A STUDY OF THE AETIOLOGY AND CONTROL OF RAINBOW TROUT GASTROENTERITIS

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by

JORGE DEL POZO GONZALEZ BVM MRCVS MSc

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To Begoña García, with all my love

I could have never done it without you

Declaration

I declare that this thesis has been composed in its entirety by me. Except where specifically acknowledged, the work described in this thesis has been conducted by me and has not been submitted for any other degree.

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Signature of supervisor: _____

Date: _____

Abstract

Disease has been identified as a major problem in the aquaculture industry for the welfare of the fish stocked as well as for its economic impact. The number of diseases affecting cultured fish has increased significantly during recent years with the emergence of several conditions that have added to the overall impact of disease on the industry. Frequently, a lack of scientific knowledge about these diseases is compounded by an absence of effective treatment and control strategies. This has been the case with rainbow trout gastroenteritis (RTGE), an emerging disease of rainbow trout (*Oncorhynchus mykiss* Walbaum). This study investigated several aspects related to its aetiology and control.

A retrospective survey of UK rainbow trout farmers was undertaken to ascertain the extent and severity of RTGE in the UK as well as to identify RTGE risk factors at the site level. Participants in this study accounted for over 85% of UK rainbow trout production in 2004. It was found that the total number of RTGE-affected sites had risen from 2 in the year 2000 to 7 in 2005. The disease was only reported from sites producing more than 200 tonnes of trout/year for the table market. Analysis of risk factors associated with RTGE at the site level showed that this syndrome was associated with large tonnage and rapid production of rainbow trout for the table market. The data collected during this study enabled the identification of those sites that were most likely to present with RTGE the following year and this information was used to study the epidemiology of RTGE at the unit level.

A prospective longitudinal study was undertaken in 12 RTGE-affected UK sites. It described in detail the impact, presentation, current control strategies and spread pattern of RTGE within affected UK sites. The risk factors associated with RTGE presence and severity were also investigated. Data were collected for each productive unit (*i.e.* cage, pond, raceway or tank) on the mortalities, fish origin, site management and environmental factors. RTGE was identified using a case definition based on gross pathological lesions. Analysis of these data revealed that RTGE behaved in an infectious manner. This conclusion was supported by the presence of a pattern typical of a propagating epidemic within affected units. Also, the risk of an unaffected unit becoming RTGE positive was increased if it had received fish from or was contiguous to a RTGE-affected unit. The presentation also suggested an incubation period of 20-25 days. Risk factor analysis identified management and environmental risk factors for RTGE, including high feed input and stressful events, which could be used to generate a list of control strategies.

A study of the histopathological and ultrastructural presentation of RTGE was conducted. The location of segmented filamentous bacteria (SFB) and pathological changes found in affected fish were examined. Pyloric caeca were the digestive organ where SFB were found more frequently and in higher numbers, suggesting that this was the best location to detect SFB in RTGE-affected trout. Scanning and transmission electron microscopy revealed a previously undescribed interaction of SFB with the mucosa of distal intestine and pyloric caeca and this included the presence of attachment sites and SFB engulfment by enterocytes, as previously described in other host species. The SFB were not always adjacent to the pathological changes observed in the

digestive tract of RTGE-affected trout. Such changes included cytoskeletal damage and osmotic imbalance of enterocytes, with frequent detachment. These observations suggested that if SFB are indeed the cause of RTGE their pathogenesis must involve the production of extracellular products.

Analysis of the gross presentation and blood biochemistry in RTGE-affected fish was used to examine the patho-physiologic mechanisms of RTGE. To enable identification of positive RTGE cases for this study, a case definition was created from the information available on RTGE gross presentation in the literature. This case definition was assessed in a sample including 152 fish cases and 152 fish controls from 11 RTGE-affected UK sites, matched by unit of origin. The analysis of these fish using bacteriology, packed cell volume (PCV) and histopathology revealed that RTGE occurred simultaneously with other parasitic and bacterial diseases in a percentage of fish identified with this case definition. With the information gained after analysing the gross presentation, RTGE-affected fish without concurrent disease were selected for the study of the pathogenesis, which included blood biochemical analyses. These analyses revealed a severe osmotic imbalance, and a reduced albumin/globulin ratio suggesting selective loss of albumin, typical for a protein losing enteropathy.

The role of the SFB "*Candidatus* arthromitus" in the aetiology of RTGE was assessed using a newly developed "*C.* arthromitus"-specific polymerase chain reaction assay (PCR) in conjunction with histological detection. This technique was applied to eight different groups of trout, including an RTGE-affected group and seven negative control groups. This analysis was conducted on DNA extracted from paraffin wax-embedded tissues as well as fresh intestinal

contents. The results revealed the presence of "*C.* arthromitus" DNA in apparently healthy fish from sites where RTGE had never been reported. Additionally, SFB were observed histologically in two trout from an RTGE-free hatchery. These findings do not permit the exclusion of "*C.* arthromitus" as the aetiological agent for RTGE, although they suggest that the presence of these organisms in the digestive system of healthy trout is not sufficient to cause clinical disease, and therefore other factors are necessary.

In conclusion, this study has used a multidisciplinary approach to the study of RTGE which has generated scientific information related to the epidemiology, pathogenesis and aetiology of this syndrome. The results of this project have suggested priority areas where further work is required, including experimental transmission of RTGE, field assessment of the control strategies proposed and further investigation into the aetiology of RTGE.

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CHAPTER 1. General Introduction

1.1. The relevance of rainbow trout gastroenteritis

1.1.1. Aquaculture in the world, Europe and the UK

Fish are an integral part of the human diet, and evidence of widespread global use of sea foods by humans is reported as early as 20,000 years ago in the fossil record (Scarre 1993). Fisheries production has been increasing ever since (Figure 1.1), as a direct result of the growth of the world's human population, especially during the last six decades (FAO 2006). Most of the fisheries production is destined for human nutrition and in 2005, 75% of the fisheries production was used for this purpose (FAO 2006).

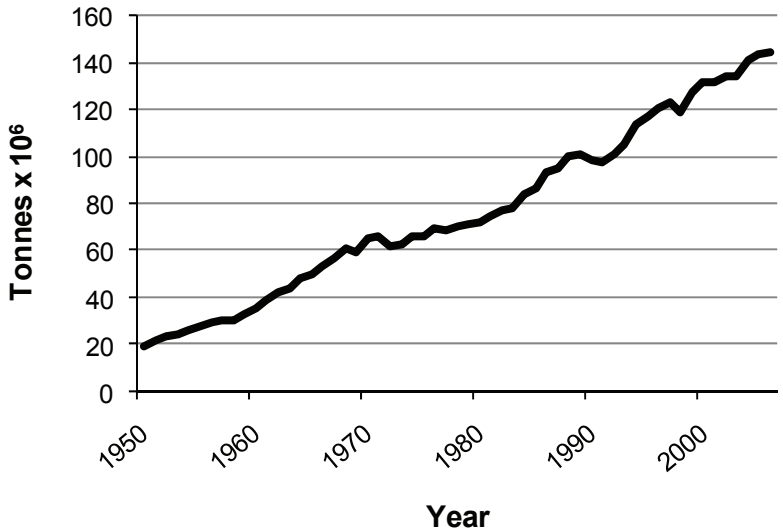

Figure 1.1. Global fisheries production trend from 1950 to 2006. Production is expressed as millions of tonnes. Data provided by the Food and Agriculture Organization (FAO) of the United Nations.

Before aquaculture, humans relied exclusively in the capture of wild fish from the environment, but eventually learnt how to rear fish in captivity, perhaps as a result of sedentarism (Rabanal 1988). It is believed that fish culture practices were first developed in China during the period from 2000-1000 B.C., where

carp, *Cyprinus carpio* L were cultured initially by the emperors (Rabanal 1988; Hickling 1971). Now, aquaculture practices have extended all over the world and the relative contribution of aquaculture to the global fisheries production has grown steadily over the last two decades (Figure 1.2). In 2006, aquaculture production contributed to 35% of the global fishery production, which was approximately 144 million tonnes (FAO 2008a; FAO 2008b). Aquaculture has grown more rapidly than all other animal food-producing sectors, with an average annual growth rate for the world of 8.8% per year since 1970, compared with only 1.2% for capture fisheries and 2.8% for terrestrial farmed meat production systems (FAO 2006). However, there are signs that the rate of growth for global aquaculture may have peaked, and although the growth in production of the different major species groups continues, the increases seen so far this decade are less dramatic than the extraordinary growth rates achieved in the 1980s and 1990s. Thus, while the trend for the near future appears to be one of continued increases in production, the rate of these increases may be declining (FAO 2006).

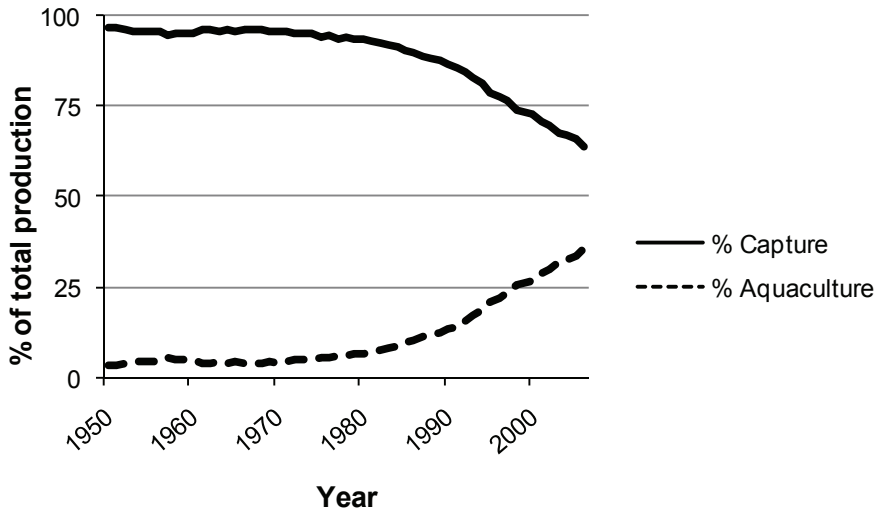

Figure 1.2. Relative contribution of capture and aquaculture to the global fisheries production from the year 1950 to the year 2006. Data provided by the Food and Agriculture Organization of the United Nations (FAO).

In Europe, fishery and aquaculture production follow similar trends, although the relative contribution of aquaculture to the fisheries production has not been as marked as it has been globally, and in 2006 aquaculture contributed to only 16% of the European fisheries production, which was approximately 16 million tonnes (FAO 2008a; FAO 2008b). Nonetheless, aquaculture in Europe produces a significant volume of fish per year and in 2006 it produced 2.2·million tonnes, with an approximate value of 4000 million British pounds (FAO 2008a).

The United Kingdom (UK) is a special example within the global trend, as although aquaculture production has grown markedly since the 1980s. Before that, the UK aquaculture production was negligible (FAO 2008a). This has resulted in a relatively fast rate of growth of the UK aquaculture industry when compared with the rest of the world, which is reflected in a faster increase in the relative contribution of aquaculture to the UK fisheries production (Figure 1.3). This trend also reflects an apparent drop in the growth of the UK aquaculture industry, commencing in the year 2005. It is possible that this drop indicates a trend or a transient effect, although overall, the aquaculture production in the UK in the year 2006 totalled 0.2 million tonnes with a value of nearly 450 million British pounds and represented 22% of the total UK fisheries production.

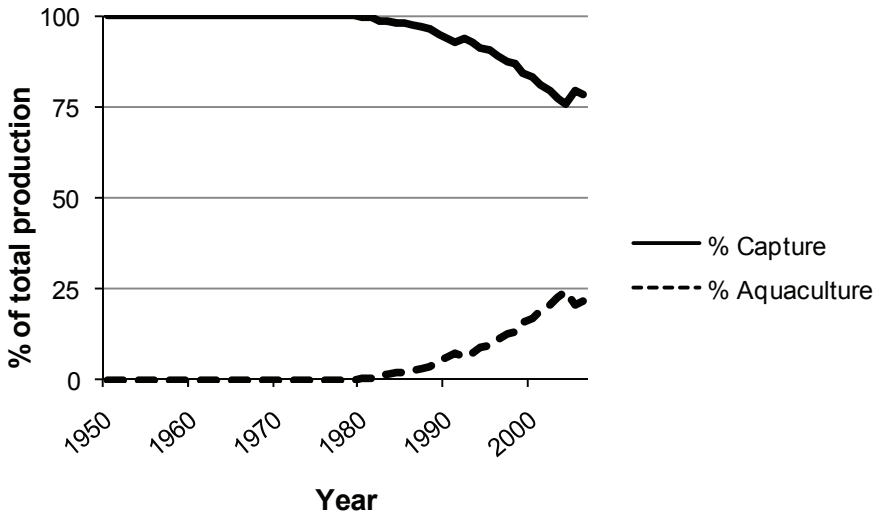

Figure 1.3. Relative contribution of capture and aquaculture to the UK fisheries production from the year 1950 to the year 2006. Data provided by the Food and Agriculture Organization of the United Nations (FAO).

In the UK, most aquaculture production takes place in salt water (94%), with the remainder of the production taking place in fresh water (FAO 2008a). This is reflected in the production of each cultured species (Figure 1.4). The majority of the production of the UK is comprised of Atlantic salmon (*Salmo salar* Linnaeus) and blue mussel (*Mytilus edulis* Linnaeus), both harvested at sea. Rainbow trout (*Oncorhynchus mykiss* Walbaum) was third in production volume in 2006. The UK rainbow trout industry will be the focus of interest in this study.

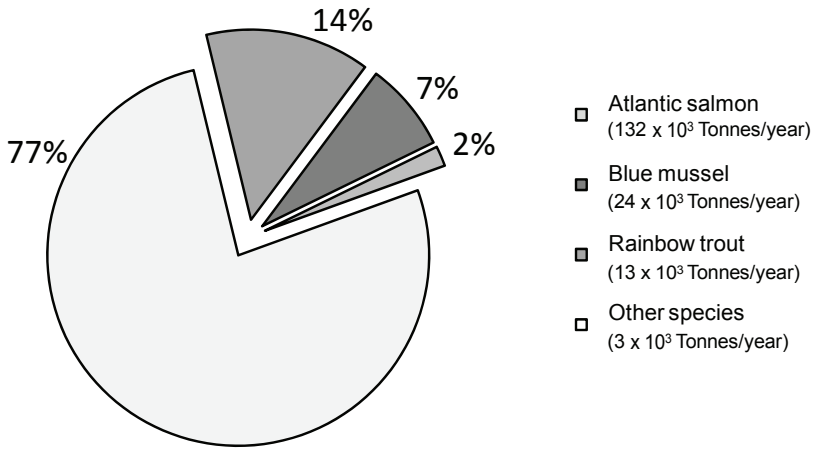

Figure 1.4. Relative contribution of each farmed species to the total UK aquaculture production for 2006 (Total 2006 production: 172,000 tonnes). Food and agriculture organization of the United Nations (FAO).

1.1.2. Rainbow trout in aquaculture

Rainbow trout are ray-finned teleost fish included in the family Salmonidae (Behnke 1992). As such, they have an elongated, fusiform body shape and present a forked tail, pelvic fins located far back and a dorsal adipose fin. Their colouration can vary with habitat, size, and sexual condition, but generally present a blue to olive green colour above a pink band along the lateral line and silver colour below (Figure 1.5). Taxonomically, the species was originally named *Salmo mykiss* by Johann Julius Walbaum in 1792 based on type specimens from Kamchatka peninsula (Russia). Richardson named a specimen of this species *Salmo gairdneri* in 1836, and in 1855, W. P. Gibbons found a population and named it *Salmo iridia*, later corrected to *Salmo irideus*. However, both *S. gairdneri* and *S. irideus* scientific names were deprecated once it was determined that Walbaum's type description was conspecific. More recently, DNA studies showed rainbow trout are genetically closer to Pacific salmon (*Oncorhynchus* species) than to brown trout (*Salmo trutta*) or Atlantic salmon (*S. salar*), so the genus was changed and today this species is known as *Oncorhynchus mykiss* Walbaum (Behnke 1992).

Figure 1.5. Drawing of a rainbow trout (*Oncorhynchus mykiss* W.) illustrating the main morphological features of this species as well as the typical colouration. Public domain image from the U.S. Fish and Wildlife Service Source (http://images.fws.gov/)

Rainbow trout are native to the Pacific drainages of North America, from Alaska to Mexico, the Russian Kamchatka peninsula and the Okhothsk sea (Behnke 1992). Since 1874 this species has been introduced to waters on all continents except Antarctica, for recreational angling and aquaculture purposes (MacCrimmon 1971).

Rainbow trout are suitable for aquaculture for a variety of reasons (Cowx 2005; Sedgwick 1990). It is hardy, easy to spawn, fast growing, tolerant of a wide range of environments and handling, and the large fry can be easily weaned on to an artificial diet. Pelleted feed is available for all the life stages, enabling the production of high-protein and high-energy diets with 35-45% protein and 22% or more fat, which rainbow trout convert efficiently, often at food conversion ratios (FCRs) close to 1:1. Also, it is possible to produce populations of all-female rainbow trout, therefore avoiding early maturation of male fish. Additional advantages of all-female trout include higher FCRs and resistance to handling and disease (Cowx 2005). Finally, there are several outputs for rainbow trout culture, including food products, live fish for recreational game fishing and the sale of eggs and fry from hatcheries to other farms.

The commercial rearing of rainbow trout was facilitated in early 1900s by a Danish trout farmer who designed a production site where fresh water flowed through each fish pond, a design that radically improved the productivity simultaneously reducing the environmental challenge to the fish stocked (Sedgwick 1990). The global rainbow trout aquaculture has grown exponentially since the 1950s, initially in Europe and more recently in Chile (FAO 2008a; FAO 2006). In 2006, rainbow trout was produced in a total of 58 countries with Chile the largest producer (Figure 1.6). In this country the

majority of rainbow trout is produced in salt or brackish water. Other major producing countries included Norway, Iran, Denmark, France, Italy, United States of America (USA), Spain, Germany, Poland, China and the UK, which was the 12th highest producer worldwide in 2006 (FAO 2008a).

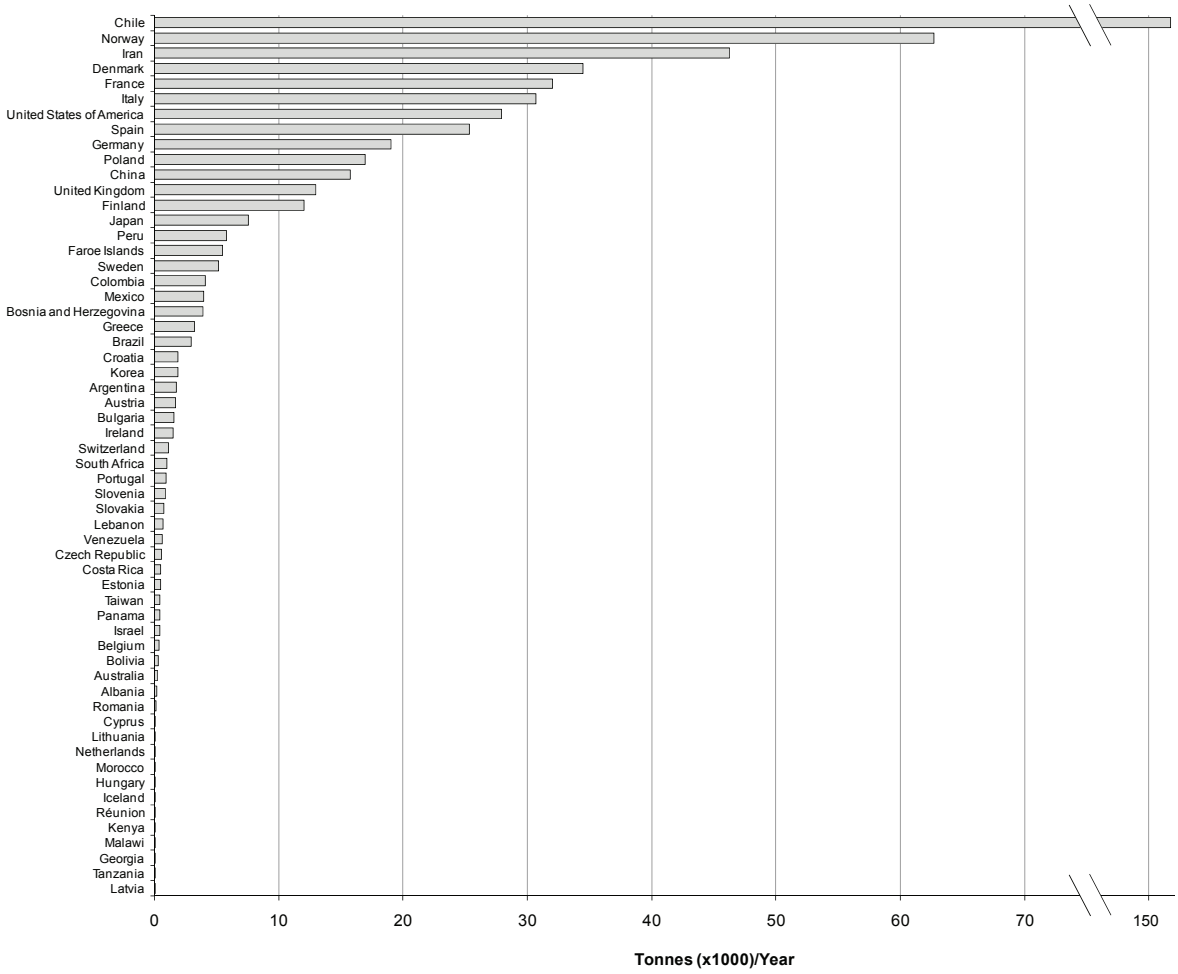

Figure 1.6. Total rainbow trout (*Oncorhynchus mykiss* W.) aquaculture production for 2006 by country (total=550,000 tonnes). Food and agriculture organization of the United Nations (FAO).

Until the end of the Second World War the UK trout industry consisted of less than 20 restocking farms (*i.e.* sites producing trout destined to stock rivers or lakes to facilitate fishing). In 1950, a Danish entrepreneur opened the first table farm in Lincolnshire (*i.e.* producing trout for human consumption), and in 1960 pelleted feeds were introduced. This last development contributed to the

expansion of the rainbow trout industry in the UK, which has a size of almost 360 trout farms at present (Read 2008).

Most UK aquaculture trout production (Tyson 2008; FAO 2008a) is comprised of rainbow trout (96.3%), although other trout species are also produced, including brown trout (*S. trutta* L.; 3.6%) and brook trout (*Salvelinus fontinalis* L.; 0.1%). When examining the trend of rainbow trout aquaculture production for the UK from 1970 (Figure 1.7), one can observe a phase of exponential growth from the 1980s to the 1990s, and a period of slower growth thereafter, suggesting that the industry has reached stability. In 2006, approximately 18,000 tonnes of rainbow trout were produced in the UK with an approximate total value of 36 million British pounds. Of this, most of the production (78%) was destined for the table market and the rest (22%) was destined for restocking (FAO 2008a).

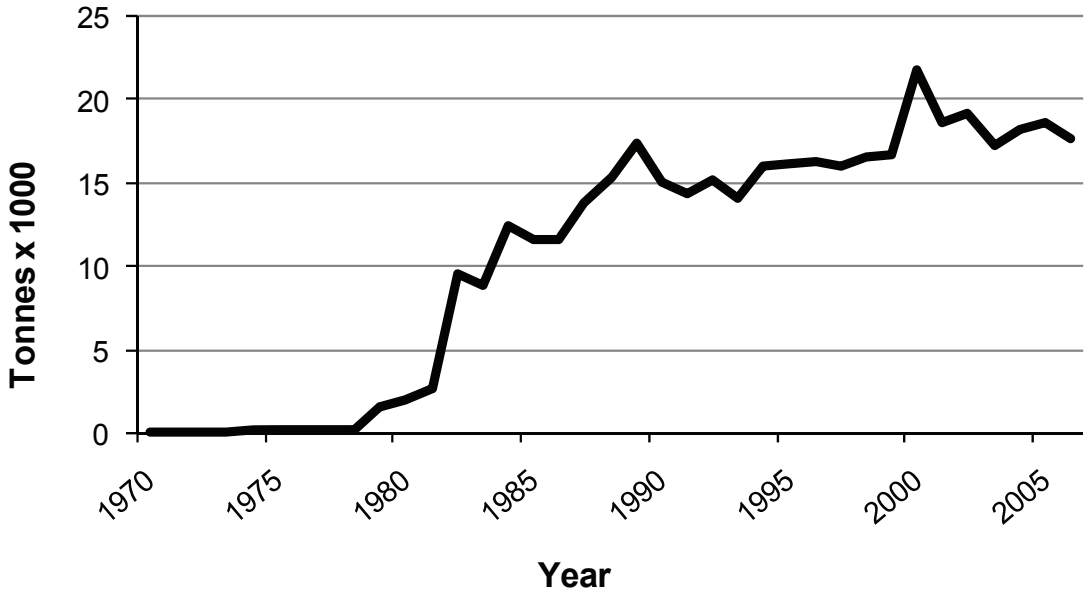

Figure 1.7. UK rainbow trout (*Oncorhynchus mykiss* W.) aquaculture production from 1970 to 2006. Food and Agriculture Organization of the United Nations (FAO).

These statistics suggest that rainbow trout aquaculture in the UK is a successful industry, although over the course of its history, several ongoing issues have been identified that have hindered further growth. Among these issues, the profit margins, the environmental impact of trout sites, decreased availability of protein sources for feed and fish disease were most important (Cowx 2005).

As with any business, rainbow trout farms aim to increase revenue and reduce expenditure. This can be done by using the best value feed, seed and materials, and achieving an efficient FCR. The average cost of production stems mainly from the cost of the fish, husbandry, feed and veterinary and medicine costs, and has been estimated at 0.7-1.1 British pounds/kg (Cowx 2005). Monoculture is the most common practice and intensive systems are considered necessary in most situations to make the operation economically attractive (Cowx 2005).

In the UK, the size of farm considered viable for table trout production has been moving upwards (Read 2008). Trout farms impact their surrounding environment in different ways (Cowx 2005), including the biochemical alteration of water and sediment by uneaten feed, fish excreta and disease treatment chemicals, the transmission of disease to vulnerable wild populations and the potential impact of escapees on wild stocks. The need to maintain these factors within acceptable levels imposes several costly requirements on the design of trout farms, as well as a limit to the productivity of each site, both of which are enforced in the UK by the environment agency (EA) in England and Wales and the Scottish environment protection agency (SEPA) in Scotland.

The decreasing availability and increasing cost of fish meal, the major source of protein in commercial trout diets, have led to the widespread consideration of alternative sources of protein in the formulation of feeds for use in the fish farming industry (Cowx 2005). Soybean protein is one of the most promising plant protein sources as a fish meal substitute. Recommendations for soya product incorporation in salmonid diets vary among the studies. Excessively high inclusion levels of soya products have been found to affect palatability, feed conversion and intestinal integrity, although the exact causal links between these effects are not yet known (Bakke-McKellep *et al.*, 2007; Kaushik 2007; Sanden *et al.*, 2005).

The impact of fish disease to the UK rainbow trout industry is directly relevant to understand the importance this study for the industry and is explained in detail in the following section (1.1.3.).

1.1.3. Impact of diseases on the UK trout farming industry

The presence of disease in rainbow trout-producing sites has a negative influence on the industry for several reasons, including economic, welfare, environmental and public health. In fact, disease has been recognised as one of the greatest challenges facing trout farmers in the UK at the current time (MacIntyre 2008; Read 2008; North *et al.* 2008; Wall 2008).

The economic impact of fish mortalities stems from fish that have died at the site, which cannot be sold for human consumption (Regulation (EC) No 1774/2002 2002), or fish that have been rejected or downgraded at the processing plant as well as the additional costs for appropriate disposal of these fish. Loss of investment is higher the closer the fish is to complete the

productive cycle, as the investment in feed input and husbandry is continuous for each fish during the whole cycle. Some diseases also cause reduction in the growth rate of diseased trout (Speare *et al.* 1998; Beamish *et al.* 1996). A recent study has shown that the primary risk factor associated with deteriorating rainbow trout welfare in the UK is disease, irrespective of which disease is involved and how many diseases the population has been exposed to (MacIntyre 2008). Diseases can have important environmental consequences through their impact on wild fish populations (Murray & Peeler 2005). Public health issues related directly to rainbow trout disease are rare although both *Clostridium botulinum* and *Lactococcus garviae* have been diagnosed in rainbow trout in the UK (Scott 2002; Bark & McGregor 2001; Cann & Taylor 1982). In the former the risk to public health was considered low, as the toxin was never isolated from the flesh of affected fish (Cann & Taylor 1982). Human disease in connection with these outbreaks has not been reported, although these two conditions are potentially zoonotic (Sobel 2005; Fefer *et al.* 1998; Elliot *et al.* 1991).

For all of these reasons, the reduction or elimination of disease is in the best interest of the UK rainbow trout industry. Several factors have conspired against this, including a low number of licensed treatments (Read 2008) as well as the lack of scientific information on specific rainbow trout diseases. The importance of research on fish disease for the aquaculture industry is high, as shown by the distribution of the UK aquaculture research and development (R&D) investment from 1999 to 2006, where fish disease research received 56% of all the expenditure (James 2006). The present study aimed to contribute

to this effort by supplying further scientific evidence on a specific rainbow trout disease.

Several disease entities have been reported in the UK rainbow trout industry, including infectious and non infectious diseases. The most important diseases are infectious and parasitic (Table 1.1) and together cost the industry approximately 5 million British pounds/year (Read 2008).

Table 1.1. List of common diseases of farmed rainbow trout in the UK (DEFRA; http://defra.gov.uk).

Common disease name	*Abbreviation*	*Aetiology*	*Type*
Enteric redmouth	ERM	*Yersinia ruckeri*	Bacterial
Furunculosis	"Furunc"	*Aeromonas salmonicida*	Bacterial
Proliferative kidney disease	PKD	*Tetracapsuloides bryosalmonae*	Parasitic
Rainbow trout fry syndrome	RTFS	*Flavobacterium psychrophilum*	Bacterial
White spot	"Ich"	*Ichthyophthirius multifiliis*	Parasitic

Taken from the DEFRA website on 31st January 2009. Detailed pathological descriptions for each one of these rainbow trout diseases can be found in Austin (2007), Ferguson (2006) and Roberts (2001).

Despite the importance of the diseases listed in Table 1.1, these are not the only diseases in the UK rainbow trout industry and notifiable and emerging diseases have been also reported in UK sites.

An updated list of notifiable diseases of fish was supplied by DEFRA in their website (http://www.defra.gov.uk/corporate/regulat/forms/fish/DOF21.pdf). The regulations designed to control disease impose large costs in terms of trade restrictions, surveillance, and control or eradication programmes (Murray & Peeler 2005). In 2006, an outbreak of viral haemorraghic septicaemia (VHS) was diagnosed in a single UK site on the river Ouse. The positive diagnosis of VHS at this site resulted in the culling and disposal of all fish on the farm, the disinfection and fallowing of the site and the restriction of movements of live and

dead fish from all the fish farms in the catchment (VHS National Control Centre 2006).

An ‘‘emerging disease’’ has been defined as a new disease, a new presentation of a known disease (*e.g.* increased severity or appearance in a new species) or an existing disease that appears in a new geographical area (Brown 2000). Numerous diseases have emerged as serious economic or ecological problems in aquaculture through pathogen exchange with wild populations, evolution from non-pathogenic micro-organisms and anthropogenic transfer of stocks (Murray & Peeler 2005). In the UK rainbow trout industry, perhaps the most relevant emerging diseases of recent years have included sleeping disease (McLoughlin & Graham 2007), strawberry disease/red mark syndrome (Ferguson *et al.* 2006); (Irving *et al.* 2006) and rainbow trout gastroenteritis (Branson 2003). The establishment of these has been facilitated by intensive aquaculture practices, which frequently result in high population densities and stressful factors (such as concurrent disease) which increase the risk of infection establishment and spread (North *et al.* 2006; Pickering & Pottinger 1989). For all the reasons previously explained, it is necessary to reduce the impact of these emerging diseases and this can be achieved by the application of biosecurity programmes together with the use of adequate treatments (Murray & Peeler 2005). Information on the risk factors, aetiology and pathogenesis associated with a specific disease is required for the appropriate design of treatments and control strategies (Treves-Brown 2000) and the present project has tried to increase this information, focusing on a specific emerging disease of rainbow trout: rainbow trout gastroenteritis (RTGE).

1.2. Current knowledge of rainbow trout gastroenteritis

1.2.1. Terminology

The term rainbow trout gastroenteritis (RTGE) was chosen in 1999 to accommodate an enteric syndrome that had been regularly reported since 1995 during the summer (Branson 2003). The defining feature of this syndrome was the accumulation of large quantities of segmented filamentous organisms (SFB) within the distal intestine of affected fish (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001), something that has not been reported in any other enteritis of rainbow trout (Appendix I).

1.2.2. History

The first report of RTGE took place in 1992 in France, affecting a small number of farms (Sanz 2000) and has been present in this country since then (Toranzo 2004; Michel *et al.* 2002). In Spain, RTGE was first detected in 1995 and by 1999 it was affecting approximately 60% of the rainbow trout farms (Branson 2003; Sanz 2000). As reported by Sanz (2000), the number of sites affected by RTGE in France and Spain increased steadily from 1994 to 1999 (Figure 1.8), and the disease has been considered to have the highest impact on the rainbow trout industry of both these countries (Sanz 2000). In Italy, RTGE was diagnosed in the year 2000 for the first time, and a similar increase has been observed (Cervellione, F., personal comm.)

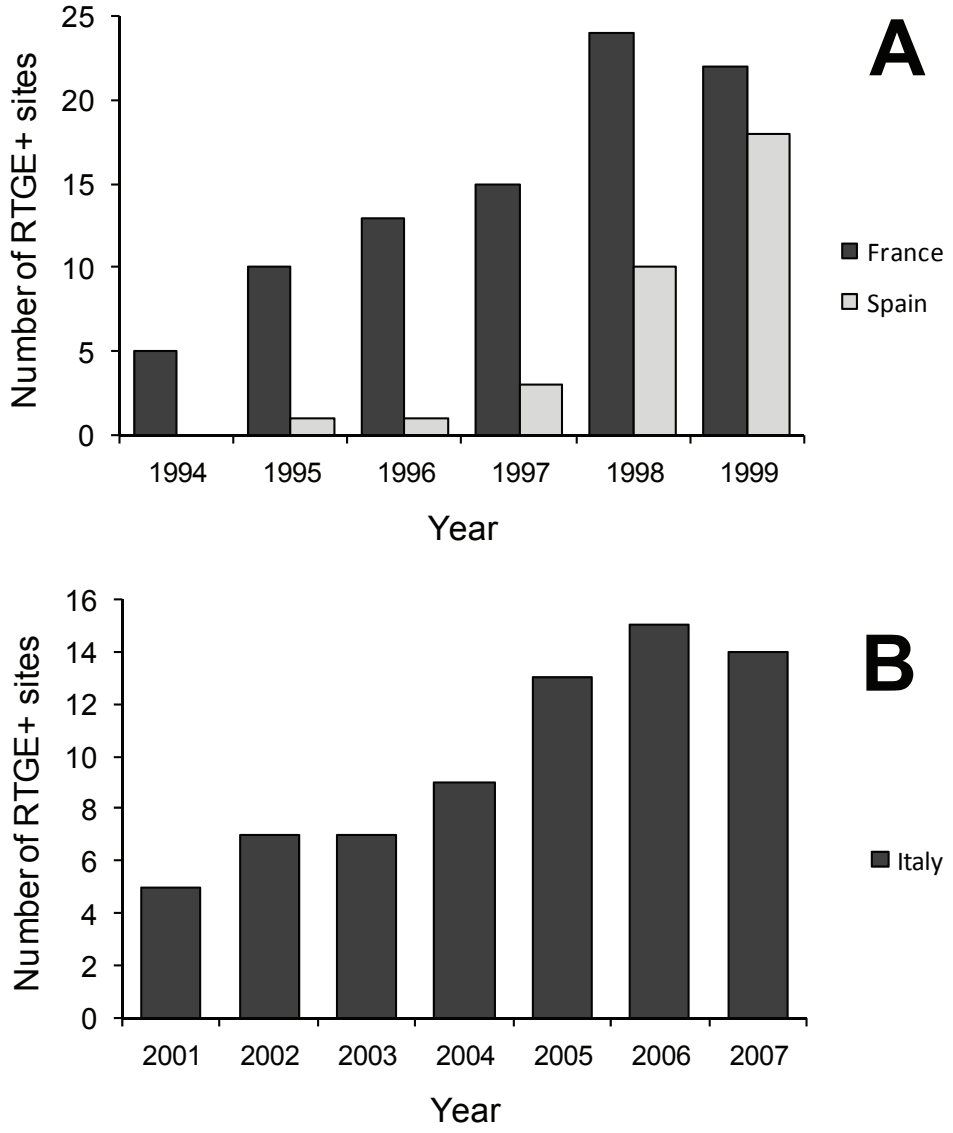

Figure 1.8. A: Number of RTGE-affected sites per year in France and Spain from 1994 to 1999. Data from Sanz (2000). B: Number of RTGE-affected sites per year in Italy from 2001 to 2007 (Cervellione, F., personal comm.).

The first case of RTGE in the UK was diagnosed in 2000, and it was observed again in the same farm in 2001, as well as in other rainbow trout farms (Denham 2004; Branson 2003). One report is available on the presence of RTGE in Croatia (Toranzo 2004). In all these reports, the diagnosis of RTGE was based on the observation of external and internal pathological changes, microscopical examination of fresh smears and histopathology (Toranzo 2004).

1.2.3. Epidemiology

Several authors have reported on the mortality due to RTGE in affected sites and despite an overall similarity, there are differences between these reports (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). None of these reports presents information about the sample size used to obtain any of the figures provided. The proportion of productive units affected by RTGE within affected sites ranged between 10-40% (Urdaci *et al.* 2001). All reports included daily mortality figures and it is likely that these were calculated as the mean of the daily mortalities during the outbreaks, although this was not clear in any report. Daily mortality figures ranged between 0.3-1% (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001; Sanz 2000). In one report, the authors recorded peak daily mortalities of 3-4% (Michel *et al.* 2002). There was variablilty regarding the weight range affected by RTGE: two reports agreed fish between 50-500g were affected (Branson 2003; Urdaci *et al.* 2001), whereas the third recorded RTGE most frequently in fish larger than 800g (Michel *et al.* 2002). In two cases, information about duration of RTGE mortalities was also provided. This information was consistent between the two reports and suggested the presence of outbreaks lasting 2-4 wk, with mortalities peaking on the second week after onset (Branson 2003; Michel *et al.* 2002). In the first UK case, RTGE mortalities were restricted to only some of the ponds and started three weeks after introduction of naive fish (Branson 2003). All this information is consistent with RTGE being an infectious condition with an incubation time of approximately 3 weeks.

All published RTGE reports also suggested factors that may have influenced the presentation of this syndrome. None of the suggestions was supported by data (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). All reports have

suggested that this syndrome was present in the summer preferentially and that water temperature may have played an important role in its development (Denham 2004; Toranzo 2004; Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001; Sanz 2000). They have also suggested that there may be a lower temperature threshold for RTGE although there is disagreement on the minimum temperature at which RTGE was observed ranging from 12-16ºC (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001; Sanz 2000). One exception was RTGE presence observed at 9ºC (Branson 2003). Overall, this information suggests that despite the importance of water temperature, there may not be a constant temperature threshold for RTGE. Anecdotal observations by field workers have suggested that RTGE appears to be triggered by stress factors with the first mortalities following episodes of environmental perturbation and handling. Disappearance of clinical RTGE after changes in diet or environment was also observed (Michel *et al.* 2002). Additional suggestions include an association of low-energy diets with RTGE (Urdaci *et al.* 2001).

1.2.4. Gross presentation

All reports are consistent in the description of RTGE presentation (Denham 2004; Toranzo 2004; Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001; Sanz 2000). Clinical appearance of RTGE occurred suddenly, with lethargy and loss of appetite. Fish tended to gather near the pond outlet and uncoordinated swimming occasionally occurred, reminiscent of neuropathological toxic mechanisms. In many cases, the accumulation of large amounts of yellow mucoid faeces (or faecal casts) in affected ponds was the first sign of disease noticed by fish farmers (Toranzo 2004; Branson 2003; Michel *et al.* 2002).

Gross lesions included abdominal distension, and mucous content of the intestine, which could extrude from the anus. In some cases, RTGE-affected fish presented with a dyschromia characterized by dark streaks scattered along the flanks and/or with yellowish mucoid excretions from the vent (Branson 2003; Michel *et al.* 2002; Sanz 2000). Internal lesions in diarrhoeic trout included signs of acute haemorrhagic enteritis predominantly in the hind gut, with a haemorrhagic and oedematous appearance of the intestinal mucosa (Figure 1.9). The entire digestive tract, including the enlarged stomach and pyloric caeca, was filled with a straw-coloured mucoid material which could accumulate as a dense occluding plug in the terminal portion (Branson 2003; Michel *et al.* 2002; Sanz 2000).

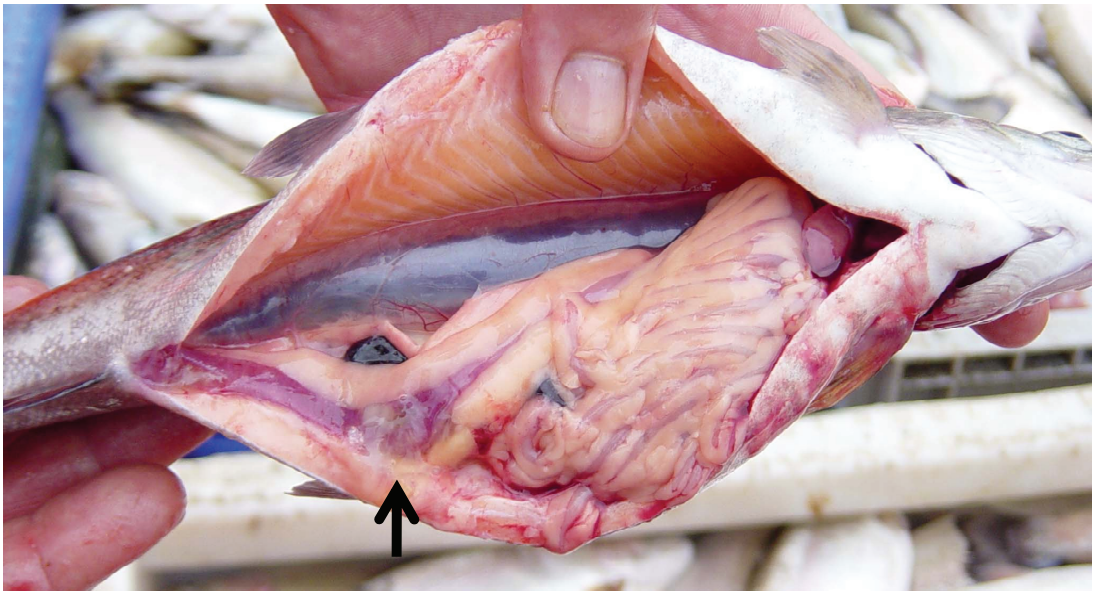

Figure 1.9. Internal gross presentation of rainbow trout gastroenteritis (RTGE), including acute hemorrhagic enteritis of the hind gut and yellow and viscous intestinal contents flowing out of the ruptured intestine (arrow). (Picture courtesy of Prof. Turnbull, J. F.).

1.2.5. Histopathology

Large quantities of SFB have been observed in smears of the intestinal contents of RTGE-affected fish (Michel *et al.* 2002; Urdaci *et al.* 2001) and this may be the most defining clinical feature of this syndrome. Enteritic changes are found as part of the presentation of other conditions caused by several parasitic, fungal, bacterial and viral agents (Austin B. & Austin D.A. 2007; Ferguson 2006; Michel *et al.* 2002; Urdaci *et al.* 2001; Roberts 2001), although none of these conditions is reported to be associated with SFB (Appendix I).

The SFB found within the digestive tract of RTGE-affected trout were non-branched with an approximate width of 1µm (0.6-0.12µm) and up to 60µm long, a length that was made up of a variable number of 1.2-2.6µm long segments (Urdaci *et al.*, 2001). Several forms, apparently corresponding to different stages of maturation, could be seen in wet mounts or in preparations stained with metachromatic toluidine blue. These organisms are Gram variable, but generally Gram positive, and produce spores that stain readily with malachite green (Ferguson 2006; Michel *et al.* 2002; Urdaci *et al.* 2001). Previous reports vary on the preferred location of SFB in the digestive system of RTGE-affected trout. Some (Michel *et al.* 2002) reported their presence throughout the digestive system, whereas others (Branson 2003) observed SFB more frequently in the distal intestine.

Histopathological lesions in the digestive system of affected trout were more severe in the pyloric stomach and caeca (Ferguson 2006; Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001; Sanz 2000). The mucosa was congested and the intestinal wall thickened by oedema, but perhaps the most severe change was

epithelial necrosis with extensive mucosal detachment. Histopathological changes in other organs have not been reported.

1.2.6. Aetiology

The aetiological agent of RTGE has not been identified (Toranzo 2004; Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). This has hindered the development of control strategies targeted at the primary cause of RTGE, including treatments and diagnostic tests (Toranzo 2004). An apparent reduction in mortalities after treatment with antibiotics (Urdaci *et al.* 2001; Sanz 2000) suggests that there is at least some bacterial component in the pathogenesis of this syndrome. Most authors have proposed SFB as the most likely aetiology for RTGE (Michel *et al.* 2002; Urdaci *et al.* 2001), although the inability to culture them *in vitro* (Angert 2005) has hindered confirmation. It is therefore not possible to exclude other potential aetiological candidates, such as a viral agent.

1.2.6.1. Segmented filamentous bacteria

The filamentous bacteria observed in the distal intestine of rainbow trout affected with RTGE have been identified as part of the "*Candidatus* arthromitus" group of segmented filamentous bacteria. This identification was achieved by fluorescent *in situ* hybridization of SBF 16S rRNA sequences, using an oligonucleotide probe specifically designed to react with known SBF 16S rRNA sequences from chicken, rat and mouse (Michel *et al.* 2002; Urdaci *et al.* 2001).

Although SFB cannot be cultured outside of the host gastrointestinal tract (Angert 2005), populations can be maintained as mono-associations with mice and this was the method chosen to sequence the mouse SBF 16S rRNA gene

(Klaasen *et al.* 1991a). This enabled comparison of 16S rRNA sequences, which demonstrated that SFB found in the ileum of mice were closely related to the genus *Clostridium* (Snel *et al.* 1994), and further work by the same authors determined that SFB from rats, mice and chickens formed a distinct phylogenetic group and proposed the inclusion of these organisms in the "*C.* arthromitus" group (Snel *et al.* 1995). "*Candidatus*" is a provisional status used for incompletely described prokaryotes (Murray & Stackebrandt E. 1995) and the term "arthromitus" was coined by Leidy (1881) from the greek *arthron* (=joint) and *mitos* (=thread).

"*Candidatus* arthromitus" form a group of bacteria with similar morphology and ecological niches. Gram positive, endospore-forming SFB have been found attached to the intestine of a wide range of vertebrates (Smith 1997; Lowden & Heath 1995; Klaasen *et al.* 1992; Allen 1992; Goodwin *et al.* 1991; Sanford 1991; Angel *et al.* 1990b; Davis & Savage 1974) and invertebrates (Klaasen *et al.* 1992; Margulis *et al.* 1990; Leidy 1881). A single study reported the presence of SFB, using light microscopy, in the intestines of healthy individuals from 13 vertebrate species, including the carp, concluding that SFB are ubiquitous in the animal kingdom (Klaasen *et al.* 1993).

Repeated lack of success in the transmission of chicken or rat SBF to mice suggested host specificity of SFB, which also presented slight morphological differences depending on the host species (Allen 1992; Tannock *et al.* 1984) and consequent phylogenetic 16S rRNA analysis of "*C.* arthromitus" from crab-eating monkey, rats, mice and chickens confirmed these were in fact different species (Imaoka *et al.* 1997; Snel *et al.* 1995). As a result the most accepted theory today is that different species of SFB colonize different host species

specifically. These phylogenetic differences were also found with trout SFB (Figure 1.10 A, B), suggesting that these also represent a different species within the "*C.* arthromitus" group (Urdaci *et al.* 2001).

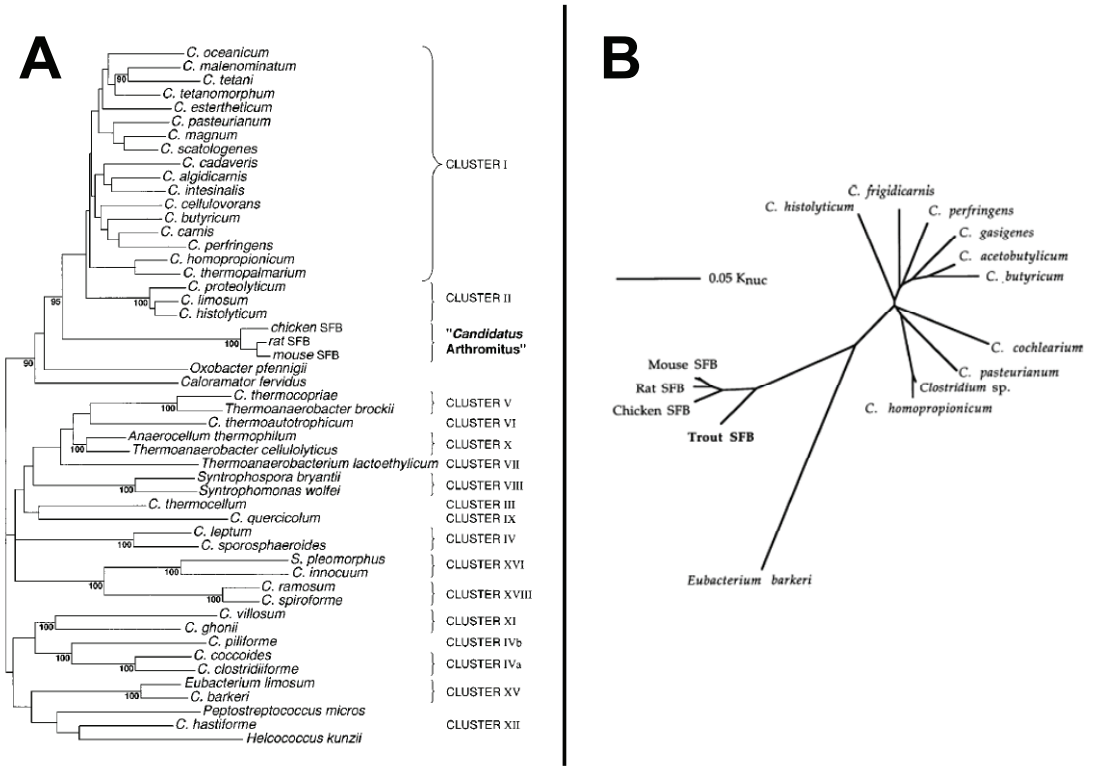

Figure 1.10. Phylogenetic trees illustrating the position of "*Candidatus* arthromitus" respecting other bacterial species. A: phylogenetic position of the SFB within the *Clostridium* subphylum of the gram-positive bacteria (C.= *Clostridium*). Snel *et al.* (1995), with permission; B: relative phylogenetic position of trout "*C.* arthromitus", the closest *Clostridium* species and "*C.* arthromitus"from other species. Urdaci *et al.* (2001), with permission.

The life cycle and multiplication of "*C.* arthromitus" have been examined by several authors (Angert 2005; Ferguson D.J. & Birch-Andersen 1979; Chase & Erlandsen 1976; Davis & Savage 1974). In "*C.* arthromitus", endospore formation is coordinated with the production of active intracellular offspring for reproduction or for dispersal through harsh environments such as aerobic conditions. These spores have been shown to participate in the spread mechanism of SFB between susceptible chickens and mice (Ali & Reynolds 1996; Klaasen *et al.* 1992) and have been observed in trout "*C.* arthromitus"

(Michel *et al.* 2002). It has been noted that the duration of a whole growth cycle in mice SFB should occur within the 20-30h villous transit time of epithelial cells, unless attachment retards or inhibits cell migration (Angert 2005), and it is likely that this may also be the case in trout "*C.* arthromitus". Factors affecting "*C.* arthromitus" numbers have been studied in mice and an increase in SFB numbers has been linked to low immunoglobulin A concentrations (Suzuki *et al.* 2004) whereas a decrease in numbers was associated with age, activation of the mucosal immune system and administration of *Lactobacillus plantarum* to immunodepressed mice (Fuentes *et al.* 2008; Snel *et al.* 1998). Goodwin *et al.* (1991) suggested that diet composition, environmental stress and antimicrobial drugs also play a role in SFB colonization in chickens, turkeys and quails.

At present, it is not clear if the "*C.* arthromitus" found in RTGE-affected trout are responsible for its aetiology, or if their proliferation is just a consequence of the changes caused by this syndrome. Arguments for the aetiological role of "*C.* arthromitus" include the fact that these organisms have never been reported in the digestive system of healthy trout, whereas they are consistently observed in RTGE-affected trout (Denham 2004; Toranzo 2004; Michel *et al.* 2002; Urdaci *et al.* 2001; Sanz 2000). However, "*C.* arthromitus" has never been reported as an aetiological cause of disease in other animal species and although it was initially associated with diarrhoea, malabsorption, fluid and gas-filled intestines in poultry (Goodwin *et al.* 1989), the same workers concluded that SFB are not necessarily pathogens, and that they might in fact be part of the normal microflora of the intestine (Goodwin *et al.* 1991). Other arguments against the aetiological role of "*C.* arthromitus" in RTGE include the observation of SFB in the intestine of healthy carp using light microscopy (Klaasen *et al.* 1993). Other

reason include an stimulatory effect of "*C.* arthromitus" on the immune system and intestinal transit of mice (Umesaki *et al.* 1999; Jiang *et al.* 1998; Snel *et al.* 1996) and a possible correlation with lower *Salmonella enteritidis* colonization of the murine intestine (Garland *et al.* 1982). All of the above suggest a non-pathogenic role for these bacteria.

"*Candidatus* arthromitus" could also be normal part of the native flora of rainbow trout, which has not been previously detected. Klaasen *et al.* (1993) emphasized that the inability to detect mucosa-associated SFBs in any species could relate to the gut being only poorly or irregularly colonized by these organisms, as previously suggested for other bacterial species (Davis & Balish 1979). Additionally, when sampling of SFB positive animals takes place more than 3h post-mortem, they may not be detected, possibly due to destruction of SFB attachment sites by autolytic enzymes (Davis 1980). The observation of impaired "*C.* arthromitus" membranes in the intestine of RTGE-affected trout (Michel *et al.* 2002) may be an indication of the fragility of trout SFB. However, the application of molecular techniques to the study of rainbow trout flora may help to provide a better understanding of the contribution of non-culturable organisms to the microflora of trout, as suggested by the work by Pond et al. (2006).

1.2.6.2. Viral aetiology

There are several parallels between trout RTGE and "stunting syndrome", an infectious enteric disorder of turkey poults (Angel *et al.* 1990b; Bracewell & Randall 1984). In this syndrome, SFB were only observed in turkey poults with clinical signs of diarrhoea, but not in the intestine of clinically normal birds

(Angel *et al.* 1990b). This led to the hypothesis that SFB were associated with the aetiology of this syndrome.

This situation was clarified firstly by the development of a transmission protocol using homogenised intestines from affected poults as the inocula (Angel *et al.* 1990a). This enabled the experimental manipulation of the syndrome in the laboratory, and it was found that an inoculum without bacteria, obtained using 0.45 or 0.20µm microfilters, was also successful in the transmission of the stunting syndrome to naive poults (Sell *et al.* 1992). This finding supported the hypothesis of a viral agent, and indeed a virus was subsequently isolated and characterized (Ali & Reynolds 1997).

1.2.7. Control strategies

The only information available on possible management strategies for RTGE in the literature is based on anecdotal observations. Sudden changes in diet and/or environment and feeding restriction have been associated with reduced RTGE losses (Branson 2003; Michel *et al.* 2002). The treatment of RTGE at affected sites has been based on trial and error, as a result of a lack of scientific knowledge on its aetiology and pathogenesis. Treatments used have included different antibiotic treatments and in-feed liquid paraffin or sodium chloride (NaCl) at various concentrations. Fasting for 7 days or more has also been used in Italy (Sarti *et al.* 2008).

The reported antibiotic treatment of RTGE consisted of in-feed administration of tetracycline or oxytetracycline over 6 days, after a 3-4 days fasting period (Urdaci *et al.* 2001). The outcome of this treatment strategy was not reported. Another report suggests that treatment with the antibiotics amoxycillin,

oxytetracycline and potentiated sulphonamide was effective in alleviating the condition, but that it often recurred once treatment was completed (Branson 2003). This observation suggests that the cause of RTGE is not eliminated completely from the population by the treatment, or that affected populations are continuously exposed to the cause of RTGE. There was no information on the time elapsed from the end of the treatment to recurrence. Administration of flumequine, a fluoroquinolone, has been recently used in Italy in repeated treatments at 25-day intervals, although the efficacy of this strategy is not reported (Sarti *et al.* 2008; Treves-Brown 2000).

Penicillin, placed in drinking water, eliminated SFB from the ileum of healthy mouse and rat in 10 h, but recolonization was observed 4 to 5 weeks after the penicillin treatment was stopped (Davis & Savage 1976). In further studies, the influence of several antimicrobial drugs on SFB in the ileum of mice was tested. The drugs tested included amoxycillin, bacitracin, cefotaxim, ciprofloxacin, clindamycin, cotrimoxazole, doxycyclin, gentamicin, metronidazole, neomycin, polymyxin, streptomycin, trimethoprim, and vancomycin. All of these drugs reduced the number of SFB in the ileum, although to different degrees, suggesting that SFB are sensitive to antimicrobial drugs (Klaasen *et al.* 1991b; Koopman *et al.* 1987).

1.3. Conclusion

Rainbow trout gastroenteritis has been present in the UK since 2000 (Branson 2003), although there is no information on the number of sites affected. It is reported as the rainbow trout disease with the highest impact in France and Spain, where the number of sites affected has escalated during recent years

(Sanz 2000). Despite the importance of RTGE as an emerging disease, scientific information on this syndrome is very limited. Data on the prevalence, spread, aetiology and pathogenesis are required to enable effective prevention and control of this disease. The study presented here was designed to contribute to that information.

1.4. Project outline

This study was jointly funded by the Department of Environmental, Food and Rural Affairs (DEFRA, project nº: FC1173), the Scottish Aquaculture Research Forum (SARF, project nº: SARF016) and the British Trout Association (BTA). The primary aim of the project was to identify potential control strategies for RTGE through the investigation of the epidemiological, pathogenic and aetiological aspects of this syndrome. The ultimate aim was to support the sustainability of UK trout farming by controlling RTGE. The scope for each one of the chapters is reviewed below.

Chapter 2. *Retrospective epidemiological study:* This study included the production of definitive evidence for the distribution and severity of RTGE in the UK trout industry. It also identified risk factors at the site level and populations at risk for the prospective study. The tool chosen was a questionnaire-based retrospective survey. This is the only chapter which has been sent for publication before the submission of this thesis and it has been accepted by the journal “Aquaculture” (Elsevier, The Netherlands).

Chapter 3. *Prospective epidemiological study:* This study described in detail the impact, pattern of spread, and risk factors associated with RTGE within a population of sites affected by this syndrome. It was designed as a prospective longitudinal study.

Chapter 4. *Histopathological and ultrastructural description:* This study provided a detailed description of the patterns associated with SFB presentation in RTGE fish, as well as a description of the pathological changes found in RTGE-affected fish. It was based on a sample library created from fish sampled from 11 UK sites affected by RTGE.

Chapter 5. *Study of the pathogenesis of RTGE:* This study aimed to examine the pathogenesis of RTGE using gross examination, histology and blood biochemistry on the sample library above mentioned.

Chapter 6. *Study of the role of SFB:* This study assessed the association of SFB with RTGE-affected fish. A molecular tool for specific detection of SFB was designed, assessed and finally applied to describe the distribution of SFB in the digestive system of healthy trout, as well as RTGE-affected trout.

The chapters in this thesis take the form of a series of draft manuscripts readied for publication. The contribution of Jorge del-Pozo to all of the chapters includes the totality of the field sampling, data collection, laboratory work, statistical analyses and writing of the manuscripts. All other authors, including Prof. James F. Turnbull, Dr. Margaret Crumlish and Prof. Hugh W. Ferguson, provided assistance with the experimental design, guidance and proof reading for all the chapters.

1.5. References

Ali, A. & Reynolds, D. L. 1996, 'Primary cell culture of turkey intestinal epithelial cells'. *Avian diseases*, vol. 40, pp. 103-108.

Ali, A. & Reynolds, D. L. 1997, 'Stunting syndrome in turkey poults: isolation and identification of the etiologic agent'. *Avian diseases*, vol. 41, pp. 870-881.

Allen, P. C. 1992, 'Comparative study of long, segmented, filamentous organisms in chickens and mice'. *Laboratory Animal Science*, vol. 42, pp. 542-547.

Angel, C. R., Sell, J. L. & Trampel, D. W. 1990a, 'Stunting syndrome in turkeys. Development of an experimental model'. *Avian diseases*, vol. 34, pp. 447-453.

Angel, C. R., Sell, J. L., Fagerland, J. A., Reynolds, D. L. & Trampel, D. W. 1990b, 'Long-segmented filamentous organisms observed in poults experimentally infected with stunting syndrome agent'. *Avian diseases*, vol. 34, pp. 994-1001.

Angert, E. 2005, 'Alternatives to binary fission in bacteria'. *Nature Reviews: Microbiology*, vol. 3, pp. 214-224.

Austin B. & Austin D.A. 2007, *Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish*. Fourth Ed., Springer-Praxis, Chichester.

Bakke-McKellep, A. M., Koppang, E. O.; Gunnes, G., Sanden, M., Hemre, G-I., Landsverk, T. & Krogdahl, A. 2007, 'Histological, digestive, metabolic, hormonal and some immune factor responses in Atlantic salmon, *Salmo salar* L., fed genetically modified soybeans'. *Journal of fish diseases*, vol. 30, pp. 65-79

Bark, S. & McGregor, D. 2001, 'The first occurrence of Lactococcosis in farmed trout in England'. *Trout News*, vol. 31, pp. 9-11.

Beamish, F. W. H., SitjaBobadilla, A., Jebbink, J. A. & Woo, P. T. K. 1996, 'Bioenergetic cost of cryptobiosis in fish: Rainbow trout *Oncorhynchus mykiss* infected with *Cryptobia salmositica* and with an attenuated live vaccine'. *Diseases of Aquatic Organisms*, vol. 25, pp. 1-8.

Behnke, R. J. 1992, *Native trout of western North America. AFS monographs 6*. First Ed., American Fisheries Society, Bethesda, MA, USA.

Bracewell, C. D. & Randall, J. J. 1984, 'The infectious stunting syndrome'. *World's Poultry Science Journal*, vol. 40, pp. 31-37.

Branson, E. 2003, 'Rainbow trout Gastro-Enteritis (RTGE)-first diagnosis in the UK'. *Fish Veterinary Journal*, vol. 7, pp. 71-76.

Brown, C. 2000. 'Emerging diseases of animals: An overview'. In: *Emerging diseases of animals*, C. Brown & C. Bolin, (Eds.), American Society for Microbiology, Washington DC, USA, p. 324.

Cann, D. C. & Taylor, L. Y. 1982, 'An Outbreak of Botulism in Rainbow-Trout, *Salmo-Gairdneri* Richardson, Farmed in Britain'. *Journal of Fish Diseases*, vol. 5, pp. 393-399.

Chase, D. G. & Erlandsen, S. L. 1976, 'Evidence for a complex life cyle and endospore formation in the attached, filamentous, segmented bacterium from murine ileum'. *Journal of Bacteriology*, vol. 127, pp. 572-583.

Cowx, I. G. Cultured Aquatic Species Information Programme: *Oncorhynchus mykiss* (Walbaum, 1792). FAO Fisheries and Aquaculture Department [online] . 2005. http://www.fao.org/fishery/culturedspecies/Oncorhynchus_mykiss (Accessed on 31/01/2009).

Davis, C. P. 1980. 'Post-mortem alterations of bacterial localization'. In: *Scanning Electron Microscopy*, A. M. F. O'Hare, (Ed.), SEM inc., Chicago, pp. 523-526.

Davis, C. P. & Balish, E. 1979. 'Bacterial localization in the gastrointestinal tract of athymic (nude) mice'. In: *Scanning Electron Microscopy*, A. M. F. O'Hare, (Ed.), SEM inc., Chicago, pp. 189-195.

Davis, C. P. & Savage, D. C. 1974, 'Habitat, succession, attachment, and morphology of segmented, filamentous microbes indigenous to the murine gastrointestinal tract'. *Infection and Immunity*, vol. 10, pp. 948-956.

Davis, C. P. & Savage, D. C. 1976, 'Effect of penicillin on the succession, attachment, and morphology of segmented, filamentous microbes in the murine small bowel'. *Infection and Immunity*, vol. 13, pp. 180-188.

Denham, K. 2004, 'Fish health controls: The activities of the fish health inspectorate in England and Wales'. *Trout News*, vol. 38, pp. 29-31.

Elliot, J. A., Collins, M. D., Piggot, N. E. & Facklam, R. R. 1991, 'Differentiation of *Lactococcus lactis* and *Lactococcus garvieae* from humans by comparison of whole-cell protein patterns'. *Journal of clinical microbiology*, vol. 29, pp. 2731-2734.

FAO 2006, *The state of World fisheries and Aquaculture: 2006*. Food and Agriculture Organization of the United Nations, Rome, Italy.

FAO. Aquaculture production (1950-2006). 2008a. Fisheries and Aquaculture Information and Statistics Service (FIES), Rome, Italy, http://www.fao.org/fishery/statistics/software/fishstat (Accessed on 20/01/2009).

FAO. Total fishery production (1950-2006). 2008b. Fisheries and Aquaculture Information and Statistics Service (FIES), Rome, Italy, http://www.fao.org/fishery/statistics/software/fishstat (Accessed on 20/01/2009).

Fefer, J. F., Ratzan, K. R., Sharp, S. E. & Saiz, E. 1998, '*Lactococcus garvieae* endocarditis: Report of a case and review of the literature'. *Diagnostic Microbiology and Infectious Disease*, vol. 32, pp. 126-130.

Ferguson D.J. & Birch-Andersen, A. 1979, 'Electron microscopy of a filamentous, segmented bacterium attached to the small intestine of mice from a laboratory animal colony in Denmark'. *Acta Pathologica et Microbiologica Scandinavica, section B (Microbiology)*, vol. 87, pp. 247-252.

Ferguson, H. W. 2006, *Systemic pathology of fish: A text and atlas of normal tissues in teleosts and their responses in disease*. Second Ed., Scotian Press, London, UK.

Ferguson, H. W., Girons, A., Rizgalla, G., LaPatra, S., Branson, E., McKenzie, K., Davies, M., Collins, R. O., Diab, A. & Crumlish, M. 2006, 'Strawberry disease in rainbow trout in Scotland: pathology and association with *Flavobacterium psychrophilum*'. *Veterinary Record*, vol. 158, pp. 630-632.

Fuentes, S., Egert, M., Jimenez-Valera, M., Monteoliva-Sanchez, M., Ruiz-Bravo, A. & Smidt, H. 2008, 'A strain of *Lactobacillus plantarum* affects segmented filamentous bacteria in the intestine of immunosuppressed mice'. *FEMS Microbiology Ecology*, vol. 63, pp. 65-72.

Garland, C. D., Lee, A. & Dickson, M. R. 1982, 'Segmented filamentous bacteria in the rodent small intestine: Their colonization of growing animals and possible role in host resistance to *Salmonella*'. *Microbial Ecology*, vol. 8, pp. 181-190.

Goodwin, M. A., Cooper, G. L., Brown, J., Bickford, A. A., Waltman, W. D. & Dickson, T. G. 1991, 'Clinical, pathological, and epizootiological features of long-segmented filamentous organisms (bacteria, LSFOs) in the small intestines of chickens, turkeys, and quails.'. *Avian diseases*, vol. 35, pp. 872-876.

Goodwin, M. A., Cooper, G. L. & Waltman, W. D. 1989, 'Diarrhea associated with long-segmented filamentous organisms (LSFO) in the small intestines of turkeys, chickens and quails'. In: *38th Western Poultry Disease Conference*, pp. 103-109.

Hickling, C. F. 1971, *Fish culture*. Faber and Faber, London, UK.

Imaoka, A., Okada, Y., Matsumoto, S., Setoyama, H. & Umesaki, Y. 1997, '16S Ribosomal DNA Sequence Divergence of Segmented Filamentous Bacteria with Special Reference to Inter-species and Within-species Variation of Host Animals'. *Systematic and Applied Microbiology*, vol. 20, pp. 418-422.

Irving, S., Feist, S., Stone, D., Verner-Jeffreys, D., Bateman, K., Martin, P., Thrush, M., Longshaw, M., Roberts, E. & Rimmer, G. 2006, 'New and emerging diseases in fish in England and Scotland'. *Finfish News*, vol. 2, pp. 9-12.

James, M. 2006, 'Grass roots aquaculture R&D and important notes for bean counters'. *Finfish News*, vol. 1, pp. 29-34.

Jiang, H.-Q., Snel, J., Talham, G. L. & Cebra, J. J. 1998, 'Segmented filamentous bacteria (SFB) stimulate gut mucosal immune system of formerly germ-free mice'. *FASEB Journal*, vol. 12, p. A912.

Kaushik, S. J. 2007. 'Soybean products in salmonid diets'. In: *Use of alternative protein sources in aquaculture diets*, C. Lim, C. D. Webster, & C. S. Li, (Eds.), The Haworth Press, Inc., New York USA.

Klaasen, H. L. B. M., Koopman, J. P., Poelma, F. G. J. & Beynen, A. C. 1992, 'Intestinal, segmented, filamentous bacteria'. *FEMS Microbiology Reviews*, vol. 88, pp. 165-180.

Klaasen, H. L. B. M., Koopman, J. P., Van den Brink, M. E., Bakker, M. H., Poelma, F. G. J. & Beynen, A. C. 1993, 'Intestinal, segmented, filamentous bacteria in a wide range of vertebrate species.'. *Laboratory Animals*, vol. 27, pp. 141-150.

Klaasen, H. L. B. M., Koopman, J. P., Van den Brink, M. E., Van Wezel, H. P. N. & Beynen, A. C. 1991a, 'Mono-association of mice with non-cultivable, intestinal, segmented, filamentous bacteria.'. *Archives of Microbiology*, vol. 156, pp. 148-151.

Klaasen, H. L. B. M., Koopman, J. P., Vollard, E. J., Theeuwes, A. G. M., Van den Brink, M. E., Scholten, P. M., Bakker, M. H. & Beynen, A. C. 1991b, 'Influence of antimicrobial drugs on segmented filamentous bacteria in the ileum of mice'. *Microbial Ecology in Health and Disease*, vol. 4, pp. 391-397.

Koopman, J. P., Scholten, P. M., Van Heumen T.J.C. & Van Druten, J. A. M. 1987, 'The influence on gastro-intestinal ecology of some antibiotics used for the decontamination of mice'. *Zeitschrift fuer Versuchstierkunde*, vol. 30, pp. 137-141.

Leidy, J. 1881, 'The parasites of termites'. *Journal of the Academy of Natural Sciences of Philadelphia*, vol. 8, pp. 425-447.

Lowden, S. & Heath, T. 1995, 'Segmented filamentous bacteria associated with lymphoid tissues in the ileum of horses'. *Research in Veterinary Science*, vol. 59, pp. 272-274.

MacCrimmon, H. R. 1971, 'World distribution of rainbow trout (*Salmo gairdneri*)'. *Journal of the Fisheries Research Board of Canada*, vol. 28, pp. 663-704.

MacIntyre, C. M. 2008, *Water quality and welfare assessment on United Kingdom trout farms*. PhD thesis, University of Stirling, Stirling, UK.

Margulis, L., Olendzenski, L. & Afzelius, B. A. 1990, 'Endospore forming filamentous bacteria symbiotic in termites: ultraestructure and growth in culture of arthromitus'. *Symbiosis*, vol. 8, pp. 95-116.

McLoughlin, M. F. & Graham, D. A. 2007, 'Alphavirus infections in salmonids - a review'. *Journal of Fish Diseases*, vol. 30, pp. 511-531.

Michel, C., Bernardet, J. F., Daniel, P., Chilmonczyk, S., Urdaci, M. C. & de Kinkelin, P. 2002, 'Clinical and aetiological aspects of a summer enteritic syndrome associated with the sporulating segmented filamentous bacterium "*Candidatus* arthromitus" in farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum)'. *Journal of Fish Diseases*, vol. 25, pp. 533-543.

Murray, A. G. & Peeler, E. J. 2005, 'A framework for understanding the potential for emerging diseases in aquaculture'. *Preventive Veterinary Medicine*, vol. 67, pp. 223-235.

Murray, R. G. E. & Stackebrandt E. 1995, 'Taxonomic note: Implementation of the provisional status *Candidatus* for incompletely described prokaryotes'. *International Journal of Systematic Bacteriology*, vol. 45, pp. 186-187.

North, B. P., Ellis, T., Knowles, T., Bron, J. E. & Turnbull, J. F. 2008. 'The use of stakeholder focus groups to identify indicators for the on-farm assessment of trout welfare'. In: *Fish Welfare*, E. J. Branson, (Ed.), Blackwell Publishing, Oxford, UK, pp. 243-247.

North, B. P., Turnbull, J. F., Ellis, T., Porter, M. J., Migaud, H., Bron, J. & Bromage, N. R. 2006, 'The impact of stocking density on the welfare of rainbow trout (*Oncorhynchus mykiss*)'. *Aquaculture*, vol. 255, pp. 466-479.

Pickering, A. D. & Pottinger, T. G. 1989, 'Stress responses and disease resistance in salmonid fish: Effects of chronic elevation of plasma cortisol'. *Fish Physiology and Biochemistry*, vol. 7, pp. 253-258.

Pond, M. J., Stone, D. M. & Alderman, D. J. 2006, 'Comparison of conventional and molecular techniques to investigate the intestinal microflora of rainbow trout (*Oncorhynchus mykiss*)'. *Aquaculture*, vol. 261, pp. 194-203.

Rabanal, H. 1988, *History of aquaculture*. FAO document repository, Manila, Philippines.

Read, N. 2008. 'Fish farmer's perspective of welfare'. In: *Fish welfare*, E. J. Branson, (Ed.), Blackwell Publishing, Oxford, UK, pp. 101-110.

Regulation (EC) No 1774/2002, Chapter II; article 5; Paragraph 1(e). Health rules concerning animal by-products not intended for human consumption.

Roberts, R. J. 2001, *Fish Pathology*. Third Ed., W.B. Saunders, Edinburgh, UK.

Sanden, M., Berntssen, M. H. G., Krogdahl, A., Hemre, G-I., Bakke-McKellep, A. M. 2004, 'An examination of the intestinal tract of Atlantic salmon (*Salmo salar* L.) parr fed different varieties of soy and maize'. *Journal of Fish Diseases*, vol. 28, pp. 317-330.

Sanford, S. E. 1991, 'Light and electron microscopic observations of a segmented filamentous bacterium attached to the mucosa of the terminal ileum of pigs'. *Journal of Veterinary Diagnostic Investigation*, vol. 3, pp. 328-333.

Sanz, F. 2000, 'La gastroenteritis de la trucha (Language: Spanish)'. *Trouw Informa*, vol. Spring, pp. 16-18.

Sarti, M., Cervellione, F., Giusto, C., Manzano, M., Pecchini, A. & Luzzana, U. 2008, 'Intestinal microflora modulation as a strategy for the control of rainbow trout gastroenteritis (RTGE) (Language: Italian)'. In: *XV conference of the Italian Society of Fish Pathology*, Erice, Italy, p. 25.

Scarre, C. 1993, *Smithsonian Timelines of the Ancient World: A Visual Chronology from the Origins of Life to AD 1500*. First Ed., DK ADULT, Washington, DC, USA.

Scott, A. 2002, 'Fish health controls: The activities of the fish health inspectorate in England and Wales 2001'. *Trout News*, vol. 34, pp. 24-27.

Sedgwick, S. D. 1990, *Trout farming handbook*. Fifth Ed., Fishing News Books (Blackwell Science), Oxford, England.

Sell, J. L., Reynolds, D. L. & Jeffrey, M. 1992, 'Evidence that bacteria are not causative agents of stunting syndrome in poults'. *Poultry Science*, vol. 71, pp. 1480-1485.

Smith, T. M. 1997, 'Segmented filamentous bacteria in the bovine small intestine'. *Journal of Comparative Pathology*, vol. 117, pp. 185-190.

Snel, J., Blok, H. J., Kengen, M. P., Kudwig, W., Poelma, F. G. J., Koopman, J. P. & Akkermans, A. D. L. 1994, 'Phylogenetic Characterization of *Clostridium* Related Segmented Filamentous Bacteria in Mice Based on 16S Ribosomal RNA Analysis'. *Systematic and Applied Microbiology*, vol. 17, p. 172.

Snel, J., Heinen, P. P., Blok, R. J., Carman, R. J., Duncan, A. J., Allen, P. C. & Collins, M. D. 1995, 'Comparison of 16S rRNA sequences of segmented filamentous bacteria isolated from mice, rats, and chickens and proposal of "*Candidatus* arthromitus"'. *International Journal of Systematic Bacteriology*, vol. 45, pp. 780-782.

Snel, J., Hermsen, H. J., Smits, H. J., Bos, N. A., Eling, W. M. C., Cebra, J. J. & Heidt, P. J. 1998, 'Interactions between gut-associated lymphoid tissue and colonization levels of indigenous, segmented, filamentous bacteria in the small intestine of mice'. *Revue Canadienne de Microbiologie*, vol. 44, pp. 1177-1182.

Snel, J., Van den Brink, M. E., Bakker, M. H., Poelma, F. G. J. & Heidt, P. J. 1996, 'The influence of indigenous segmented filamentous bacteria on small intestinal transit in mice'. *Microbial Ecology in Health and Disease*, vol. 9, pp. 207-214.

Sobel, J. 2005, 'Botulism'. *Clinical Infectious Diseases*, vol. 41, pp. 1167-1173.

Speare, D. J., Daley, J., Markham, R. J. F., Sheppard, J., Beaman, H. J. & Sanchez, J. G. 1998, '*Loma salmonae*-associated growth rate suppression in rainbow trout, *Oncorhynchus mykiss* (Walbaum), occurs during early onset

xenoma dissolution as determined by in situ hybridisation and immunohistochemistry'. *Journal of Fish Diseases*, vol. 21, pp. 345-354.

Suzuki, K., Meek, B., Doi, Y., Muramatsu, M., Chiba, T., Honjo, T. & Fagarasan, S. 2004, 'Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut'. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, pp. 1981-1986.

Tannock, G. W., Miller, J. R. & Savage, D. C. 1984, 'Host specificity of filamentous, segmented microorganisms adherent to the small bowel epithelium in mice and rats'. *Applied and Environmental Microbiology*, vol. 47, pp. 441-442.

Toranzo, A. E. 2004. 'Report about bacterial diseases'. In: *Mediterranean Aquaculture Laboratories*, P. Alvarez-Pellitero et al., (Eds.), CIHEAM/FAO, pp. 49-89.

Treves-Brown, K. M. 2000, *Applied fish pharmacology*. Kluwer Academic Press, Dordretch, The Netherlands.

Tyson, P. 2008, '2006 Summary of UK trout production'. *Finfish News*, vol. 5, p. 41.

Umesaki, Y., Setoyama, H., Matsumoto, S., Imaoka, A. & Itoh, K. 1999, 'Differential Roles of Segmented Filamentous Bacteria and Clostridia in Development of the Intestinal Immune System'. *Infection and Immunity*, vol. 67, pp. 3504-3511.

Urdaci, M. C., Regnault, B. & Grimont, P. A. D. 2001, 'Identification by in situ hybridization of segmented filamentous bacteria in the intestine of diarrheic rainbow trout (*Oncorhynchus mykiss*)'. *Research in Microbiology*, vol. 152, pp. 67-73.

VHS National Control Centre 2006, 'VHS in Yorkshire'. *Finfish News*, vol. 2, pp. 5-9.

Wall, T. 2008. 'Disease control, vaccination and medicine availability related to welfare'. In: *Fish Welfare*, E. J. Branson, (Ed.), Blackwell Publishing, Oxford, UK, pp. 195-201.

CHAPTER 2. A Retrospective Cross-Sectional Study on Rainbow Trout Gastroenteritis (RTGE) in the UK

Del-Pozo, Jorge*; Crumlish, Margaret; Ferguson, Hugh W.; Turnbull James F.

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2.1. Abstract

Rainbow trout gastroenteritis (RTGE) is a summer enteritic syndrome affecting farmed rainbow trout which has been reported since 1992 in France, Spain, Italy, and Croatia. RTGE was first reported in the UK in the year 2000 and limited information has been available about its epidemiology since this report. Our work aims to contribute to this knowledge with a retrospective cross-sectional study devised to determine the extent and severity of RTGE in the UK as well as to identify RTGE risk factors at the site level. Eighty-four sites participated in the study, representing 88% of the 2004 rainbow trout production by weight in the UK. It was found that RTGE had been present in at least 11 (13%) of these sites from 2000 to 2005 and the total number of sites affected by RTGE per year had increased over time, with prevalence values in the sample ranging from 2.4% during the year 2000 to 8.3% during 2005. Univariable analysis of the whole dataset revealed a confounding effect of high average production, leading to stratification of all the analysis. Several management and environmental variables were significantly associated with RTGE, including higher mean production, use of a major processing plant, lower residence times, lower harvest weights, use of diploid fish, systematic vaccination, water oxygenation, the use of automatic and/or demand feeding systems and higher maximum water temperature. Also, a number of fry sources were associated

with RTGE, but further analysis suggested that a common source of RTGE via fry was unlikely. None of the egg sources or feed types included in the study was associated with RTGE and production for the restocking market presented a protective association. Multivariable logistic regression identified the use of a major processing plant and lower residence time as the two variables with the strongest association with RTGE presence. Overall, the results of this study have confirmed RTGE in the UK as a major disease problem that is linked to a high productivity level and production of fish for the portion-size market.

2.2. Introduction

Since 1995, an unusual form of mortality has been regularly reported in farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum). This condition was first described in France in 1992 and since then has been reported in Spain, Italy, Croatia and the UK (Denham 2004; Toranzo 2004; Branson 2003). In Spain, the first report was in 1995 and by 1999 it was affecting approximately 60% of the Spanish rainbow trout farms (Branson 2003). Similarly, the first case in the UK was diagnosed in a single site in 2000 and the following year the condition was observed in more locations (Denham 2004; Branson 2003). The presentation of the condition was consistent in these reports and the term rainbow trout gastroenteritis (RTGE) was proposed in 1999 to describe this syndrome (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001).

Rainbow trout gastroenteritis was reported to predominantly occur in the summer and to have a significant impact to the production of affected sites (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). Daily mortalities in the order of 0.5-1.0% and higher were common, generally affecting relatively big

fish (≥0.8kg). Mortalities could be observed for as long as 15-30 days, resulting in significant economic losses for affected sites (Michel *et al.* 2002).

Michel *et al.* (2002) reported that fish affected by RTGE generally exhibit lethargy and reduced appetite but can also develop dark striping of the flanks. Frequently, accumulation of mucoid faeces on the bottom of ponds and nervous signs were also included in the presentation. Internal gross lesions include severe congestion and oedema of the intestinal wall with the intestinal lumen containing relatively large quantities of mucoid material. Histologically, this presentation is accompanied by accumulation of large numbers of segmented filamentous bacteria (SFB) (Michel *et al.* 2002). A genetic probe targeted at specific 16S rRNA regions of SFB from rat, mouse and chicken was used to identify the SFB in the intestine of RTGE-affected trout and as a result these were included in the "*Candidatus* arthromitus" group, closely related to *Clostridium* phylogenic group I (Urdaci *et al.* 2001; Snel *et al.* 1995; Murray & Stackebrandt 1995; Snel *et al.* 1994). "*Candidatus* arthromitus" has been suggested as a possible aetiological agent for RTGE, as they are always observed in trout presenting RTGE pathological changes (Urdaci *et al.* 2001), although failure to culture SFB *in vitro* has hindered confirmation of this hypothesis (Angert 2005; Klaasen *et al.* 1992; Davis & Savage 1974). The role of SFB in intestinal illnesses of other animal species is still unclear and although their presence has been reported in poultry with diarrhoea (Morishita *et al.* 1992; Angel *et al.* 1990; Goodwin *et al.* 1989), a direct aetiological role has never been reported and various authors consider these organisms as part of the normal intestinal microflora (Talham *et al.* 1999; Jiang *et al.* 1999; Umesaki *et al.* 1995; Klaasen *et al.* 1993; Goodwin *et al.* 1991).

At the start of this study, no information was available on the epidemiology of RTGE at the site level in the UK. Knowledge of risk factors associated with a health condition is essential for the design of control strategies for that condition (Thrusfield 2006) and these risk factors can be identified either through observational studies or experimental/clinical trials. In the earlier stages of research, observational studies are commonly used to generate hypotheses and establish association between possible risk factors and the presence of clinical disease. Cross-sectional studies are observational studies that make few assumptions about the disease status of the population of interest, allowing large numbers of potential risk factors to be screened. A cross-sectional design was used in this study, since limited information was available on RTGE within the UK, allowing the prevalence to be established (Thrusfield 2006; Dohoo *et al.* 2003). The objectives of this study were (1) to estimate the extent and severity of RTGE in the UK rainbow trout industry and (2) to identify risk factors associated with RTGE at the level of the farm or site.

2.3. Materials and Methods

A cross-sectional retrospective study of UK rainbow trout farms was conducted from April to June 2006, with data collected referring to the time interval between the years 2000 and 2005. The year 2000 was selected due to the first report of RTGE in the UK (Branson 2003). In this study the unit of interest was the whole site (*i.e.* A collection of productive units owned and operated as a single unit), and risk factors were those associated with RTGE positive sites.

2.3.1. Participants

Participants included members of the British Trout Association (BTA) and non members. All members of the BTA are listed and their contact details (telephone, address and e-mail) available in the website of this organization, accessible to the general public (www.britishtrout.co.uk). Non BTA members' details are more difficult to obtain, as they are not listed in a single public source. To obtain a representative list of all the rainbow trout farms in the UK, a list of all BTA members was amalgamated with a list of rainbow trout farms constructed from sources of data in the public domain. The resulting list consisted of 169 salmonid farms distributed throughout the UK. Further selection was made on the list by eliminating rainbow trout sites that were not operating from 2000 to 2005 and sites that did not stock rainbow trout. After the selection process, a total of 126 rainbow trout sites were included in the final list. To ensure confidentiality, each site was assigned a unique randomly generated number.

2.3.2. Questionnaire design and validation

The survey questionnaire (Appendix II) was designed to include closed questions, which provide a limited range of options (Thrusfield 2006; Oppenheim 1992). Closed questions were considered appropriate for this survey, as those surveyed had the option to respond by telephone or mail. The information required for the survey was compiled from literature searches on RTGE and "*C.* arthromitus" as well as information available on the usual management procedures of rainbow trout sites. All this information was synthesised into a set of questions that were then arranged by subject, from generic to more specific. After the preliminary design, the questionnaire was

pilot-tested in two rainbow trout sites (not included in the study) where the time length, layout and absence of ambiguity of the questionnaire were assessed. All the variables included are displayed in Table 2.1 and a copy of the questionnaire can be found in Appendix II.

Table 2.1. Variables included in the RTGE retrospective cross-sectional study conducted between April and June 2006.

Category	*Variable (possible responses)*
Outcome	Presence of RTGE from 2000 to 2005
Environmental Variables	Water use (m^3/day) *†; Water source (borehole/lake/river); Water type (hard/soft); Water reuse (yes/no); Maximum yearly water temperature (°C); Water Treatment (no/aeration/oxygenation); Substrate (earth/concrete/metal/fibreglass); Contiguous Sites ‡ (yes/no)
Production Variables	Mean Production (tonnes/year), Target market (table/restocking); System type (cages/ponds/raceways/tanks); Feeding system (automated/demand/manual); Closed Production § (yes/no); Mean Trout Residence Time (months); Mean Stocking Density (kg/m^3); Weight at Stocking (g); Weight at Harvest (g); Fallowing (yes/no); Type of Feed (feed 1 to 14 **); Scheduled Treatments (no/vaccination 1 to 4 **); Use of Processing Plant (processing plant 1 to 7 **); Share of Facilities (workers/equipment)
Biological Variables	Source of Fry (fry source 1 to 34 **); Source of eggs (egg source 1 to 11 **); Ploidy (Diploid/Triploid); Other Animals on Site (Dog/Sheep/Cattle/Cat); Predator Presence (Cormorant/Heron/Mink/Otter/Seagull)

*=Based on the site abstraction license. †=Only applied to pond sites. ‡=This variable indicated the presence of another rainbow trout producing site in the same water system within 5 miles. §= Closed systems produce their own fry. **=Random number assigned to maintain confidentiality.

2.3.3. Survey approach and participation

A standard letter containing information on the purpose of the project and the funding bodies involved was mailed to all 126 sites. After a minimum interval of one week, all sites were contacted by mail (once) and telephone (weekly) to request their participation. These data collection procedures were implemented during a period of three months (April-June 2006) to ensure the inclusion of the maximum number of participants possible.

2.3.4. Data Management and Analysis

A database was created in the software package EpiInfo™ (Dean *et al.* 2007) and used to collate all survey data, create several calculated variables (mean production (tonnes/year), summer water usage (m^3/day) and mean stocking density (kg/m^3)). Continuous variables were categorised into high and low through their median value and also included as dichotomous variables in the analysis. The identification of risk factors was based in statistical association with whether or not there was a case of RTGE. This was defined as: *"A case of RTGE is a rainbow trout producing site that had RTGE diagnosed by an independent fish health expert on fish samples from one or more of its production units at any moment between the beginnings of the year 2000 to the end of the year 2005"*.

The analysis of the data was firstly descriptive, which provided information on the background of RTGE in the UK. This was followed by risk factor univariable and multivariable analyses. The data were not normal and univariable analysis used Fisher Exact and Kruskall-Wallis tests for dichotomous and continuous variables respectively, with results with $p<0.05$ considered as statistically significant. During analysis, a correlation was observed between several possible risk factors and a higher mean production. In order to minimize the confounding effect of mean production, data were stratified into low and high productivity level through the median of the mean production (70 tonnes/year). All cases were located in the upper stratum and as a result the final univariable and multivariable analysis were performed only in this stratum. Multivariable analysis involved the use of logistic regression modelling. Univariable logistic regression was used for the selection of variables for multivariable analysis, and

all variables that showed a Wald statistic $p<0.25$ through univariable logistic regression were kept for multivariable analysis. Before model building, the assumption of linearity of the logit of continuous variables with the dependent variable was tested using the Box-Tidwell transformation test and continuous variables that did not meet this assumption were included as dichotomous variables only. These variables were also tested for multicolinearity using Pearson correlation and variables presenting significant correlation ($p<0.05$) were not included in the same model. In these cases, the variable judged as most biologically plausible was used as a candidate in the multivariable analysis. Both forward and backward stepwise model building approaches were used and the best model was determined by a significant reduction in the -2*log likelihood statistic. The Hosmer-Lemeshow test was applied to all candidate models to ensure adequate goodness of fit and all possible interaction terms were tested (Thrusfield 2006; Dohoo *et al.* 2003). All the univariable analysis, correlation tests and multivariable logistic regression models were conducted using both SPSS™ (SPSS Inc.) and EpiInfo™ (CDC) computer packages.

2.4. Results

2.4.1. Study response and participant sites

Of 126 UK sites initially contacted, 84 sites agreed to take part in the study. This sample did not include sites from Northern Ireland and as an indicator for the response rate, the sum of the yearly production of all the sites participating in this study during 2004 (13,539 tonnes) represented 88% by weight of the total UK production (15,917 tonnes), excluding Northern Ireland (DEFRA 2006).

Of these 84 sites, 58 (69%) were BTA members and 26 (31%) were non BTA members. The sample included river-based as well as lake-based sites utilising pond, raceways, tanks or cage systems and their mean yearly production ranged from 3 to 1000 tonnes/year. Participant sites were located in England, Wales and Scotland but not Northern Ireland (Figure 2.1).

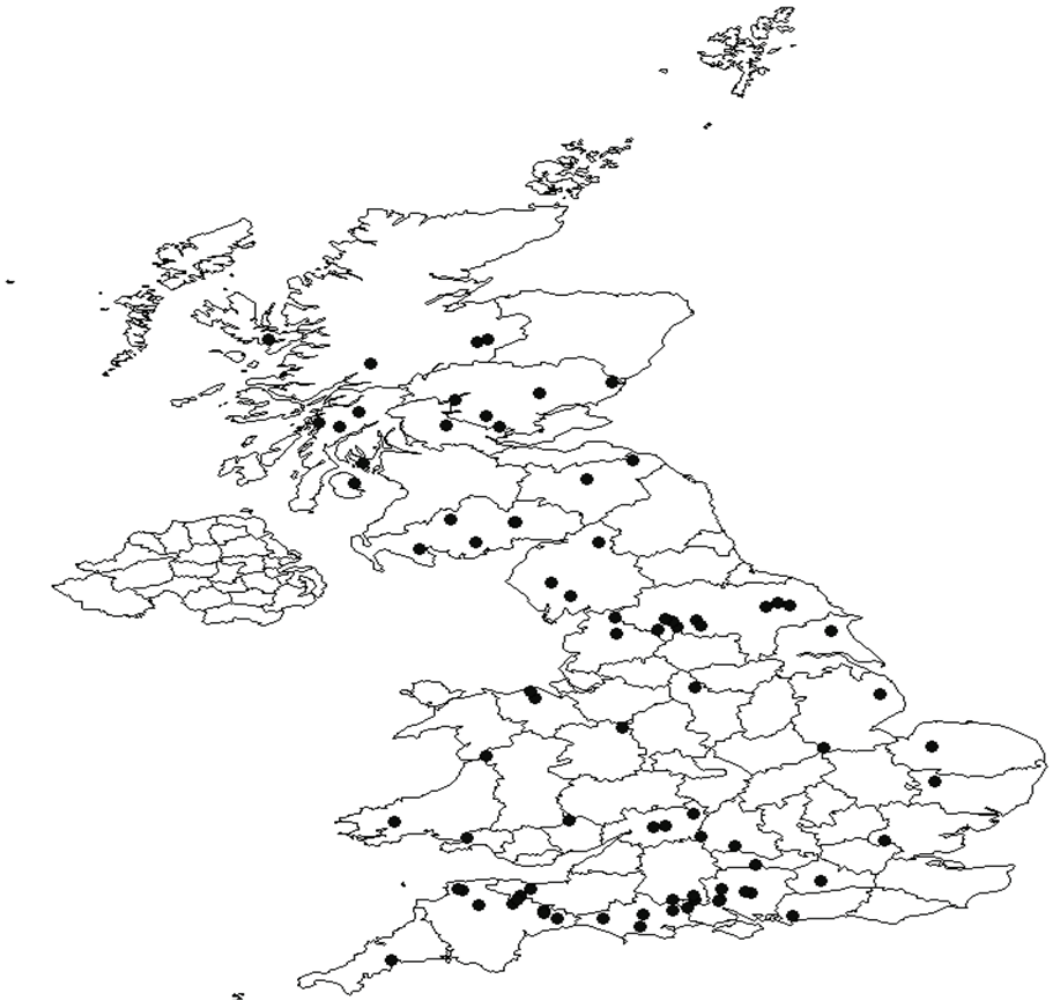

Figure 2.1. Map of the UK showing the approximate location of the rainbow trout producing sites (n=84) which participated in the rainbow trout gastroenteritis (RTGE) retrospective cross-sectional study.

Of all the sites included 29 (35%) produced exclusively for the table market, 34 (40%) for the restocking market, 17 (20%) produced for both markets and the remaining 4 (5%) were fry only producers. The residence time of trout at each

one of the sites ranged from 5 to 36 months (mean= 15mo). Trout were harvested at weights ranging from 5 to 3300 grams (mean= 698g). More than half of the sites stocked only rainbow trout, although 43% stocked other species as well, namely brown trout (*Salmo trutta*), brook trout (*Salvelinus fontinalis*) or carp. One feed company supplied 83% of the sites, with the remaining 17% being supplied by any of the other 13 companies. Only 11 sites (13%) used closed production systems *i.e.* eggs were produced from their own broodstock, hatched on site and no external fry were purchased.

2.4.2. Impact and distribution of RTGE in the UK

The survey results revealed a total of 11 RTGE cases (13% of sites) from 2000 to 2005 (Table 2.2). Only one of the cases had RTGE diagnosed every year from 2000 to 2005 and the rest presented RTGE during one to four years. All cases were located in two distinct areas, either the south of England or Scotland. Cases in the south of England were river-based and clustered in a relatively small area whereas Scottish cases were both river-based and loch-based sites and distributed throughout Scotland. All cases were high-level producers, producing 200 to 1000 tonnes/year for the table market, using externally supplied fry (non-closed system), with the exception of a single site that combined production for restocking as well.

Table 2.2. Number of participant sites on the RTGE retrospective epidemiology study divided by region and presence or absence of RTGE from 2000 to 2005.

REGION	*RTGE Absent (%)*	*RTGE Present (%)*	*TOTAL*
England	55 (93%)	4 (7%)	59
Scotland	12 (63%)	7 (37%)	19
Wales	6 (100%)	0 (0%)	6
TOTAL	73	11	84

The site-level prevalence of RTGE in the surveyed population ranged from 2% during 2000 to 8% during the year 2005 (Figure 2.2). An increasing trend in the total number of cases was observed, with new cases observed every year although none of the farms had RTGE present on all the years of the study.

All sites located within five miles in the same water system to RTGE sites were also positive for RTGE. As a result, it was not possible to analyse the risk associated with close geographical proximity to RTGE cases.

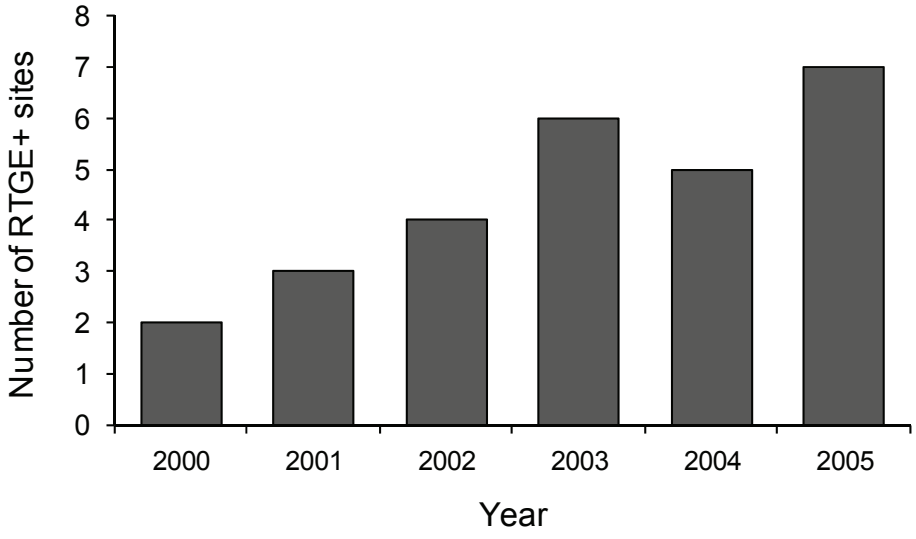

Figure 2.2. Bar chart displaying the total number of Rainbow trout gastroenteritis (RTGE) positive cases per year for all participant sites of the UK retrospective cross-sectional study (n=84).

2.4.3. Univariable analysis

The preliminary univariable analysis of the whole dataset revealed the presence of a confounding effect of mean production on several variables. To minimize this effect, stratified analyses were conducted by dividing the data into high and low producers. Since cases were only present in the stratum of high producers it would have not been possible to adjust for confounding by mean production

and the rest of the analysis was performed on a dataset including only high producers, which comprised 41 sites (11 cases and 30 controls).

The univariable analysis identified a total of 17 variables statistically associated with RTGE cases, of which 12 were categorical and 5 were continuous (Table 2.3).

Table 2.3. Dichotomous and continuous variables statistically associated with RTGE cases identified through univariable analysis of higher producers stratum data (>70 tonnes/year, n=41, p<0.05) from Rainbow trout gastroenteritis (RTGE) retrospective cross-sectional survey in the UK. Variables not significantly associated with RTGE were not included in this table.

Dichotomous Variable (Variable type)	*Crude Odds Ratio*	*95% Confidence interval (T)*	*P (FE)*
Fry Source 3 (Yes/No)	18.0	3.3-97.8	<0.001
Use of Processing plant 1 or 2 (Yes/No)	18.8	3.2-110.3	<0.001
Mean Production (High/Low)*	18.2	2.0-161.4	0.002
Fry Source 12 (Yes/No)	17.1	1.6-178.1	0.01
Fry Source 16 (Yes/No)	17.1	1.6-178.1	0.01
Automated and/or Demand Feeders (Yes/No)	11.3	1.0-123.2	0.05
Diploid vs. Triploid	8.2	1.0-72.4	0.03
ERM I.P. Vaccination†	6	1.4-26.6	0.02
Fry Source 8 (Yes/No)	3.1	1.7-39.5	0.01
Aeration vs. Oxygenation	0.2	0.05-0.9	0.04
Earth Substrate‡	0.2	0.1-1.0	0.05
Production for Restocking Only	0.1	0.01-0.8	0.01

(T) Taylor series, (FE) Fisher exact. *High>217 tonnes/year. †: ERM= Enteric red mouth disease; I.P.=Intraperitoneal. ‡: Only applies to river-based sites

Continuous Variable (Measure Unit)	*Median (mean) RTGE cases*	*Median (mean) non-RTGE cases*	*P (KW)*
Water Use (Million Gallons/day)*	20(21.5)	5(9.5)	0.002
Mean Production (tonnes/year)	300(452.7)	155(244.1)	0.003
Trout Residence Time (Months)	8(9.2)	12(14.3)	0.005
Trout Weight at Harvest (grams)	400(390)	600(823.4)	0.02
Maximum Water Temperature (^{0}C)	20(21.4)	18.5(18.3)	0.05

(KW)=Kruskal-Wallis analysis; * only applies to river based sites.

It was not possible to calculate the odds ratio for instances where there were no cases (or non-cases) exposed to the independent variable. For example, all cases harvested the fish at a weight lower than the median of the sample (median=469g), produced fish for the market table and used externally produced fry, and none of the cases used water from a borehole. Further analysis of fry sources significantly associated with RTGE did not reveal one or more sources that had been shared by all RTGE cases. Fry sources 12 and 16 were only present in cases from the South of England, whereas fry sources 3 and 8 were present in sites from both areas.

2.4.4. Multivariable analysis

The reduction of the sample size due to stratification did not affect the number of variables that could be included in the final multivariable model as the number of cases stayed constant, although the number of cases available (n=11) limited the maximum number of variables that could be included in a logistic regression model to 2 (Dohoo *et al.* 2003). Correlation analysis revealed significant multicolinearity between several variables significantly associated with RTGE (Table 2.4). Significantly correlated variables were not included in the same model. Forward and backward stepwise model building approaches yielded several acceptable models. The final model presented in Table 2.5 had the lowest -2*log likelihood ratio value of all the potential candidates.

Table 2.4. Inter-correlation between selected variables to be offered to the multivariable logistic regression model (P-values for Pearson correlation). These variables were identified by univariable logistic regression in the higher producers stratum data (>70 tonnes/year, n=41) from a Rainbow trout gastroenteritis (RTGE) retrospective cross-sectional survey conducted in the UK.

Variables	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*
a- Trout Residence time (months)	--								
b-Use of Processing plant 1 or 2	0.13	--							
c-Production for Restocking Only	**0.008**	**0.006**	--						
d-Trout Weight at Harvest (grams)	**<0.001**	0.07	0.48	--					
e-Automated and/or Demand Feeders	0.61	0.62	0.72	0.83	--				
f-Diploid vs. Triploid	0.22	0.34	0.10	0.84	0.42	--			
g-ERM I.P. Vaccination†	0.11	**<0.001**	**0.01**	0.10	0.32	0.34	--		
h-Use of Oxygenation	**0.01**	0.14	0.39	**0.03**	0.32	0.34	0.56	--	
i-Maximum Water Temperature (°C)	**0.005**	**0.002**	0.10	**<0.001**	0.71	0.72	**0.01**	0.09	--
j-Mean Production (Tonnes/year)	**0.004**	0.270	**0.01**	0.86	0.19	0.11	0.27	0.16	0.68

Headers a-j: Letters are used to abbreviate the table headers and correspond to each variable in the first column. †: ERM= Enteric red mouth disease; I.P.=Intraperitoneal.

The results of Hosmer-Lemeshow test (χ^2=2.15; d.f.=8; p=0.98) suggest that this model fits the data adequately. The constant was not significantly different from zero, as the first variable in the model is a risk factor whereas the second variable is protective (*i.e.* Higher residence time presents lower odds of belonging to the group of RTGE cases). The interaction between these two predictor variables was not significant. The confidence intervals around the point estimates were wide, suggesting that caution should be taken when drawing inference from this model.

Table 2.5. Multivariable logistic regression model for higher producers stratum data (>70 tonnes/year, n=41) from a Rainbow trout gastroenteritis (RTGE) retrospective cross-sectional survey conducted in the UK against the outcome RTGE case=Yes/No.

Variables (Unit)	*Odds Ratio (95% C.I.)*	*Z*	*P*
Use of Processing Plant 1 or 2 (Yes/No)	24.93(2.33-266.87)	2.66	0.008
Trout Residence Time (Months)	0.59(0.59-0.97)	-2.08	0.04
CONSTANT	*	1.25	0.21

-2*Log Likelihood=25.6, Degrees of Freedom=2, P<0.001

2.5. Discussion

The presence of RTGE in the UK was first confirmed in a rainbow trout-producing site in England in 2000 (Branson 2003) and no information has been available on the epidemiology of this condition within the UK rainbow trout industry since this report. This study aimed to appraise the extent and severity of RTGE in the UK rainbow trout industry and to identify the risk factors associated with RTGE at the site level.

The response rate to the survey was good with respondents representing 88% of UK trout production by weight in 2004 (DEFRA 2006). The sample was considered to be representative of the rainbow trout industry of the UK and comprised most types of farming systems, spread throughout the UK with the exception of Northern Ireland. Several sites did not participate and the remaining 12% of the production came from a relatively large number of smaller sites (42 or more), suggesting that the sample may have been biased to larger producers. Despite this, the inclusion of both large and small producers, including BTA members and non-BTA members suggest this is not the case, although this possibility should be considered when interpreting the findings of this study.

The survey identified a total number of 11 sites in the UK which had RTGE diagnosed by a fish health expert between 2000 and 2005. The number of cases by year suggested an increasing trend in the prevalence of RTGE positive sites from 2000 to 2005 in the survey sample. The inclusion of cases that were diagnosed by a fish health expert ensured an acceptable specificity of the screening method although the sensitivity of this design was not guaranteed, as health expert advice may not have been summoned if a

transitory problem due to RTGE did not present a significant impact on the overall site production. It is also possible that an increasing awareness on the presence of this disease in the UK could have raised the diagnosed/reported cases. There was therefore possible underreporting of the condition in the survey, although it is reasonable to expect that all significant RTGE outbreaks were included and the increasing trend is likely to be real. The results of the survey demonstrate that RTGE is not linked to a single geographical location with cases distributed in two distinct areas (*i.e.* South of England and Scotland) which have markedly different environmental and production characteristics. All sites in the same water system within 5 miles of a case were also cases, suggesting the possibility of local spread of this condition. However, it was not possible to test this hypothesis further.

The univariable analysis considered potential risk factors related to farming system, biotic and abiotic factors. RTGE was not restricted to a single farming system although it was strongly associated with high productivity level and even within the subset of high producers a significative association was found between RTGE cases and high mean production. It was not possible to eliminate completely the possibility of confounding by the mean production and several variables correlated with high production were also associated with RTGE cases in the high stratum only, including the use of oxygenation, vaccination strategies against enteric redmouth disease (ERM) and several fry and egg sources. Variables confounded by high production which applied exclusively to pond sites included higher water turnover and the use of a non-earth based substrate.

All RTGE cases produced rainbow trout for the table market and production for the restocking market was negatively associated with RTGE. This was reflected in the association of RTGE with lower trout turnover times, use of diploid trout instead of triploid and lower fish weights at harvest, all consistent with the production of portion-sized trout for the table market. None of the feed types was associated with RTGE, although the association of the use of automated and/or demand feeding systems with RTGE suggests that feeding strategies may play an important role in its development. Fish stocking management strategies, such as stocking density, weight of fish at stocking and the use of systematic fallowing were not associated with RTGE.

Univariable analysis also suggested an important role of a higher maximum water temperature in the summer, which was significantly associated with RTGE cases. The nature of the data collected did not allow the definition of a specific temperature range for the condition, as time interval data would have been required, but this finding is consistent with previous reports of the condition, which confirmed the presence of RTGE only when water temperatures were over 12-16°C (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001), although in one of these reports it was suggested that in Scottish farms the temperature threshold can be as low as 9°C (Branson 2003). RTGE was not associated with water calcium levels and none of the cases used borehole water. All cases bought fry from outside sources and four fry sources were significantly associated with RTGE cases although none of these was common to all cases, suggesting the absence of a common source of RTGE via fingerling introduction in the surveyed population. The presence of any other

animal species on site with potential contact with the fish was not associated with RTGE.

Multivariable logistic regression modelling of the dataset identified two factors strongly associated with RTGE cases, namely the use of one or both of two processing plants and lower trout turnover times. These two variables generated the best model, but a significant correlation with other variables associated with RTGE and the presence of other acceptable models suggested that these variables may also be interpreted as indicators of a specific type of site. The limited sample size of the dataset resulted in inflated odds ratios and confidence intervals, suggesting that the model has limited predictive power, although the presence of the factors included in the model are indicative of a higher risk of being a RTGE case (Dohoo *et al.* 2003). The use of one or both of two processing plants was associated with RTGE and further analysis revealed that these sites were an important subpopulation of the highest producers in the sample, strongly suggesting that RTGE may be exclusively associated with the subset of highest producers of the UK rainbow trout industry. Intensive aquaculture practices present many situations where stress and physical injury can strongly increase susceptibility to naturally occurring pathogens and the quantity of fish movements within and into intensive sites is necessarily higher, increasing the likelihood of disease introduction and spread (Ashley 2007; Bucke 1980). Transmission between sites via shared harvest equipment and vehicles from processing plants was not suggested by this analysis, as sharing of workers and equipment was not associated with RTGE cases, although the apparently increasing prevalence of RTGE within the UK together with the possibility of local spread observed in this study suggest an

important role of biosecurity in RTGE control, as previously suggested for other fish diseases (Danner & Merrill 2006; Scott 2004; St-Hilaire *et al.* 2002). In any case, more data is necessary to verify the hypothesis that RTGE is infectious. Shorter fish residence times were also strongly associated with RTGE and positive cases had residence times of 6 to 13 months. This type of productive cycle is characteristic of rainbow trout farms that produce for the portion size table market, as shown by the presence of correlation with production for the table market within the sample. Lower residence times have also been linked with intensive production and increased stress (Ashley 2007) and a relatively higher feed input is necessary to achieve the productive targets, a factor that could play an important role in the development of RTGE.

Concluding, this retrospective cross-sectional study has shown that RTGE was present in the UK in at least 11 sites from 2000 to 2005 and that it is likely that its prevalence in the UK has increased during this period. RTGE was associated with high productivity and production of fish for the portion size market. More research will be required to clarify the influence of the risk factors identified on the presentation of RTGE, including transmission trials, pathogenesis studies and epidemiology studies at the productive unit level.

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2.7. References

Angel, C. R., Sell, J. L., Fagerland, J. A., Reynolds, D. L. & Trampel, D. W. 1990, 'Long-segmented filamentous organisms observed in poults experimentally infected with stunting syndrome agent'. *Avian diseases*, vol. 34, pp. 994-1001.

Angert, E. 2005, 'Alternatives to binary fission in bacteria'. *Nature Reviews: Microbiology*, vol. 3, pp. 214-224.

Ashley, P. J. 2007, 'Fish welfare: Current issues in aquaculture'. *Applied Animal Behaviour Science*, vol. 104, pp. 199-235.

Branson, E. 2003, 'Rainbow trout Gastro-Enteritis (RTGE)-first diagnosis in the UK'. *Fish Veterinary Journal*, vol. 7, pp. 71-76.

Bucke, D. 1980, 'Fish farming and disease: Behavioural aspects'. *Applied Animal Ethology*, vol. 6, pp. 87-94.

Danner, G. R. & Merrill, P. 2006. 'Disinfectants, disinfection and biosecurity in aquaculture'. In: *Aquaculture Biosecurity. Prevention, Control, and Eradication of Aquatic Animal Disease*, A. D. Scarfe, C.-S. Lee, & P. J. O'Bryen, (Eds.), Blackwell Publishing, Oxford, pp. 91-128.

Davis, C. P. & Savage, D. C. 1974, 'Habitat, succession, attachment, and morphology of segmented, filamentous microbes indigenous to the murine gastrointestinal tract'. *Infection and Immunity*, vol. 10, pp. 948-956.

Dean, A. G., Arner, T. G., Sunki, G. G., Friedman, R., Lantinga, M., Sangam, S., Zubieta, J. C., Sullivan, K. M., Brendel, K. A., Gao, Z., Fontaine, N., Shu, M., Fuller, G., Smith, D. C., Fagan, R. F. & Nitschke, D. A. Epi Info™, a database and statistics program for public health professionals. [3.4.3]. 2007. Atlanta, Georgia, USA, Centers for Disease Control and Prevention.

DEFRA 2006, 'Summary of UK rainbow trout production in 2004'. *Finfish News*, vol. 1, p. 40.

Denham, K. 2004, 'Fish health controls: The activities of the fish health inspectorate in England and Wales 2003'. *Trout News*, vol. 38, pp. 29-31.

Dohoo, I., Martin, W. & Stryhn, H. 2003, *Veterinary Epidemiological Research*. 1st Ed., AVC Publishing, Charlottetown, Prince Edward Island, Canada.

Goodwin, M. A., Cooper, G. L., Brown, J., Bickford, A. A., Waltman, W. D. & Dickson, T. G. 1991, 'Clinical, pathological, and epizootiological features of long-segmented filamentous organisms (bacteria, LSFOs) in the small intestines of chickens, turkeys, and quails.'. *Avian diseases*, vol. 35, pp. 872-876.

Goodwin, M. A., Cooper, G. L. & Waltman, W. D. 1989, 'Diarrhea associated with long-segmented filamentous organisms (LSFO) in the small intestines of turkeys, chickens and quails'. In: *38th Western Poultry Disease Conference*, pp. 103-109.

Jiang, H.-Q., Bos, N. A. & Cebra, J. J. 1999, 'A comparison of some innocuous commensal gut bacteria and an enteric virus in stimulating the specific and non-specific development of IgA production in the gut of formerly germ-free mice'. *Immunology letters*, vol. 69, p. 188.

Klaasen, H. L. B. M., Koopman, J. P., Poelma, F. G. J. & Beynen, A. C. 1992, 'Intestinal, segmented, filamentous bacteria'. *FEMS Microbiology Reviews*, vol. 88, pp. 165-180.

Klaasen, H. L. B. M., Koopman, J. P., Van den Brink, M. E., Bakker, M. H., Poelma, F. G. J. & Beynen, A. C. 1993, 'Intestinal, segmented, filamentous bacteria in a wide range of vertebrate species.'. *Laboratory Animals*, vol. 27, pp. 141-150.

Michel, C., Bernardet, J. F., Daniel, P., Chilmonczyk, S., Urdaci, M. C. & de Kinkelin, P. 2002, 'Clinical and aetiological aspects of a summer enteritic syndrome associated with the sporulating segmented filamentous bacterium "*Candidatus* arthromitus" in farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum)'. *J.Fish Dis.*, vol. 25, pp. 533-543.

Morishita, T., Lam, K. & McCapes R.H. 1992, 'Isolation of two filamentous bacteria associated with enteritis in turkey poults'. *Poultry Science*, vol. 71, pp. 203-207.

Murray, R. G. E. & Stackebrandt, E. 1995, 'Taxonomic note: Implementation of the provisional status *Candidatus* for incompletely described prokaryotes'. *International Journal of Systematic Bacteriology*, vol. 45, pp. 186-187.

Oppenheim, A. N. 1992, *Questionnaire design, interviewing and attitude measurement*. Pinter Publishing Ltd., London.

Scott, A. 2004, 'Sleeping disease: A lesson in biosecurity'. *Trout News*, vol. 37, pp. 17-23.

Snel, J., Blok, H. J., Kengen, M. P., Kudwig, W., Poelma, F. G. J., Koopman, J. P. & Akkermans, A. D. L. 1994, 'Phylogenetic Characterization of *Clostridium* Related Segmented Filamentous Bacteria in Mice Based on 16S Ribosomal RNA Analysis'. *Systematic and Applied Microbiology*, vol. 17, p. 172.

Snel, J., Heinen, P. P., Blok, R. J., Carman, R. J., Duncan, A. J., Allen, P. C. & Collins, M. D. 1995, 'Comparison of 16S rRNA sequences of segmented filamentous bacteria isolated from mice, rats, and chickens and proposal of "*Candidatus* arthromitus"'. *International Journal of Systematic Bacteriology*, vol. 45, pp. 780-782.

St-Hilaire, S., Ribble, C. S., Stephen, C., Anderson, E., Kurath, G. & Kent, M. L. 2002, 'Epidemiological investigation of infectious hematopoietic necrosis virus in salt water net-pen reared Atlantic salmon in British Columbia, Canada'. *Aquaculture*, vol. 212, pp. 49-67.

Talham, G. L., Jiang, H.-Q., Bos, N. A. & Cebra, J. J. 1999, 'Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system'. *Infection and Immunity*, vol. 67, pp. 1992-2000.

Thrusfield, M. V. 2006, *Veterinary Epidemiology*. Third Ed., Blackwell Publishing, Oxford.

Toranzo, A. E. 2004. 'Report about bacterial diseases'. In: *Mediterranean Aquaculture Laboratories*, P. Alvarez-Pellitero et al., (Eds.), CIHEAM/FAO, pp. 49-89.

Umesaki, Y., Okada, Y., Matsumoto, S., Imaoka, A. & Setoyama, H. 1995, 'Segmented filamentous bacteria are indigenous intestinal bacteria that activate intraepithelial lymphocytes and induce MHC class II molecules and fucosyl asialo GM1 glycolipids on the small intestinal epithelial cells in the ex-germ free mouse'. *Microbiology and Immunology*, vol. 39, pp. 555-562.

Urdaci, M. C., Regnault, B. & Grimont, P. A. D. 2001, 'Identification by in situ hybridization of segmented filamentous bacteria in the intestine of diarrheic rainbow trout (*Oncorhynchus mykiss*)'. *Research in Microbiology*, vol. 152, pp. 67-73.

CHAPTER 3. Prospective Longitudinal Study of "*Candidatus* arthromitus"-Associated Rainbow Trout Gastroenteritis in the UK

Del-Pozo, Jorge*; Crumlish, Margaret; Ferguson, Hugh W.; Turnbull, James F.

3.1. Abstract

Rainbow trout gastroenteritis (RTGE) is an emerging disease of farmed rainbow trout (*Oncorhynchus mykiss*) reported in Croatia, France, Italy, Spain and the UK. The impact of RTGE at the site level varies and daily mortalities of 0.5-1% are common. The gross lesions seen in affected fish include severe enteritis with congestion and oedema. The segmented filamentous bacterium "*Candidatus* arthromitus" has been suggested as a possible aetiological agent of RTGE. A limited number of epidemiological observations on RTGE are available in the literature and climatic and stress factors such as water temperature and fish handling have been indicated as possible risk factors. This prospective longitudinal epidemiology study was designed to describe the impact, presentation, current control strategies and spread pattern of RTGE within affected UK sites. Risk factors associated with both the presence and severity of the syndrome were also investigated. For this purpose, a longitudinal study at the population level was designed and data collected prospectively from June to November 2006 from 12 RTGE positive UK sites. This information was used to describe RTGE presentation and pattern of spread, as well as the treatments used during outbreaks. Conditional multivariable logistic regression (CLR) and general linear modelling (GLM) were used to identify potential risk factors associated with the presence and severity

of RTGE. The results of the descriptive analysis strongly suggested that RTGE is infectious, an observation supported by risk analysis of fish transfers and unit location. The CLR analysis identified eight variables significantly associated with the presence of RTGE, including two models with four environmental and four management factors. Additionally, GLM analysis identified a significant association of mean feed input per fish during an outbreak with RTGE cumulative mortalities.

3.2. Introduction

Rainbow trout gastroenteritis (RTGE) is an enteric syndrome affecting commercially reared rainbow trout *Oncorhynchus mykiss* (Walbaum) during the summer (Michel *et al.* 2002; Urdaci *et al.* 2001). The clinical presentation of the syndrome includes severe congestion and oedema of the intestinal wall associated with the presence of large numbers of the segmented filamentous bacterium "*Candidatus* arthromitus" (Michel *et al.* 2002). RTGE has been reported in several European countries (Toranzo 2004; Cervellione, personal comm.; Branson 2003; Michel *et al.* 2002). In France, Spain and the UK, RTGE has spread beyond the site where it was first detected and has become a significant problem for the rainbow trout industry (Branson 2003; Chapter 2; Michel *et al.* 2002; Sanz 2000). A retrospective epidemiological study of RTGE in the UK (Chapter 2) reported an increase from two to seven in the total number of RTGE positive sites between 2000 and 2005. In this study, RTGE was associated with high levels and rapid rates of production of fish for the portion size table market. The impact of RTGE on the sites affected is economically significant, and daily mortalities of 0.5-1% of relatively big fish (≥0.8 kg) have been reported (Michel *et al.* 2002). Mortalities peak

approximately 10-14 days after onset, and cease approximately 3-4 weeks later, after the fish reach 160-200 g or when water temperatures drop below 12°C (Branson 2003). Anecdotal observations have led to the suggestion of several risk factors that could be associated with RTGE.

Firstly, in a single land-based site, RTGE was observed within a limited group of ponds receiving first-use water but never in ponds receiving second-use water, even if the water originated from an affected unit (Branson 2003). Other suggested factors have included stressors, such as handling (Michel *et al.* 2002). All the authors agree on the importance of water temperature, but there is disagreement between reports on the minimum temperature at which RTGE is observed (12-16°C) and none of these reports provides information on the number of outbreaks used to extract these figures (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). Additionally, RTGE has been reported once at water temperatures of 9°C in a single Scottish site (Branson 2003), suggesting there may be variability in the presentation and therefore a relatively large sample is required.

Other anecdotal epidemiological observations include the disappearance of clinical RTGE after changes in diet or environment and an apparent association of low-energy diets with RTGE (Michel *et al.* 2002; Urdaci *et al.* 2001). Several approaches have been used for the treatment of RTGE, although there are no reports of the efficacy of any of these treatments. These have included 3-4 days fasting followed by 6 days of tetracycline or oxytetracycline oral administration (Urdaci *et al.* 2001), flumequine treatments every 25 days (Sarti *et al.* 2008) and various percentages of sodium chloride (NaCl) mixed in with the feed (McKenzie, K. personal comm.). The review of the literature available

on RTGE at the beginning of this study revealed that most of the epidemiological information on RTGE was based on anecdotal observations.

An epidemiological study was designed with the aim of describing the impact, pattern of spread and risk factors associated with RTGE within a population of sites affected by this syndrome. The ultimate aim was to provide data applicable to the prevention and control of this disease in rainbow trout productive systems (Hedrick 1998).

3.3. Materials and Methods

3.3.1. Study design

A prospective longitudinal study was designed to investigate the presence and severity of RTGE, with the productive unit as the observational unit. A productive unit was defined as a population of rainbow trout stocked in the same cage/tank/pond/raceway at a specific point in time. The timeframe was June to November 2006.

3.3.2. Study population and inclusion criteria

The study involved 17 rainbow trout producing sites chosen during a pre-sampling phase. All 17 sites had participated previously in a retrospective study of RTGE in the UK (Chapter 2) and agreed to participate in the study on the basis of confidential handling of the data collected. The number of sites selected was as high as practicable while allowing for regular visits to all the sites. Every one of the selected sites produced more than 200 tonnes per year of rainbow trout for the table market and RTGE had been reported previously in at least 11 of them (Chapter 2). These criteria were chosen so RTGE could be

reasonably expected to occur in a proportion of these sites during the study. The timeframe of the study was chosen to include the time of the year when RTGE had been previously observed, adding one month before and afterwards to ensure inclusion of complete RTGE outbreaks (Branson 2003). At the end of the study a total of 12 sites were positive for RTGE and analysis was performed only in data from these sites.

Descriptive analyses were performed on all the data from the 12 affected sites for reference to the total number of units. For further statistical analysis, only relevant cases and controls were included. To ensure that units from the population at risk were included as negative controls, only units that had been stocked for at least a month and fed at least once during the study were included as controls. These criteria filtered out the units that were used for fish transfer management only (*i.e.* grading, harvest, *etc.*). The cases did not include units with concurrent diseases and/or RTGE daily mortalities that did not exceed 0.05%.

3.3.3. Data Collection

All the data used in the study were collated from farm records. These records were kept on paper or computer and included information collected daily from June to October 2006 for every unit in the study. Computer records were exported from FarmControl™ (Maritech, UK) and DJournal™ (Skretting, UK) software, using standard exporting features of these packages and paper records were manually entered onto spreadsheets. All the data were finally collated in a single spreadsheet for convenience of analysis.

3.3.3.1. RTGE case definition and recording

During the study the following case definition was used to identify affected fish: *"RTGE is a condition of rainbow trout, associated with daily mortalities of 0.5% or more and present during the summer. Affected moribund fish usually present a lighter colouration as well as a generally swollen appearance externally, while internally, their lower intestine is distended, congested, oedematous and has a yellow viscous content. Other organs appear apparently unchanged"*. This case definition was created from literature searches and preliminary field observations (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001) and facilitated the selection of RTGE cases based on gross lesions, while minimising the presence of concurrent disease in the sample (Chapter 4). This case definition was used to create RTGE diagnosis guidelines that were sent to each participant site before the start of the study, in order to enable recognition of RTGE by site staff with consistency across the sites (Appendix III). In addition, all participant sites were visited regularly during the period of the study to verify RTGE diagnosis and recording by the site workers. This was assessed by collection and analysis of samples from all suspected RTGE positive units at the time of the visit for gross examination and bacteriological analysis. A unit was only considered a case when RTGE had been recorded during the period of the study. Both the presence and cumulative mortalities of RTGE were considered outcomes for subsequent analysis.

3.3.3.2. Independent variables

All the continuous variables were included in the analysis both as recorded, summarized (minimum, mean and maximum) and categorized through the median value (Table 3.1).

Table 3.1. Variables recorded during a RTGE prospective longitudinal study. Each variable was recorded daily for each one of the stocked units in all sites.

Category	*Variable*	*Possible Values*
Outcome Variables	RTGE presence	Yes/No
	RTGE cumulative mortality (%)	Continuous, Min, Max, Mean
Site Management	Number of days stocked	Continuous, Min, Max, Mean
	Aeration System	Yes/No
	Oxygenation System	Yes/No
	System Type	Cages Ponds Raceways Tanks
	Feeding system	Automated Demand Hand
	Feed input*	Continuous, Min, Max, Mean
	Feed type	1 to 14
	Pellet size (mm)	Continuous, Min, Max, Mean
	Treatments	Starvation In-feed NaCl mixed on site Commercial in-feed NaCl In-feed Liquid Paraffin In-feed Oxytetracycline Other in-feed Antibiotic Chloramin T in water + In-feed Liquid paraffin Chloramin T in water Formalin in water
	Number of Movements	Continuous, Min, Max, Mean
	Fish transfer	RTGE to RTGE RTGE to Healthy Healthy to RTGE Healthy to Healthy Stocking Harvest
	Contiguity	Contiguity to RTGE cases Downstream to RTGE cases**
	Water Usage (number of times)	Continuous**
Environment	Water temperature (°C)	Continuous, Min, Max, Mean
	Bottom material	Concrete Earth Fibreglass Metal Net
Fish Variables	Total biomass (kg)	Continuous, Min, Max, Mean
	Mean Weight (g)	Continuous, Min, Max, Mean
	Stocking Density (kg/m^3)	Continuous, Min, Max, Mean
	Source of fry	1 to 34
Other Mortality Causes	Bacterial Kidney Disease (BKD)	Yes/No
	Costiasis	Yes/No
	Enteric Red Mouth (ERM)	Yes/No
	Furunculosis	Yes/No
	Handling mortalities	Yes/No
	Predation	Yes/No
	Proliferative Kidney Disease	Yes/No
	Rainbow Trout Fry	Yes/No
	Sleeping Disease (SD)	Yes/No
	Unspecified mortalities	Yes/No
	White Spot (“Ich”)	Yes/No

* Feed input was expressed as a percentage of the individual fish weight; **: Only applies to land-based sites

3.3.3.2.1. Environment

Mean daily temperature was recorded in °C as part of standard site management procedures by all participant sites. Measurements were taken in a single location within the site. Information on the number of times water was used before entering specific units was obtained from analysis of the layout of all land based sites. Information on aeration systems and bottom material used for each unit was collected on site visits.

3.3.3.2.2. Feeding practices

A categorical variable was created to record the feeding system used in each one of the units included in the study. In addition, feed type (Type 1 to 15), pellet size and weight fed were recorded daily for each unit. In order to take into account variations in feed input due to the number of fish in the unit as well as the mean weight across the fish in the unit, feed input was expressed in the analysis as a percentage of individual fish weight.

3.3.3.2.3. Fish stocking data

Information on the source of fry was available for all the fish present in a site and coded as categorical variables. Each one of the 34 fry sources used by all sites was assigned a random number for confidentiality. In the event of mixing of fish from different batches, all fry sources of the fish stocked in the unit were considered simultaneously in the analysis. The mean fish weight was recorded by site staff by regular weighing of samples consisting of 100 individuals from each unit. These figures were then recorded in a database, which then estimated the daily variation between samples according to the feed input. This figure was also expressed as stocking density (number of fish/m^3) during the analysis.

3.3.3.2.1. Disease management

Several variables were created to record all the treatments given to the fish during the period of the study, including treatment type (1 to 17) and length of the course of treatment. Dosage was also recorded as a percentage of the feed for in-feed treatments.

3.3.3.2.2. Mortalities

Other causes of mortality were recorded during the study both in RTGE cases and controls and were included in the analysis as dichotomous variables (*i.e.* presence or absence) to examine the possible effect that they may have had in the presence and prevalence of RTGE. Causes of observed mortality other than RTGE included unspecified diseases, predation and losses due to fish handling.

3.3.3.2.3. Movement and contiguity data

All fish movements, including intra-site movements, stockings and harvests were recorded as part of normal management procedures. This information was collated in a single spreadsheet and each movement categorized according to the RTGE status of the source and receiving unit: healthy to healthy, healthy to RTGE, RTGE to healthy, RTGE to RTGE, stocking and harvesting. For receiving units that eventually became cases, only inward movements previous to outbreaks were included in the analysis.

A map of each site was used to record units that were contiguous to or downstream (only land based) from previous cases. The map was not available for one site, which was not included in the analysis and the first RTGE case in each site (*i.e.* "index" case) was not considered for contiguity analysis.

3.3.4. Statistical analysis

All data management and analyses in this study were conducted in MS Excel™ (Microsoft, USA), SPSS™ (SPSS Inc., USA) and EpiInfo™ (CDC, USA) computer packages.

3.3.4.1. Incubation period descriptive analysis

Two different approaches were taken to describe the incubation period of RTGE on the 12 sites included in the study. The first one involved the selection of previously empty units that had been stocked with fish from outside the site during the period of the study and then undergone an outbreak. Intra-site transfers were not considered for this analysis to reduce the risk of including fish that may have been already incubating the disease. Once the selection was made, histograms of the time elapsed between stocking and RTGE onset were plotted and the observed frequencies analysed. The second approach involved analysis of the time in days between RTGE outbreaks in units that had suffered two consecutive RTGE outbreaks during the period of the study. These two approaches allowed the estimation of the incubation plus the time course of the disease, as the first day when RTGE mortalities were recorded was the reference value.

3.3.4.2. Descriptive analysis of RTGE treatments

All treatments used 15 days before and during RTGE outbreaks were described, including summary statistics and analysis of mortality plots in respect of treatment days.

3.3.4.3. Analysis of RTGE spread pattern

Intra-site movements and contiguity data were included in the case-control study as well as analysed separately in order to obtain further information on

the role of these two variables in the spread pattern of RTGE within sites. Preliminary analysis suggested clear differences in the spread pattern of RTGE between water-based sites and land-based sites, which led to the performance of stratified analyses. In addition, the simultaneous presentation of RTGE across most units in a cage site suggested the fish movement data from this site were not suitable for this part of the study. During the analysis, the risk of receiving units becoming cases after fish transfer from an affected unit was examined using contingency tables. Then, the differences in the RTGE relative risk of receiving units depending on the timing of transfer from RTGE positive units were examined. This analysis was conducted on a dataset including only outgoing movements from RTGE positive units, which were classified according to a 15-day time series, as shown in Table 3.3. All fish transfer data seven weeks before and seven weeks after RTGE outbreaks were used as a reference and all other time series groups were compared with this reference using contingency tables. A Fisher Exact test was used to assess the significance of the Mantel Haenszel-adjusted odds ratios, with a significance level of $p<0.05$.

Table 3.2. Fifteen-day time series used for analysis of the RTGE risk of receiving units depending on the timing of fish transfer relative to the RTGE outbreak on the outgoing unit. All the frequency data from groups -6 to 5 were compared against data from the control groups -7 and 7

TIME SERIES	*Weeks relative to RTGE outbreak***
-7*	7 & MORE WEEKS PRE-OUTBREAK
-6	5 & 6 WEEKS PRE-OUTBREAK
-4	3 & 4 DAYS PRE-OUTBREAK
-2	1 & 2 WEEKS PRE-OUTBREAK
0	FIRST TWO WEEKS OF OUTBREAK
00	3 WEEKS TO END OF OUTBREAK
1	1 & 2 WEEKS POST-OUTBREAK
3	3 & 4 WEEKS POST-OUTBREAK
5	5 & 6 WEEKS POST-OUTBREAK
7*	7 & MORE WEEKS POST-

*Data from these groups were used as reference for analysis

** Time in relation to RTGE outbreak in outgoing unit

The analysis of contiguity data was conducted in a similar fashion and both the odds of contiguity and downstream location to RTGE cases were calculated using contingency tables stratified by earth-based and water-based sites and tested with Fisher Exact as explained above. In addition, Kaplan-Meier analysis of survival was used to evaluate differences in the time from the beginning of the study to the onset of RTGE, harvest or the end of the study. These differences were assessed for units that were contiguous to cases for all site types or for downstream/upstream location to cases for land based sites only.

Finally, both fish movements and contiguity to cases were included in an unconditional multivariable logistic regression model. The first RTGE cases on each site were eliminated from the analysis and only units for which data on both aspects were available were included, resulting in a final number of 15 cases and 32 controls. These variables were previously tested for correlation using Pearson product-moment correlation coefficient and all possible interaction terms in the model were tested (Thrusfield 2006; Dohoo *et al.* 2003).

3.3.4.4. Case-control study

A case-control study was performed on the data from the selected RTGE cases and healthy units from the population at risk. Cases were matched to controls in system type and geographical location. System type was chosen due to its confounding association with several management variables and to account for the differences found between earth-based and water-based sites, whereas geographical location (England/Scotland) was chosen for its association with all the environmental variables. A ratio of two controls per case was used for all matches by random selection of cases and controls within each match, leading to a final number of 43 cases and 86 controls. All continuous variables were

then summarized and minimum, maximum and mean values were included in the analysis both as continuous and categorised through the median value.

Firstly, univariable analysis was conducted on the resulting data. Continuous independent variables were compared using Kruskal-Wallis test and categorical variables were analysed with Fisher's exact test and stratified by system type and geographical location. Mantel-Haenzel adjusted odds ratios (ORs) were used for measurement of the strength of associations. Variables with a $p< 0.25$ in univariable analyses were included in multivariable conditional logistic regression (CLR) models (Rahman *et al.* 2003) where controls were matched to cases by system type and geographical location. Before model building, the assumption of linearity of the logit of continuous variables with the dependent variable was tested using the Box-Tidwell transformation test and continuous variables that did not meet this assumption were included as dichotomous variables only. All variables were also tested for correlation and all variables with Pearson product-moment correlation coefficient over 0.7 were not included in the same model. The maximum number of explanatory variables included in the final model was limited by the number of events per variable (Bagley *et al.* 2001; Peduzzi *et al.* 1996) and both forward and backward stepwise model building approaches were used. The best model was determined by a significant reduction in the -2*log likelihood statistic. All the interaction terms within the models were evaluated (Thrusfield 2006; Dohoo *et al.* 2003).

3.3.4.5. General linear model

A general linear model (GLM) was used to assess the effect of management practices during the outbreak on the severity of RTGE, expressed as the cumulative mortality percentage of each RTGE outbreak. To achieve this, a spreadsheet was created only including data collected during RTGE outbreaks in all selected cases.

Examination of RTGE cumulative mortality indicated over dispersion and skewing to the right of the untransformed dependent variable. As a consequence of this, logarithmic transformation (ln) was applied, which resulted in a normally distributed outcome suitable for GLM analysis (KS=0.072; $p>0.15$). This transformation was also applied to the independent variables when appropriate. Additionally, four outliers were eliminated from the data, where cumulative mortalities had been overestimated as a result of fish movements into these units during the outbreak. This action resulted in a final dataset including 69 RTGE cases. Univariable GLM was used to select the variables and those with $p<0.05$ were retained as covariates for multivariable GLM analysis. In every model, the site variable was included in the model as a random factor in order to account for intrinsic differences between the sites. Correlated variables were not included simultaneously in the same model and both forward and backward stepwise procedures were used to find the most appropriate model, which presented the highest adjusted R^2 value. All possible variable interactions were tested and the final model appropriateness was finally confirmed by leverage-point analysis and normality testing of the model's residuals.

3.4. Results

3.4.1. Descriptive results

3.4.1.1. Site characteristics and RTGE impact at the site level

The presence of RTGE was confirmed in 12 UK rainbow trout producing sites. Some characteristics of the units in these sites are summarized in Table 3.3.

Table 3.3. Summary statistics of all stocked productive units (cage/tank/pond/raceway) from 12 RTGE+ UK sites (n=420).

	Min	*Mean*	*Max*	*SD*
Stocking days (d)	4	113.9	153	38.3
N° Fish	97	30430.4	148474	26758.3
Mean Fish Weight (g)	4	144.8	2859	225.0
Stocking Density (kg/m^3)	0.3	17.6	63	13.0
Water Temperature (°C)	7	15.2	24	0.9
N° Times water is used	1	1.3	8	0.9
N° fish movements*	0	8.8	63	10.1

*Includes all movements in or out of the units. SD: Standard deviation

Within these sites, RTGE was observed and recorded in 164 productive units (39% of all stocked units). The number of units affected by RTGE varied from 2 (5.7%) to 44 (95.7%) within a site. Fish losses due to RTGE totalled 61.4 tonnes representing 27% of the total mortality weight of all selected sites during the period of the study. Total cumulative RTGE mortalities at the unit level ranged 0.02-77.9% with daily RTGE mortalities of 0.002-21.9%.

3.4.1.2. RTGE presentation in affected units

RTGE was first reported in June in southern sites and in July in northern sites. A total of 73 units were selected as cases for further analysis. RTGE represented 53.8% of the total number of mortalities recorded in the selected units. Mortalities due to disease occurring at different times to RTGE included common summer parasitic, bacterial and viral infections. The characteristics of the selected cases are shown in Table 3.4.

Table 3.4. Summary statistics of 73 RTGE-affected units from 12 RTGE+ sites. During RTGE outbreaks, concurrent diseases were absent and daily RTGE mortalities exceeded 0.05%.

Variable	*Min*	*Mean*	*Max*	*SD*
Number of RTGE Outbreaks	1	1.2	2	0.4
Total Number of Days Stocked (d)	42	125.5	153	30.0
Total Number of Fish Movements*	0	10.4	63	10.5
Stocking Density (kg/m^3)†	0.8	25.9	58.9	12.7
Fish Mean Weight (g)†	16	216.5	564	103.9
Water Temperature (°C)†	9.8	15.9	22.0	2.0
Water Temperature (°C)‡	12.0	16.1	20.7	1.7

SD: Standard deviation. * All movements in or out of the units. † During RTGE outbreaks. ‡ During first day RTGE outbreaks.

RTGE was more commonly observed within ponds and raceways in land-based sites. It was observed only twice in tanks as an apparently concurrent cause of mortalities with proliferative kidney disease (PKD) and for this reason, these cases were not used for further analysis.

The mean length of RTGE outbreaks was 25.5 days, although it ranged from 3 to 63 days. For visual examination of the epidemic curves rolling mean mortality values were plotted, as mortalities were not recorded daily and the plotting of raw data could have obscured the underlying presentation. These plots frequently (51%) revealed an epidemic pattern involving a lower primary peak of mortality numbers followed by a higher secondary peak, with 20-25d of separation between peaks (Figure 3.1 A). In 41% of the cases, a single mortality peak could be observed (Figure 3.1 B). These two presentation types were not clearly delimited in several longer outbreaks, but this occurrence was infrequent (8%).

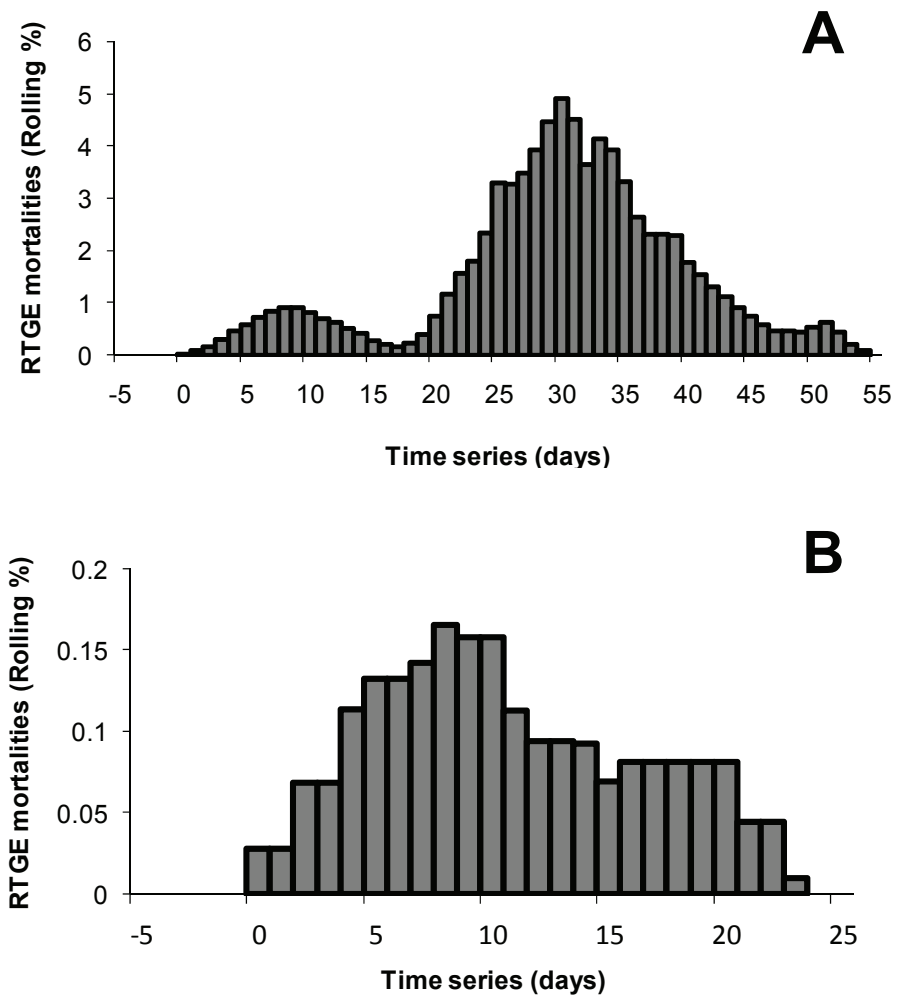

Figure 3.1. Examples of two typical presentations of RTGE outbreaks. Day 0 indicates the first day of the outbreak. A: Primary peak in mortalities followed by a higher secondary peak and a smaller tertiary peak; B: Single mortality peak. Note a shorter outbreak with lower mortalities in B.

3.4.1.3. RTGE incubation period

The mean time elapsed between stocking of previously empty units to RTGE onset was 23.7 days (4 to 48 days; SD=11.7). The mean time between repeated outbreaks was 22.6 days (19 to 26 days; SD=2.2).

3.4.1.4. RTGE treatments

A total of 66 units were treated at least once during RTGE outbreaks of which 11 (16.7%) received more than one type of treatment. Six different treatments were used against RTGE (Figure 3.2). These treatments included: (a) 4% NaCl

mixed manually with standard commercial feed and externally covered with fish oil for 5 days; (b) 4% NaCl included within a specially formulated commercial pelleted feed for 10-15 days; (c) 2% liquid paraffin mixed manually with the feed for 2-3 days; (d) Chloramin-T bath treatment at 6ppm and (e) 1% in-feed oxytetracycline for 10 days. Treatments (d) and (e) were never given in isolation and were always combined with in-feed salt or in-feed liquid paraffin.

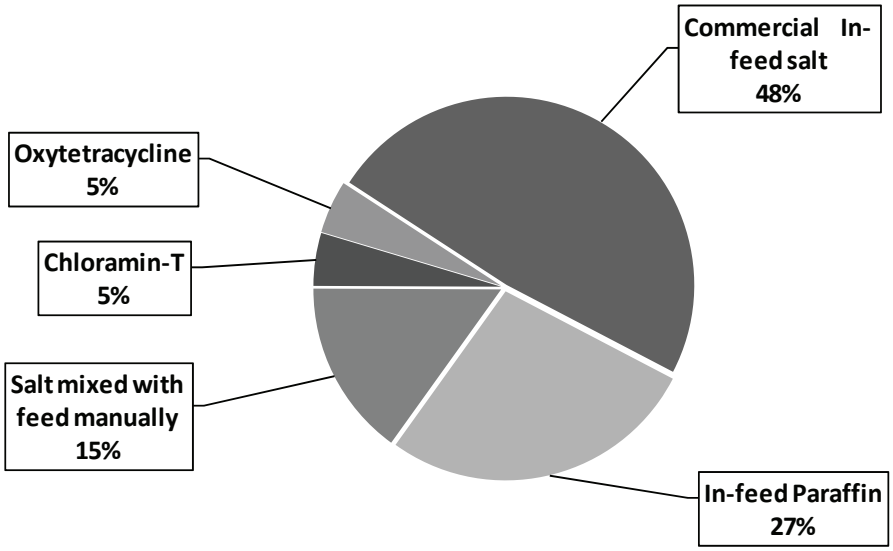

Figure 3.2. Frequency of usage of 6 different treatments targeted RTGE during 2006 in 12 RTGE-affected UK sites.

None of the treatments eliminated the condition completely in every usage and secondary outbreaks were observed in 16 units treated previously against RTGE (24.2%). In addition, RTGE occurred shortly after treatment with NaCl or liquid paraffin in units that were previously unaffected, suggesting neither of these two treatments prevented RTGE.

The effect of the treatments on RTGE mortality plots was generally inconsistent and varied between units, although several patterns were observed. These included an apparent reduction in RTGE mortalities **during** treatment with both

types of in-feed NaCl. This was observed in 79% of the units, although RTGE mortalities were still present after treatment in 58% units (Figure 3.3 A). This reduction in mortalities was more frequently observed (83% units) when in-feed NaCl was administered during periods of 10-15 days as opposed to periods of 3 days (60% units). An anecdotal observation was made in a single site, which used commercial in-feed NaCl with an alternating regime (*i.e.* 15 days on supplemented diet followed by 15 days on non-supplemented diet). In this site, RTGE outbreaks occurred in the periods when non-supplemented diet was given to the fish (Figure 3.3 B).

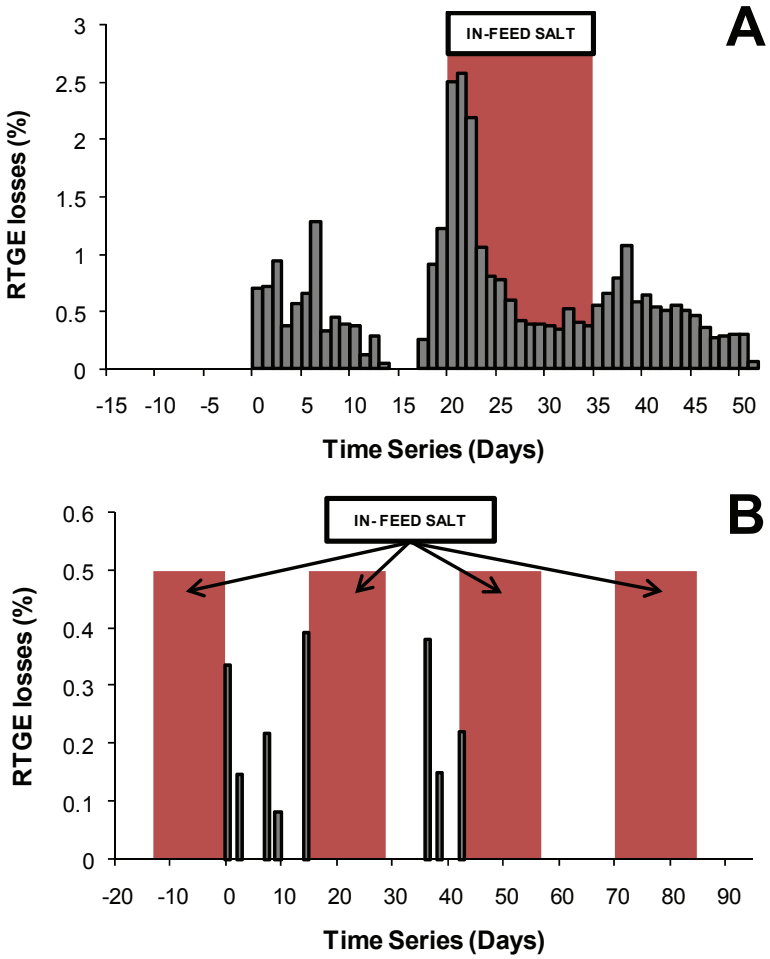

Figure 3.3. RTGE epidemic curves of two selected units (12 sites). A: Sharp decrease in RTGE mortalities during in-feed NaCl treatment (highlighted). Note a temporary increase of RTGE mortalities after the end of the treatment. B: Example of mortality pattern observed in a site using an alternating regime of in-feed NaCl (highlighted).

A reduction in mortalities due to RTGE was observed in only 13.3% of all the units treated with liquid paraffin.

3.4.1.5. Pattern of spread

The results of initial analysis of fish transfer data in respect to the RTGE status of outgoing and receiving units are displayed in Table 3.5. A total number of 935 intra-site fish transfers were examined during this analysis and significant increases in the odds of being RTGE+ after receiving fish from a RTGE positive unit were observed in land-based sites, whereas the differences were not significant in cage sites. An epidemic pattern consistent with Figure 3.1 A was observed in 31% of the units that became positive after transfer.

Table 3.5. Results of the analysis of intra-site fish transfers in respect to the RTGE status of outgoing and receiving units and stratified by site type (12 sites).

	INTRA-SITE FISH MOVEMENT FREQUENCIES						
	Healthy to Healthy	***RTGE to Healthy***	***Healthy to RTGE***	***RTGE to RTGE***	***Odds ratio****	***95% CI***	***P (FE)***
Land Based	375	140	26	34	3.5	1.96-6.27	<0.001
Water Based	224	45	79	12	0.76	0.36-1.57	0.5

*Odds of being RTGE positive after incoming fish transfer from a RTGE positive unit. FE: Fisher Exact (threshold P<0.05)

No fish movements out of RTGE+ units undergoing an outbreak were made in cage sites, making time series analysis impossible for this type of site. However, this analysis was possible for land-based sites and it was found that the relative RTGE risk of receiving units was significantly increased only if the fish introduced originated from a case unit that was undergoing an RTGE outbreak at the time of the transfer (Figure 3.4). The relative risks ranged from

2.58 (95% CI: 1.82-3.65) if transfer took place during the first two weeks of the outbreak to 2.2 (95% CI: 1.63-2.97) during the rest of the outbreak.

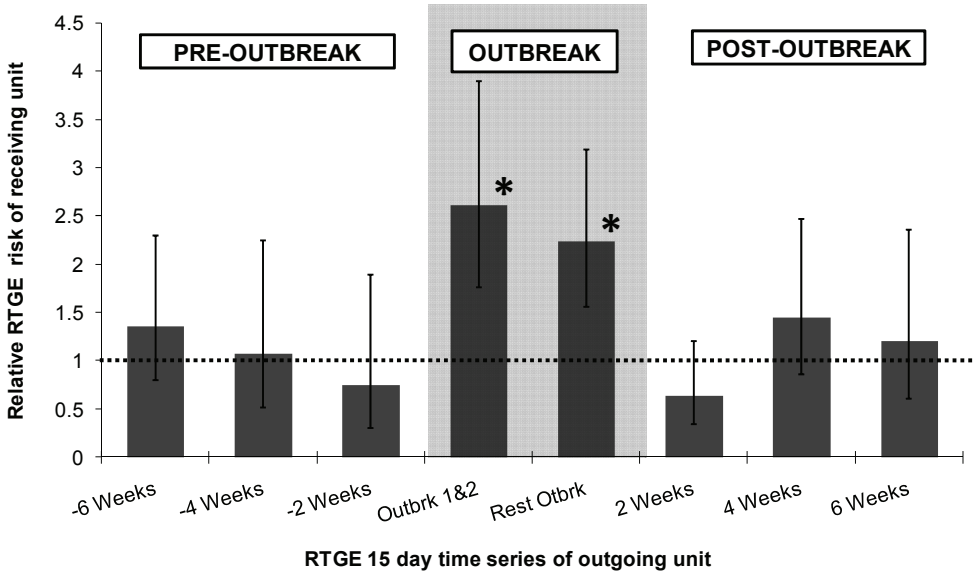

Figure 3.4. Bar chart of the RTGE relative risk of previously unaffected units that received fish from a RTGE-affected unit, depending on the outbreak status of the latter at the time of transfer (12 sites). Significant increases in the relative risk are highlighted ($p<0.05$; Fisher Exact) and error bars display 95% confidence intervals.

The results of the analysis of contiguity to cases are shown in Table 3.6. Significantly increased odds of a unit becoming RTGE positive were observed for both earth and cage sites when the unit was contiguous to previous cases. Additional analysis in land-based sites revealed significantly increased RTGE odds ratios only for units located downstream to RTGE cases (OR=5.09 (1.08-27.19); p=0.03). This increase was not observed when the units were located upstream to RTGE cases (OR=2.19 (0.67-7.28); p=0.23).

Table 3.6. Analysis of the RTGE risk of previously unaffected units depending on their contiguity to previous RTGE cases (12 sites).

	CONTIGUITY TO RTGE CASES						
	Contiguous and RTGE+	***Contiguous and healthy***	***Non contiguous and RTGE+***	***Non contiguous and healthy***	***Odds ratio****	***95% CI***	***P(FE)***
Land based	28	15	1	13	24.8	2.8-546.1	<0.001
Water	8	6	6	29	6.4	1.4-32.9	0.01
All Sites**	36	21	7	42	11.1	3.2-36.0	<0.001

*Odds of becoming RTGE positive after contiguity to a RTGE positive unit. **Stratified by site type using Mantel-Haenzel adjusted odds ratio. FE: Fisher exact test (Confidence level p<0.05).

The survival analysis shown in Figure 3.5 confirmed a significantly faster onset of RTGE from the first day of the study between units contiguous to cases and non contiguous units to cases for all site types (Wilcoxon=24.16; p<0.001). This was also observed in units located downstream to RTGE cases for land based sites only (Wilcoxon=10.65; p=0.001).

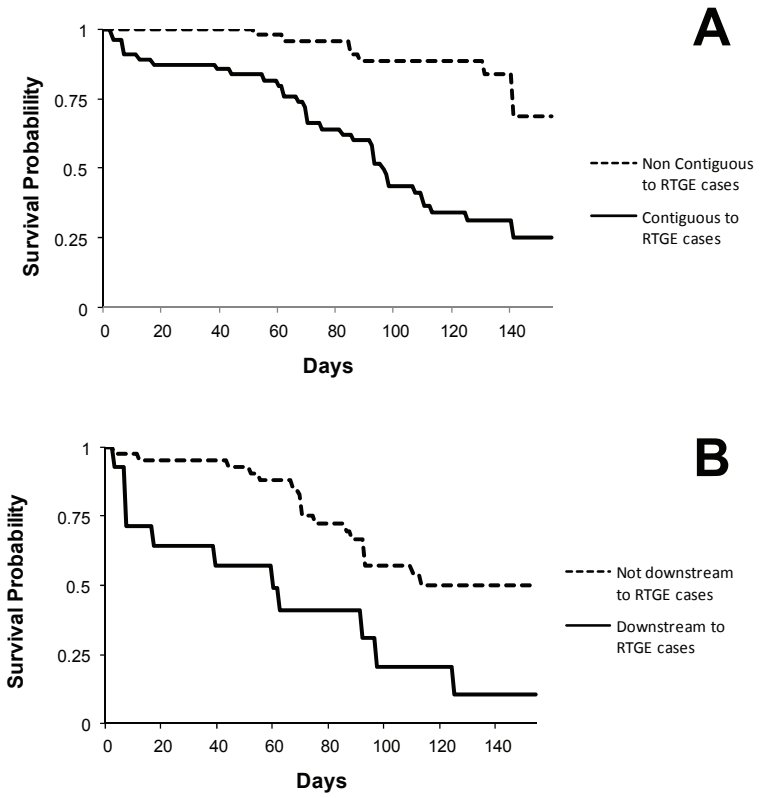

Figure 3.5. Kaplan Meier survival plots of the onset of RTGE (12 sites). A: Significantly lower survival probability for units contiguous to RTGE cases in all site types (Wilcoxon=24.2; p<0.001). B: Significantly lower survival probability for units downstream to previous RTGE cases in pond sites (Wilcoxon=10.7; p<0.001).

Finally, contiguity to cases and fish transfer from RTGE positive units were identified as risk factors for RTGE by multivariable logistic regression. The interactions between the two variables were not significant, suggesting an independent effect of each variable in the model. This model is displayed in Table 3.7.

Table 3.7. Multivariable logistic regression model of the association of contiguity and fish transfer from cases with RTGE onset (cases: 15 units; controls: 32 units).

Variables	*Odds Ratio (95% C.I.)*	*Z*	*P*
Contiguous to RTGE case (Yes/No)	8.3(1.8-38.6)	2.7	0.007
Received fish from RTGE (Yes/No)	8.9(1.5-54.2)	2.4	0.017
CONSTANT	*	-3.4	<0.001

-2*Log Likelihood=42.9, Degrees of Freedom=2, p<0.001

3.4.2. Cases and controls study

A total number of 34 variables with p<0.25 in Mantel-Haenzel stratified univariable analysis were included in multivariable CLR analysis. Each case was matched to two controls by system type and geographical location. The maximum number of variables in the final model was reduced to four in order to maintain an acceptable number of events per variable (case n=43) (Dohoo *et al.* 2003; Peduzzi *et al.* 1996). Two different models were fitted to the data, one for environmental variables and one for management variables (Table 3.8). Forward and backward stepwise model building approaches yielded several acceptable models and the final models selected presented the lowest -2*log likelihood ratio compared with the other candidate models.

Table 3.8. Conditional multivariable logistic regression models of the association of environmental and management variables with RTGE presence in a unit (12 sites; n=129; 43 cases/86 controls).

Environmental Variables	*Odds Ratio (95% C.I.)*	*Z*	*P*
Mean stocking density (kg/m^3)	23.9 (1.3-444.7)	2.1	0.03
Mortalities due to predation (Yes/No)	19.3(1.2-300.3)	2.1	0.03
Mean temperature of water (°C)	15.8 (1.1-218.0)	2.1	0.04
Contiguous to RTGE case (Yes/No)	11.1 (1.2-104.1)	2.1	0.03

-2*Log Likelihood=26.3, Degrees of Freedom=4, P<0.001

Management Variables	*Odds Ratio (95% C.I.)*	*Z*	*P*
Received fish from RTGE cases (Yes/No)	5.5 (1.7-18.2)	2.8	0.005
Mean daily Feed Input per Fish (High/Low)*	5.4 (1.7-17.2)	0.6	0.005
Mortalities due to handling (Yes/No)	3.0 (1.1-8.7)	0.5	0.04
No use of aeration	0.2 (0.04-0.7)	-2.4	0.02

*Categorised through median (=0.9% of individual fish weight). -2*Log Likelihood=67.6, Degrees of Freedom=4, p<0.001

The specified -2*Log likelihood ratio statistic for the models was calculated by comparison with an empty model and a $p<0.05$ for each variable indicated that the coefficient for that predictor variable was significantly different from zero.

3.4.3. General linear model

Table 3.9 shows all significant management variables in multivariable GLM along with coefficients that indicate the direction and magnitude of the effect of each variable on the cumulative RTGE mortality during the outbreak (cases n=69).

Table 3.9. General linear model of the variables associated with the natural logarithm of the RTGE cumulative mortalities (12 sites). Site was included as a random factor in the model to account for intrinsic site differences (n=69).

Model term	*Factor Type*	*Coefficient*	*SE*	*P*
Site	Random			<0.001
Ln Outbreak Length (Days)	Covariate	1.21	0.12	<0.001
Ln Mean (Daily Feed Input (kg)/Fish Number)	Covariate	0.54	0.13	<0.001
Constant		1.18	0.93	0.21

Adjusted R^2=85.5%. SE: Standard error

The model (Table 3.9) adequately fitted the data, as suggested by a normal distribution of the residuals (KS=0.09; p>0.15) and the absence of outlier leverage points. This model accounted for 85.5% of the variation in the dataset and included two significant variables, namely outbreak length in days and the mean daily feed input per fish expressed in kg. Both these variables were log transformed and presented a positive effect on the log of the cumulative RTGE mortality. The magnitude of the coefficients also indicated a higher influence of outbreak length on RTGE cumulative mortalities.

3.5. Discussion

This study has described the impact, pattern of spread and risk factors associated with the presence and severity of RTGE within affected rainbow trout producing sites. The epidemiological design chosen was a prospective longitudinal study, as this design allows examination of the association between exposure to a potential cause and development of disease, even if these are separated by a period of time (Thrusfield 2006). This approach has been successfully used for identifying risk factors associated with the presence of disease in Atlantic salmon, dairy cattle, poultry, rainbow trout and shrimp (Corsin *et al.* 2005; Corsin *et al.* 2001; Rose *et al.* 1999; Ortega *et al.* 1996; Kaneene & Miller 1995; Jarp *et al.* 1995; McVicar 1987).

A total number of 12 sites were positive for RTGE during this study, five more sites than reported in 2005 (Chapter 2), suggesting the prevalence of RTGE positive sites in the UK had increased. This syndrome was recorded from June to October 2006, and outbreaks started more than a month later in Scottish sites, compared with English sites. RTGE represented 27% of the total number

of fish mortalities recorded in these sites and affected relatively larger fish, resulting in significant impact on the production of the sites. The proportion of affected units varied greatly between sites suggesting that differences in fish, management and environmental variables played an important role in the presentation of RTGE at the unit level. To avoid possible confounding effects due to intrinsic differences between sites this factor was considered in the analysis by using Mantel Haenzel adjusted odds ratios, case-control matching in CLR and the inclusion of site as random effect in GLM.

Clearly delimited outbreaks of RTGE were observed in 45% of the units affected, whereas in the remaining units, mortalities due to RTGE were relatively low and/or other conditions were observed simultaneously. Data from the latter were not included in further analysis. These observations suggest that during previous years, RTGE was unlikely to have been present, undetected, at low mortality levels. Therefore, previous underreporting of RTGE at the country level is unlikely, a possibility previously suggested (Chapter 2).

Water temperatures recorded during RTGE outbreaks were consistent with previous reports (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). The spread distribution of temperature data did not allow the estimation of a temperature threshold, suggesting that temperature is not the only factor influencing the onset of RTGE.

Descriptive analysis of the duration of RTGE outbreaks revealed great variability, although analysis of their presentation was consistent with an infectious nature of RTGE. Most outbreaks presented multiple mortality peaks in the epidemic curve, a presentation that is consistent with a propagating

epidemic, with the disease spreading between fish in the affected unit (Thrusfield 2006). The mean time between mortality peaks, recurrent outbreaks and the onset of RTGE in newly stocked units suggested an incubation period of 20-25 days. This is also consistent with previous anecdotal observations and treatment approaches based on these: Branson (2003) observed in a single site that RTGE mortalities started in batches of newly stocked fish about 3 weeks after their arrival, and in Italy flumequine treatments are repeated every 25 days to avoid recurrence (Sarti *et al.* 2008).

Several treatments were used for RTGE during this study. The two main strategies for RTGE treatment used during this study were 4% NaCl or 2% liquid paraffin, both mixed in with the feed. In both cases, the supplemented feed input and length of the treatments were variable, both between and within sites. The presence of recurrent outbreaks after treatment suggested that RTGE was not consistently eliminated from the population by these treatments and more information on the pathogenesis of RTGE is needed in order to understand the potential effects of these treatments on RTGE-affected fish.

A sharp reduction in the number of RTGE mortalities was commonly observed during in-feed NaCl treatment, although the presence of RTGE was not totally eliminated and in 58% occasions RTGE mortalities were still present at the end of the treatment. These observations suggest that although in-feed NaCl may have a palliative effect on RTGE-affected fish, it does not eliminate it. The intermittent presentation of RTGE in the site where in-feed NaCl was alternated with non-supplemented feed could have been an effect of the NaCl treatment or a result of the incubation period of RTGE, although the lack of reference controls at this site (all the units were given the treatment), did not allow further

examination of these possibilities. A sharp reduction of mortalities during or after treatment was observed in only 13% of the units treated with in-feed liquid paraffin, suggesting this treatment may have been ineffective.

Other treatments were used during in this study, including oral oxytetracycline and chloramin-T bath, but these were always combined with in-feed salt or liquid paraffin and therefore it was not possible to define the effects of these treatments in isolation. Antibiotic treatments previously used for RTGE have included amoxycillin, oxytetracycline and potentiated sulphonamides, which have been found to alleviate the condition, although recurrence was observed after treatment (Branson 2003). Urdaci *et al.* (2001), reported the use of oxytetracycline in a treatment lasting 6 days, followed by 3-4 days fasting with no further detail on the outcome (Urdaci *et al.* 2001).

Fish transfers are a biosecurity concern (Danner & Merrill 2006; Scott 2004) and several known rainbow trout infectious diseases have been transmitted in cohabitation challenge (Ogut 2004; Madetoja *et al.* 2000; McCarthy *et al.* 1996). Frequency analysis of intra-site fish transfers in RTGE-affected sites revealed increased odds of a unit becoming RTGE positive after receiving fish from an affected unit only in land-based sites. The presence of propagating RTGE outbreaks in 31% of the units that became positive after receiving fish from previous cases was consistent with horizontal transmission of RTGE between fish in these units. Additional time-series analysis of these data revealed that the relative risk of receiving units was increased only when fish were introduced from a batch undergoing an active RTGE outbreak. This type of movement was observed only in land-based sites and never in cage sites and this is likely to have been the reason for the non-association of RTGE and intra-site fish

movements in cage sites. The results of these analyses suggested that RTGE was transmitted through cohabitation although transmission may have occurred only from fish undergoing clinical disease. However, this mechanism did not explain all the outbreaks, suggesting that other factor or factors had an influence on the intra-site spread of RTGE.

To test the possibility of spread via water, the effect of contiguity to cases was examined. This analysis confirmed significantly increased odds of becoming RTGE positive for units that were contiguous to cases in both land-based and cage sites. Furthermore, in land-based sites, a significantly increased risk of becoming RTGE positive was also observed for units located downstream to cases but not for units located upstream. A significantly more rapid onset of RTGE was also observed for units contiguous or downstream to cases using survival analysis. The results of the analyses of the site layouts in respect to the spread of RTGE were consistent with spread via water of RTGE. These results apparently contradict previous anecdotal observations by Branson (2003), who reported that RTGE was never observed in ponds receiving second-use water from an affected unit in a single site. It is possible that fish transfers could have played a predominant role in this specific case, although this possibility was not examined in the study.

An independent effect of fish transfers and contiguity to cases was confirmed with logistic regression analysis, suggesting that RTGE is infectious and spreads within affected sites both by fish transfers from clinically affected units and via water. Large accumulation of yellow mucoid faeces have been observed in RTGE-affected ponds (Michel *et al.* 2002) and it is possible that that faecal-oral route is the mechanism of transmission of RTGE, although

experimental transmission experiments will be required to confirm this possibility.

Conditional logistic regression analysis of risk factors identified several variables associated with the presence of RTGE at the unit level using two models that related to both the environment and management within RTGE-positive sites. Neither of these models identified a significant association of fry source or feed type with the presence of RTGE, suggesting that RTGE outbreaks do not have a common source, although the possibility cannot be dismissed due to the limited data available. None of the interactions between the variables included in both models was significant, suggesting an independent effect of these on the outcome.

Four environmental variables were positively associated with RTGE presence, namely higher mean stocking densities, presence of mortalities due to predation, higher mean water temperatures and contiguity to previous RTGE cases. The influence of higher stocking densities is probably a result of the increased contact rate between an infectious agent and susceptible individuals within the unit, therefore facilitating the transmission of disease (Thrusfield 2006). Predation is likely to induce stress in the stocked fish (Huntingford *et al.* 2006) and it is not possible to eliminate the possibility of a direct or indirect role of predators in the transmission and spread of RTGE. Higher water temperature has been linked with the onset and presence of RTGE in previous reports (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001) and the results of this study are consistent with those observations. The effect of increased water temperature is likely to be complex, and increased temperatures have been linked to variations in the immune system, higher metabolic rates, reduced

dissolved oxygen and changes in the intestinal microflora (Bowden *et al.* 2007; Pond *et al.* 2006; Evans & Claiborne 2006). In addition, the multiplication or pathogenicity of a hypothetical RTGE bacterial agent may also be affected by temperature. The inclusion of contiguity to previous cases supported previous observations.

The management model identified three risk factors for RTGE: reception of fish from affected units, higher mean feed input and mortalities due to handling. The model also included one protective factor: lack of an aeration system. The inclusion of contiguity to previous cases also supported previous observations. Higher feed input levels are associated with shorter production cycles for the table market, both risk factors for RTGE at the site level (Chapter 2). The mechanism behind the association of higher feed input with RTGE is likely to be complex and could involve increased environmental organic load, increased metabolic stress and influences on the bacterial flora (Pond *et al.* 2006; Woo *et al.* 2003; Reddy & Leatherland 1998). High feeding rates and/or rapid growth have been associated with a range of pathological conditions in salmonids, including bacterial gill disease (BGD), columnaris disease, vibriosis, acute cardiomypathy and water belly syndrome (Speare 1998; Ferguson *et al.* 1990; Staurnes *et al.* 1990). The presence of mortalities associated with fish handling is also associated with increased stress (Ashley 2007) and may have resulted in predisposition of fish to RTGE. A protective effect of the absence of an aeration system could have been related to the water movement created by these systems. This may have helped to keep potentially infectious material suspended in the water column, thus facilitating contact with susceptible individuals, or it may have reflected a lower level of intensification.

The results of the GLM identified a significant influence of higher daily feed input/fish during outbreaks. This was observed after adjustment for confounding by outbreak length, and suggested that reducing the feeding input during RTGE outbreaks could help to reduce the impact that this condition has on affected units. This analysis has confirmed anecdotal observations by Branson (2003) who reported a decrease of RTGE mortalities after feed restriction as well as providing an epidemiological basis for a control strategy previously reported, consisting of fasting affected units for 7 or more days (Sarti *et al.* 2008).

Overall, the results of this study strongly suggested an infectious nature of RTGE and despite the limitations of a relatively small dataset it allowed an in-depth description and analysis of the impact, presentation, spread and current control strategies. It also identified risk factors associated with both the presence and severity of this syndrome within affected sites. As in all observation-based studies the risk factors identified should be tested in intervention strategies. An experimental model for RTGE would greatly increase our capacity to investigate these observations further in experimental studies.

3.6. Acknowledgements

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3.7. References

Ashley, P. J. 2007, 'Fish welfare: Current issues in aquaculture'. *Applied Animal Behaviour Science*, vol. 104, pp. 199-235.

Bagley, S. C., White, H. & Golomb, B. A. 2001, 'Logistic regression in the medical literature: Standards for use and reporting, with particular attention to one medical domain'. *Journal of Clinical Epidemiology*, vol. 54, pp. 979-985.

Bowden, T. J., Thompson, K. D., Morgan, A. L., Gratacap, R. M. L. & Nikoskelainen, S. 2007, 'Seasonal variation and the immune response: A fish perspective'. *Fish & Shellfish Immunology*, vol. 22, pp. 695-706.

Branson, E. 2003, 'Rainbow trout Gastro-Enteritis (RTGE)-first diagnosis in the UK'. *Fish Veterinary Journal*, vol. 7, pp. 71-76.

Corsin, F., Turnbull, J. F., Hao, N. V., Mohan, C. V., Phi, T. T., Phuoc, L. H., Tinh, T. N. & Morgan, K. L. 2001, 'Risk factors associated with white spot syndrome virus infection in a Vietnamese rice-shrimp farming system'. *Diseases of Aquatic Organisms*, vol. 47, pp. 1-12.

Corsin, F., Turnbull, J. F., Mohan, C. V., Hao, N. V. & Morgan, K. L. 2005, 'Pond-level risk factors for white spot disease outbreaks'. In: *Diseases in Asian Aquaculture 5*, pp. 24-28.

Danner, G. R. & Merrill, P. 2006. 'Disinfectants, Disinfection, and Biosecurity in Aquaculture'. In: *Aquaculture Biosecurity.Prevention, Control, and Eradication of Aquatic Animal Disease*, A. D. Scarfe, C.-S. Lee, & P. J. O'Bryen, (Eds.), Blackwell Publishing, Oxford, pp. 91-128.

Dohoo, I., Martin, W. & Stryhn, H. 2003, *Veterinary Epidemiological Research*. 1st Ed., AVC Publishing, Charlottetown, Prince Edward Island, Canada.

Evans, D. H. & Claiborne, J. B. 2006, *The physiology of fishes*. 3rd Ed., Taylor & Francis, New York.

Ferguson, H. W., Poppe, T. & Speare, D. J. 1990, 'Cardiomiopathy in farmed norwegian salmon'. *Diseases of Aquatic Organisms*, vol. 8, pp. 225-231.

Hedrick, R. P. 1998, 'Relationships of the host, pathogen, and environment: Implications for diseases of cultured and wild fish populations'. *Journal of Aquatic Animal Health*, vol. 10, pp. 107-111.

Huntingford, F. A., Adams, C., Braithwaite, V. A., Kadri, S., Pottinger, T. G., Sandoe, P. & Turnbull, J. F. 2006, 'Current issues in fish welfare'. *Journal of Fish Biology*, vol. 68, pp. 332-372.

Jarp, J., Gjevre, A., Olsen, A. & Bruheim, T. A. 1995, 'Risk factors for furunculosis, infectious pancreatic necrosis and mortality in post-smolt of Atlantic salmon, *Salmo salar* L'. *Journal of Fish Diseases*, vol. 18, pp. 67-78.

Kaneene, J. B. & Miller, R. 1995, 'Risk factors for metritis in Michigan dairy cattle using herd- and cow-based modelling approaches'. *Preventive Veterinary Medicine*, vol. 23, pp. 183-200.

Madetoja, J., Nyman, P. & Wiklund, T. 2000, '*Flavobacterium psychrophilum*, invasion into and shedding by rainbow trout *Oncorhynchus mykiss*'. *Diseases of Aquatic Organisms*, vol. 43, pp. 27-38.

McCarthy, I. D., Carter, C. G., Houlihan, D. F., Johnstone, R. & Mitchell, A. I. 1996, 'The performance of all-female diploid and triploid Atlantic salmon smolts on transfer together to sea water'. *Journal of Fish Biology*, vol. 48, pp. 545-548.

McVicar, A. H. 1987, 'Pancreas disease of farmed Atlantic salmon, *Salmo salar*, in Scotland: Epidemiology and early pathology'. *Aquaculture*, vol. 67, pp. 71-78.

Michel, C., Bernardet, J. F., Daniel, P., Chilmonczyk, S., Urdaci, M. C. & de Kinkelin, P. 2002, 'Clinical and aetiological aspects of a summer enteritic syndrome associated with the sporulating segmented filamentous bacterium '*Candidatus* arthromitus' in farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum)'. *Journal of Fish Diseases*, vol. 25, pp. 533-543.

Ogut, H. 2004, 'Early Kinetics of Infectious Hematopoietic Necrosis Virus(IHNV) Infection in Rainbow Trout'. *Journal of Aquatic Animal Health*, vol. 16, pp. 152-160.

Ortega, C., Muzquiz, J. L., Fernandez, A., Ruiz, I., De Blas, I., Simon, M. C. & Alonso, J. L. 1996, 'Water quality parameters associated with *Aeromonas* spp-affected hatcheries'. *Veterinary Research*, vol. 27, pp. 553-560.

Peduzzi, P., Concato, J., Kemper, E., Holford, T. R. & Feinstein, A. R. 1996, 'A simulation study of the number of events per variable in logistic regression analysis'. *Journal of Clinical Epidemiology*, vol. 49, pp. 1373-1379.

Pond, M. J., Stone, D. M. & Alderman, D. J. 2006, 'Comparison of conventional and molecular techniques to investigate the intestinal microflora of rainbow trout (*Oncorhynchus mykiss*)'. *Aquaculture*, vol. 261, pp. 194-203.

Rahman, M., Sakamoto & Fukui, T. 2003, 'Conditional versus unconditional logistic regression in the medical literature'. *Journal of Clinical Epidemiology*, vol. 56, pp. 101-102.

Reddy, P. K. & Leatherland, J. F. 1998. 'Stress physiology'. In: *Fish diseases and disorders*, vol. II J. F. Leatherland & P. T. K. Woo, (Eds.), CABI publishing, New York, pp. 279-301.

Rose, N., Beaudeau, F., Drouin, P., Toux, J. Y., Rose, V. & Colin, P. 1999, 'Risk factors for *Salmonella enterica* subsp. enterica contamination in French broiler-chicken flocks at the end of the rearing period'. *Preventive Veterinary Medicine*, vol. 39, pp. 265-277.

Sanz, F. 2000, 'La gastroenteritis de la trucha (Language: Spanish)'. *Trouw Informa*, vol. Spring, pp. 16-18.

Sarti, M., Cervellione, F., Giusto, C., Manzano, M., Pecchini, A. & Luzzana, U. 2008, 'Intestinal microflora modulation as a strategy for the control of rainbow trout gastroenteritis (RTGE) (Language: Italian)'. In: *XV conference of the Italian Society of Fish Pathology*, Erice, Italy, p. 25.

Scott, A. 2004, 'Sleeping disease: A lesson in biosecurity'. *Trout News*, vol. 37, pp. 17-23.

Speare, D. J. 1998. 'Non infectious disorders associated with intensive aquaculture husbandry'. In: *Fish diseases and disorders*, vol. II J. F. Leatherland & P. T. K. Woo, (Eds.), CABI publishing, New York, pp. 303-333.

Staurnes, M., Andorsdottir, G. & Sundby, A. 1990, 'Distended, water-filled stomach in sea-farmed rainbow trout'. *Aquaculture*, vol. 90, pp. 333-343.

Thrusfield, M. V. 2006, *Veterinary Epidemiology*. Third Ed., Blackwell Publishing, Oxford.

Toranzo, A. E. 2004. 'Report about bacterial diseases'. In: *Mediterranean Aquaculture Laboratories*, P. Alvarez-Pellitero et al., (Eds.), CIHEAM/FAO, pp. 49-89.

Urdaci, M. C., Regnault, B. & Grimont, P. A. D. 2001, 'Identification by in situ hybridization of segmented filamentous bacteria in the intestine of diarrheic rainbow trout (*Oncorhynchus mykiss*)'. *Research in Microbiology*, vol. 152, pp. 67-73.

Woo, P. T. K., Bruno, D. W. & Lim, L. H. S. 2003, *Diseases and disorders of finfish in cage culture*. 1 Ed., CABI publishing, New york.

CHAPTER 4. Histopathology & Ultrastructure of "*Candidatus* arthromitus"-Associated Rainbow Trout Gastroenteritis

Del-Pozo, Jorge*; Crumlish, Margaret; Turnbull, James F.; Ferguson, Hugh W.

4.1. Abstract

Rainbow trout gastroenteritis (RTGE) is linked to the presence of large numbers of the segmented filamentous bacterium "*Candidatus* arthromitus" (SFB) within the lower digestive system, although the aetiological role of this bacterium has yet to be clarified. The present study examined the histopathological changes and ultrastructure of the digestive tract of fish with typical gross lesions, and assessed the preferred locations of SFB. Histopathology showed that 85% of the fish affected with RTGE had SFB in distal intestine and/or pyloric caeca. The presence and number of SFB were always significantly higher ($p<0.001$) in pyloric caeca, suggesting that this is the preferred site for SFB. Additionally, SFB degradation was observed in distal intestine with apparent osmotic damage, reduced stain retention and filament fragmentation. Histopathological changes included enterocyte detachment and congestion of the lamina propria and adventitial layers. These changes were relatively more frequent in distal intestine ($p<0.001$), mirroring the gross presentation. Scanning and transmission electron microscopy revealed a close interaction of SFB with the mucosa of distal intestine and pyloric caeca. These interactions included the presence of SFB attachment sites and alterations of the apical membrane of enterocytes which suggested that engulfment of SFB had taken place. Despite these close interactions, SFB were most commonly seen in the lumen rather

than adjacent to the areas with pathological changes, suggesting that if these organisms do indeed play a role in the pathogenesis of RTGE extracellular products may be involved. Ultrastructural changes included loss of microvillar structure, membrane blebbing, hydropic mitochondrial damage and basal hydropic degeneration of enterocytes, which frequently resulted in disruption of tight junctions and enterocyte detachment. As a result, relatively large areas of lamina propria were exposed, probably resulting in compromise of the host osmotic balance and facilitation of the entry of secondary pathogens. Changes in other cell types included an apparent secretory activity of rodlet cells, which is also described.

4.2. Introduction

Rainbow trout gastroenteritis (RTGE) is a term suggested in 1999 to describe a specific syndrome of rainbow trout, *Oncorhynchus mykiss* W. (Branson 2003). This condition has been reported in Croatia, France, Italy, Spain and the UK during the summer (Toranzo 2004; Branson 2003, Cervellione personal comm.; Michel *et al.* 2002; Urdaci *et al.* 2001). The economic impact of RTGE is significant, and daily mortalities of 0.5-1% were common during outbreaks (Branson 2003; Michel *et al.* 2002).

The presentation of RTGE includes severe enteritis with massive accumulation of segmented filamentous bacteria (SFB) within the lower digestive system (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). These SFB have been classified as "*Candidatus* arthromitus", a group of bacteria closely related phylogenetically to *Clostridium* (Urdaci *et al.* 2001; Snel *et al.* 1995). Trout SFB have been suggested as the aetiological agent for RTGE, although this possibility has not been confirmed, in part due to the current inability to culture

SFB *in vitro* (Angert 2005; Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). The gross presentation of RTGE has been described in several studies. It included externally abdominal dilation, whereas internally severe distal enteritis with congestion and oedema of the intestinal wall were found (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). The intestine contains a yellow and viscous fluid, in which large numbers of SFB can be seen under light microscopy (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). Histopathological changes include severe enterocyte detachment, congestion and SFB accumulation in both distal intestine and pyloric caeca (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). Previous reports on the preferred location of SFB in the digestive system of enteritic trout vary; Michel *et al.* (2002) reported their presence throughout the digestive system, whereas Branson (2003) observed SFB preferentially in the distal intestine.

Enteritides other than RTGE have been described in rainbow trout, including bacterial, parasitic, fungal and viral conditions (Appendix I; Ferguson 2006; Weber 2005; Roberts 2001; Austin *et al.* 1992) although isolated enteritis is infrequent in fish and is most often part of systemic disease (Ferguson 2006). Of all the enteritides of rainbow trout, RTGE is the only one that is reported to be associated with SFB, suggesting that this condition is a distinct disease entity, despite the generic term used to describe it.

There are several reports on the pathological changes accompanying RTGE at both the histopathological and ultrastructural level, but most of these have focused on the detection and description of SFB rather than the host responses. no numerical data have been provided in any report to support observations on where SFB are preferentially found in RTGE-affected fish (Branson 2003;

Michel *et al.* 2002). This study aimed to contribute to these areas by applying a statistical approach to the study of SFB histopathological presentation and using scanning and transmission electron microscopy to complement previous ultrastructural studies on the presentation of RTGE.

4.3. Materials and methods

4.3.1. RTGE gross case definition

All the RTGE-affected fish (RTGE+) in this study were identified by a case definition created from previous literature and based on the gross presentation (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). The case definition was: *"RTGE is a condition of rainbow trout, observed in units with daily cumulative mortalities of 0.5% or more and present during the summer. Affected fish present a distended abdomen externally while internally their lower intestine is dilated, congested and oedematous, containing a yellow viscous substance"*.

4.3.2. Fish sampling and sample processing

The rainbow trout in this study were sampled from 1st June to 31st September 2006 at 11 UK sites with RTGE presence. At each site visited, two groups of fish were sampled from a single productive unit (*i.e.* cage, pond or raceway) during an RTGE outbreak: (a) As many moribund fish showing external signs of RTGE as practically possible (moribund RTGE+) and (b) 30 randomly sampled apparently healthy fish. The sample size of group b was chosen to enable disease detection with 95% confidence if the prevalence was at 10% or more. This strategy enabled the random detection of cohabiting fish consistent with the case definition, which were processed and included in the analysis as "subclinical" RTGE-positive fish ("subclinical" RTGE+).

All fish were sampled on-site, euthanized prior to sampling with benzocaine (SIGMA E1501) at 250mg/l (AVMA 2001), and fish origin, fork length, weight and the presence of any external or internal gross signs were recorded. Samples for histopathology were taken from three locations in the digestive tract; the pyloric end of the stomach (one 1cm tubular section), proximal pyloric caeca (10-15 whole caeca) and distal intestine (one 1cm tubular section). All the digestive tissues sampled were sectioned longitudinally before placement in fixative to allow rapid penetration, with the aim of minimizing artifacts caused by autolysis. All histology samples were placed in 10% buffered formalin and left for at least 24h before processing using standard protocols and embedding in individual paraffin wax blocks. Finally 5µm sections from these blocks were cut, placed on slides and stained with haematoxylin and eosin (H&E). Sections positive for SFB were also Gram-stained following standard protocols.

4.3.3. "*Candidatus* arthromitus" histological presence

The digestive tissues of all fish consistent with the case definition for RTGE were assessed for the presence of SFB and histopathological changes. All the histology slides were read "blind" by the same person and SFB were identified as bacteria approximately 0.6-1.2µm in diameter and up to 60µm in length, with apparent segmentation every 1.2-1.6µm (Urdaci *et al.* 2001). SFB presence was noted in each tissue after scanning the entire section at objective x10 and if present, the number of SFB was recorded as the mean number of SFB counted in three microscopic fields at x20 (area=0.95 mm^2). The microscopic fields were systematically chosen for SFB abundance and a value of 100 was recorded for SFB numbers equal to or exceeding 100 bacteria/field. Additionally, any histopathological changes observed were noted. Finally, the

statistical differences regarding histological presence and quantity of SFB were assessed with respect to the organ (*i.e.* distal intestine or pyloric caeca) and clinical status (*i.e.* moribund or "subclinical"). The statistical tests used were Fisher exact (FE) for SFB presence and Kruskal Wallis (KW) for SFB numbers, with significance assumed under p=0.05.

4.3.4. Ultrastructural description of RTGE

Samples from six moribund rainbow trout with gross presentations consistent with RTGE were also processed for scanning and transmission electron microscopy. These fish were sampled from a single site at one point in time and only pyloric caeca and distal intestine samples were taken, which were also incised to allow rapid fixation. The samples were placed for 24h in a fixative comprising 2.5% (v/v) glutaraldehyde in 0.1M sodium cacodylate. Scanning electron microscopy samples were then trimmed into 9 mm squares of no more than 1 mm thickness, post-fixed for 2h in 1% (v/v) osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.3, and then dehydrated in increasing concentrations of ethanol (30-100%). The pieces were then dried with carbon dioxide in a Bal-Tec 030™ critical point drier (Leica™, Wetzlar, Germany), mounted on a metal stub with colloidal silver, coated with a thin conductive film of gold in an Edwards S150B™ sputtering coater (Edwards™, Crawley, UK) and examined with a Jeol JSM6460LVN™ scanning electron microscope (Jeol, Welwyn Garden City, UK), operated at 5-10kV. Transmission electron microscopy samples were trimmed to 1mm^2 cubes, rinsed in 0.1 M sodium cacodylate buffer, pH 7.3 for 12h and post-fixed for 1h in 1% (v/v) osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.3. All samples were then washed with distilled water and en-bloc stained in the dark for 2h with 2% (v/v)

uranyl acetate in 30% (v/v) acetone. The samples were then dehydrated with increasing concentrations of acetone (60-100%), infiltrated and blocked with agar low viscosity resin, cut at 50-70 nm with a Reichert Ultracut-E™ ultramicrotome (Leica™, Wetzlar, Germany) and contrasted with 5% uranyl acetate and lead citrate. These samples were examined with a Tecnai G2 Spirit Bio Twin™ transmission electron microscope (Tecnai™, Eindhoven, Netherlands) operated at 120 kV.

4.4. Results

A total of 464 rainbow trout were sampled from 11 sites in the UK from June to September 2006. Of these, 134 were moribund fish consistent with the case definition for RTGE (moribund RTGE+) and 330 were randomly sampled fish from the same units. During post-mortem examination, consistency with RTGE case definition was recorded in 18 of the randomly sampled fish and these fish were included in the analyses as “subclinical” RTGE+ fish. This resulted in a total number of 152 RTGE+ fish (“subclinical” + moribund) in which the analyses were performed. The remaining 312 fish were not consistent with RTGE case definition and were not included in this study.

Externally, “subclinical” RTGE+ fish presented with milder dilation of the abdomen but internally with all gross signs included in RTGE case definition. In 22% of the “subclinical” RTGE+ fish, the yellow viscous intestinal content was mixed with feed.

4.4.1. "*Candidatus* arthromitus" histological presence

A total of 152 RTGE positive fish (134 moribund and 18 "subclinical") were included in the study, with a mean weight of 218(±90.5; SD) grams. All of these fish had gross signs consistent with the case definition for RTGE both internally and externally (Figure 4.1 A, C). Microscopic examination of the yellow viscous fluid from the vent revealed large quantities of sporulating SFB in most fish (Figure 4.1 B).

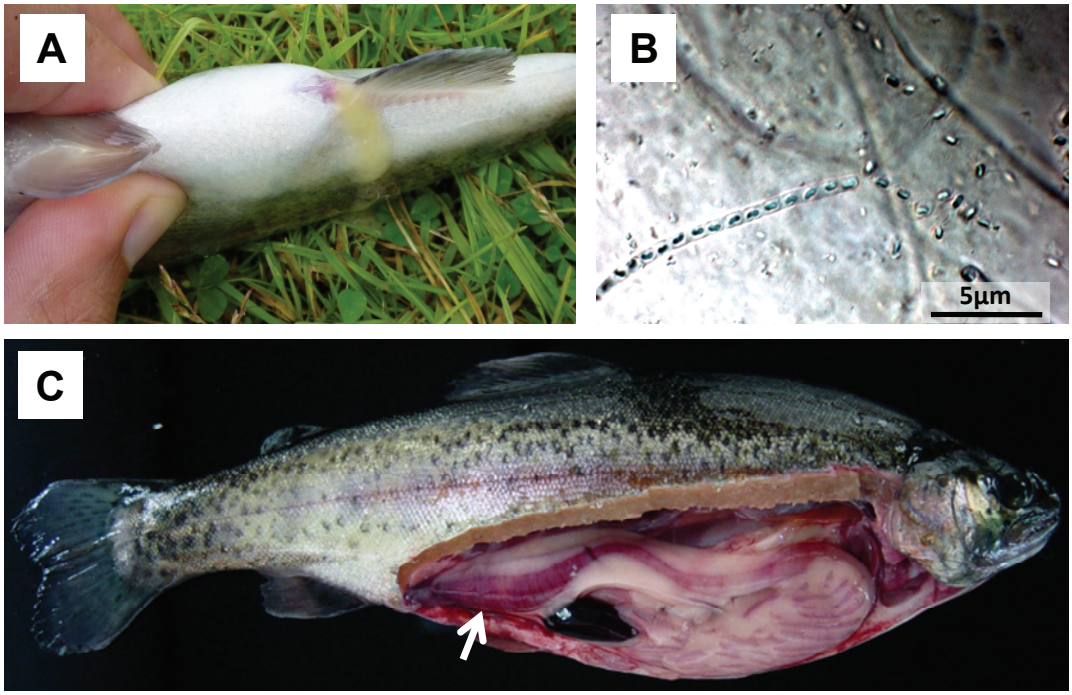

Figure 4.1. Gross and microscopic changes in trout positive for rainbow trout gastroenteritis (RTGE). A: Abdominal distension and release of a yellow and viscous substance from the vent when pressure is applied to the abdomen. (B): Microscopic examination of intestinal contents reveals large quantities of sporulating segmented filamentous bacteria (B, x20, unstained smear). C: Internally, there is congestion and oedema of the intestinal wall (C, arrow).

Histologically, it was possible to observe SFB in the pyloric caeca and/or distal intestine of 85% of all RTGE positive fish, both attached to the mucosa and free within the lumen (Figure 4.2 A, B). The SFB were never observed in any of the sections of pyloric stomach. No significant differences were observed between

moribund and apparently healthy RTGE+ fish in either the presence of SFB ($p=0.7$; FE) or in the SFB numbers observed ($p=0.4$; KW). Marked differences were observed, however, depending on the organ and SFB detection was significantly more frequent in pyloric caeca than in distal intestine of all RTGE-positive fish ($p<0.001$; FE). The average number of detectable SFB per x20 field was also significantly higher in pyloric caeca than in distal intestine ($p<0.001$; KW), with SFB counts of 45.9 (±2.9; SE) SFB/field in the former and 6.5 (±1.3; SE) SFB/field in the latter. Moreover, apparent degradation of SFB was observed in the distal intestine of 10 RTGE+ fish, as evidenced by reduced stain retention and disruption of the filamentous structure (Figure 4.2 C, D). Gram variability of SFB was observed in the Gram-stained sections, although most SFB were Gram-positive.

The histopathological changes observed in RTGE-positive fish included generalised enterocyte detachment (Figure 4.2 E) and congestion of the adventitial layers and the lamina propria in distal intestine and/or pyloric caeca (Figure 4.2 E, F). Histopathological changes were significantly more frequent in distal intestine ($p<0001$; FE) than in pyloric caeca. The bar chart in figure 4.3 summarizes, by organ, the frequency of SFB observations with the observed frequency of enterocyte detachment and congestion of the intestinal wall in all RTGE positive fish.

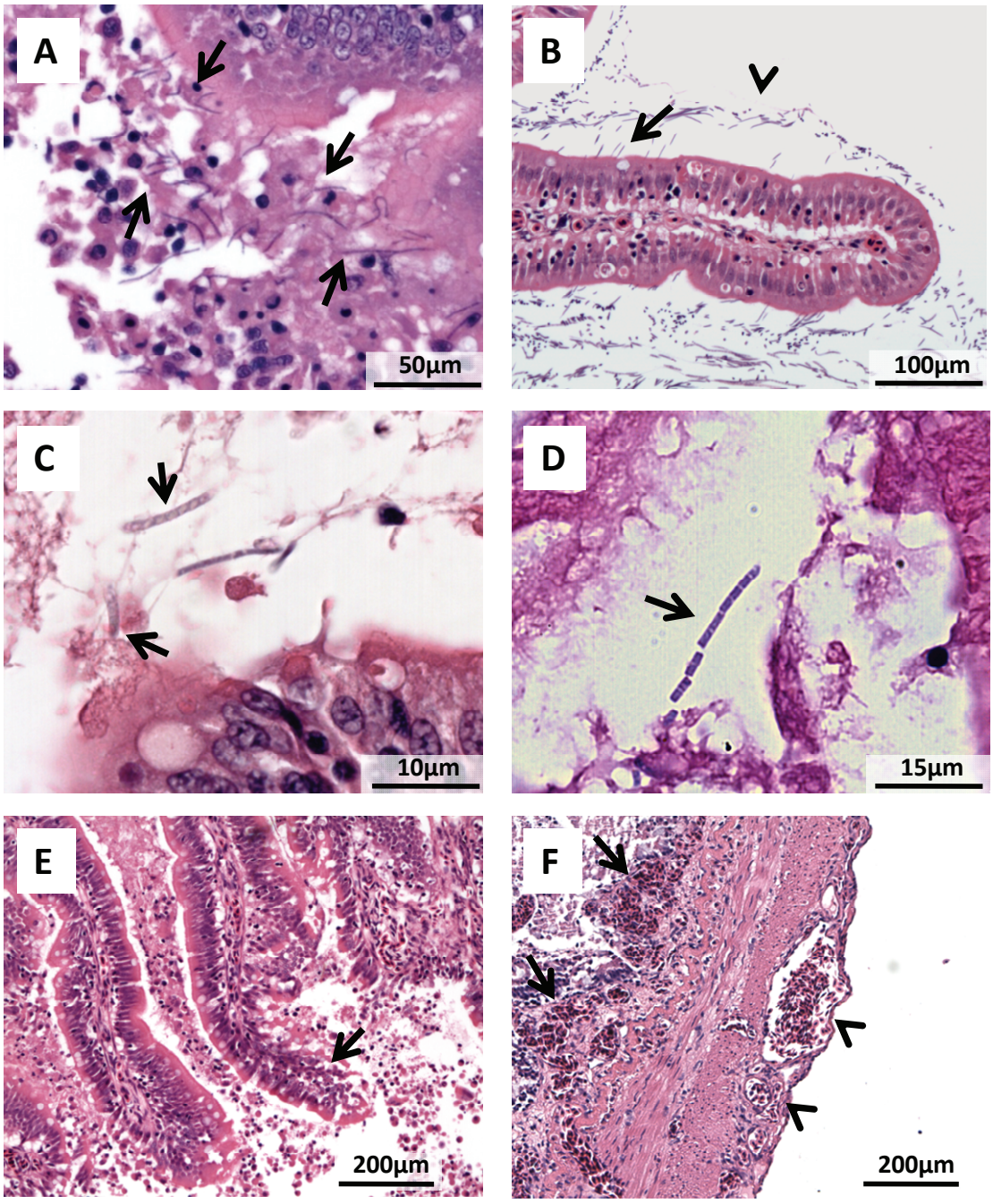

Figure 4.2. Histological presentation of rainbow trout gastroenteritis (RTGE) in distal intestine. A: Digestive contents frequently presented a mixture of segmented filamentous bacteria (SFB) and detached enterocytes (H&E; x20). B: SFB could be present in large quantities attached to the mucosa (arrow) or floating in the lumen (arrowhead; H&E, x10). Damaged SFB could be observed, with reduced stain retention (C; H&E; x40) and fragmentation (D; H&E; x100). Histopathological changes included enterocyte detachment (E; H&E; x100) and congestion of the lamina propria (F, arrow) and adventitial layer (F, arrowhead; H&E; x100).

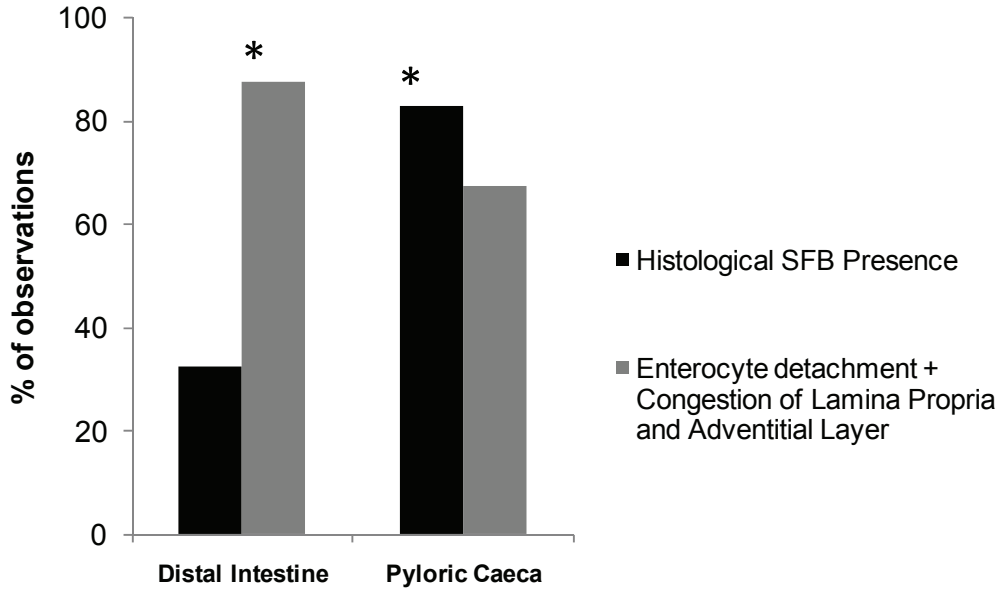

Figure 4.3. Observed frequencies by organ of segmented filamentous bacteria (SFB) presence and histopathological changes in RTGE-affected fish (n=152). Asterisks represent a significantly higher frequency ($p<0.001$; FE).

4.4.2. Ultrastructural description of RTGE

4.4.2.1. Scanning electron microscopy

Large numbers of SFB were always present in distal intestine and/or pyloric caeca of the 6 RTGE-affected fish sampled for electron microscopy. These bacteria could be seen free-floating in the lumen as well as attached to the intestinal mucosa in both these locations (Figure 4.4 A, B), although epithelial-associated SFB were not ubiquitous and appeared to be restricted to specific locations. All SFB were approximately 1µm wide, of variable length and were clearly segmented (Figure 4.4 C). In the distal intestine, SFB occasionally had structures consistent with propagation by budding at the distal end (Figure 4.4 D). Enterocytes interacted with SFB frequently, and these interactions resulted in changes in their apical membrane, which folded around sections of SFB and presented apparent “trails” suggesting that engulfment of SFB by enterocytes had taken place (Figure 4.4 E, F).

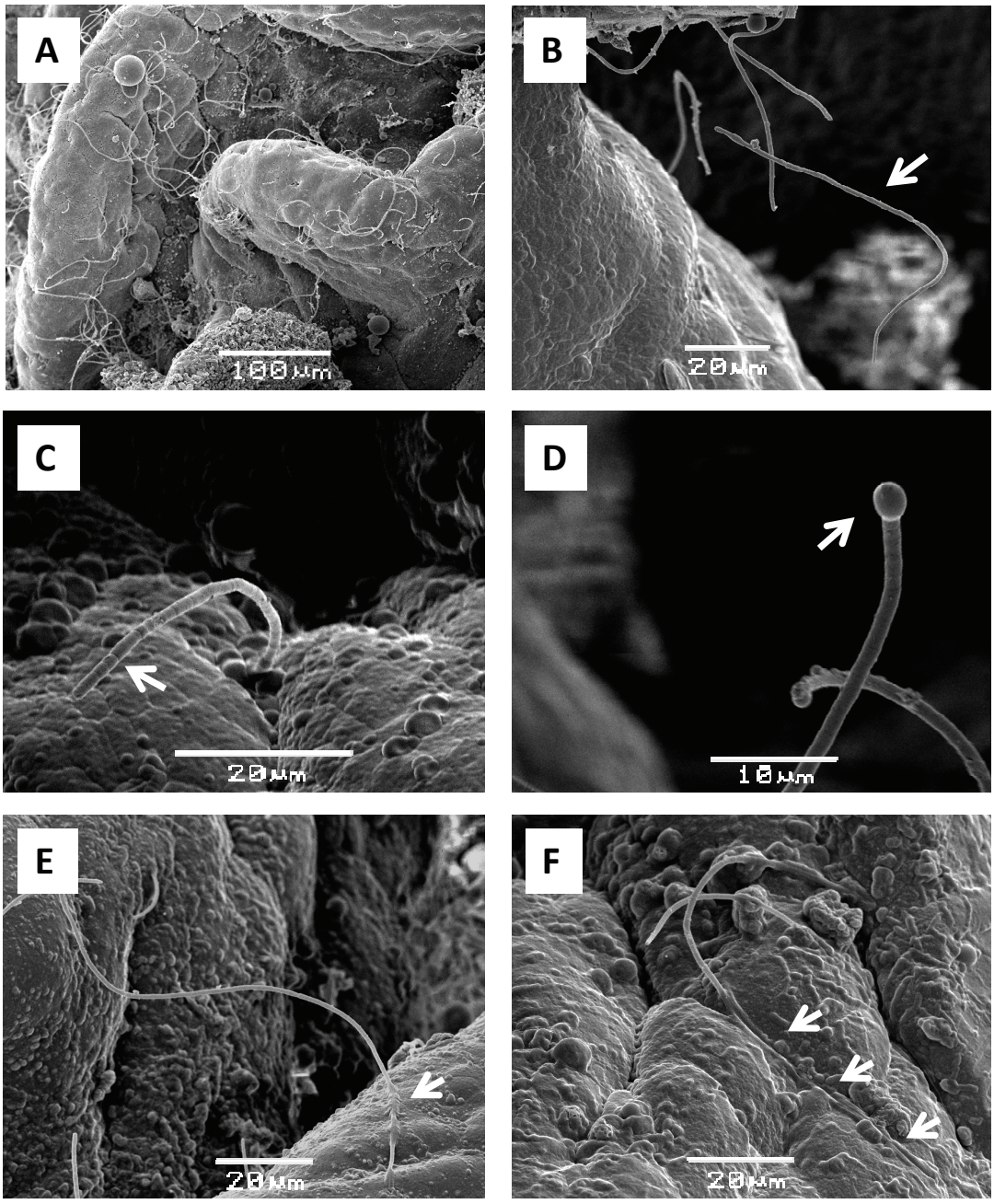

Figure 4.4. Scanning electron microscopy observations of SFB in fish with RTGE. (A): Large numbers of SFB were observed attached to the intestinal mucosa in specific locations. These SFB were also free-floating in the lumen (B, arrow), had clear segmentation (C, arrow) and occasionally budding at their distal end (D, arrow). Interaction of the enterocyte surface was frequent and included wrapping of SFB by the apical membrane of enterocytes (E, arrow) which presented folds suggestive of SFB engulfment (F, arrows).

Pathological changes observed included multifocal detachment of the mucosal layer and pronounced apical blebbing of enterocytes (Figure 4.5 A, B, C). The former always resulted in direct exposure of the lamina propria to the digestive lumen whereas the latter could be associated or not with SFB proximity (Figure 4.5 B, C).

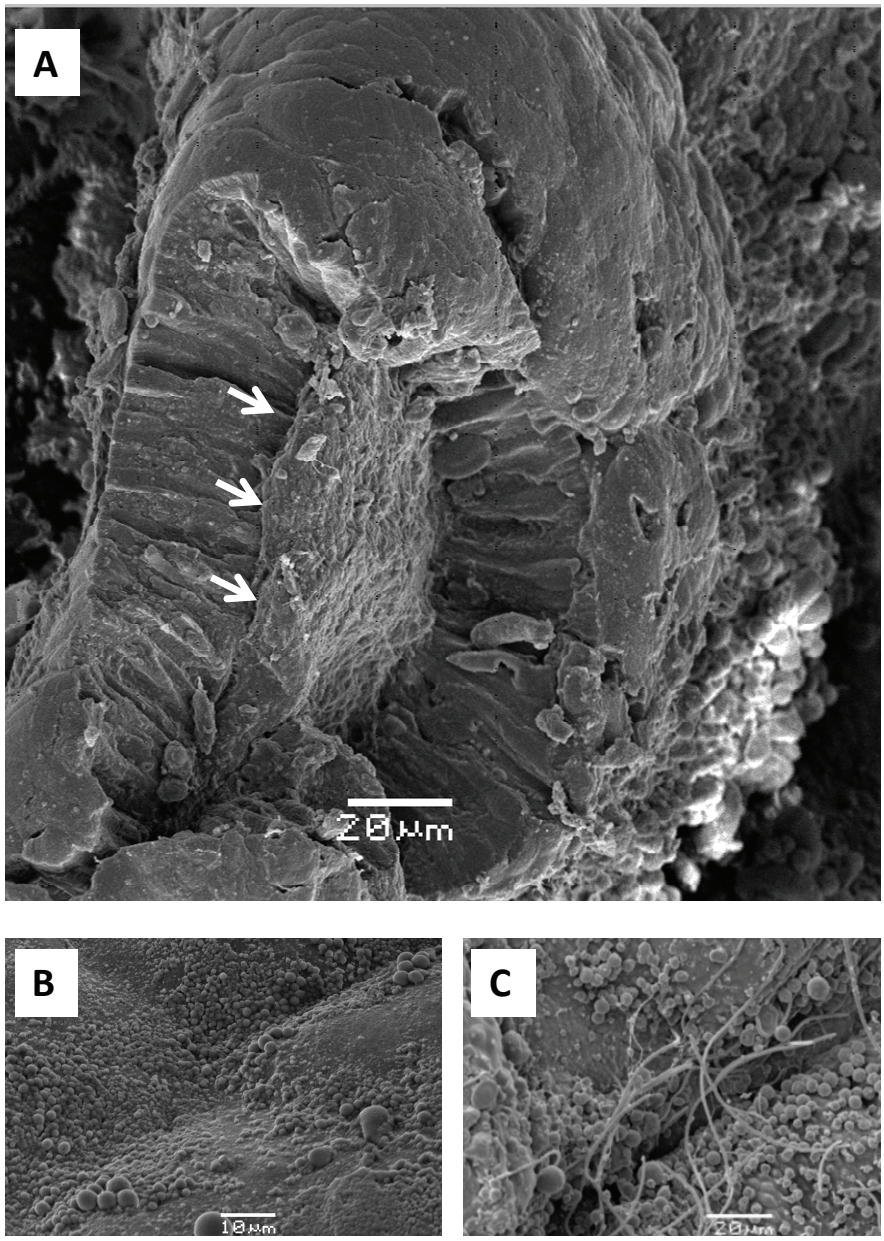

Figure 4.5. Scanning electron microscopical observations of pathological changes within distal intestine of fish with RTGE. Focal detachment of the mucosal layer resulted in direct exposure of the lamina propria to the lumen (A, arrows). Pronounced apical blebbing of distal intestinal mucosa was observed both non-associated (B) and associated (C) with SFB proximity.

4.4.2.2. Transmission electron microscopy

Different developmental stages were observed within single SFB filaments, including vegetative, dividing and sporulating stages (Figure 4.6 A, B). Loss of cellular structure resulted in spore release (Figure 4.6 C), as previously reported (Michel *et al.* 2002). The proximal segment at SFB attachment sites presented a pear-shaped appearance (Figure 4.6 D). These segments were always extracellular and surrounded by an electron dense area within the cytoplasm of the adjacent enterocytes (Figure 4.6 E). No other cellular reaction was observed and no direct evidence of SFB phagocytosis was observed in TEM.

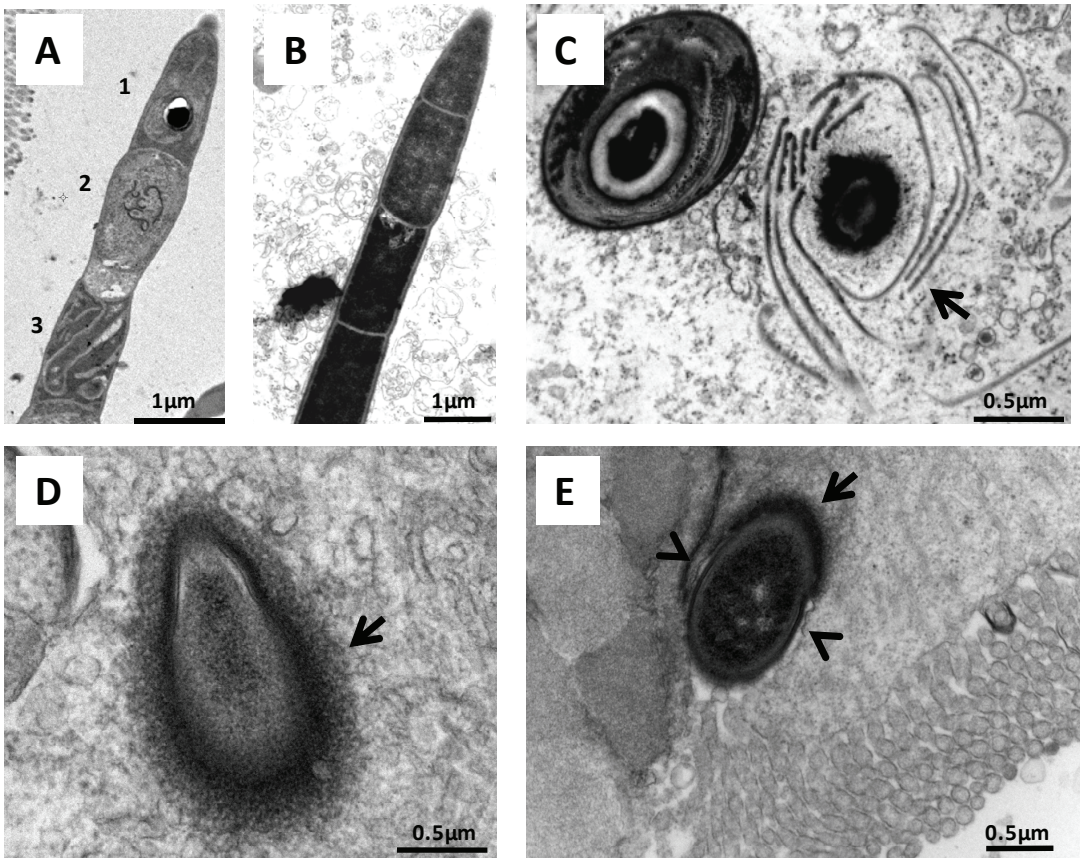

Figure 4.6. Transmission electron microscopical observations of SFB within distal intestine of fish with RTGE. A: Different developmental stages were present within SFB filaments, including sporulation (A, 1), cell division (A, 2&3) and vegetative (B). Loss of SFB cellular structure resulted in spore release (arrow, C). Pear-shaped segments were found at SFB attachment sites. These segments were always extracellular and surrounded by electron dense areas in the adjacent host cytoplasm (D, arrow). Note the integrity of host membrane surrounding SFB at the attachment site (E, arrowhead).

Ultrastructural changes indicative of osmotic imbalance and cytoskeletal damage were frequently observed both at the apical and basal poles of enterocytes. At the apical pole these changes included cytoplasmic membrane blebbing and loss of microvilli structure (Figure 4.7 A, B, C & D). The presentation of membrane blebbing suggested that this occurred initially close to tight junctions and progressed to the rest of the apical pole while some blebs appeared to cast off from the cell. Hydropic degeneration with cytoplasmic dilution was seen at the basal pole, and the cytoplasmic membrane appeared intact (Figure 4.7 E). Other enterocyte lesions included hydropic degeneration of mitochondria. A large number of enterocytes had lost the tight junction integrity and were detaching from the enteric mucosa (Figure 4.7 F). Changes in other cell types were also seen; for example, rodlet cells were abundant and always present adjacent to an area of fluid accumulation, suggesting they may have been actively secreting (Figure 4.7 G).

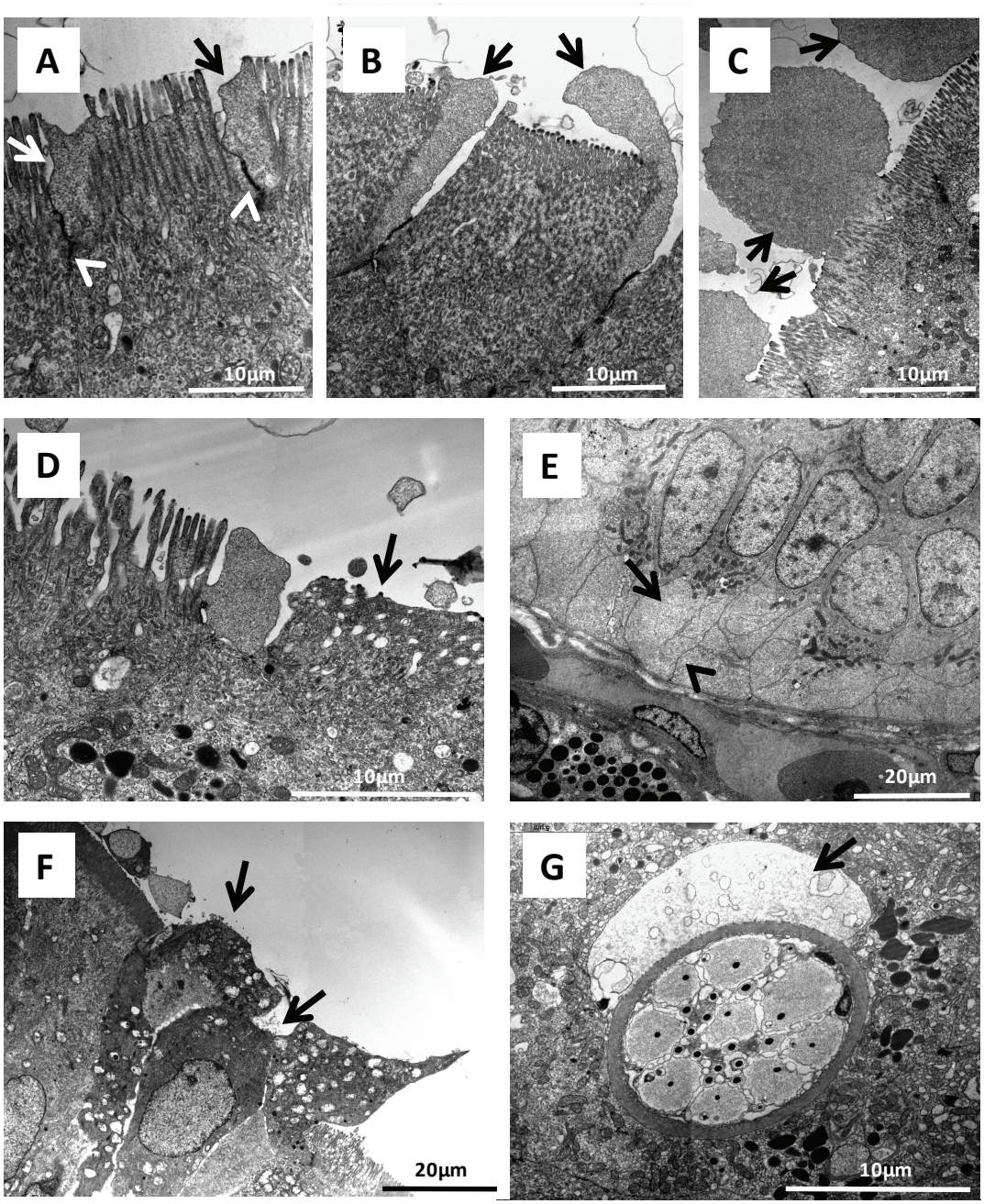

Figure 4.7. Transmission electron microscopy of pathological changes within distal intestine of fish with RTGE. Membrane blebbing, initially located near tight junctions (arrows, A-C), and structural loss of microvilli (arrow, D) were observed in the apical pole of enterocytes, whereas hydropic degeneration with cytoplasmic dilution was present in the basal pole (arrowhead, E), where membrane continuity was intact (arrow, E). Hydropic mitochondria and enterocyte detachment (arrows, F) were frequent as well as an apparent secretory activity of rodlet cells (arrow, G).

4.5. Discussion

Accumulation of large numbers of "*C.* arthromitus" have been observed in the lower digestive tract of rainbow trout with RTGE (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). In this study, most RTGE-affected fish presented with large numbers of SFB in their lower digestive tract, detectable by histopathology or electron microscopy. By contrast with other reports, however (Michel *et al.* 2002), the presence of SFB was never confirmed in any of the pyloric stomach sections examined in this study. Although the histopathological presence of SFB was significantly associated with RTGE-affected fish, these organisms were also observed in several apparently healthy controls from the same units (data not shown). This was probably a result of generalised increase of SFB load within affected units or subclinical infection, although all of these fish were actively feeding and did not present any of the gross changes associated with RTGE. No significant differences were observed in the presence and quantity of SFB between moribund and "subclinical" RTGE+ fish, but SFB were more commonly observed and in higher numbers in pyloric caeca than in distal intestine. Both these observations suggest that the pyloric caeca are the preferred site for histological detection of SFB in RTGE-affected fish. Previous reports on the preferred location of trout SFB do vary; Michel *et al.* (2002) reported their presence throughout the digestive system, whereas Branson (2003) observed SFB more frequently in the distal intestine. It is not possible to contrast our findings with these reports, as none of them included numerical data. The differences between organs were significant in our study and it is possible that the conditions in pyloric caeca are more favourable for SFB. This possibility is supported by the observation of reduced staining and loss of

structure (degeneration) of SFB noted in several distal intestines from RTGE-affected fish, an observation consistent with SFB membrane impairment as previously reported (Michel *et al.* 2002). Congestion and enterocyte detachment were more frequent in distal intestine, and it is possible to hypothesize that SFB degradation in the distal intestine could have coincided with sporulation, cellular toxicity or autolysis during fixation. Also, this may have been the reason for the failure to detect these organisms histologically in 15% of RTGE-affected fish. The degradation of SFB could also have a bearing on RTGE pathogenesis, as "*C.* arthromitus" are closely related to *Clostridium* (Urdaci *et al.* 2001; Snel *et al.* 1994) and may produce endotoxin, which would be released after cytoplasmic membrane damage (Michel *et al.* 2002).

All analytical methods used in this study revealed SFB both free within the lumen and attached to the digestive mucosa. On the mucosal surface, SFB appeared to concentrate in specific areas, whereas other locations were devoid of these organisms. Morphological differences between mucosal areas with and without SFB were not noted in this study, although they may exist and several authors have noted a tropism of SFB for lymphoid tissues and specific locations in other animals. Preferred locations include the ileum of mice (Davis & Savage 1974), the ileum and caecum of the chicken (Klaasen *et al.* 1992) and ileal Peyer's patches of several species (Meyerholz *et al.* 2002; Smith 1997; Lowden & Heath 1995; Jepson *et al.* 1993; Sanford 1991; Pearson *et al.* 1982). All the SFB observed in this study had an appearance consistent with previous reports, both in size, segmentation, sporulation and the presence of different developmental stages within a single filament (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). There was no evidence for spore germination in

either intestine or pyloric caeca, as previously observed (Michel *et al.* 2002). This observation suggests that these spores may constitute the form of dispersal between hosts, as has been shown for SFB in mice (Klaasen *et al.* 1992). There was close interaction of SFB with the digestive mucosa of RTGE-affected rainbow trout and SFB attachment sites, and apparent engulfment of SFB by enterocytes was observed. Attachment sites were similar to those described for SFB in mice and poultry, with an electron-dense area resulting from actin accumulation, implying a cell metabolic response (Jepson *et al.* 1993), although the nipple-like appendages reported in other species (Yamahuchi & Snel 2000; Jepson *et al.* 1993; Chase & Erlandsen 1976; Davis & Savage 1974) were not observed in the distal segments of trout SFB, which presented a pear-like shape. It is possible that this absence resulted from the plane of the section, although morphological differences have been observed between SFB in different animal species (Heczko *et al.* 2000; Smith 1997; Lowden & Heath 1995; Klaasen *et al.* 1993; Allen 1992; Goodwin *et al.* 1991; Sanford 1991) and it is possible that trout SFB do not present this feature. Apparent engulfment of SFB by adjacent enterocytes was suggested by SEM observations, although this was not directly confirmed by TEM. It is not possible to discard this possibility, and intracellular bodies structurally similar to SFB were observed occasionally in TEM (data not shown), but phagocytosis was never observed directly. Phagocytosis of SFB has been reported in poultry and has been related to the role of these in the activation of the mucosal immune system (Yamahuchi & Snel 2000).

The role of SFB in the aetiology of RTGE is unclear (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001) and this study also failed to demonstrate a role.

None of the pathological changes observed in the digestive system of RTGE-affected fish was exclusively associated with closely apposed SFB, suggesting that if SFB are indeed the cause of RTGE, they are inducing pathological changes without direct contact, for example by toxin release (Michel *et al.* 2002). An aetiological role of SFB has never been demonstrated in other species and although accumulation of large numbers of SFB were associated with stunting syndrome in turkey poults, this syndrome was subsequently shown to be caused by a virus (Ali & Reynolds 1997; Angel *et al.* 1990). No inclusion bodies or viral particles were seen in the digestive system of RTGE-affected fish in this or other studies.

Severe pathological changes were observed in the digestive mucosa of rainbow trout affected with RTGE, including loss of microvilli, apical blebbing, hydropic change to mitochondria and basal hydropic degeneration. All these changes suggest cytoskeleton damage and severe osmotic imbalance at the enterocyte level. The blebs observed were consistent with zeosis, as their matrix was devoid of cytoplasmic organelles and filled with ribosomes only (Ghadially 1997). The cytoplasmic blebbing of enterocytes has been reported in other species to be associated with local ischaemia, with the presence of enterotoxin and apoptosis (Kamaras & Murrel 2001; Mills *et al.* 1999; Malorni *et al.* 1990; Wagner *et al.* 1979) and can precede enterocyte detachment (Barros *et al.* 2003; Wagner *et al.* 1979). In RTGE+ fish, large numbers of affected enterocytes lost the integrity of their tight junctions and were shed to the enteric lumen. Mucosal loss was extensive and resulted in direct exposure of a large area of the lamina propria to the digestive lumen. This would probably have compromised osmotic balance and facilitated the entry of opportunistic

pathogens, as the digestive osmotic barrier is located at the tight junctions and is part of the passive immune system (Evans & Claiborne 2006). Finally, the frequent presence of rodlet cells with adjacent fluid accumulation suggests that these cells were being recruited and were actively secreting. Rodlet cells are exclusive to teleosts and it is believed that they have a role as part of the host response to tissue damage, stress and noxious agents, including parasites, toxic metals and acid exposure (Reite & Evensen 2006; Reite 2005). It is therefore possible that rodlet cell recruitment and activity may be a common feature of fish enteritis, although there are no reports in this respect. Degranulation of eosinophilic granular cells was not seen in RTGE fish, in line with reports of an independent action of these two cell types (Reite 2005).

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4.7. References

Ali, A. & Reynolds, D. L. 1997, 'Stunting syndrome in turkey poults: isolation and identification of the etiologic agent'. *Avian diseases*, vol. 41, pp. 870-881.

Allen, P. C. 1992, 'Comparative study of long, segmented, filamentous organisms in chickens and mice'. *Laboratory Animal Science*, vol. 42, pp. 542-547.

Angel, C. R., Sell, J. L., Fagerland, J. A., Reynolds, D. L. & Trampel, D. W. 1990, 'Long-segmented filamentous organisms observed in poults experimentally infected with stunting syndrome agent'. *Avian diseases*, vol. 34, pp. 994-1001.

Angert, E. 2005, 'Alternatives to binary fission in bacteria'. *Nature Reviews: Microbiology*, vol. 3, pp. 214-224.

Austin, B., Stobie, M. & Robertson, P. A. W. 1992, '*Citrobacter freundii*: the cause of gastro-enteritis leading to progressive low level mortalities in farmed rainbow trout, *Oncorhynchus mykiss* Walbaum, in Scotland'. *Bulletin of the European Association of Fish Pathologists*, vol. 12, pp. 166-167.

AVMA 2001, '2000 Report of the AVMA Panel on Euthanasia'. *Journal of the American Veterinary Medical Association*, vol. 218, pp. 669-696.

Barros, L. F., Kanaseki, T., Sabirov, R., Morishima, S., Castro, J., Bittner, C. X., Maeno, E., Ando-Akatsuka, Y. & Okada, Y. 2003, 'Apoptotic and necrotic blebs in epithelial cells display similar neck diameters but different kinase dependency'. *Cell Death and Differentiation*, vol. 10, pp. 687-697.

Branson, E. 2003, 'Rainbow trout Gastro-Enteritis (RTGE)-first diagnosis in the UK'. *Fish Veterinary Journal*, vol. 7, pp. 71-76.

Chase, D. G. & Erlandsen, S. L. 1976, 'Evidence for a complex life cyle and endospore formation in the attached, filamentous, segmented bacterium from murine ileum'. *Journal of Bacteriology*, vol. 127, pp. 572-583.

Davis, C. P. & Savage, D. C. 1974, 'Habitat, succession, attachment, and morphology of segmented, filamentous microbes indigenous to the murine gastrointestinal tract'. *Infection and Immunity*, vol. 10, pp. 948-956.

Evans, D. H. & Claiborne, J. B. 2006, *The physiology of fishes*. 3rd Ed., Taylor & Francis, New York.

Ferguson, H. W. 2006, *Systemic pathology of fish: A text and atlas of normal tissues ain teleosts and their responses in disease*. Second Ed., Scotian Press, London, UK.

Ghadially, F. N. 1997, *Ultrastructural pathology of the cell and matrix*. IV Ed., Butterworth-Heinemann, Oxford, UK.

Goodwin, M. A., Cooper, G. L., Brown, J., Bickford, A. A., Waltman, W. D. & Dickson, T. G. 1991, 'Clinical, pathological, and epizootiological features of long-segmented filamentous organisms (bacteria, LSFOs) in the small intestines of chickens, turkeys, and quails.'. *Avian Diseases*, vol. 35, pp. 872-876.

Heczko, U., Abe, A. & Finlay, B. B. 2000, 'Segmented filamentous bacteria prevent colonization of enteropathogenic *Escherichia coli* O103 in rabbits'. *The Journal of Infectious Diseases*, vol. 181, pp. 1027-1033.

Jepson, M. A., Clark, M. A., Simmons, N. L. & Hirst, B. H. 1993, 'Actin accumulation at sites of attachment of indigenous apathogenic segmented filamentous bacteria to mouse ileal epithelial cells.'. *Infection and Immunity*, vol. 61, pp. 4001-4004.

Kamaras, J. & Murrel, W. J. 2001, 'The effect of bacterial enterotoxins implicated in SIDS on the rabbit intestine'. *Pathology*, vol. 33, pp. 187-196.

Klaasen, H. L. B. M., Koopman, J. P., Poelma, F. G. J. & Beynen, A. C. 1992, 'Intestinal, segmented, filamentous bacteria'. *FEMS Microbiology Reviews*, vol. 88, pp. 165-180.

Klaasen, H. L. B. M., Koopman, J. P., Van den Brink, M. E., Bakker, M. H., Poelma, F. G. J. & Beynen, A. C. 1993, 'Intestinal, segmented, filamentous bacteria in a wide range of vertebrate species.'. *Laboratory Animals*, vol. 27, pp. 141-150.

Lowden, S. & Heath, T. 1995, 'Segmented filamentous bacteria associated with lymphoid tissues in the ileum of horses'. *Research in Veterinary Science*, vol. 59, pp. 272-274.

Malorni, V., Fiorentini, C., Paradisi, S., Giuliano, M., Mastrantonio, P. & Donelli, G. 1990, 'Surface blebbing and cytoskeletal changes induced in vitro by toxin B from Clostridium difficile: an immunochemical and ultrastructural study'. *Scandinavian Journal of Gastroenterology*, vol. 52, pp. 340-356.

Meyerholz, D. K., Stabel, T. J. & Cheville, N. F. 2002, 'Segmented filamentous bacteria interact with intraepithelial mononuclear cells'. *Infection and Immunity*, vol. 70, pp. 3277-3280.

Michel, C., Bernardet, J. F., Daniel, P., Chilmonczyk, S., Urdaci, M. C. & de Kinkelin, P. 2002, 'Clinical and aetiological aspects of a summer enteritic syndrome associated with the sporulating segmented filamentous bacterium "*Candidatus* arthromitus" in farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum)'. *Journal of Fish Diseases*, vol. 25, pp. 533-543.

Mills, J. C., Stone, N. L. & Pittman, R. N. 1999, 'Extranuclear Apoptosis: The Role of the Cytoplasm in the Execution Phase'. *The Journal of Cell Biology*, vol. 146, pp. 703-708.

Pearson, G. R., McNulty, M. S., McCracken, R. M. & Curran, W. 1982, 'Scanning electron microscopic observations of segmented filamentous bacteria

in the small intestine of domestic fowl'. *Veterinary Record*, vol. 111, pp. 365-366.

Reite, O. B. 2005, 'The rodlet cells of teleostean fish: their potential role in host defence in relation to the role of mastcells/eosinophilic granule cells'. *Fish & Shellfish Immunology*, vol. 19, p. 267.

Reite, O. B. & Evensen, E. 2006, 'Inflammatory cells of teleostean fish: A review focusing on mast cells/eosinophilic granule cells and rodlet cells'. *Fish & Shellfish Immunology*, vol. 20, pp. 192-208.

Roberts, R. J. 2001, *Fish pathology*. Third Ed., W.B. Saunders, Edinburgh, UK.

Sanford, S. E. 1991, 'Light and electron microscopic observations of a segmented filamentous bacterium attached to the mucosa of the terminal ileum of pigs'. *Journal of Veterinary Diagnostic Investigation*, vol. 3, pp. 328-333.

Smith, T. M. 1997, 'Segmented filamentous bacteria in the bovine small intestine'. *Journal of Comparative Pathology*, vol. 117, pp. 185-190.

Snel, J., Blok, H. J., Kengen, M. P., Kudwig, W., Poelma, F. G. J., Koopman, J. P. & Akkermans, A. D. L. 1994, 'Phylogenetic Characterization of *Clostridium* Related Segmented Filamentous Bacteria in Mice Based on 16S Ribosomal RNA Analysis'. *Systematic and Applied Microbiology*, vol. 17, p. 172.

Snel, J., Heinen, P. P., Blok, R. J., Carman, R. J., Duncan, A. J., Allen, P. C. & Collins, M. D. 1995, 'Comparison of 16S rRNA sequences of segmented filamentous bacteria isolated from mice, rats, and chickens and proposal of "*Candidatus* arthromitus"'. *International Journal of Systematic Bacteriology*, vol. 45, pp. 780-782.

Toranzo, A. E. 2004. 'Report about bacterial diseases'. In: *Mediterranean Aquaculture Laboratories*, P. Alvarez-Pellitero et al., (Eds.), CIHEAM/FAO, pp. 49-89.

Urdaci, M. C., Regnault, B. & Grimont, P. A. D. 2001, 'Identification by in situ hybridization of segmented filamentous bacteria in the intestine of diarrheic rainbow trout (*Oncorhynchus mykiss*)'. *Research in Microbiology*, vol. 152, pp. 67-73.

Wagner, R., Gabbert, H. & Höhn, P. 1979, 'The mechanism of epithelial shedding after ischemic damage to the small intestinal mucosa'. *Virchows Archiv B Cell Pathology Zell-pathologie*, vol. 30, pp. 25-31.

Weber, E. S. 2005, 'Gastroenterology for the piscine patient'. *Veterinary Clinics of North America: Exotic Animal Practice*, vol. 82, pp. 247-276.

Yamahuchi, K. & Snel, J. 2000, 'Transmission electron microscopic demonstration of phagocytosis and intracellular processing of segmented filamentous bacteria by intestinal epithelial cells of the chick ileum'. *Infection and Immunity*, vol. 68, pp. 6496-6504.

CHAPTER 5. A Study of Gross, Histological and Blood Biochemical Changes in Rainbow Trout (*Oncorhynchus mykiss* W.) with Rainbow Trout Gastroenteritis (RTGE)

Del-Pozo, Jorge*; Crumlish, Margaret; Turnbull, James F.; Ferguson, Hugh W.

5.1. Abstract

Rainbow trout gastroenteritis (RTGE) is an emerging disease syndrome of farmed rainbow trout (*Oncorhynchus mykiss* W.) in several European countries. The clinical presentation includes severe distal enteritis with congestion, oedema and mucosal detachment. Affected fish present with accumulation of large numbers of the segmented filamentous bacteria "*Candidatus* arthromitus" (SFB) in the lower digestive system. These organisms have been suggested as the aetiology of RTGE, although confirmation has been hindered by the inability to culture SFB *in vitro*. Alterations in the microflora and the presence of a toxin have also been proposed, but the mechanisms behind the pathogenesis of RTGE are still unknown. This study examined the macroscopic and microscopic changes in trout with RTGE (RTGE+), as well as the blood chemistry. The aims of the study included assessment of a case definition for RTGE, refinement of its clinical description and study of its pathogenesis using blood biochemical analysis. The case definition was created from previous literature on RTGE and was based on the gross presentation. A total of 464 rainbow trout sampled from 11 sites in the UK were included in this study, comprising 152 RTGE+ fish and 330 random, apparently healthy fish. Using this sample, the case definition was assessed by analysis of its agreement with

three laboratory tests: histopathology, packed cell volume and kidney bacteriology. Cluster analysis was used for refinement of the clinical description and this analysis indicated the presence of three distinct presentations within the population of RTGE+ fish. Cluster A included gross signs associated with moribund RTGE+ fish and it is likely that these signs were associated with the final clinical stages of RTGE. Clusters B and C identified gross signs consistent with concurrent diseases, notably furunculosis, enteric red mouth and proliferative kidney disease. These results confirmed the presence of concurrent disease in a sample of fish identified according to the reported gross presentation for RTGE. These criteria were used to choose fish with RTGE only for the analysis of the pathogenesis. The blood chemistry of RTGE+ fish without concurrent disease indicated a severe osmotic imbalance, and a reduced albumin/globulin ratio indicative of selective loss of albumin. These findings are compatible with a protein losing enteropathy (PLE).

5.2. Introduction

Rainbow trout gastroenteritis (RTGE), present in several European countries (Denham 2004; Toranzo 2004; Cervellione, personal comm.; Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001; Sanz 2000), has been spreading since its first report in 1992 (Michel *et al.* 1999). This is a syndrome that affects farmed rainbow trout *Oncorhynchus mykiss* (Walbaum) predominantly during the summer (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). The impact of RTGE varies both in number of cages, tanks or ponds affected and number of mortalities within affected units (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). Outbreaks last an average of 25.5 days and usually present a pattern

consistent with a propagating epidemic, suggesting this condition is infectious (Chapter 3).

The aetiology of RTGE is still unclear, although segmented filamentous bacteria (SFB), have been implicated (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). Both the gross and histopathological presentation of RTGE have been described by several authors (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001): Externally, clinical onset is sudden with lethargy and loss of appetite. Yellow mucoid material is often excreted from the vent of affected fish and it is possible to observe an accumulation of large amounts of similar material in the bottom of ponds. Occasionally, uncoordinated swimming is observed. Some fish present with characteristic dischromic changes consisting of dark, vertical stripes along the flanks. Internally signs of acute enteritis are observed, with generalised dilation of the digestive system and mucosal congestion and oedema. The lower digestive tract contains a yellowish mucoid material that accumulates to form an occluding plug in the anal area. Histologically, SFB accumulation, congestion and enterocyte detachment are present in both distal intestine and pyloric caeca. Michel *et al.* (2002) also described these histopathological changes in the pyloric end of the stomach.

Several hypotheses have been proposed regarding the pathogenesis of RTGE, including alterations in the microflora and/or the presence of a toxin or toxic component (Michel *et al.* 2002). There is lack of published information on the pathogenesis of RTGE, and the work presented here aims to contribute to that information by investigating the pathogenesis of RTGE.

5.3. Materials and Methods

5.3.1. RTGE case definition

Positive fish (RTGE+) were identified according to a case definition created from previous literature and based on the gross presentation, (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). The case definition was: *"RTGE is a condition of rainbow trout, observed in units with daily cumulative mortalities of 0.5% or more and present during the summer. Affected fish present a distended abdomen externally, while internally their lower intestine is dilated, congested and oedematous, and has a yellow viscous content"*. During the study, apparently healthy individuals used as negative controls were identified by a general absence of gross abnormalities.

5.3.2. Fish sampling

All the rainbow trout in this study were sampled from 1st June to 31st September 2006 at 11 UK sites with RTGE. Two categories of fish were sampled from a single unit undergoing an RTGE outbreak: (1) As many moribund fish showing external signs of RTGE as practically possible and (b) 30 randomly sampled apparently healthy fish. The sample size of (b) was chosen to enable disease detection with 95% of confidence if the prevalence was at 10% or more.

All fish were sampled on-site, euthanized prior to sampling with benzocaine (SIGMA E1501) at 250mg/l (AVMA 2001), and fish origin, fork length, weight and the presence of any external or internal gross signs were recorded. Blood samples were taken from the caudal vein with a 2 ml pre-heparinised (1000 IU/ml) syringe with a 23G needle (Terumo™, Leuven, Belgium) and posterior kidney samples were aseptically taken with a sterile plastic loop. Samples for

histology were taken from three locations in the digestive tract, the pyloric end of the stomach (one 1cm tubular section), proximal pyloric caeca (10-15 whole caeca) and distal intestine (one 1cm tubular section).

5.3.3. Sample processing

Samples for histology were placed in 10% (v/v) neutral buffered formalin and left for a minimum period of 24h before processing using standard protocols and embedding in individual paraffin wax blocks. Finally 5µm sections from these blocks were cut, placed on slides and stained with haematoxylin and eosin (H&E). All the histology slides were examined "blind" by the same person and SFB were identified as bacteria approximately 0.6-1.2µm in diameter and up to 60µm in length, with apparent segmentation every 1.2-1.6µm (Urdaci *et al.* 2001). SFB presence was recorded in each tissue after scanning the entire section at objective x10. Any histopathological changes were noted and the presence of lamina propria and/or adventitial congestion and epithelial detachment was recorded.

Blood samples were loaded into heparinised haematocrit tubes that were subsequently centrifuged (20,000 g, 5 min) and the packed cell volume (PCV) read as a percentage of the total volume of the sample. Posterior kidney samples were inoculated onto a tryptone soya agar (TSA, Oxoid™, UK) plate and incubated at 22°C. When bacterial growth was present, colony morphology and Gram staining were recorded following standard microbiological procedures (Buller 2004).

5.3.1. RTGE case definition assessment

The case definition of RTGE was assessed by analysis of its agreement with three laboratory tests, namely PCV, histopathology and kidney bacteriology in TSA, as well as all the possible combinations of these. These analyses were conducted on data from all the RTGE+ fish in the sample, matched individually with healthy negative controls from the same unit. A receiver operating characteristic (ROC) curve was plotted to determine the most appropriate threshold PCV value to separate RTGE+ fish from negative controls, which was chosen as the point in the curve which was nearest to sensitivity=1 and 1-specificity=0 (Thrusfield 2006). Histological sections were considered positive for RTGE if there was presence of SFB, congestion of the adventitial and lamina propria and detachment of enterocytes (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). A fish was considered positive for histopathological RTGE if distal intestine or pyloric caeca were positive. Bacterial cultures were considered negative for bacterial growth if a total absence of bacterial colonies was noted after incubation for 15 days. Sensitivity and specificity values for the three laboratory tests were calculated with their 95% confidence intervals (Thrusfield 2006).

Further analysis was made on the different clinical presentations seen exclusively in RTGE+ fish. For this purpose, the tools used were the analysis of the statistical association of each gross sign with the results of the laboratory tests and the cluster analysis of gross presentations using Ward's linkage method (Hill & Lewicki 2007).

5.3.2. Weight and condition factor in RTGE fish

To complement the findings of the cluster analysis, a subset of the data was selected for univariable conditional logistic regression (CLR) analysis of the differences in weight and condition factor between RTGE+ fish without concurrent disease and apparently healthy fish. A group of RTGE+ fish which were negative for bacteriology and did not present enlarged kidneys were selected and matched by unit of origin with three negative controls. Before CLR, the linearity of the logit of continuous variables with the dependent variable was tested for all variables using the Box-Tidwell transformation test. A cut-off point of p=0.05 was applied in CLR analysis (Dohoo *et al.* 2003).

5.3.3. Blood Biochemistry

Biochemical analyses were conducted on plasma from a sample of 30 fish (20 RTGE+ and 10 controls) taken from a single unit in one of the sites affected. None of the RTGE+ fish sampled was positive for bacteriology or had an enlarged kidney. The quantity of plasma obtained from RTGE+ fish was very small as a result of the increase in PCV and two groups of RTGE+ fish were required to ensure a sufficient quantity of plasma for the analyses. Blood samples were taken from the caudal vein with a 2 ml pre-heparinised (1000 IU/ml) syringe with a 23G needle (Terumo™, Leuven, Belgium) and centrifuged at 3000g for 10m. Plasma was then transferred to a cryovial and snap-frozen in liquid nitrogen for transport and storage. All samples were analysed by Scottish Agricultural College, Penicuik, UK. Total protein and albumin were measured for the first group of RTGE+ fish (n=10) while sodium, chloride and potassium were measured for the second group of RTGE+ fish (n=10); all parameters were measured for the control group (n=10).

5.3.4. Statistical analysis

All the fish data with the exception of the blood biochemistry dataset were collated in a single spreadsheet, where a calculated variable for condition factor was created (CF= (100*Weight (g))/Forklength^3 (cm)). If not specified previously, the statistical association was tested with Fisher Exact (FE) for dichotomous variables and non-parametric Kruskal-Wallis (KW) for continuous variables, with significance assumed below p=0.05. All data management and analyses were conducted in MS Excel 2007™ (Microsoft, USA), Statistica™ (Statsoft Ltd., Bedford, UK) and EpiInfo™ (CDC, Atlanta, USA).

5.4. Results

5.4.1. Fish sampling

A total of 464 rainbow trout were sampled from 11 sites in the UK (7 in Scotland and 4 in England) from June to September 2006. Of these, 134 were moribund fish consistent with the RTGE case definition (moribund RTGE+) and 330 were randomly selected fish from the same units. An average of 42 (range=31-81) fish were sampled from a RTGE+ unit in each site, of which a mean of 12 (range=1-51) were moribund RTGE+ and 30 were random apparently healthy fish. During post-mortem examination, 18 randomly sampled fish were consistent with the case definition and these fish were included in the analyses as “subclinical” RTGE+ fish. Externally, these fish presented with less obvious dilation of the abdomen and internally with all gross signs included in the RTGE case definition. In 22% of the “subclinical” RTGE+ fish, the yellow viscous intestinal content was mixed with feed.

5.4.2. RTGE case definition assessment

The dataset included all 152 RTGE+ fish matched with 152 negative controls. The PCV values of RTGE+ fish were significantly higher ($p<0.001$, KW), with a mean PCV=55% (range=19-92%), whereas negative controls presented a mean PCV of 44% (range=7-68%). The surface area under the ROC curve for PCV was 75% and the apparent shoulders found in the extremes of the plot indicated the presence of relatively lower, physiological and higher PCV values in RTGE+ fish (Figure 5.1). The nearest point of the curve to sensitivity=1 and 1-specificity=0 was PCV=51%, which was chosen as the threshold for the PCV laboratory test.

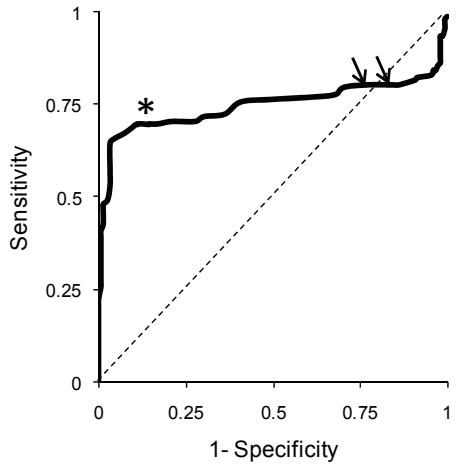

Figure 5.1. Receiver operating characteristic (ROC) curve used to determine the threshold where the sensitivity and specificity of packed cell volume (PCV) were highest for the detection of RTGE (PCV=51%). Cases and controls were sampled from the same productive units simultaneously (n=304; 152 RTGE+). A star indicates the cut-off point where the distance to sensitivity and specificity=1 was minimum (51%), whereas the two arrows indicate the physiological range (CPV=32-45%).

The sensitivity and specificity of both positive and negative kidney bacteriology were assessed, as only 15% of RTGE+ fish were positive for this test. The values for sensitivity and specificity for each test are shown in Table 5.1.

Table 5.1. Sensitivity and specificity of three laboratory tests applied to a sample of RTGE+ cases and apparently healthy negative controls from the same units (n=304; 152 RTGE+).

Laboratory test	*Sensitivity (95% CI)*	*Specificity (95% CI)*	*Relative distance**
Histological RTGE	93.6 (87.9-96.7)	80.4 (74-85.6)	0.21
PCV>51%	84.8 (77.5-90)	74.3 (67.4-80.1)	0.30
Pos. K. Bacteriology	92 (75-97.8)	53.8 (47.9-59.5)	0.47
Neg. K. Bacteriology	46.2 (40.5-52.1)	8.0 (2.2-25)	1.07

* Euclidean distance relative to the point where sensitivity and specificity equal 1 in a scatter plot where Y=Sensitivity and X=1-Specificity. CI: Confidence Interval; PCV: % packed cell volume; Pos. K. Bact.: Positive kidney bacteriology; Neg. K. Bact.: Negative kidney bacteriology; Histological RTGE (*i.e.* SFB presence, enterocyte detachment and congestion in intestine or caeca).

Descriptive analysis of the gross clinical presentation revealed that RTGE+ fish had other external and internal gross signs that were not included in the case definition of RTGE. Several of these pathological changes were significantly more prevalent in moribund RTGE+ fish (Table 5.2). The stomach of all the fish presenting gastric dilation contained a clear, slightly viscous fluid.

Table 5.2. External and internal gross signs in 152 RTGE+ fish. Gross signs included in RTGE case definition are not included. The statistical significance of the association of each gross sign with moribund fish is indicated.

	Description	*Frequency (%)*	*Relative % Moribund**	*OR (95% CI)*	*P (FE)*
External Gross Signs	Swollen Appearance	107 (70%)	96%	18.8 (5.9-60.1)	<0.001
	Lighter Colouration	81 (53%)	100%	N/A	<0.001
	Striping of Flanks	47 (31%)	98%	12.2 (1.6-93.4)	0.001
	Gill Pallor	9 (6%)	89%	1.5 (0.2-12.2)	0.6
	Darker Colouration	7 (5%)	100%	N/A	<0.001
	Haemorrhagic Gills	5 (3%)	100%	N/A	<0.001
	Skin Lesions	1 (1%)	100%	N/A	<0.001
Internal Gross Signs	Pyl. Caeca Congestion	97 (64%)	90%	2.7 (1.1-6.6)	0.03
	Gastric Dilation	61 (40%)	98%	19.1 (2.5-146.2)	<0.001
	Enlarged Kidney	16 (11%)	81%	0.8 (0.2-2.9)	0.4
	Splenomegaly	16 (11%)	88%	1.3 (0.3-6.0)	0.5
	Hepatic Pallor	8 (5%)	88%	1.3 (0.2-10.9)	0.6
	Hepatomegaly	5 (3%)	80%	0.7 (0.1-6.6)	0.6
	Hepatic Haemorrhage	5 (3%)	80%	0.7 (0.1-6.6)	0.6

* Relative to the total number of fish where each pathological change was observed. OR: Odds ratio of the association with moribund RTGE + fish; 95% CI: 95% Confidence Intervals; FE: Fisher Exact.

After incubation of kidney samples on TSA for 15d at 22°C, 23 (15%) RTGE+ fish were positive for bacterial growth. Analysis of colony morphology and Gram staining (Buller 2004) indicated that 18(78%) of these isolates were consistent with *Aeromonas salmonicida*, and 3 (13%) with *Yersinia ruckeri,* while the remaining 2 (9%) were mixed cultures. To account for the potential presence of concurrent bacterial diseases in RTGE+ fish, positive kidney bacteriology results were included in the cluster analysis. A percentage of RTGE+ were not positive in histopathology (20%) and the range of PCV values found in RTGE+ fish included physiological reference values (PCV=32-45% (Stoskopf 1993)), as well as relatively higher and lower values. Additionally, proliferative kidney disease (PKD), a condition that results in anaemia (Clifton-Hadley *et al.* 1987) was reportedly endemic in at least 5 of the sampling sites. For these reasons, kidney bacteriology, histopathology and physiological, higher and lower PCV values were included in the cluster analysis. This analysis identified three clusters in the data (Figure 5.2).

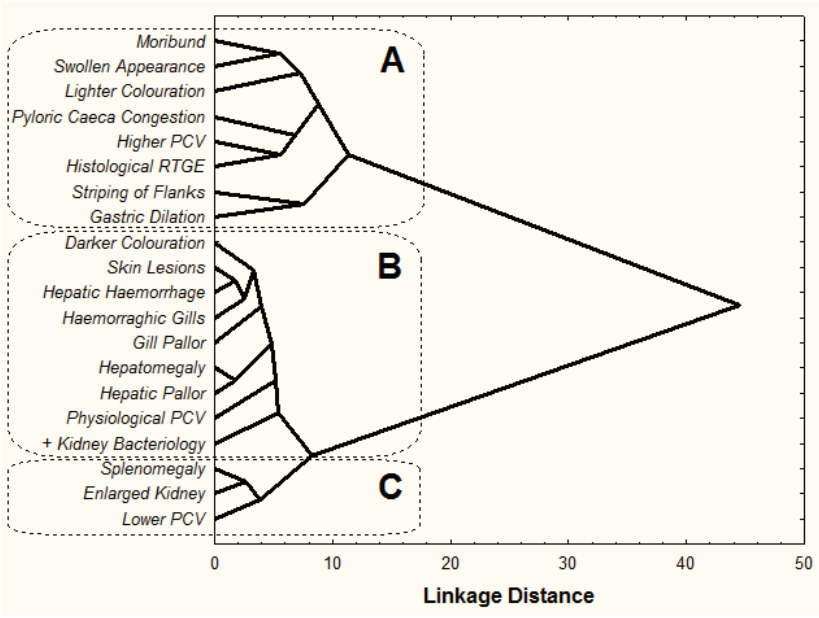

Figure 5.2. Cluster analysis of laboratory tests and gross signs presented by rainbow trout with RTGE. Gross signs part of the case definition used are not included. Gross signs observed in cluster A were present in 31-75% of the fish, those of cluster B in 11-16% and those of cluster C in 1-6% (n=152).

Pathological changes included in clusters B and C appeared in 1 to 16% of the RTGE+ fish and include signs that can be found in other disease entities, including bacterial and parasitic conditions (Ferguson 2006; Roberts 2001). Pathological changes found in cluster A were presented by 31-75% of the RTGE+ fish and were significantly more frequent in the RTGE+ moribund fish (Table 5.2). These pathological changes are not commonly reported in other fish disease conditions in the literature (Figure 5.3 A, B, C & D).

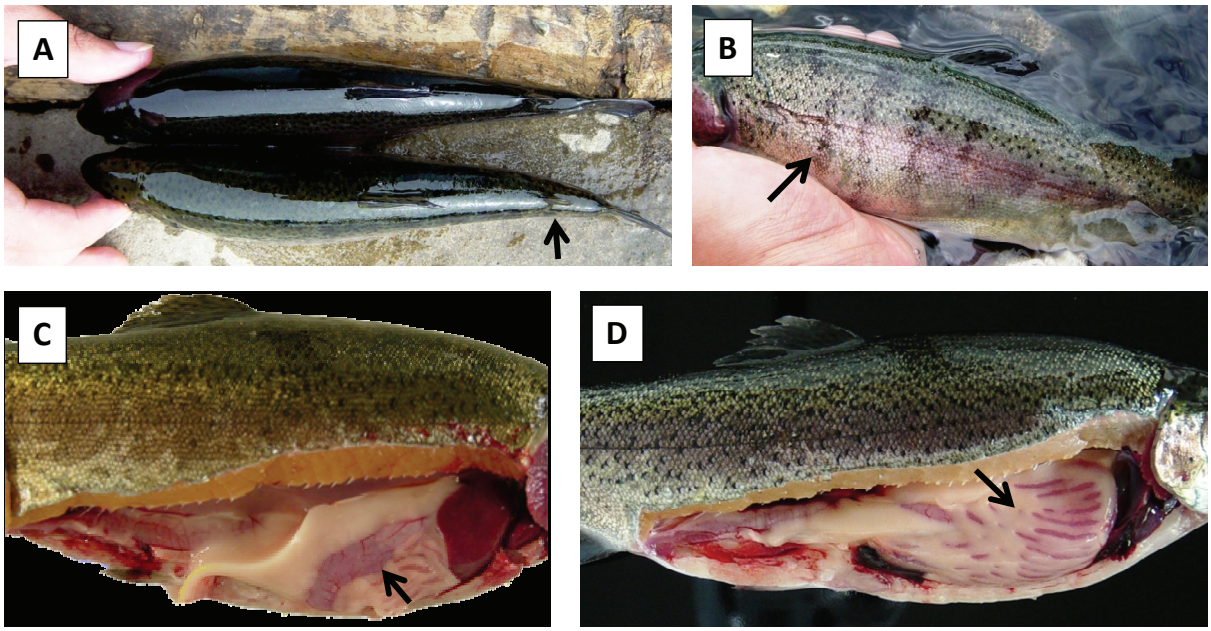

Figure 5.3. External and internal pathological changes frequently observed in moribund fish consistent with RTGE case definition. A: generally swollen appearance with lighter colouration (arrow) when compared with apparently healthy fish above; B: dischromic changes with striped marking of the flanks (arrow); C: dilation of the stomach with clear fluid contents (arrow); D: pyloric caeca with severe congestion and oedema (arrow).

5.4.3. Weight and condition factor in RTGE fish

For comparative analysis of fish weight and condition factor in RTGE+ fish, the matching of cases and controls resulted in a final dataset of 276 fish including 90 RTGE+ fish. The results of the Box Tidwell transformation test revealed fish weight was not linear with the logit of the outcome, therefore this variable was

included as dichotomous in the final analysis. Finally, no significant differences were found between RTGE+ and apparently healthy negative controls with regards to weight or condition factor (Table 5.3).

Table 5.3. Univariable conditional logistic regression (CLR) analysis of the differences in weight and condition factor (CF) between RTGE positive fish and apparently healthy negative control trout sampled from the same unit.

VARIABLE	*RTGE+ Mean (Range)*	*RTGE- Mean (Range)*	*Conditional logistic regression*	
			OR (95%CI)	*P*
Fish weight (g)*	192.0 (24.0-390.0)	222.6 (63.0-500.0)	1.0 (0.5-1.8)	0.94
CF †	1.3 (1.0-2.6)	1.4 (0.9-4.0)	1.0 (0.4-2.4)	0.99

OR: Odds ratio; * this variable failed Box Tidwell transformation test and was included as a dichotomous variable divided through the median (192g); † CF: Condition factor = (100*Weight (g))/Forklength^3 (cm).

5.4.4. Blood Biochemistry

Both RTGE+ groups had significantly higher PCV values than the apparently healthy group, similar to previous results. Significantly higher levels of total plasma proteins were recorded in the first group of RTGE+ fish when compared with the apparently healthy group ($p<0.001$; KW). A significant increase in both albumin and globulin fractions ($p<0.001$; KW) was also found. Further analysis revealed a lower albumin/globulin ratio in RTGE+ fish ($p=0.02$; KW). Significantly lower concentrations of sodium and chloride ions were recorded in the plasma of RTGE+ fish when compared with the group of apparently healthy fish, although no differences were noted in the plasma concentration of potassium between these two groups (Table 5.4).

Table 5.4. Comparative values of biochemical parameters of blood plasma from apparently healthy fish RTGE-affected fish.

VARIABLE	*RTGE– Mean (SE)*	*RTGE+ (a) Mean (SE)*	*RTGE+ (b) Mean (SE)*	*P (KW)*	*Reference values**
Sodium (mmol/l)	146.5 (2.4)	-	89.4 (1.5)	<0.001*	123-164†
Chloride (mmol/l)	123.1 (2.2)	-	45.5 (2.0)	<0.001*	120-147†
Potassium (mmol/l)	3.3 (0.2)	-	3.3 (0.2)	0.9095	3.3-3.5†
Total Protein (g/l)	43 (1.5)	83.1 (3.5)	-	<0.001*	28-60§
Albumin (g/l)	18.6 (0.7)	32.2 (1.9)	-	<0.001*	17-19§
Globulin (g/l)	24.4 (1.1)	50.9 (2.7)	-	<0.001*	5-41§
Albumin/Globulin (g/g)	0.8 (0.03)	0.6 (0.04)	-	0.02*	N/A

SE: Standard Error. (a) & (b): RTGE+ experimental groups. KW: Kruskal-Wallis. All groups n=10 fish. †: Powell (2006). §: Bowser (1993)

5.5. Discussion

A case definition is a list of criteria used to identify diseased individuals within affected populations, thereby enabling the diagnostic consistency necessary for the scientific study of diseases in the field (Dohoo *et al.* 2003). The initial case definition for RTGE used in this study was created from previous reports on the clinical presentation and epidemiology of this syndrome (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). This case definition aimed to enable the identification of RTGE during field sampling and provide the site managers with a tool for identifying RTGE. For both these reasons, this case definition was based on the gross presentation. It was necessary to assess the case definition before its use for the research of RTGE, but the absence of a "gold standard" diagnostic test for RTGE and the lack of available prevalence complicated this task. The former did not allow the use of traditional methodologies of sensitivity and specificity calculation (Thrusfield 2006) and the latter made the use of latent class models inadequate (Dohoo *et al.* 2003). To overcome these problems, an

indirect approach was used, and the case definition was indirectly assessed by comparison with three laboratory tests in a sample of cases and controls.

The inclusion of PCV for the case definition assessment tool was a result of the apparently higher PCV values observed in RTGE+ fish during a preliminary phase of the experiment (data not shown). This observation was confirmed in the sample and a significantly higher PCV was observed in RTGE+ fish. For the estimation of a threshold to separate cases from non cases using PCV, a ROC curve was plotted, using the assessed case definition as reference. A ROC plot is a visual representation of the changes in sensitivity (*i.e.* % true positives) and 1-specificity (*i.e.* 1-% true negatives) for each of the possible thresholds of the test (Thrusfield 2006). The area under the curve indicated that PCV is an acceptable predictor for RTGE in this dataset, and the coordinates of the plot were used to calculate the threshold that would maximize both sensitivity and specificity. The representation of the ROC curve also suggested the presence of PCV values that did not follow the main pattern.

Bacterial samples from the kidney were used as an indicator of the possible presence of bacterial septicaemia and both apparently healthy and RTGE+ fish were predominantly negative. However, a relatively small percentage of RTGE+ fish were positive for bacterial growth, a result that suggested the presence of bacterial septicaemia in these individuals.

Histopathology has been one of the main diagnostic tools used for RTGE and histopathological changes found in RTGE+ fish included SFB presence, enterocyte detachment and congestion in distal intestine or pyloric caeca (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). These features of the

histopathological presentation of RTGE were used for its diagnosis also in this study. In the majority of cases, there was agreement with the gross presentation of RTGE, and most fish positive by histopathology were also positive for RTGE case definition. Despite this, a relatively high number of fish did not present with histological evidence of RTGE, despite having gross lesions consistent with the case definition. These can be considered false negatives. All the above described criteria had to be present in a section for a positive histopathological diagnosis, and it is possible that these negative results could have been caused by failure to detect any of the histopathological changes, as a result of human error, SFB degradation, tissue autolysis or the presence of focal lesions. More importantly, it is also possible that these results were caused by the existence of other enteritic conditions resulting in signs mimicking the RTGE gross case definition, which would result in misclassification of these fish as RTGE+.

The results of the case definition assessment using three laboratory tests revealed the presence of two or more subpopulations in the RTGE+ fish sample. Most RTGE+ fish had significantly increased PCV, negative kidney bacteriology and positive histopathology but a relatively small percentage could present differing results for one or more of these tests. Cluster analysis was used to analyse the possibility of different gross presentations found in association with these laboratory tests and it revealed three different clinical presentations in the RTGE+ fish sample.

Cluster A was comprised of pathological changes which were frequent in the sample and included increased PCV values and positive histopathology. Further analysis revealed that the gross signs included in cluster A were

significantly associated with moribund RTGE+ fish, suggesting that this presentation reflects the final clinical stages of RTGE. As a result of this, cluster A presentation has special diagnostic importance for RTGE, as disease diagnosis is most frequently attempted in moribund fish (Ferguson 2006).

Cluster B and cluster C presentations included pathological changes that were infrequent in the RTGE+ sample. Pathological changes in cluster B were grouped with positive kidney bacteriology and physiological PCV, as well as gross signs consistent with the presence of bacterial septicaemia in these fish (Austin & Austin 2007; Ferguson 2006; Roberts 2001). The isolates were mostly consistent with *Y. ruckeri* and *A. salmonicida*, suggesting that these RTGE+ fish had concurrent enteric redmouth (ERM) and furunculosis, respectively. Cluster C signs are consistent with infection with *Tetracapsuloides bryosalmonae*, the causative agent of PKD, an observation that is supported by the relatively low PCV values of these fish, splenomegaly and enlarged kidneys, all consistent with this disease (Clifton-Hadley et al. 1987). It is possible that these two presentations had been a reflection of false positives or the presence of concurrent diseases in RTGE+ fish. Several infectious agents and parasites have been reported to cause histopathologically obvious enteritis in trout, although this feature is normally part of a systemic presentation and not associated with SFB presence (Austin & Austin 2007; Ferguson 2006; Roberts 2001). A proportion of fish positive for RTGE histopathology was observed for both fish positive for bacteriology (26%) and fish with physiological/lower PCV values (77%). It is possible that fish negative for RTGE histopathologically had been misclassified by the case definition, although this possibility is unlikely for fish that were positive by histopathology.

Concluding, these results suggest that a population of fish identified using the proposed RTGE case definition will be comprised of a majority of fish with RTGE exclusively and a smaller percentage of RTGE+ fish with concurrent diseases, including PKD, furunculosis or ERM. Moreover, this case definition is not 100% specific and it is possible that a relatively small percentage of the fish consistent with this case definition are non-RTGE enteritides that have been misclassified. The information gained on the gross presentations found in these groups enables the design of a case definition for the identification of RTGE+ fish that do not present concurrent disease by excluding fish that present with any of the gross signs included in cluster B or C. This might be useful in situations where the presence of other diseases is unwanted, for example pathogenesis studies using field samples or transmission attempts using challenge with neat RTGE+ digestive contents.

Michel *et al.* (2002) made several suggestions regarding the possible pathogenic mechanisms of RTGE, specifically focusing on the role of the bacteria. The study presented here has examined the pathophysiological changes observed in RTGE+ fish. Fish weight and condition factor appeared unchanged in RTGE+ fish, suggesting that these parameters do not alter susceptibility to the disease within an affected unit. There was no apparent loss of weight during the clinical phase of RTGE, although affected fish stop feeding after the onset of clinical RTGE (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). This may reflect a relatively short clinical phase, although it is also possible that weight loss was counterbalanced by fluid uptake, as evidenced by the gross swelling observed in RTGE+ fish.

Changes in haematological parameters have been used to examine the pathophysiology of several diseases of salmonids (Rehulka & Minarik 2007; Powell *et al.* 2006; Ackerman & Iwama 2001; Byrne *et al.* 1995; Clifton-Hadley *et al.* 1987). In the case of RTGE, the results of the haematological analysis indicated the presence of severe osmotic imbalance. During the study, the negative control fish values for total protein, albumin, globulin, Na^+, Cl^- and K^+ were comparable with previously published rainbow trout reference values (Powell 2006; Bowser 1993), suggesting cohabiting fish used as controls were healthy. Significantly higher PCVs and total protein concentration indicated haemoconcentration in RTGE+ fish (Stockham & Scott 2002). As a result, both albumin and globulin were increased, although a significantly reduced albumin/globulin ratio was observed. An increase in globulin is unlikely, given the acute nature of RTGE and the relatively longer time required for the synthesis of high molecular weight proteins (Stockham & Scott 2002). By contrast, selective loss of albumin through the digestive system is consistent with the severe enterocyte detachment observed in RTGE+ fish and it is therefore the likely cause for the observed effect.

Selective albumin loss is typical of protein losing enteropathies (PLE), although in other animal species PLE involves hypoproteinemia (Stockham & Scott 2002). It is possible that the presentation of PLE in rainbow trout differs to that in mammals. Capillary hydraulic and oncotic pressures are considerably lower in fish and their capillary membranes are relatively permeable to protein (Evans & Claiborne 2006). Additionally, the role of albumin in the maintenance of the plasma oncotic pressure is not as important as it is in mammals (Evans & Claiborne 2006). It still has some influence, however, as shown by relatively

small differences observed in circulating volume recovery between splenectomized fish that were injected with plasma or saline solution (Olson *et al.* 2003). Transcapillary fluid transfer is also fast in rainbow trout, which can replace their entire plasma volume with interstitial fluid approximately half an hour post-haemorrhage (Olson *et al.* 2003; Duff & Olson 1989). Additionally, rainbow trout living in fresh water regulate their plasma at a higher osmolality than their environment (Evans & Claiborne 2006). Disruption of the intestinal mucosal osmotic barrier would result in fast fluid transfer from the digestive lumen into the fish. In RTGE+ fish, this fluid uptake may occur at the same time as a fast transfer of fluid from the vascular to the interstitial compartment, triggered by the increase in hydrostatic pressure together with a reduction in the oncotic pressure within the capillaries. The differences in hydrostatic pressure between the interstitial and the vascular spaces (Evans & Claiborne 2006) will result in fluid retention in the former and haemoconcentration in the latter. It is possible that the balance could be quickly restored, but the gross presentation of moribund fish with RTGE suggests these fish are drinking, and therefore there is a constant influx of hypotonic fluid into RTGE+ fish. This hypothesis is supported by the presence of a swollen but histopathologically normal stomach containing a clear fluid. It is possible that this behaviour is triggered by the renin-angiotensin system (Fuentes & Eddy 1998), stimulated by the sudden decrease in blood volume and blood pressure resulting from the haemoconcentration, although perhaps there are other mechanisms involved. The concurrent hyponatraemia and hypochloraemia reflect the electrolyte loss through the intestine and the excessive intake of hypotonic water. It is also

possible that the apparent normokalaemia is in reality a hypokalaemia masked by the haemoconcentration (Stockham & Scott 2002).

The pathogenesis model proposed in this study would also help to explain the apparent reduction of RTGE mortalities observed during the treatment with in-feed salt (Chapter 3). An increase in the intake of electrolytes would contribute to increase the osmolality of the intestinal lumen, therefore restoring the Na^+/Cl^- balance within the vascular space and limiting the osmotically driven intake of hyposmotic fluid from the environment. As a result, this treatment effectively provides osmotic support to RTGE+ fish, as long as in-feed salt is ingested. The interstitial accumulation of fluid is consistent with the gross swollen appearance observed in RTGE+ fish. Despite this, there were no clear signs of the presence of ascites and it is possible that the secondary (lymphatic) circulation, which may be involved in regulation of the circulating volume (Olson 1996), is also involved. Also, the accumulation of fluid in the interstitial space may have caused cerebral oedema, with functional compromise of the central nervous system, resulting in the uncoordinated swimming observed by Michel et al (2002).

Bacterial enteritis in fish is not common (Lumsden 2006; Roberts 2001), but RTGE may prove to be a model suitable to study the pathophysiology of these mucosal changes. Further research will be necessary to uncover the reasons for the initial epithelial detachment and the physiological consequences of the severe osmotic imbalance that ensues.

5.6. Acknowledgements

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5.7. References

Ackerman, P. A. & Iwama, G. W. 2001, 'Physiological and Cellular Stress Responses of Juvenile Rainbow Trout to Vibriosis'. *Journal of Aquatic Animal Health*, vol. 13, pp. 173-180.

Austin B. & Austin D.A. 2007, *Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish*. Fourth Ed., Springer-Praxis, Chichester.

AVMA 2001, '2000 Report of the AVMA Panel on Euthanasia'. *Journal of the American Veterinary Medical Association*, vol. 218, pp. 669-696.

Bowser, P. R. 1993. 'Clinical pathology of salmonid fishes'. In: *Fish medicine*, M. K. Stoskopf, (Ed.), Saunders W.B., London, UK, pp. 327-332.

Branson, E. 2003, 'Rainbow trout Gastro-Enteritis (RTGE)-first diagnosis in the UK'. *Fish Veterinary Journal*, vol. 7, pp. 71-76.

Buller, N. B. 2004, *Bacteria from fish and other aquatic animals: A practical identification manual*. CABI publishing, Oxford, UK.

Byrne, P. J., Ostland, V. E., Lumsden, J. S., MacPhee, D. D. & Ferguson, H. W. 1995, 'Blood chemistry and acid-base balance in rainbow trout *Oncorhynchus mykiss* with experimentally-induced acute bacterial gill disease'. *Fish Physiology and Biochemistry*, vol. 14, pp. 509-518.

Clifton-Hadley, R. S., Richards, R. H. & Bucke, D. 1987, 'Further consideration of the haematology of proliferative kidney disease (PKD) in rainbow trout, *Salmo gairdneri* Richardson'. *Journal of Fish Diseases*, vol. 10, pp. 435-444.

Denham, K. 2004, 'Fish health controls: The activities of the fish health inspectorate in England and Wales'. *Trout News*, vol. 38, pp. 29-31.

Dohoo, I., Martin, W. & Stryhn, H. 2003, *Veterinary Epidemiological Research*. 1st Ed., AVC Publishing, Charlottetown, Prince Edward Island, Canada.

Duff, D. W. & Olson, K. R. 1989, 'Response of rainbow trout to constant-pressure and constant-volume hemorrhage'. *The American Journal of Physiology*, vol. 257, pp. 1307-1314.

Evans, D. H. & Claiborne, J. B. 2006, *The physiology of fishes*. 3rd Ed., Taylor & Francis, New York.

Ferguson, H. W. 2006, *Systemic pathology of fish: A text and atlas of normal tissues in teleosts and their responses in disease*. Second Ed., Scotian Press, London, UK.

Fuentes, J. & Eddy, F. B. 1998, 'Cardiovascular responses in vivo to angiotensin II and the peptide antagonist saralasin in rainbow trout *Oncorhynchus mykiss*'. *Journal of Experimental Biology*, vol. 201, pp. 267-272.

Hill, T. & Lewicki, P. 2007, *STATISTICS Methods and Applications*. Statsoft, Tulsa, Oklahoma, USA.

Lumsden, J. S. 2006. 'Gastrointestinal tract, swimbladder, pancreas and peritoneum'. In: *Systemic pathology of fish: A text and atlas of normal tissues in teleosts and their responses in disease*, Second edn, H. W. Ferguson, (Ed.), Scotian Press, London, UK, pp. 169-199.

Michel, C., Antonio, D. & Hedrick, R. P. 1999, 'Production of viable cultures of *Flavobacterium psycrophilum*: approach and control'. *Research in Microbiology*, vol. 150, pp. 351-358.

Michel, C., Bernardet, J. F., Daniel, P., Chilmonczyk, S., Urdaci, M. C. & de Kinkelin, P. 2002, 'Clinical and aetiological aspects of a summer enteritic syndrome associated with the sporulating segmented filamentous bacterium "*Candidatus* arthromitus" in farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum)'. *Journal of Fish Diseases*, vol. 25, pp. 533-543.

Olson, K. R. 1996, 'Secondary circulation in fish: Anatomical organization and physiological significance'. *Journal of Experimental Zoology part A: Comparartive Experimental Biology*, vol. 275, pp. 172-185.

Olson, K. R., Kinney, D. W., Dombkowski, R. A. & Duff, D. W. 2003, 'Transvascular and intravascular fluid transport in the rainbow trout: revisiting Starling's forces, the secondary circulation and interstitial compliance'. *Journal of Experimental Biology*, vol. 206, pp. 457-467.

Powell, M. D. 2006. 'Physiological and clinical pathology'. In: *Systemic pathology of fish: A text and atlas of normal tissues in teleosts and their responses in disease*, Second edn, H. W. Ferguson, (Ed.), Scotian Press, London, UK, pp. 336-355.

Powell, M. D., Speare, D. J. & Becker, J. A. 2006, 'Whole body net ion fluxes, plasma electrolyte concentrations and haematology during a *Loma salmonae*

infection in juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum)'. *Journal of Fish Diseases*, vol. 29, pp. 727-735.

Rehulka, J. & Minarik, B. 2007, 'Blood parameters in brook trout *Salvelinus fontinalis* (Mitchill, 1815), affected by columnaris disease'. *Aquaculture Research*, vol. 38, pp. 1182-1197.

Roberts, R. J. 2001, *Fish pathology*. Third Ed., W.B. Saunders, Edinburgh, UK.

Sanz, F. 2000, 'La gastroenteritis de la trucha (Language: Spanish)'. *Trouw Informa*, vol. Spring, pp. 16-18.

Stockham, S. L. & Scott, M. A. 2002, *Veterinary clinical pathology*. First Ed., Wiley Blackwell, Iowa, USA.

Stoskopf, M. K. 1993, *Fish medicine*. First Ed., W.B. Saunders, Philadelphia, USA.

Thrusfield, M. V. 2006, *Veterinary Epidemiology*. Third Ed., Blackwell Publishing, Oxford.

Toranzo, A. E. 2004. 'Report about bacterial diseases'. In: *Mediterranean Aquaculture Laboratories*, P. Alvarez-Pellitero et al., (Eds.), CIHEAM/FAO, pp. 49-89.

Urdaci, M. C., Regnault, B. & Grimont, P. A. D. 2001, 'Identification by in situ hybridization of segmented filamentous bacteria in the intestine of diarrheic rainbow trout (*Oncorhynchus mykiss*)'. *Research in Microbiology*, vol. 152, pp. 67-73.

CHAPTER 6. A Comparative Molecular Study of the Presence of "*Candidatus* arthromitus" in the Digestive System of Healthy Rainbow Trout *Oncorhynchus mykiss* (Walbaum) Affected with Rainbow Trout Gastroenteritis (RTGE)

Del-Pozo, Jorge*; Turnbull James; Ferguson, Hugh; Crumlish, Margaret

6.1. Abstract

Observations were made using histopathological techniques in conjunction with a nested PCR protocol for the specific detection of "*Candidatus* arthromitus"on DNA extracted from formalin-fixed paraffin wax-embedded tissues. Samples positive for "*C.* arthromitus" DNA included fish with rainbow trout gastroenteritis (RTGE), clinically normal co-habiting fish, and apparently healthy controls from both RTGE positive and RTGE negative sites. The results obtained from the PCR were confirmed by nucleotide sequencing. "*Candidatus* arthromitus" DNA was found in distal intestine as well as in sections of pyloric caeca, suggesting that both these locations are appropriate for molecular detection of "*C.* arthromitus" DNA in trout. Furthermore, rainbow trout fry distal intestinal samples from two different hatcheries where RTGE had not been reported were also positive for "*C.* arthromitus" DNA. Differences in "*C.* arthromitus" DNA detection between paraffin wax-embedded and fresh digestive content samples from the same fish suggested that these may be predominantly epithelium-associated in healthy trout. Parallel histopathological observations indicated that pyloric caeca are the preferred site for visualizing segmented filamentous

bacteria (SFB) in trout with RTGE. The results of this study showed that the presence of SFB was not invariably associated with clinical disease and that more information is required to understand the role of these organisms.

6.2. Introduction

Fish enteritides are part of a systemic presentation in most cases (Austin B. & Austin D.A. 2007; Ferguson 2006; Weber 2005; Roberts 2001). This is not the case with rainbow trout gastroenteritis (RTGE), which is an exclusively enteritic syndrome of rainbow trout *Oncorhynchus mykiss* (Walbaum), reported in several European countries (Denham 2004; Toranzo 2004; Ghittino, personal comm.; Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001; Sanz 2000). Clinically, fish present with congestion and oedema of the intestinal wall (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). The presence of RTGE in a rainbow trout producing site usually results in significant economic loss for affected sites, and daily mortalities of 0.5-1.0% are common (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001).

RTGE has been linked to the accumulation of large numbers of segmented filamentous bacteria (SFB) within the digestive tract of enteritic rainbow trout (Michel *et al.* 2002; Urdaci *et al.* 2001), a phenomenon that has not been reported in healthy trout. These SFB are described as Gram-positive bacteria 0.6-1.2µm in diameter and up to 60µm long, with apparent segmentation every 1.2-1.6µm. In the longest filaments, vegetative segments can be observed often containing spores, which are readily stained with malachite green (Michel *et al.* 2002; Urdaci *et al.* 2001). A comparative study of 16S rRNA gene sequences has shown that SFB found in the ileum of mice (Klaasen *et al.* 1991)

are closely related to the genus *Clostridium* (Snel *et al.* 1994). These bacteria have been included in a separate group called "*Candidatus* arthromitus", which includes SFB found in the digestive system of several species (Snel *et al.* 1995). The most recent addition to this group has been the SFB in trout (Urdaci *et al.* 2001).

At present, the role of "*C.* arthromitus" in the aetiology of RTGE is not clear: Urdaci et al. (2001) suggested that the presence of other pathogens cannot be excluded although in their study SBF were always observed in enteritic trout. An additional possibility is that SFB are part of the natural microbial flora of rainbow trout, but have not previously been detected.

It is largely accepted that "*C.* arthromitus" cannot be cultured *in vitro* (Angert 2005; Klaasen *et al.* 1992; Davis & Savage 1974) making it impossible to study SFB using routine bacteriology methods. Molecular techniques have been proposed as a way to identify fastidious and non-culturable micro-organisms (Fenollar & Raoult 2004) and have been successfully used for several fish pathogens (Fouz *et al.* 2006; Chang *et al.* 2002; Toyama *et al.* 1994). The use of molecular techniques using DNA extracted from paraffin wax-embedded tissues has made possible the use of archive material and to relate the molecular analysis to specific histopathological lesions in fish (Crumlish *et al.* 2007). The purpose of the present study was to use DNA extracted from fresh intestinal contents and paraffin wax-embedded tissue samples for the specific detection of "*C.* arthromitus" 16S rRNA gene using a nested polymerase chain reaction (PCR). The ultimate objective of the research was to describe and compare the presence of "*C.* arthromitus" in the pyloric caeca and distal intestine of fish clinically affected by RTGE, as well as in clinically normal fish, in

an attempt to clarify the role of "*C.* arthromitus" in the aetiology of this syndrome.

6.3. Material and methods

6.3.1. Experimental design

6.3.1.1. Primary experimental groups

Four groups of fish samples from two UK rainbow trout farms both positive (Site A) and negative (Site B) for the presence of RTGE were used (Table 6.1). These four groups comprised: (a) fish displaying RTGE signs, (b) apparently healthy cohabiting fish, (c) negative controls from RTGE-free units of site A and (d) negative controls from site B. Molecular analyses were performed on both paraffin wax-embedded tissues and fresh contents of distal intestine and pyloric caeca. There were six fish per experimental group and all the samples were also processed for histology.

Table 6.1. Experimental groups analyzed histopathologically and with nested PCR for specific detection of "*Candidatus* arthromitus".

Fish id	*Group*	*Site*	*Site RTGE status*	*Production unit RTGE status*	*Fish RTGE status*
1 to 6	a	A	**POSITIVE**	**POSITIVE**	**POSITIVE**
7 to 12	b	A	**POSITIVE**	**POSITIVE**	**NEGATIVE**
13 to 18	c	A	**POSITIVE**	**NEGATIVE**	**NEGATIVE**
19 to 24	d	B	**NEGATIVE**	**NEGATIVE**	**NEGATIVE**

6.3.1.2. Additional experimental groups

In order to complement the primary findings of the main experimental design, five more experimental groups were included in the study (Table 6.2): (1) fry from a UK hatchery (Hatchery A) that supplied fish to RTGE-positive sites, (2) fry from a UK hatchery (Hatchery B) that supplied fish to sites in which RTGE

had never been reported, (3) fry from Northern Ireland (Hatchery C) and (4) ongrowing rainbow trout from Northern Ireland (Site C). For these additional groups, histopathological and molecular analyses were performed in paraffin wax-embedded samples of distal intestine only. Clinical RTGE had never been reported in any of the rainbow trout producing sites included in groups 1 to 4.

Table 6.2. Additional experimental groups analyzed histopathologically and with "*Candidatus* arthromitus" specific nested PCR.

Fish id	*Group*	*Site*	*Site RTGE status*	*Fish RTGE status*
25 to 30	1	Hatchery A	**NEGATIVE**	**NEGATIVE**
31 to 36	2	Hatchery B	**NEGATIVE**	**NEGATIVE**
37 to 42	3	Hatchery C	**NEGATIVE**	**NEGATIVE**
43 to 46	4	Site C	**NEGATIVE**	**NEGATIVE**

6.3.2. Fish sampling and tissue fixation

All the fish were killed using an overdose of benzocaine (Sigma-Aldrich™, Gillinham, UK) at 250mg/l (AVMA 2001). Distal intestine and pyloric caeca were sampled separately and sequentially. Fresh digestive contents were collected in sterile cryovials by sterile sectioning of each digestive area distal to the collection point. Using a different set of sterile forceps for each sample, pressure was exerted in the proximal area to transfer the contents into the cryovial, which was then snap-frozen in liquid nitrogen. Following this, the tissues sampled for histology were directly placed in 10% (v/v) neutral buffered formalin after sterile dissection. No incision was made in the digestive tract before fixation and tissue samples were kept in the formalin fixative for at least 24 h before further processing. Fixed tissues were then trimmed and placed in plastic cassettes in an automatic tissue processor, according to the

manufacturer's protocol (Citadel™ Tissue processor, Pennsylvania, USA). After processing, cassettes were removed from the tissue processor and embedded in paraffin wax. The excess wax surrounding the embedded tissue was trimmed away to maximize the proportion of tissue-to-wax in each block, which then was cut using a manual rotary microtome.

6.3.3. DNA extraction

Fresh digestive contents were processed for DNA extraction with a DNEasy™ kit (Qiagen™, Crawley, UK) following the manufacturer's protocol. For paraffin wax-embedded tissues the protocol described by Crumlish *et al* (2007) was applied with modifications, whereby each paraffin wax-embedded tissue sample was cut at 1µm of thickness to further improve the extraction of bacterial DNA (Loeschke *et al.* 2005).

6.3.4. Nested PCR procedure

A Buffer IV PCR kit was used for the reaction (ABGENE™, Epsom, UK). PCR reactions were assembled in thin-walled 0.2ml PCR tubes and the reaction volume (50 µL) contained 500ng of template DNA for the first step, 10 mM of each primer, 2.5 mM of each dNTP, 5 µL of 10× PCR reaction buffer (ABGENE), 1.5 mM $MgCl_2$, 2.5 U of Taq DNA polymerase (ABGENE) and miliQ water up to 50µl. The tubes were placed in a thermocycler (Biometra T gradient™, Goettingen, Germany) and subjected, for the first step, to an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1 min 30 s, extension for 72°C for 1 min 30 s and a single extension cycle of 72°C for 5 min. For the second PCR reaction all the parameters were identical, except only 1µl of the first round PCR product was added and the thermocycler programme was modified for annealing (58°C for 1

min) and extension (72°C for 1 min). Two sets of primers targeted to 16S rRNA were used in the two steps of the nested PCR (MWG Oligo™, London, UK). For the first step universal primers, complementary to conserved regions of most eubacterial 16S rRNA, were used: 20F (5'-AGAGTTTGATCATGGCTCAG-3') and 1500R (5'GGTTACCTTGTTACGACTT-3') (Weisburg *et al.* 1991). The second step of the nested PCR was performed using primers SFB779F (5'-TGTGGGTTGTGAATAACAAT-3') and SFB1380R (5'-GGTTAGCCCACAGGTTTCGG-3'), specific for "*C.* arthromitus" (Urdaci *et al.* 2001). Negative controls with no DNA were used for each reaction and the results were visualised using a 1 % (w/v) tris acetate buffer (TAE) agarose gel with 0.5 µg/ml ethidium bromide run in an electrophoresis well set at 80V. A Trackit™ 100bp ladder (Invitrogen™, Paisley, UK) was used for reference.

6.3.5. DNA sequencing procedure

Nucleotide sequencing was used to identify the final amplification products of the nested PCR technique. For the primary experimental groups, the samples sequenced included two randomly chosen amplification products from each group of paraffin wax-embedded tissues (16 samples) and all the positive samples from the fresh intestinal content samples. For the additional experimental groups all the positive samples were sequenced. Each sample was purified using a Qiaquick™ DNA kit (Qiagen™, Crawley, UK) following the manufacturer's protocol and sequenced using a CEQ Beckman 2000™ sequencer following the manufacturer protocol (Beckman Coulter™, High Wycombe, UK). All the sequences obtained were contrasted with other 16S rRNA sequences in the EMBL database by BLAST analysis (Altschul *et al.* 1997).

6.3.6. Histology processing

Two 5µm thick paraffin wax-embedded sections of each sample were stained using both haematoxylin and eosin and Gram stains. Each section was then examined using light microscopy for the presence or absence of autolytic changes in the tissue and compatibility of any pathological changes observed in the section with the current histopathological description of RTGE (Michel *et al.* 2002). Also, the whole section was screened at x20 for the presence of Gram-positive filamentous bacteria and, if present, a description of these bacteria was made. This included segmented appearance, Gram variability and interaction/association with enterocytes. The bacteria were considered consistent with SFB if they were approximately 0.6-1.2µm in diameter and up to 60µm long, with apparent segmentation every 1.2-1.6µm (Urdaci *et al.* 2001).

6.4. Results

6.4.1. Molecular detection of "*C.* arthromitus"

All of the samples from all the experimental groups were positive for the presence of eubacterial DNA, as shown by the presence of a 1480bp amplification product in the primary PCR (data not presented). No amplification products were observed in any of the negative control samples (no template DNA).

6.4.1.1. Primary experimental groups

The results of the second step of the nested PCR for paraffin wax-embedded tissues are displayed in Figure 6.1, where a PCR product at the expected weight band of 601bp can be observed for most samples from all fish groups (A to D) in both distal intestine and pyloric caeca, with the exception of two distal

intestine samples from RTGE-positive fish (lanes 5 and 6) and one pyloric caeca sample from an apparently healthy individual cohabiting with RTGE-positive fish (lane 36).

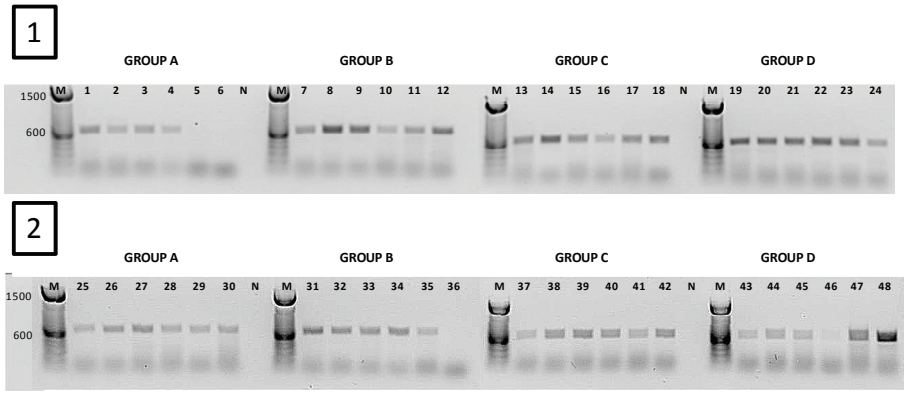

Figure 6.1. PCR products from paraffin wax-embedded tissues obtained with a "*Candidatus* arthromitus"-specific nested PCR. Group A: RTGE-affected fish; Group B: Apparently healthy fish cohabiting with affected; Group C: Fish from an apparently healthy unit in an affected site; Group D: healthy fish from a random unit in an unaffected site. (n=6 samples per group; gel 1: distal intestine samples; gel 2: pyloric caeca samples; M: marker; N: negative control).

A different detection pattern was observed after the second PCR step on fresh digestive contents (Figure 6.2), where RTGE+ fish (group A) were positive in all cases for both distal intestine and pyloric caeca. Three samples from cohabiting fish were positive for both organs (lanes 7, 11, 12, 31, 35 and 36) and only one pyloric caeca sample from site B was positive (lane 45).

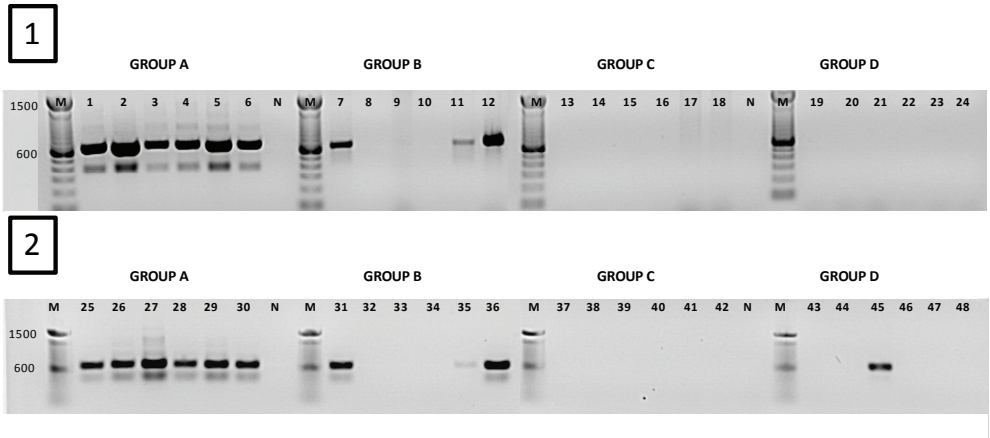

Figure 6.2. PCR products from fresh digestive contents obtained with a "*Candidatus* arthromitus"-specific nested PCR. Group A: RTGE-affected fish; Group B: Apparently healthy fish cohabiting with affected; Group C: Fish from an apparently healthy unit in an affected site; Group D: healthy fish from a random unit in an unaffected site. (n=6 samples per group; gel 1: distal intestine samples; gel 2: pyloric caeca samples; M: marker; N: negative control).

6.4.1.2. Additional sample groups

In the case of groups 1 to 5, only two fish from group 1 (hatchery A; lanes 51 and 54) and one fish from group 2 (hatchery B; lane 55) were positive in the PCR test (Figure 6.3). Sample 52 produced a non-specific amplification product, which did not appear in two subsequent repetitions of the analysis using the same sample and was therefore not considered when interpreting the results. All the rainbow trout from Northern Ireland were negative for "*C.* arthromitus" DNA (Data not shown).

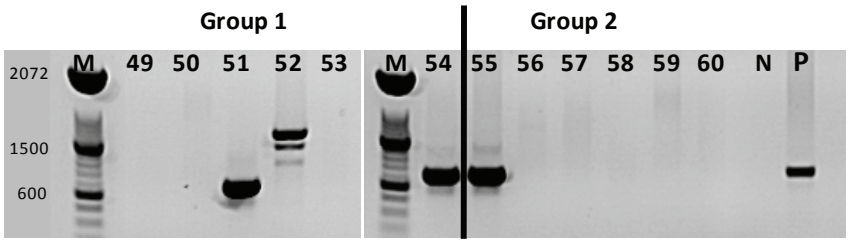

Figure 6.3. PCR products from paraffin wax-embedded distal intestine obtained with a "*Candidatus* arthromitus"-specific nested PCR. Group 1: hatchery A; group 2: Hatchery B. M: marker; N-: no template DNA; P: positive control.

The BLAST search of all the sequences obtained from the amplification products of this study showed 98 to 100% consistency with the published sequence of trout SFB with EMBL accession number AY007720 (Urdaci *et al.* 2001).

6.4.2. Histopathology

Gram-variable SFB were observed in distal intestine and pyloric caeca sections of both RTGE-affected and apparently healthy trout. These bacteria were not observed in any of the distal intestine samples of any fish with RTGE and evidence of severe tissue lysis was observed in these sections. This was not the case with pyloric caeca from the same fish (Figure 6.4 A), where autolytic changes were less pronounced and Gram variable segmented filamentous bacteria were always present in large numbers both attached to the mucosal

layer of the intestine as well as apparently free within the lumen (Figure 6.4 A, B).

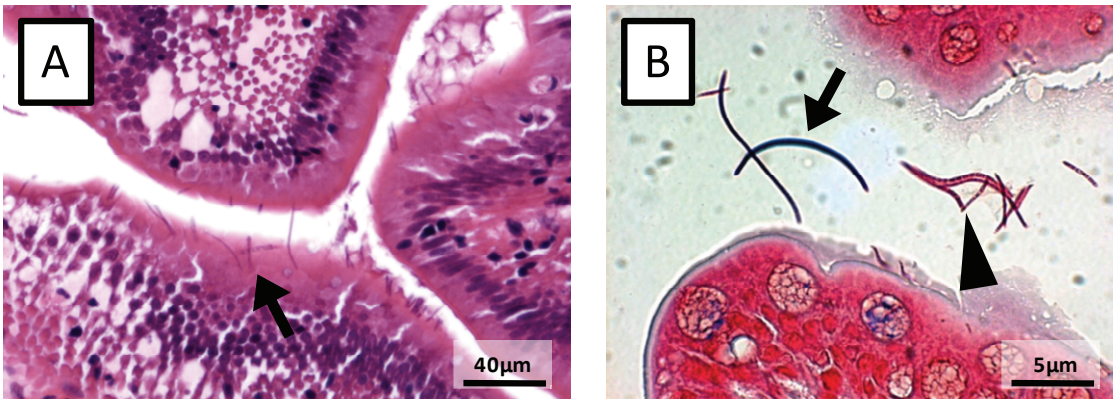

Figure 6.4. Images of SFB in the pyloric caeca of RTGE-affected trout. A: SFB (arrow) closely associated with enterocytes (H&E; x20). B: SFB were clearly segmented (arrowhead) and displayed Gram-variability (Gram counterstained with Neutral Red; x100) and could present positive (arrow) or negative (arrowhead) staining. These sections were positive in "*Candidatus* arthromitus"-specific PCR.

Regarding the presence of SFB within the digestive system of apparently healthy fish, these were observed in the distal intestine of two fish cohabiting with RTGE-affected individuals and one fry from hatchery A (Figure 6.5). In this case, SFB were never observed interacting closely with the enterocytes, although close interaction with feed particles was observed in several occasions. SFB were not present in large numbers in any of these samples (Figure 6.5).

When contrasting the results of the histopathology and the "*C.* arthromitus"-specific PCR, it was found that all sections presenting SFB were also positive in the PCR test. Gram-positive filamentous bacteria were observed in several tissue sections that were negative in PCR for "*C.* arthromitus" DNA. None of these bacteria were Gram-variable or segmented.

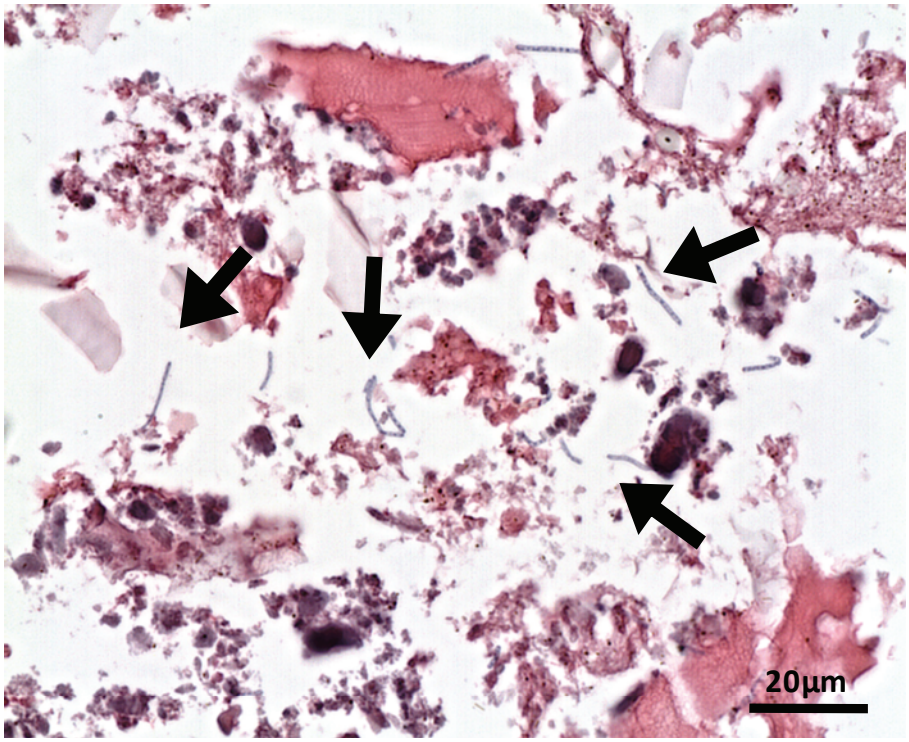

Figure 6.5. Distal intestine of apparently healthy rainbow trout with SFB presence (arrows; H&E; x20). This section was positive in "*Candidatus* arthromitus" specific PCR.

6.5. Discussion

This study has demonstrated the presence of "*C.* arthromitus" DNA in both RTGE positive samples and negative controls in formalin-fixed wax-embedded tissues and frozen digestive contents from rainbow trout digestive system. "*Candidatus* arthromitus" DNA was found in both distal intestine and pyloric caeca, suggesting that samples from both locations are equally appropriate for determining the presence of SFB using molecular microbiological techniques. Histologically, SFB were present in large numbers within pyloric caeca of RTGE-affected fish, although they were not observed in distal intestine sections even in the same fish, despite being detected by PCR in all but two occasions. The inconsistency of the results between paraffin wax-embedded and fresh intestinal samples in this case may have been due to the limitations of the

technique used for DNA extraction in wax-embedded tissues. This technique targeted a very specific section of the intestine (approximately 100µm in total thickness) which in conjunction with an irregular mucosal colonisation by SFB and DNA degradation due to autolytic changes, may have led to false negative results (Klaasen *et al.* 1993b; Davis & Savage 1974).

In addition, caution is recommended in the interpretation of negative results in PCR performed on DNA extracted from formalin-fixed paraffin-wax embedded tissues, due to the harshness of the procedures required for this technique (Cataloluk *et al.* 2003; Srinivasan *et al.* 2002). Nevertheless, the technique used in this study did provide positive results, which were confirmed by sequencing and suggested that the PCR technique used here was able to detect "*C.* arthromitus" DNA in paraffin wax-embedded sections.

Attachment of SFB to the enteric mucosa was observed histologically in enteritic trout, although evident differences between mucosal areas with and without SFB were not noted. A preference of SFB for mucosal lymphoid epithelium has been described in rodents (Jepson *et al.* 1993; Abrams 1977) and horses (Lowden & Heath 1995). In both species SFB are strongly anchored to the epithelial cells of Peyer's patches, as well as to the small intestinal epithelium. This is not a possibility in rainbow trout, where a structure comparable to mammalian Peyer patches has not been identified, although trout do have an intraepithelial lymphoid population (Bernard *et al.* 2006; McMillan & Secombes 1997).

More relevant to the aetiological role of "*C.* arthromitus" in the development of RTGE is the fact that SFB DNA was found in the digestive system of trout with

clinical RTGE and in apparently healthy fish. In addition, bacteria consistent with SFB were observed histologically within the digestive system of apparently healthy trout. No previously published reports are available on the histological observation of SFB in healthy rainbow trout, and their presence has been consistently associated only with fish affected with RTGE (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). The present observations do suggest, however, that SFBs can also be found in apparently healthy fish, albeit more infrequently and in lower numbers than in fish affected with RTGE. Additionally, the differences in SFB detection between fresh digestive contents and wax-embedded tissues from the same fish suggest that SFB could be mostly epithelium associated in healthy trout, with sporadic presence in the digestive lumen. In RTGE-affected fish the apparent increase in SFB numbers (Michel *et al.* 2002) would result in a consistent presence of these organisms floating freely in the digestive tract lumen. A higher frequency of SFB presence in the digestive contents of healthy cohabiting fish, as opposed to healthy fish from other sites, suggests that the load of SFB is increased within the affected units, although it is also possible that these fish were subclinically affected with RTGE.

Several factors may have contributed to the absence of histological observation of SFB in healthy rainbow trout, including the location, degradation and the presentation of these bacteria. Several authors have observed that the inability to detect mucosa-associated SFBs in any species is linked to the intestine being only poorly or irregularly colonized by these organisms (Klaasen *et al.* 1993b; Davis & Savage 1974). Davis (1980) noted that if sampling of SFB-positive animals takes place more than 3h post-mortem, SFBs may not be

detected, possibly due to destruction of SFB attachment sites by autolytic enzymes. Additionally, the filamentous structure of the bacteria decays quickly after suspension in saline and even when the samples are refrigerated. Structural degradation of trout SFB occurs within a few hours (Michel *et al.* 2002), a length of time that may well be reduced if the intestine is not incised to allow rapid fixation. It is also possible that trout SFB could also be present in another form in healthy trout. For example, *Bacillus cereus* normally presents a flagellated rod shape but also has an "arthromitus" stage within the digestive system of healthy arthropods (Margulis *et al.* 1998). These organisms are also spore forming (Angert 2005) and could have been present as spores. It is therefore possible that SFB have gone undetected in clinically healthy rainbow trout and although SFB could still play a role in the aetiology of RTGE, this would not involve a straightforward infection that conforms easily to Koch's postulates. These observations are compatible with two possibilities: a) that SFB are a previously undetected part of the native intestinal microflora of rainbow trout or b) that SFB are endemic in specific trout populations.

Looking at the first of these, it is possible that "*C.* arthromitus" are part of the microflora of normal rainbow trout, as this group of bacteria is considered by some authors to be ubiquitous in the animal kingdom (Klaasen *et al.* 1993a). A lack of SFB pure culture methodologies (Angert 2005) may have contributed to their non-detection. However other methodologies are possible, and light microscopy analysis has been useful for the detection of SFB presence within the intestine of healthy carp (Klaasen *et al.* 1993a). Several reports have indicated a significant variability of rainbow trout intestinal microflora and have suggested that external factors such as feeding, season and antibiotic

administration have an effect on stability of the intestinal microflora even at the individual level (Huber *et al.* 2004; Spanggaard *et al.* 2000). More recently, a study demonstrated that the microflora are more stable when fish are kept in constant conditions, but this would hardly be the case in rainbow trout production sites, which are regularly subjected to sometimes quite pronounced environmental changes (Pond *et al.* 2006).

The other possibility is the existence of rainbow trout populations where SFB are endemic and this position is consistent with the negative PCR results observed in rainbow trout from Northern Irish sites. However, the number of individuals included in the study was not statistically sufficient to declare freedom from SFB presence. The endemic status of naive stocks would be reached after contact with infected trout or infective material and then would be maintained through horizontal transmission. It is likely that spores are essential for the horizontal transmission of SFB as observed in mice and chicken (Ali & Reynolds 1996; Klaasen *et al.* 1991), as "*C.* arthromitus" are considered to be unculturable and species-specific suggesting a limited adaptability to environmental conditions (Angert 2005; Allen 1992; Tannock *et al.* 1984). If this is the case, the spore survival time should be long enough to ensure the presence of receptive individuals within the population. If SFB are endemic, they could either be present in all the individuals of the population or exclusively in apparently healthy carrier individuals, where they would be able to colonize and multiply. In this case carrier trout may release infectious material either continuously or sporadically which would not infect other trout in the population until favourable conditions for the bacteria were met.

A consistent increase in the number of SFB has been previously reported in RTGE-affected fish (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). This was confirmed by the results of this study, although SFB were observable only in the pyloric caeca of these fish. This increase could occur immediately before or during the course of RTGE and it could be a result of exposure to an as yet unknown trigger. This could be either external to the fish (*e.g.* diet, treatment, environmental conditions, etc.) or arise from within the fish (*e.g.* presence of a different infectious agent, changes in digestive motility, immunosuppressed status, etc.). Ultimately the increased SFB presence may play or not a significant role in the aetiology of RTGE but would certainly facilitate histological detection.

Factors affecting SFB numbers have been studied in mice and an increase in SFB numbers has been linked to low IgA concentrations (Suzuki *et al.* 2004). Decrease in numbers was associated with age, activation of the mucosal immune system and administration of *Lactobacillus plantarum* to immunodepressed mice (Fuentes *et al.* 2008; Snel *et al.* 1998). Goodwin *et al.* (1991) suggested that diet composition, environmental stress and antimicrobial drugs also play a role in SFB colonization.

As suggested by previous work, an increased number of SFB in RTGE-affected trout is not necessarily linked with the aetiology of RTGE (Michel *et al.* 2002; Urdaci *et al.* 2001). In other animal species, SFB have been reported as commensal as well as being associated with various enteritides. In poultry, SFB have been associated with inflammation of intestinal mucosa and vacuolation of enterocytes (Goodwin *et al.* 1989). However, it was concluded at a later date that SFB are not necessarily pathogens and in fact they might be

part of the normal intestinal microflora of poultry (Goodwin *et al.* 1991). SFB were initially associated with the aetiology of stunting syndrome in turkey poults (Angel *et al.* 1990), an infectious enteric disorder that was subsequently shown to be caused by virus (Ali & Reynolds 1997; Sell *et al.* 1992). The proliferation of SFB was observed only in turkey poults with clinical signs of diarrhoea, but not in the intestine of symptom-free birds, a situation similar to RTGE (Michel *et al.* 2002; Urdaci *et al.* 2001).

A positive effect of SFB on their host has been recognized in mammals. These organisms are considered to be non-pathogenic potent microbial stimuli of the murine gut mucosal immune system and to competitively exclude pathogens from the murine small bowel (Umesaki *et al.* 1999; Talham *et al.* 1999; Umesaki *et al.* 1995; Klaasen *et al.* 1993b; Garland *et al.* 1982). All of these positions are compatible with SFB being a previously unrecognised non-pathogenic part of the normal microflora of healthy rainbow trout intestine. Nevertheless, it is not possible to exclude a direct or indirect involvement of SFB in a complex pathogenesis picture with the evidence available. Increased concentrations of a hypothetical SFB toxin could play an important role, perhaps even being the cause of the disease itself. The possible involvement of a toxin in RTGE pathogenesis is not a new concept (Michel *et al.* 2002), and the phylogenetic proximity of "*C.* arthromitus" to the *Clostridium* genus of bacteria, which comprises several toxin-producing species, would make this hypothesis feasible (Wilson *et al.* 2002; Snel *et al.* 1994). If this was true and SBF are directly or indirectly related to RTGE pathogenesis, only trout with SFB in their digestive system would be susceptible to developing RTGE.

Concluding, this study has reported the presence of "*C.* arthromitus" DNA in apparently healthy and RTGE-affected rainbow trout using molecular probes specific for this bacterial species. It also has explored several hypotheses regarding the aetiological role of "*C.* arthromitus" in the pathogenesis of RTGE. Parallel histopathological observations have confirmed the presence of SFB in RTGE-affected and clinically healthy rainbow trout. Further work is required to clarify several of the points raised, namely quantitative studies of SFB in both sick and healthy rainbow trout, in situ studies of SFB morphology in apparently healthy individuals, SFB culture attempts and further research on the pathogenic mechanisms behind RTGE.

6.6. Acknowledgements

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6.7. References

Abrams, G. D. 1977, 'Microbial effects on mucosal structure and function'. *The American Journal of Clinical Nutrition*, vol. 30, pp. 1880-1886.

Ali, A. & Reynolds, D. L. 1996, 'Primary cell culture of turkey intestinal epithelial cells'. *Avian Diseases*, vol. 40, pp. 103-108.

Ali, A. & Reynolds, D. L. 1997, 'Stunting syndrome in turkey poults: isolation and identification of the etiologic agent'. *Avian Diseases*, vol. 41, pp. 870-881.

Allen, P. C. 1992, 'Comparative study of long, segmented, filamentous organisms in chickens and mice'. *Laboratory Animal Science*, vol. 42, pp. 542-547.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Dipman, D. L. 1997, 'Gapped BLAST and PSI-BLAST: A new generation of protein database search programs'. *Nucleic Acid Research*, vol. 25, pp. 3389-3402.

Angel, C. R., Sell, J. L., Fagerland, J. A., Reynolds, D. L. & Trampel, D. W. 1990, 'Long-segmented filamentous organisms observed in poults experimentally infected with stunting syndrome agent'. *Avian Diseases*, vol. 34, pp. 994-1001.

Angert, E. 2005, 'Alternatives to binary fission in bacteria'. *Nature Reviews: Microbiology*, vol. 3, pp. 214-224.

Austin B. & Austin D.A. 2007, *Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish*. Fourth Ed., Springer-Praxis, Chichester.

AVMA 2001, '2000 Report of the AVMA Panel on Euthanasia'. *Journal of the American Veterinary Medical Association*, vol. 218, pp. 669-696.

Bernard, D., Six, A., Rigottier-Gois, L., Messiaen, S., Chilmonczyk, S., Quillet, E., Boudinot, P. & Benmansour, A. 2006, 'Phenotypic and Functional Similarity of Gut Intraepithelial and Systemic T Cells in a Teleost Fish'. *The Journal of Immunology*, vol. 176, pp. 3942-3949.

Branson, E. 2003, 'Rainbow trout Gastro-Enteritis (RTGE)-first diagnosis in the UK'. *Fish Veterinary Journal*, vol. 7, pp. 71-76.

Cataloluk, O., Cakmak, E. A., Buyukberber, N. & Barlas, O. 2003, 'Formalin fixing and paraffin embedding may lead to extra band development in PCR'. *The new microbiologica : official journal of the Italian Society for Medical, Odontoiatric, and Clinical Microbiology (SIMMOC)*, vol. 26, pp. 193-198.

Chang, P. H., Lin, C. W. & Lee, Y. C. 2002, '*Lactococcus garvieae* infection of cultured rainbow trout, *Oncorhynchus mykiss* in Taiwan and associated biophysical characteristics and histopathology'. *Bulletin of the European Association of Fish Pathologists*, vol. 22, pp. 319-327.

Crumlish, M., Diab, A. M., George, S. & Ferguson, H. W. 2007, 'Detection of the bacterium *Flavobacterium psychrophilum* from a natural infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum), using formalin-fixed, wax-embedded fish tissues'. *Journal of Fish Diseases*, vol. 30, pp. 37-41.

Davis, C. P. 1980. 'Post-mortem alterations of bacterial localization'. In: *Scanning Electron Microscopy*, A. M. F. O'Hare, (Ed.), SEM inc., Chicago, pp. 523-526.

Davis, C. P. & Savage, D. C. 1974, 'Habitat, succession, attachment, and morphology of segmented, filamentous microbes indigenous to the murine gastrointestinal tract'. *Infection and Immunity*, vol. 10, pp. 948-956.

Denham, K. 2004, 'Fish health controls: The activities of the fish health inspectorate in England and Wales'. *Trout News*, vol. 38, pp. 29-31.

Fenollar, F. & Raoult, D. 2004, 'Molecular genetic methods for the diagnosis of fastidious microorganisms'. *APMIS*, vol. 112, pp. 785-807.

Ferguson, H. W. 2006, *Systemic pathology of fish: A text and atlas of normal tissues in teleosts and their responses in disease*. Second Ed., Scotian Press, London, UK.

Fouz, B., Zarza, C. & Amaro, C. 2006, 'First description of non-motile *Yersinia ruckeri* serovar I strains causing disease in rainbow trout, *Oncorhynchus mykiss* (Walbaum), cultured in Spain'. *Journal of Fish Diseases*, vol. 29, pp. 339-346.

Fuentes, S., Egert, M., Jimenez-Valera, M., Monteoliva-Sanchez, M., Ruiz-Bravo, A. & Smidt, H. 2008, 'A strain of *Lactobacillus plantarum* affects segmented filamentous bacteria in the intestine of immunosuppressed mice'. *FEMS Microbiology Ecology*, vol. 63, pp. 65-72.

Garland, C. D., Lee, A. & Dickson, M. R. 1982, 'Segmented filamentous bacteria in the rodent small intestine: Their colonization of growing animals and possible role in host resistance to *Salmonella*'. *Microbial Ecology*, vol. 8, pp. 181-190.

Goodwin, M. A., Cooper, G. L., Brown, J., Bickford, A. A., Waltman, W. D. & Dickson, T. G. 1991, 'Clinical, pathological, and epizootiological features of long-segmented filamentous organisms (bacteria, LSFOs) in the small intestines of chickens, turkeys, and quails.'. *Avian Diseases*, vol. 35, pp. 872-876.

Goodwin, M. A., Cooper, G. L. & Waltman, W. D. 1989, 'Diarrhea associated with long-segmented filamentous organisms (LSFO) in the small intestines of turkeys, chickens and quails'. In: *38th Western Poultry Disease Conference*, pp. 103-109.

Huber, I., Spanggaard, B., Appel, K. F., Rossen, L., Nielsen, T. & Gram, L. 2004, 'Phylogenetic analysis and in situ identification of the intestinal microbial community of rainbow trout (*Oncorhynchus mykiss*, Walbaum)'. *Journal of Applied Microbiology*, vol. 96, pp. 117-132.

Jepson, M. A., Clark, M. A., Simmons, N. L. & Hirst, B. H. 1993, 'Actin accumulation at sites of attachment of indigenous apathogenic segmented filamentous bacteria to mouse ileal epithelial cells.'. *Infection and Immunity*, vol. 61, pp. 4001-4004.

Klaasen, H. L. B. M., Koopman, J. P., Poelma, F. G. J. & Beynen, A. C. 1992, 'Intestinal, segmented, filamentous bacteria'. *FEMS Microbiology Reviews*, vol. 88, pp. 165-180.

Klaasen, H. L. B. M., Koopman, J. P., Van den Brink, M. E., Bakker, M. H., Poelma, F. G. J. & Beynen, A. C. 1993a, 'Intestinal, segmented, filamentous bacteria in a wide range of vertebrate species.'. *Laboratory Animals*, vol. 27, pp. 141-150.

Klaasen, H. L. B. M., Koopman, J. P., Van den Brink, M. E., Van Wezel, H. P. N. & Beynen, A. C. 1991, 'Mono-association of mice with non-cultivable, intestinal, segmented, filamentous bacteria.'. *Archives of Microbiology*, vol. 156, pp. 148-151.

Klaasen, H. L. B. M., Van der Heijden, P. J., Stok, W., Poelma, F. G. J., Koopman, J. P., Van den Brink, M. E., Bakker, M. H., Eling, W. M. C. & Beynen, A. C. 1993b, 'Apathogenic, intestinal, segmented, filamentous bacteria stimulate the mucosal immune system of mice'. *Infection and Immunity*, vol. 61, pp. 303-306.

Loeschke, S., Goldmann, T. & Vollmer, E. 2005, 'Improved detection of mycobacterial DNA by PCR in formalin-fixed, paraffin-embedded tissues using thin sections'. *Pathology, Research and Practice*, vol. 201, pp. 37-40.

Lowden, S. & Heath, T. 1995, 'Segmented filamentous bacteria associated with lymphoid tissues in the ileum of horses'. *Research in Veterinary Science*, vol. 59, pp. 272-274.

Margulis, L., Jorgensen, J. Z., Dolan, S., Kolchinsky, R., Rainey, F. A. & Lo, S.-C. 1998, 'The arthromitus stage of *Bacillus cereus*: Intestinal symbionts of animals'. *Proceedings of the National Academy of Sciences*, vol. 95, pp. 1236-1241.

McMillan, D. & Secombes, C. 1997, 'Isolation of rainbow trout (*Oncorhynchus mykiss*) intestinal intraepithelial lymphocytes (IEL) and measurement of their cytotoxic activity'. *Fish & Shellfish Immunology*, vol. 7, pp. 527-541.

Michel, C., Bernardet, J. F., Daniel, P., Chilmonczyk, S., Urdaci, M. C. & de Kinkelin, P. 2002, 'Clinical and aetiological aspects of a summer enteritic syndrome associated with the sporulating segmented filamentous bacterium "*Candidatus* arthromitus" in farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum)'. *Journal of Fish Diseases*, vol. 25, pp. 533-543.

Pond, M. J., Stone, D. M. & Alderman, D. J. 2006, 'Comparison of conventional and molecular techniques to investigate the intestinal microflora of rainbow trout (*Oncorhynchus mykiss*)'. *Aquaculture*, vol. 261, pp. 194-203.

Roberts, R. J. 2001, *Fish pathology*. Third Ed., W.B. Saunders, Edinburgh, UK.

Sanz, F. 2000, 'La gastroenteritis de la trucha (Language: Spanish)'. *Trouw Informa*, vol. Spring, pp. 16-18.

Sell, J. L., Reynolds, D. L. & Jeffrey, M. 1992, 'Evidence that bacteria are not causative agents of stunting syndrome in poults'. *Poultry Science*, vol. 71, pp. 1480-1485.

Snel, J., Blok, H. J., Kengen, M. P., Kudwig, W., Poelma, F. G. J., Koopman, J. P. & Akkermans, A. D. L. 1994, 'Phylogenetic Characterization of *Clostridium* Related Segmented Filamentous Bacteria in Mice Based on 16S Ribosomal RNA Analysis'. *Systematic and Applied Microbiology*, vol. 17, p. 172.

Snel, J., Heinen, P. P., Blok, R. J., Carman, R. J., Duncan, A. J., Allen, P. C. & Collins, M. D. 1995, 'Comparison of 16S rRNA sequences of segmented filamentous bacteria isolated from mice, rats, and chickens and proposal of "*Candidatus* arthromitus"'. *International Journal of Systematic Bacteriology*, vol. 45, pp. 780-782.

Snel, J., Hermsen, H. J., Smits, H. J., Bos, N. A., Eling, W. M. C., Cebra, J. J. & Heidt, P. J. 1998, 'Interactions between gut-associated lymphoid tissue and colonization levels of indigenous, segmented, filamentous bacteria in the small intestine of mice'. *Revue Canadienne de Microbiologie*, vol. 44, pp. 1177-1182.

Spanggaard, B., Huber, I., Nielsen, J., Nielsen, T., Appel, K. F. & Gram, L. 2000, 'The microflora of rainbow trout intestine: a comparison of traditional and molecular identification'. *Aquaculture*, vol. 182, pp. 1-15.

Srinivasan, M., Sedmak, D. & Jewell, S. 2002, 'Effect of Fixatives and Tissue Processing on the Content and Integrity of Nucleic Acids'. *American Journal of Pathology*, vol. 161, pp. 1961-1971.

Suzuki, K., Meek, B., Doi, Y., Muramatsu, M., Chiba, T., Honjo, T. & Fagarasan, S. 2004, 'Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut'. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, pp. 1981-1986.

Talham, G. L., Jiang, H.-Q., Bos, N. A. & Cebra, J. J. 1999, 'Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system'. *Infection and Immunity*, vol. 67, pp. 1992-2000.

Tannock, G. W., Miller, J. R. & Savage, D. C. 1984, 'Host specificity of filamentous, segmented microorganisms adherent to the small bowel epithelium in mice and rats'. *Applied and Environmental Microbiology*, vol. 47, pp. 441-442.

Toranzo, A. E. 2004. 'Report about bacterial diseases'. In: *Mediterranean Aquaculture Laboratories*, P. Alvarez-Pellitero et al., (Eds.), CIHEAM/FAO, pp. 49-89.

Toyama, T., Kita-Tsukamoto, K. & Wakabayasi, H. 1994, 'Identification of *Cytophaga psychrophila* by PCR targeted 16S ribosomal RNA'. *Fish Pathology*, vol. 29, pp. 271-275.

Umesaki, Y., Okada, Y., Matsumoto, S., Imaoka, A. & Setoyama, H. 1995, 'Segmented filamentous bacteria are indigenous intestinal bacteria that activate intraepithelial lymphocytes and induce MHC class II molecules and fucosyl asialo GM1 glycolipids on the small intestinal epithelial cells in the ex-germ free mouse'. *Microbiology and Immunology*, vol. 39, pp. 555-562.

Umesaki, Y., Setoyama, H., Matsumoto, S., Imaoka, A. & Itoh, K. 1999, 'Differential Roles of Segmented Filamentous Bacteria and Clostridia in Development of the Intestinal Immune System'. *Infection and Immunity*, vol. 67, pp. 3504-3511.

Urdaci, M. C., Regnault, B. & Grimont, P. A. D. 2001, 'Identification by in situ hybridization of segmented filamentous bacteria in the intestine of diarrheic rainbow trout (*Oncorhynchus mykiss*)'. *Research in Microbiology*, vol. 152, pp. 67-73.

Weber, E. S. 2005, 'Gastroenterology for the piscine patient'. *Veterinary Clinics of North America: Exotic Animal Practice*, vol. 82, pp. 247-276.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. 1991, '16S ribosomal DNA amplification for phylogenetic study'. *Journal of Bacteriology*, vol. 173, pp. 697-703.

Wilson, M., McNab, R. & Henderson, B. 2002. 'Bacterial exotoxins'. In: *Bacterial disease mechanisms*, 1st edn, M. Wilson, R. McNab, & B. Henderson, (Eds.), Cambridge University Press, Cambridge, UK, pp. 466-513.

CHAPTER 7. General Discussion

The primary aim of this study was to identify potential control strategies for rainbow trout gastroenteritis (RTGE). Scientific information on this syndrome had mainly focused on case reporting (Toranzo 2004; Branson 2003; Sanz 2000) and the study of the segmented filamentous bacteria "*Candidatus.* arthromitus" in affected fish (Michel *et al.* 2002; Urdaci *et al.* 2001). There was a lack of information in several areas of the syndrome including its epidemiology, pathogenesis and aetiology. Such information is needed for a rational approach to control. In order to provide knowledge in each of these fields, the study was designed using a multidisciplinary approach whereby RTGE was studied at the country, site, fish and aetiological levels.

7.1. RTGE at the national level (Chapter 2)

The methodology chosen was a retrospective survey, which revealed a relatively rapid rise in the number of RTGE-affected sites in the UK. This mirrored the pattern observed in France, Spain and Italy (Sarti *et al.* 2008; Sanz 2000). In France and Spain, RTGE is considered the most damaging disease for the rainbow trout industry (Sanz 2000). It is therefore reasonable to think that without adequate control strategies, the impact of RTGE on the UK rainbow trout industry could escalate to the levels observed in these countries. This is not the case at present and other diseases have a higher impact to the UK trout industry (Read 2008). However, the importance of RTGE in other countries suggests it is necessary to have appropriate control and prevention measures in place. In the UK, the presence of RTGE was associated with high and rapid production of trout for the table market and all the sites affected were relatively

large farms by UK standards, producing more than 200 tonnes of trout/year. A general increase in production has been identified as one of the key processes underlying disease emergence in aquaculture (Murray & Peeler 2005). This could be related to the higher stocking densities and frequent movement of live fish to and from producing sites (Murray & Peeler 2005). Altogether, the results of this study support the idea that disease monitoring at high productivity sites is of special relevance to the detection of emerging diseases in the UK. This is currently being developed at the Institute of Aquaculture (University of Stirling, UK) in the form of a scheme for monitoring sentinel farms in the UK trout industry (SARF028).

The retrospective survey allowed the identification of UK sites previously affected by RTGE as well as the risk factors associated at the site level. Additional information on a wide sample of sites from the UK rainbow trout industry was collected. With these data, it was possible to generate a list of sites which were likely to have RTGE in the following year, enabling the collection of data prospectively.

7.2. RTGE at the site level (Chapter 3)

A prospective longitudinal study was used to describe in detail the impact, pattern of spread and risk factors associated with RTGE within a population of 11 sites in the UK. The results of this study revealed an economic loss from RTGE on these sites comparable to that described for sites in Spain and France (Michel *et al.* 2002; Urdaci *et al.* 2001), thus reinforcing the importance of this disease for the UK.

This study also provided epidemiological evidence supporting the idea that RTGE behaves as an infectious condition that spreads within affected sites via fish movements and water. Transmission via fish transfer was more likely to occur if the fish had been transferred from a unit undergoing a clinical outbreak, suggesting this condition may not be highly contagious. The importance of fish transfers for the intra-site spread of disease is not a new concept (Danner & Merrill 2006; Scott 2004), but the data provided by this study has enabled the establishment of relevant control strategies for RTGE before identification of the aetiological agent is achieved. These strategies would include the avoidance of fish transfers and isolation of affected units during RTGE outbreaks, which if followed could reduce the intra-site spread of RTGE.

Identifying the hypothetical incubation period of 20-25 days also has direct repercussions for controlling RTGE, as it provides a temporal reference for the expected recurrence after treatment and may be used for the design of future treatment strategies. This finding has provided an epidemiological basis for a treatment which has been used against RTGE, comprising flumequine treatments repeated every 25 days (Sarti *et al.* 2008; Treves-Brown 2000). However, the efficacy of this treatment is not reported and this medicine is not licensed for aquaculture use in the UK (NOAH 2009). Additionally, this approach may lead to the development of antibiotic resistance. Field studies to assess the effects of different antibiotic treatments on RTGE-affected fish are necessary before considering the implementation of such strategies in the UK.

Previous suggestions on the importance of temperature on the onset of RTGE were confirmed by the results of the study (Toranzo 2004; Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001; Sanz 2000). The distribution of the data

suggests an absence of a common threshold temperature, which may have resulted in the observed disagreement between published reports. Directly relevant to the control of RTGE is the identification of management and environmental risk factors associated at the site level. Avoiding high feeding rates and stressful events can be directly included as part of a preventative programme. High feeding rates have been associated with a range of pathological conditions in salmonids although there is no information on the mechanisms behind this association, which could perhaps be related to increased organic loading or increased metabolic stress (Speare 1998; Staurnes *et al.* 1990). In this study it was also found that higher feed input during RTGE outbreaks was associated with higher cumulative mortalities, confirming previous anecdotal observations (Branson 2003) and this suggests that feed restriction during RTGE outbreaks is likely to reduce the impact of RTGE once it is present within a unit. This observation has also provided epidemiological basis for another treatment strategy that has been used against RTGE, comprised of fasting affected fish for 7 days or more (Sarti *et al.* 2008).

The appraisal of RTGE treatments used in UK affected sites revealed that treatments are based on trial and error and are applied with an inconsistent methodology. This is probably due to the fact that RTGE is an emerging syndrome where there is lack of scientific information. Descriptive analysis revealed an apparent reduction in RTGE mortalities when sodium chloride (NaCl) was fed to the fish during outbreaks. The use of NaCl as an in-feed treatment has not been reported before, although it has been studied with reference to osmoregulatory and nutritional aspects (Salman 2007; Perry *et al.*

2006). However, the results of this study suggested that the use of NaCl during outbreaks may reduce the impact of RTGE.

7.3. RTGE pathogenesis (Chapters 4 & 5)

An attempt to study the pathogenesis of RTGE consisted of analysis of gross lesions and blood biochemistry of RTGE-affected fish (Chapters 4 & 5). The diagnosis of RTGE in laboratories has been based mainly on the observation of external and internal gross lesions, microscopical examination of fresh smears and histopathology (Toranzo 2004). However, the lack of information on the aetiology of RTGE has resulted in the absence of a "gold standard" test for RTGE diagnosis. This study applied three laboratory tests to a sample of RTGE-affected fish in order to test a case definition derived from the information available on RTGE gross lesions. These analyses included bacteriology, histopathology and packed cell volume (PCV) and revealed that some RTGE-affected fish in the sample had concurrent disease. These fish had large numbers of segmented filamentous bacteria (SFB) in their digestive tract, suggesting that they had not been wrongly classified. Concurrent disease could have resulted from the entrance of secondary pathogens facilitated by loss of the intestinal mucosal integrity, although it was not possible to examine this possibility further here. However, this finding suggested that gross lesion-based field identification of RTGE fish for experimental studies is likely to result in sampling fish with concurrent disease, which may hinder research on RTGE, as this is currently based exclusively on field samples. For example, the presence of concurrent disease has been the reason for previous unsuccessful attempts at RTGE transmission (Verner-Jeffreys, personal comm.). To avoid this possibility in the study of RTGE pathogenesis, the information gained through

these analyses was applied to the design of a case definition in which concurrent diseases were filtered out

This modified case definition was then used for the sampling of RTGE-affected fish. Comparative analysis of the blood biochemistry of RTGE-affected and apparently healthy fish from the same unit, produced a profile consistent with a protein losing enteropathy, as described in other animal species (Stockham & Scott 2002). This pathogenic mechanism was consistent with the gross presentation as well as the observed response to in-feed NaCl. The results of this study are consistent with a palliative effect of in-feed NaCl on RTGE-affected fish, if fed **during** RTGE outbreaks.

Ultrastructural studies (Chapter 4) revealed cytoskeletal damage and severe osmotic imbalance at the level of the enterocyte. This could have been a result of the exposure of enterocytes to a toxin (Michel *et al.* 2002). In other species these changes have been reported to be associated with local ischaemia, with the presence of enterotoxin and with apoptosis (Kamaras & Murrel 2001; Malorni *et al.* 1990).

7.4. RTGE aetiology (Chapters 4 &6)

The aetiological role of "*C.* arthromitus" was assessed with molecular tools and ultrastructural analysis. Molecular analysis revealed the presence of "*C.* arthromitus" DNA in populations of healthy trout from sites where RTGE had never been reported, and SFB were observed histologically in two of these trout. These results suggested that if "*C.* arthromitus" are indeed involved in the aetiology of RTGE, they may be necessary, but not sufficient to cause clinical disease. Despite this, large numbers of SFB were observed only in RTGE-

affected fish, in agreement with previous observations (Toranzo 2004; Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001). Additionally, the close interaction of SFB with enterocytes was described for the first time in rainbow trout, confirming a reaction of enterocytes to the presence of SFB. Despite this, SFB were not always adjacent to the pathological changes observed in enterocytes, suggesting that if SFB are indeed the cause of RTGE, the pathogenesis requires the involvement of an extracellular product.

7.5. Further work

The multidisciplinary approach used in this study has been successful in providing scientific information regarding the epidemiology, pathogenesis and aetiology of RTGE, with direct applications to the prevention and control of RTGE at the site level (Appendix IV). Rainbow trout gastroenteritis is still a challenging and worthwhile subject, and the results from this study suggest that priority should be placed on specific areas for future research, including:

Transmission studies: The adaptation to RTGE of the challenge protocols used in the research of stunting syndrome (Angel *et al.* 1990) would allow testing experimental transmission of RTGE in the laboratory. Fresh digestive contents or intestinal homogenates from RTGE-affected fish without concurrent disease could be used as the challenge material (Chapter 5). The ability to manipulate RTGE in the laboratory would result in a faster and more accurate acquisition of scientific information on this syndrome.

Field intervention studies: Aimed at the testing of the control measures suggested by the results of this study (Appendix IV), as well as to assess formally the effectiveness of different antibiotic treatments and different

concentrations of in-feed NaCl in a semi-controlled environment. It may be especially interesting to assess the effect of prolonged fasting of affected fish and the use of antibiotic treatments. These types of intervention studies are likely to present several challenges, which should be considered during the design of the experiment, including site participation and a natural variability in RTGE prevalence between units.

Further aetiological studies: These could include the application of culture independent methodologies (*e.g.* denaturing gradient electrophoresis) to the screening of intestinal contents of RTGE-affected fish to detect the presence of bacterial aetiological agents other than SFB. Also, it would be possible to use gradient centrifugation of diluted intestinal contents of RTGE-affected fish for detecting viral particles. If the experimental transmission of RTGE were successful, it would be possible to test the extent of the bacterial involvement in RTGE pathogenesis through the use of filtered and unfiltered intestinal contents. This would also alleviate the need for *in vitro* culture methods.

SFB culture studies: These would include the culture *in vitro* of trout "*C.* arthromitus" using different anaerobic media, perhaps enriched with rainbow trout intestinal homogenate. This possibility is likely to be challenging, as all previous attempts have been unsuccessful (Angert 2005), although the availability of pure "*C.* arthromitus" cultures would enable direct testing of their aetiological role in RTGE, through the challenge of apparently healthy trout with large quantities of pure SFB.

7.6. References

Angel, C. R., Sell, J. L. & Trampel, D. W. 1990, 'Stunting syndrome in turkeys. Development of an experimental model'. *Avian Diseases*, vol. 34, pp. 447-453.

Branson, E. 2003, 'Rainbow trout Gastro-Enteritis (RTGE)-first diagnosis in the UK'. *Fish Veterinary Journal*, vol. 7, pp. 71-76.

Danner, G. R. & Merrill, P. 2006. 'Disinfectants, Disinfection, and Biosecurity in Aquaculture'. In: *Aquaculture Biosecurity.Prevention, Control, and Eradication of Aquatic Animal Disease*, A. D. Scarfe, C.-S. Lee, & P. J. O'Bryen, (Eds.), Blackwell Publishing, Oxford, pp. 91-128.

Kamaras, J. & Murrel, W. J. 2001, 'The effect of bacterial enterotoxins implicated in SIDS on the rabbit intestine'. *Pathology*, vol. 33, pp. 187-196.

Malorni, V., Fiorentini, C., Paradisi, S., Giuliano, M., Mastrantonio, P. & Donelli, G. 1990, 'Surface blebbing and cytoskeletal changes induced in vitro by toxin B from Clostridium difficile: an immunochemical and ultrastructural study'. *Scandinavian Journal of Gastroenterology*, vol. 52, pp. 340-356.

Michel, C., Bernardet, J. F., Daniel, P., Chilmonczyk, S., Urdaci, M. C. & de Kinkelin, P. 2002, 'Clinical and aetiological aspects of a summer enteritic syndrome associated with the sporulating segmented filamentous bacterium "*Candidatus* arthromitus" in farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum)'. *Journal of Fish Diseases*, vol. 25, pp. 533-543.

Murray, A. G. & Peeler, E. J. 2005, 'A framework for understanding the potential for emerging diseases in aquaculture'. *Preventive Veterinary Medicine*, vol. 67, pp. 223-235.

NOAH 2009, *Compendium of Data Sheets for Animal Medicines*. National Office for Animal Health, London, UK.

Perry, S. F., Rivero-Lopez, L., McNeill, B. & Wilson, J. 2006, 'Fooling a freshwater fish: how dietary salt transforms the rainbow trout gill into a seawater gill phenotype'. *Journal of Experimental Biology*, vol. 209, pp. 4591-4596.

Read, N. 2008. 'Fish farmer's perspective of welfare'. In: *Fish welfare*, E. J. Branson, (Ed.), Blackwell Publishing, Oxford, UK, pp. 101-110.

Salman, N. 2007, 'Some nutritional and osmoregulatory responses of fish to dietary salt'. *Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology*, vol. 146, p. 10.

Sanz, F. 2000, 'La gastroenteritis de la trucha (Language: Spanish)'. *Trouw Informa*, vol. Spring, pp. 16-18.

Sarti, M., Cervellione, F., Giusto, C., Manzano, M., Pecchini, A. & Luzzana, U. 2008, 'Intestinal microflora modulation as a strategy for the control of rainbow

trout gastroenteritis (RTGE) (Language: Italian)'. In: *XV conference of the Italian Society of Fish Pathology*, Erice, Italy, p. 25.

Scott, A. 2004, 'Sleeping disease: A lesson in biosecurity'. *Trout News*, vol. 37, pp. 17-23.

Speare, D. J. 1998. 'Non infectious disorders associated with intensive aquaculture husbandry'. In: *Fish diseases and disorders*, vol. II J. F. Leatherland & P. T. K. Woo, (Eds.), CABI publishing, New York, pp. 303-333.

Staurnes, M., Andorsdottir, G. & Sundby, A. 1990, 'Distended, water-filled stomach in sea-farmed rainbow trout'. *Aquaculture*, vol. 90, pp. 333-343.

Stockham, S. L. & Scott, M. A. 2002, *Veterinary Clinical Pathology*. First Ed., Wiley Blackwell, Iowa, USA.

Toranzo, A. E. 2004. 'Report about bacterial diseases'. In: *Mediterranean Aquaculture Laboratories*, P. Alvarez-Pellitero et al., (Eds.), CIHEAM/FAO, pp. 49-89.

Treves-Brown, K. M. 2000, *Applied fish pharmacology*. Kluwer Academic Press, Dordretch, The Netherlands.

Urdaci, M. C., Regnault, B. & Grimont, P. A. D. 2001, 'Identification by in situ hybridization of segmented filamentous bacteria in the intestine of diarrheic rainbow trout (*Oncorhynchus mykiss*)'. *Research in Microbiology*, vol. 152, pp. 67-73.

Appendix I: Other enteritides of rainbow trout

The table below contains a list of enteritides of rainbow trout other than rainbow trout gastroenteritis (RTGE) for reference. Although this list is not exhaustive, it contains the most common conditions observed in farmed rainbow trout. The presence of SFB is not reported in the literature for any of these conditions and the aetiological agent for all of them has been identified.

Type	*Aetiology*	*Common Name*	*Presentation in intestine*	*Systemic component*
Viral	Birnavirus	Infectious Pancreatic Necrosis (IPN)	Enterocyte detachment and catarrhal enteritis	Yes
	Rhabdovirus	Infectious Haemopoietic Necrosis (IHN)	Eosinophilic granular cell necrosis, enterocyte detachment	Yes
	Rhabdovirus	Viral Haemorrhagic Septicaemia (VHS)	Multifocal haemorrhage and enterocyte necrosis	Yes
Bacterial	*Yersinia ruckeri*	Enteric redmouth (ERM)	Petechial haemorrhage and congestion	Yes
	Aeromonas salmonicida	Furunculosis	Primary lesion: Acute enteritis with congestion	Yes
	Piscirickettsia salmonis	Piscirickettsiosis	Macrophage infiltration and ischaemic necrosis	Yes
	Citrobacter freundii	N/A	Dilation and watery contents	Yes
	Lactococcus garvieae	Lactococcossis	Epithelial detachment and multifocal haemorrhage	Yes
	Streptococcus spp.	Streptococcosis	Epithelial detachment and multifocal haemorrhage	Yes
Fungal	*Ichthyophonus hoferi*	N/A	Granulomatous inflamation	Yes
Metazoan	Cestodes	Tapeworm	Focal necrosis and granulomatous responses with mild catarrhal enteritis	No
	Acantocephalans	N/A	Focal necrosis and granulomatous responses	No
Protozoan	Flagellates (*Spironucleus*)	Hexamitiasis	Catarrhal enteritis	No
	Myxozoa *(Ceratomyxa*)	N/A	Diffuse, necrotizing and granulomatous enteritis with loss of folds	No
Nutritional	Excessive soya protein	Soya-induced enteritis	Short and widened folds and inflammatory infiltrate	No

- Austin B. & Austin D.A. (2007). Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish. Fourth Ed., Springer-Praxis, Chichester.
- Austin, B. *et al.* (1992). Bulletin of the European Association of Fish Pathologists, vol. 12, pp. 166-167.
- Eldar, A. & Ghittino, C. (1999). Diseases of Aquatic Organisms, vol. 36, pp. 227-231.
- Ferguson, H. W. (2006). Systemic pathology of fish: A text and atlas of normal tissues in teleosts and their responses in disease. Second Ed., Scotian Press, London, UK.
- Roberts, R. J. (2001). Fish pathology. Third Ed., W.B. Saunders, Edinburgh, UK.
- Weber, E. S. (2005). Veterinary Clinics of North America: Exotic Animal Practice, vol. 82, pp. 247-276.

Appendix II: RTGE Retrospective Questionnaire

RTGE Epidemiological questionnaire conducted by:
Jorge del Pozo MRCVS, *Institute of Aquaculture, University of Stirling, Scotland*

RAINBOW TROUT GASTROENTERITIS (RTGE, Gastro): EPIDEMIOLOGICAL STUDY QUESTIONNAIRE

PLEASE NOTE ALL THE INFORMATION PROVIDED WILL BE TREATED AS STRICTLY CONFIDENTIAL

INSTRUCTIONS:
To fill the questionnaire using the computer: If the square grey boxes are clicked, a cross will appear which means a positive answer to the question. The rectangular boxes are text fields where the answer can be written.
To fill the questionnaire by hand: Print the document and fill the forms normally.

Farm name:

What is the type of system/s present in the farm?

Ponds ☐ Raceways ☐ Cages ☐ Tanks ☐ Any other

What are these systems made of?

Earth ☐ Concrete ☐ Metal ☐ Fibreglass ☐ Any other

Purpose of production?

Table market ☐ Restocking ☐ Hatchery ☐ Fishery ☐

Systematic Fallowing? (i.e. emptying and disinfecting the ponds between batches or in any regular basis)

Yes ☐ No ☐ Details

Approximate yearly production in tons (if not in tons, what is the measure unit?)

2003 2004 2005

Water abstraction licence Million Gallons/day
What percentage of this water do you use during the summer? (approx) %

Maximum water temperature in the summer? (approximate) ^{0}C

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RTGE Epidemiological questionnaire conducted by:
Jorge del Pozo MRCVS, *Institute of Aquaculture, University of Stirling, Scotland*

Average stocking density at time of stocking (fill in what is applicable) in **Kg trout/m^3**
(if not in Kg/m3, what is the measure unit?)

Ponds Raceways Cages Tanks Any other

Trout weigh at time of stocking (fill in what is applicable) in **grams**
(if not in grams, what is the measure unit?)

Ponds Raceways Cages Tanks Any other

Average stocking density at time of harvest (fill in what is applicable) in **Kg trout/m^3**
(if not in Kg/m3, what is the measure unit?)

Ponds Raceways Cages Tanks Any other

Trout weigh at time of harvest (fill in what is applicable) in **grams**
(if not in grams, what is the measure unit?)

Ponds Raceways Cages Tanks Any other

Average turnover time of trout in the farm in months: months
i.e What is the average time since any trout is stocked in the farm to the time of harvest or sale?

Type of rainbow trout cultured

Diploid ☐ Triploid ☐ Mixed ☐

All female? ☐

Other species in farm

Brown trout ☐ Brook trout ☐ Other

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RTGE Epidemiological questionnaire conducted by:
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Please list all the egg sources of the farm (if applicable)

Please list all the fry sources of the farm (if applicable)

Farm water source

River ☐ Borehole ☐ Lake ☐ Other

Water type

Hard ☐ Normal ☐ Soft ☐

Any second use of water? ☐

Details

Any aeration system on farm ☐

Any oxygenation system on farm on farm ☐ **When?**

Feed supplier **Type/s of feed used**

Feed method

Hand ☐ Automated ☐ Demand ☐ Other

Any specific summer feeding strategy? ☐
(i.e. reduced feeding when water reaches a specific temperature timing of feeding, etc)

Details #

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RTGE Epidemiological questionnaire conducted by:
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Use of feed additives during the summer

Immunostimulants ☐ Booster ☐ Other ☐ Details

Any systematic treatment? ☐ Details
(i.e. a treatment that is done to the fish every year whether they are sick or not)

Any systematic vaccination? ☐ Details

Any other fish farm in the same water system within 5 miles? ☐

What is the name of the neighboring farm?

What processing plant do the harvested fish go to?

What predators are present in the farm?

Do you have any other animals in the farm? What animals?

Do you share any of the following with other farms?

Equipment ☐ Workers ☐

What farms?

RTGE Epidemiological questionnaire conducted by:
Jorge del Pozo MRCVS, *Institute of Aquaculture, University of Stirling, Scotland*

Do you think you would recognize RTGE (Rainbow Trout Gastroenteritis or Gastro) if you saw it? ☐

Please describe briefly what would you expect to find in a trout with RTGE

Have you ever had problems with RTGE on your farm?

Yes ☐ **No** ☐

If yes, would you agree to be contacted for further study? ☐
(Further study would involve a visit to the farm to gather specific information about the RTGE outbreaks, at a time arranged to the farmer convenience)

General comments

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RTGE Epidemiological questionnaire conducted by:
Jorge del Pozo MRCVS, *Institute of Aquaculture, University of Stirling, Scotland*

What to do now?

Return the questionnaire by e-mail:
Once the questionnaire is finished click on the **File** menu and then click **Save as**. In the dialog that will appear, you can give the file a name, click **Save** and the resulting file can then be attached to an e-mail.

Return the questionnaire by post:
Post the completed questionnaire to:

Mr Jorge del Pozo
Institute of Aquaculture,
Pathfoot building
University of Stirling
STIRLING FK9 4LA

Alternatively you can print the form and fill it by hand and contact Jorge del Pozo on jd24@stir.ac.uk or 07816 279221 who will send you a prepaid envelope.

THANK YOU FOR YOUR COOPERATION WITH THIS INVESTIGATION

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Appendix III: Prospective Epidemiology RTGE case definition for farmers

RTGE Prospective study
RTGE (Gastro) IDENTIFICATION

UNIVERSITY OF STIRLING

GENERAL GUIDELINES FOR RTGE (Gastro) IDENTIFICATION

EXTERNAL SIGNS

- Apathy
- Not feeding
- Lighter color
- Swollen appearance
- Dead or moribund

INTERNAL SIGNS

- Other organs look apparently normal
- Intestinal reddening (Predominantly in the hind gut).
- Yellow and gelatinous intestinal contents

Appendix IV: RTGE control guidelines

RAINBOW TROUT GASTROENTERITIS

RESEARCH ON RTGE

A research project has now been underway for 3 years conducted through the Institute of Aquaculture, Stirling and funded by DEFRA, SARF and the BTA. The disease is very complex and this document does not aim to present all the findings. Our intention here is to present the most important information. While we are confident in these results; we are circulating them to you **before publication** in the hope they will help you to manage RTGE better this summer.

WHAT IS RAINBOW TROUT GASTROENTERITIS?

RTGE or "Gastro" is a contagious disease of rainbow trout. This disease usually develops as water temperatures rise above 15°C, although it has been observed at 10°C. It is usually observed in farmed trout produced for the table market, from 16g to pre-harvest weight. Affected fish do not feed and accumulate at pond outlets or the edge of cages. They have a swollen appearance with lighter colouration, sometimes accompanied with striping of the flanks. Internally, they have an inflamed and reddened intestine containing a yellow viscous material with no feed. Frequently, pyloric caeca are also affected but other organs look normal. An increasing number of sites have had problems due to RTGE over the last 7 years, with the largest, most intensive producers usually most severely affected. An average of 5% of fish die in affected production units but losses can be as high as 78% of the fish.

KEY FINDINGS

- RTGE behaves like a simple infection – moving fish from a unit (pond, cage, etc.) with RTGE is very likely to result in a disease outbreak in the unit which receives the fish. In addition, water movement between cages or on land based sites also appear to play a role in the spread of RTGE – i.e. the causative agent is water borne.
- High feeding levels tend to increase the severity of RTGE outbreaks.
- Salt treatment appears to provide osmotic support to affected fish rather than preventing the problem – feeding salt to fish during the outbreaks appears to reduce the overall losses but not the duration of the outbreak. Other possible actions of in-feed salt cannot be accounted for with the knowledge available.
- Liquid paraffin does not appear to have a significant effect.
- We do not have enough data to comment on other treatments such as antibiotics and Chloramin T.
- A single source of infection from eggs, fry or feed has not been identified during this project.

WHAT CAN YOU DO?

We have not field tested any control strategies and therefore can give no guarantees but have the following suggestions which you may wish to consider in the context of your own business. This applies to farms that have had RTGE, have high levels of production for the table market or consider that they might be at risk.

- Isolate units affected with RTGE and do not move the fish unless absolutely necessary.
- If possible avoid reuse of water from units suffering from RTGE.
- Addition of salt to the diet is most effective when it is commenced prior to an outbreak and maintained throughout the duration of the outbreak. The options are to have premixed diet on site and start feeding as soon as you see or suspect RTGE or you may consider using diets with added salt over the entire high risk period.
- Avoid very high levels of feeding if possible and reduce feeding at the first sign of an outbreak.