is followed by corresponding different ITS sequences. Some authors have even suggested that a 1% difference in ITS is indicative of separate species status [7]. Even though such a yardstick is probably impossible to define, there seems to be very little intra-specific variation in ITS in the genus. Thus, once a sequence of a species is known and submitted to a public database, a subsequent ID can be made by comparison (*e.g.* a BlastN search of

GenBank). The inter-specific length variation of ITS, however, is often large, rendering alignment and subsequent calculations of genetic distances difficult, if not impossible, and because of this, this fragment is not ideal as a phylogenetic marker [9]. The differentiation of *G. longipes* from *G. orecchiae* can, in addition to the differentiation by morphological analyses, also be easily done by comparing their ITS sequences. The fragments containing ITS1 and 2 and the 5.8S of the two species are of very different sizes (1002 and 1074 bp in *G. longipes* and *G. orecchiae*, respectively). The K2-parameter distance calculated in this study (0.39) is *e.g.* equivalent to the distance between the well defined South American species *Gyrodactylus turnbulli* Harris, 1986 and *Gyrodactylus bullatarudis* Turnbull, 1956.

The phylogenetic reconstruction shows low support for many of the basal clades which was also noted by previous authors [9]. There are no obvious common factors explaining the apparent close relationship of the species clustering with *G. longipes*. Most are marine species colonising coastal waters but *G. mariannae*, which is described from the freshwater host *Cottus poecilopus* Heckel, is an exception to this. Rokicka et al. [18], however, speculated that common evolutionary lineages between geographically separated species might be explained by parasites on marine hosts crossing oceans and then subsequently moving into rivers, where the parasites then transfer onto indigenous freshwater hosts. There is largely no overlap in the normal habitat ranges of the marine fish hosts listed in the group. For example, the group includes two species described from Antarctic hosts (*G. coriicepsi* from *Notothenia coriiceps* Richardson and *Gyrodactylus nudifronsi* Rokicka, Lumme *et* Ziętara, 2009 from *Lepidonotothen nudifrons* (Lönnberg)), *Gyrodactylus robustus* Malmberg, 1957, which has been described from two species of *Platichthys* and *Clupea pallasi* Valenciennes collected from Swedish waters and from the Pacific, *Gyrodactylus perlucidus* Bychowsky *et* Poljansky, 1953 is described from *Zoarces viviparus* (L.) which has a northern distribution (NE Atlantic, Baltic and White Seas) and finally *Gyrodactylus flesi* Malmberg, 1957 has been recorded from at least nine different hosts [14], of which the natural range of some extend into the Mediterranean. While the identification of *Gyrodactylus* species in historical records is assumed to be correct, absolute confidence rests with those studies where the morphological identification is supported by molecular characterisation. For example, only two of the nine reported hosts for *G. flesi, i.e. Platichthys flesus* (L.) [9] and *Pleuronectes platessa* L. [17], have been confirmed by molecular studies; the remaining records [14] await further confirmation.

While the findings from this study and those of Paladini et al. [2] suggest that *G. longipes* and *G. orecchiae* appear to co-occur in the Adriatic and Tyrrhenian Seas, there are also reports of an unidentified species of *Gyrodactylus* on *S. aurata* from Spain [19] and from Turkey [20], and these require indentifying to confirm the potential distribution of each *Gyrodactylus* species throughout the Mediterranean. The citation of *Gyrodactylus* sp. from *S. aurata* made by Santamarina et al. [19], however, seems to be incorrect due to a misspelling of the fish species *Carassius auratus* (L.), which was tested in the study of Goven & Amend [49].

High numbers of *G. orecchiae* (1000+ gyrodactylids/fish) associated with the mortality of juvenile *S. aurata* [2] raises concerns regarding the disease potential this species poses to juvenile populations of *S. aurata*. The finding of a second species, *G. longipes*, also requires close monitoring to define its risk to juvenile *S. aurata* within the Mediterranean.

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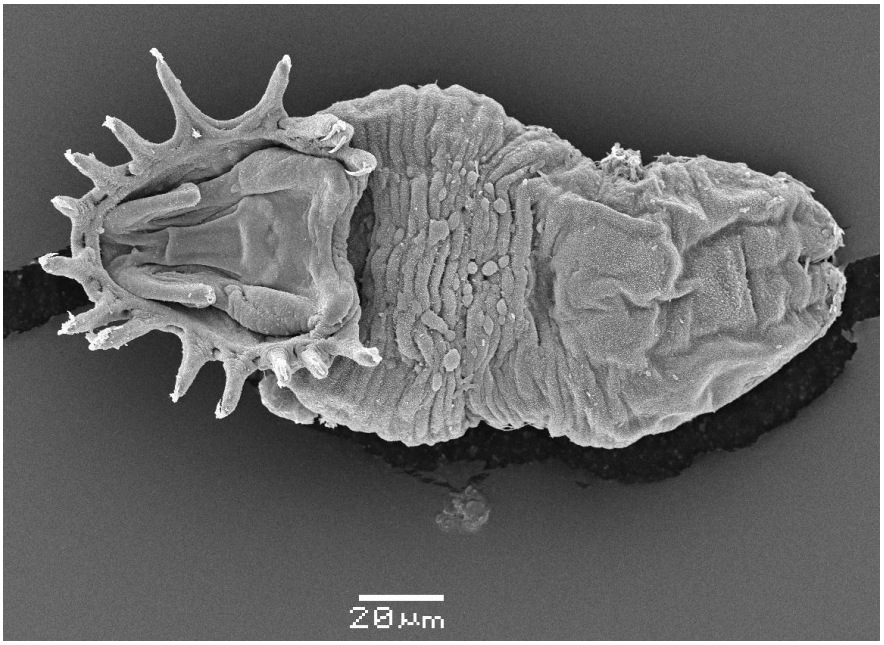
Chapter 5

Other significant pathogens of farmed salmonids: *Gyrodactylus salmonis* in North America



Gyrodactylus salaris Malmberg, 1957 haplotype A [original image].

Paper IV

Rubio-Godoy M., **Paladini G.**, Freeman M., García-Vásquez A., Shinn A.P. (2012). Morphological and molecular characterisation of *Gyrodactylus salmonis* (Platyhelminthes, Monogenea) isolates collected in Mexico from rainbow trout (*Oncorhynchus mykiss* Walbaum). *Veterinary Parasitology*, 186: 289–300.

Aspects of this paper were presented as:

Rubio-Godoy M., **Paladini G.**, Freeman M.A., García-Vásquez A., Shinn A.P. (2011). Description of a new strain of *Gyrodactylus salmonis* (Platyhelminthes, Monogenea) collected in Mexico from rainbow trout (*Oncorhynchus mykiss* Walbaum): morphological and molecular characterization. *Proceedings of the 86th Annual Meeting, American Society of Parasitologists, Anchorage, Alaska, 1st-4th June 2011*: 92 (talk).

Paladini G., Rubio-Godoy M., Freeman M.A., García-Vásquez A., Shinn A.P. (2011). *Gyrodactylus salmonis*: a strained relationship. *Proceedings of the VIII International Symposium of Fish Parasites (ISFP8), Viña del Mar, Chile, 26th-30th September 2011*: 74 (talk).

5.1. General introduction of Paper IV

The following paper has been published in *Veterinary Parasitology* and it describes a new strain/isolate of *Gyrodactylus salmonis* (Yin *et* Sproston, 1948), using a combination of morphological and molecular analyses, from a Mexican population of feral rainbow trout, *Oncorhynchus mykiss* Walbaum. Although this species seems to be widespread in northern North America, there is also an unconfirmed report of its occurrence on rainbow trout in Croatia (Zrnčić & Oraić, 2008). *Gyrodactylus salmonis* is another significant pathogen of farmed salmonids. It also exhibits low host specificity and is pathogenic to brook trout, *Salvelinus fontinalis* (Mitchill) (see Cone & Odense, 1984; Cusack & Cone, 1986; Rubio-Godoy *et al*., 2012). Given the impacts that this parasite has on salmonid stocks, it can be regarded as the North American counterpart to *G. salaris* and, therefore, finding its occurrence outside its normal geographic range is worth reporting. This is the furthest south that this species has been found and highlights once again the risks of translocating pathogens into new areas with the movement of fish stocks.

5.2. Authors' contribution

This published study is the result of an on-going collaboration between a Mexican colleague, Dr Miguel Rubio-Godoy from the Instituto de Ecologia, Xalapa, and researchers at Institute of Aquaculture, Stirling. Dr Rubio-Godoy collected the feral rainbow trout samples from Mexico and on finding specimens of *Gyrodactylus* contacted Dr Andrew P. Shinn and myself to carry out the morphological analyses and to identify the material. I personally processed the gyrodactylid material, took the morphometric measurements, took part of the light microscope images and produced the drawings. Dr Shinn and Dr Adriana García-Vásquez, a former PhD student at the University of Stirling, produced part of the figure plates and assisted with the morphological identification. Dr Mark A. Freeman, from the University of Malaysia and formerly from the University of Stirling, performed the

molecular characterisation. Dr Rubio-Godoy drafted the first version of the manuscript, with certain sections being written by myself and Dr Shinn. Subsequently drafts of the manuscript were revised by myself and the other co-authors. All authors read and approved the final version of the manuscript.

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Morphological and molecular characterisation of *Gyrodactylus salmonis* (Platyhelminthes, Monogenea) isolates collected in Mexico from rainbow trout (*Oncorhynchus mykiss* Walbaum)

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ABSTRACT

Gyrodactylus salmonis (Yin et Sproston, 1948) isolates collected from feral rainbow trout, *Oncorhynchus mykiss* (Walbaum) in Veracruz, southeastern Mexico are described. Morphological and molecular variation of these isolates to *G. salmonis* collected in Canada and the U.S.A. is characterised. Morphologically, the marginal hook sickles of Mexican isolates of *G. salmonis* closely resemble those of Canadian specimens – their shaft and hook regions align closely with one another; only features of the sickle base and a prominent bridge to the toe permit their separation. The 18S sequence determined from the Mexican specimens was identical to two variable regions of SSU rDNA obtained from a Canadian population of *G. salmonis*. Internal transcribed spacer (ITS) regions (spanning ITS1, 5.8S and ITS2) of Mexican isolates of *G. salmonis* are identical to ITS sequences of an American population of *G. salmonis* and to *Gyrodactylus salvelini* Kuusela, Ziętara et Lumme, 2008 from Finland. Analyses of the ribosomal RNA gene of Mexican isolates of *G. salmonis* show 98–99% similarity to those of *Gyrodactylus gobiensis* Gläser, 1974, *Gyrodactylus salaris* Malmberg, 1957, and *Gyrodactylus rutilensis* Gläser, 1974. Mexican and American isolates of *G. salmonis* are 98% identical, as assessed by sequencing the mitochondrial cox1 gene. *Oncorhynchus mykiss* is one of the most widely-dispersed fish species in the world and has been shown to be an important vector for parasite/disease transmission. Considering that Mexican isolates of *G. salmonis* were collected well outside the native distribution range of all salmonid fish, we discuss the possibility that the parasites were translocated with their host through the aquacultural trade. In addition, this study includes a morphological review of *Gyrodactylus* species collected from rainbow trout and from other salmonid fish of the genus *Oncorhynchus* which occur throughout North America.

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1. Introduction

The native distribution range of rainbow trout, *Oncorhynchus mykiss* (Walbaum), in North America spans from the Aleutian Islands in Alaska to near the U.S.A.–Mexico border in Baja California (Froese and Pauly, 2010). The latter region is also the northernmost distribution of unambiguously native Mexican trouts, which range southwards to at least the Río Culiacán, Sinaloa, Mexico (Hendrickson et al., 2002). Several salmonid fish occur either in sympatry or in close vicinity in this extended territory, which practically encompasses the whole western seaboard of North America (*i.e.*, Canada, the U.S.A. and Mexico). Rainbow trout is one of the most widely translocated fish species in the world, and has been introduced to numerous countries for sport and commercial aquaculture (Froese and Pauly, 2010; ISSG, 2010). *Oncorhynchus mykiss* was first introduced to central Mexico (*i.e.*, southern North America) for aquacultural purposes in the late 1880s (Hendrickson et al., 2002). Different varieties of *O. mykiss* and other salmonids were subsequently introduced during the 20th century, and the following were recorded by Rosas (1976) as in use in Mexican fish culture in the 1970s: *O. mykiss*; Mexican golden trout, *Oncorhynchus chrysogaster* (Needham et Gard); cutthroat trout, *Oncorhynchus clarkii clarkii* (Richardson); and brook trout, *Salvelinus fontinalis* (Mitchill).

Apart from the recognised ecological impact of introduced rainbow trout, including negative effects on native fish species through predation and competition (ISSG, 2010), several factors potentially make *O. mykiss* a major vector for disease/parasite transmission and/or introduction: first, a variety of diseases and infections affect rainbow trout, caused by viral, bacterial, protistan and metazoan pathogens (Buchmann et al., 1995; F.A.O., 2005); second, the occurrence of sympatric salmonid fish species within the vast natural distribution range of *O. mykiss* in North America raises the possibility that rainbow trout acquired several parasite species through host transfers; similarly, *O. mykiss* may have acquired low specificity parasites from other fish host families; finally, the widespread anthropogenic introduction of rainbow trout might have facilitated dispersal of several of these pathogens – *e.g.*, the decline of various wild salmonid populations in the U.S.A. has been linked to whirling disease caused by the introduction of the myxozoan parasite *Myxobolus cerebralis* (Höfer, 1903) with translocated *O. mykiss* (see Granath et al., 2007).

Monogenean flatworms of the genus *Gyrodactylus* von Nordmann, 1832 include important fish pathogens that affect aquaculture and potentially endanger the survival of wild fish stocks; examples include Gyrodactylus salaris Malmberg, 1957 infecting salmonids (Bakke et al., 2007) and *Gyrodactylus cichlidarum* Paperna, 1968 infecting cichlids (García-Vásquez et al., 2010). Eleven *Gyrodactylus* species have been described from Mexican fish (Table 1), although several more are likely to be found as many undescribed gyrodactylids have been recorded from different teleost fish families (Salgado-Maldonado, 2006; Pérez-Ponce de León et al., 2010; Rubio-Godoy et al., 2010). Previously, *Gyrodactylus* sp. have been collected from *O. mykiss* in central Mexico, from fish farms located in Distrito Federal (Mexico City) and Estado de México (Armijo, 1980; Flores-Crespo and Flores, 1993). Worldwide, fourteen valid *Gyrodactylus* species have been recorded from *O. mykiss* (Table 2). Salmonid fish in North America are parasitised by at least five species of *Gyrodactylus*: *G. avalonia* Hanek et Threlfall, 1969; *G. brevis* Crane et Mizelle, 1967; *G. colemanensis* Mizelle et Kritsky, 1967; *G. nerkae* Cone, Beverley-Burton, Wiles et McDonald, 1983; and, *G. salmonis* (Yin et Sproston, 1948). Of these five species, *G. colemanensis* and *G. salmonis* are the most geographically widespread in North America, occurring in fish farms across the U.S.A. and Canada (Gilmore et al., 2010).

In this paper, Mexican isolates of *G. salmonis* collected from feral rainbow trout in the State of Veracruz, southeastern Mexico are described. In addition, this study includes a morphological review of *Gyrodactylus* species collected from rainbow trout and from other salmonid fish of the genus *Oncorhynchus* which occur naturally throughout North America.

2. Materials and methods

2.1. Specimen collection

Forty feral rainbow trout, *O. mykiss* fingerlings (mean standard length ± 1 S.D., 4.8 ± 0.35 cm; mean weight ± 1 S.D., 1.8 ± 0.43 g) and six 1+ fish (mean standard length ± 1 S.D., 17.4 ± 2.01 cm; mean weight ± 1 S.D., 56.1 ± 16.9 g) were collected by electrofishing in May 2007 in the Río Pixquiac around Xalapa, Veracruz, Mexico (19°28′39″N, 96°57′00″W), and transported live to the laboratory. Fish were killed by pithing and inspected under a dissection microscope. Ectoparasites were detached from the fish surface and preserved in 80% ethanol until analysed.

2.2. Specimen preparation

Ten representative gyrodactylid specimens were prepared as whole mounts in ammonium picrate glycerine following the procedure detailed by Malmberg (1970) to study taxonomic features of the haptor, male copulatory organ (MCO) and pharynx. Further specimens had their haptors excised using a scalpel and were subjected to proteolytic digestion as described previously (Paladini et al., 2009), to release the attachment hooks from enclosing tissue. The corresponding bodies were stored in 96% ethanol for subsequent molecular sequencing. The hooks were mounted in a 1:1 formalin: glycerine mix and the edges of the coverslip were then sealed with the permanent mounting medium Pertex (Histolab Products AB, Gothenburg, Sweden).

2.3. Morphological analysis

For the morphological study, images of the haptoral attachment 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. Each gyrodactylid specimen was subjected to morphometric analysis taking 22 point–to–point measurements on the haptoral hooks

Table 1
Gyrodactylids recorded in Mexican fish.

Gyrodactylus spp.	Host (Family)	Reference
G. bullatarudis Turnbull, 1956	*Poecilia mexicana* (Poeciliidae)	Rubio-Godoy et al. (2010)
G. cichlidarum Paperna, 1968	*Oreochromis mossambicus* (Cichlidae)	García-Vásquez et al. (2010)
	Oreochromis niloticus (Cichlidae)	García-Vásquez et al. (2010)
G. elegans von Nordmann, 1832[a]	*Girardinichthys multiradiatus* (Goodeidae)	Salgado-Maldonado et al. (2001); Sánchez-Nava et al. (2004)
G. jarocho Rubio-Godoy, Paladini, García-Vásquez et Shinn, 2010	*Xiphophorus hellerii* (Poeciliidae)	Rubio-Godoy et al. (2010)
G. lamothei Mendoza-Palmero, Sereno-Uribe et Salgado-Maldonado, 2009	*Girardinichthys multiradiatus* (Goodeidae)	Mendoza-Palmero et al. (2009)
G. mexicanus Mendoza-Palmero, Sereno-Uribe et Salgado-Maldonado, 2009	*Girardinichthys multiradiatus* (Goodeidae)	Mendoza-Palmero et al. (2009)
G. neotropicalis Kritsky et Fritts, 1970	*Astyanax fasciatus* (Characidae)	Mendoza-Franco et al. (1999)
G. niloticus Cone, Arthur et Bondad-Reantaso, 1995	*Oreochromis niloticus* (Cichlidae)	López-Jiménez (2001)
	Oreochromis mossambicus (Cichlidae)	Salgado-Maldonado et al. (2005)
	Oreochromis aureus (Cichlidae)	Salgado-Maldonado et al. (2005)
G. spathulatus Mueller, 1936	*Catostomus nebuliferus* (Catostomidae)	Pérez-Ponce de León et al. (2010)
	Gila conspersa (Cyprinidae)	Pérez-Ponce de León et al. (2010)
	Ictalurus cf. *pricei* (Ictaluridae)	Pérez-Ponce de León et al. (2010)
G. xalapensis Rubio-Godoy, Paladini, García-Vásquez et Shinn, 2010	*Heterandria bimaculata* (Poeciliidae)	Rubio-Godoy et al. (2010)
G. yacatli García-Vásquez, Hansen, Christison, Bron et Shinn, 2011	*Oreochromis niloticus* (Cichlidae)	García-Vásquez et al. (2011)

[a] These species were identified by Salgado-Maldonado et al. (2001) and by Sánchez-Nava et al. (2004) as *Gyrodactylus* cf. *elegans* von Nordmann, 1832; however, later analysis of the samples indicates these are in fact two undescribed species (Salgado-Maldonado, 2006). Valid fish names checked in FishBase, July 2011.

Table 2
Gyrodactylus species recorded from *Oncorhynchus mykiss* and other *Oncorhynchus* species.

Oncorhynchus sp.	*Gyrodactylus* sp.	Localities
O. mykiss Walbaum	*G. avalonia* Hanek et Threlfall, 1969[a]	Newfoundland[1] and Nova Scotia[2], Canada
	G. bohemicus Ergens, 1992	South Bohemia, Czech Republic[3]
	G. brachymystacis Ergens, 1978	China[4]
	G. brevis Crane et Mizelle, 1967	California, USA[5]
	G. colemanensis Mizelle et Kritsky, 1967	California[6], Arkansas[2] and Colorado[7], USA; Newfoundland[2] and Nova Scotia[8], Canada
	G. derjavinoides Malmberg, Collins, Cunningham et Jalali, 2007	Tidaholm, Sweden[9]; Denmark[9]
	G. gobii Schulman, 1953	Brandenburg and Thuringia, Germany[10]
	G. lavareti Malmberg, 1957	Sweden[11]; Baltic Sea, Finland[12]; Arctic Ocean[12] and Kola Peninsula[13], Russia
	G. lenoki Gussev, 1953	China[14]
	G. masu Ogawa, 1986	Japan[15]
	G. salaris Malmberg, 1957	*e.g.*, Sweden[11]; Norway[16]; Denmark[17]; Finland[18]; Germany[10]; Russia[12]; Spain[18]; Poland[19]; Italy[20]
	G. salmonis (Yin et Sproston, 1948)	British Columbia[2] and Nova Scotia[2], Canada; Montana[2], Idaho[2] and Arkansas[2], USA
	G. teuchis Lautraite, Blanc, Thiery, Daniel et Vigneulle, 1999	Brittany and Western Pyrenees, France[21]
	G. truttae Gläser, 1974	Czech Republic[22]
	Gyrodactylus sp. Morph 8 Shinn et al., 1995	UK[23]
O. aguabonita Jordan	*G. salmonis* (Yin et Sproston, 1948)	California[2], USA
O. clarkii clarkii Richardson	*G. salmonis* (Yin et Sproston, 1948)	British Columbia[2], Canada
O. keta Walbaum	*G. somnaensis* Ergens et Yukhimenko, 1990	Amur river basin, Russia[24]
O. kisutch Walbaum	*G. salmonis* (Yin et Sproston, 1948)	British Columbia[2], Canada
O. masou masou Brevoort	*G. masu* Ogawa, 1986	Japan[15]
O. nerka Walbaum	*G. masu* Ogawa, 1986	Japan[25]
	G. nerkae Cone, Beverley-Burton, Wiles et MacDonald, 1983	British Columbia[2], Canada
O. rhodurus Jordan et McGregor	*G. masu* Ogawa, 1986	Japan[13]

References: [1] Hanek and Threlfall, 1969; [2] Cone et al., 1983; [3] Ergens, 1992; [4] You et al., 2006; [5] Crane and Mizelle, 1967; [6] Mizelle and Kritsky, 1967; [7] Hathaway and Herlevich, 1973; [8] Cone and Wiles, 1989; [9] Malmberg et al., 2007; [10] Lux, 1990; [11] Malmberg, 1957; [12] Koski and Malmberg, 1995; [13] Karasev et al., 1997; [14] Wang et al., 1997; [15] Ogawa, 1986; [16] Johnsen and Jensen, 1991; [17] Buchmann and Bresciani, 1997; [18] Malmberg, 1993; [19] Rokicka et al., 2007; [20] Paladini et al., 2009; [21] Lautraite et al., 1999; [22] Gläser, 1974; [23] Shinn et al., 1995; [24] Ergens and Yukhimenko, 1990; [25] Ogawa, 1994.

[a] *Gyrodactylus avalonia* Hanek et Threlfall, 1969 is suspected to be a junior synonym of *Gyrodactylus arcuatus* Bychowsky, 1933, but this species is considered as valid until molecular characterisation of *G. avalonia* is conducted (J. Lumme and S.D. King, pers. comm.).

Table 3
Morphological measurements of the haptoral structures of Mexican isolates of *Gyrodactylus salmonis* from rainbow trout, *Oncorhynchus mykiss*, and of other *Gyrodactylus* species collected from the same host. Values shown in μm are means ± 1 S.D., and ranges in parentheses.

Measurement	Mexican isolates of *G. salmonis* (Yin et Sproston, 1948) This study (n = 10)	*G. avalonia* Hanek et Threlfall, 1969 Holotype USNPC 70439 (n = 1)[1]	*G. bohemicus* Ergens, 1992 (n = 20)[2]	*G. brachymystacis* Ergens, 1978 (n = ?)[3]	*G. brevis* Crane et Mizelle, 1967 USNPC 61635 paratypes (951–4; 1169–15; 1171–4/8) (n = 3)[4]	*G. colemanensis* Mizelle et Kritsky, 1967 from Shinn et al. (1995) and Shinn (1993) (n = 10)[5]	*G. derjavinoides* Malmberg, Collins, Cunningham et Jalali, 2007 (n = 20)	*G. gobii* Schulman, 1953 (n = 1)[6]
Hamulus								
Total length	64.4 ± 1.7 (61.3–67.7)	39.1	87–91	90–96	38.4 ± 1.5 (37.1–40.1)	47.2 ± 1.0 (45.3–48.2)	56.1 ± 3.5 (50.3–60.9)	52.3
Shaft length	43.5 ± 0.8 (42.5–44.7)	25.4	63–66	66–69	20.9 ± 1.3 (19.6–22.1)	32.3 ± 0.9 (30.5–33.0)	33.7 ± 2.0 (30.9–37.3)	34.5
Point length	35.7 ± 0.7 (34.5–36.6)	25.4	42–43	42–45	17.4 ± 0.4 (17.0–17.8)	22.9 ± 0.7 (21.5–23.8)	30.8 ± 1.1 (28.6–33.0)	27.2
Root length	22.1 ± 1.2 (19.9–24.0)	11.8	30–32	31–36	13.5 ± 1.0 (12.7–14.6)	17.8 ± 0.8 (16.6–19.0)	19.4 ± 1.7 (16.1–21.7)	15.7
Proximal shaft width	11.0 ± 0.6 (10.3–12.1)	7.8	–	–	6.2 ± 0.3 (6.1–6.6)	7.0 ± 0.2 (6.8–7.3)	9.2 ± 0.4 (8.2–9.7)	8.1
Distal shaft width	6.5 ± 0.3 (6.1–6.8)	3.9	–	–	3.0 ± 0.1 (2.9–3.2)	–	6.0 ± 0.4 (5.2–6.8)	5.3
Aperture angle (°)	35.8 ± 2.1 (31.5–39.2)	46.3	–	–	37.2 ± 1.9 (35.9–39.4)	–	29.9 ± 1.7 (27.2–34.4)	32.8
Hamulus aperture	24.0 ± 1.3 (21.6–25.9)	17.8	–	–	12.4 ± 0.3 (12.2–12.8)	17.5 ± 0.7 (16.5–18.5)	16.8 ± 1.5 (14.7–20.7)	18.6
Ventral bar								
Total length	23.2 ± 2.1 (20.2–26.0)	28.1	10–11	10–12	**18.4**	–	23.4 ± 1.4 (21.1–25.9)	22.2
Total width	25.5 ± 1.1 (24.1–27.1)	23.6	32–36	31–34	**13.3**	–	31.6 ± 2.3 (26.2–34.9)	21
Process to mid-length	2.2 ± 0.6 (1.3–2.9)	8.9	–	–	**1.7**	–	2.8 ± 1.1 (1.1–4.6)	3.4
Median length	8.5 ± 0.9 (7.0–10–1)	5.0	–	–	**5.6**	–	8.2 ± 1.1 (6.4–10.1)	5.9
Process length	2.1 ± 0.3 (1.7–2.6)	5.6	–	–	**0.8**	–	3.5 ± 0.6 (2.9–4.8)	2.6
Membrane length	13.4 ± 1.4 (11.6–15.1)	14.8	20–22	22–24	**11.1**	–	13.2 ± 1.5 (11.2–16.8)	13.1
Marginal hooks								
Total length	44.6 ± 0.8 (43.8–46.5)	**23**	43–45	42–46	20.9 ± 1.1 (20.1–21.7)	31.1 ± 0.5 (30.3–31.9)	32.5 ± 1.7 (28.8–36.6)	42.8
Shaft length	37.0 ± 0.6 (36.3–38.4)	–	–	–	15.4 ± 1.2 (14.5–16.2)	25.8 ± 0.6 (24.9–26.8)	26.2 ± 1.3 (22.9–28.6)	34.6
Sickle length	7.9 ± 0.2 (7.7–8.3)	**4**	9–10	8–9	6.8 ± 0.0 (6.8–6.8)	6.0 ± 0.2 (5.7–6.3)	6.5 ± 0.3 (5.9–7.0)	8.4
Sickle proximal width	4.8 ± 0.2 (4.5–5.0)	–	–	–	4.6 ± 0.1 (4.5–4.6)	4.2 ± 0.1 (4.0–4.4)	5.1 ± 0.4 (4.4–5.8)	5.7
Toe length	1.8 ± 0.1 (1.5–2.0)	–	–	–	2.4 ± 0.1 (2.3–2.4)	1.6 ± 0.1 (1.3–1.7)	1.8 ± 0.3 (1.3–2.7)	2.0
Sickle distal width	6.0 ± 0.2 (5.7–6.3)	–	–	–	3.1 ± 0.3 (2.9–3.3)	4.1 ± 0.3 (3.6–4.5)	5.0 ± 0.3 (4.5–5.6)	5.6
Aperture	6.0 ± 0.2 (5.7–6.3)	–	–	–	5.5 ± 0.1 (5.5–5.6)	4.6 ± 0.2 (4.2–4.8)	5.0 ± 0.4 (4.4–5.8)	7.1
Instep/arch height	0.8 ± 0.1	–	–	–	0.6 ± 0.1	–	0.5 ± 0.1	0.4

Table 3
(*continued*)

Measurement	*G. lavareti* Malmberg, 1957 (*n* = ?)[6]	*G. lenoki* Gussev, 1953 (*n* = ?)[7]	*G. masu* Ogawa, 1986 This study (n = 5)	*G. salaris* Malmberg, 1957 This study (n = 31)	*G. salmonis* (Yin et Sproston, 1948) This study (n = 3)[8]	*G. teuchis* Lautraite, Blanc, Thiery, Daniel et Vigneulle, 1999 This study (n = 13)	*G. truttae* Gläser, 1974 This study (n = 41)	*Gyrodactylus* sp. Morph 8 (from Shinn et al., 1995) (n = 2)[9]	
Hamulus									
Total length	84	82–91	75.4 ± 1.5 (73.3–77.3)	76.0 ± 2.3 (72.0–81.7)	65.7 ± 1.7 (63.7–66.8)	67.5 ± 2.1 (64.7–72.4)	64.8 ± 1.8 (60.3–68.8)		–
Shaft length	58–59	65–72	46.2 ± 1.9 (42.9–47.6)	47.0 ± 1.9 (42.2–51.0)	44.5 ± 1.5 (42.7–45.5)	44.1 ± 1.1 (42.3–46.4)	39.7 ± 0.9 (37.9–41.7)		–
Point length	36	41–45	40.0 ± 1.1 (38.9–41.8)	39.5 ± 1.3 (37.1–41.9)	35.6 ± 0.9 (35.1–36.6)	34.7 ± 0.9 (34.9–38.5)	33.2 ± 0.9 (30.2–34.5)		–
Root length	26–27	26–33	27.0 ± 1.8 (25.0–28.7)	26.2 ± 2.1 (22.2–30.6)	21.0 ± 1.4 (20.1–22.7)	20.2 ± 1.0 (18.6–22.3)	21.6 ± 1.1 (19.2–23.6)		–
Proximal shaft width	–	–	13.6 ± 0.8 (12.8–14.7)	11.7 ± 0.8 (9.9–13.3)	11.4 ± 0.3 (11.1–11.7)	10.8 ± 1.0 (8.7–11.8)	9.8 ± 0.6 (8.7–11.0)		–
Distal shaft width	–	–	8.0 ± 1.4 (6.5–9.5)	7.3 ± 0.8 (6.0–9.8)	6.1 ± 0.1 (6.0–6.2)	6.2 ± 0.8 (4.4–7.2)	5. 9 ± 0.3 (5.3–6.7)		–
Aperture angle (°)	–	–	33.0 ± 2.2 (30.6–36.2)	36.4 ± 1.9 (33.7–41.5)	33.2 ± 1.7 (31.2–34.5)	33.2 ± 1.2 (30.3–34.6)	31.6 ± 1.3 (28.5–34.6)		–
Hamulus aperture	–	–	24.9 ± 1.3 (22.9–26.3)	27.7 ± 1.7 (24.5–32.9)	24.0 ± 1.5 (23.0–25.8)	23.2 ± 0.9 (21.1–24.5)	20.1 ± 0.8 (18.5–22.3)		–
Ventral bar									
Total length	–	9–14	28.7 ± 0.8 (28.0–29.8)	31.7 ± 2.1 (27.4–36.6)	25.5 ± 1.0 (24.7–26.7)	27.7 ± 2.7 (23.6–33.4)	27.2 ± 1.6 (23.6–31.1)		–
Total width	29	26–33	36.3 ± 2.8 (32.9–39.8)	31.0 ± 3.7 (27.0–40.7)	32.8 ± 4.5 (27.8–36.3)	29.4 ± 2.4 (22.8–31.6)	31.9 ± 1.9 (27.6–35.2)		–
Process to mid-length	–	–	4.1 ± 0.6 (3.3–4.8)	3.1 ± 0.9 (1.1–4.6)	2.2 ± 0.4 (2.0–2.8)	2.9 ± 0.8 (1.2–4.2)	3.8 ± 0.9 (2.2–6.3)		–
Median length	8.4	–	7.6 ± 1.2 (6.5–9.4)	10.3 ± 1.4 (7.6–13.9)	9.3 ± 1.1 (8.1–10.1)	11.0 ± 1.5 (8.8–12.9)	8.9 ± 0.9 (7.4–10.8)		–
Process length	–	–	4.7 ± 0.3 (4.3–5.0)	2.0 ± 1.1 (0.24–4.7)	1.8 ± 0.3 (1.6–2.2)	2.8 ± 0.8 (1.3–4.9)	3.8 ± 1.0 (2.4–7.0)		–
Membrane length	18	23–26	16.9 ± 0.3 (16.5–17.1)	18.3 ± 2.0 (14.2–22.0)	13.5 ± 1.1 (12.4–14.5)	14.9 ± 2.3 (12.1–19.6)	15.2 ± 1.6 (12.3–21.0)		–
Marginal hooks									
Total length	40	43–45	41.0 ± 0.6 (40.2–41.4)	39.1 ± 2.1 (33.6–43.0)	37.5 ± 4.7 (33.1–42.4)	35.8 ± 1.0 (34.5–37.6)	32.1 ± 1.2 (29.8–36.1)	26.9 ± 0.6 (26.5–27.3)	
Shaft length	33	–	33.4 ± 0.5 (32.7–33.8)	32.2 ± 1.6 (29.6–34.5)	29.8 ± 4.6 (25.4–34.6)	28.5 ± 0.9 (27.5–30.3)	25.7 ± 1.0 (23.6–29.1)	21.1 ± 0.6 (20.6–21.5)	
Sickle length	7.7	10–11	7.8 ± 0.4 (7.3–8.2)	7.9 ± 0.3 (7.3–8.6)	8.0 ± 0.1 (7.9–8.2)	7.7 ± 0.3 (7.2–8.3)	6.9 ± 0.2 (6.5–7.3)	6.0 ± 0.03 (6.0–6.01)	
Sickle proximal width	–	–	5.3 ± 0.4 (4.9–5.8)	5.7 ± 0.3 (4.9–6.2)	5.1 ± 0.2 (4.9–5.2)	5.4 ± 0.2 (5.0–5.6)	5.1 ± 0.3 (4.5–5.6)	3.4 ± 0.04 (3.3–3.4)	
Toe length	–	–	2.2 ± 0.2 (1.9–2.4)	2.1 ± 0.2 (1.6–2.5)	2.2 ± 0.3 (1.9–2.4)	2.2 ± 0.2 (1.8–2.6)	1.6 ± 0.2 (1.2–2.1)	1.4 ± 0.08 (1.3–1.4)	
Sickle distal width	–	–	5.7 ± 0.3 (5.5–6.1)	6.3 ± 0.3 (5.9–7.2)	5.6 ± 0.2 (5.4–5.7)	7.0 ± 0.4 (6.3–7.6)	5.5 ± 0.3 (4.9–6.1)	4.4 ± 0.4 (4.1–4.6)	
Aperture	–	–	7.2 ± 0.3 (6.7–7.5)	6.5 ± 0.3 (6.0–7.4)	6.4 ± 0.2 (6.3–6.6)	6.5 ± 0.5 (6.0–7.9)	5.3 ± 0.3 (4.5–6.0)	4.6 ± 0.1 (4.5–4.6)	
Instep/arch height	–	–	0.7 ± 0.2 (0.4–0.8)	0.8 ± 0.2 (0.5–1.1)	0.6 ± 0.1 (0.5–0.7)	0.6 ± 0.2 (0.4–1.0)	0.7 ± 0.1 (0.5–1.1)	–	

Notes: 1, Marginal hooks not visible in holotype; figures in bold taken from Hanek and Threlfall, 1969; 2, Measurements taken from Ergens, 1992; 3, Measurements taken from Ergens, 1983; 4, Ventral bar not visible in paratypes; illustrative figures shown in bold measured from USNPC 73549 paratype 1169–16/17, collected from California roach, *Hesperoleucus symmetricus* Baird et Girard; marginal hooks visible in two paratypes only; 5, Measurements are based on 10 hamuli and 10 marginal hooks released by proteolytic digestion from a pooled sample of gyrodactylids; 6, Measurements extrapolated from drawing in Pugachev et al., 2009. 6, Taken from Kuusela et al., 2008; samples obtained from *Coregonus lavaretus* (L.) in Finland; 7, Measurements taken from Ergens, 1983; 8, samples obtained from *Salvelinus fontinalis* (Mitchill) in Nova Scotia, Canada; 9, Morph 8 was identified on the basis of marginal hook sickles released by proteolytic digestion from a pooled sample of gyrodactylids.

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 the gyrodactylid–specific Point–R macro (Bron & Shinn, University of Stirling) written within the KS300 (ver.3.0) (Carl Zeiss Vision GmbH, 1997) image analysis software. The 22 point–to–point measurements (shown in Table 3) are given in micrometres as the mean ±1 standard deviation followed by the range in parentheses, and were selected from those described in Shinn et al. (2004).

The following *Gyrodactylus* type material and specimens were obtained for the current study: *G. avalonia* holotype (USNPC 70439); *G. brevis* four paratypes (USNPC 061635 – three slides; 073549 – one slide); *G. colemanensis* two paratypes (USNPC 061691); ethanol fixed specimens of *G. masu* Ogawa, 1986 were provided by Kazuo Ogawa from Tokyo University, Japan; photographs of the *G. nerkae* holotype (NMCIC(P)1983–0168) deposited in the National Museum of Natural Sciences, Ottawa, Canada made by Judith Price; photographs of specimens of *G. lavareti* Malmberg, 1957 and of *G. salvelini* Kuusela, Ziętara et Lumme, 2008 from the private collection of Jaakko Lumme from the University of Oulu, Finland were sent for evaluation; ethanol fixed specimens of *G. salmonis* were donated by Stanley King from Dalhousie University, Canada and David Cone from St Mary's University, Canada. Additional specimens of *Gyrodactylus* parasitising *O. mykiss* were collected by the authors from several sites across Europe; the details and measurements of the identified specimens are listed in Tables 3 and S1. When no material was available for morphological analysis, additional measurements not given in original descriptions were extrapolated from published images.

2.4. Molecular analysis

DNA extractions were performed on four individual ethanol-fixed gyrodactylid monogeneans that had been removed from four different feral *O. mykiss* specimens collected near Xalapa, Mexico. All PCRs were performed in quadruplicate (amplifications from four different worms) and all PCR products were sequenced in both forward and reverse directions. DNA was extracted using a GeneMATRIX kit (EURx Poland) following the tissue protocol. Three gene regions were targeted using previously described oligonucleotide primers with additional more specific primers designed during this study. The small subunit ribosomal DNA (SSU rDNA) was amplified using the universal primers 18e, 390f, 870f, 870r and 18gM, for which primers sequences and PCR conditions have been previously described (Freeman and Ogawa, 2010; Morris and Freeman, 2010). In addition to the universal SSU rDNA primers, an additional specific forward primer, Gyro-300f 5′ CTTGTTGTCGGCGACGGATC 3′ was used with the reverse primer 870r to confirm the initial sequence reads. The internal transcribed spacer 1 (ITS1), 5.8S ribosomal DNA and internal transcribed spacer 2 (ITS2) regions of the ribosomal RNA gene were amplified using the primers ITS1F and ITS2R (Ziętara et al., 2002). The cytochrome c oxidase subunit I (cox1) gene was amplified using the primers and PCR conditions described by Kuusela et al. (2008), as well as the newly designed primer pair Gyro-coxF 5′ TCCAAGGGTAGGTACCGAC 3′ and Gyro-coxR 5′ TATACCAGTAGGCACTGC 3′ that utilised the same PCR conditions. All PCR bands of the expected sizes were recovered from the PCR products using a GeneMATRIX PCR products extraction kit (EURx Poland). Sequencing reactions were performed using BigDyeTM Terminator Cycle Sequencing chemistry utilising the same oligonucleotide primers that were used for the original PCRs. Individual sequence reads were each confirmed as monogenean using nucleotide BLAST searches in GenBank (Altschul et al., 1990). Contiguous sequences were obtained manually using CLUSTAL_X (Thompson et al., 1997) and BioEdit (Hall, 1999). For phylogenetic analyses, taxa were chosen from BLAST searches that had high similarities to the novel sequence. CLUSTAL_X was used for the initial sequence alignments and manually edited using the BioEdit sequence alignment editor and percentage divergence matrices constructed in CLUSTAL_X using the neighbor-joining method (Saitou and Nei, 1987). Phylogenetic analyses were performed using maximum parsimony methodologies in PAUP*4.0 beta10 (Swofford, 2002).

3. Results

Forty-six feral rainbow trout were collected. Eighteen gyrodactylids were found on the fins of 5 out of the 6 1+year old fish (mean abundance ± 1 S.D., 3.0 ± 1.67 worms/host; range, 0–5 worms/host).

Gyrodactylids collected in Mexico exhibited limited but constant morphological and molecular variation when contrasted to *G. salmonis* from Canada. In the following sections, we present the morphological and molecular description of Mexican isolates of *G. salmonis*. Marginal hook sickle morphology is the key to the separation and discrimination of all the gyrodactylids parasitising salmonids, and we provide graphical (Figs. 1 and 2 and S1) and morphological data (Table 3) to distinguish between the fourteen previously recorded gyrodactylid species from *O. mykiss* (Table 2) and Mexican isolates of *G. salmonis*. We also provide a graphical comparison of the marginal hook sickle morphology of Mexican isolates of *G. salmonis* and other *Gyrodactylus* species recorded from other native and introduced salmonids which occur throughout North America (Fig. S2).

3.1. Taxonomic description

Gyrodactylus salmonis (Mexican isolates)
(Figs. 1 and 2, S1 and S2; Table 3)

Host: *Oncorhynchus mykiss* Walbaum ("rainbow trout", "trucha arcoíris").

Site of infection: Fins.

Locality: Río Pixquiac, Xalapa, Veracruz, Mexico (19°28′39″N, 96°57′00″W).

Reference material: Ten specimens were prepared for light microscopical analyses. Five voucher specimens are deposited in the Colección Nacional de Helmintos (CNHE reg. no. 7541), Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City. An additional five voucher specimens are deposited in the Parasitic Worm

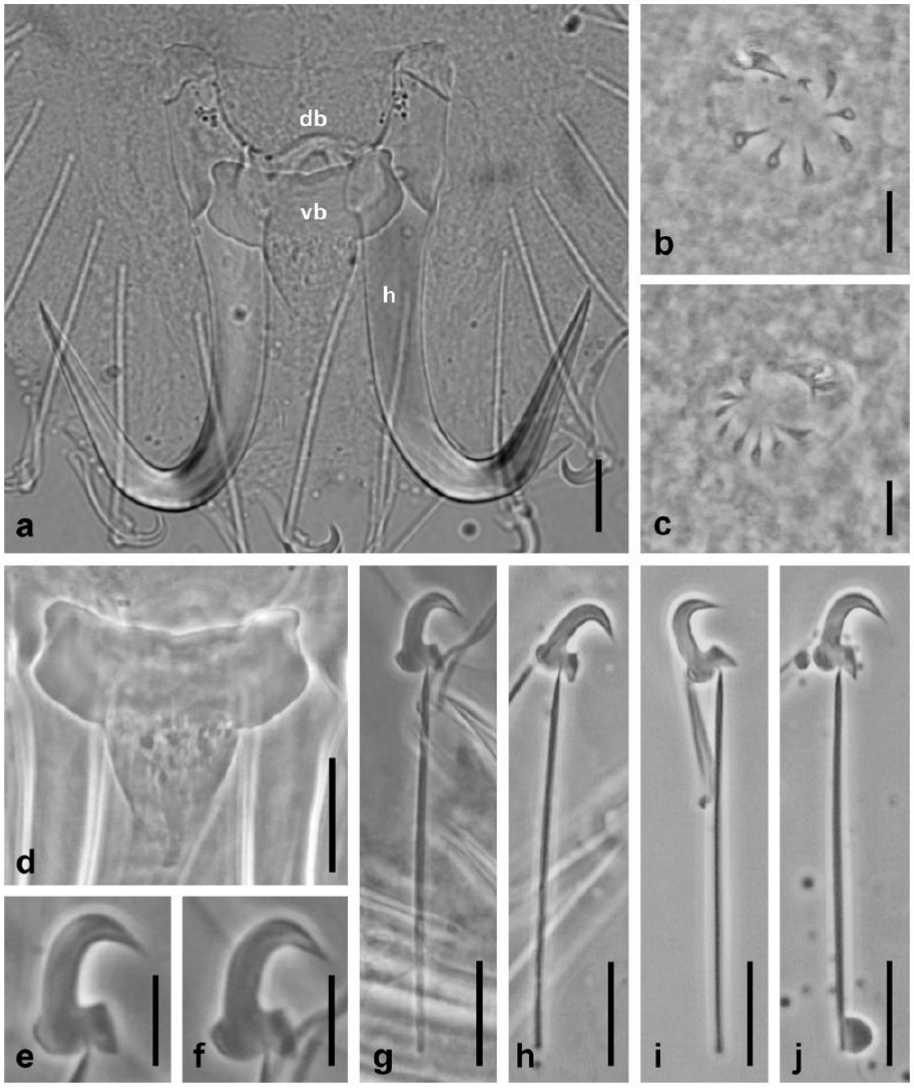

Fig. 1. The haptoral armature and male copulatory organ of Mexican isolates of *Gyrodactylus salmonis* from *Oncorhynchus mykiss* (Walbaum). (a) Central haptoral hook complex of two hamuli (h) linked by a dorsal bar (db) and a ventral bar (vb); (b) developing male copulatory organ; (c) developed male copulatory organ showing a 1+8 arrangement of spination; (d) ventral bar; (e and f) marginal hook sickle proper; (g–j), marginal hooks. Scale bars: a, d, g–j = 10 μm; b–c, e–f = 5 μm.

Collection at The Natural History Museum (BMNH reg. no. 2011.10.19.1-5), London, UK.

DNA reference sequences: Three sequences are deposited in GenBank: 1) partial 18S gene (1914 bp) deposited under accession number JN230350; 2) partial ITS1 (656 bp), 5.8S (157 bp), partial ITS2 (417 bp) under accession number JN230351; and, 3) partial cytochrome c oxidase subunit I (cox1) mitochondrial gene (1718 bp) under accession number JN230352.

Description: Whole body measurements, given as the mean and the range in parentheses, taken from 6 specimens only. Body 507 (410–590) long; 159 (100–210) wide at the level of the uterus. Haptor longitudinally ovate, clearly delineated from the body, 97.1 (87.5–105) long × 116.7 (100–132) wide. Anterior pharynx bulb 27.5 (23.3–34.0) long × 38.0 (28.8–45.7) wide; posterior pharynx bulb 20.8 (17.2–23.0) long × 63.5 (54.7–75.1) wide. Intestinal crura extending below the testes. Male copulatory organ (MCO), observed on 3 specimens, ventro-lateral, posterior to the posterior pharyngeal bulb, 18.7 (17.6–20.5) long × 19.0 (17.7–20.7) wide, spherical, armed with one large principal hook 4.3–5.3 long and a single ring of 6–8 spines 3.2–4.3 long (Figs. 1 and 2b, c, c). Measurements of the haptoral armature (mean only) are based on ten specimens (see Table 3 for full measurement details). Hamulus total length 64.4; shaft length 43.5; point 35.7 long with a 35.8° aperture; proportionately short straight roots 22.1 long. Dorsal bar simple, attaching close to the anterior edge of the dorsal

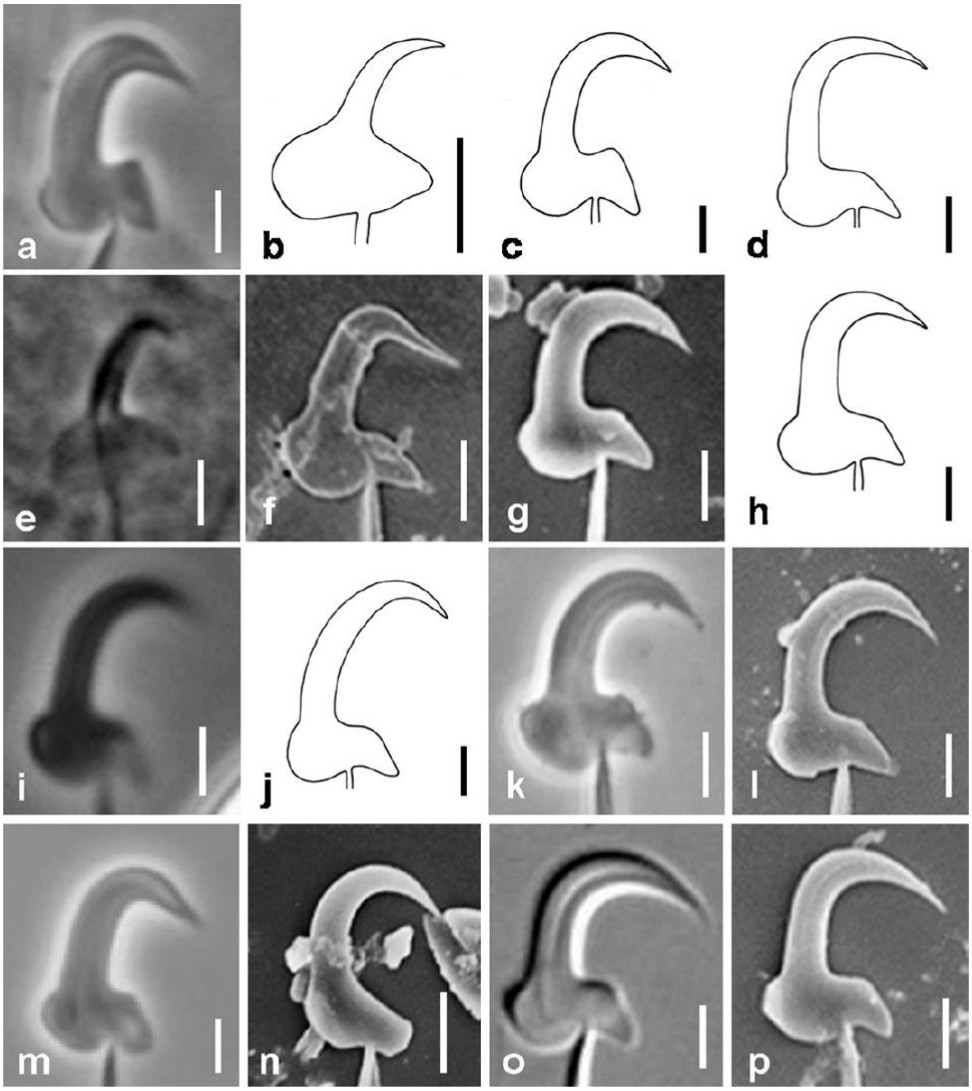

Fig. 2. Species of *Gyrodactylus* von Nordmann, 1832 recorded from *Oncorhynchus mykiss* (Walbaum). (a) Mexican isolate of *G. salmonis*; (b) *G. avalonia* Hanek et Threlfall, 1969 (redrawn from Hanek and Threlfall, 1969); (c) *G. bohemicus* Ergens, 1992 (redrawn from Ergens, 1992); (d) *G. brachymystacis* Ergens, 1978 (redrawn from Ergens, 1983); (e) *G. brevis* Crane et Mizelle, 1967 (original); (f) *G. colemanensis* Mizelle et Kritsky, 1967 (original); (g) *G. derjavinoides* Malmberg, Collins, Cunningham et Jalali, 2007 (original); (h) *G. gobii* Shulman, 1954 (redrawn from Gusev, 1985); (i) *G. lavareti* Malmberg, 1957 (image courtesy of J. Lumme, University of Oulu, Finland); (j) *G. lenoki* Gussev, 1953 (redrawn from Ergens, 1983); (k) *G. masu* Ogawa, 1986 (original); (l) *G. salaris* Malmberg, 1957 (original); (m) *G. salmonis* (Yin et Sproston, 1948) (image reproduced courtesy of S. R. Gilmore, C. L. Abbott and D. K. Cone, and Wiley Blackwell); (n) *Gyrodactylus* sp. Morph 8 *sensu* Shinn et al., 1995 (original); (o) *G. teuchis* Lautraite, Blanc, Thiery, Daniel et Vigneulle, 1999 (original); (p) *G. truttae* Gläser, 1974 (original). Scale bar = 2.5 μm.

bar attachment point on each hamulus, 27.1 long, 2.2 wide. Dorsal bar attachment points large, covering one third of the width of the hamulus. Ventral bar 23.2 long; 25.5 wide. Ventral bar processes small. Median portion of the ventral bar, stout, slightly curved, length representing approximately half the total length of the ventral bar. Ventral bar membrane triangular, 13.4 long. Marginal hook 44.6 long; shaft length 37.0; sickle proper length 7.9. Sickle proximal width 4.8. Toe pointed, triangular, steeply faced, 1.8 long. Toe point below the lower level of the heel. Bridge of the toe with prominent peak, which slopes towards the base of the sickle shaft. Heel not pronounced, gently curved. Sickle shaft, broad, gently curving into a broad tip of moderate length that terminates beyond the limit of the toe. Distal width 6.0. Line described by inner curve of the marginal hook sickle, trapezoid. Sickle aperture 6.0 long. Instep height 0.8.

Comments: Of the marginal hook sickles presented in Fig. 2, the Canadian isolate of *G. salmonis* (Fig. 2m) is the closest morphologically to that of the Mexican isolate of *G. salmonis* (Fig. 2a). Although the size of these is similar in both cases (7.9 μm Mexican isolate of *G. salmonis vs.* 8.0 μm

Canadian isolate of *G. salmonis*), when the invariantly sized hooks of both are overlaid one another (Fig. S1d–f), then it is evident that sickle base features permit their discrimination from each other. The sickle heel of the Canadian specimen of *G. salmonis* is larger but has a shorter, rounded, downward projecting toe (Fig. S1e, f). The toe of the Mexican isolate of *G. salmonis* by comparison, is larger, pointed and steeply sloped with a characteristic peak to the top of the top bridge which then slopes down towards the sickle shaft (Fig. S1b, d). The shape of the inner curve, therefore, is distinctly trapezoid in the Mexican isolate of *G. salmonis* and rectangular in the Canadian isolate of *G. salmonis*. The approximate shape and size of the sickle shaft and tip regions for both gyrodactylids coincide closely with each other. The approximate dimensions of all the features, except the ventral bar total width, extracted from the haptoral hard parts for both gyrodactylids are closely matched.

3.2. Molecular characterisation

PCR amplification and gene sequencing for the three different gene regions were successfully achieved for all four individual Mexican isolates of *G. salmonis*. Contiguous sequences were constructed and deposited in GenBank under the accession numbers JN230350-52.

Due to 18S data being currently unavailable for *G. salmonis*, the 18S sequence determined from the Mexican specimens were compared with two variable regions of SSU rDNA obtained from a Canadian population of *G. salmonis* (Gilmore, Abbott and Cone, unpublished data). The regions spanned bases 422–864 and 1252–1740 from the submitted 18S sequence for the Mexican specimens and had 100% homology over all bases. The SSU (18S) rDNA sequence of 1914 base pairs (JN230350) was also similar to *Gyrodactylus gobiensis* Gläser, 1974 (99.1%) and Gyrodactylus rutilensis Gläser (1974) (98.3%). The ITS1, 5.8S and ITS2 region of the rDNA (1230 bp; JN230351) was identical to sequences from *Gyrodactylus salmonis* isolate s1 (GQ368233) collected from Washington, U.S.A. and *Gyrodactylus salvelini* isolate Inari (EF113106) collected from Lake Inarijarvi, Barents Sea basin, Finland with 1230/1230 identities and no gaps in both cases. The cox1 gene of 1718 bp (JN230352) was most similar to the American isolates of *G. salmonis* ranging between 98% and 99.5% identities depending on the isolate. Table 4 shows a percentage identity matrix based on cox1 alignments of 1527 nucleotides for the Mexican isolate from the present study and 8 other gyrodactylids identified using blast searches. The highest sequence identity to Mexican isolates of *G. salmonis* was American *G. salmonis* isolate s1, with an identity of 99.48%; *G. salvelini* had an identity of 97.25%. The other American strains of *G. salmonis* also had a similar percentage identity (96.92–97.38%) to *G. salvelini* (see Table 4).

The phylogenetic tree (Fig. 3) shows that the known American isolates of *G. salmonis* (s1, d4 and d1) group together with the sequence from the present study from Mexico. This grouping is well supported with a bootstrap value of 91. The Mexican isolate forms a well-supported clade with the isolate s1, which is a sister clade to isolate d4, with isolate d1 as the basal isolate of the *G. salmonis* group. The next known most related species is *G. salvelini*.

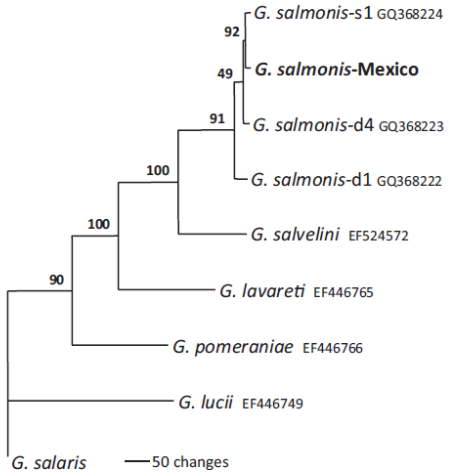

Fig. 3. Maximum parsimony phylogenetic tree based on an alignment of 1527 nucleotides of mitochondrial gene cox 1 sequence data. Bootstrap values were obtained from 1000 resamplings; scale bar represents 50 nucleotide base changes.

4. Discussion

The isolate of *G. salmonis* described here is the first gyrodactylid to be formally identified from *O. mykiss* in Mexico using both morphological and molecular data. Prevalence and abundance of the Mexican *G. salmonis* isolate was low, and infected fish did not show evident damage; further, there are no reports of fish morbidity or mortality associated to gyrodactylid infection in rainbow trout farms in Veracruz, which would suggest a stable host-parasite interaction.

Gyrodactylus salmonis and *G. colemanensis* have virtually pan-North American distributions, as both species have been recorded from farmed *O. mykiss* across both the U.S.A. and Canada (Cone et al., 1983; Shinn et al., 2011) (Table 1). *Gyrodactylus salmonis* has low host specificity, as it has also been recorded in the same region on farmed salmonid fish introduced from Europe, such as *Salmo trutta fario* L. (see Malmberg, 1993), *Salmo salar* L. (see Cone and Cusack, 1988; Malmberg, 1993) and *S. fontinalis* (see Cone and Cusack, 1988; Wells and Cone, 1990; Malmberg, 1993); as well as on native *Oncorhynchus* species like golden trout *Oncorhynchus aguabonita* (Jordan) (see Cone et al., 1983), coho salmon *Oncorhynchus kisutch* (Walbaum) (see Cone et al., 1983) and *O. clarkii clarkii* (see Cone et al., 1983), whose distribution ranges overlap with the ancestral distribution range of *O. mykiss*. However, Gilmore et al. (2010) proposed that *G. salmonis* is a natural parasite of fish of the genus *Salvelinus*; a proposal strengthened by the recent observation in the South River watershed in Nova Scotia, Canada, that this gyrodactylid mostly parasitised brook trout, *S. fontinalis*, despite the fact that three other salmonids (*O. mykiss*, *S. trutta* and *S. salar*) occurred in the same river (You et al., 2011). A further gyrodactylid parasite that has been found on sympatric salmonids in the northern Pacific is *G. masu*, which has been recovered from *O. mykiss*, masu

Table 4
Percentage identity matrix of selected *Gyrodactylus* spp., based on an alignment of 1527 nucleotides of mitochondrial cox1 gene sequence data.

	Taxa	1	2	3	4	5	6	7	8	9
1.	*G. salmonis* (Mexican isolates)	*100*	99.48	98.82	98.56	97.25	87.43	82.51	79.24	79.04
2.	*G. salmonis*-s1	99.48	*100*	98.82	98.69	97.25	87.23	82.51	79.57	79.11
3.	*G. salmonis*-d4	98.82	98.82	*100*	98.62	97.38	87.16	82.19	79.76	79.11
4.	*G. salmonis*-d1	98.56	98.69	98.62	*100*	96.92	87.10	82.84	79.83	79.50
5.	*G. salvelini*	97.25	97.25	97.38	96.92	*100*	87.03	81.60	79.37	78.65
6.	*G. lavareti*	87.43	87.23	87.16	87.10	87.03	*100*	82.06	79.31	79.76
7.	*G. pomeraniae*	82.51	82.51	82.19	82.84	81.60	82.06	*100*	79.37	79.63
8.	*G. lucii*	79.24	79.57	79.76	79.83	79.37	79.31	79.37	*100*	78.98
9.	*G. salaris*	79.04	79.11	79.11	79.50	78.65	79.76	79.63	78.98	*100*

salmon *Oncorhynchus masou masou* (Brevoort) (see Ogawa, 1986), and amago salmon *Oncorhynchus rhodurus* Jordan et McGregor (see Ogawa, 1994). Considering that rainbow trout is also sympatric with chum salmon *Oncorhynchus keta* (Walbaum) and sockeye salmon *Oncorhynchus nerka* (Walbaum), it is plausible that *G. salmonis* infects these two salmonids; and, conversely, that *O. mykiss* and other sympatric *Oncorhynchus* species harbour *G. somnaensis* Ergens et Yukhimenko, 1990 found on *O. keta* (see Ergens and Yukhimenko, 1990) and *G. nerkae* found on *O. nerka* (see Cone et al., 1983). In brief, it is possible that all *Oncorhynchus* species whose distribution range overlaps with that ancestral of *O. mykiss* (*i.e.*, *O. aguabonita*, *O. chrysogaster*, *O. kisutch*, *O. keta*, *O. clarkii clarkii* and *O. nerka*) exchange gyrodactylids.

Rainbow trout and other non-native salmonids were introduced in the late 19th century to central Mexico (Hendrickson et al., 2002), where they did not occur naturally. Although there are native trouts in Mexico, these are distributed in the northwest of the country, mainly in river systems west of the continental divide of the Sierra Madre Occidental mountain range, which drain into the Sea of Cortés and the Pacific Ocean (Hendrickson et al., 2002; Camarena-Rosales et al., 2008). In the Pleistocene, the distribution range of native salmonid fish extended some 400 km further south of today's range, as illustrated by the occurrence of the fossil species *Salmo australis* Cavender et Miller in Lake Chapala, Jalisco, at nearly 20° N latitude (Cavender and Miller, 1982); but salmonid fish never occurred naturally in central Mexico, *i.e.*, southern North America. Therefore, the most plausible explanation for the occurrence of *G. salmonis* on rainbow trout in Veracruz (southeastern Mexico, on the Gulf of Mexico slope) is that the parasite was originally introduced with its translocated fish host. The question whether rainbow trout acquired the parasite from other salmonids (possibly of the genus *Salvelinus*) prior to translocation or once in Mexico remains open – and will probably not be solved given the extensive anthropogenic movement of fish stocks and presumably their parasites.

Mexican isolates of *G. salmonis* are both morphologically and molecularly very similar to the American and Canadian specimens of *G. salmonis*. Morphological analysis shows that features of the sickle base of the marginal hooks permit the discrimination of Mexican *G. salmonis* from Canadian *G. salmonis* specimens. Molecular analysis indicated that alignment of the partial 18S sequence from the Mexican isolate of *G. salmonis* with those determined by Gilmore et al. (unpublished data) from a Canadian population of *G. salmonis* are identical. An alignment with the Mexican 18S sequence with other *Gyrodactylus* species listed in GenBank indicated that it was very similar (98–99%) to that of *G. gobiensis* and other gyrodactylids such as *G. salaris* and *G. rutilensis*. Similarly, the ITS regions of the Mexican and American isolates of *G. salmonis* were found to be identical to each other and also to those of *G. salvelini* [1230/1230 bases]. Considering the general lack of suitability of 18S for discriminating *Gyrodactylus* species, and the 100% homology found across the ITS regions, the mitochondrial marker cox1 was also sequenced. Cox1 of the Mexican isolate of *G. salmonis* shows higher variability compared to the ITS region, with 1589/1597 bases identical with no gaps to the American isolate of *G. salmonis*, *i.e.* 99.5% similar. There is less similarity to *G. salvelini* with 1635/1684 bases identical with no gaps (97% similarity). The American isolate of *G. salmonis* is also 97% similar to *G. salvelini* with 1554/1597 (no gaps) of comparable sequence data. Cox1 sequences of the American isolates of *G. salmonis* exhibit a range of variation; as Mexican isolates fit into this range of variation, their identity as *G. salmonis* is confirmed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetpar.2011.11.005.

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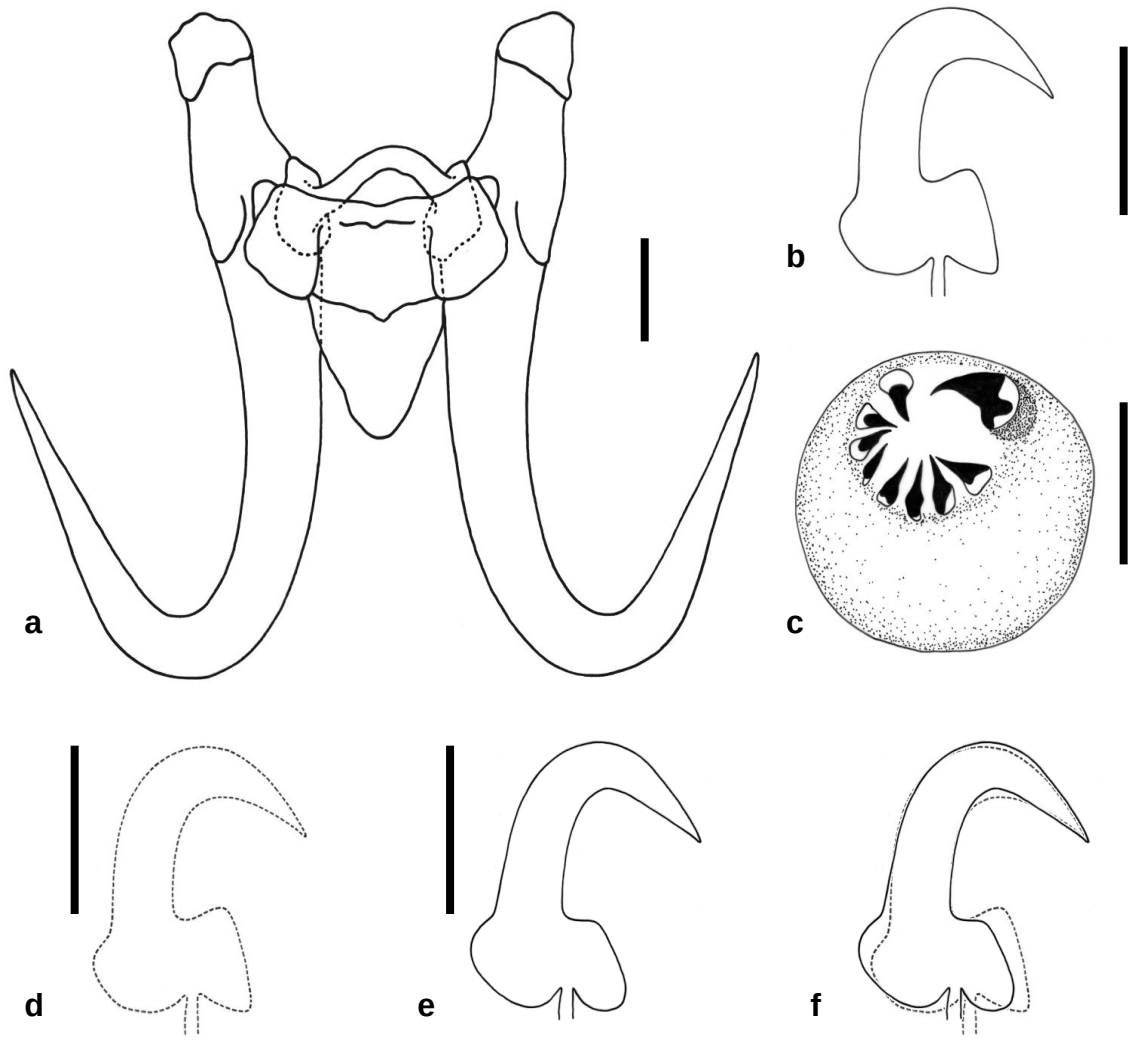


Fig. S1. Graphic illustration of the haptoral armature and male copulatory organ of Mexican isolates of *Gyrodactylus salmonis* from *Oncorhynchus mykiss* (Walbaum). (**a**) central haptoral hook complex of two hamuli linked by a dorsal bar and a ventral bar; (**b**) marginal hook sickle; (**c**) male copulatory organ with 1+8 arrangement of spination; (**d**) marginal hook sickle (broken line) of the Mexican isolate of *G. salmonis*; (**e**) marginal hook sickle of *G. salmonis*; (**f**) size invariant overlay of Mexican isolate of *G. salmonis* (broken line) with that of *G. salmonis* (solid line) Scale bars: a, b, d-f = 10 μm; c = 5 μm.

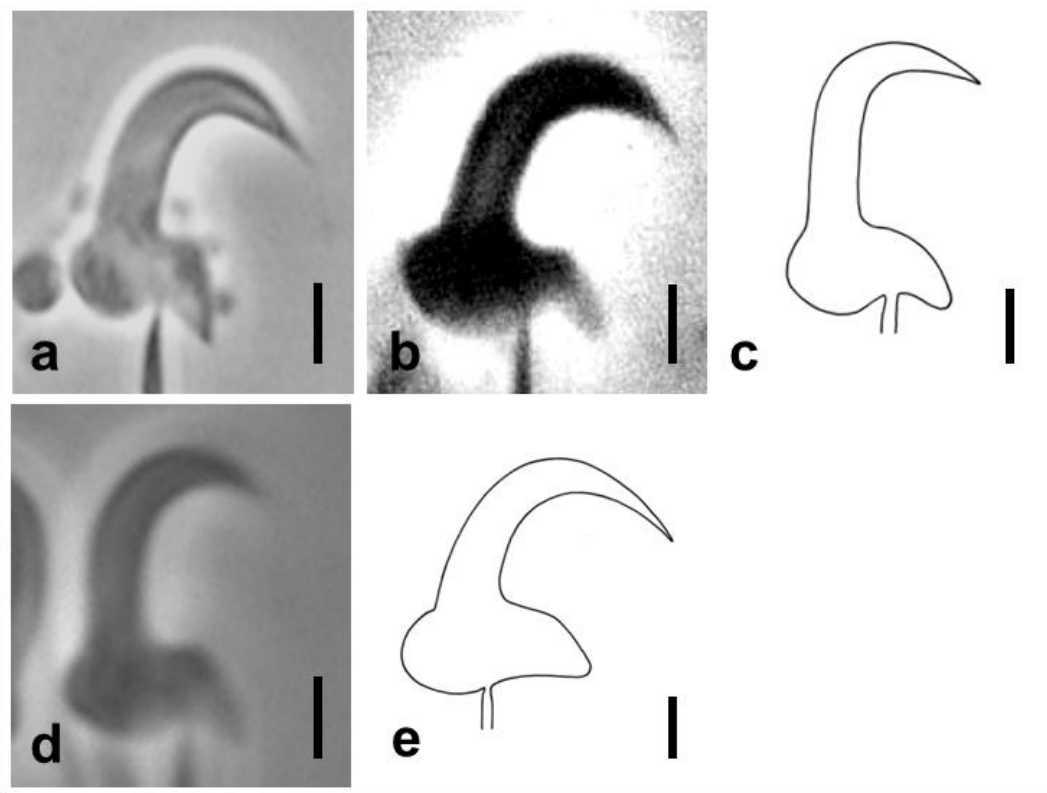


Fig. S2. Species of *Gyrodactylus* von Nordmann, 1832 recorded from other salmonids, populations of which occur throughout North America. (**a**) Mexican isolate of *G. salmonis*; (**b**) *G. derjavini* Mikailov, 1975 (image reproduced courtesy of Malmberg *et al*., 2007 and the Polish Institute of Parasitology); (**c**) *G. nerkae* Cone, Beverley-Burton, Wiles *et* McDonald, 1983 from a Canadian population of *Oncorhynchus nerka* (Walbaum) (redrawn from Cone *et al*., 1983); (**d**) *G. salvelini* Kuusela, Ziętara *et* Lumme, 2008 from a Finnish population of *Salvelinus alpinus alpinus* (L.) (image courtesy of J. Lumme, University of Oulu, Finland); (**e**) *G. somnaensis* Ergens *et* Yukhimenko, 1990 from *Oncorhynchus keta* (Walbaum) from China / Russia (redrawn from Ergens and Yukhimenko, 1990). Scale bars = 2.5 μm.

TABLE PROVIDED AS SUPPLEMENTARY INFORMATION

Table S1. Sample details of *Gyrodactylus* species, reported in the literature as infecting *Oncorhynchus* spp., that were obtained for a comparative morphological study with Mexican isolates of *Gyrodactylus salmonis* collected from a population of *Oncorhynchus mykiss* from Veracruz, Mexico.

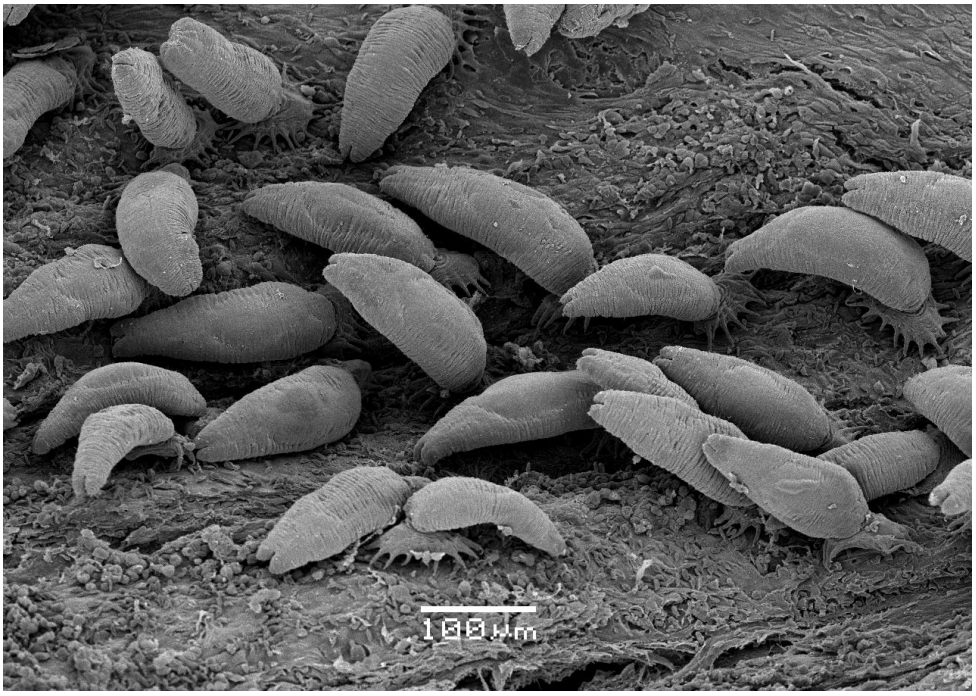
Species	Site	Longitude / Latitude	Host	Microhabitat (No. specimens)
G. bohemicus	South Bohemia, Czech Republic (taken from Ergens, 1992)	48°44'19''N; 14°29'47''E	*O. mykiss* *S. fontinalis*	Fins, skin and gills
G. brevis *	Navarro River, California, U.S.A.	39°05'47''N; 123°29'48''W		
G. colemanensis	Nova Scotia, Canada (Material kindly provided by D. K. Cone)	No details	*O. mykiss*	
G. derjavini	River Sardab-rud, nr New-sahr, N. Iran	No details	*S. trutta caspius*	Fins
G. derjavinoides	Refsgaard nr Vejle, Jutland, Denmark	55º36'57"N; 9º43'37"W	*O. mykiss*	Fins (8)
	Strathclyde, Scotland (confidential location)		*O. mykiss*	Fins (9)
	River Nera, Umbria, Italy	42°51'41"N; 12°58'49"E	*O. mykiss*	Mucus scrape (1)
	River Bann, N. Ireland	54°20'30"N; 6°13'30"W	*S. salar*	Fins (1)
	River Colebrooke, N. Ireland	54°22'40"N; 7°16'00"W	*S. salar*	Fins (1)
G. gobii *	Brandenburg and Thuringia, Germany (taken from Schulman, 1953)	No details	*O. mykiss*	Skin, fins and gills
G. lavareti *	Sweden (taken from (Malmberg, 1957)	No details	*O. mykiss*	
G. masu	Japan (Specimens kindly provided by K. Ogawa)	No details	*O. mykiss*	
G. nerkae	Holotype CMNPA 1983-0168 – measurements extracted from photographs provided by J. Price	No details	*O. nerka*	
G. salaris	Mosbjerg, Denmark	57º30'10"N; 10º16'5"W	*O. mykiss*	Fins (6)
	Jyvaskyla, Finland (confidential location)	No details	*O. mykiss*	Fins (2)
	River Sile, Veneto, Italy	45°38'23"N; 12°08'14"E	*O. mykiss*	Mucus scrape (6)
	Avisio Torrent, Trentino Alto Adige, Italy	46°16'43"N; 11°26'40"E	*O. mykiss*	Mucus scrape (4)

	River Sérchio, Tuscany, Italy	44°02'52"N; 10°27'40"E	*O. mykiss*	Mucus scrape (10)
	Clitunno Fountain, Umbria, Italy	42°44'42"N; 12°42'25"E	*O. mykiss*	Mucus scrape (1)
	River Nera, Umbria, Italy	42°51'41"N; 12°58'49"E	*O. mykiss*	Mucus scrape (2)
G. salmonis	Nova Scotia, Canada (Material kindly provided by D. K. Cone and S.D. King)	No details	*S. fontinalis*	
G. teuchis	Clitunno Fountain, Umbria, Italy	42°44'42"N; 12°42'25"E	*O. mykiss*	Mucus scrape (5)
	River Nera, Umbria, Italy	42°51'41"N; 12°58'49"E	*O. mykiss*	Mucus scrape (8)
G. truttae	River Ordie, Scotland	56°29' 7"N; 3°30'37"W	*S. trutta fario*	Body & fins (5)
	River Tweed, Scotland	55°28' 4"N; 2°54'6"W	*S. trutta fario*	Body & fins (8)
	Shockie Burn, Scotland	56°27' 1"N; 3°30'56"W	*S. trutta fario*	Body & fins (7)
	Ballinderry River, N. Ireland	54°38'25"N; 6°46'30"W	*S. trutta fario*	Body & fins (10)
	Six Mile Water, N. Ireland	54°46'25"N; 5°57'00"W	*S. trutta fario*	Body & fins (5)
	Enler River, N. Ireland	54°20'30"N; 5°47'10"W	*S. trutta fario*	Body & fins (6)
G. sp. morph 8 (Shinn *et al.*, 1995)	River South Esk, Scotland (based on two marginal hooks from a pool of specimens that were processed by sonication)	No details	*O. mykiss*	

Note: * when type material was not available for measurement, additional morphological data not given in original descriptions was extrapolated from drawings/photographs presented in published papers.

Chapter 6

The experimental susceptibility of English and Welsh salmonids to *Gyrodactylus salaris* (Platyhelminthes, Monogenea)



Experimental infection of Welsh Atlantic salmon, *Salmo salar* L., with *Gyrodactylus salaris* Malmberg, 1957 [original image]

Aspects of this work were presented as:

Paladini G. (2012). *Gyrodactylus*: tales of invasion, resistance and control strategies. *Aquaculture UK 2012, Aviemore, Scotland, 23rd-24th May 2012* (talk).

Paladini G., Williams C., Hansen H., Taylor N.G.H., Rubio-Mejía O.L., Denholm S.J., Hytterød S., Bron J.E., Shinn A.P. (2012). *Gyrodactylus salaris*: the good, the bad and the ugly. *Institute of Aquaculture 3rd PhD Research Conference, Stirling, Scotland, 24th October 2012*: 16 (talk).

6.1. Introduction

There are over 430 species of *Gyrodactylus*, small ectoparasitic monogenean worms principally infecting fish, some species of which are highly pathogenic (Harris *et al*., 2004; Shinn *et al*., 2012a, b; www.gyrodb.net; www.monodb.org). While most species of *Gyrodactylus* are non-pathogenic, causing little harm to their hosts, other species like *Gyrodactylus salaris* Malmberg, 1957 - which is a listed pathogen of Atlantic salmon, *Salmo salar* L., for OIE (Office International des Epizooties) - has had catastrophic effects on juvenile salmon populations in 46 Norwegian rivers (Johnsen *et al*., 1999; Bakke *et al*., 2007). Uncontrolled increases in the size of the parasite population on resident salmon populations have necessitated extreme measures, such as the use of the biocide rotenone to kill-out entire river systems in order to remove the whole fish population within a river and with them the infecting *G. salaris* (see Bakke *et al*., 2007). Given the impact that *G. salaris* has had in Norway and elsewhere in Scandinavia and Russia (Rintamäki, 1989; Ieshko *et al*., 1995; Alenäs, 1998; Alenäs *et al*., 1998), Norway and the UK, now have mandatory surveillance programmes, the latest screening wild salmonid populations (*i.e.* brown trout *Salmo trutta fario* L., Arctic charr *Salvelinus alpinus alpinus* (L.), grayling *Thymallus thymallus* (L.), Atlantic salmon, *etc*) for the presence of the notifiable pathogen *G. salaris*, while Norway and Sweden screen only wild Atlantic salmon populations. The current study sets out to make a contribution to national *G. salaris* contingency planning by determining the responses of different English and Welsh salmonids to pathogenic strains of *G. salaris*. The study also aims to assess the extent to which laboratory conditions might affect the results of infection experiments, and gauge whether extrapolation from existing results is appropriate for UK contingency planning.

The recent reports of *G. salaris* in Italy (Paladini *et al*., 2009a; Chapter 2 of the current thesis) and Poland (Rokicka *et al*., 2007) purportedly linked to the movement of

salmonid stocks across borders, emphasise the biosecurity risk this pathogen poses. Great Britain and Northern Ireland (forming the United Kingdom) are currently recognised *G. salaris*-free states (Platten *et al*., 1994; Shinn *et al*., 1995). Given the value of their respective salmonid industries (total Scottish and Northern Ireland salmon production 157,385 tonnes in 2011, worth ~£540 million; ICES, 2012) and recreational salmon and sea tout fishing, which throughout the UK is worth in excess of £230 million, it is important the UK's *G. salaris*-free status is upheld. Coarse and game angling figures for Scotland in 2010 were estimated at over £100 million (www.scotland.gov.uk), whilst recreational and commercial salmon and sea trout fisheries in England and Wales in 2001 (last figures available) had a capital value of £130 million (www.cefas.defra.gov.uk).

Existing UK dispersion models and contingency plans for the containment of *G. salaris* are based on the assumption that British stocks of Atlantic salmon would be vulnerable to *G. salaris* and therefore at risk (see Bakke *et al*., 1990; Bakke & MacKenzie, 1993); that brown trout would be entirely resistant to infection and unaffected (see Jansen & Bakke, 1995; Bakke *et al*., 1999; Harris *et al*., 2000); and that grayling would be relatively resistant (see Soleng & Bakke, 2001a). Brown trout and grayling, following models determined for Scandinavian populations of these hosts (see review in Bakke *et al*., 2007), are thought to harbour low-level infections for a few weeks, not displaying the exponential increase in numbers seen on Atlantic salmon. Native UK stocks of brown trout and grayling, however, have been separated from their Scandinavian counterparts since the last period of glaciation (Halvorsen & Hartvigsen, 1989), and their relative patterns of susceptibility and/or resistance may therefore differ from those predicted from Norwegian studies. If these differences are demonstrated, then current contingency plans would require redrafting and dispersion models re-designed and re-analysed.

Assumptions that UK salmon are susceptible to *G. salaris* are derived from an earlier study by Bakke and MacKenzie (1993), which tested the susceptibility of two

Scottish populations of Atlantic salmon (*i.e.* from the Rivers Shin and Conon) to a Norwegian strain of *G. salaris*. The experimental exposure of other British salmonids (*i.e.* brown trout, grayling, *etc*.) to *G. salaris* has not been conducted to date. To ensure that *G. salaris* infections on English and Welsh salmonids follow the same infection dynamics as their Scandinavian counterparts, and that current national *G. salaris* contingency plans within England and Wales are appropriate, it was imperative that these trials were conducted to guarantee completeness of the existing contingency policy. The current study sets out to verify whether these assumptions were correct. To determine this, Atlantic salmon, brown trout and grayling eggs, stripped from wild fish, were reared in English and Welsh Government-run hatcheries and then transported to a secure research facility in Norway for experimental challenge with a strain of *G. salaris* (haploptype A) known to be pathogenic to Norwegian and Scottish Atlantic salmon.

6.2. Materials and methods

6.2.1. Origin of experimental salmonid populations

i) *Salmo salar* from the River Dee, Wales

In 2010/2011, eggs from wild Atlantic salmon caught in the River Dee, northern Wales, were stripped, fertilised and reared to 0+ parr in the Environment Agency Wales' (EAW) Maerdy Hatchery, Corwen, Conwy, Wales (52º59'18.18" N; 3º27'48.18" W). The eggs began hatching around mid-January 2011. The fish were reared on ambient water (av. 2.7 ºC) from the Afon Ceirw, in a natural photoperiod regime, and with a 1% body wt day^{-1} daily ration of feed (Skretting Nutra parr 02). The fish weighed a mean of 3.4 ± 0.3 g at the time they were shipped to Norway.

ii) *Salmo trutta fario* from the River Tyne, England

On the 11th November 2010, adult sea trout broodstock were collected from the River Rede, a tributary of the River Tyne, Northumberland, northern England. The ripe female fish were stripped and fertilised on the 12th, 20th and 30th November 2010 and the eggs maintained at the EAW's Kielder Hatchery (55º14'00.45" N; 2º34'39.69" W). Egg hatching occurred over the period 19th March to the 2nd April 2011. The eggs and fish were maintained at ambient water temperatures (0–18.5ºC), with natural photoperiod conditions and a 0.1–2.8% body wt day^{-1} daily feed ration (Skretting Emerald Fry 00, 01 and 02 crumb) over the 303–316 days they were maintained until shipped to Norway. The fish had a mean weight of 4.45 ± 0.4 g at the time they were shipped.

iii) *Thymallus thymallus* from the River Nidd, England

Grayling broodstock originating from the River Nidd, Knaresborough, England were stripped and the eggs raised in the EAW's Calverton Fish Farm (53º02'01.43" N; 1º03'05.95" W). Egg hatching began on approximately the 13th April 2011. The fish were reared on borehole water (mean 10 ± 1ºC), and a constant natural photoperiod (05.00-21.30 without adjustment). First *ad libitum* feed was *Artemia salina* for approximately two weeks, then gradual weaning on Coppens TroCo Crumble Top and HE feed. Throughout rearing phase, dried diet was supplemented by gamma-radiated chironomids. The grayling had a mean length of 111.7 ± 0.8 mm and weight 12.7 g at the time of shipping to Norway in January 2012.

iv) *Salmo salar* from the River Lærdalselva, Norway, as a control

The Atlantic salmon stock used as a control was originating from the River Lærdalselva, Norway (approximately coordinates: 61º02' N; 7º36' W) and maintained in

the research aquarium of the Norwegian Veterinary Institute (NVI), Oslo (Norway). A single tank of 10 fish (mean weight 5.5 ± 0.5 g) was used during the trial.

6.2.2. Transportation of salmonids to Norway

In January 2012, 70 Atlantic salmon originating from the Welsh River Dee, 70 brown trout from the English River Tyne, and 70 grayling from the English River Nidd were shipped to the NVI in Oslo. Each population of fish was prepared by EAW staff at the hatchery, by double-bagging the fish in polythene bags and placing them on chill packs, to ensure a stable temperature during shipping. These were sealed in International Air Transport Association (IATA)-approved robust polystyrene boxes, each of which measured 65 cm (depth) × 58 cm (length) × 49 cm (width). The polystyrene boxes were then placed inside a double-walled cardboard outer to ensure protection during transportation. The relevant permissions from the Chief Veterinary Officer in the UK and in Norway, from the Norwegian authorities (The Directorate for Nature Management and the Food Safety Authorities) and from the NVI, were obtained before the fish were shipped. The experimental procedure was approved by the Ethics Committee within the University of Stirling, UK and, additionally, was monitored by senior government officials and fish biologists within Defra (Department of the Environment, Fisheries and Rural Affairs), London, EAW (Environment Agency Wales), Brampton and at Cefas (Centre for Environment, Fisheries & Aquaculture Science), Weymouth Laboratory, UK. Despite the UK's bacterial kidney disease (BKD) status, as the fish were being flown to a secure experimental facility no additional TRACES (TRAde Control and Expert System; http://europa.eu/legislation_summaries/food_safety/veterinary_checks_and_food_hygiene/f84009_en.htm) documentation was required. The fish were flown using the specialist live animals courier, Gulf Agency Company (GAC) Logistics, through Manchester International airport to Gardermoen Airport, Oslo, Norway. Following their clearance by

the veterinary surgeon at Oslo, the fish were transported immediately by van to the NVI, Oslo research facility. The fish, still within their plastic bags, were transferred to 0.6 m (diameter) × 0.7 m (depth) fibreglass tanks supplied with a constant 11 ± 1°C water flow rate of 0.2 L min^{-1} and additional aeration, and the temperature of the water in the bags was allowed to adjust to that of the tank, before the bags were opened and the fish released. No fish were lost during the transportation exercise, which lasted in total for 6 hours, by the time of packing, to the arrival at the NVI. The fish were left to acclimate for a further 7 days before the *G. salaris* infection trial was started. The source of the water used within the aquarium was from the Oslo city domestic supply, which was passed through a particle filter (Structural C-2160-F7 composite, 310 L) and an activated carbon filter (GAK 170) prior to use.

6.2.3. Source of Gyrodactylus salaris *used for the trial*

The *G. salaris* strain used in the experiment was obtained from wild Atlantic salmon juveniles, sampled by electrofishing in the River Fusta, Northern Norway. Based on sequencing of mitochondrial cytochrome oxidase I, this *G. salaris* population is characterised as haplotype A and it has been shown to be pathogenic to Atlantic salmon (see Hytterød *et al*., 2011).

6.2.4. Gyrodactylus salaris *infection procedure*

Thirty fish from each population were randomly selected and then infected by transferring them to a static 30 L tank with aeration into which approximately 3000 *G. salaris* had been added by gently scraping the excised fins of heavily infected aquarium-held fish. This approach has been used effectively in the past (T.A. Bakke, *pers. comm.*) to ensure an infection of 50-70 parasites $fish^{-1}$ over a 24 h exposure period. This technique assumes that the 50% of parasites will successfully transfer to the new host (*e.g.*

introducing 3000 parasites in a tank with 30 fish, the 50% will transfer, therefore 1500 parasites, which if divided by 30 fish, it will give an infection of ~50 parasites fish^{-1}). Following the exposure period, each fish was lightly anaesthetised in Finquel® Vet. 100% (50 mg Finquel L^{-1}), tattooed with a unique mark using alcian blue (40 mg ml^{-1}), and the total number of *G. salaris* on each fin and body zone was counted (Fig. 6.1). Alcian blue marking was preferred as a rapid, reliable, easy, and long-lasting method (Bridcut, 1993), over fin clipping, as fins are the preferred microhabitat of *G. salaris*.

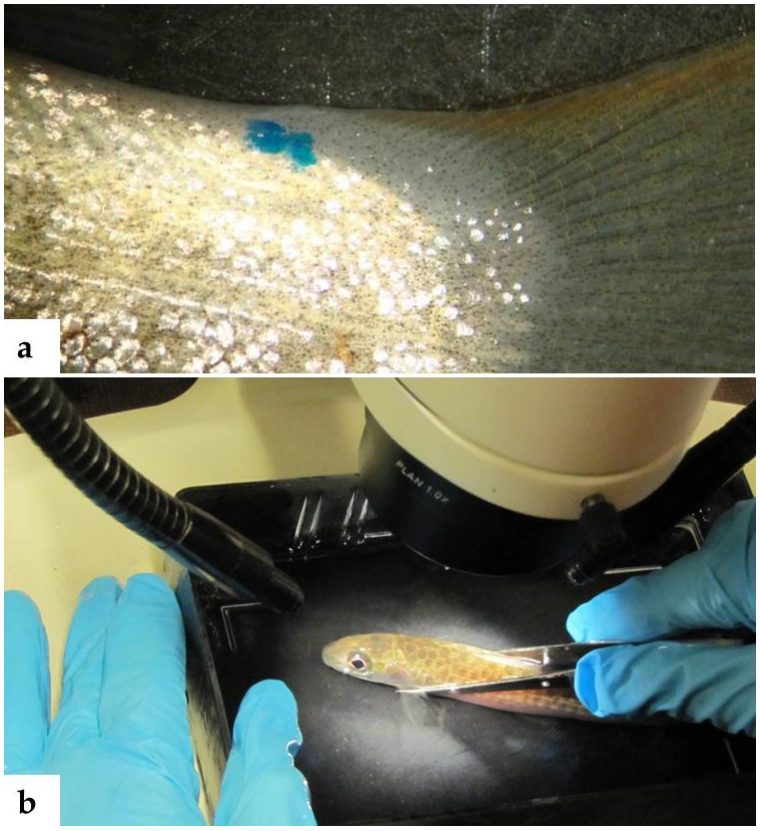


Figure 6.1. (**a**) An example of a unique tattoo mark using Alcian blue; (**b**) The *G. salaris* burden on an anaesthetised grayling being assessed under a dissecting microscope [original images].

Each fish was then randomly assigned to one of three recovery tanks (5 L circular, flow-through 200 ml min^{-1} tanks). Each population was tested in triplicate (each replicate n = 10 juvenile fish), with the exception of the River Lærdalselva Norwegian Atlantic salmon control, which was already a standardised model previously tested in several trials

by the same research aquarium, and for which only a single tank of 10 fish was infected. The brown trout population, however, showed to be highly aggressive when separated into the three small tanks of 10 fish each. For this reason, a single 0.6 × 0.7 m fibreglass tank containing all 30 fish was used for the brown trout trial.

Seven days later, each tank of fish was anaesthetised and the number of *G. salaris* on each individually marked fish determined by manual counting with the aid of a Leica MZ7.5 stereo-microscope. The fish were sampled approximately every 7 days until day 48 and then every 14 days thereafter. The fish were fed once every two weeks.

6.3. Results

The dynamics of *G. salaris* infection on each of the three salmonid populations originating from England and Wales were compared against an infection of *G. salaris* on Norwegian Atlantic salmon over trials lasting up to 110 days. The parasite numbers on each individually marked fish are presented in Figures 6.2-6.3, while the mean parasite burden and the range of parasite number for each population of fish at each sampling time point are shown in Table 6.1 and Figures 6.4-6.5. The initial *G. salaris* infection burdens 24 hours post-infection (p.i.) was 88.0 parasites $fish^{-1}$ (29–218) on the Welsh salmon from the River Dee; 80.9 parasites $fish^{-1}$ (47–110) on the Norwegian control; 65.3 parasites $fish^{-1}$ (32–221) on brown trout; and 60.7 parasites $fish^{-1}$ (28–149) on grayling (see Table 6.1).

The results obtained demonstrate that the Welsh salmon are highly susceptible to *G. salaris* infection (average intensity ~4000 parasites $fish^{-1}$ in 40 d; see Fig. 6.2.A), when compared against the Norwegian control tank of fish which had a mean intensity of ~2000 parasites $fish^{-1}$ over the same time period (Fig. 6.2.B). These fish were unable to initiate a successful defence against the parasite and the experiment was terminated on day 40 p.i. due to concerns for fish welfare.

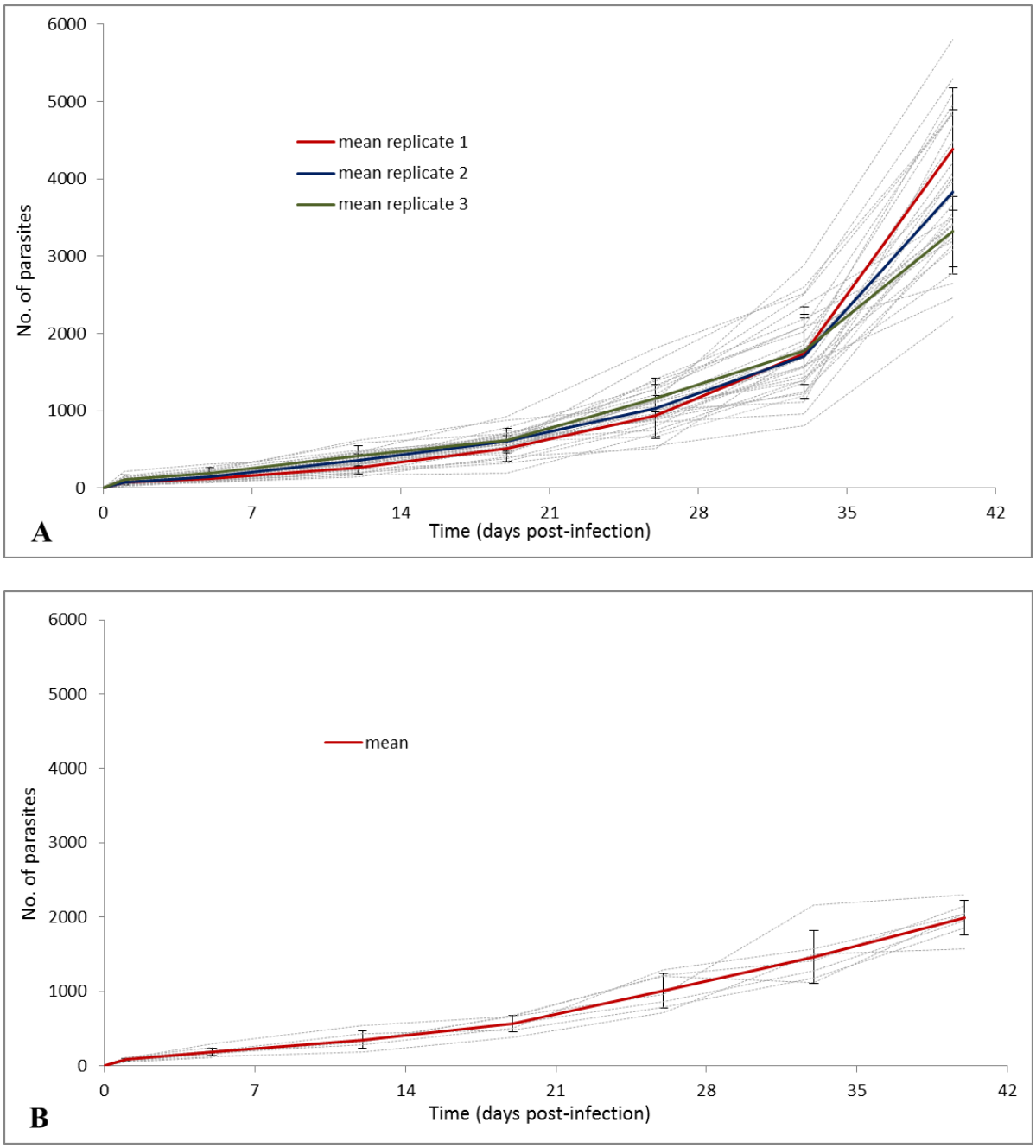


Figure 6.2. Experimental infection of *Gyrodactylus salaris* Malmberg, 1957 (Fusta strain, haplotype A) on (**A**) Atlantic salmon, *Salmo salar* L. (n = 30; three replicates of 10 fish each), from the River Dee in Wales, UK; and (**B**) the control group of Norwegian Atlantic salmon (n = 10) from the River Lærdalselva. Parasite numbers on the Welsh Atlantic salmon (**A**) rapidly increased to ~4000 *G. salaris* per fish by day 40 post-infection, while on the control (**B**) increased to ~2000 parasites per fish. By day 40 p.i. the experiment was terminated due to fish welfare concerns. The growth on the two hosts (Welsh and Norwegian salmon populations) is shown on the same scale for direct comparison.

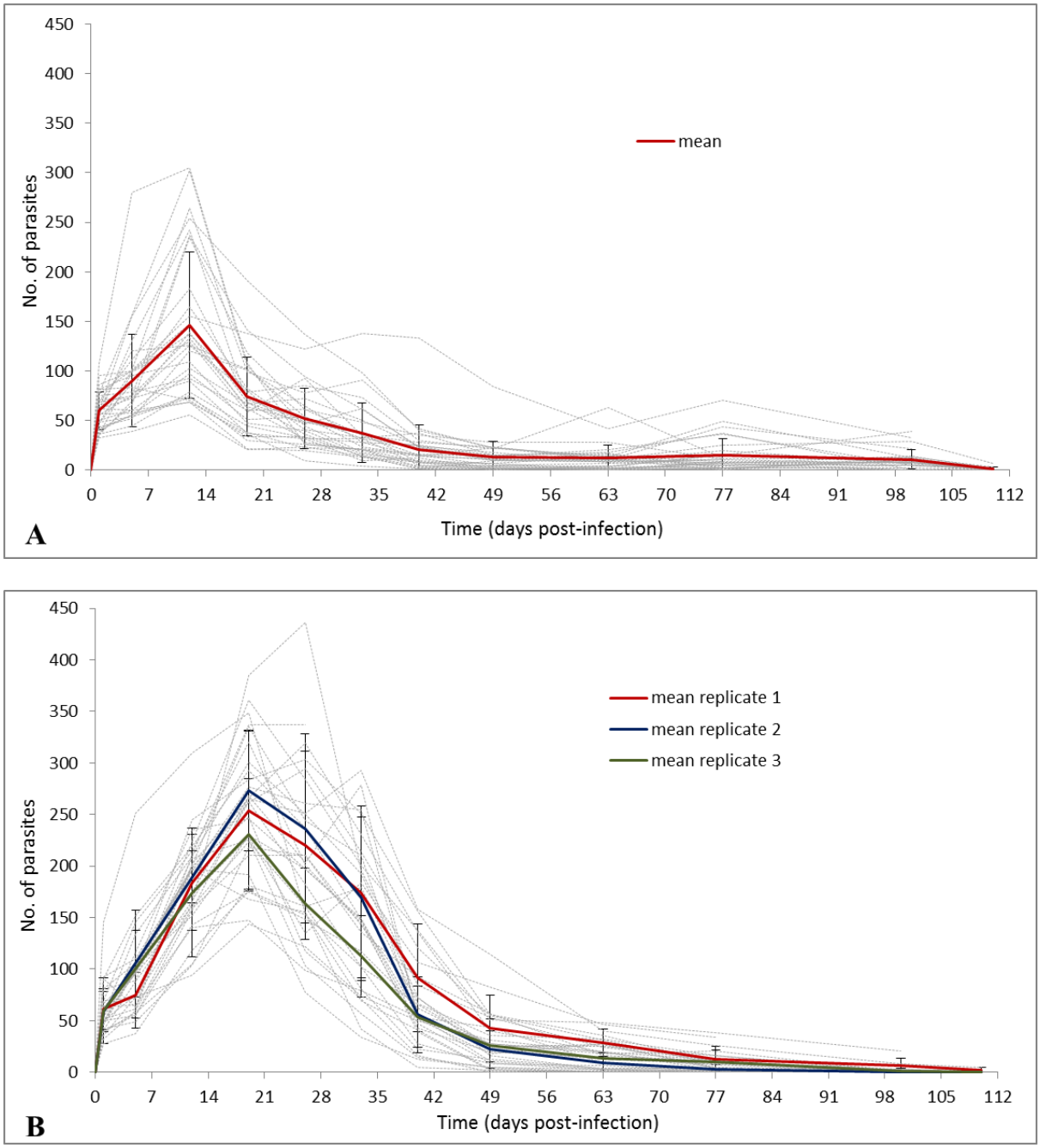


Figure 6.3. Experimental infection of *Gyrodactylus salaris* Malmberg, 1957 (Fusta strain, haplotype A) on a population of (**A**) brown trout, *Salmo trutta fario* L. (n = 30), from the River Tyne in England, UK; and (**B**) grayling, *Thymallus thymallus* (L.) (n = 30; three replicates of 10 fish each), from the River Nidd in England, UK. Brown trout and grayling were able to carry a *G. salaris* infection for at least 110 days (*i.e.* 7 of the 30 brown trout were still infected with 1-6 *G. salaris* each; and only two grayling were still infected, one with five *G. salaris*, the other fish with a single parasite) when the experiment was terminated. The growth on brown trout and grayling is shown on the same scale for direct comparison.

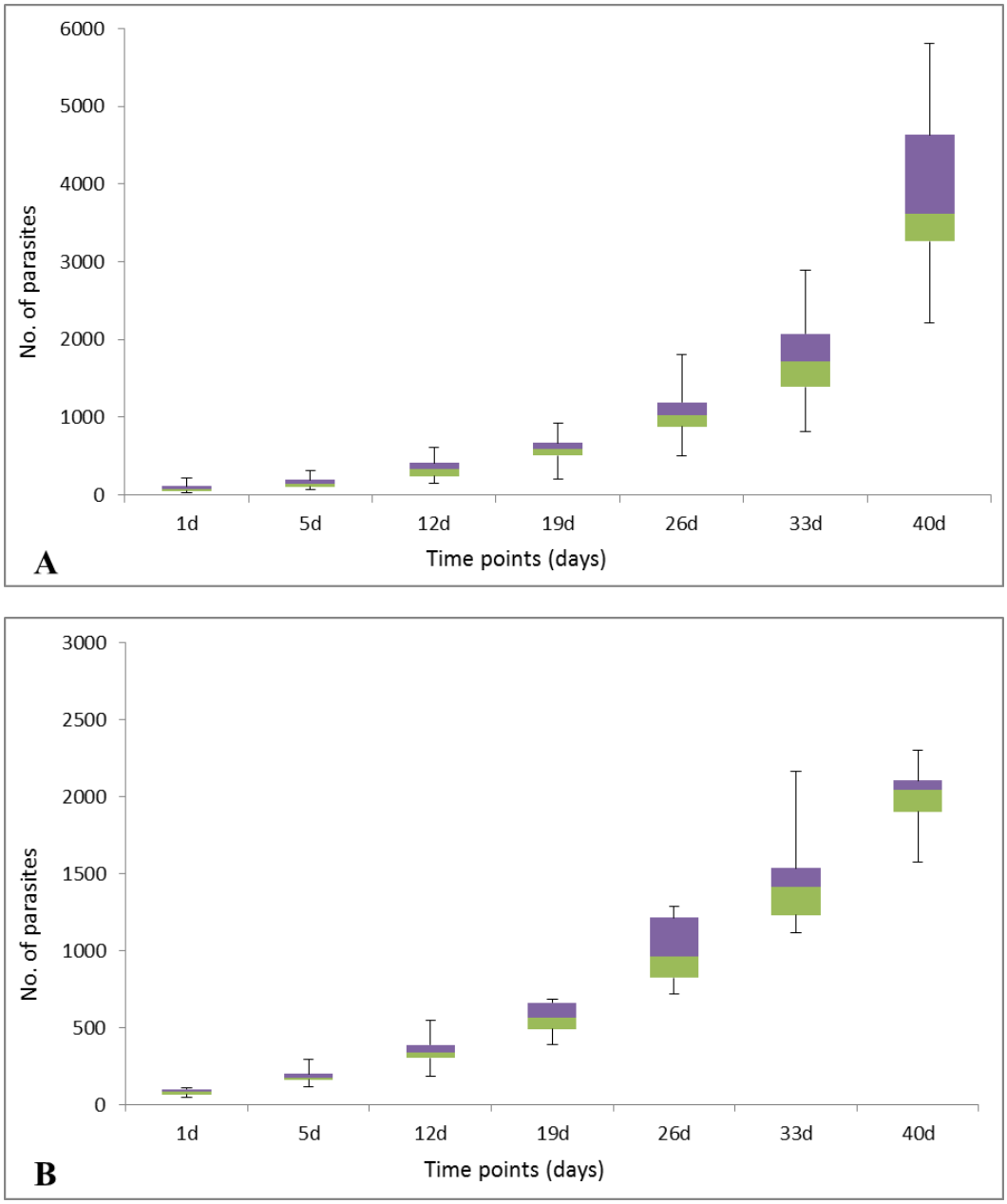


Figure 6.4. Box-and-whisker plots illustrating the population dynamics of *Gyrodactylus salaris* Malmberg, 1957 (Fusta strain, haplotype A) during the 40 day-experimental infection on (**A**) Atlantic salmon, *Salmo salar* L. (n = 30), from the River Dee in Wales, UK; and on (**B**) the control group of Norwegian Atlantic salmon (n = 10) from the River Lærdalselva. Upper and lower whiskers represent maximum and minimum values, respectively; boxes represent the 25th (purple) and the 75th percentiles (green), with the median value between them.

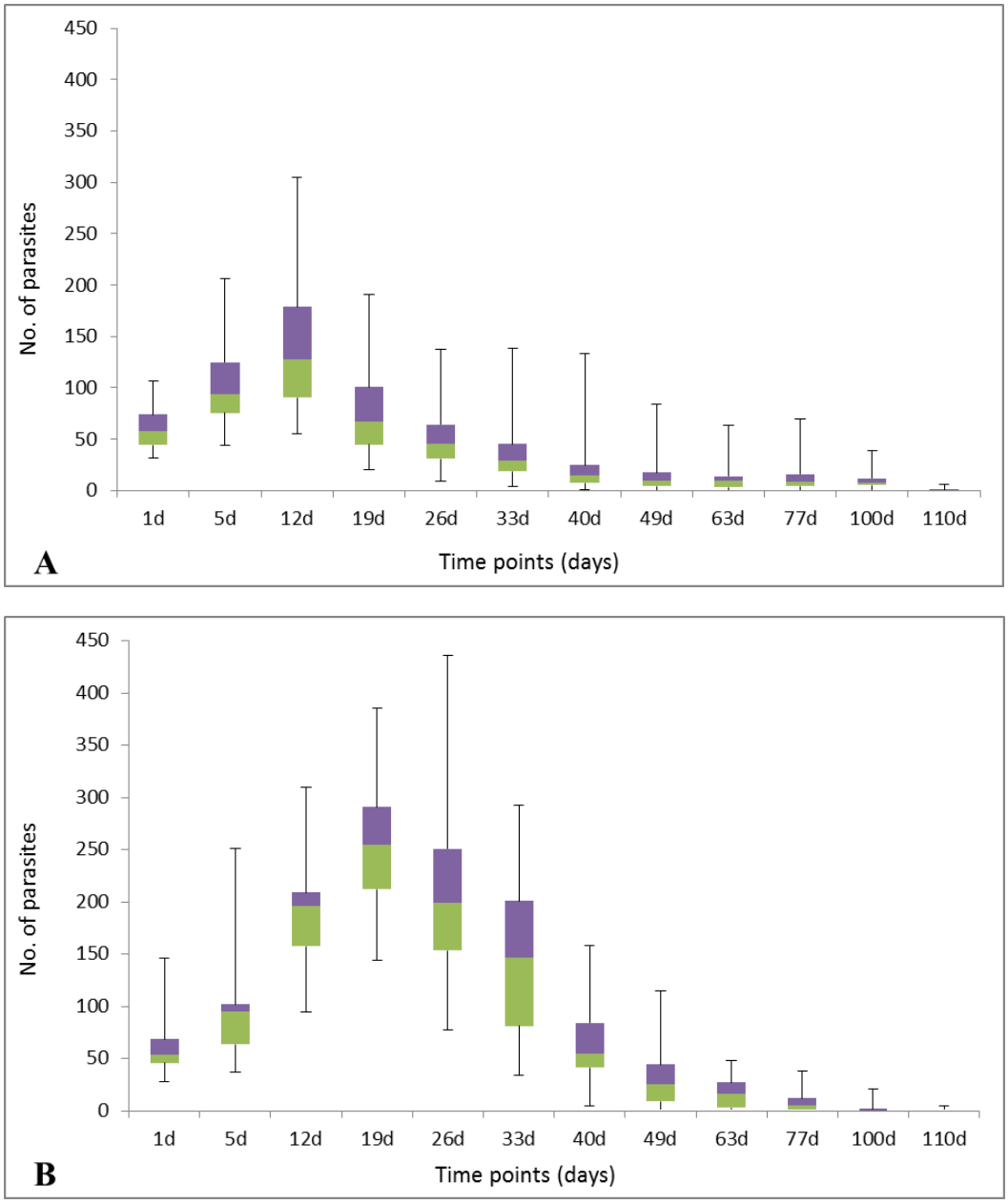


Figure 6.5. Box-and-whisker plots illustrating the population dynamics of *Gyrodactylus salaris* Malmberg, 1957 (Fusta strain, haplotype A) during the 110 day-experimental infection on (**A**) brown trout, *Salmo trutta fario* L. (n = 30), from the River Tyne in England, UK; and on (**B**) grayling, *Thymallus thymallus* (L.) (n = 30), from the River Nidd in England, UK. The growth on brown trout and grayling is shown on the same scale for direct comparison. Upper and lower whiskers represent maximum and minimum values, respectively; boxes represent the 25th (purple) and the 75th percentiles (green), with the median value between them.

The infections of *G. salaris* on the brown trout from the River Tyne peaked after ~12 days (mean intensity 145.9 parasites fish^{-1}; Fig. 6.3.A), whilst those on the River Nidd grayling peaked after ~19 days (mean intensity 252.6 parasites fish^{-1}; Fig. 6.3.B). Thereafter, the size of parasitic infection decreased on both hosts. The *G. salaris* infection had almost disappeared on both sets of fish by the time the experiment was terminated on day 110 post-infection. The population of *G. salaris* on three of the 30 grayling that were tested appeared to display two peaks of infection on days 19 (av. 238.0 ± 49.4 parasites fish^{-1}) and 33 (av. 250.3 ± 62.2 parasites fish^{-1}) p.i., with a subsequent steady decrease in parasite numbers from day 26 p.i. until the experiment was terminated on day 110 p.i. Brown trout showed a similar response, with three brown trout displaying two peaks of infection on days 12 (av. 119.3 ± 14.2 parasites fish^{-1}) and 26 (av. 83.0 ± 10.1 parasites fish^{-1}) p.i., with a subsequent steady decrease in numbers from day 19 p.i. onwards.

The experiment was terminated on day 110 p.i. for three reasons. First, that by day 110 p.i., the infection on most fish had disappeared; only seven of the brown trout were still infected (range 1–6 parasites fish^{-1}; see Fig. 6.3.A), and only two of the 30 grayling were infected (*i.e.* one with one parasite, the other with five *G. salaris*; see Fig. 6.3.B). Second, the experiment was terminated out of welfare concerns for the fish, in that sufficient data had been collected to inform the likely response of these populations of fish to a *G. salaris* (haplotype A) infection and that prolonging the infection was unlikely to result in additional information. Finally, the decision to terminate the experiment was based on the operational costs of the experiment.

A power outage on day 69 p.i., which resulted in a temporary cessation in water flow to the grayling and brown trout tanks, resulted in the loss of five brown trout and five grayling; the stress induced on the remaining populations is the most likely explanation for the observed small increase in parasite numbers on day 76 p.i. Figures 6.6-6.7 show the average distribution of *G. salaris* across the body and fins of each fish species throughout

the experimental infection. The graphs show the importance of the fins as the preferred site of infection.

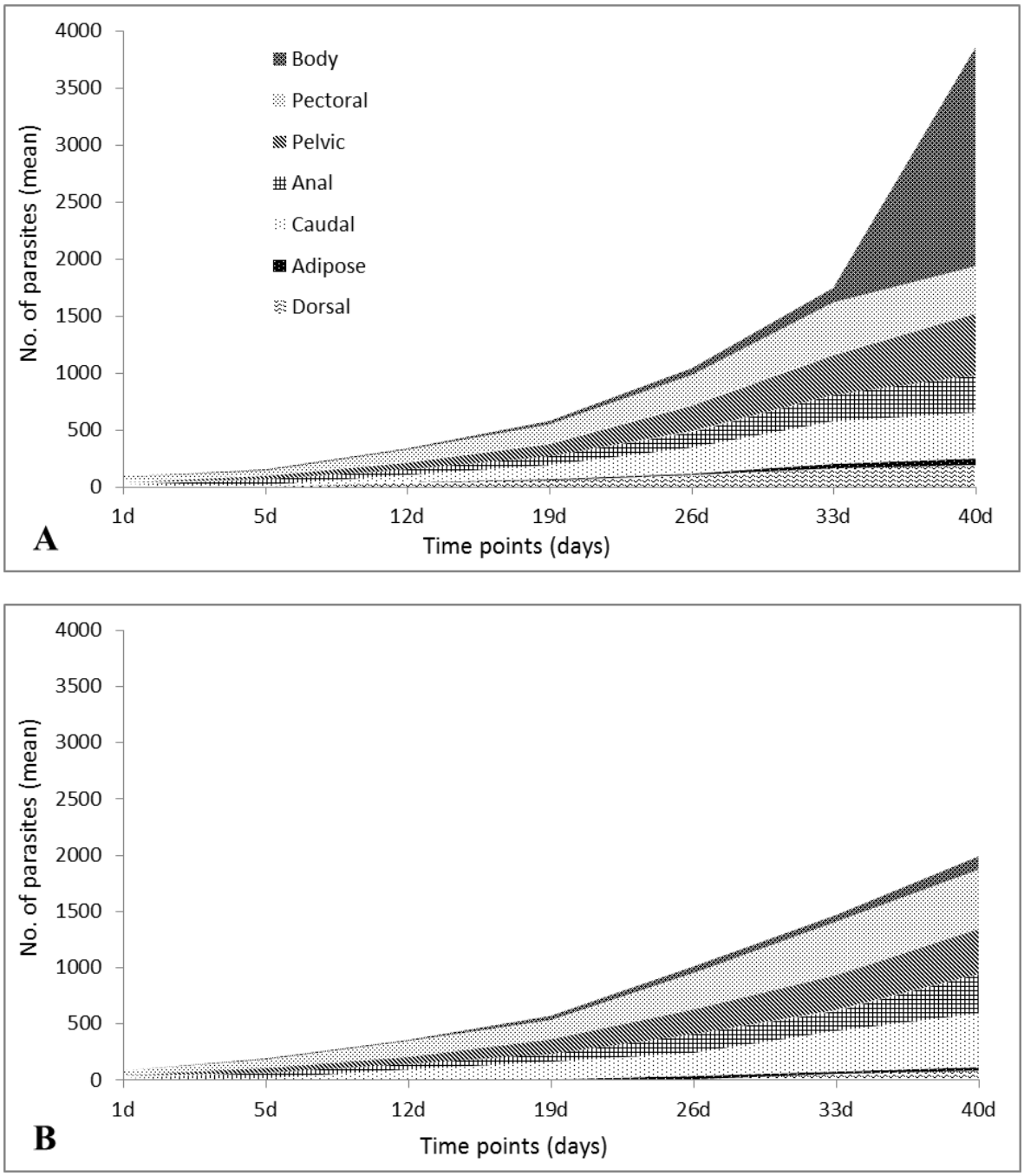
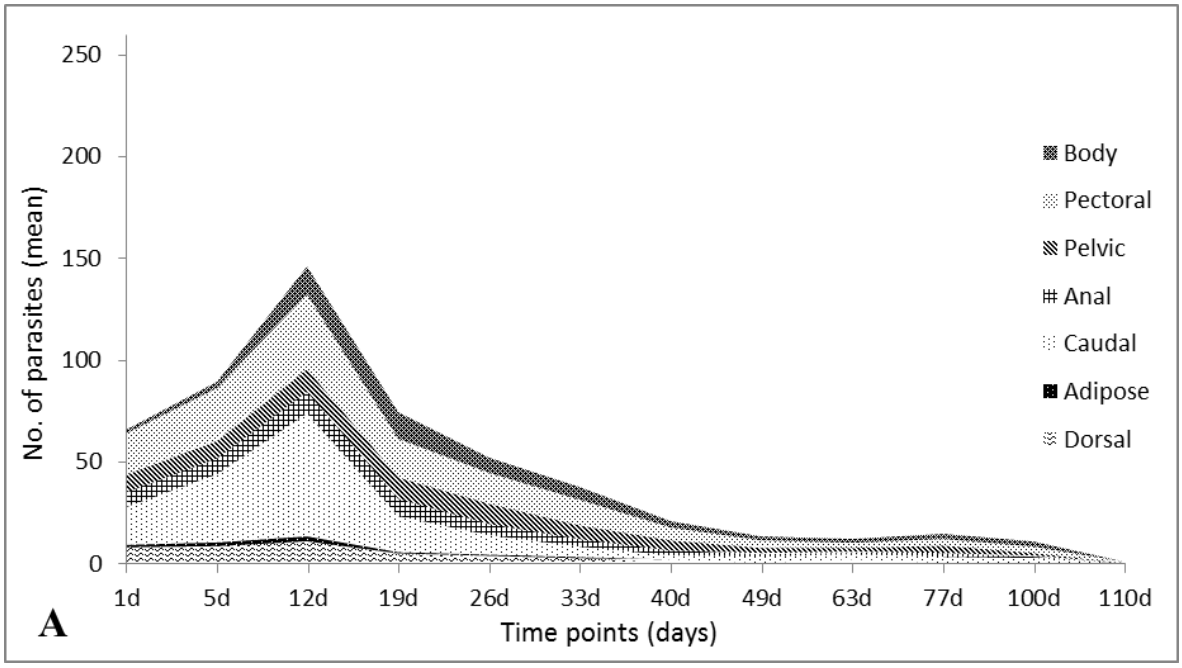


Figure 6.6. The distribution of *Gyrodactylus salaris* Malmberg, 1957 (Fusta strain, haplotype A) on the fins and body of Atlantic salmon, *Salmo salar* L., from (**A**) the Welsh River Dee (n=30) and (**B**) the control from the River Lærdalselva, Norway (n=10), throughout the 40 day-infection and population-growth trial. The growth on the two Atlantic salmon populations is shown on the same scale for direct comparison.



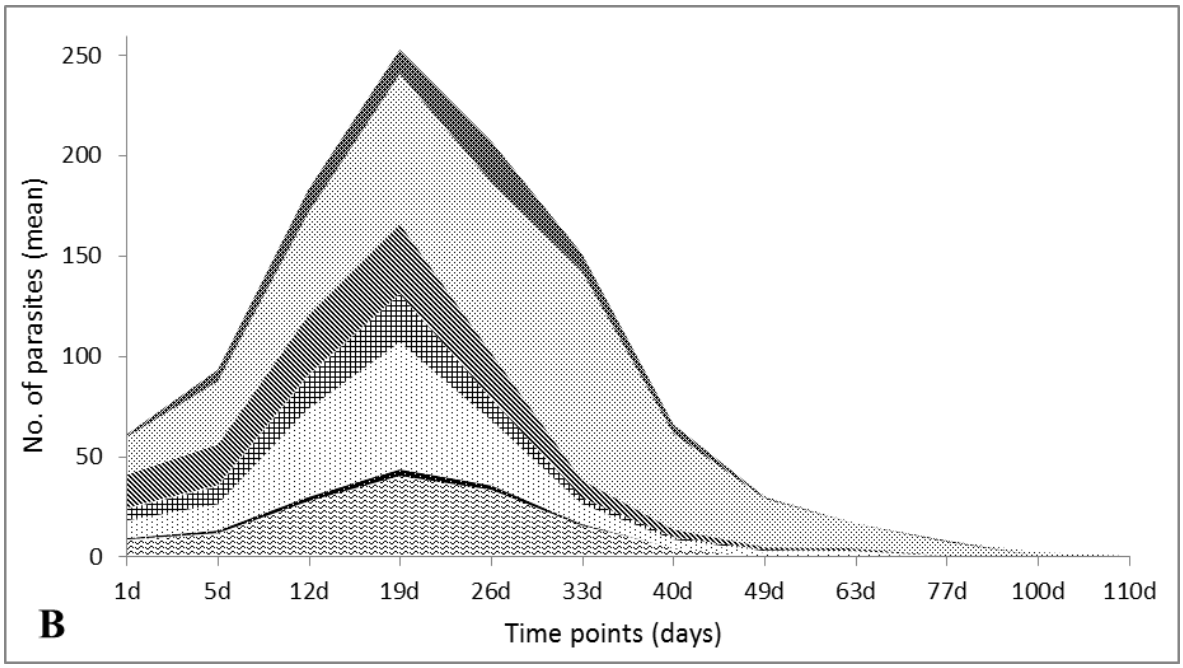


Figure 6.7. The distribution of *Gyrodactylus salaris* Malmberg, 1957 (Fusta strain, haplotype A) on the fins and body of a population of (**A**) brown trout, *Salmo trutta fario* L., from the English River Tyne (n=30) and (**B**) grayling, *Thymallus thymallus* (L.), from the English River Nidd (n=30), throughout the duration of the 110 day-experiment. The growth on brown trout and grayling is shown on the same scale for direct comparison.

Numbers on the Welsh salmon from the River Dee suggest that when the infection reaches ~2000 *G. salaris* per fish, there is no remaining space for further increases on the fins, and the numbers on the body subsequently rapidly increase (Fig. 6.6.A). The distribution of *G. salaris* on grayling and brown trout, for example, indicates that parasites have a preference towards occupying the pectoral fins and caudal fin, respectively (Fig. 6.7.A-B).

Table 6.1. Intensity of *Gyrodactylus salaris* Malmberg, 1957 (River Fusta haplotype A strain) infection on *Salmo salar* L. from the River Dee, Wales and from the Laerdalselva, Norway (control group), from *Salmo trutta fario* L. from the River Tyne, England and from *Thymallus thymallus* (L.) from the River Nidd, England. The mean intensity ±1 standard deviation and the range in parentheses are presented for each time point post-infection (p.i.) and host. Parasite numbers on the two groups of *S. salar* increased rapidly, the numbers by day 40 post-infection were such that the experiment was terminated out of welfare concerns for the fish. Although a small number of *S. trutta fario* and *T. thymallus* were still infected with a low number of *G. salaris* on day 110 p.i., the experiment was nevertheless terminated at this point.

Time points (days)	***Salmo salar* (R. Dee, Wales)**	***Salmo salar* (control) Laerdalselva, Norway**	***Salmo trutta fario* (R. Tyne, England)**	***Thymallus thymallus* (R. Nidd, England)**
1	88.0 ± 44.9 (28–215)	80.9 ± 20.9 (47–110)	59.7 ± 19.4 (32–107)	60.7 ± 24.0 (28–149)
5	157.4 ± 61.4 (76–314)	183.4 ± 53.6 (114–291)	90.1 ± 46.7 (39–280)	93.4 ± 40.9 (37–251)
12	343.6 ± 116.5 (151–615)	349.1 ± 113.1 (184–544)	145.9 ± 73.6 (55–305)	182.5 ± 46.2 (94–310)
19	581.6 ± 156.9 (200–923)	560.4 ± 111.6 (385–679)	74.0 ± 39.4 (20–191)	252.6 ± 64.3 (144–385)
26	1043.5 ± 296.2 (511–1812)	1003.1 ± 231.3 (714–1284)	52.0 ± 30.5 (9–137)	206.7 ± 81.1 (77–436)
33	1741.5 ± 510.2 (810–2890)	1459.7 ± 352.6 (1114–2165)	37.3 ± 30.0 (4–138)	151.1 ± 73.0 (34–293)
40	3850.7 ± 898.2 (2210–5805)	1988.9 ± 233.5 (1570–2300)	20.7 ± 24.3 (1–133)	66.1 ± 42.6 (5–158)
49	-	-	13.1 ± 15.6 (0–84)	29.7 ± 26.7 (0–115)
63	-	-	11.8 ± 13.2 (0–63)	16.9 ± 14.7 (0–48)
77	-	-	14.5 ± 16.7 (0–70)	8.5 ± 10.2 (0–38)
100	-	-	10.6 ± 9.9 (0–39)	2.1 ± 4.5 (0–21)
110	-	-	0.9 ± 1.6 (0–6)	0.3 ± 1.2 (0–5)

6.4. Discussion

Great Britain and Northern Ireland are currently considered to be *G. salaris* free (see Chapter 3). Although it has been almost two decades since the first major surveys of

Platten *et al.* (1994) and Shinn *et al.* (1995), on-going surveillance programmes of natural water courses, recreational fisheries and fish farms by the relevant fish health inspectorate in each country of the UK screen specifically for *G. salaris*, among other notifiable pathogens, and to date, no specimens of *G. salaris* have been found. Current national contingency plans in the UK assume that the dynamics of *G. salaris* infection on native English and Welsh salmonids will follow those already modelled in Scandinavia. These Scandinavian studies suggest that Atlantic strains of salmon are susceptible to infection, whilst grayling are innately resistant, though *G. salaris* can survive and reproduce on Scandinavian grayling for 143 days, and that brown trout are entirely resistant to infection.

These definitions, relating to the relative susceptibility of fish to *G. salaris* infection, follow those detailed by Bakke *et al.* (2002), and consider that fish can either be: 1) *Susceptible* - when *G. salaris* is capable of colonising and reproducing on a host, and the parasite population continues to grow to levels at which the host might die; 2) *Responding* - when the *G. salaris* infection grows initially and appears to be non-pathogenic; the host's immune system is able to respond, decreasing and eliminating the parasitic infection within few weeks; and, 3) *Innately resistant* - when the parasite population fails to grow, and the infection persists for only a short period without increasing and then disappears.

While evaluating the relative susceptibility and response of each population of fish to *G. salaris*, it is also important to detail which strain of *G. salaris* is used.

6.4.1. Earlier studies investigating the susceptibility of British salmonids to G. salaris

Only a few studies of the susceptibility of British salmonids to *G. salaris* exist in the scientific literature. These studies are limited to the exposure of two Scottish populations of Atlantic salmon from the Rivers Conon and Shin (see Bakke & MacKenzie, 1993; Dalgaard *et al*., 2003, 2004). The trials conducted by Bakke & MacKenzie (1993)

investigated *G. salaris* infections of these fish, both held communally in tanks and also held individually, over a period of 50 days. These infections employed *G. salaris* collected from the River Figga and, therefore, most likely haplotype A (see Table 6.2). During the experimental period, none of the fish were able to completely eliminate their *G. salaris* infection. Peak infections (*i.e.* ~1500 parasites fish^{-1}) were seen on days 22 and 36 p.i., and towards the end of the experiment, some of the fish appeared to mount a response and markedly reduce the size of their parasitic burdens. Other fish within each group, however, were unable to respond and died as a consequence of rising parasite numbers (Bakke & MacKenzie, 1993). Dalgaard *et al*. (2003) similarly evaluated the susceptibility of River Conon Atlantic salmon from Scotland to a strain of *G. salaris* originating from the River Lærdaselva, Norway (most likely haplotype F, see Hansen *et al*., 2003). Aged 0+ fish were either infected with *G. salaris*, following the standard procedures detailed in the works of Bakke and colleagues, or treated with corticosteroids to induce a level of stress in the fish before they were exposed to *G. salaris*. As expected, the treated salmon were more susceptible to infection with a mean intensity of ~280 parasites fish^{-1} by the end of the 8 week experiment. The untreated salmon, by comparison, had an average of 98 parasites fish^{-1}; there was 40% fish mortality in both populations of fish (Dalgaard *et al*., 2003). A subsequent trial by Dalgaard *et al*. (2004), again using Atlantic salmon from the River Conon, investigated their relative susceptibility to *G. salaris* haplotype F (see Table 6.2) alongside Atlantic salmon from Canada, Denmark and Sweden and also rainbow trout from Denmark. The Atlantic salmon from River Conon displayed the same high susceptibility described by Dalgaard *et al*. (2003).

Table 6.2. A summary of the *Gyrodactylus salaris* Malmberg, 1957 haplotypes used in previous experiments conducted in Norway ascertaining the susceptibility of different strains of *Salmo salar* L. (A = Atlantic strain; B = Baltic strain). The haplotypes marked with an asterisk are tentatively proposed based on their geographic origin and their relative proximity to defined strains (see Hansen *et al*., 2003). The host response to *G. salaris* infection in each case is presented using the three categories defined by Bakke *et al.* (2002); *i.e.* susceptible, responding or innately resistant.

Reference	Origin of *Salmo salar* tested in previous studies (rivers)	Origin of the *G. salaris* strain	*G. salaris* haplotype	Parasite population dynamics	Host response
Bakke (1991)	A: Alta, Lone, Drammenselva and Lierelva (Norway)	R. Drammenselva	F	exponential growth	susceptible
	B: Neva (Russia)	R. Drammenselva	F	declining after 3 weeks	responding
Bakke & MacKenzie (1993)	A: Conon and Shin (Scotland) and Lierelva (Norway)	R. Figga	A*	exponential growth	susceptible
Bakke *et al*. (1990)	A: Alta and Lone (Norway)	R. Drammenselva	F	exponential growth	susceptible
	B: Neva (Russia)	R. Drammenselva	F	declining after 3 weeks	innately resistant and responding
Bakke *et al*. (1998)	A: Akerselva (Norway)	unknown	unknown	exponential growth	susceptible
Bakke *et al*. (1999)	A: Alta (Norway)	R. Lierelva	F	exponential growth	susceptible
	♀A×♂brown trout hybrids: Alta (Norway) × Fossbekk (Norway)	R. Lierelva	F	declining after 3 weeks	innately resistant and susceptible
	♂A×♀brown trout hybrids: Alta (Norway) × Fossbekk (Norway)	R. Lierelva	F	elimination in 2 weeks	innately resistant
Bakke *et al*. (2002)	A: Lierelva (Norway)	R. Rauma	A	exponential growth	susceptible
	A: Lierelva and Batnjjordselva (Norway)	R. Batnfjordselva and Steinkjerselva	A and A*	exponential growth	susceptible
	A: Namsen and Alta (Norway)	R. Lierelva	F	exponential growth	susceptible
	A×B hybrids: Imsa (Norway) × Neva (Russia)	R. Lierelva	F	declining after 4 weeks	responding
	B: Neva (Russia)	R. Lierelva	F	declining after 3 weeks	responding
Bakke *et al*. (2004)	A: Lierelva (Norway)	R. Figga	A*	exponential growth	susceptible
	B: Indalsälv (Sweden)	R. Figga	A*	slightly declining after 4 weeks	responding and susceptible
Cable *et al*. (2000)	A: Alta and Lierelva (Norway)	R. Lierelva	F	exponential growth	susceptible
	B: Neva (Russia)	R. Lierelva	F	declining after 3 weeks	innately resistant and responding
Dalgaard *et al*. (2003)	A: Conon (Scotland)	R. Lærdalselva	F	exponential growth	susceptible
	B: Lule (Sweden)	R. Lærdalselva	F	declining after 6 weeks	responding
Dalgaard *et al*. (2004)	A: Conon (Scotland), Skjern (Denmark) and Bristol Cove (Canada)	R. Lærdalselva	F	exponential growth	susceptible
	B: Mörrum (Sweden)	R. Lærdalselva	F	exponential growth	susceptible
Jansen *et al*. (1991)	A: Imsa (Norway)	R. Lierelva	F	exponential growth	susceptible
	♀A×♂B hybrids: Imsa (Norway) × Neva (Russia)	R. Lierelva	F	exponential growth	susceptible
current study	A: Dee (Wales), Lærdalselva (Norway)	R. Fusta	A	exponential growth	susceptible

6.4.2. Susceptibility of other salmonids to G. salaris

Gyrodactylus salaris has been demonstrated to colonise and reproduce on a large number of salmonids, other than *S. salar*, and, under experimental studies, on a number of non-salmonid species as well (Mo, 1987; Bakke *et al*., 1990; Bakke & Sharp, 1990; Bakke *et al*., 1991b; Soleng & Bakke, 1998). Within the family Salmonidae, there are three subfamilies: the Coregoninae, the Salmoninae and the Thymallinae, which collectively embrace ten genera. Of these, only five genera have been evaluated for their susceptibility to *G. salaris*, and these are detailed in Table 6.3.

The lack of clinical signs of disease on some of these hosts may mean that *G. salaris* infections may go undetected. This is well demonstrated by the study of Paladini *et al.* (2009a; also see Chapter 2), who on finding *G. salaris* in Italy for the first time then looked at formalin-preserved material in farm archives and found that the parasite had most likely been in the region for at least 9 years prior to discovery. Such asymptomatic hosts may represent a serious problem in that they can serve as significant carriers of the parasite and may also play an important role in the epidemiology and dispersal of *G. salaris* across Europe (Bakke *et al*., 2002; Peeler & Thrush, 2004; Peeler & Oidtmann, 2008). Establishing the factors associated with the transmission and differential susceptibility of fish hosts to *G. salaris*, therefore, is central to the rational formulation of national contingency plans, regulating salmonid movements within Europe and developing programmes of management and control. The research agenda for the past two decades for European states with strong salmonid industries, therefore, has been to focus on each of these.

Table 6.3. The host subfamilies, genera and species of Salmonidae that have been experimentally tested with respect to their susceptibility to *Gyrodactylus salaris* Malmberg, 1957. The table also lists the genera that have yet to be assessed.

Subfamily	Genus	Host species tested for susceptibility	Representative references
Coregoninae	*Coregonus* L.	*Coregonus lavaretus* (L)	Soleng & Bakke (2001b)
	Prosopium Jordan	-	
	Stenodus (Güldenstädt)	-	
Salmoninae	*Brachymystax* Günther	-	
	Hucho (Günther)	-	
	Oncorhynchus Suckley	*Oncorhynchus mykiss* (Walbaum)	Bakke *et al*. (1991a); Lindenstrøm *et al*. (2000); Dalgaard *et al*. (2004)
	Salmo (L.)	*Salmo salar* L.	Bakke *et al*. (1990); current study
		S. trutta fario L.	Jansen & Bakke (1995); current study
	Salvelinus Richardson	*Salvelinus alpinus alpinus* (L)	Bakke & Jansen (1991a); Bakke *et al*. (1996); Robertsen *et al*. (2007); Winger *et al*. (2008)
		S. fontinalis (Mitchill)	Bakke *et al*. (1992b)
		S. namaycush (Walbaum)	Bakke *et al*. (1992c)
	Salvethymus Chereshnev *et* Skopets	-	
Thymallinae	*Thymallus* Linck	*Thymallus thymallus* (L.)	Bakke & Jansen (1991b); Soleng & Bakke (2001a); current study

6.4.3. The infection of G. salaris *on English and Welsh salmonids*

Understanding how each salmonid population could respond to *G. salaris*, in the event of its introduction, can help support national contingency plans by confirming whether current assumptions are correct and that the remedial action that would be taken is appropriate. While standard operating procedures (SOPs) for the processing and

identification of *G. salaris* in a given sample of *Gyrodactylus* specimens were recently addressed by Shinn *et al*. (2010), based on the information from the current study, it is suggested that some amendment to current contingency planning is required.

The infection of Welsh Atlantic salmon from the River Dee followed the expected infection trajectory with fish being highly susceptible to *G. salaris* infection. The trial found infections rapidly rose to ~4000 parasites per fish in just 40 days. This finding is in close agreement with the response of Atlantic salmon (Atlantic strain) populations from elsewhere, including those tested from Scotland (Bakke & MacKenzie, 1993). The rate of parasite population increase (*i.e.* 17% d^{-1}) on the River Dee salmon, however, was markedly faster than that on the Norwegian control group of salmon (*i.e.* 5% d^{-1}). The number of *G. salaris* observed on the eyes of the Welsh salmon, although not given as a specific category in Figure 6.6, was seen to increase throughout the duration of the trial. The eye may represent an immunologically-privileged site (Barber & Crompton, 1997), given that the immune response to parasitic infection is believed to be lower in the eye (Cox, 1994). The observed increase in the number of parasites may reflect parasites moving away to avoid the host's immune response, as also suggested by other researchers (*e.g.* Price, 1987; Sudhdeho & Mettrick, 1987). In Figures 6.2.A and 6.6.A, the number of parasites on the body was shown to change dramatically after day 33 p.i. While the fins are the favoured site of *G. salaris* infection, one explanation for this marked increase on the body is that as space on the fins becomes limited, the parasites then move on to the body where there is more space and less competition for resources. Buchmann & Bresciani (1998), however, suggested that this distribution on the host may be a consequence of differing mucous cell densities in different parts of the fish (Pickering, 1974) and that the parasites avoid the mucous-cell-rich areas during the response phase and escape localised immune reactions (Richards & Chubb, 1996; Buchmann & Bresciani, 1997; Buchmann & Uldal, 1997; Buchmann, 1998a, b). A third explanation is that, as the fins become

damaged, the parasites move away from these areas as secure attachment could be compromised, and also since sites of damage are likely to be subject to localised host immune responses.

The River Tyne brown trout and the River Nidd grayling were both responsive to *G. salaris* infection, with parasite numbers increasing and then subsequently declining to near extinction over the 110 days the trial was run. There were no mortalities as a direct result of parasitic infection and there was no discernible change in fish behaviour. Brown trout were also observed to harbour a low infection of the ciliate protozoan *Trichodina* sp. that subsequently disappeared after the first two weeks. The Welsh salmon were found to be infected with the flagellate protozoan *Ichthyobodo necator* (Henneguy) Pinto, 1928, which was present throughout the duration of the experimental trial (see Fig. 6.8). The co-occurrence of protozoans, such as *I. necator* and *Trichodina* sp., alongside *Gyrodactylus* infections, however, is a common finding on wild fish (*personal observation*) and has been specifically commented upon by Rintamäki (1989), working on *G. salaris* on a salmon farm in the Baltic region of Finland.

6.4.4. The experimental infection procedure

The period of experimental exposure used in the current study was 24 h and follows the methodology used in other *G. salaris* infection trials (see for example Bakke *et al*., 1999; Cable *et al*., 2000; and Soleng & Bakke, 2001a). There is, however, no standard exposure period, and the times reported in the scientific literature appear to vary markedly, *e.g.* 48 h as used by Jansen *et al*. (1991), Bakke *et al*. (2004) and Dalgaard *et al*. (2004); 72 h as employed by Bakke & MacKenzie (1993); and up to 2 weeks in the study by Bakke *et al*. (1990). The experimental exposure period used in the current trial, however, was shown to be effective, as demonstrated by the prevalences obtained, with 100% of fish successfully infected with *G. salaris*.

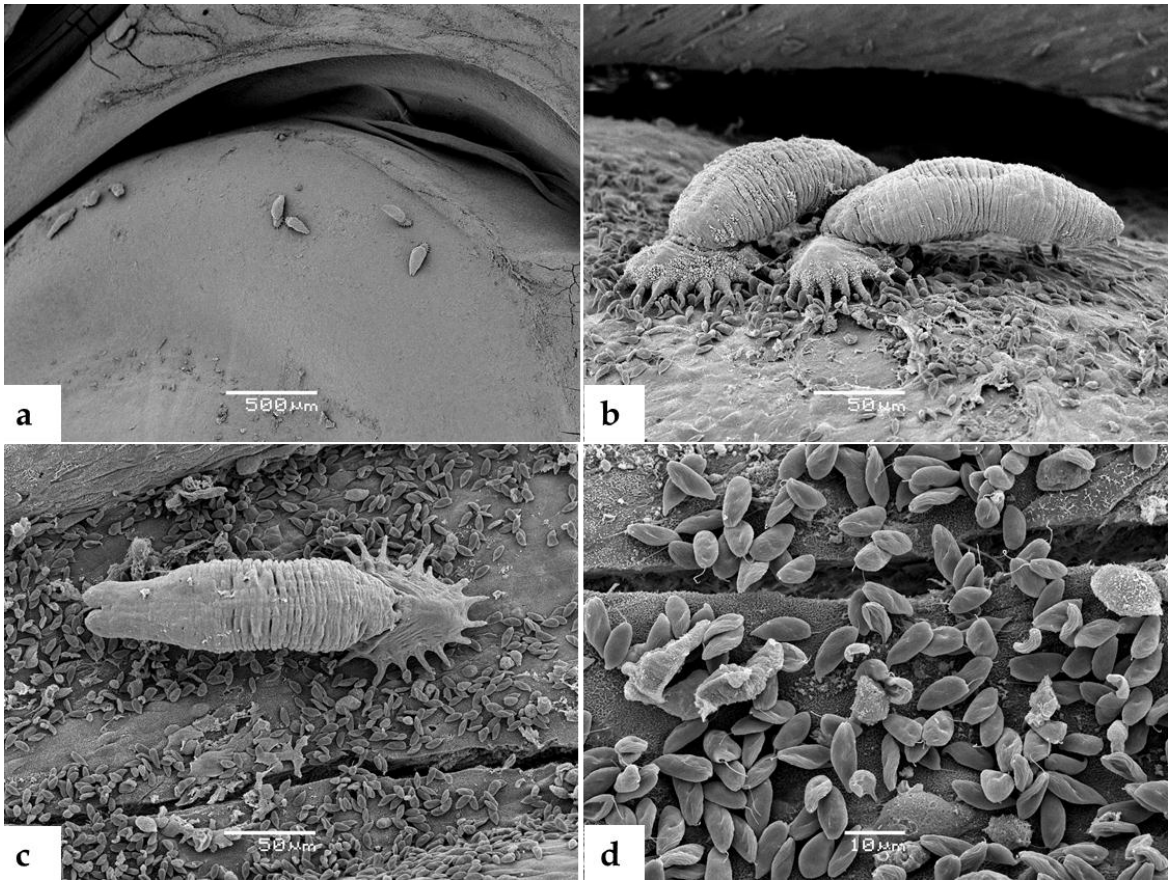


Figure 6.8. Scanning electron micrographs (SEM) showing a co-infection of *Gyrodactylus salaris* Malmberg, 1957 and *Ichthyobodo necator* (Henneguy) Pinto, 1928 on the eye of an Atlantic salmon, *Salmo salar* L. from the River Dee, Wales. (**a**) low resolution image of *G. salaris* on the top of the eye; (**b**), (**c**) *G. salaris* attached to the cornea of the eye in regions where the density of *I. necator* was marked; (**d**) close up of *I. necator* clearly showing the terminal flagella [original images].

6.4.5. The importance of including grayling in the current trial

English and Welsh grayling are commonly infected with *Gyrodactylus thymalli* Žitňan, 1960, a congener morphologically and genetically similar to, and commonly confused with, *G. salaris* (see McHugh *et al*., 2000; Shinn *et al*., 2004). It has been suggested that *G. thymalli* may be conspecific with *G. salaris* (see Hansen *et al*., 2006; OIE, 2012), however Scandinavian grayling are unable to support experimental infections of *G. salaris* suggesting that *G. thymalli* is not conspecific with *G. salaris* (see Soleng & Bakke, 2001b). Given the debate regarding their conspecificity, that *G. thymalli* exists

within the UK and that the UK has been separated from mainland Europe for ~200,000 years (Gupta *et al*., 2007), the inclusion and experimental exposure of British grayling to *G. salaris* was important. Earlier trials with Scandinavian populations of grayling using the Lierelva strain of *G. salaris* suggested that infections could persist for anything between 35 (Soleng & Bakke, 2001b) and 143 days (Sterud *et al*., 2002). In both studies, the experiments were terminated with a low number of parasites still on their hosts. There was value, therefore, in determining the response of English grayling to infection. Likewise, the infections of *G. salaris* on grayling in the current study were not completely outside the expected response, with a low level of parasites remaining on fish for the duration of the 110-day experiment. Only two out of the 30 grayling, however, were still infected at the end of the trial. The finding that English grayling can carry infections for long periods of time gives cause for concern in that they may play a role in extending the infection window for other more susceptible hosts.

6.4.6. The importance of including brown trout in the current trial

Perhaps the most interesting findings from the current trial arise from the infection of *G. salaris* on the population of brown trout from the River Tyne. Prior to this study, brown trout had been considered resistant to *G. salaris* infection. Jansen and Bakke (1995), for example, infecting both single and pooled samples of brown trout with the strain of *G. salaris* from the River Lierelva (haplotype F), found that fish could carry an infection for up to 50 days. The current study found that when a pool of brown trout were each given an initial infection of ~70 *G. salaris* per fish, then the *G. salaris* infections on these fish persisted for at least 110 days, when the experiment was terminated. Of these, 7 of the 30 fish were still infected with between 1 and 6 parasites each.

Brown trout parr naturally infected with *G. salaris* at low intensities have been reported by a number of authors (Tanum, 1983; Mo, 1988; Malmberg & Malmberg, 1991;

Johnsen & Jensen, 1992). The studies by Tanum (1983) and Mo (1988) also demonstrated that brown trout were able to maintain their *G. salaris* infections when cohabited with infected salmon. A study by Bakke *et al*. (1999) found that brown trout, exposed to infected fins of Atlantic salmon for 24 h and subsequently held in isolation, eliminated their *G. salaris* infections in less than two weeks, suggesting that they could be innately resistant. Harris *et al*. (2000) also considered brown trout to be innately resistant to *G. salaris* when, after exposing groups of fish to infected salmon fins for 24 h, the fish lost their infections within 42 days. In a survey by Jansen and Bakke (1995), anadromous brown trout from the River Lierelva were cohabited with heavily infected Atlantic salmon from the Lierelva for 5 days, and then either isolated and held individually or maintained as a group. In both cases, the infections of *G. salaris* on the brown trout persisted for approximately 49 days p.i. In a repeat trial using a stock of brown trout from Lake Tunhovd, the infection of *G. salaris* on the isolated brown trout (n = 21) persisted for 28 days, whilst the infection on grouped fish (n = 21) lasted for 21 days p.i. These trials suggested that brown trout can serve as a carrier for disseminating the parasite, although it is not able to support an infection with *G. salaris* for long periods (Jansen & Bakke, 1995).

The current study, however, found that English brown trout can carry an infection of *G. salaris* for 110 days, and this finding appears to contradict those of previous studies (*i.e.* Jansen & Bakke, 1995; Bakke *et al*., 1999; Harris *et al*., 2000). This might be explained by a potential different pathogenicity between *G. salaris* haplotypes; *i.e.* Jansen and Bakke (1995), Bakke *et al*. (1999) and Harris *et al*. (2000) used haplotype F, while in the current experiment we used haplotype A. Additional studies, therefore, are required to elucidate this further.

6.4.7. Findings in support of national contingency plans

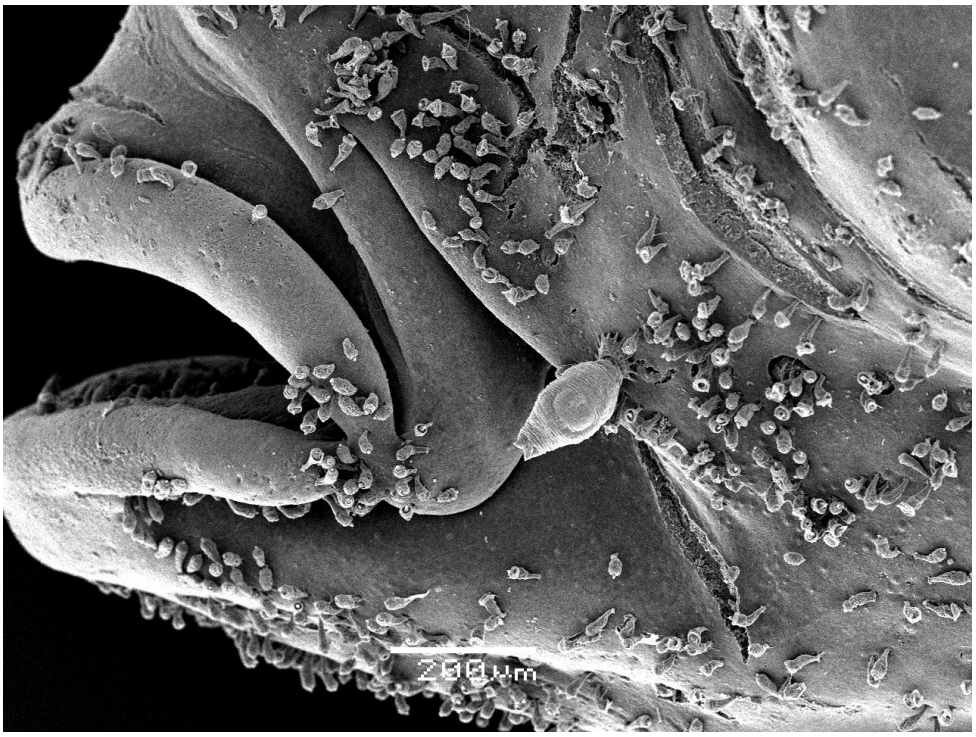
Although every precaution was taken to ensure fish welfare was upheld throughout the duration of the current susceptibility trial, the level of stress placed upon each population of fish during their transportation from the UK to Norway and in their experimental tanks is not known. Whilst the 110 day period of infection may not accurately reflect how British populations of brown trout in the wild would respond to *G. salaris*, if introduced into the UK, the trial has shown that the River Tyne population of brown trout are able to manage infections and keep numbers to a low level, even under periods of anticipated stress. Although there were no *G. salaris*-related brown trout mortalities, the concern is that populations of brown trout under stress may extend the period over which individuals can carry an infection of *G. salaris*, therefore, increasing the possible risk of parasite transfer to other fish species. Most of Bakke *et al.*'s experimental findings are based on studies using *G. salaris* "originating" from the River Lierelva (Norway), *i.e.* haplotype F according to the study of Hansen *et al*. (2003). This haplotype has been commonly found on rainbow trout, Atlantic salmon and Arctic charr (see Hansen *et al*., 2003; Hansen *et al*., 2007a; Robertsen *et al*., 2007). The study conducted by Bakke & MacKenzie (1993) on Scottish salmon, however, used a strain of *G. salaris* originating from the River Figga, Norway, most likely corresponding to haplotype A (though not stated, this is interpreted from the map of haplotype distribution presented in Hansen *et al*., 2003), which is also known to be pathogenic to *S. salar*. The strain of *G. salaris* used in the current study was derived from the River Fusta in the Vefsna region of Norway and corresponds to haplotype A. It is not possible, therefore, to ascertain whether the observed differences displayed by the brown trout compared with previous studies are due to genetic differences between each population of trout, or due to the strain of *G. salaris* that was used.

The findings from this trial are significant in that they demonstrate that Welsh salmon, as with Scottish salmon, are also susceptible to *G. salaris*, that grayling respond in a similar manner to their Scandinavian counterparts and carry infections for up 110 days, and that English brown trout are responsive to a *G. salaris* infection, but can harbour infections for longer than those reported for Norwegian populations, *i.e.* 110+ days as opposed to 50 days. These extended windows of infection and the interpretation of "resistance" need to be considered carefully in terms of the role that brown trout could play within the context of national contingency planning and subsequent management in the event of a *G. salaris* outbreak.

Current national surveillance programmes for *G. salaris* focus on areas where Atlantic salmon are dominant, with relevant sites being sampled on a regular basis *i.e.* at least once a year. Other sites, perhaps through limitations of manpower and other resources, are sampled less frequently. The demonstration from this study that *G. salaris* can persist on brown trout for long periods would suggest that during a suspected outbreak, the surveillance of brown trout farms and of watercourses inhabited by brown trout, especially where the two salmonids co-exist, should be increased. Given the suggested association of rainbow trout movements and emerging *G. salaris* infections, it is also suggested that during a suspected outbreak, brown trout in and around rainbow trout sites are carefully monitored. Current national contingency plans may, therefore, benefit from a clarification of the potential role that brown trout could play in the spread of *G. salaris*.

Chapter 7

Alternative chemical strategies to control *Gyrodactylus salaris*



Head of a three-spined stickleback, *Gasterosteus aculeatus aculeatus* L., with a natural infection of *Gyrodactylus arcuatus* Bychowsky, 1933 and *Apiosoma* sp. [original image]

Aspects of this work were presented as:

Paladini G. (2012). *Gyrodactylus*: tales of invasion, resistance and control strategies. *Aquaculture UK 2012, Aviemore, Scotland, 23rd-24th May 2012* (talk).

Declaration

The statistical analysis component of this study was conducted by Dr Nicholas G.H. Taylor during a visit to Cefas, Weymouth Laboratory.

7.1. Introduction

While a range of integrated pest-management strategies are used in aquaculture to control parasite infections of stock, the use of chemicals remains the preferred method (Brooks, 2009; Shinn & Bron, 2012). Although a large number of studies have been conducted to identify compounds suitable for control of parasite infections in both farmed and ornamental populations of fish (see review by Schelkle *et al*., 2009, 2010 for those tested against *Gyrodactylus*), there are, unfortunately, only a small number of efficacious, licensed treatments that can be employed, with the permission for use of these being governed by the regulatory authorities within each country (Shinn & Bron, 2012).

The impact of *Gyrodactylus salaris* Malmberg, 1957 in Norway currently costs £38 million p.a., of which £23 million are linked to loss of tourism and angling restrictions, while the remaining £15 million represent the cost of on-going surveillance programmes and river treatments. Although this parasite is easily controlled under farming conditions using formalin or other licenced products, infections on wild fish present a range of larger logistical problems.

Gyrodactylus salaris infections in Norway are currently managed through the use of the biocide rotenone ($C_{23}H_{22}O_6$), which is an extract from the roots and stems of the plant *Derris elliptica*. Rotenone is a broad-spectrum biocide that is used to kill-out the entire fish population within the river system under treatment. Rotenone has an impact on the respiratory system and acts by interfering with the electron transport chain in mitochondria (Marking & Bills, 1976; Eriksen *et al*., 2009). The "treatment" of wild stocks using rotenone requires the handling of large volumes of chemical and heavy manpower to ensure that all parts of the water system are treated effectively. There are, however, considerations regarding the loss of non-target species, the impact on biodiversity and the environmental impact and cost more widely. Ideally, a suitable alternative should be equally as effective in removing the parasite, have a low environmental impact (short half-

life, safe breakdown products, *etc*), and be able to reduce the problem of fish losses and impacts on non-target species, *i.e.* impacts on biodiversity. The alternative compound, however, may not circumvent the problems of chemical and manpower costs.

Aqueous aluminium sulfate (100 μg L^{-1} $Al_2(SO_4)_3$; given continuously over a 10–14 day-period), followed by a rotenone treatment, is also being trialled for use in control of *G. salaris* infections in Norwegian river systems (Soleng *et al*., 1999; Poléo *et al.*, 2004). There are, however, human health concerns regarding the use of both compounds. Rotenone has been suggested to be responsible for behavioural and pathological symptoms of Parkinson's disease (Giasson & Lee, 2000; Newhouse *et al*., 2004; Cannon *et al*., 2009), whilst the use of aluminium sulfate has been identified as a risk factor in the development of Alzheimer's Disease (WHO, 1998). The UK Government also has concerns regarding the use of either product. The UK's largest water poisoning incident resulted when undiluted aluminium sulfate was accidentally added to the domestic water supply via the water treatment plant at Camelford, UK, with a recorded maximum concentration of 620,000 μg L^{-1}, which was 3100 times higher than the maximum concentration admissible, *i.e.* 200 μg L^{-1} (http://en.wikipedia.org/wiki/Camelford_water_pollution_ incident).

Although rotenone and aluminium sulfate are regarded by some as unsuitable treatment options for the management of *G. salaris* infections in the wild, there has, unfortunately, been little effort to look for alternatives. Schelkle *et al.* (2009) provided a recent review of compounds that have been tested on gyrodactylids and then, in 2010, took a closer look at the impact of different concentrations of salt (NaCl) on the infection dynamics of *Gyrodactylus bullatarudis* Turnbull, 1956 and *G. turnbulli* (see Schelkle *et al.*, 2010). More recently, Brooker *et al.* (2011) studied the effect of octopaminergic receptor agonists/antagonists, *i.e.* compounds that elicit a response by binding to a post-synaptic receptor (on muscles or nerves), which mimic or block natural transmitters, and found that different concentrations affect the ability of gyrodactylids to attach or locate a

host when detached, and also their survival. The utility of these compounds, however, is merely of scientific interest at this stage, and these studies provide greater knowledge concerning whether these classes of compound are efficacious in affecting *Gyrodactylus* species, rather than suggesting that they be used as replacements to rotenone and aluminium sulfate.

Bronopol (2-bromo-2-nitropropane-1,3-diol), marketed under the trade name of Pyceze^{TM} (Novartis Animal Vaccines Ltd.), is a broad-spectrum disinfectant, which has been demonstrated to cause membrane damage in microbial organisms through the inhibition of membrane-bound enzymes (Stretton & Manson, 1973; Shepherd *et al*., 1988). Bronopol is commonly used in aquaculture and is currently licensed within the EU for use against infections of the oomycete *Saprolegnia parasitica* (Coker) (see Branson, 2002; Novartis, 2002, 2006), and has been shown to be effective in the control of other ectoparasitic species, such as the ciliate protozoan *Ichthyophthirius multifiliis* Fouquet, 1876 (see Picón-Camacho *et al*., 2012; Shinn *et al*., 2012c).

UK contingency plans are currently based on a large number of experimental studies conducted within Scandinavia. These include the implicit assumption that the pattern of *G. salaris* infection and population growth observed in the laboratory environment, accurately reflect the dynamics of infection in the wild. If the experimental data are to be used to inform national contingency planning, *i.e*. with regard to surveillance, containment, treatment and management, then it is imperative that the influence of water chemistry on *G. salaris* infections is investigated. Almost all our current understanding of *G. salaris* population dynamics is derived from a single experimental protocol carried out using a common domestic water supply in Oslo (Soleng *et al*., 1999). It is known, however, that subtle deviations in water composition can affect the course of gyrodactylid infection, notably the addition of aqueous aluminium sulfate, which, as noted above, is being trialled as a remedial measure for the control of *G. salaris* (see Soleng *et*

al., 1999; Poléo *et al.*, 2004). Permissible levels of other heavy metals, *i.e.* cadmium and zinc, typically found in UK domestic tap water (*i.e.* <5 µg L^{-1}), have also been shown to impact significantly on the pattern of population growth of *Gyrodactylus turnbulli* Harris, 1986 on guppies held in research aquaria (Carter, 2003; Gheorghiu *et al*., 2007).

Given the apparent importance of water chemistry in the establishment of *Gyrodactylus*, the influence of different tannins and humic substances on the survival of gyrodactylids is also of interest. This natural organic material is produced from decaying vegetation and its concentration as dissolved organic carbon (DOC) in water can range from 4.3 to 14.5 mg L^{-1} (Sharp *et al*., 2006). Tannic acid ($C_{76}H_{52}O_{46}$) is a polyphenol that is ubiquitous in plants, including tea. Its astringent properties are used in the formulation of several pharmaceutical anti-diarrhoeal, haemostatic and anti-haemorrhoidal products (Ashok & Upadhyaya, 2012). Tannins, by way of generalisation, have the ability to inhibit enzymes, precipitate proteins, and to scavenge free radicals. Given these properties, their use as anti-viral (*e.g.* HIV, see Lin *et al*., 2004), anti-bacterial (*e.g. Staphylococcus aureus* and *Helicobacter pylori*, see Akiyama *et al*., 2001; Funatogawa *et al*., 2004) and anti-parasitic (*e.g. Leishmania*, see Kolodziej & Kiderlen, 2005) agents have been explored.

The current study represents a preliminary investigation looking for alternative compounds for use against *Gyrodactylus* spp., and begins by assessing the potential suitability of broad-spectrum disinfectants, *e.g.* bronopol, and of natural compounds, *e.g.* tannic acid. This study evaluates the impact of bronopol on the survival of *G. salaris* and *Gyrodactylus arcuatus* Bychowsky, 1933, a common species of three-spined sticklebacks, *Gasterosteus aculeatus aculeatus* L., and then assesses the use of tannic acid against one species only, *G. salaris*.

7.2. Materials and methods

7.2.1. Origin of experimental fish and parasite populations

7.2.1.1. Source of fish and parasites for the bronopol trials

The *in vitro* efficacy of bronopol was initially tested in the UK against *G. arcuatus*, a common species parasitising three-spined sticklebacks, and then subsequently against *G. salaris* from Atlantic salmon in Norway. *Gyrodactylus arcuatus* was selected as the species for chemical assessment in the UK because this species is easily acquired and is found in a wide range of aquatic habitats from freshwater to marine, and it therefore represents a good model for testing.

Forty specimens of three-spined sticklebacks (each 3-6 cm in total length) naturally infected with *G. arcuatus* were collected from a tributary of the River Allan in Stirlingshire and transported to the Institute of Aquaculture, University of Stirling, UK. The fish were held in small tank (60×30×40 cm) with oxygen, under 12 h light:12 h dark photoperiod regime, at 6±1°C, fed on bloodworms and left to acclimate for one month to allow parasite numbers on the captive-held fish to increase.

For the trials using *G. salaris* (haplotype F) conducted in the Natural History Museum, Department of Zoology, University of Oslo (Norway), two groups, each of 10 Atlantic salmon (each 10-15 cm in total length; weight ranging 5-20 g), were sampled from the Rivers Glitra and Lierelva, and subsequently maintained in 200 L aquaria supplied with 6±1ºC dechlorinated Oslo tap water. The fish were fed *ad libitum* with a commercial pellet food, and kept under a photoperiod regime of 12 h light:12 h dark. The fish stock from the River Glitra had been maintained in the aquarium for a period of five months prior to the experiment, whilst the population of salmon collected from the River Lierelva had been kept in the University of Oslo research aquarium for a period of two years. The aim of the

study, using two different *G. salaris* populations, was to test if any host-parasite adaptation to aquarium condition may occur.

7.2.1.2. Source of fish and parasites for the tannic acid trials

The *in vitro* efficacy of tannic acid was tested at the Natural History Museum, Department of Zoology, University of Oslo (Norway), against one population of *Gyrodactylus salaris* only. The population of *G. salaris* (haplotype F) used in this study originated from the River Lierelva and was maintained and used in the same way as described under section 7.2.1.1.

7.2.2. Bronopol exposure procedure

Two sets of trials were conducted: a continuous-exposure trial and a 1 hour-exposure to bronopol trial. For the continuous chemical-exposure trial, a fresh batch of bronopol was prepared at the following concentrations: 0, 25, 50, 100, 150, 250, 375, 500, 625 and 750 ppm of bronopol, using water feeding the experimental tanks, filtered through a 0.2 µm filter. For the trials, a heavily *Gyrodactylus*-infected three-spined stickleback and a heavily *Gyrodactylus*-infected Atlantic salmon from each of the Norwegian populations (Glitra and Lierelva strains) were killed using a UK Home Office Schedule 1 method, and small pieces of fin, each with 10 *Gyrodactylus* specimens attached, were selected, removed and placed into a 5 cm Petri dish (Fig. 7.1) containing 10 ml of the relevant concentration of bronopol and maintained at 6±1°C. Each concentration was tested in triplicate, the parasites in each dish were assessed every hour, and the number of dead and live parasites determined. For the 1 hour-exposure trial, only three doses of bronopol were tested in addition to a control, *i.e.* 25, 50, 100 ppm (also in triplicate) on the two stocks of *G. salaris* (n = 50 parasites per replicate).

7.2.3. Tannic acid exposure procedure

Trials involving continuous exposure to tannic acid, as well as a 10 minute-exposure, were conducted. A 1 mM solution of tannic acid (Sigma-Aldrich, Steinheim, Germany) was prepared using 0.2 µm filtered, dechlorinated Oslo tap water at 6 ± 1°C and then serially diluted to give concentrations of 0.5, 0.25, 0.1, 0.075 mM and a control (0 mM). Ten *G. salaris* haplotype F were placed into a 5 cm Petri dish containing 10 ml of each concentration, run in triplicate, including a control set. For the 10 minute-exposure trial, only one dose of tannic acid was tested, *i.e.* 0.5 mM (also in triplicate), on the same stock of *G. salaris* (n = 50 parasites per replicate).

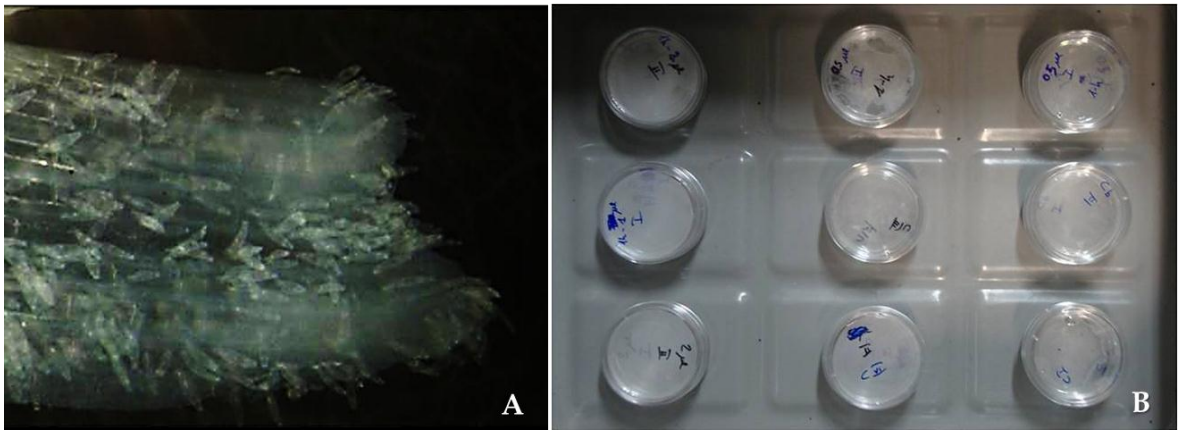


Figure 7.1. A: A fin of Atlantic salmon, *Salmo salar* L., with heavy infection of *Gyrodactylus salaris* Malmberg, 1957 (haplotype F). Ten specimens were removed and placed into each Petri dish used in the trial; **B**: 5 cm Petri dishes, each containing a relevant dose of either bronopol (Pyceze^{TM}) or tannic acid for the *in vitro* assessments [original images].

7.2.4. Parasite survival/mortality assessment

Parasite survival was assessed by monitoring the plates every hour during the treatment process. When the parasite is affected by the chemical, its body start twitching and subsequently the parasite detaches from the piece of fin. The parasite is considered dead when no movements occur and the body, initially transparent, become whitish in colour.

7.2.5. Statistical analyses

Statistical analysis of the bronopol data was conducted by Dr N.G.H. Taylor during a visit to the Cefas Weymouth Laboratory.

The influence of different doses of bronopol (Pyceze^{TM}) on the survival of the different *Gyrodactylus* species and strains was compared using *Cox* proportional hazards regression. The analysis was conducted in R v2.13 using the *coxph* function in the survival library and the *coxme* function in the *coxme* library, to allow each replicate within each treatment to be incorporated as a random effect. Models were built by first fitting the maximal model, where dose was included as a covariate, and the species or strain of *Gyrodactylus* as a factor. An interaction term was also included between the continuous exposure dose of bronopol (ppm) and species or strain, as was an interaction term between dose and time, and species and time, to establish whether the association between these variables and the dependent variable changed over time. Interactions and explanatory variables with a probability of significance (*p*) greater than 0.05 were then systematically removed from the model (least significant first), until only variables significant at $p<0.05$ remained. To account for variability between replicates, the influence of including a random intercept and random slope term in the model was also assessed. Where little variance was attributed to the random intercept term and its inclusion did not improve model fit (this was assessed graphically and through comparison of the Akaike's Information Criterion (AIC) and parameter estimates obtained between models), it was assumed that there was little variance between the replicates, and a fixed effects model was therefore used in which data from the replicates were pooled.

The influence of a short term (1 h), low-dose exposure (25, 50 and 100 ppm), as opposed to continuous exposure, on the subsequent survival of *G. salaris*, was assessed using the same methods. LD50s associated with a 1 h period of exposure to bronopol were then estimated for each of the *Gyrodactylus* species or strains that were tested using

logistic regression models, *i.e.* a generalised linear model assuming a binomial (or quasibinomial) error distribution and logit link function, where the proportion of parasites surviving was the dependent variable, and the dose to which they were exposed to, the explanatory covariate. Model fit was assessed by plotting the relationship between the logit of the dependant variable and various transformations of the explanatory covariate, and assessing reduction in residual deviance compared to the baseline model. As in the other analyses, the variability occurring between the replicates was assessed by including replicate as a random intercept term in the model, this time using the *lmer* function from the *lme4* library of R. Where little variability was observed between replicates, a fixed effects model was used. The model providing the best fit (according to the greatest reduction in residual deviance) was then used to predict the LD50 for that exposure time.

7.3. Results

This study explores the potential of two compounds in killing specimens of different species and strains of *Gyrodactylus*. Given the *Gyrodactylus* material available for investigation, larger data sets were obtained from the trials using bronopol and the results of these will be looked at in greater detail. The trials for tannic acid are based on smaller datasets and can only be considered as preliminary; the results nonetheless are interesting and worthy of reporting.

7.3.1. Bronopol

The results of the bronopol trials are shown in Tables 7.1-7.3 where it can be seen that the action of bronopol on *G. salaris* (only the results for the *G. salaris* maintained on Glitra salmon are shown as both sets of results were similar) was markedly better than on *G. arcuatus*.

Table 7.1. The treatment efficacy of 0-750 ppm (mg L^{-1}) bronopol (Pyceze^{TM}) against *Gyrodactylus arcuatus* Bychowsky, 1933, against time (h) when continuously exposed. Yellow boxes highlight where all (*i.e.* 100%) the parasites were killed, while the light blue boxes highlight where 50-99% of the parasites were killed. Figures represent the combined results from three replicate dishes per dose, each containing 10 parasites (total n = 30).

***G. arcuatus* from 3-spined stickleback from the River Allan, UK continuous exposure (% dead)**								
DOSE (ppm)	**1h**	**2h**	**3h**	**4h**	**5h**	**6h**	**7h**	**24h**
0 (control)	0	0	0	0	0	0	0	0
25	3.3	10	13.3	13.3	13.3	13.3	13.3	100
50	0	0	0	0	6.7	6.7	6.7	100
100	0	0	0	3.3	10	10	16.7	100
150	0	0	0	0	0	0	20	100
250	0	0	0	10	10	30	70	100
375	3.3	3.3	6.7	13.3	16.7	70	93.3	100
500	3.3	10	80	90	100			
625	3.3	6.7	90	90	100			
750	6.7	76.7	96.7	100				

Table 7.2. The treatment efficacy of 0-750 ppm (mg L^{-1}) bronopol (Pyceze^{TM}) against *Gyrodactylus salaris* Malmberg, 1957 (Lierelva strain, haplotype F), against time (h) when continuously exposed. Yellow boxes highlight where all (*i.e.* 100%) the parasites were killed, while the light blue boxes highlight where 50-99% of the parasites were killed. Figures represent the combined results from three replicate dishes per dose, each containing 10 parasites (total n = 30).

***G. salaris* from Atlantic salmon from the River Glitra, Norway continuous exposure (% dead)**								
DOSE (ppm)	**1h**	**2h**	**3h**	**4h**	**5h**	**6h**	**7h**	**24h**
0 (control)	0	0	0	0	0	0	0	0
25	0	0	6.7	30	40	56.7	70	100
50	0	26.7	40	50	63.3	80	93.3	100
100	20	30	83.3	100				
150	30	40	86.7	100				
250	46.7	70	100					
375	46.7	70	100					
500	50	100						
625	100							
750	100							

Table 7.3. The treatment efficacy of 0-100 ppm (mg L^{-1}) bronopol (Pyceze^{TM}) against *Gyrodactylus salaris* Malmberg, 1957 (Lierelva strain, haplotype F) against time (h) when parasites are exposed for just one hour, after which the bronopol was replaced with fresh, dechlorinated, filtered Oslo tap water. Yellow boxes highlight where all (*i.e.* 100%) the parasites were killed, while the light blue boxes highlight where 50-99% of the parasites were killed. Figures represent the combined results from three replicate dishes per dose, each containing 50 parasites (total n = 150).

***G. salaris* from *S. salar* from the River Glitra, Norway 1 h-exposure (% dead)**								
DOSE (ppm)	**1h**	**2h**	**3h**	**4h**	**5h**	**6h**	**7h**	**24h**
0 (control)	0	0	0	0	0	0	0	0
25	0	0	6	17.3	40	48	50.7	78
50	7.3	15.3	21.3	38.7	63.3	70	83.3	100
100	10	20	27.3	48.7	66	100		

7.3.1.1. Exposure of Gyrodactylus *to a continuous dose of bronopol (Pyceze^{TM})*

The *Cox* proportional hazards regression test found that there was variability between the replicates and, therefore, a fixed effects model was used where the data were pooled between replicates. The results of Table 7.4 show that there was a significant relationship between dose and the likelihood of dying *i.e.* as the dose increases, so the likelihood of dying increases. There was also a significant interaction effect between dose and time, showing that the effect of increasing the dose of bronopol was reduction of the survival of *Gyrodactylus* as time increased. Both populations of *G. salaris* had a significantly higher mortality rate than that of *G. arcuatus*. The *G. salaris* population from Lierelva had the highest mortality rate but this was not significantly higher than the population of *G. salaris* collected from Glitra. There was no interaction effect between dose and species suggesting that an increased dose of bronopol led to the same increase over the baseline mortality rate in all three species, *i.e.* the treatment was equally effective at each dose on all species. There was also a significant interaction between dose and time suggesting that as time progressed, the influence of the dose of bronopol had less effect on the mortality rate.

Table 7.4. A summary of the *Cox* proportional hazards regression looking at the influence of different doses of bronopol on the survival of the different strains (*G. salaris* from Glitra and from Lierelva) and species of *Gyrodactylus* (*G. arcuatus* and *G. salaris*). All model terms are included in the table below (n = 660; number of events = 600). The reference level for the strain effect was *G. arcuatus*.

	coef	**se(coef)**	**z**	**Pr(>|z|)**
Dose (ppm)	0.005	0.0002	23.244	2×10^{-16}
***G. salaris* (Glitra)**	0.829	0.120	6.908	4.92×10^{-12}
***G. salaris* (Lierelva)**	0.952	0.123	7.754	8.88×10^{-15}
Dose (ppm) *vs* Time (h)	-0.0002	0.0001	-3.444	0.001

R^2 = 0.67 (max possible = 1)

7.3.1.2. The proportion of Gyrodactylus *specimens surviving at 1 h-continuous bath of bronopol*

A generalised linear model assuming a binomial (or quasibinomial) error distribution was used in R to determine the proportion of *Gyrodactylus* specimens surviving at 1 h-exposure to different doses of bronopol (Pyceze^{TM}), *i.e.* the probability of death by the end of a 1 h-exposure event. The approach used in R is presented in Table 7.5.

Table 7.5. The output from a generalised linear model used to determine the concentration of bronopol (ppm ± SE) needed to kill different proportions of two populations of *G. salaris* and a population of *G. arcuatus* when continuously exposed to bronopol for one hour. All model terms are included in the table below.

The coefficients were:

	Estimate	**Std.Err**	**t value**	**Pr(>|t|)**
(Intercept)	7.409	1.096	6.758	5.64×10^{-9}
Dose (ppm)	-0.009	0.001	-6.816	4.47×10^{-9}
***G. salaris* (Glitra)**	-3.957	0.830	-4.767	1.17×10^{-5}
***G. salaris* (Lierelva)**	-4.785	0.903	-5.298	1.64×10^{-6}

The dispersion parameter for the quasibinomial family was taken to be 3.34.

Null deviance: 581 (65 df); residual deviance: 102 (62 df)

The analysis found that both strains of *G. salaris* had significantly higher mortality rates than *G. arcuatus* (*i.e.* $p<0.001$), and as dose increased, the probability of their survival reduced significantly (*i.e.* $p<0.001$). The dose (± SE) of bronopol (ppm) needed to kill

different proportions (*i.e.* p=50% to 95%) of the *G. salaris* populations after a 1 h-continuous exposure are presented in Table 7.6, while the concentrations needed to kill different proportions of *G. arcuatus* are presented in Table 7.7.

Table 7.6. Dose (± SE) of bronopol (ppm) needed to kill different proportions (*i.e.* p=50% to 95%) of the two *G. salaris* strains after a continuous exposure of one hour, as estimated by the R "dose.p" function.

Proportion (p)	Dose (ppm)	SE
0.5	384	26.47
0.9	659	46.81
0.95	753	56.59

Table 7.7. Concentrations of bronopol (ppm ± SE) needed to kill different proportions of *Gyrodactylus arcuatus*, as estimated by the R "dose.p" function.

Proportion (p)	Dose (ppm)	SE
0.5	810	30.49
0.9	1051	39.45
0.95	1132	44.04

Higher doses of bronopol were required in this case because *G. arcuatus* was significantly less sensitive to treatment than both strains of *G. salaris* were, either due to inherently reduced susceptibility or due to differences in aspects of water quality or chemistry employed in the trial.

7.3.1.3. Treatment of G. salaris *using bronopol: continuous* vs *1 hour-exposure*

A *Cox* proportional hazards regression was also used to investigate what doses of bronopol (Pyceze^{TM}) when presented continuously would be needed to kill the two populations of *G. salaris*. The approach used in R is presented in Table 7.8.

Table 7.8. The output from the *Cox* proportional hazards regression used to determine the concentration of bronopol (ppm ± SE) needed to kill different proportions of two populations of *G. salaris* when presented continuously. All model terms are included in the table below (n = 1190; number of events = 797). Abbreviations: conc. = concentration; h = hour.

	coef	**se(coef)**	**z**	**Pr(>|z|)**
Dose (ppm)	0.039	0.002	22.247	$<2\times10^{-16}$
***G. salaris* (Lierelva)**	-0.447	0.104	-4.312	1.62×10^{-5}
Treatment (conc.)	1.782	0.143	12.440	$<2\times10^{-16}$
Dose (ppm) *vs* Treatment (conc.)	-0.030	0.002	-19.172	$<2\times10^{-16}$
***G. salaris* (L) *vs* Treatment (conc.)**	0.430	0.144	2.981	0.003
Dose (ppm) *vs* Time (h)	-0.003	0.0002	-13.386	$<2\times10^{-16}$

$R^2 = 0.72$ (max possible = 1)

The results from Table 7.8 show that as in the previous analysis there was a significant increase in the mortality rate of *G. salaris* as the dose of bronopol increased, but as time progressed the influence of dose on mortality decreased. A continuous dose of bronopol, not surprisingly, was more effective than a 1 h-exposure, but the difference between these two types of treatment regime (*i.e.* continuous exposure *vs* 1 hour-exposure) got smaller as the dose of bronopol increased – this is demonstrated by the significant negative interaction effect between dose and treatment type. The population of *G. salaris* from the Lierelva had a significantly lower mortality rate than the population of *G. salaris* from the Glitra, and this difference in mortality rate was more pronounced under the continuous exposure regime when compared to the one hour-exposure approach, *i.e.* the *G. salaris* Glitra strain was more sensitive to the continuous exposure than was the population of *G. salaris* from the Lierelva.

7.3.2. Tannic acid

Table 7.9 shows the efficacy of tannic acid in killing *G. salaris* haplotype F (Lierelva strain) when continuously exposed to tannic acid, demonstrating that low doses of tannic acid result in high percentage kills. A repeat trial using 0.5 mM tannic acid for only 10 minutes resulted in 90% parasite mortality within the first hour post-exposure (Table 7.10). The reason why only the dose of 0.5 mM tannic acid has been tested for the 10 minute-trial is because 0.5 mM was the lowest dose killing the 100% of *G. salaris* within the first hour (see Table 7.9). The effect of tannic acid on *G. salaris* is shown in Fig. 7.2, presenting clear evidence for swelling of the parasite and lifting off of the parasite tegument.

Table 7.9. The treatment efficacy of 0-1 mM tannic acid against *Gyrodactylus salaris* (Lierelva strain haplotype F) against time (h) when continuously exposed. Yellow boxes highlight where all (*i.e.* 100%) the parasites were killed, while the light blue boxes highlight where 50-99% of the parasites were killed. Figures represent the combined results from three replicate dishes per dose, each containing 10 parasites (total n = 30).

***G. salaris* Lierelva strain haplotype F continuous exposure (% dead)**				
DOSE (mM)	**1h**	**2h**	**3h**	**4h**
0 (control)	0	0	0	0
0.075	0	70	83.3	100
0.1	50	96.7	100	
0.25	73.3	100		
0.5	100			
1	100			

Table 7.10. The treatment efficacy of 0-0.5 mM tannic acid against *Gyrodactylus salaris* (Lierelva strain haplotype F) against time (h) when parasites are exposed for just 10 minutes, after which the tannic acid was replaced with fresh, dechlorinated, filtered Oslo tap water. Yellow boxes highlight where all (*i.e.* 100%) the parasites were killed, while the light blue boxes highlight where 50-99% of the parasites were killed. Figures represent the combined results from three replicate dishes per dose, each containing 50 parasites (total n = 150).

***G. salaris* Lierelva strain haplotype F 10 minute-exposure (% dead)**								
DOSE (mM)	**1h**	**2h**	**3h**	**4h**	**5h**	**6h**	**7h**	**24h**
0 (control)	0	0	0	0	0	0	0	0
0.5	90	92.7	97.3	98	98.7	99.3	99.3	100

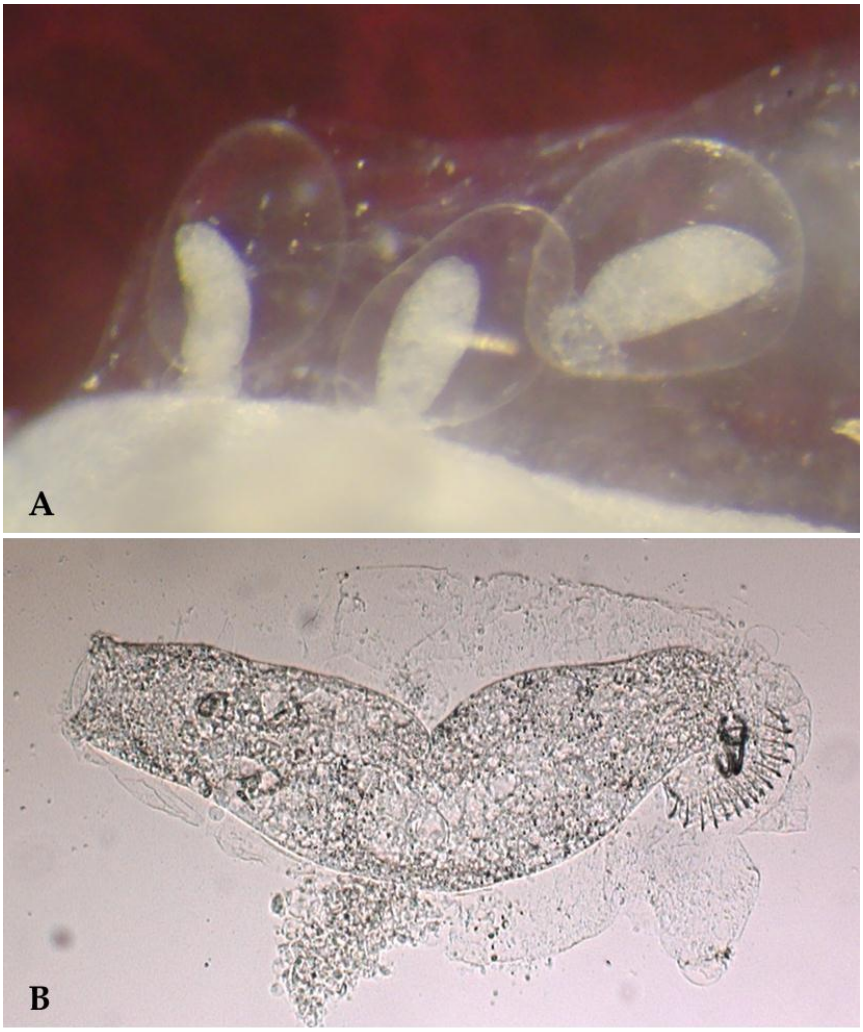


Figure 7.2. The effect of 0.5 mM tannic acid on *Gyrodactylus salaris* Malmberg, 1957 which causes swelling and lifting off of the tegument. **A**: dead parasites under the dissecting microscope; **B**: dead parasite mounted on a slide [original images].

7.4. Discussion

Bronopol, licensed as Pyceze^{TM}, is used extensively throughout the UK in the aquaculture industry for the control of the oomycete *S. parasitica* infecting salmonid fish and eggs (Pottinger & Day, 1999; Branson, 2002; Aller-Gancedo & Fregeneda-Grandes, 2007); for the ciliated protozoan *I. multifiliis* (see Picon-Camacho *et al*., 2012; Shinn *et al*., 2012c), and elsewhere for the dinoflagellate *Amyloodinium ocellatum* (Brown, 1931) Brown *et* Hovasse, 1946, the causative agent of "velvet disease" (Roberts-Thomson,

2007). Against *Gyrodactylus*, however, the efficacy of bronopol has never been tested prior to this study. Although these results demonstrate that bronopol could be used to control infections of *G. salaris* in confined aquaria, this does not mean that this advocates its use in river systems as there are a plethora of logistic, economic (*e.g.* 1 L of Pyceze™ costs about £35) and environmental considerations to take into account. This does, however, take important steps towards investigating alternative control agents for use in the event of an outbreak. The differences in susceptibility between *G. salaris* and *G. arcuatus* are very interesting. The reason why *G. arcuatus* is less sensitive than *G. salaris* to the treatment with bronopol may be explained by the fact that its host, *Gasterosteus aculeatus aculeatus*, is able to adapt to a wide range of environmental conditions, and so its parasites. This behaviour has also been observed in *Gyrodactylus salinae* Paladini, Huyse *et* Shinn, 2011, which can survive on its host, *Aphanius fasciatus* (Valenciennes), despite massive changes of water temperature and salinity (Paladini *et al*., 2011b). Another explanation could be that the water conditions under which the study was carried out were different in the two trials, although both parasite species, *G. arcuatus* and *G. salaris*, were tested keeping the same water temperature of the environment where they were collected from, in order to reduce at minimum the stress. The tolerance of its host to changes in environmental conditions may explain the higher doses needed to kill *G. arcuatus*. If the responses of *G. arcuatus* are correct, then this species it may serve as a useful species in the future for the evaluation of other anti-parasitic and anti-monogenean treatments. It is important to stress that the current study using bronopol, at this stage, must be considered as exploratory only, rather than a study that set out to define concentrations to subsequently deploy in a river.

Tannic acid has never been employed in aquaculture to control parasitic infections and this is the first time that this has been evaluated in the current pilot study. No statistical analyses were conducted because larger datasets are required for a robust interpretation and

conclusion as to the efficacy of this compound. The study does suggest, however, that low doses do produce high percentage kills in relatively short periods of time, a feature perhaps imparted by its multiple phenyl groups (Figure 7.3) which are known to participate in the protein precipitation process. Further work, however, is needed to see whether these results can be repeated using larger numbers of specimens, and whether it is as effective as rotenone at equal or lower doses to those used when continuously deployed over longer time periods (*i.e.* up to 10 days as it is used for aluminium sulfate). Any future trials, however, must be supported by toxicity trials on a range of fish species and other indicator species such as *Daphnia* to begin to have a clearer understanding of what sort of impact these chemicals might have on biodiversity and species composition in rivers.

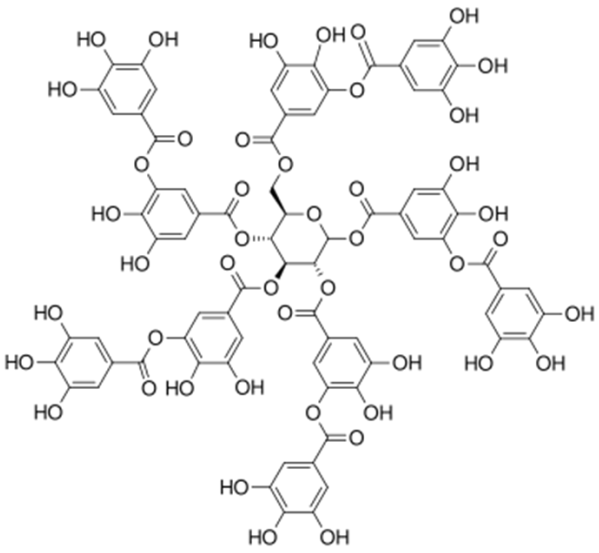
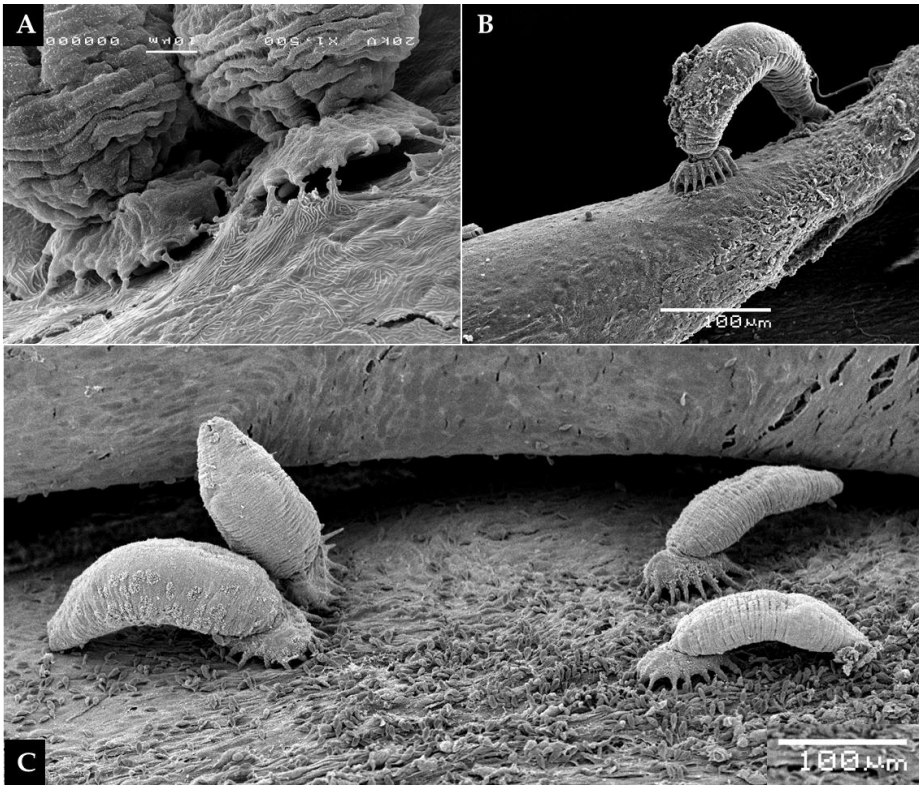


Figure 7.3. The molecular structure of tannic acid ($C_{76}H_{52}O_{46}$), a plant polyphenol. Its precise formulation varies depending on the plant source it is extract from, but its multiple phenyl groups (C_6H_5) attached to the hydroxyl groups (OH) may explain its ability to kill *Gyrodactylus* at low doses [image taken from Wikipedia; http://en.wikipedia.org/wiki/File:Tannic_acid.svg].

Chapter 8

Summary of findings and general discussion



Scanning electron micrographs (SEMs) of (**A**) *Gyrodactylus orecchiae* Paladini, Cable, Fioravanti, Faria, Di Cave *et* Shinn, 2009, (**B**) *Gyrodactylus arcuatus* Bychowsky, 1933 and (**C**) *Gyrodactylus salaris* Malmberg, 1957 [original images].

8.1. General discussion

The main objective of this research project was to investigate several lines of research surrounding *Gyrodactylus salaris* Malmberg, 1957 in order to provide a better understanding of the risks that this species poses to British salmonid stocks, and to determine the accuracy of assumptions dictating current practices, *e.g.* surveillance of wild salmonid stocks, estimates of the probability of detecting *G. salaris* in any given sample, and the related topic of national contingency planning. The most important objective was to determine the susceptibility of English and Welsh salmonids to *Gyrodactylus salaris* by conducting a series of infection trials, following the infection dynamics on individual fish to see if the trajectories and duration were similar to those seen on their Scandinavian counterparts. Current surveillance in the UK focuses on Atlantic salmon and rainbow trout. The role of brown trout and grayling thus need determining and this will be discussed later in this chapter.

This closing discussion chapter, however, will take a chronological walk through the PhD, discussing the order in which each study was conducted rather than the final order they are presented in this thesis.

8.1.1. Sampling of Italian salmonids for Gyrodactylus *material*

As the PhD was starting, it was necessary to become familiar with the biology, systematics and morphology of *Gyrodactylus*. A trip to Italy in the first months of the study was used to access a large number of rainbow trout, *Oncorhynchus mykiss* (Walbaum), samples that had been collected from fish farms throughout four regions of Italy in 2005 (see Chapter 2; Paladini *et al*., 2009a). Although gyrodactylosis represents a common and economically significant parasitic disease of rainbow trout farmed in Italy (Fioravanti & Caffara, 2007), a study of the *Gyrodactylus* species was lacking. Only a single species, *Gyrodactylus derjavinoides* Malmberg, Collins, Cunningham *et* Jalali, 2007

(reported as *Gyrodactylus derjavini* Mikhailov, 1975 prior to its later reclassification), was already known from Italian brown trout and rainbow trout (Malmberg, 1993). Mucus scrapes taken from a sample of 10–40 cm rainbow trout taken at each site subsequently resulted in the discovery of four known species of *Gyrodactylus*, including the OIE-notifiable pathogen, *G. salaris*. This was the first record of *G. salaris* from Italy and the Italian authorities were duly informed via OIE. The other three species were *G. derjavinoides*, *Gyrodactylus teuchis* Lautraite, Blanc, Thiery, Daniel *et* Vigneulle, 1999 and *Gyrodactylus truttae* Gläser, 1974, which were the same four species previously reported from rainbow trout in Denmark (Buchmann & Bresciani, 1997; Lindenstrøm *et al*., 1999; Buchmann *et al*., 2000; Nielsen & Buchmann, 2001; Lindenstrøm *et al*., 2003). The subsequent examination of archive material, fixed in formalin, from a rainbow trout farm in Veneto region dating back to 2000 also revealed the presence of *G. salaris*, suggesting that this species has been present in Italy, undetected, for many years. The unobserved presence of *G. salaris* prior to the first official report (Paladini *et al*., 2009) may be due to three potential hypotheses: 1) a stable host-parasite relationship is established; 2) *G. salaris* was found in a fish farm, where fish are usually treated intensively, controlling therefore the infection; and 3) it is a non-pathogenic form of *G. salaris*. This finding highlights the need for more rigorous biosecurity control measures in the trade and transfer of salmonid stocks from one country to another. Given the Italian history of imported rainbow trout from Denmark and Spain, it is likely that *G. salaris* has been introduced via rainbow trout trade from Denmark, rather than a transfer from local indigenous fish species. Further evidence to support this hypothesis is provided from a molecular analysis of the specimens, which revealed the *G. salaris* to be haplotype F, together with the discovery of a new haplotype from River Nera (haplotype named here as "*Sal* Nera"). Haplotype F is commonly found on rainbow trout from Denmark, among

other countries (Hansen *et al*., 2003), and this therefore suggests that *G. salaris* in Italy may have been introduced from Denmark.

8.1.2. Geographic distribution of G. salaris *throughout Europe*

The last review of the *G. salaris* distribution across Europe was made in 2007 (Bakke *et al*., 2007) in which *G. salaris* was reported as valid from 8 EU countries. The publication of the first record of *G. salaris* from Poland (Rokicka *et al*., 2007) prior to the start of this PhD, plus the new finding of *G. salaris* in Italy, suggested that the distribution of *G. salaris* across Europe required updating. A thorough search of the literature, much of it not in English, indicates that *G. salaris* has been reported from 23 out of ~50 recognised European states (see Chapter 3). Of these, the reports from only 17 countries are considered valid, as they have been identified by either morphology (n = 4), molecular (n = 3) or a combination of both techniques (n = 10; Table 3.1 in Chapter 3). The records of *G. salaris* from France, Portugal, Spain and Slovakia, are all believed to have been misidentified, the first three with a morphologically similar species, *i.e. G. teuchis*, which was subsequently described from France (see Lautraite *et al*., 1999), while the record from Slovakia might represent a confusion with *G. truttae*. In order to provide an additional contribution to this study, re-evaluation of existing specimens and the collection of new *Gyrodactylus* material from salmonids from five states, *i.e.* Finland, Germany, Italy, Portugal and Spain were made. Finland was already listed as a *G. salaris*-positive country; Germany was reported as being *G. salaris*-positive; however, there were some personal concerns regarding the validity of this record. The *G. salaris* status of Portugal and Spain is assumed to have been based on misidentifications and we believe the parasite is absent from both these territories. The specimens collected from rainbow trout from Germany and Italy were both confirmed as *G. salaris*. Additional material from Italy was used to extend the current distribution in the country from 4 to 7 regions, suggesting that this species is

widely spread throughout the country (Paladini *et al*., 2009a; Paladini *et al*., 2010b). The additional specimens obtained from Spain and Portugal, however, consisted of a single species only, *G. teuchis*, lending further support to the hypothesis that *G. salaris* is absent from both states.

The finding of a new *G. salaris* haplotype ("*Sal* Nera") on Italian rainbow trout (see Chapter 2; Paladini *et al*., 2009a) highlights the wide haplotype diversity that exists for *G. salaris* and raises further questions regarding the pathogenicity of this strain to other salmonids. Knowledge of differences in disease patterns by haplotype differentiation would help in discriminating the pathogenic strains from the non-pathogenic strains, and would hopefully allow for a clearer understanding of the risks of importing certain salmonids between areas in Europe. The majority of studies that have looked at the susceptibility of several salmonids to *G. salaris* have used haplotype F, originating from the Rivers Drammenselva, Lierelva and Lærdalselva, whilst only a few other experimental challenges have been carried out using haplotype A, collected from the Rivers Figga, Batnfjordselva, Steinkjerselva and Rauma (see Table 6.2). The identification of haplotype A originating from the Norwegian Rivers Steinkjerselva (see Bakke *et al*., 2002) and Figga (see Bakke & MacKenzie, 1993; Bakke *et al*., 2004), however, is only tentatively proposed based on their geographic location and their relative proximity to defined strains, according to the map that is presented in Hansen *et al*. (2003). It is important to stress that *G. salaris* haplotype A was used in the current study (see Chapter 6) to challenge English and Welsh salmonids. The same haplotype was also used by Bakke and MacKenzie (1993) in their study, which assessed the susceptibility of Scottish populations of Atlantic salmon from the Rivers Conon and Shin to *G. salaris.* Two subsequent studies were carried out by Dalgaard *et al*. (2003, 2004) on the same two Scottish salmon populations using haplotype F originating from the River Lærdalselva, and were able to show that these populations were equally as susceptible to this haplotype as they were to haplotype A, although not

pointed out. Difference in haplotype pathogenicity, however, has never been tested consciously and no studies have been conducted since the discovery of different haplotype strains by Hansen *et al*. (2003).

8.1.3. Taxonomic description of Gyrodactylus *species*

To correctly identify *G. salaris*, a comprehensive understanding of gyrodactylid taxonomy and proficiency in discriminating species was required during the current study. Considering that the genus *Gyrodactylus* contains a large number of species (~450), part of the PhD training required that specimens of *Gyrodactylus* coming through the Aquatic Parasitology Laboratory within the Institute of Aquaculture could be accurately identified. Some of the material submitted was associated with the mortality of aquaculture stocks, and so this material was of particular interest in terms of determining which species were responsible and under what conditions they were causing problems. As a consequence of investigating this material, eight new species of *Gyrodactylus* and two new genera of Monogenea were subsequently described and published. Only three of these studies are presented in this thesis as they fall within the remit of the PhD framework; the others are detailed in the Appendix at the back of this thesis.

Gyrodactylus orecchiae Paladini, Cable, Fioravanti, Faria, Di Cave *et* Shinn, 2009 is the first species of this genus to be officially described from gilthead seabream, *Sparus aurata* L., farms in Croatia and Albania, where it was responsible for the loss of up to 10% of the juvenile fish stocks (see Chapter 4; Paladini *et al*., 2009b). Identification was performed using both morphological and molecular analyses. Subsequently, additional samples of gilthead seabream, but this time from Bosnia-Herzegovina and Italy, revealed a second new species, *Gyrodactylus longipes* Paladini, Hansen, Fioravanti *et* Shinn, 2011 (see Chapter 4; Paladini *et al*., 2011a). The gilthead seabream from the Italian site, however, carried a mixed infection of both new species, with *G. longipes* being found

principally on the gills, whilst *G. orecchiae* was found on the skin. Following the description of *G. longipes*, unconfirmed farm reports from the same Italian site where the co-infection was recorded suggest that a 5–10% mortality of juveniles gilthead seabream was associated with a *Gyrodactylus* infection, suspected to be *G. longipes*. A subsequent sample of skin and fins mucus scrape from gilthead seabream collected from the north of France (undisclosed location) revealed the presence of *G. longipes,* extending further the geographical distribution of this species across Europe.

The discovery of these two new species of *Gyrodactylus* in such a short time was surprising, given that gilthead seabream culture is well established in the Mediterranean and that samples had been screened by the Laboratory of Fish Pathology of the University of Bologna, Italy, among other diagnostic laboratories, for many years, but a *Gyrodactylus* infection had never been seen before. Possible explanations for their appearance and impact in the Mediterranean include local climatic changes, imposing additional stresses on aquaculture stock and the possible migration of fish species carrying the parasite into the area from which the parasite transferred onto a more susceptible host, *i.e.* gilthead seabream.

Over 430 species of *Gyrodactylus* have been described so far, but only about 20% of these have been sequenced, principally their 18S small ribosomal internal transcribed spacer units (ITS 1 & 2). The growing number of species descriptions allows researchers to have a larger picture of the phylogenetic relationships between species. Earlier this year, a new species, *Gyrodactylus chileani* Ziętara, Lebedeva, Muñoz *et* Lumme, 2012 described from *Helcogrammoides chilensis* (Cancino) was published. A molecular evaluation of this species found that it clustered with *G. orecchiae* and an undescribed species from black goby, *Gobius niger* L., from the North Sea. A new lineage group called "the *Gyrodactylus orecchiae* lineage" was created, which extends from the Mediterranean and the North Sea to the South-Eastern Pacific, where *G. chileani* was collected from (Ziętara *et al*., 2012).

While *G. orecchiae* clusters with other marine species, the geographical area that these three species cover is enormous and ideally more marine species within this area are required before more detailed comments regarding their inter-relationships and the possible origins of *G. orecchiae* can be made.

The description of *G. longipes* from Italy and Bosnia-Herzegovina represents the seventh and first marine species of *Gyrodactylus* respectively to be reported from each country.

Sequences of the internal transcribed spacer regions (ITS1 and 2) have been widely used as species-specific reference sequences (barcodes) in the genus *Gyrodactylus*. To date, more than 100 *Gyrodactylus* species have been sequenced, and partial or complete ITS1 and 2 sequences are available in GenBank. This marker seems to match well to morphological markers, *i.e.* morphologically different species can be separated by the corresponding different ITS sequences. The discrimination of *G. longipes* from *G. orecchiae* can be easily obtained by morphological analyses, but also by comparing their ITS sequences, where the fragments containing ITS1 and 2 and the 5.8S of *G. longipes* are 1002 bp and those of *G. orecchiae* are 1074 bp (see Chapter 4; Paladini *et al*., 2011a). While identifications of *Gyrodactylus* species based only on morphological studies are assumed to be correct, absolute confidence can only be attributed to those descriptions where the morphology is supported by molecular characterisation.

Gilthead seabream that are heavily infected with *G. orecchiae* (1000+ gyrodactylids $fish^{-1}$) associated with the mortality of juvenile stocks raises concerns regarding the potential pathogenicity that this species may have in the gilthead seabream industry throughout the Mediterranean. *Gyrodactylus orecchiae* is currently known from three countries within the Mediterranean (Albania, Croatia and Italy), but it is possible that its distribution may be wider than this. Given the impact this species has already had, it would be worth evaluating further samples from elsewhere in the Mediterranean and

monitoring this species carefully until more can be learnt about its host specificity and conditions underlying its pathogenicity. The same is also true for *G. longipes*, presently reported from three countries within the Mediterranean (Italy and Bosnia-Herzegovina) and the English Channel (France), which also appears to be associated with fish mortality (see Chapter 4; Paladini *et al*., 2011a).

The new strain/isolate of *Gyrodactylus salmonis* (Yin *et* Sproston, 1948) described in Chapter 5 (Rubio-Godoy *et al*., 2012) is the first species of *Gyrodactylus* to be formally identified from Mexican populations of *Oncorhynchus mykiss* using a combination of morphological and molecular analyses. The intensity of infection of *G. salmonis*, at the time the samples were taken, was low and the infected fish did not show signs of damage, suggesting a stable host-parasite relationship. *Gyrodactylus salmonis*, however, is considered a threatening species as it is specifically highly pathogenic to brook trout, *Salvelinus fontinalis* (Mitchill), causing extensive fin damage as a consequence of the parasite's feeding and hook attachment, which penetrates deep into its host's epidermis (Cusack & Cone, 1986; Cone & Odense, 1984).

Gyrodactylus salmonis is known to have a low host specificity and it has been recorded from several other salmonids, *i.e.* brown trout, *Salmo trutta fario* L. (see Malmberg, 1993), Atlantic salmon, *Salmo salar* L. (see Cone & Cusack, 1988; Malmberg, 1993) and *Salvelinus fontinalis* (Mitchill) (see Cone & Cusack, 1988; Wells & Cone, 1990; Malmberg, 1993); as well as on native Mexican species, such as golden trout, *Oncorhynchus aguabonita* (Jordan) (see Cone *et al*., 1983), coho salmon *Oncorhynchus kisutch* (Walbaum) (see Cone *et al*., 1983) and *Oncorhynchus clarkii clarkii* (Richardson) (see Cone *et al*., 1983). This study represents the first report of *Gyrodactylus salmonis* from Mexican rainbow trout populations and the most reasonable explanation for its occurrence on this host is that the parasite was originally introduced with its translocated

fish host. It is not clear, however, whether rainbow trout acquired the parasite from other salmonids prior to its introduction or once in Mexico.

This new Mexican isolate of *G. salmonis* has been confirmed by molecular analysis, although subtle morphological differences in the marginal hook shape allow for the *G. salmonis* from Mexico to be discriminated from those in the USA and Canada, where this species is normally distributed. The alignment of the partial 18S sequence from the Mexican isolate of *G. salmonis* with those determined by Gilmore *et al*. (*unpublished results*) from a Canadian *G. salmonis* confirmed that they were identical matches.

Given that there is a general lack of suitability of using the 18S gene for discriminating certain *Gyrodactylus* species, and the 100% homology found across the ITS regions for these two populations of *G. salmonis*, the mitochondrial marker COI was also sequenced as a precautionary measure to provide further confidence in the results that were obtained. The COI showed a higher degree of variability when compared with the ITS region, but 1589 of the 1597 bases were identical with those of the American isolate of *G. salmonis*, *i.e.* 99.5% similar, confirming the identity of the Mexican isolate as *G. salmonis* (see Chapter 5; Rubio-Godoy *et al*., 2012).

8.1.4. The susceptibility of English and Welsh salmonids to G. salaris

To contribute to the British *G. salaris* contingency plans, one of the central issues in this study was to determine the relative susceptibilities of English and Welsh salmonid populations, in particular Atlantic salmon, brown trout and grayling, to *G. salaris*. Surveillance programmes in the UK, which are focused on the sampling of Atlantic salmon and monitoring of rainbow trout sites, have been based on the assumption that British salmonids would follow the same dynamics as their Scandinavian counterparts. While assessing the relative susceptibility and response of each population of salmonid to *G. salaris*, it was also important to consider which strain of *G. salaris* should be used. The

current study used *G. salaris* haplotype A, derived from the River Fusta in the Vefsna region of Norway, a strain which is commonly known to be pathogenic to Atlantic salmon as shown by a number of earlier studies (see Bakke & MacKenzie, 1993; Cable *et al*., 2000; Bakke *et al*., 2002, 2004). The *G. salaris* infection of Welsh salmon from the River Dee followed the predicted projected infection trajectory for Atlantic salmon with parasites increasing exponentially until the burden of parasites on the fish were at a level where the experiment was terminated on health and welfare grounds (*i.e.* a mean intensity ~4000 parasites $fish^{-1}$ in only 40 days). The rate of parasite increase 17% d^{-1}) on the River Dee salmon, however, was noticeably faster than that on the Norwegian control group of salmon (*i.e.* 5% d^{-1}). The brown trout population from the English River Tyne and grayling from the English River Nidd, in contrast, were both able to respond to their *G. salaris* infection. Peak infections were reached on 12 days post-infection (p.i.) for brown trout and 19 days p.i. for grayling; thereafter the number of parasites declined to near extinction over the remaining period of the trial which was terminated 110 days p.i.

The findings from this study are potentially important and although they demonstrate that brown trout can manage to control an infection of *G. salaris*, they can harbour low parasite intensities for longer periods than those reported for Norwegian stocks, *i.e.* 110+ days as opposed to 50 days. Given these extended windows of infection, the interpretation of the term "resistance" needs to be clarified and considered wisely, in terms of the role that brown trout could play in the event of a *G. salaris* outbreak in the UK. Currently, the relevant fish inspectorates throughout the UK regularly monitor the health of fish at high profile Atlantic salmon sites and rainbow trout farms. Any specimens of *Gyrodactylus* found on these fish will be identified to ensure that they are not *G. salaris*. Other sites throughout the UK are sampled less regularly; however, the results from this study suggest that brown trout should be considered carefully during a suspected outbreak.

Gyrodactylus salaris has been experimentally demonstrated to colonise several salmonid hosts other than Atlantic salmon (see *e.g.* Bakke *et al*., 1991a; Bakke & Jansen, 1991a, b; Bakke *et al*., 1992b, c; Bakke *et al*., 1996), but most of the records of *G. salaris* from across Europe are from rainbow trout (see Chapter 3), notably from countries where Atlantic salmon is not present, *e.g.* Italy. Given that the rainbow trout trade represents the largest risk in the dissemination of *G. salaris* between countries, this study suggests that it would be advisable, during a suspected outbreak, to ensure that brown trout sites, especially those in close proximity to rainbow trout farms are also carefully monitored. The potential role of brown trout in maintaining or spreading infections now needs consideration and to be factored into current management plans for containing or treating an infection, should it establish in the UK.

8.1.5. Alternative compounds for the treatment of G. salaris

The current cost of on-going surveillance programmes, river treatments and the impact on tourism from *G. salaris* in Norway is estimated to be about US$ 57M p.a. While farm-held stock can be readily treated using products such as formalin, infections in the wild represent a more challenging problem. The broad-spectrum biocide rotenone is widely used to eliminate parasites and hosts from infected rivers, which are consequentially either restocked with uninfected fish or allowed to repopulate by adult salmon returning to their native rivers to spawn. The environmental impact and loss of biodiversity through the use of rotenone though cannot be estimated. Although aluminium sulfate is now being trialled, which is given as a 10–14 day treatment to remove the parasite but not kill the salmon, the long-term impact that these sorts of treatments will have on the environment requires full evaluation. There is therefore a need for alternative compounds that are equally as effective but have minimal deleterious effects on the environment and non-target species. Much of the problem underlying the application of new compounds is linked to their licensing and

environmental impact. Many current studies of compounds are more academic in nature than directly practically applicable (see Brooker *et al.*, 2011). Such studies, however, provide a better knowledge of whether certain classes of compound could be used to control *Gyrodactylus* infections.

The current study set out to explore the potential utility of bronopol (Pyceze^{TM}), a product that is licensed in the UK for use in the aquaculture industry for the control of the oomycete *Saprolegnia parasitica* infecting salmonid fish and eggs (Pottinger & Day, 1999; Branson, 2002; Aller-Gancedo & Fregeneda-Grandes, 2007). Its use as a potential treatment for whitespot, *Ichthyophthirius multifiliis* Fouquet, 1876 (see Picon-Camacho *et al*., 2012; Shinn *et al*., 2012c), and the causative agent of "velvet disease", *Amyloodinium ocellatum* (Brown, 1931) Brown *et* Hovasse, 1946 (see Roberts-Thomson, 2007) has also been recently assessed. Although bronopol is a broad-spectrum disinfectant, it has not been tested on monogeneans before.

The findings from the trials conducted in this study show that significant reductions in the *G. salaris* population, when tested *in vitro*, can be achieved within an hour (*i.e.* 1 hour LC50 for *G. salaris* was ~384 ppm bronopol) although larger doses were required to effect the same percentage kill of *Gyrodactylus arcuatus* Bychowsky, 1933 a species commonly found on three-spined sticklebacks (*i.e.* 1 hour LC50 was ~810 ppm bronopol). Further work is now needed to test the efficacy of bronopol on infected hosts *in vivo* and to determine what concentrations are needed when deployed over longer periods (*i.e.* 10+ days) to obtain the same level of treatment as that achieved through the use of aluminium sulfate.

The greater sensitivity displayed by *G. salaris* is interesting and suggests that *G. arcuatus*, a species which is readily available in the UK, may serve as an appropriate laboratory model given that live cultures of *G. salaris* are not permitted in the UK. Likewise, the pilot trials conducted with tannic acid appear to show some promise and

these ideally now need repeating on larger numbers of specimens, by testing on *G. arcuatus*, and by exploring a number of delivery regimes to reduce the amount of chemical needed to control infections. Nevertheless, tannic acid, a naturally occurring polyphenolic compound derived from the breakdown of plant material is worthy of further study as a potentially efficacious compound for controlling *Gyrodactylus* infections, and more extensive testing, as detailed for bronopol, is needed. While these compounds do show some promise, their study in this PhD does not advocate their use in rivers, but hopefully this study does begin to provide more data on "other alternative compounds for consideration".

8.2. Future work

8.2.1. Investigating the presence of Gyrodactylus salaris *in other European countries*

The current distribution of *G. salaris* within Europe highlights the potential role of trading fish species in the spreading of this notifiable pathogen through countries. The *G. salaris* status in many European countries, however, remains unknown and the assessment of gyrodactylid material collected from these countries would help in determining the actual geographical distribution range of *G. salaris*. In each case, a combination of morphological and molecular methods, as recommended by OIE, should be used to confirm the identity of specimens and to remove any potential doubt regarding their confusion with morphologically similar species such as *G. thymalli* or *G. teuchis*.

8.2.2. Pathogenicity of Gyrodactylus *strains*

The difference in pathogenicity of *G. salaris* mitochondrial haplotypes has never been tested. While it was hoped that there might be an opportunity to compare haplotypes A and F on the same hosts in this study, sufficient parasite material to conduct the trial was

unavailable. A study investigating the potential variable pathogenicities resulting from infection by different haplotypes on the same host would be informative regarding the potential risk that translocated haplotype strains might have on naïve populations. Much of our current understanding results from assessing the susceptibility of different salmonids to a single haplotype (*i.e.* F, see Table 6.2 in Chapter 6). As detailed in the introduction of this thesis, a total of 15 *G. salaris* haplotypes have been identified using COI analysis (Hansen *et al.*, 2003; Meinilä *et al*., 2004; Kuusela *et al*., 2005; Hansen *et al*., 2007a, b; Robertsen *et al*., 2007; Paladini *et al*., 2009a); other strains may also exist and the pathogenicity that each of these causes to Atlantic salmon and other hosts should be evaluated. Likewise, the discovery of two new, potentially pathogenic, species of *Gyrodactylus* from gilthead seabream within the Mediterranean urgently requires evaluation to have a full appreciation of the risk they pose to aquaculture and wild stocks. At this time, it is not known whether gilthead sea bream is the principal host to these species or whether they originate from another economically important marine host species that is frequently farmed alongside gilthead seabream, *i.e.* European seabass, *Dicentrarchus labrax* (L.), or other sparids such as sharpsnout seabream, *Diplodus puntazzo* (Walbaum). The pathogenic potential of these two new *Gyrodactylus* species on these other hosts also requires establishing.

8.2.3. Treatment development

The results from the two treatment compounds that were trialled in this study appear encouraging and are worthy of further investigation. Further trials should not only employ larger numbers of specimens, and assess their effect on infected hosts, but also examine the mechanism of treatment delivery. Aluminium sulfate is currently deployed at low concentrations, <100 ppb $Al_2(SO_4)_3$, over a period of 10–14 days. Similar or better results may be produced using the current compounds; however, factors such as their

toxicity on the host, cost, impact on the environment and non-target species, and half-life require establishing.

8.2.4. Transcriptomics

In Chapter 6, the susceptibility of different salmonids to *G. salaris* was investigated. Understanding the mechanisms of host resistance to infection during the infection cycle is clearly of interest, since it may inform the development of techniques to help protect susceptible species. Broad-scale transcriptomic analysis techniques provide tools that could help dissect the mechanisms of host-pathogen interaction and of the host's defences against infection. The two major technologies that could be used to investigate this are oligo-microarray and RNA-seq, the latter of which uses high-throughput sequencing. The first steps in this direction have already been carried out as part of the current work and the results are being analysed and will be reported on in the near future.

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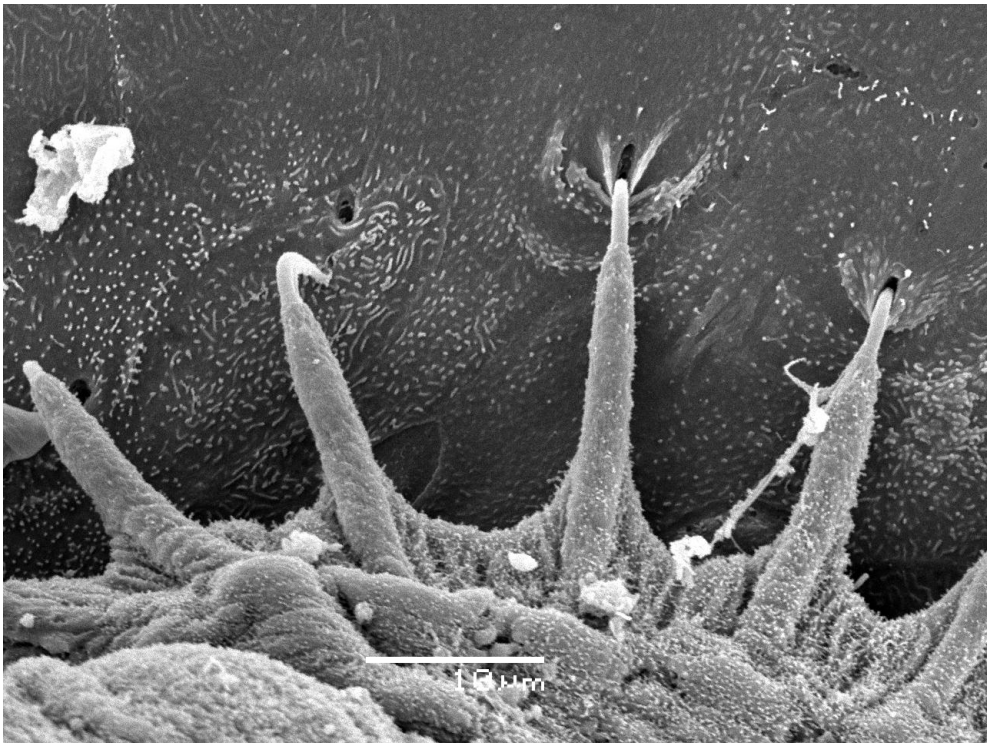

Scanning electron micrograph (SEM) of the marginal hooks of *Gyrodactylus salaris* Malmberg, 1957 penetrating the skin of Atlantic salmon, *Salmo salar* L. [original image]

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List of papers produced

and

Collaborative posters and oral communications delivered at conferences during the PhD

Giuseppe Paladini, *Homo sapiens* L. (Mammalia, Hominidae) [original image].

Awarded second prize as "Best Student Presentation" for the talk entitled "*Gyrodactylus*: tales of invasion, resistance and control strategies" presented at the conference *Aquaculture UK 2012, Aviemore, Scotland, 23rd-24th May 2012.*

Awarded second prize as "Best Poster Presentation" for the poster entitled "The potential impact of monogeneans on Italian fish stocks" presented at the *6th International Symposium on Monogenea (ISM6), Cape Town, South Africa, 2nd-7th August 2009.*

LIST OF PUBLISHED PAPERS DURING THE PHD

1. Yoon G.H., Al-Jufaili S., Freeman M.A., Bron J.E., **Paladini G.**, Shinn A.P. (2013). *Omanicotyle heterospina* n. gen. *et* n. comb. (Monogenea: Microcotylidae) from the gills of *Argyrops spinifer* (Forsskål) (Teleostei: Sparidae) from the Sea of Oman. *Parasites & Vectors*, 6: 170.

2. Rubio-Godoy M., **Paladini G.**, Freeman M., García-Vásquez A., Shinn A.P. (2012). Morphological and molecular characterisation of *Gyrodactylus salmonis* (Platyhelminthes, Monogenea) isolates collected in Mexico from rainbow trout (*Oncorhynchus mykiss* Walbaum). *Veterinary Parasitology*, 186: 289–300.

3. Schelkle B., **Paladini G.**, Shinn A.P., King S., Johnson M., van Oosterhout C., Mohammed R., Cable J. (2011). *Ieredactylus rivuli* gen. *et* sp. nov. (Monogenea, Gyrodactylidae) from *Rivulus hartii* (Cyprinodontiformes, Rivulidae) in Trinidad. *Acta Parasitologica*, 56 (4): 360–370.

4. **Paladini G.**, Hansen H., Fioravanti M.L., Shinn A.P. (2011). *Gyrodactylus longipes* n. sp. (Monogenea: Gyrodactylidae) from farmed gilthead seabream (*Sparus aurata* L.) from the Mediterranean. *Parasitology International*, 60: 410–418.

5. **Paladini G.**, Huyse T., Shinn A.P. (2011). *Gyrodactylus salinae* n. sp. (Platyhelminthes: Monogenea) infecting the south European toothcarp *Aphanius fasciatus* (Valenciennes) (Teleostei, Cyprinodontidae) from a hypersaline environment in Italy. *Parasites & Vectors*, 4: 100.

6. Grano-Maldonado M.I., Gisbert E., Hirt-Chabbert J., **Paladini G.**, Roque A., Bron J.E., Shinn A.P. (2011). An infection of *Gyrodactylus anguillae* Ergens, 1960 (Monogenea) associated with the mortality of glass eels (*Anguilla anguilla* L.) on the north-western Mediterranean Sea board of Spain. *Veterinary Parasitology*, 180: 323–331.

7. Rubio-Godoy M., **Paladini G.**, García-Vásquez A., Shinn A.P. (2010). *Gyrodactylus jarocho* sp. nov. and *Gyrodactylus xalapensis* sp. nov. (Platyhelminthes: Monogenea) from Mexican poeciliids (Teleostei: Cyprinodontiformes), with comments on the known gyrodactylid fauna infecting poeciliid fish. *Zootaxa*, 2509: 1–29.

8. Shinn A.P., Collins C., García-Vásquez A., Snow M., Matějusová I., **Paladini G.**, Longshaw M., Lindenstrøm T., Stone D.M., Turnbull J.F., Picon-Camacho S., Vázquez Rivera C., Duguid R.A., Mo T.A., Hansen H., Olstad K., Cable J., Harris P.D., Kerr R., Graham D., Monaghan S.J., Yoon G.H., Buchmann K., Taylor N.G.H., Bakke T.A., Raynard R., Irving S., Bron J.E. (2010). Multi-centre testing and validation of current protocols for *Gyrodactylus salaris* (Monogenea) identification. *International Journal for Parasitology*, 40: 1455–1467.

9. **Paladini G.**, Cable J., Fioravanti M.L., Faria P.J., Shinn A.P. (2010). The description of *Gyrodactylus corleonis* sp. n. and *G. neretum* sp. n. (Platyhelminthes: Monogenea) with comments on other gyrodactylids parasitising pipefish. *Folia Parasitologica*, 57: 17–30.

10. **Paladini G.**, Gustinelli A., Fioravanti M.L., Hansen H., Shinn A.P. (2009). The first report of *Gyrodactylus salaris* Malmberg, 1957 (Platyhelminthes, Monogenea) on Italian cultured stocks of rainbow trout (*Oncorhynchus mykiss*). *Veterinary Parasitology*, 165: 290–297.

11. **Paladini G.**, Cable J., Fioravanti M.L., Faria P.J., Di Cave D., Shinn A.P. (2009). *Gyrodactylus orecchiae* sp. n. (Monogenea: Gyrodactylidae) from farmed population of gilthead seabream (*Sparus aurata*) in the Adriatic Sea. *Folia Parasitologica*, 56: 21–28.

12. **Paladini G.**, Tarsi L., Minardi D., Fioravanti M.L. (2009). The parasite fauna of *Scomber scombrus* L. caught off the Adriatic Sea and of imported *Scomber japonicus* Houttuyn. *Ittiopatologia*, 6: 211–219 [In Italian, with English summary].

13. **Paladini G.**, Quaglio F., Fioravanti M.L. (2008). New knowledge on fish microsporidiosis. *Ittiopatologia*, 5: 179–186 [In Italian, with English summary].

14. Minardi D., **Paladini G.**, Whittington I.D., Fioravanti M.L. (2008). Taxonomic study on Capsalidae (Monogenea), parasites of teleosts and elasmobranchs held in captivity. *Ittiopatologia*, 5: 171–178 [In Italian, with English summary].

15. **Paladini G.**, Gustinelli A., Fioravanti M.L., Minardi D., Prearo M. (2008). Redescription of *Thaparocleidus vistulensis* (Monogenea: Ancylodiscoididae) from Wels catfish (*Silurus glanis* L.) from Po river and taxonomic status of the genus. *Ittiopatologia*, 5: 129–138 [In Italian, with English summary].

LIST OF PAPERS IN ADVANCED STAGE OF PREPARATION

1. **Paladini G.**, Williams C., Hansen H., Taylor N.G.H., Rubio O.L., Denholm S.J., Hytterød S., Bron J.E., Shinn A.P. (*in prep.*). The experimental susceptibility of English and Welsh salmonids to *Gyrodactylus salaris* (Platyhelminthes, Monogenea).

2. **Paladini G.**, Bron J.E., Shinn A.P. (*in prep.*). Geographical distribution of *Gyrodactylus salaris* Malmberg, 1957 (Monogenea, Gyrodactylidae).

3. **Paladini G.**, Taylor N.G.H., Harris P.D., Shinn A.P. (*in prep.*). Bronopol *vs Gyrodactylus* spp.: an *in vitro* treatment.

4. Rubio-Godoy M., García-Vásquez A., **Paladini G.**, Freeman M., Shinn A.P. (*in prep.*). Morphological and molecular description of *Gyrodactylus tomahuac* n. sp. (Monogenea) from the blackfin goodea, *Goodea atripinnis* (Cyprinodontiformes: Goodeidae); including a comparison to congeners parasitising poeciliid fish.

5. Shinn A.P., **Paladini G.** (*in prep.*). *Gyrodactylus abruptus* n. sp. and *Gyrodactylus pilittae* n. sp. (Gyrodactylidae: Monogenea): two new species rediscovered.

COLLABORATIVE POSTERS AND ORAL COMMUNICATIONS DELIVERED AT CONFERENCES DURING THE PHD

1. **Paladini G.** (2012). *Gyrodactylus*: tales of invasion, resistance and control strategies. *Aquaculture UK 2012, Aviemore, Scotland, 23rd-24th May 2012* (talk). **Awarded second prize as "Best Student Presentation"**

2. **Paladini G.**, Rubio-Godoy M., Domingues M.V., Whittington I.D., Shinn A.P., Bron J.E. (2011). A beginner's guide to "flukology". *Proceedings of the VIII International Symposium of Fish Parasites (ISFP8), Viña del Mar, Chile, 26th-30th September 2011*: 73-74 (talk).

3. **Paladini G.**, Rubio-Godoy M., Freeman M.A., García-Vásquez A., Shinn A.P. (2011). *Gyrodactylus salmonis*: a strain-ed relationship. *Proceedings of the VIII International Symposium of Fish Parasites (ISFP8), Viña del Mar, Chile, 26th-30th September 2011*: 74 (talk).

4. Shinn A.P., **Paladini G.**, Rubio-Godoy M., Whittington I.D., Domingues M.V., Bron J.E. (2011). MonoDb: work in progress for a centralized web-based resource for the class Monogenea. *Proceedings of the 86th Annual Meeting, American Society of Parasitologists, Anchorage, Alaska, 1st-4th June 2011*: 44 (talk).

5. Rubio-Godoy M., **Paladini G.**, Freeman M.A., García-Vásquez A., Shinn A.P. (2011). Description of a new strain of *Gyrodactylus salmonis* (Platyhelminthes, Monogenea) collected in Mexico from rainbow trout (*Oncorhynchus mykiss* Walbaum): morphological and molecular characterization. *Proceedings of the 86th Annual Meeting, American Society of Parasitologists, Anchorage, Alaska, 1st-4th June 2011*: 92 (talk).

6. **Paladini G.**, Fioravanti M.L., Caffara M., Minardi D., Righetti M., Prearo M. (2010). The distribution of *Gyrodactylus salaris* Malmberg, 1957 (Monogenea, Gyrodactylidae) in northern and central Italy. Parassitologia, 52 (1-2): 361. (*XXVI Congresso Nazionale SoIPa (Società Italiana di Parassitologia), Perugia, Italy, 22nd-25th June 2010* - talk)

7. Minardi D., **Paladini G.**, Righetti M., Arsieni P., Pavoletti E., Prearo M. (2010). Ritrovamento di *Aspidogaster limacoides* Diesing, 1835 in *Rutilus pigus* (Lacepède, 1803) proveniente dal Lago Maggiore, Italia. *Proceedings of the XVI Convegno Nazionale SIPI (Società Italiana di Patologia Ittica), Orvieto (TR), Italy, 27th-29th May 2010*: 53 (poster).

8. **Paladini G.**, Hansen H., Fioravanti M.L., Shinn A.P. (2009). The potential impact of monogeneans on Italian fish stocks. *Proceedings of the 6th International Symposium on Monogenea (ISM6), Cape Town, South Africa, 2nd-7th August 2009*: P15 (poster). **Awarded second prize as "Best Poster Presentation"**

9. Minardi D., **Paladini G.**, Prearo M., Freeman M.A., Shinn A.P. (2009). Morphological and molecular characterisation of *Gussevia asota* Kritsky, Thatcher *et* Boeger, 1989: new notes on an old friend. *Proceedings of the 6th International Symposium on Monogenea (ISM6), Cape Town, South Africa, 2nd-7th August 2009*: P11 (poster).

10. Akoll P., Scheimer F., Fioravanti M.L., **Paladini G.**, Shinn A.P., Konecny R., Rutaisire J. (2009). Cichlidogyrus Paperna, 1960 fauna of Nile Tilapia (*Oreochromis niloticus* L.) from Lake Kyoga and Lake Victoria, Uganda. *Proceedings of the 6th International Symposium on Monogenea (ISM6), Cape Town, South Africa, 2nd-7th August 2009*: P2 (poster).

11. **Paladini G.**, García-Vásquez A., Shinn A.P. (2009). Tilapia – the "Aquatic chicken" and its monogenean parasites: food for thought. *Proceedings of the 6th International Symposium on Monogenea (ISM6), Cape Town, South Africa, 2nd-7th August 2009*: C61 (talk).

12. Shinn A.P., **Paladini G.**, Whittington I.D., Harris P.D., Littlewood T., Bakke T.A., Cable J., Christison K.W., Domingues M.V., Rubio-Godoy M., Conway D., Bron J.E. (2009). MonoDb: First steps in the provision of a centralised web-based resource for the Monogenea. *Proceedings of the 6th International Symposium on Monogenea (ISM6), Cape Town, South Africa, 2nd-7th August 2009*: I5 (talk).

13. Minardi D., Whittington I.D., Kearn G., **Paladini G.**, Shinn A.P., Fioravanti M.L., Bron J.E. (2009). Preliminary observations on the reproductive system of *Neoentobdella diadema* (Monticelli, 1902 Kearn *et* Whittington, 2005 (Monogenea: Capsalidae). *Proceedings of the 6th International Symposium on Monogenea (ISM6), Cape Town, South Africa, 2nd-7th August 2009*: C13 (talk).

14. **Paladini G.**, Florio D., Caffara M., Gustinelli A., Quaglio F., Fioravanti M.L. (2008). Descrizione di *Loma acerinae* (Microsporidia) in Gobidi pescati nel Mar Adriatico. *Proceedings of the XV Convegno Nazionale SIPI (Società Italiana di Patologia Ittica), Erice (TP), Italy, 22nd-24th October 2008*: 33 (talk).

Metamorphosis of a PhD student

First day (end of 2008)

Submission day (end of 2012)