FACTORS AFFECTING EXPERIMENTAL *STREPTOCOCCUS AGALACTIAE* INFECTION IN TILAPIA, *OREOCHROMIS NILOTICUS*

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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.

Signature: _____

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Date: _____

Abstract

Streptococcus agalactiae infection is one of the major disease problems affecting farmed tilapia (*Oreochromis niloticus*) worldwide. Tilapia are highly susceptible to this disease which results in mortality of up to 70% over a period of around 7 days and significant economic losses for farmers. Affected tilapia commonly present with an irregular behaviour associated with meningoencephalitis and septicaemia. Currently, factors affecting the virulence and transmission of *S. agalactiae* in fish including tilapia are poorly understood. Reports from natural outbreaks of *S. agalactiae* infection on tilapia farms have suggested larvae and juvenile or fish smaller than 20 g are not susceptible. In addition, there is variability in individual response to experimental inflammatory challenge associated with coping styles (bold, shy) in common carp (*Cyprinus carpio*). The central hypotheses of this thesis were that weight, age and coping style might affect the development and progression of this bacterial disease. This study investigated these three factors with experimental *S. agalactiae* infection in Nile tilapia.

A range of bacterial isolates recovered from farmed tilapia, presenting with clinical sign of streptococcosis during natural disease outbreaks were identified and characterised as *S. agalactiae* by standard conventional methods, biochemical characteristic tests, Lancefield serogrouping and species-specific PCR assay. These isolates were Gram-positive cocci, either β- or non-haemolytic (γ), non-motile, oxidase negative and all of serogroup B. In addition, they were able to grow on Edwards medium (modified) agar as blue colonies

and growth was observed in broth from 22 to 37 ^{o}C and with 0.5-5% NaCl. The biochemical profiles showed some differences in reactions while all the PCR samples showed similarities to the *S. agalactiae* type strain. These data confirmed that these strains were identified as group B *S. agalactiae*.

A challenge model for *S. agalactiae* in Nile tilapia was developed and the LD_{50} estimated prior to performing subsequent experimental challenge studies. Two exposure routes, immersion and intraperitoneal injection (i.p.), were tested with various concentrations of *S. agalactiae*. Only i.p. injection produced significant mortalities (9×10^8 CFU/ml = 48% mortality, 9×10^7 = 48% and 8×10^6 = 26%). *Streptococcus agalactiae* was recovered and identified from all the dead and moribund fish during these experiments, where affected fish showed similar clinical signs and pathology to those reported from natural *S. agalactiae* infections. The study results showed that an experimental i.p. challenge model for *S. agalactiae* infection had successfully infected healthy Nile tilapia. In the immersion challenges, only 1 fish died despite testing a range of bacterial concentrations, exposure times, stocking density, water system and bacterial preparations.

The experimental studies were conducted to investigate the association between weight or age of fish and susceptibility to *S. agalactiae* infection in Nile tilapia. This was performed under experimental conditions including control groups and a single population of 8 months old fish from one set of parents divided into 7 weight categories. These fish received a single i.p. injection of 6 ×

10^7 CFU/ml of *S. agalactiae*. Controls and fish of 4 or 8 months old with a mean weight of 5 g received an i.p. injection of 7×10^7 CFU/ml of *S. agalactiae*. Clinical signs, lesions and histopathological changes in the affected fish were consistent with those reported in natural infection. *Streptococcus agalactiae* was recovered and identified from all moribund or dead fish. The mortality in the study of different weights varied from 0 to 33% between the groups but the association with weight was weak ($R^2 = 0.02$). In the study of different ages the 4 months old fish group had a total mortality of 24%, and the 8 months old fish group a total mortality of 4%. This study produced no evidence for an association between the weight and susceptibility to *S. agalactiae* infection but suggested an association between the age or growth rate of fish and this disease.

Different coping styles and susceptibility to *S. agalactiae* infection in Nile tilapia was examined. Fish were screened and scored depending on their risk-taking behavioural responses to a range of different environmental conditions. Individual differences in behavioural responses were evident but only consistent across behavioural trials for some individuals. A selection of fish with consistent responses across trials was exposed to the 6×10^7 CFU/ml of *S. agalactiae* by i.p. injection. Fewer bold than shy fish died suggesting that the bold fish might be less susceptible to the infection than shy fish.

In conclusion, this study characterised a number of *S. agalactiae* isolates and developed an experimental bacterial challenge model. Subsequent experiments suggested that age (or growth rate) and coping style in fish but not the fish weight may affect susceptibility to *S. agalactiae* infection in Nile tilapia.

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1.1 Introduction

Streptococcus agalactiae (synonym *S. difficile*) is described as a Gram-positive, cocci-shaped bacterium which commonly occurs in pairs or in long chains. They produce small, translucent, round, and slightly raised, pinpoint colonies, measuring 1-2 mm in diameter and appear yellowish to grey in colour when grown on solid agar (Plumb, 1999; Buller, 2004). Strains belonging to *S. agalactiae* are described as α-, β- or non-haemolytic (γ) when cultured on blood agar (Kitao et al., 1981; Buller, 2004). They are described as non-motile, non-capsulated, non-spore forming and are negative for the presence of oxidase and catalase enzymes. These bacteria are able to grow at pH 9.6 but not at 10°C nor at 45°C nor in the presence of 40% (v/v) bile salts or in the presence of 6.5% NaCl (w/v) (Inglis et al., 1993; Plumb, 1999; Buller, 2004). This bacterium is classified as belonging to the group B *Streptococcus* (GBS) species using the Lancefield serogrouping method (Devriese, 1991; Facklam, 2002). At present, based on the composition of the capsular polysaccharide antigen, GBS organisms have been classified into ten serotypes (Ia, Ib and II to IX) (Chaffin et al., 2000; Persson et al., 2004; Slotved et al., 2007). Those strains with molecular serotype Ia, Ib, II and III have been previously reported in Nile tilapia (*Oreochromis niloticus*) in Thailand and China, red tilapia (*Oreochromis* spp.) in Thailand, wild fish in bays along the Florida and Alabama

Gulf Coast and wild fish in northern Queensland, Australia (Plumb et al., 1974; Vandamme et al., 1997; Suanyuk et al., 2008; Rodkhum et al., 2011; Ye et al., 2011; Bowater et al., 2012). Recently, Evans et al. (2008, 2009) have demonstrated that the human GBS serotype Ia is able to infect fish. *Streptococcus agalactiae* of human and bovine origin can infect and cause clinical disease in Nile tilapia by i.p. and/or immersion routes (Pereira et al., 2010). GBS can be pathogenic, virulent and infective across a diverse range of species; however, the zoonotic potential of GBS of piscine origin has not yet been adequately investigated.

The *S. agalactiae* bacteria appear able to naturally infect a wide range of hosts including humans, terrestrial and aquatic animals. Members of this bacterial species have been associated with numerous clinical disease outbreaks and *S. agalactiae* has been identified as the causative agent of neonatal meningitis, sepsis, pneumonia, osteomyelitis and soft tissue infections in humans (Wilkinson et al., 1973; Baker, 1980; Jones et al., 2003; Brochet et al., 2006; Johri et al., 2006). It is a potential threat for pregnant women and elderly people as well as a serious cause of mortality for immune-compromised adults, especially those with diabetes mellitus, malignancies, liver cirrhosis and a history of previous surgery (Farley, 2001; Bolanos et al., 2005).

Streptococcus agalactiae is also able to colonize mammary glands of various ruminants, resulting in clinical and sub-clinical mastitis in cattle which can seriously affect milk quality (Wilkinson et al., 1973; Keefe, 1997; Phuektes et al., 2001). Moreover, it has also been isolated from various other animals presenting with a disease including mice, cats, dogs, hamsters, guinea pigs, chickens, horses, emerald monitors (*Varanus prasinus*), monkeys, camels, frogs, bottlenose dolphins (*Tursiops truncatus*) and captive saltwater crocodiles (*Crocodylus porosus*) (see Amborski et al., 1983; Elliott et al., 1990; Wagner and Kaatz, 1997; Yildirim et al., 2002a, 2002b; Hetzel et al., 2003; Zappulli et al., 2005; Evans et al., 2006c; Bishop et al., 2007).

In fish, this disease has been reported in wild and cultured fish species including both freshwater and marine animals in natural outbreaks throughout the world (Plumb, 1999; Buller, 2004; Austin and Austin, 2007). Recently, *S. agalactiae* has been isolated from fish presenting with bacterial septicaemia and meningoencephalitis in a wide range of fish species from 12 countries listed in Table 1.1.

Table 1.1 *Streptococcus agalactiae* infections and the fish species they affect reported in the scientific literature.

Fish species	Source	References
Nile tilapia, *Oreochromis niloticus* (L.)	Israel	Eldar et al. (1994)
	Thailand	Suanyuk et al. (2005, 2008)
	Japan	Evans et al. (2006a)
	China	Zhang et al. (2008), Ye et al. (2011)
	Brazil	Salvador et al. (2005), Mian et al. (2009)
Red tilapia, *Oreochromis* spp.	Thailand	Suanyuk et al. (2008)
	Malaysia	Siti-Zahrah et al. (2008), Musa et al. (2009), Abuseliana et al. (2010), Zamri-Saad et al. (2010)
	Vietnam	Oanh and Phuong (2011)
	Columbia	Hernández et al. (2009), Jiménez et al. (2011)
Rainbow trout, *Oncorhynchus mykiss* (Walbaum)	Israel	Eldar et al. (1994)
	Iran	Pourgholam et al. (2011)
Ya-fish, *Schizothorax prenanti*	China	Geng et al. (2011)
Golden shiners, *Notemigonus crysoleucas* (Mitchill)	USA	Robinson and Meyer (1966)
Striped bass, *Morone saxatilis* (Walbaum), bluefish, *Pomatomus saltatrix* L., grey weakfish, *Cynoscion regalis* (Bloch & Schneider)	USA	Baya et al. (1990)
Bullminnows, *Fundulus grandis* (Baird & Girard)	USA	Rasheed and Plumb (1984)
Silver pomfret, *Pampus argenteus* (Euphrasen)	Kuwait	Duremdez et al. (2004)
Wild mullet, *Liza klunzingeri* (Day),	Kuwait	Evans et al. (2002)
Seabream, *Sparus auratus* L.		Al-Marzouk et al. (2005)
Silvery croaker, *Otolithes argenteus* (Nowaiby), striped grunt, *Rhonciscus stridens*	Kuwait	Al-Marzouk et al. (2005)
Giant sea catfish, *Arius thalassinus* (Ruppell),	Kuwait	Al-Marzouk et al. (2005)
	Australia	Bowater et al. (2012)
Wild gulf menhaden, *Brevoortia patronus* (Goode), Hardhead sea catfish, *Arius felis* L., striped mullet, *Mugil cephalus* L., Pinfish, *Lagodon rhombodies* L., Atlantic croaker, *Micropogonias undulates* L., spot, *Leiostomus xanthurus* (Lacepede), stingray, *Dasyatis* sp., silver weakfish, *Cynoscion nothus* (Holbrook)	USA	Plumb et al. (1974)
Wild giant Queensland grouper, *Epinephelus lanceolatus* (Bloch), Javelin grunter, *Pomadasys kaakan* (Cuvier), wild stingrays, estuary rays, *Dasyatis fluviorum* (Ogilby), Mangrove whipray, *Himantura granulata* (Macleay), eastern shovelnose ray, *Aptychotrema rostrata* (Shaw)	Australia	Bowater et al. (2012)

1.2 *Streptococcus agalactiae* infection in tilapia

Although *S. agalactiae* infections have been reported in many fish species and in a wide range of aquatic environments, it is regarded as a significant pathogen affecting warm-water fish species (Eldar et al., 1994; Evans et al., 2002). This disease usually occurs during periods of higher water temperatures often above 15 ^{o}C, hence in temperate climates clinical outbreaks are often regarded a problem in the summer months (Eldar et al., 1994; Kawamura et al., 2005; Siti-Zahrah et al., 2008). It is a recognised pathogen in global aquaculture and disease outbreaks have resulted in significant fish losses resulting in serious economic losses in tilapia species (*Oreochromis* spp.) (Figure 1.1) (Eldar et al., 1994; Salvador et al., 2005; Suanyuk et al., 2005; Mian et al., 2009; Abuseliana et al., 2010; Zamri-Saad et al., 2010).

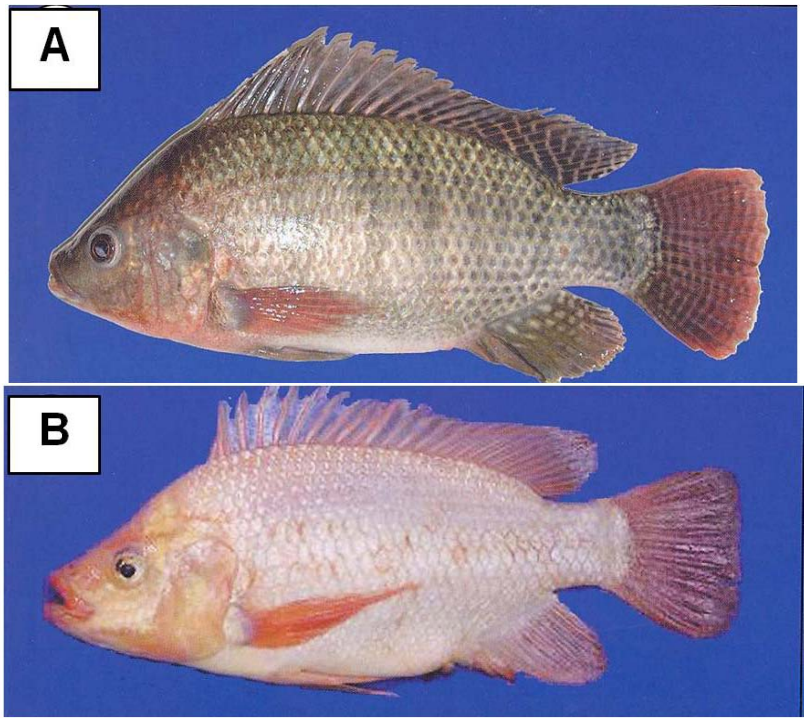

Figure 1.1 The tilapia species that are most commonly reared in aquaculture. A, Nile tilapia, *Oreochromis niloticus*; B, Red tilapia, *Oreochromis* spp. These images are taken from Morrison et al. (2006).

Mortality of up to 30% during a single natural outbreak has been reported in Nile tilapia farms in Thailand and China (Suanyuk et al., 2008; Zhang et al., 2008) and in red tilapia farms in Malaysia (Musa et al., 2009). The affected fish all presented grossly with typical signs of streptococcosis. Furthermore, a single outbreak of *S. agalactiae* infection reported in tilapia in Malaysia, resulted in fish losses between 60 and 70% of the stocked cages (Siti-Zahrah et al., 2005). It would appear that *S. agalactiae* is one of the major bacterial species affecting the sustainable production of tilapias in the world.

1.3 Transmission studies

Many studies have investigated the transmission of this pathogen within a farm site. Naturally occurring infections within farms have shown that *Streptococcus* spp. can occur through the water as direct contact between fish. The uptake of the pathogen and the disease occurrence appeared to be exacerbated if the fish were held in crowded or intensive culture conditions or if they had small abrasions, wounds or external injuries to the skin, fin or scales (Plumb, 1999; Nguyen et al., 2001b; Evans et al., 2002). Nguyen et al. (2002) showed that the bacteria were excreted in the faeces of infected fish where they can survive in the water column leading to further infection within the surrounding fish population through the faecal-oral route. An additional transmission route is orally, through cannibalism of dead or moribund animals. Studies by Minami (1979) and Kim et al. (2007) showed that using infected trash fish as feed could introduce streptococcosis outbreaks into yellowtail and flounder farms. However, little information is available describing the transmission in tilapia

during natural *S. agalactiae* infections. Hernández et al. (2009) and Jiménez et al. (2011) found that there was no vertical transmission of *S. agalactiae* disease in tilapia as the bacteria were not detected in the larvae or juvenile fish derived from the infected parent fish. Therefore, the horizontal transmission of the pathogens between fish is believed to be the most common mechanism of spreading the disease between individuals.

Recently, *S. agalactiae* was isolated from infected tilapia in natural outbreaks and shown to be pathogenic to the tilapia experimentally by varied routes. Four experimental transmission routes including intraperitoneal (i.p.) injection (Evans et al., 2004b; Filho et al., 2009; Mian et al., 2009; Pretto-Giordano et al., 2010a; Abuseliana et al., 2011), immersion (Mian et al., 2009; Ng et al., 2009; Rodkhum et al., 2011), cohabitation and gill inoculation (Mian et al., 2009) have successfully been reported in tilapia with *S. agalactiae*. Moreover, there are other exposure routes for experimental challenge studies with streptococcal infection in fish that could infect healthy fish including; intramuscular injections, bath, oral with food containing the bacteria and via a plastic catheter or gavage and nare inoculation (Robinson and Meyer, 1966; Rasheed and Plumb, 1984; Eldar et al., 1995a; Perera et al., 1997; Bromage et al., 1999; Evans et al., 2000; Shoemaker et al., 2000; Evans et al., 2001; Nguyen et al., 2001a; Bromage and Owens, 2002; Evans et al., 2002; McNulty et al., 2003; Lahav, 2004; Al-Marzouk et al., 2005).

1.4 Factors influencing infectious disease

In all bacterial diseases in fish, the surrounding environmental conditions can influence the uptake, colonisation and establishment of the bacterial diseases within the susceptible fish species because fish are reliant on their environmental conditions to support their homoeostasis and sub-optimal or variable conditions. There are few studies reporting the range of environment factors and how these have contributed towards the development of *S. agalactiae* infection in tilapia. Among the conditions that were suspected of favouring this disease were high water temperature, low dissolved oxygen (DO) and the weight and / or the age of fish.

Siti-Zahrah et al. (2008), Mian et al. (2009) and Rodkhum et al. (2011) suggested that high water temperature (≥27^{o} C) influenced the occurrence of *S. agalactiae* infection causing mortality in both natural and experimental outbreaks within tilapia. It is considered that both the non-specific and specific immune responses of fish are significantly decreased when fish are subjected to high temperature stress or temperatures above the normal water temperature range of the fish (Le Morvan et al., 1998; Ndong et al., 2007). Therefore, high water temperature, which will incidentally favour bacterial growth, was considered to be a stress factor that increased the susceptibility of tilapia to *S. agalactiae.* However, the severity of the disease may be further influenced by the rate of bacterial growth and expression of virulence factors which can also be influenced by the environmental water temperatures. So a single environmental variable such as water temperature can influence the

disease progression affecting the host and the bacterium which may lead to individual fish susceptibility (Rodkhum et al., 2011). This may also contribute towards the variation in mortality rates reported during different clinical outbreaks.

In addition, Evan et al. (2003) demonstrated that long periods of low DO level (up to 1 mg DO per litre) in the water increased a stress response in the fish which led to impaired immune response resulting in decreased resistance against *S. agalactiae* in experimental Nile tilapia. Generally, low DO may be due to algal blooms, high density of fish, high water temperature or high nutrient levels in the farm. It has been shown that sublethal DO levels cause hypersecretion of catecholamines and corticosteroids in fish producing changes in blood glucose levels (Mazeaud et al., 1977; Wedemeyer and McLeay, 1981). Detectable blood glucose is considered a reliable indicator of stress responses in fish (Thomas and Robertson, 1991; Rotllant and Tort, 1997). Hyperglycemia is a result of changes in liver glycogenolysis, which causes the increased conversion of reserved glycogen to glucose (Mazeaud and Mazeaud, 1981). This imposes severe energy demands due to the depletion of reserve glycogen on the stressed fish. The severe energy demand causes an energy crisis that is believed to result in the impairment of resistance to pathogens (Wedemeyer, 1976; Schreck, 1981). Among the possible explanation are the impairment of actions of phagocytes or cytotoxic cells and antibody production due to the energy crisis.

It has been hypothesised that fish weight and / or age may be a major factor affecting the establishment of *S. agalactiae* infections in farmed tilapia (Hernández et al., 2009). The weight and / or age of fish was considered a critical condition that predisposed tilapia to outbreaks of *S. agalactiae* infection according to a randomly sampled prevalence study (Siti-Zahrah et al., 2008; Suanyuk et al., 2008; Hernández et al., 2009; Mian et al., 2009; Zamri-Saad et al., 2010; Jiménez et al., 2011; Amal and Zamri-Saad, 2011). However, the link between fish weight and / or age and *S. agalactiae* susceptibility has not been demonstrated yet and others have linked the infectivity to the immune response by different fish (Evans et al., 2004a), high stocking density (11.2 - 22.4 g/L) (Shoemaker et al., 2000), variation in farm management, environment conditions and other factors associated with co-infections. Further work is required to establish if weight and / or age is a true risk factor associated with *S. agalactiae* infections in tilapia species.

It would appear from published literature that environment stressors and sub-optimal water quality factors including high un-ionized ammonia (UIA) concentration (≥2 mg/L) (Plumb et al., 1974, Eldar et al., 1995a; Hurvitz et al., 1997; Evans et al., 2006b), high nitrite concentration (Bunch and Bejerano, 1997; Bowser et al., 1998), high salinity and alkalinity (pH>8) (Chang and Plumb, 1996a; Perera et al., 1997), as well as high stocking density (Shoemaker et al., 2000) contribute to the development of natural disease outbreaks of *S. agalactiae* infections in tilapia species. Such factors are commonly associated with intensive aquaculture practise and some have been

shown to cause a stress response in the fish resulting in a suppression of the immune system in fish. Further studies have also investigated the role of the pathogen as well as the susceptibility of the host, during the on-set of a streptococcal infection in fish. The key influencing factors reported that can vary the severity of the infections include the bacterial strain used or virulence expression from the bacterium, the bacterial concentration, the fish species, individual fish response, route of infection, stock density, fluctuating environment condition and management variation as well as other factors associated with multiple or co-infections (Shoemaker et al., 2000; Austin and Austin, 2007; Agnew and Barnes, 2007; Bromage and Owens, 2009).

The presence of secondary invaders such as other microbes may also influence the establishment of *S. agalactiae* infections in farmed conditions. Few studies have been published on co-infections, the study by Xu et al. (2007, 2009), however, demonstrated that a concurrent infection of Nile tilapia by either *Gyrodactylus niloticus* or *Ichthyophthirius multifiliis* with *Streptococcus* spp. resulted in increased host susceptibility and mortality following exposure to the bacterial pathogen. Evans et al. (2007) found that infection with *Trichodina* sp. increased the susceptibility and mortality of fish to streptococcal disease caused by either *S. iniae* or *S. agalactiae*. Their results suggested an enhancement of bacterial invasion by ectoparasites promoting a significant mortality increase due to multiple infections. This may be due to the parasite damaging the fish's epithelium and providing portals of entry for invasive bacteria (Cusack and Cone, 1986). The parasite may also act as a vector for

bacteria since bacterial colonies were found on the tegumental surface of the parasites (Busch et al., 2003).

1.5 Clinical signs and lesions

Infection by *S. agalactiae* in tilapia leads to various clinical signs, including the presence of external and internal lesions. Affected tilapia presents nervous with behavioural abnormalities and systemic bacterial infection. The classical clinical signs reported with *S. agalactiae* infections in tilapia include erratic swimming (such as spiraling or spinning), uni- or bi-lateral exophthalmia also known as “pop-eye”, corneal opacity, and haemorrhages in the eye, at the base of the fins and in the opercula. Darkening of the skin, distended abdomen and body curvature or vertebral deformity have also been reported in affected tilapia (Plumb, 1999; Salvador et al., 2005; Suanyuk et al., 2005; Austin and Austin, 2007; Siti-Zahrah et al., 2008; Abuseliana et al., 2010, 2011). Not all of these clinical signs are present in all of the affected fish and in some cases, the affected fish showed no obvious clinical signs before sudden death (Eldar et al., 1995a; Musa et al., 2009; Pretto-Giordano et al., 2010a; Rodkhum et al., 2011; Ye et al., 2011).

Internally, the disease appears to affect the liver, spleen, kidney, heart, eyes and brain, where abnormalities are visible grossly. The affected fish show congestion and haemorrhage of the liver, spleen, kidney and brain. The spleen and liver are often enlarged and the liver is pale in colour, inflammation around the heart and kidney has been reported as well as softening of the brain and

the occasional accumulation of fluid within the abdominal cavity or ascites (Eldar et al., 1994, 1995a; Salvador et al., 2005; Suanyuk et al., 2005; Musa et al., 2009; Pretto-Giordano et al., 2010a).

1.6 Pathogenesis

The pathogenesis of *S. agalactiae* infection in tilapias is not yet fully described or understood. The initial pathological changes in naturally infected fish were first observed in the blood vessels, bacterial colonies and exotoxin were observed in association with tissue lesions particularly in the liver, spleen, kidney and brain (Chen et al., 2007; Suanyuk et al., 2008; Zamri-Saad et al., 2010). Bacteria led to local necrosis, enter and multiply within macrophages and subsequent invasion of the blood stream (Eldar et al., 1994; Evan et al., 2002; Musa et al., 2009). Macrophages may act as a vehicle for *S. agalactiae*, allowing the bacterium to cross the blood-brain barrier and enter the central nervous system and to be more easily disseminated to other organs and tissues described as a bacterial septicemia (Evans et al., 2001; Nguyen et al., 2001b; Bowater et al., 2012). Failure of initial phagocytosis and killing of the bacteria by the host immune response will allow the establishment of disease.

Histopathological changes in *S. agalactiae*-infected tilapia were observed in several internal organs, particularly the spleen, eyes and brain. The liver and spleen were congested and vacuolated with focal necrosis (Suanyuk et al., 2008; Filho, et al., 2009; Zamri-Saad et al., 2010). The kidneys were severely congested and haemorrhagic with extensive interstitial nephritis (Suanyuk et al.,

2008; Zamri-Saad et al., 2010). Granulomas were found in the brain (Suanyuk et al., 2008; Hernández et al., 2009), and also found in the spleen, kidney and ovary (Chang and Plumb, 1996b; Chen et al., 2007; Abuseliana et al., 2011; Rodkhum et al., 2011). Severe mononuclear infiltration in the heart, spleen, kidney, liver, intestine and eyes were also observed (Chang and Plumb, 1996b; Filho et al., 2009). The meninges were thickened by the infiltration of macrophages and lymphocytes resulting in meningoencephalitis (Eldar et al., 1994, 1995a; Chang and Plumb, 1996b; Chen et al., 2007; Filho et al., 2009; Mian et al., 2009; Zamri-Saad et al., 2010; Abuseliana et al., 2011; Rodkhum et al., 2011). Bacteria phagocytised by macrophages were seen in the spleen, heart and brain (Chang and Plumb, 1996b; Chen et al., 2007; Hernández et al., 2009; Zamri-Saad et al., 2010).

The pathological findings in the brains and eyes of diseased fish correlated with the clinical behavioural abnormalities; for example, the presence of meningitis would explain the erratic pattern of swimming or central nervous system involvement (Eldar et al., 1995a; Chang and Plumb, 1996b; Chen et al., 2007; Filho et al., 2009). In addition, heterophil infiltration into the periorbital tissues, choroid, and oedematous or inflammatory exudates exerting pressure were corresponding to gross lesions of the exophthalmoses and corneal opacity (Rasheed et al., 1985; Filho et al., 2009).

1.7 Diagnosis

Clinical disease diagnoses of bacterial infections in fish species follow the same principles as for other vertebrate animals. During a disease outbreak the optimal approach would be to take an outbreak history combined with fish tissues of affected animals with clear clinical signs of disease and corresponding apparently normal fish from the same site/pond/cage. Diagnosis of *S. agalactiae* infection in tilapia should be based on typical clinical signs, lesions, and demonstration of Gram-positive coccal bacteria, isolated from internal organs of affected fish, pathological findings and confirmation of the bacterial species with other laboratory methods. The pathogen is routinely isolated from the spleen, kidney, eyes and brain using media such as tryptone soya agar (TSA), brain heart infusion agar (BHIA), Todd-Hewitt broth agar (THBA), blood agar or selective agar containing thallium acetate-oxolinic acid (Buller, 2004; Austin and Austin, 2007). The incubation period is reported at between 24-48 hr at 25-35°C (Inglis et al., 1993; Plumb, 1999).

The bacterial isolates are then characterised by biochemical tests including API 20 Strep system, API Rapid Strep 32 system (Kitao et al., 1981; Plumb, 1999). Beside this, Lancefield serogrouping should be performed by using the appropriate specific antisera (Lancefield, 1933), as *S. agalactiae* belong to the group B serogroup. Selected organs from affected fish including kidney, spleen, eyes, brain, liver, intestine, gills, heart and muscle should be fixed in 10% (v/v) neutral buffered formalin, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin (H&E) for histopathology (Roberts, 2001) and

immunohistochemistry (Hetzel et al., 2003; Hernández et al., 2009). In addition, a rapid optical immunoassay has also been used to detect and identify group B *Streptococcus* antigen from bacterial culture and clinically-infected fish specimens (Evans et al., 2010). Recently, molecular techniques such as 16Sr PCR have been usefully applied as part of the diagnostic procedure to confirm the presence of the suspected aetiological agent (Berridge et al., 2001; Phuektes et al., 2001; Duremdez et al., 2004; Mata et al., 2004; Jiménez et al., 2011; Pourgholam et al., 2011; Ye et al., 2011).

The diagnosis of streptococcal infections in fish has been complicated in the past because similar clinical signs are seen in the same fish species due to other Gram positive bacterial pathogens. There are several other closely related Gram-positive cocci that share similar features with *S. agalactiae* and in natural infections may present similar gross clinical signs of disease. These include *Streptococcus iniae*, *Lactococcus garviae*, *L. piscium*, *Vagococcus salmoninarum* and *Enterococcus* sp. (Kusuda et al., 1991; Inglis et al., 1993; Eldar et al., 1994; Buller, 2004; Austin and Austin, 2007). Therefore, the identification of *S. agalactiae* in tilapia should include a combination of standard conventional methods, biochemical characteristics, Lancefield serogrouping and species-specific PCRs to ensure that the right aetiological agent is identified.

1.8 Treatment

The most common treatment strategy during a confirmed bacterial disease outbreak in farmed fish populations is to administer antibiotics. These are predominantly administered in the feed. Overall, most strains of *S. agalactiae* have been shown to be susceptible to a variety of antibiotics in many fish species (Robinson and Meyer, 1966; Baya et al., 1990; Evans et al., 2002; Duremdez et al., 2004; Al-Marzouk et al., 2005; Geng et al., 2011). Published literature has described *S. agalactiae* isolates recovered from tilapia which were sensitive to various antimicrobial agents (Table 1.2). Differences in resistance and sensitivity to antibiotics among the same bacterial species could be due to serotype variety and frequent or inappropriate use of chemotherapy such as inadequate concentration or duration of these drugs in fish farms (Musa et al., 2009; Abuseliana et al., 2010).

Table 1.2 Comparison of antibiotic sensitivities of *Streptococcus agalactiae* isolated from naturally infected tilapia reported in the scientific literature.

Sensitive/ resistant to	Antibiotic drugs	References
Sensitive	oxytetracycline	Suanyuk et al. (2005), Jantawan et al. (2007), Musa et al. (2009)
	amoxicillin	Jantawan et al. (2007), Musa et al. (2009), Abuseliana et al. (2010)
	ampicillin, erythromycin	Eldar et al. (1994), Suanyuk et al. (2005), Jantawan et al. (2007), Musa et al. (2009), Abuseliana et al. (2010)
	chloramphenicol	Eldar et al. (1994), Jantawan et al. (2007), Musa et al. (2009), Abuseliana et al. (2010)
	tetracycline, vancomycin	Eldar et al. (1994), Abuseliana et al. (2010)
	lincomycin	Musa et al. (2009), Abuseliana et al. (2010)
	penicillin	Eldar et al. (1994), Suanyuk et al. (2005)
	ciprofloxacin, cefalotin	Eldar et al. (1994), Jantawan et al. (2007)
	nitrofurantoin	Eldar et al. (1994), Musa et al. (2009)
	mezlocillin, methicillin, cefuroxime, ofloxacin, fusidic acid	Eldar et al. (1994)
	doxycycline, enrofloxacin	Jantawan et al. (2007)
	flumequin, novobiocin, fosfomycin, oleandomycin	Musa et al. (2009)
	rifampicin, gentamicin*	Abuseliana et al. (2010)
	sulphamethoxazole/ trimethoprim*	Eldar et al. (1994), Jantawan et al. (2007), Abuseliana et al. (2010)
Resistant	oxolinic acid	Suanyuk et al. (2005), Jantawan et al. (2007), Musa et al. (2009)
	nalidixic acid	Eldar et al. (1994), Suanyuk et al. (2005), Jantawan et al. (2007), Musa et al. (2009)
	kanamycin, streptomycin	Musa et al. (2009), Abuseliana et al. (2010)
	neomycin	Jantawan et al. (2007), Abuseliana et al. (2010)
	amikacin	Eldar et al. (1994), Abuseliana et al. (2010)
	sulphamethoxazole	Jantawan et al. (2007), Musa et al. (2009)
	colistin	Eldar et al. (1994)
	polymicin B	Jantawan et al. (2007)
	oleandomycin	Musa et al. (2009)
	gentamicin*	Eldar et al. (1994)
	sulphamethoxazole/ trimethoprim*	Suanyuk et al. (2005)

**The sensitivity of the isolates to gentamicin and sulphamethoxazole/trimethoprim is variable.*

If the clinical outbreak is reported quickly with appropriate samples taken and laboratory confirmation of the pathogen combined with antibiogram then the prescribed treatment should work if a therapeutic dose is provided. Although antibiotics or synthetic and natural compounds including herbs have demonstrated activity in *in vitro* and *in vivo* studies against pathogens, their efficacy is not always similar when used under field conditions. There are numerous reasons for this but the lack of response to a therapeutic dose. This is probably because of the rapid onset of anorexia in the sick animals and the appearance of drug resistant strains (Smith et al., 1994). Moreover, drug residues and withdrawal periods are also of concern in farmed fish destined for human consumption and antibiotics may also be harmful to environment. Therefore, antibiotic therapy may not always be successful, but improvement in stock density, water quality, environment and management will help to mitigate the problem. Therefore a combined approach is more effective.

1.9 Prevention and control

Improving water quality and environmental conditions, and reduction of overcrowding are the usual preventive measures to limit *S. agalactiae* infection in intensively farmed tilapia. Avoiding overfeeding, minimising unnecessary handling or transportation, and the prompt removal of moribund and dead fish, periodic cleaning of the tanks and adequate disinfection of all production unit and utensils should also be done to decrease the transmission of pathogen and to reduce the risk of disease outbreak. Moreover, vaccination and the use of herbs, synthetic compounds, probiotics, non-specific immunostimulants are all

thought to have some potential in aquaculture for controlling streptococcosis (Inglis et al., 1993; Plumb, 1999; Buller, 2004).

Vaccination of fish by immersion and oral routes are widely practiced in aquaculture as they are relatively easy to deliver, less labour intensive, less time consuming, and thought to be less stressful to the fish; although, the injection vaccination is feasible using semi-automatic vaccination devices or by hand. There are few studies on vaccination of tilapia against *S. agalactiae* infection. Eldar et al. (1995c), Pasnik et al. (2005), Tengjaroenkul and Yowarach (2009) and Pretto-Giordano et al. (2010b) have developed an injectable modified-killed *S. agalactiae* vaccine composed of whole cell and bacterial protein for the prevention of streptococcosis in tilapia. This vaccine gave a relative percent of survival (RPS) of between 49 and 100%, indicating that these vaccines were efficient in experimental studies against the infection in Nile tilapia. Evans et al. (2004a, 2005) showed the efficiency of the formalin-killed *S. agalactiae* vaccine when administered by intraperitoneal (i.p.) injection. In this study, the RPS was 80% in the 30g tilapia and 25% in the 5g tilapia, respectively, whereas the RPS value of bath immunisation (34%) was lower than IP vaccination. Oral delivery of the killed whole cell *S. agalactiae* vaccine incorporated in feed has also been tried against infection by *S. agalactiae* in tilapia (Firdaus-Nawi et al., 2011). Therefore, there are varied responses to the different types of vaccines produced as well as the different delivery methods. Currently, the AQUAVAC® Strep Sa commercial vaccine (MERCK Animal Health) has been developed providing protection against *S. agalactiae* biotype

II strain infections in tilapia farms. It is an inactivated, oil-adjuvanted vaccine which is administered intraperitoneally as a single injection dose to fish weighing no less than 15 grams. The vaccine showed that high levels of protection develop by 21 days post-vaccination (at 28°C water temperature) and that protection lasts for at least 30 weeks under experimental conditions.

Herbs have also been reported as effective in controlling diseases in aquaculture. Research on using herbs to control *S. agalactiae* in tilapia is increasing with the demand for more environmentally friendly aquaculture processes. For example, Borisutpeth et al. (2005), Wongthai et al. (2011) and Pirarat et al. (2012) reported the *in vitro* antibacterial activity of 4 herb extracts, *Hibiscus sabdariffa, Cassia fistula, Citrus grandis* (*C. maximus*) and Red Kwao Krua (*Butea superb* Roxb.) against *S. agalactiae* isolated from diseased Nile tilapia. Rattanachaikunsopon and Phumkhachorn (2009, 2010) showed reduced mortality of *S. agalactiae* infected Nile tilapia when fed a diet supplemented with the herb *Andrographis paniculata* or *Cratoxylum formosum* extracts. Moreover, the results of Rattanachaikunsopon and Phumkhachorn (2010) suggest that the aqueous extract of *C. formosum* has potential to be used as an immunostimulant to prevent *S. agalactiae* infection. The study showed that an aqueous extract of *C. formosum* added to the fish's diet improved their innate immune responses including phagocytic, lysozyme and respiratory burst activities in tilapia. Similarly, feeding with dried extract of rosemary leaves (*Rosmarinus officinalis*) and *Pseuderatherum palatiferum* leaf significantly reduced mortality following infection with *S. agalactiae* in tilapia

under experimental conditions (Zilberg et al., 2010; Suebsomran and Taveekitjakan, 2011).

Currently, there is considerable interest in the use of many synthetic compounds and bacteria in fish diets to control *S. agalactiae* infection in tilapia. For example, Samrongpan et al. (2008) showed the benefit of mannan-oligosaccharide (MOS) as a feed supplement for Nile tilapia fry in terms of improved growth and enhanced disease resistance against *S. agalactiae*. Ng et al. (2009) reported that red hybrid tilapia fed with 0, 1, 2 or 3 g/kg organic acid-added to their diets showed significantly higher survival rates (66.7-83.4%) than the control group (41.7%) after challenged by immersion with 10^5 CFU/ml *S. agalactiae*. Probiotics have also been investigated; a study by Srisapoome et al. (2011) demonstrated that mortality decreased in tilapia fed on a diet supplemented with the bacterium *Bacillus pumilus*. These studies showed the potential to enhance disease resistance caused by *S. agalactiae* in tilapia.

However, much more work is required in the efficacious control and treatment of aquatic *S. agalactiae* infections. The effectiveness of these vaccines, herbs, synthetic compounds and probiotics *in vivo* is dependent on the bacterial serotype, target fish species, route of administration, concentration, composition, type, culture conditions and other factors. At present the pathogenesis of the infection is poorly understood in farmed fish. Therefore, it is very difficult to prevent the disease, especially as it appears when the fish are stressed by poor water quality and management conditions in farm as well as in

multiple infections. Moreover, the bacterium is considered ubiquitous making eradication near-impossible thus it does not seem possible to eliminate the pathogen from the fish and aquatic environment.

1.10 Conclusion

Streptococcus agalactiae is an important pathogen affecting a wide range of fish species including both freshwater and marine animals throughout the world. Moreover, it is regarded as one of the most significant pathogens affecting warm-water fish species. Tilapia culture is important for global food security and this fish is highly susceptible to *S. agalactiae* infection resulting in serious economic losses. Affected tilapia present with a wide range of nervous signs and gross pathological signs resulting in a systemic bacterial infection. Histologically, the affected fish show congestion, haemorrhages and inflammation in several internal organs, particularly the liver, heart, spleen, kidney, eyes and brain. Disease diagnosis should be based on typical clinical signs, including lesions, viable bacterial isolation/recovery from affected fish and aetiological identification through subsequent laboratory methods including standard conventional methods, biochemical characteristic tests, Lancefield serogrouping, histopathology, with immunohistochemistry and molecular techniques, as appropriate. Although chemotherapy and vaccination may not be always successful, good water quality, proper management and environment condition are necessary to prevent the outbreak and spread of disease in intensively farmed tilapia.

1.11 Project outline

The main objective of this study was to investigate a range of factors affecting variability in experimental *S. agalactiae* infections in Nile tilapia (*O. niloticus*). The specific tasks involved were to:

- Identify and characterise as *S. agalactiae* with a range of laboratory based tests
- Assess whether the *S. agalactiae* isolated could infect healthy Nile tilapia using two exposure routes of infection including; immersion and intraperitoneal (i.p.) injection
- Investigate whether the weight or age of fish associate the severity of *S. agalactiae* infection in Nile tilapia
- Develop methodologies to determine the risk-taking phenotype in Nile tilapia and examine whether the different coping styles influence the susceptibility to *S. agalactiae* infection in Nile tilapia

Chapter 2 - Identification and characterisation of *Streptococcus agalactiae* recovered from farmed tilapia

2.1 Abstract

The purpose of this study was to identify and characterise bacterial isolates recovered from farmed tilapia during natural disease outbreaks where affected animals presented with clinical signs of streptococcosis. These bacteria were identified and characterised as *Streptococcus agalactiae* by standard conventional methods, biochemical tools including the API 20 Strep system, Lancefield serogrouping and species-specific PCR assay. A growth curve and standard curve were used to determine the growth patterns of two bacterial isolates. The results demonstrated that the isolates were Gram-positive cocci, either β- or non-haemolytic (γ), non-motile, oxidase negative and serogroup B. In addition, they were able to grow on Edwards medium (modified) agar as blue colonies and growth was observed in TSB from 22 to 37 ^{o}C and in TSB with 0.5-5% NaCl. The biochemical profiles showed some differences in the reactions while all the PCR samples showed similarities to the *S. agalactiae* type strain. Based on those results, these isolates were identified as group B *S. agalactiae*.

2.2 Introduction

Streptococcus agalactiae is an important bacterial pathogen associated with fish losses and high morbidity and is the aetiological agent of fish streptococcosis (Baya et al., 1990; Eldar et al., 1994; Evans et al., 2002; Duremdez et al., 2004). Clinically, the affected fish present grossly with exophthalmoses, erratic swimming and high mortality, and infections have been reported at water temperatures greater than 15 ^{o}C (Eldar et al., 1994; Kawamura et al., 2005). This infectious disease affects a variety of wild and cultured fish in both freshwater and marine environments. In particular, it has become a major disease problem in intensive aquaculture systems resulting in significant economic losses in cultured tilapia (*Oreochromis* spp.) world-wide (Salvador et al., 2005; Suanyuk et al., 2005; Suanyuk et al., 2008; Mian et al., 2009; Musa et al., 2009; Zamri-Saad et al., 2010).

Streptococcus sp. is a Gram-positive, coccus bacterium, which mostly occurs in long chains. The colonies appear small, yellowish to grey, translucent, rounded, slightly raised, when grown on solid agar. They are approximately 0.1-1 mm in diameter on tryptone soya agar (TSA) when incubated at 25-35 ^{o}C for 24 to 48 h that can show either α-, β- or non-haemolysis (γ) on blood agar (Kitao et al., 1981; Buller, 2004). In addition, they are non-motile, non-capsulated, non-spore forming and negative for oxidase and catalase (Inglis et al., 1993; Plumb, 1999). The phenotypic characterisation is rather problematic for primary identification during disease outbreaks as other Gram-positive cocci also associated with disease outbreaks in fish can give similar identification profiles,

leading to mis-diagnosis. This is particularly true for *Lactococcus* sp. and *Enterococcus* sp. (Kusuda et al., 1991; Buller, 2004). Therefore, it is suggested that the bacterial identification of streptococci should include a combination of conventional phenotypic, biochemical characteristics and Lancefield serogrouping (Kitao et al., 1981; Lancefield, 1933; Plumb, 1999). In addition, many species-specific PCRs of the different isolates have been produced to assist confirmation and these may be useful at the tertiary identification level (Phuektes et al., 2001; Chen et al., 2001; Mata et al., 2004a; Roach et al., 2006).

In the present study, a range of *S. agalactiae* isolates were identified and two bacterial isolates recovered from farmed tilapia, presenting with clinical signs of streptococcosis during natural disease outbreaks were fully characterised.

2.3 Materials and methods

2.3.1 Bacterial strain recovery and identification tests

A range of bacterial isolates including 14 *S. agalactiae* tested, 1 *S. agalactiae* type strain (National Collection of Industrial and Marine Bacteria; NCIMB 701348), 1 *Streptococcus iniae* type strain (American Type Culture Collection; ATCC 29178), 1 *Lactococcus garviae* type strain NCIMB 70215 and 1 *Enterococcus faecium* type strain NCIMB 11508 were used in this study and are listed in Table 2.1. The 14 *S. agalactiae* isolates were originally recovered from different natural disease outbreaks in farmed tilapia within South America and Asia (Table 2.1). These isolates were from disease outbreaks reported to cause high mortality and morbidity where fish presented with clinical signs associated with streptococcal infection (pers.com. H. Ferguson & M. Crumlish). Histologically, streptococcosis was described from the clinical pathology samples provided to the Veterinary Diagnostic Laboratories, Institute of Aquaculture, Stirling, UK and the isolates were identified as streptococcal species following routine identification methods performed by staff at the Veterinary Diagnostic Laboratories, Institute of Aquaculture, Stirling, UK. Pure cultures were then stored on protect beads (Technical Service Consultants Limited, UK) at -70 ^{o}C until required for further use.

Table 2.1 *Streptococcus agalactiae* isolates included in this study.

Number	Isolate	Source
1	*S. agalactiae*	Vietnam
2	*S. agalactiae*	Columbia
3	*S. agalactiae*	Columbia
4	*S. agalactiae*	Columbia
5	*S. agalactiae*	Columbia
6	*S. agalactiae*	Columbia
7	*S. agalactiae*	Honduras
8	*S. agalactiae*	Thailand
9	*S. agalactiae*	Thailand
10	*S. agalactiae*	Kuwait
11	*S. agalactiae*	Kuwait
12	*S. agalactiae*	Kuwait
13	*S. agalactiae*	Kuwait
14	*S. agalactiae*	Kuwait
15	*S. agalactiae* type strain	NCIMB 701348
16	*S. iniae* type strain	ATCC 29178
17	*L. garviae* type strain	NCIMB 70215
18	*E. faecium* type strain	NCIMB 11508

Identification: NCIMB, National Collection of Industrial and Marine Bacteria; ATCC, American Type Culture Collection

The bacterial isolates were grown on tryptone soya agar (TSA; Oxoid, U.K.), with 5% (v/v) sheep blood agar (Oxoid, U.K.) and Edwards medium (modified) agar (Oxoid, UK), incubated for 48 h, at 28 ^{o}C. They were identified using conventional bacteriology identification methods including Gram stain, oxidase test, motility test and haemolysis test (Frerichs and Millar, 1993).

Growth characteristics of the bacterial isolates to various temperature tolerances and sodium chloride (NaCl) concentrations were determined. This was performed by placing 2-3 colonies from each bacterial strain into 2 ml of sterile (0.85% w/v) saline solution in a sterile bijoux and bacterial density was adjusted using sterile 0.85% saline solution to give a bacterial concentration equal to a MacFarland Standard no. 1. A 100 µl sample of this bacterial suspension was transferred to each test bijoux containing 5 ml of tryptone soya broth (TSB; Oxoid, U.K.) and incubated. For the temperature tolerance test, a bacterial suspension from each isolate was incubated as described above at 4, 15, 22, 28 or 37 ^{o}C. Growth tolerance in varied concentrations of NaCl was determined at 0.5, 1, 2, 3, 4, 5, 6, 6.5 and 7% (w/v) NaCl in TSB inoculated as described above and incubated at 28 ^{o}C. For both the temperature and the salt tolerance tests, negative controls (TSB only) were included. The samples were checked for turbidity daily up to 4 days after which time the results were recorded.

Biochemical profiles were produced following the manufacturers guidelines for the API 20 Strep system (BioMerieux®, U.K.) and Lancefield serogrouping B by the use of the Slidex strepto kit test (BioMerieux®, U.K.). The *S. agalactiae* NCIMB 701348 strain was used as a positive control when performing the assays. Two bacterial isolates from different geographic origins (isolate number 1 and number 2) were selected for further investigation and a growth curve and standard curve was produced.

2.3.2 Polymerase chain reaction (PCR) assay

Bacterial DNA extraction was performed following a crude DNA extraction method as described in Seward et al. (1997). Briefly, a single bacterial colony was aseptically removed from a pure culture grown on TSA and inoculated into 5 ml of TSB for 24 h at 28 ^{o}C. The bacterial suspension was centrifuged at 3,000 rpm for 15 min at 4 ^{o}C then the bacterial pellet was resuspended with 1 ml of sodium chloride-Tris-EDTA (STE) buffer (0.1 M NaCl, 10 mM Tris at pH 8, 1 mM EDTA) and centrifuged at 13,000 rpm for 1 min. The pellet was immediately resuspended in 100 μl of Tris-EDTA (TE) buffer (10 mM Tris at pH 8, 1 mM EDTA), then heated at 95 ^{o}C for 10 min and placed on ice. After that, the suspension was centrifuged at 13,000 rpm for 1 min to remove cellular debris. The DNA concentration was quantified using a spectrophotometer Nanodrop® ND-1000 (ThermoScientific, USA) and DNA aliquots were then stored at -20 ^{o}C until required.

A PCR was performed on the bacterial DNA according to Phuektes et al. (2001) with minor modifications. Each 25 μl reaction consisted of 2.5 μl of 10 X buffer, 2 μl of 25 mM $MgCl_2$, 0.5 μl of 20 mM dNTP (ThermoScientific, USA), 0.5 μl of 5 units/μl Klear Taq enzyme (KBiosciences, UK), 2 μl of bacterial DNA at approximately 500 ng/μl, 1.5 μl with 10 pmol of each primer (STRA-AgI and STRA-AgII; MWG Oligo, Germany) and 14.5 μl of milliQ ultrapure water. The primer set was *S. agalactiae*-specific STRA-AgI: 5'-AAGGAAACCTGCCATTTG-3' and STRA-AgII: 5'-

TTAACCTAGTTTCTTTAAAACTAGAA-3', which were expected to give an amplification of 270 bp. Bacterial DNA extracted from *S. agalactiae* NCIMB 701348 was used as a positive control and a negative control was included which had no DNA template.

After an initial denaturation at 95 ^{o}C for 15 min, the mixtures were amplified in 35 cycles, each consisting of denaturation at 95 ^{o}C for 30 sec, primer annealing at 55 ^{o}C for 30 sec and extension at 72 ^{o}C for 25 sec with a final extension for 10 min at 72 ^{o}C in an automated thermal cycler (Biometra®, Germany). Then 10 μl of each amplified PCR product was electrophoresed in a 1.5% w/v agarose gel (Biogene, UK), with a DNA molecular size marker (Tracklt™ 100 bp DNA ladder, Invitrogen™) in parallel. Electrophoresis in 0.5 X Tris-acetate-EDTA (TAE) buffer was performed at 100 V for 90 min. The 2% ethidium bromide stained gel was visualised under u.v. light.

2.3.3 Production of a bacterial growth curve

The bacterial strains identified as isolate number 1 or number 2 were grown on TSA for 48 h at 28 ^{o}C, then 1 colony of pure growth was aseptically removed using a sterile bacterial loop and inoculated into 10 ml of TSB for 24 h at 28 ^{o}C. This was then aseptically placed into 400 ml TSB and incubated with continual shaking (Kuhner shaker ISF-1-W, Switzerland) at 140 rpm for 28 ^{o}C. Individual inoculated suspensions were sampled at 0, 3, 6, 9, 12, 15, 24, 30, 48, 54, 72, and 96 h post inoculation, respectively. A single sterile TSB bottle was used as

the negative control and a purity check was performed as a sterility check at the end of the final incubation time. At each sampling time, 0.5 ml TSB was aseptically removed and viable bacterial colony counts performed using the Miles and Misra method (Miles et al., 1938). The results were plotted as bacterial culture density or viability versus time as a bacterial growth curve. All bacterial samples were checked on purity plates and identified as *S. agalactiae* by the identification tests and PCR assay as described in section 2.3.1 and 2.3.2.

2.3.4 Production of a bacterial standard curve

The bacteria isolate (number 1 or number 2) was subcultured onto TSA and incubated at 28 ^{o}C for 48 h. A single pure colony was inoculated into 40 ml TSB and then incubated at 28 ^{o}C at mid-log phase with continual shaking (140 rpm in Kuhner incubator). The same volume of sterile TSB without bacteria was added and used as the negative control. The bacterial suspensions were centrifuged once at 3,500 × g for 15 min at 4 ^{o}C. The supernatant was removed carefully and the pellet was resuspended with 5 ml of sterile 0.85% (w/v) saline solution and then adjusted spectrophotometrically to an optical density (OD_{610nm}) value ranging from 1 to 0.1 absorbency units. At each OD_{610nm} value the bacterial suspension was serially diluted (10-fold dilutions) in sterile 0.85% saline solution from 10^{-1} to 10^{-6} dilution series and 6 × 20 µl of bacterial dilutions at 10^{-4} to 10^{-6} were dropped onto sterile TSA plates. These were then left to dry flat at room temperature for approximately 1h, sealed using Nescofilm (Alfresa Pharma Corporation, Japan) and incubated at 28 ^{o}C for 48 h. Viable colony

counts were performed for each OD_{610nm} value obtained and a standard curve was produced by plotting the actual OD values against the number of viable bacteria (CFU/ml). All samples were also purity checked and identified as *S. agalactiae* by the standard conventional and biochemical methods as described in section 2.3.1.

2.4 Results

2.4.1 Bacterial strain recovery and identification tests

All bacterial isolates tested in this study gave pure cultures when grown on TSA media. Phenotypically, the isolates appeared slightly mucoid, white, small round as pin-point colonies on TSA, displaying either β- or non-haemolysis (γ) on sheep blood agar, blue colonies and no fermentation on Edwards medium (modified) agar after 48 h of incubation at 28 ^{o}C. They were all Gram-positive cocci, mostly in long chains, non-motile, oxidase negative and Lancefield serogroup B. In addition, these isolates were able to grow from 22 to 37 ^{o}C, but not at 4 and 15 ^{o}C. No bacterial growth was observed for any of the isolates at higher than 5% NaCl. The phenotypic characteristics of isolates tested were similar to the *S. agalactiae* type strain. However, differences were found between the growth on Edwards medium (modified) agar, temperature and salt tolerance growth test, and serogrouping when compared with other Gram positive cocci (Table 2.2).

Biochemical profiles of the isolates tested were a positive Voges-Proskauer reaction while the isolates were negative for esculin hydrolysis, pyrrolidonyl arylamidase, α-galactosidase, β-glucuronidase, β–galactosidase, arabinose, mannitol, sorbitol, inulin, raffinose, amygdalin and glycogen test. Variability was noted in these isolates for their reactions with hippurate hydrolysis, alkaline phosphatise, leucine arylamidase, arginine dihydrolase, ribose, lactose and trehalose test in the API 20 STREP system test compared with the *S. agalactiae* type strain. Moreover, the isolates tested gave predominantly different results to the type strains *S. iniae*, *L. garviae* and *E. faecium* isolates (Table 2.3).

Table 2.2 Comparison of phenotypic characteristics of the bacterial isolates tested with other *Streptococcus agalactiae*, *S. iniae*, *Lactococcus garviae* and *Enterococcus faecium.*

Test	**Isolate number**														***Type strains***			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	***S. agalactiae***	***S. iniae***	***L. garviae***	***E. faecium***
Growth on TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Haemolysis	β	β	non	non	non	non	non	β	β	β	β	β	β	β	B	β	non	non
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cell morphology	c	c	c	c	c	c	c	c	c	c	c	c	c	c	C	c	c	c
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lancefield group B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Growth on Edwards Medium (modified)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Growth on TSB at																		
4 ^{o}C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15 ^{o}C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
22 ^{o}C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
28 ^{o}C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37 ^{o}C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth on TSB in																		
0.5% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3% NaCl	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	-	+	+
4% NaCl	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	-	+	+
5% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+
6% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
6.5% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
7% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+

Identification: +, positive; -, negative; c, cocci

Table 2.3 Biochemical characteristics of the *Streptococcus agalactiae* isolates tested, and compared type strains of *S. agalactiae*, *S. iniae*, *Lactococcus garviae* and *Enterococcus faecium.*

Biochemical	**Isolate number**														***Type strains***			
reaction/enzyme	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	***S. agalactiae***	***S. iniae***	***L. garviae***	***E. faecium***
Voges–Proskauer	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
Hippurate hydrolysis	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	+
Esculin hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Pyrrolidonyl arylamidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
α-Galactosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Glucuronidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β–Galactosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Alkaline phosphatase	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-
Leucine arylamidase	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	+
Arginine dihydrolase	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	+
Utilisation of																		
Ribose	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	+
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+
Trehalose	+	+	-	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amygdalin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Glycogen	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-

Identification: +, positive; -, negative

2.4.2 Polymerase chain reaction (PCR) assay

PCR of all isolates tested were identified and showed 100% similarity to the *S. agalactiae* type strain and all gave a positive band at the correct molecular weight for the PCR reaction (270 bp, Figure 2.1). No bands were visible for *S. iniae*, *L. garviae*, *E. faecium* or for the negative control samples (Figure 2.1).

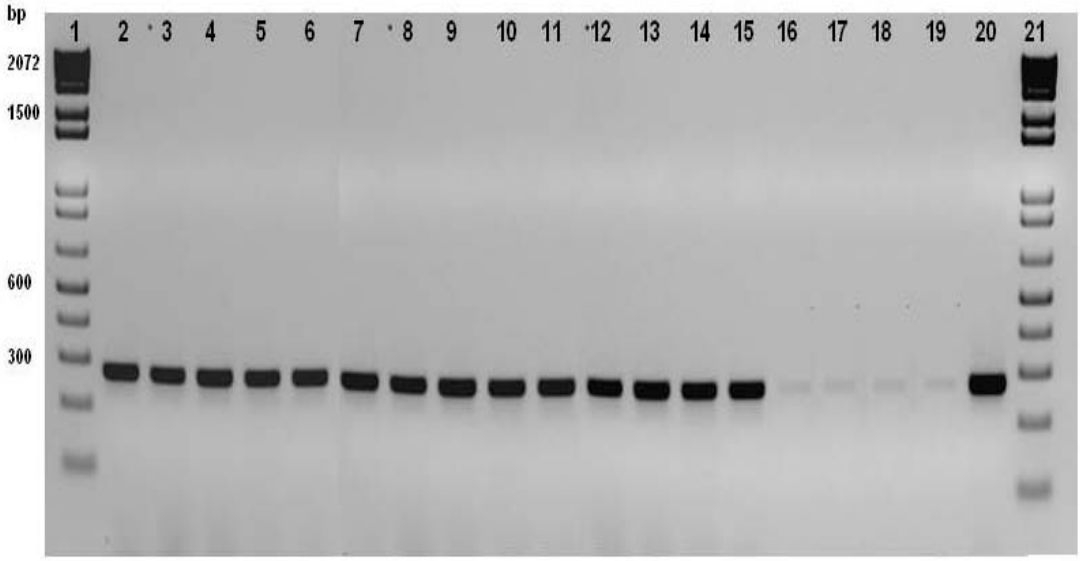

Figure 2.1 Specificity of the PCR for *Streptococcus agalactiae*. Lanes 1 & 21, 100 bp DNA ladder; Lanes 2-15, Isolates tested number 1-14 respectively; Lane 16, *S. iniae* type strain ATCC 29178; Lane 17, *Lactococcus garviae* type strain NCIMB 70215; Lane 18, *Enterococcus faecium* type strain NCIMB 11508; Lane 19, negative control (no DNA); Lane 20, positive control *S. agalactiae* type strain NCIMB 701348.

2.4.3 Bacterial growth curve

The bacterial growth curve results for *S. agalactiae* isolates number 1 and number 2 were similar and followed the typical bacterial growth phases including lag phase (0-3h), log phase (3-12h) stationary phase (12-30h) and death phase (30-96h) (Figure 2.2). No bacterial cultures were recovered from the negative control TSB sample only.

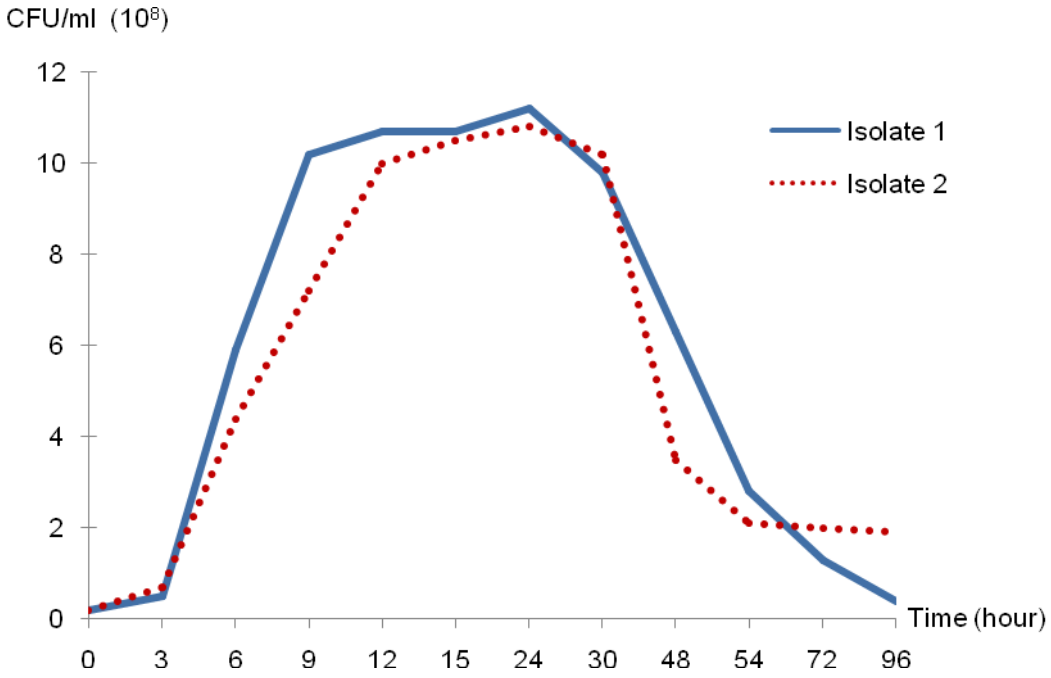

Figure 2.2 Growth curve of the *Streptococcus agalactiae* isolates tested at 28 ^{o}C, showing typical phase of growth of the number viable cells *versus* time.

The phenotypic and biochemical characteristics of both isolates tested were similar at the four different growth stages. Only pure growth on TSA was recorded from these samples. The colonies were all white, small round, Gram-positive cocci, non-motile, oxidase negative and Lancefield group B positive. The biochemical profiles at the different stages are shown in Table 2.4.

Table 2.4 Biochemical profiles of *Streptococcus agalactiae* isolates number 1 and number 2 during their growth curves.

Biochemical reaction/enzyme	**Bacterial isolates tested (hour)**									
	Number 1					**Number 2**				
	3	**6**	**9**	**24**	**48**	**3**	**6**	**9**	**24**	**48**
Voges–Proskauer	+	+	+	+	+	+	+	+	+	+
Hippurate hydrolysis	+	+	+	+	+	+	+	+	+	+
Esculin hydrolysis	-	-	-	-	-	-	-	-	-	-
Pyrrolidonyl arylamidase	-	-	-	-	-	-	-	-	-	-
α-Galactosidase	-	-	-	-	-	-	-	-	-	-
β-Glucuronidase	-	-	-	-	-	-	-	-	-	-
β–Galactosidase	-	-	-	-	-	-	-	-	-	-
Alkaline phosphatase	+	+	+	+	+	+	+	+	+	+
Leucine arylamidase	+	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	+	+	+	+	+	+
Utilisation of										
Ribose	+	+	+	+	+	+	+	+	+	+
Arabinose	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-
Trehalose	+	+	+	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-
Amygdalin	-	-	-	-	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-	-	-	-

Identification: +, positive; -, negative

All the samples of *S. agalactiae* isolate number 1 and number 2 at each time point on the growth curve were found positive for *S. agalactiae* by specific PCR assay (Figure 2.3).

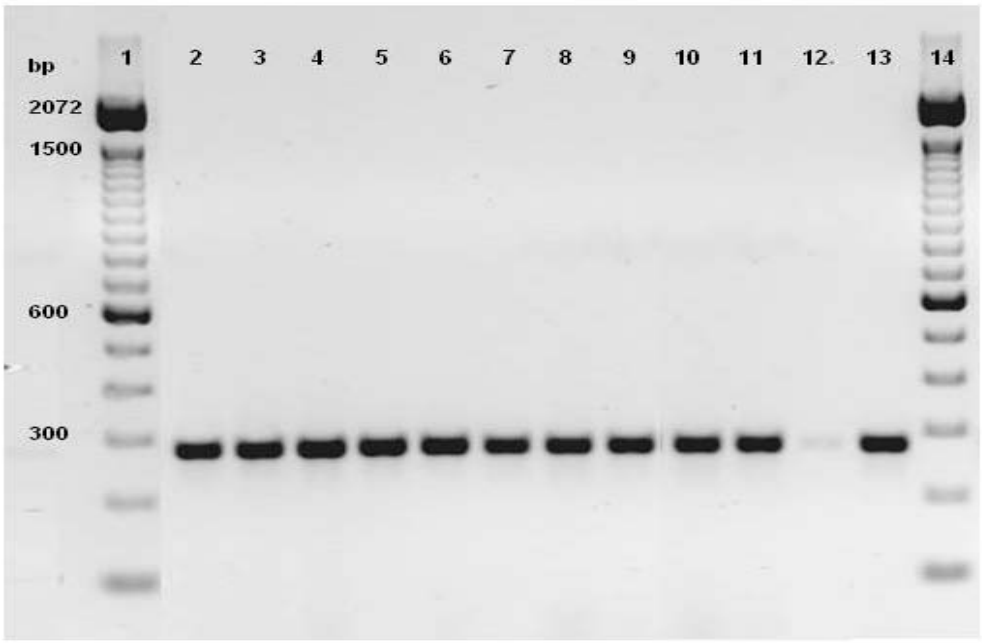

Figure 2.3 PCR amplification of samples from each time point on the growth curves. Lanes 1 & 14, 100 bp DNA ladder; Lanes 2-6, Isolates tested number 1 at 3, 6, 9, 24 and 48 h after inoculation, respectively; Lanes 7-11, Isolates tested number 2 at 3, 6, 9, 24 and 48 h after inoculation, respectively; Lane 12, negative control (no DNA); Lane 13, positive control *Streptococcus agalactiae* type strain NCIMB 701348.

2.4.4 Bacterial standard curve

A bacterial standard curve of viable CFU against absorbency was produced for both isolates of *S. agalactiae* number 1 and number 2. The standard curve patterns for each strain were very similar and the R^2 value per strain was also at an acceptable level (Figure 2.4). There was no bacterial growth recovered from the TSB negative control sample.

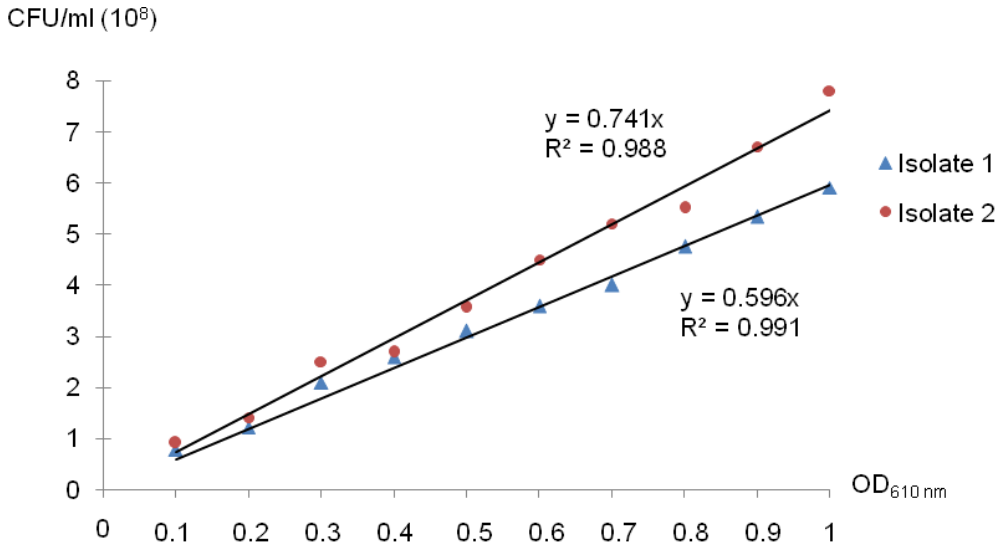

Figure 2.4 The standard curve of *Streptococcus agalactiae* isolates number 1 and number 2, at 28 ^{o}C.

All the samples of both isolates at each OD points on the standard curve were Gram-positive cocci, non-motile, oxidase negative and Lancefield group B. In addition, the biochemical characteristics of isolates tested were similar to the *S. agalactiae* type strain.

2.5 Discussion

A number of fish have been reported to be infected by *S. agalactiae* which is identified as one of the major bacterial disease pathogens affecting intensive fish farming systems and is known to cause significant economic loss in Nile and red tilapia through high fish mortalities during natural outbreaks (Suanyuk et al., 2005; Salvador et al., 2005; Musa et al., 2009; Zamri-Saad et al., 2010). Currently, there have been many reports of streptococcal identification and characterisation, which show considerable variation in methodologies applied to identify the bacterial strains (Baya et al., 1990; Eldar et al., 1994, 1995b; Vandamme et al., 1997; Yuasa et al., 1999; Colorni et al., 2002; Evans et al., 2002). The variation in the methods used combined with the heterogenic nature and variable reactions of the different *S. agalactiae* strains can be quite confusing. Whilst the haemolysis method and Lancefield serogrouping systems appear to be very useful rapid presumptive tests for the identification of streptococci, these should not be used in isolation and require validation using other reliable identification methods (Evans et al., 2002).

In this study, all the isolates that were tested and only the *S. agalactiae* type strain reacted serologically with the group B antiserum. This was in agreement with the published literature which described *S. agalactiae* as being the only streptococcal species classified to the serogroup B of the Lancefield serogrouping (Devriese, 1991; Facklam, 2002). In contrast, the *L. garviae* and *E. faecium* strains were serogroups N and D, respectively (Kusuda et al., 1991; Teixeira et al., 1996; Eldar et al., 1999a; Chen et al., 2001; Buller, 2004) whilst

S. iniae could not be classified to any serogroup (Eldar et al., 1995b; Yuasa et al., 1999; Dodson et al., 1999; Colorni et al., 2002). Although no serogrouping of the other bacterial species used in this study was performed, it would appear from the results obtained that the Lancefield serogrouping test is sensitive and specific for the individual bacterial species which is a reliable test to enable differentiation of *S. agalactiae* from other Gram-positive cocci also known to be associated with fish mortalities including *S. iniae, L. garviae* and *E. faecium*. This was shown by the reproducibility of the Lancefield serogroup B positive result from the *S. agalactiae* strains tested in this thesis over time.

Based on the results presented using the API 20 Strep system and compared with the analytical profile index of the system, the isolates tested showed some differences in the biochemical reactions. It is quite acceptable to compare the results with a reference standard identification and in this work the type strain *S. agalactiae* NCIMB 701348 was used (Eldar et al., 1994; Vandamme et al., 1997). However, other researchers have described difficulty in interpretation using the API 20 Strep system furthermore the results are sometimes not accurate or reliable when using commercial kits (Eldar et al., 1994; Lau et al., 2006; Roach et al., 2006). The main factors influencing this is the variability of bacterial isolates / strains, there may be a lack of useful information or no information available in the existing databases, differences in the age of bacteria and concentration of the bacterial inoculum used, and varied incubation temperature for enzyme reactions (Vandamme et al., 1997; Ravelo

et al., 2001; Evans et al., 2002). All of these factors have been reported as variable when comparing the identification profiles for *S. agalactiae* isolates.

The *S. agalactiae* grow well on a general purpose agar such as TSA and this was also the case for this study. Therefore TSA was thought to be a suitable agar for primary bacterial recovery in natural outbreaks as well as for use within the laboratory testing. Edwards medium (modified) agar may be used for isolating Streptococci from various samples which is convenient to detect a particular bacterium from samples containing mixed bacterial species. This agar is considered selective due to the presence of the thallium acetate and crystal violet or gentian violet and it is these chemicals that allow the differentiation of streptococci which appear blue in colour (Hardie, 1986). Using this medium in this study found that *S. agalactiae, S. iniae* and *E. faecium* were grown whilst *L. garviae* gave no colony growth. Therefore, the selective medium could be used to differentiate and identify streptococci and enterococci which are pathogenic to fish, although this test should not be used alone and further identification and characterisations are required.

Additionally, *Streptococcus* can be differentiated phenotypically from *Lactococcus* and *Enterococcus* on the basis of growth in fluid medium and in soft selective agar at 10 ^{o}C and 45 ^{o}C or in media containing 6.5% NaCl (Schleifer and Kilpper-Balz, 1987; Eldar et al., 1999a; Chen et al., 2001; Al-Marzouk et al., 2005). The salt and temperature tolerance results from this study showed that the *S. agalactiae* and *S. iniae* isolates were only able to grow in between 0.5-5% NaCl and at temperature of 22-37 ^{o}C. Consequently, the growth properties can be used to distinguish between *Streptococcus* from *Lactococcus* and *Enterococcus* spp.

A molecular technique, such as a species-specific PCR assay, is increasingly used to identify many different bacterial pathogens including the *Streptococcus* spp. (Berridge et al., 1998; Roach et al., 2006). It has been suggested that this technique could be used as an alternative method in routine diagnosis for accurate, rapid, sensitive and specific detection and identification of the pathogen from different sources (Berridge et al., 1998, 2001; Mata et al., 2004a, 2004b; Roach et al., 2006). There is no doubt that the PCR reactions can be quicker in providing a result compared with some of the more conventional bacterial identification methods. In this study, all isolates produced the same product size as *S. agalactiae* similar to the isolate obtained by Phuektes et al. (2001) and have been found to conform to the result obtained with the type strain *S. agalactiae* NCIMB 701348. Therefore, this PCR is regarded as useful in the confirmation of *S. agalactiae* within streptococcal infections. This technique may have added value as it may also provide

important information for prevention and control of this disease. The presence of pathogens may be able to be detected at earlier stages of infection and in carrier animals, when the number of bacteria in tissues may be very low.

There are many different variables that can affect bacterial growth measured both *in vitro* and *in vivo*. Laboratory studies concentrate on investigating the number of viable bacterial colonies produced within a known volume of suitable broth and under tightly controlled conditions. Measurement of bacterial growth *in vitro* has mostly concentrated on finding the more suitable growth conditions which should be as similar to those experienced during a natural outbreak. Investigation of bacterial growth has focused on the length during the phase of exponential growth. Moreover, the length of each phase is dependent on a wide range of growth factors and variables including the environmental conditions (temperature, pH, etc), type of medium, size of inoculums, time required for recovery from physical damage or shock in the transfer to new media and time required for synthesis of DNA, proteins, essential amino acids, enzymes or division factors, etc (Gross et al., 1995; Tortora et al., 2007). For example, the log phase of the two isolates tested at 28 ^{o}C in this thesis, in TSB was between 3 and 12 h, whereas for other *S. agalactiae* strains it was less than 6 h in Brain Heart Infusion (BHI) broth at 37 ^{o}C (Willett and Morse, 1966). The range of varied growth conditions published in the literature are vast but for the purpose of this work, good reliable and reproducible growth conditions were found when the bacteria were grown in TSB at 28 ^{0}C.

The use of a bacterial standard curve is a method of plotting data that is used to determine the unknown sample's concentration or number of bacteria (Gross et al., 1995). In this study the value of obtaining viable bacterial standard growth curves was to provide reliable data which could then be used to support subsequent studies performed *in vivo*. Thus, the use of a dose-response curve is extremely valuable especially for this study. Care was taken to specify at what point in a dose-response curve was measured to try to capture all phases of the growth cycle.

This study demonstrated that the range of laboratory based tests could identify group B *S. agalactiae* with confidence. Additionally, the salt and temperature tolerance test, and growth on Edwards medium (modified) agar could be used to distinguish between *Streptococcus* from *Lactococcus* and *Enterococcus* spp.

In particular, the combined positive results of Gram stain, bacterial shape, motility and oxidase results, Lancefield serogroup B and species-specific PCR could provide useful tests for the accurate identification of *S. agalactiae* from other Gram positive cocci bacteria, especially *S. iniae*. Production of the growth and standard curve were valuable to provide a useful tool for measuring accurately the number of viable CFU per ml which could then be applied in future studies to investigate the pathogenicity of the isolates *in vivo*.

Chapter 3 - Development of an experimental challenge model for *Streptococcus agalactiae* infection in Nile tilapia, *Oreochromis niloticus*

3.1 Abstract

Two challenge models using *Streptococcus agalactiae* by immersion and intraperitoneal (i.p.) injection were investigated *in vivo* in Nile tilapia (*Oreochromis niloticus*). In the immersion challenges, fish were immersed in water containing a range of bacterial concentrations from 10^4-10^7 CFU/ml *S. agalactiae* and at various exposure times from 30 seconds to 8 hours. After this time the fish were then placed into their respective tanks at a stocking density of 2, 35 or 45 g/L. In addition, immersing the fish in the original bacterial growth media and maintaining the fish in a static water system were also performed in order to get a successful challenge study. In the i.p. injection challenge, three groups of fish each at a stocking density of 45 g/L received different bacterial concentrations of 9×10^8, 9×10^7 and 8×10^6 CFU/ml *S. agalactiae* by i.p. injection, respectively. There was only 1 dead fish from the immersion challenge groups despite testing a range of bacterial concentrations, exposure times, stocking densities, water systems and bacterial preparation. In contrast, the i.p. injection produced significant mortalities (9×10^8 CFU/ml = 48% mortality, 9×10^7 = 48% and 8×10^6 = 26%), when observed over a 14 day period. Affected fish from all i.p. injected groups showed similar clinical signs

including lethargy, anorexia, erratic swimming, cloudy eyes and splenomegaly. *Streptococcus agalactiae* was recovered and identified from all the dead and moribund fish during the experiment, but was not isolated from any surviving fish of any group. Systemic infection with the presence of necrotic, inflammatory lesions in the spleen, brain and eyes from infected fish were evident. These results showed that an experimental i.p. challenge model was produced and gave similar clinical presentation to those reported from a natural *S. agalactiae* infection in tilapia.

3.2 Introduction

Streptococcus agalactiae infection has globally become one of the most economically important bacterial infections in warm water aquaculture (Evans et al., 2002; Duremdez et al., 2004; Salvador et al., 2005; Suanyuk et al., 2005; Musa et al., 2009; Pourgholam et al., 2011; Ye et al., 2011). Affected tilapia (*Oreochromis* spp.) present clinical signs including the loss of appetite, lethargy, erratic swimming, both unilateral and bilateral exophthalmia, corneal opacities and / or cloudy eyes (Eldar et al., 1995a; Plumb, 1999; Salvador et al., 2005; Suanyuk et al., 2005; Evans et al., 2009; Musa et al., 2009; Pretto-Giordano et al., 2010a). Pale liver and enlarged spleen and/or liver have also been reported in affected fish (Salvador et al., 2005; Suanyuk et al., 2005; Pretto-Giordano et al., 2010a). Histopathological changes in *S. agalactiae*-infected fish have been observed in several internal organs particularly the liver, kidney, spleen, eyes and brain (Eldar et al., 1994, 1995a; Chang and Plumb,

1996b; Suanyuk et al., 2008; Filho et al., 2009; Zamri-Saad et al., 2010; Abuseliana et al., 2011).

Infectivity studies have been used for various purposes but in aquatic disease, these are primarily used to investigate the pathogenesis of disease, routes of infection or potential treatments and vaccines. There are various infection route studies for an aquatic streptococcal infection in different fish species including a range of exposure routes: injection, immersion, bath, oral, cohabitation, gill and nares inoculation (Robinson and Meyer, 1966; Perera et al., 1997; Bromage et al., 1999; Evans et al., 2000; Shoemaker et al., 2000; Evans et al., 2001; Nguyen et al., 2001a; Bromage and Owens, 2002; McNulty et al., 2003; Lahav et al., 2004).

Previous reports have shown that *S. agalactiae* was able to cause infection in Nile tilapia (*O. niloticus*) by intraperitoneal (i.p.) injection (Evans et al., 2004b; Pasnik et al., 2005; Evans et al., 2009; Filho et al., 2009; Rattanachaikunsopon and Phumkhachorn, 2009; Pretto-Giordano et al., 2010a; Ye et al., 2011) and immersion (Mian et al., 2009; Ng et al., 2009; Rodkhum et al., 2011) where the bacterial concentration varied from 10^2 to 10^8 CFU/ml. Only a limited number of studies had been performed to evaluate the virulence and infection routes of *S. agalactiae* in Nile tilapia in terms of determining the 50% lethal dose (LD_{50}) (Evans et al., 2002; Mian et al., 2009; Abuseliana et al., 2011). Furthermore, a single robust and reliable model is not available in the published literature. Therefore, production of a challenge model using a defined bacterial strains,

exposure routes, fish populations and environmental conditions should be considered prior to performing the subsequent experimental studies.

This aim of the current study was to produce a reliable experimental challenge model by exposing *S. agalactiae* to Nile tilapia. To do this, two exposure routes were investigated: immersion and intraperitoneal (i.p.) injection to provide a lethal concentration affecting 50% of the exposed population (LC_{50}) to produce clinical signs and pathology similar to those reported in natural streptococcal infections. All fish experiements were conducted under Home Office Project Licence number 60/3949.

3.3 Materials and methods

3.3.1 Fish

Nile tilapia, *Oreochromis niloticus* with an average weight of 20 ± 10 g between 4-6 months old from the same parents were obtained from the Tropical Aquarium, Institute of Aquaculture, Stirling, UK. All the experimental challenge studies were performed in the Aquatic Research Facility (ARF), Stirling in 10 L plastic tanks using continuous flow-through water at 0.38 L/minute, a 12 h light: 12 h dark cycle and water temperature at 27^{o}C, except the sixth immersion challenge study which was conducted using a static water system with 50% water changes. Aeration was supplied through an air stone to each tank and the fish were fed with a commercial diet (Skretting Trout Nutra 25) to apparent satiation once daily.

3.3.2 Bacterial strain and passage

All studies were conducted with *S. agalacti*ae isolate number 1 which was recovered from infected Nile tilapia during a natural disease outbreak in floating cages in Vietnam. This isolate had already been identified as *S. agalactiae* and its identification profile was similar to the *S. agalactiae* type strain as described in Chapter 2.

Before performing any challenge studies, the isolate was passaged through 3 fish each by i.p. injection at high bacterial concentration to enhance virulence properties as the isolate had been in storage. The passage fish were monitored for morbidity/mortality for 2 days and sampled for bacterial recovery from the kidney onto tryptone soya agar (TSA; Oxoid, U.K.). The isolate recovered from the 3rd passage was purified as required, identified as already described (see Chapter 2), using the identification techniques as described in Frerichs and Millar (1993) and stored on protect beads (Technical Service Consultants Limited, UK) at -70 ^{o}C until required.

3.3.3 Preparation of challenge inoculums

The bacterial culture was grown in 40 mL of tryptone soya broth (TSB; Oxoid, U.K.) at 140 rpm for 28 ^{o}C (Khuner shaking incubator) to provide bacteria in the mid-log growth phase. The culture was then centrifuged (3,500 × g) for 15 min at 4 ^{o}C and the pellet resuspended in sterile 0.85% saline solution or TSB to give an optical density (OD_{610nm}) of 1.0 which gave approximately 10^8 viable colony forming units (CFU) / ml. Serial (10-fold) dilutions were performed in sterile 0.85% saline solution or TSB to 10^4 to 10^7 CFU/ml for the challenge inoculums, and viable colony counts performed following the methods of Miles & Misra (Miles et al., 1938).

3.3.4 Immersion challenge studies

All the experiments in this study are listed in Table 3.1. Fish were removed from stock tanks and immersed in 5 L of water containing the bacteria at various concentrations and exposure times. After this time the fish were then placed into their respective tanks at different stocking densities. Immersing the fish in the original bacterial growth media and maintaining the fish in a static water system were also performed. The control fish group was treated in the same way except they were exposed to sterile 0.85% saline solution or medium (TSB) without bacteria (Table 3.1).

Table 3.1 Experimental infection studies of *Streptococcus agalactiae* by immersion.

Experiment number	Bacterial isolate (passage N^0)	Bacterial concentration (CFU/ml)	Exposure time	Number of fish per tank	Number of fish per treatment group	Total number of fish per experiment	Density (g/L)	Water system	Exposure fish
1	2^{nd}	10^5, 10^6, 10^7	30 seconds	1	4	16	2	flow through	Individual
2	2^{nd}	10^4, 10^5, 10^6	1 minute	1	4	16	2	flow through	Individual
3	2^{nd}	10^7	5, 15, 30, 45, 60 minutes	1	4	24	2	flow through	Individual
4	3^{rd}	10^7	5, 15, 30, 45, 60 minutes	1	4	24	2	flow through	Individual
5	3^{rd}	10^7	1 hour	18	18	72	35	flow through	In group
6	3^{rd}	10^7	1 hour	18	18	18	35	static system	In group
				23	23	23	45		
			8 hours	18	18	18	35		
				23	23	46	45		
7	3^{rd}	10^7	8 hours	23	23	46	45	flow through	In group

Remarks: *Experiments 1-4, bacteria were resuspended in saline solution; Experiment 5, bacteria were resuspended in TSB or saline solution; Experiments 6-7, bacteria were resuspended in TSB. Each experiment had a control group whereby the fish were exposed to sterile 0.85% saline solution or TSB only.*

3.3.4.1 Experiment number 1

Individual fish were exposed to various bacterial concentrations of 10^5, 10^6 and 10^7 CFU/ml in sterile 0.85% saline solution for 30 seconds. Sixteen fish were divided into 4 groups with 4 fish in each treatment group with one fish per tank (at 2 g/L density). Three groups immersed with 10^5, 10^6 and 10^7 CFU/ml of the 2nd passage of *S. agalactiae* for 30 seconds, respectively. A control group of fish were immersed in sterile 0.85% saline solution for 30 seconds.

3.3.4.2 Experiment number 2

This study was similar to experiment number 1 but used a decreased concentration of bacteria and an increased exposure time. Individual fish were exposed to bacterial concentrations of 10^4, 10^5 and 10^6 CFU/ml in sterile 0.85% saline solution for 1 minute. Sixteen fish were divided into 4 groups with 4 fish in each treatment group and one fish per tank (at 2 g/L density). Three groups of fish were immersed with 10^4, 10^5 and 10^6 CFU/ml of the 2nd passage of *S. agalactiae* for 1 minute, respectively. A control group of fish were immersed in sterile 0.85% saline solution for 1 minute.

3.3.4.3 Experiment number 3

This study used an increased exposure time to a high concentration of bacteria. Individual fish were exposed to 10^7 CFU/ml in sterile 0.85% saline solution at various exposure times for 5, 15, 30, 45 and 60 minutes. Twenty-four fish were divided into 6 groups of 4 fish per treatment group with one fish per tank (at 2 g/L density). Five groups of fish were immersed in 10^7 CFU/ml of the 2nd

passage of *S. agalactiae* for 5, 15, 30, 45 and 60 minutes, respectively. A control group of fish were immersed in sterile 0.85% saline solution for 60 minutes.

3.3.4.4 Experiment number 4

This study repeated the approach described in experiment number 3 but used the 3rd passage of *S. agalactiae*.

3.3.4.5 Experiment number 5

This study increased the stocking density of fish and compared the bacterial preparations. Groups of fish were exposed to a 10^7 CFU/ml concentration of bacteria for 1 hour. Seventy-two fish were divided into 4 groups of 18 fish with each tank at a stocking density of 35 g/L. Two groups of fish were immersed in 10^7 CFU/ml concentration of bacteria from the 3rd passage of *S. agalactiae* prepared either as in sterile 0.85% saline solution or in TSB for 1 hour. Two control groups of fish were immersed in sterile 0.85% saline solution or in TSB for 1 hour.

3.3.4.6 Experiment number 6

This study used a different water system and then compared the exposure time to bacteria and the stocking density of fish. Groups of fish were exposed to a 10^7 CFU/ml concentration of bacteria for 1 or 8 hours at a stocking density of 35 or 45 g/L in a static water system. Thirty-six fish were divided into 2 groups of 18 fish with each tank of fish held at a stocking density of 35 g/L which were

subsequently immersed in a 10^7 CFU/ml concentration of bacteria from the 3rd passage of *S. agalactiae* in TSB for 1 or 8 hours. Another forty-six fish were divided into 2 groups of 23 fish each held at a stocking density of 45 g/L and then immersed in a 10^7 CFU/ml concentration of bacteria from of the 3rd passage of *S. agalactiae* in TSB for 1 or 8 hours. A control group of 23 fish held at a stocking density of 45 g/L were immersed in TSB for 8 hours.

3.3.4.7 Experiment number 7

This study was similar to that of experiment number 5 but used an increased exposure time and stocking density of fish. A group of fish was exposed to a concentration of 10^7 CFU/ml bacteria for 8 hours. Forty-six fish were divided into 2 groups of 23 fish each held at a stocking density of 45 g/L. One group of fish were immersed in 10^7 CFU/ml *S. agalactiae* in TSB for 8 hours. A control group of fish were immersed in TSB for 8 hours.

3.3.5 Intraperitoneal (i.p.) injection challenge study

Ninety-two fish were divided into four groups of 23 fish each held at a stocking density of 45 g/L. Three groups of fish received 0.1 ml of the 3rd passage of *S. agalactiae* suspension at different doses where the diluent was sterile 0.85% saline solution. The actual bacterial concentrations were 9×10^8, 9×10^7 and 8×10^6 CFU/ml by i.p. injection, respectively. A control group was injected with 0.1 ml of sterile 0.85% saline solution.

Fish were netted, placed into a holding tank and anaesthetised with benzocaine 50 ppm (Sigma, U.K.). Each fish was then injected with either 0.1 ml of bacteria at 9×10^8, 9×10^7 or 8×10^6 CFU/ml or sterile 0.85% saline solution and then placed back into its respective tank.

3.3.6 Mortality, clinical signs, macroscopic findings and sample evaluation

Fish were monitored daily for 14 days post bacterial exposure and checked for morbidity/mortality and gross clinical signs of disease. Any dead or moribund fish were removed and the presence of gross lesions both externally and internally was recorded and then the kidney, spleen, eye and brain were aseptically sampled for *S. agalactiae* using TSA and 5% (v/v) sheep blood agar (Oxoid, U.K.). Bacterial identification and PCR assays were performed as described in Chapter 2. At the end of the 14 day experimental period, 50% of the surviving fish in all treatment groups, including the controls, were sampled as described above.

Selected organs including kidney, spleen, eyes, brain, liver, intestine, gills, heart and muscle obtained from dead or the moribund fish, 50% of the survivors and 50% of the control fish were fixed in 10% (v/v) neutral buffered formalin, processed using standard protocols and embedded in paraffin wax blocks. Tissue sections (5 µm) were cut and stained with haematoxylin and eosin (H&E) for histopathology (Roberts, 2001).

3.4 Results

3.4.1 Immersion challenge studies

There was only one dead fish at day 3 post-exposure from the group of fish receiving *S. agalactiae* in TSB in experiment number 5. *Streptococcus agalactiae* was re-isolated from this fish in pure culture from the kidney and its identify confirmed. In addition, all the fish in experiment number 6 died within 24 hours. In contrast, no mortality, clinical signs or lesions were observed in any of the other treatment groups during the 14 day immersion-exposure (i.e. experiments number 1, 2, 3, 4 and 7). Additionally, no pathological findings were observed and no bacteria were recovered from the surviving fish in any of the treatment or the control groups of fish at the end of the study period.

3.4.2 Intraperitoneal (i.p.) injection challenge study

3.4.2.1 Mortality

The cumulative percentage daily mortalities during the experiment are provided in Figure 3.1. Mortalities were observed in the i.p. bacterial challenge groups only. The highest total mortalities (48%) were found in the 9×10^8 and the 9×10^7 CFU/ml treatment groups when observed over a 14 day period (Figure 3.1). In addition, fish exposed to bacteria in the highest concentration groups died more rapidly when compared to those exposed to lower dose of 8×10^6 CFU/ml treatment group with 26% mortality (Figure 3.1). The effects appear to be dose-dependent.

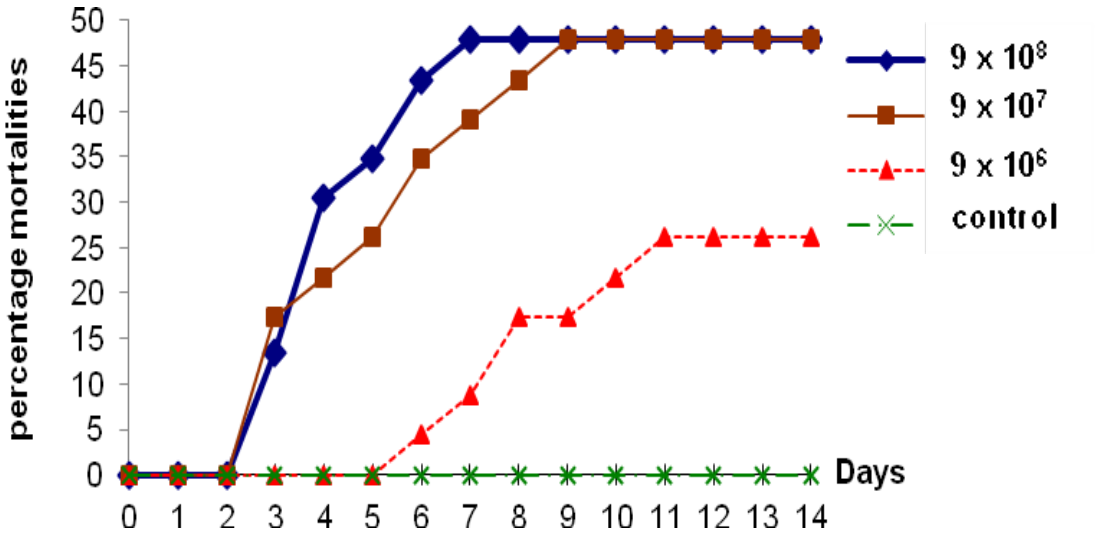

Figure 3.1 Cumulative percentage daily mortalities in fish exposed to different concentration of *Streptococcus agalactiae* administrated by i.p. injection.

3.4.2.2 Clinical signs and macroscopic findings

All affected fish showed lethargy, anorexia, erratic swimming / spiralling, and often remained stationary at the bottom of the tank by day 2 post-exposure. External gross lesions included darkening of the fish and opacity of a single eye (Figure 3.2) and internally splenomegaly (Figure 3.3), were observed in the dead and moribund fish following bacterial exposure on day 3 in both the 9 × 10^8 CFU/ml and 9 × 10^7 CFU/ml treatment groups. None of the control fish showed clinical signs of disease.

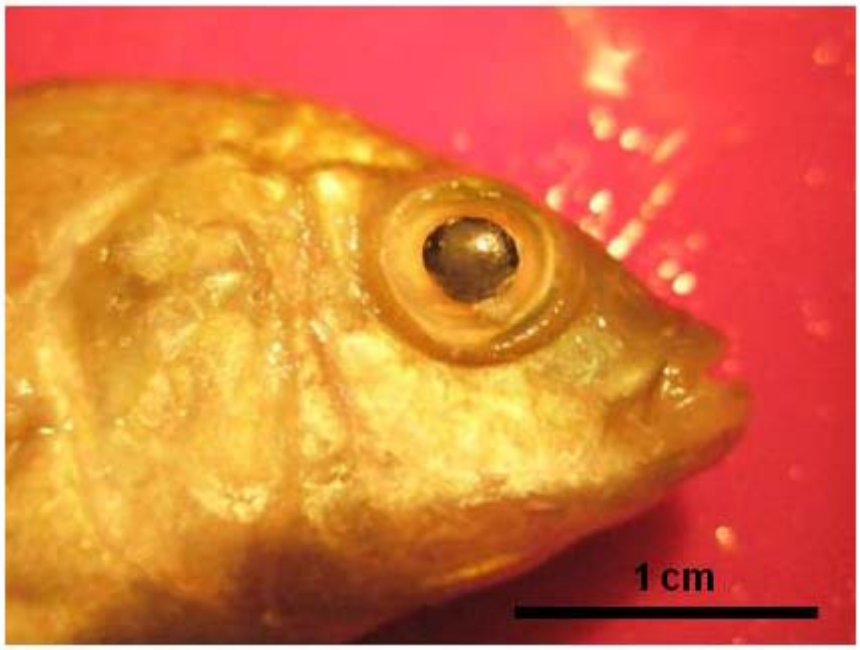

Figure 3.2 A moribund fish with corneal opacity of the eye on Day 3 from the 9 × 10^7 CFU/ml treatment group.

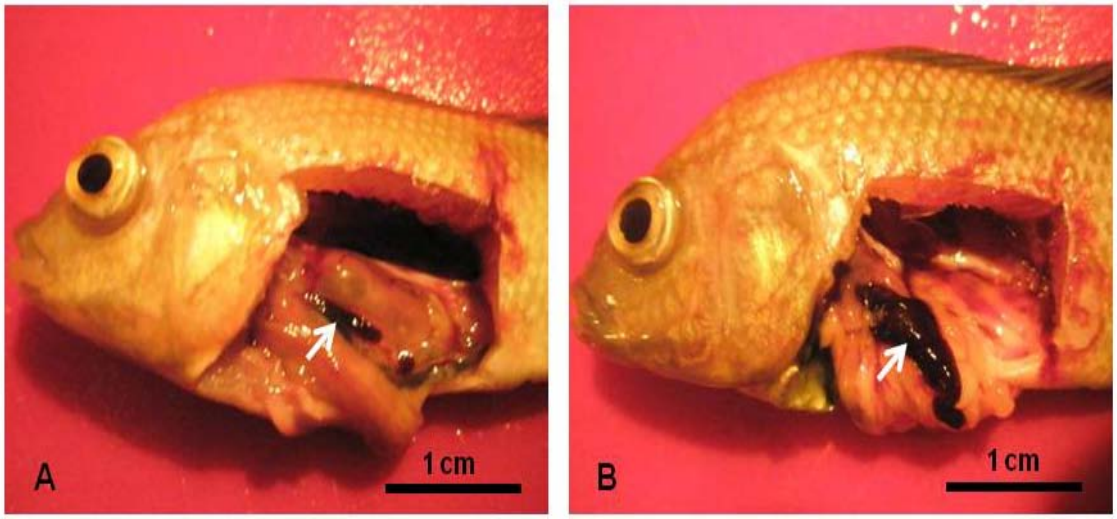

Figure 3.3 Spleen of experimental fish (arrows). A, normal sized spleen from one of the control fish at Day 14; B, splenomegaly of a moribund fish from the 9 × 10^8 CFU/ml treatment group at Day 3.

3.4.2.3 Bacterial identification and PCR assay

Streptococcus agalactiae was re-isolated in pure cultures from the kidney, spleen, eye and brain of all (i.e. 100%) of the fresh dead and moribund fish from each bacterial treatment group. No bacteria were recovered from any of the surviving fish or from the control fish that were sampled.

Bacterial colonies were identified as a Gram-positive cocci, non-motile, oxidase negative, showing β-haemolysis, and were positive for Lancefield serogroup B and positive only for Voges–Proskauer, hippurate hydrolysis, alkaline phosphatase, leucine arylamidase, arginine dihydrolase, ribose and trehalose as tested in the API 20 STREP system. Isolates were identified through primary and biochemical tests as *S. agalactiae* were then confirmed by PCR assay. The results showed that all the samples were similar to the *S. agalactiae* type strain NCIMB 701348 and all gave a positive band at the correct molecular weight of 270 bp using primer set of STRA-AgI/STRA-AgII (Figure 3.4). No bands were visible for the negative control samples.

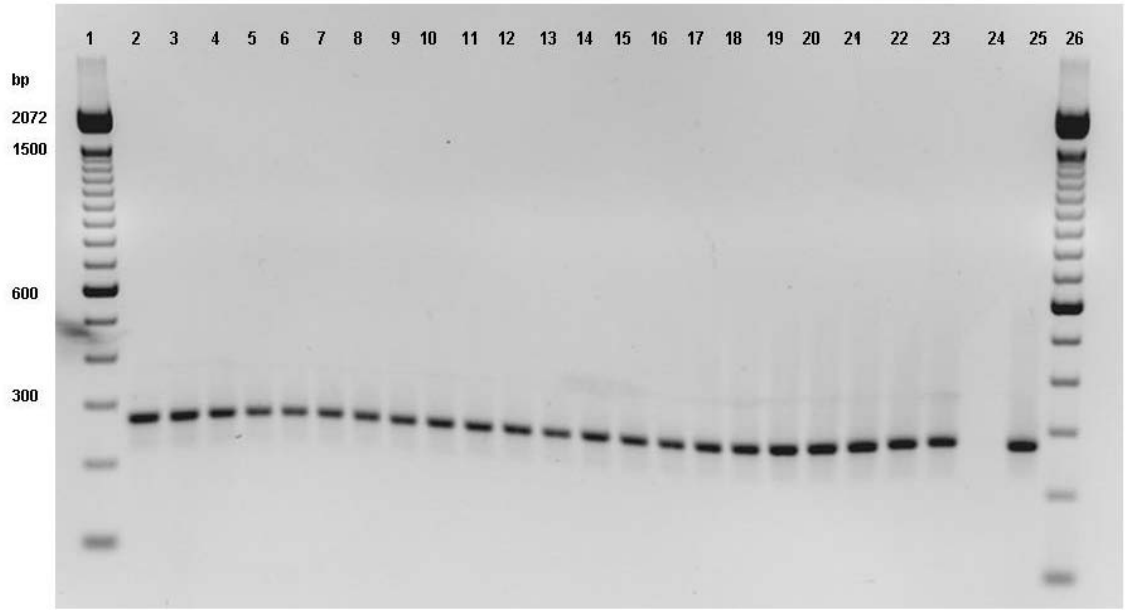

Figure 3.4 PCR amplification of samples from the dead or moribund fish from each bacterial treatment group. Lanes 1 & 26, 100 bp DNA ladder; Lanes 2-9, the 9 × 10^8 CFU/ml treatment group; Lanes 10-18, the 9 × 10^7 CFU/ml treatment group; Lanes 19-23, the 8 × 10^6 CFU/ml treatment group; Lane 24, negative control (no DNA); Lane 25, positive control *Streptococcus agalactiae* type strain NCIMB 701348.

3.4.2.4 Histopathology

The pathological changes observed in the affected fish presenting with gross lesions of streptococcosis were located mainly in the spleen, brain and eyes. Common histopathological changes included moderate to severe, diffuse, necrotic, inflammatory lesions involving lymphocytes and macrophages with visible coccal bacteria (Figure 3.5-3.8). More severe changes which included a high number of inflammatory cells and bacteria in tissues were more evident in the higher dose groups. The severe pathology occurred between days 3 and 4 post infection in both high exposure groups. While, in the lower dose group only mild inflammation was observed in all tissues and no pathology was observed in the control group.

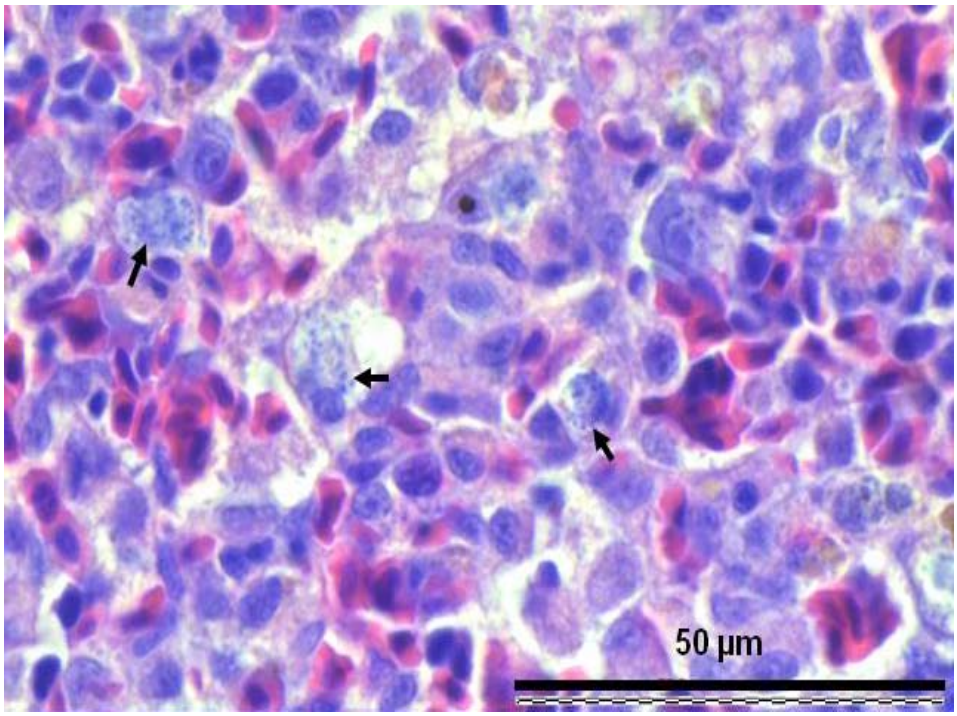

Figure 3.5 Bacteria within macrophages (arrows) in the spleen of a moribund fish from the 9×10^7 CFU/ml treatment group at day 3.

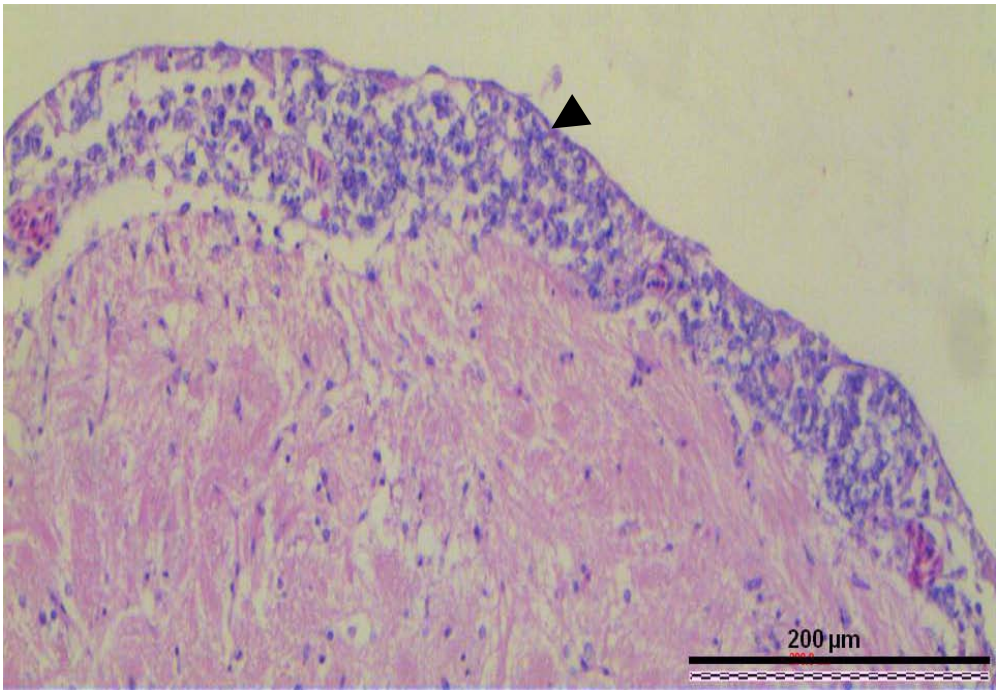

Figure 3.6 Brain of a moribund fish on Day 3 from the 9×10^7 CFU/ml treatment group showing severe and generalized meningo-encephalitis, the meninges are thickened due to the infiltration of macrophages and lymphocytes (arrowhead).

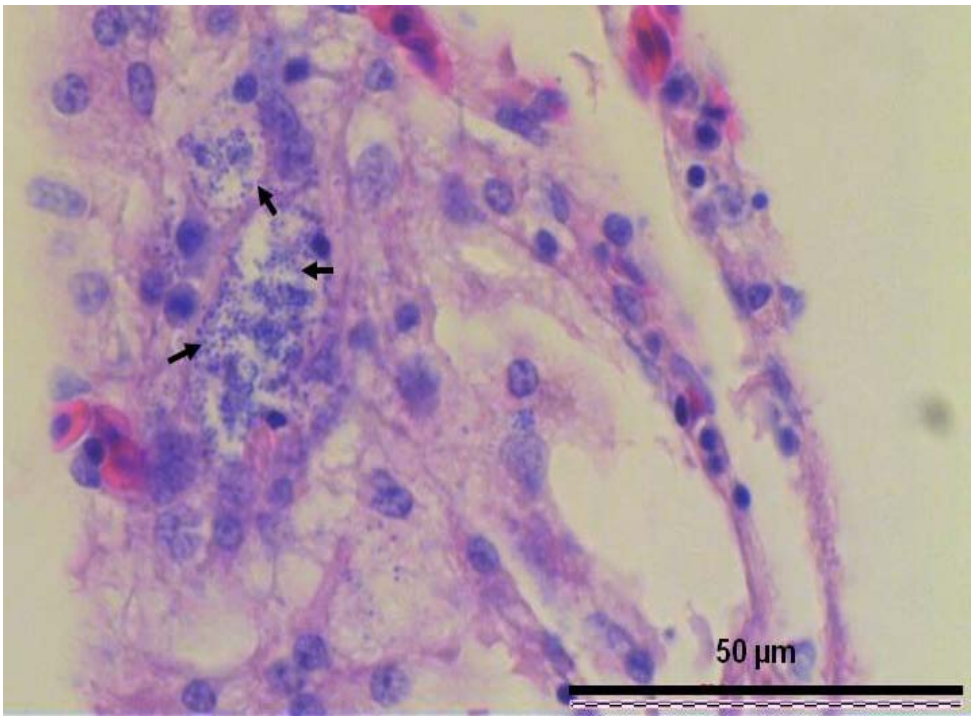

Figure 3.7 Bacteria were widely distributed in the meningeal surface (arrows) of the brain of a moribund fish on Day 3 from the 9×10^7 CFU/ml treatment group.

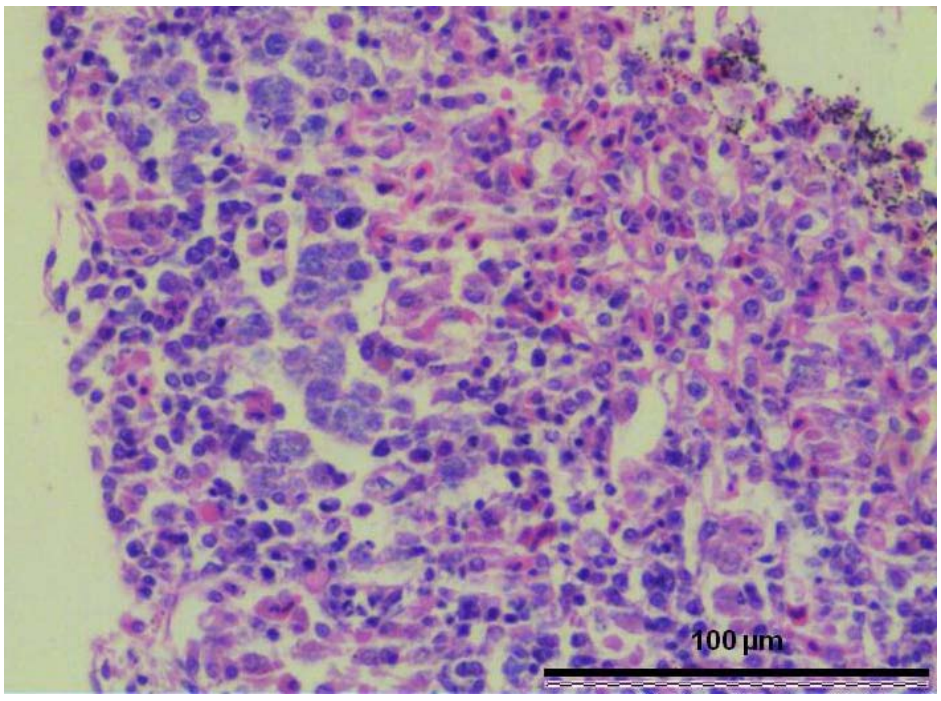

Figure 3.8 Eye of a moribund fish from the 9×10^7 CFU/ml treatment group at day 3 showing severe panopthalmitis with destruction of the eye, necrotic and inflammatory lesion by the infiltration of numerous macrophages and lymphocytes.

3.5 Discussion

Streptococcus agalactiae can cause systemic bacterial infection resulting in high mortalities with acute septicaemia (Eldar et al., 1994; Evan et al., 2002; Musa et al., 2009). Several authors have investigated challenge models with varying degrees of success (Bromage and Owens, 2002; Russo et al., 2006; Pretto-Giordano et al., 2010a). Production of a reproducible challenge model is not a simple task as the establishment of infection under experimental conditions can be influenced by numerous factors including individual fish variation, bacterial strain or species, and fluctuating environmental conditions or management variations (Bromage and Owens, 2002; Pretto-Giordano et al., 2010a). The results of this study produced an experimental challenge model against *S. agalactiae* by i.p. injection. In particular, clinical signs and histopathological findings of *S. agalactiae* infection observed in challenged fish were similar to previous reports of streptococcal infection (Salvador et al., 2005; Suanyuk et al., 2005; Filho et al., 2009; Musa et al., 2009; Zamri-Saad et al., 2010; Abuseliana et al., 2011). Moreover, the re-isolated *S. agalactiae* from the dead and moribund fish after challenging by i.p. injection combined with the presence of bacteria within tissues observed by histopathology showed that the cause of death / morbidity was due to *S. agalactiae*.

The 50% mortality observed in the current study was obtained only at the higher bacterial concentrations (9×10^7 and 9×10^8 CFU/ml) and there were differences between the onset of clinical signs and the accumulated mortality rate from that reported by Evans et al. (2004b), Pasnik et al. (2005), Evans et

al. (2009), Pretto-Giordano et al. (2010a) and Ye et al. (2011). Additionally, it could be observed that many factors can influence mortality rate and the onset of disease signs including bacterial strain / virulence, bacterial concentration, fish species, water temperature, water quality and stocking density (Chang and Plumb, 1996a; Shoemaker et al., 2000; Agnew and Barnes, 2007; Pretto-Giordano et al., 2010a). In contrast, no *S. agalactiae* was recovered from the surviving fish at the end of the 14-day study period which could suggest that a lack of organ colonisation or that the viable bacterial numbers remaining in tissues sampled may be too low to be recovered (Al-Harbi, 1996; Evans et al., 2000). It may also be that no viable bacteria were present and the fish had cleared the bacteria from their system. Also, an effective cellular immune response could be established in the later periods of infection (Filho et al., 2009), as shown in this study. Hence the bacteria may have been “walled-off” by the fish’s immune response, thus reducing the ability to recover any viable colonies. This fact explains the absence of clinical signs and lesions at the end of the experiment.

Immersion studies published by Mian et al. (2009), Ng et al. (2009) and Rodkhum et al. (2011) reported 40%, 58% and 60% mortality in Nile and red tilapia, respectively, when exposed to *S. agalactiae*. The immersion studies performed in the work presented were unable to induce any significant morbidity / mortality or bacterial recovery. The studies presented achieved similar results with no clinical signs of disease or mortality as were described previously by Abuseliana et al. (2011). The difference in bacterial strain or

virulence, fish population and environmental conditions may explain the variation of susceptibility to *S. agalactiae* in tilapia by the immersion route. In addition, Rasheed and Plumb (1984), Foo et al. (1985), Chang and Plumb (1996b), Bromage and Owens (2002) and Xu et al. (2007) suggested that damage to the integument with scraped skin or removed scales prior to bacterial immersion exposure or fin clips may predispose fish to a streptococcal infection under experimental conditions. Such a procedure prior to bacterial exposure could enhance the pathogens ability to enter its host through wounds and abrasions to the skin thus artificially promoting the establishment of the pathogen in the host (Nguyen et al., 2001b). Whilst skin abrasions or scale removal may enhance the success of disease establishment, this was not performed in this study as it is not accepted practise for UK experimental studies, following UK Home Office Guidelines.

The inability to show significant morbidity or mortality from the immersion studies performed in this work was disappointing. Several attempts were made to refine the experimental immersion study of *S. agalactiae* infection in Nile tilapia. These included the use of an increased bacterial concentration, increased exposure times to the pathogen and an increase in the number of fish exposed. In addition, other factors were attempted which included immersing the fish within the original bacterial growth media (no washing steps) and maintaining the fish in a static water system, were all used in these experiments. All of these approaches, however, were unsuccessful. The attempts to change the pathogen and host conditions to provide a successful

immersion challenge models were decided using previous information published on successful *Streptococcus* spp. immersion studies (Shoemaker et al., 2000; Mian et al., 2009; Rodkhum et al., 2011). Nevertheless, none of the attempts produced a successful immersion challenge model in the fish and was surprising given the fact that published immersion models were available. The inability to reproduce the immersion challenge model within this thesis supports the fact that successful experimental challenge models require many factors and are difficult to reproduce. Not only must the pathogenicity of the isolate be considered but care must be taken to ensure that the environmental conditions are favourable for the fish host species to succumb to the disease.

A single mortality was observed in experiment number 5, where the bacteria were provided in the broth media with no washing steps. Therefore, this suggested that although the mortality was low perhaps exposing the fish to the bacterial and broth media may produce a successful immersion challenge model and so experiment number 6 and 7 were changed to immerse fish with bacteria and growth media. The 100% fish kills within 24 hours in the static water systems used in experiment number 6, occured not due to the pathogen but due to a lack of oxygen. Inadequate water changes (only 50%) after fish were immersed with bacteria and media may have lead to elevated nutrient concentration in the water and decreased dissolved oxygen levels in these systems. This was supported by the fact that all dead fish in this experimental group were open mouthed with flared opercula without any other clinical lesions (Noga, 2010). The static water system was attempted as it was thought to

closely mimic the conditions in pond culture systems where these fish are typically recorded and may enhance further infection if the bacteria were excreted in the faeces of infected fish into the water (Nguyen et al., 2002). However, the static water system was not subsequently used since it was difficult to maintain water quality during the experiments. Other attempts were also made to produce a *Streptococcus iniae* immersion challenge model in Nile tilapia and those too were unsuccessful, but the data was not reported in this thesis.

The susceptibility of *S. agalactiae* infection in tilapia through immersion may be enhanced by suboptimal environmental conditions, such as low DO, high ammonia and nitrite levels, high water temperatures or extreme water parameters, all of which appear to be factors predisposing fish to disease (Eldar et al., 1995a; Hurvitz et al., 1997; Evans et al., 2002; Evans et al., 2003; Al-Marzouk et al., 2005; Evans et al., 2006b; Agnew and Barnes, 2007; Rodkhum et al., 2011). Therefore, there is more than just the presence of the bacteria and the fish to cause the disease and that other factors are required thus supporting the fact that establishing challenges models is not simple in aquaculture. There are advantages and disadvantages for all pathogen exposure routes where the appeal of immersion or bath exposure is that this model is considered to be more natural. Any injection model is undoubtedly giving the pathogen an advantage as it is by-passing some of the host innate immune responses, however, injection methods are acceptable techniques for experimental bacterial challenge studies in fish. The advantage of the injection route is that

this technique may be considered more reliable, reproducible, replicable and efficient than other approaches because all individual fish receive a uniform bacterial dose (Perera et al., 1997).

The results from the study produced an experimental i.p. challenge model with approximately 50% mortality at 168 and 216 h. Viable bacteria identified as *S. agalactiae* were recovered only from the dead or moribund fish exposed to the pathogen and these fish presented clinical signs and pathology similar to those described during natural infections. The production of this challenge model was essential to subsequent experimental work exploring the pathogenicity of *S. agalactiae* in tilapia.

Chapter 4 - The effect of weight and age on experimental *Streptococcus agalactiae* infection in Nile tilapia, *Oreochromis niloticus*

4.1 Abstract

Data from natural disease outbreaks have suggested that the weight and/or age of fish may influence the severity of streptococcal infections. This study investigated the association between weight or age of fish and susceptibility to *Streptococcus agalactiae* infection in Nile tilapia (*Oreochromis niloticus*). The experimental groups were control and a single population of 10-40g at 8 months old from one set of parents divided into 7 weight groups (10, 15, 20, 25, 30, 35, 40g). These fish received a single intraperitoneal (i.p.) injection of 6×10^7 CFU/ml of *S. agalactiae*. Controls and fish of 5g weight were divided into 2 groups of fish age of 4 or 8 months old and each received an i.p. injection of 7×10^7 CFU/ml of *S. agalactiae*. Mortality, clinical signs and lesions were monitored for 14 days post challenge. Recently dead or moribund fish were sampled for bacterial recovery and histopathology to evaluate bacterial infection. Clinical signs, lesions and histopathological changes in the affected fish were consistent with those reported in natural *S. agalactiae* infections in tilapia. *Streptococcus agalactiae* was recovered and identified from the kidney, spleen, eye and brain of all moribund or dead fish. The mortality in the study of different weights varied from 0 to 33% between the groups but the association with

weight was weak (R^2 = 0.02). Whilst, in the study of different ages, the 4 month old fish group had a mortality of 24% and the 8 month old fish group of 4%. This study produced no evidence for an association between the weight and susceptibility to *S. agalactiae* infection but suggested an association between the age or growth rate of fish and this disease.

4.2 Introduction

A wide range of fish species have been reported to suffer from streptococcal infections with both wild and cultured fish being affected globally (Plumb, 1999; Buller, 2004; Austin and Austin, 2007). *Streptococcus agalactiae* can be regarded as one of the main aetiological agents of streptococcal infections in tilapia (Plumb, 1999; Salvador et al., 2005; Suanyuk et al., 2005; Mian et al., 2009; Abuseliana et al., 2010; Zamri-Saad et al., 2010). Fish infected with *S. agalactiae* display a wide range of clinical signs associated with the disease including high mortality, erratic swimming, exophthalmia, cloudy eyes, septicaemia all of which can cause serious economic losses to the primary producers. Internally, the affected fish have haemorrhages and inflammatory lesions in various internal organs (Eldar et al., 1994, 1995a; Plumb, 1999; Evans et al., 2002; Suanyuk et al., 2005; Siti-Zahrah et al., 2008; Musa et al., 2009; Pretto-Giordano et al., 2010a; Zamri-Saad et al., 2010; Abuseliana et al., 2010, 2011). The increase in streptococcal disease has occurred mainly in intensive production systems, where several factors can lead to the increased incidence of disease outbreaks.

Fish weight and / or age have been suggested as possible variables that can influence *S. agalactiae* infections in fish. Published studies have reported that the weight and/or age of the fish can influence the establishment of streptococcal infections in farmed tilapia. Siti-Zahrah et al. (2008), Zamri-Saad et al. (2010) and Amal and Zamri-Saad (2011) reported that *S. agalactiae* infections in red tilapia (*Oreochromis* spp.) have an increased prevalence and severity in larger or adult fish weighing between 100-450 g. However, Mian et al. (2009) did not show the same association as they reported that Nile tilapia (*Oreochromis niloticus*) were susceptible to streptococcal infections over a wide weight range from 54 g juveniles to 1 kg adult fish. Similarly, a high mortality of farmed red tilapia and Nile tilapia were observed, affecting very small to large fish weighing from 0.3 – 500 g, including broodstock (Suanyuk et al., 2008). Additionally, Hernández et al. (2009) and Jiménez et al. (2011) reported that there were no disease incidences of *S. agalactiae* infection in larvae and juvenile of red tilapia, weighing less than 20 g.

Due to the conflicting information published in the peer-reviewed literature, this study aimed to investigate the effect of weight and age of fish on experimental *S. agalactiae* infection in Nile tilapia following intraperitoneal (i.p.) injection. All fish experiements had been done under a Home Office Project Licence number 60/3949.

4.3 Materials and methods

4.3.1 Fish

Nile tilapia, *Oreochromis niloticus* were obtained from the Tropical Aquarium, Institute of Aquaculture, Stirling, UK. A single population was pooled from the same parent in a breeding group. The experimental challenge study was performed in the Aquatic Research Facility (ARF), Stirling in 5 or 10 L plastic tanks maintained with continuous flow-through water at 0.38 L/minute, a 12 h light: 12 h dark cycle and water temperature approximately 25 ^{o}C. Aeration was supplied through an air stone to each tank and fish were fed with a commercial diet (Skretting Trout Nutra 25) to apparent satiation once daily.

4.3.2 Bacterial strain and preparation of challenge inoculums

The bacterial challenge study was conducted using the 3rd passage of *S. agalacti*ae isolate number 1. The passage and bacterial identification work are described previously in Chapters 2 and 3. The bacterial challenge inoculums were prepared as described in Chapter 3 and 0.1 ml was administered to each fish by intraperotineal (i.p.) injection. The intended concentration was 10^{7} CFU/ml, which from previous data was expected to give approximately 50% mortality at 216 h in tilapia using the challenge model described in Chapter 3.

4.3.3 Experimental challenge studies

4.3.3.1 Study of the effect of different weights

One-hundred twenty fish with a weight range of 10-40 g at 8 months old were divided into 7 weight groups (10, 15, 20, 25, 30, 35, 40 g) stocked at approximately of 45 g/L density (Table 4.1). All fish received a single i.p. injection of 0.1 ml of 6×10^7 CFU/ml of bacterial suspension in sterile 0.85% (w/v) saline solution. A single control group (40 g fish) was injected with 0.1 ml of sterile 0.85% saline solution.

Table 4.1 Experimental challenge study of the effect of different weights with *Streptococcus agalactiae.*

Treatment Group number	Number of fish per treatment group	Weight of fish (±1 g)
1	23	10
2	15	15
3	12	20
4	18	25
5	15	30
6	13	35
7	12	40
8	12	40

Remarks: *Groups 1-3, fish were kept in 5L plastic tanks, Groups 4-8, fish were kept in 10L plastic tanks. Group 8 was a control group which was injected with sterile 0.85% saline solution.*

4.3.3.2 Study of the effect of different ages

The 90 eight-month old fish were divided into 2 groups of 45 fish each with an average weight of 5 g at held at 45 g/L density. Another 90, four-month old fish were divided into 2 groups of 45 fish each with an average weight of 5 g again held at 45 g/L density. One group of 8 and one group of 4 month old fish received a single i.p. injection of 0.1 ml of 7×10^7 CFU/ml of bacterial suspension in sterile 0.85% (w/v) saline solution. All treatment groups had a control group of fish at the same weight/age and were injected with 0.1 ml of sterile 0.85% saline solution.

Fish were netted, placed into a holding tank and anaesthetised with benzocaine 50 ppm (Sigma, U.K.). Each fish was then injected with either the bacterial suspension or sterile 0.85% saline solution before being placed back into its respective tank.

4.3.4 Mortality, clinical signs, macroscopic findings and sample evaluation

Mortality and clinical signs were monitored daily for 14 days post challenge. Fresh dead or moribund fish were removed and observed the gross lesions both externally and internally and then the kidney, spleen, eye and brain was aseptically sampled for recovery of *S. agalactiae* on TSA and 5% (v/v) sheep blood agar (Oxoid, U.K.). Bacterial identification and PCR assay were performed as previously described in Chapter 2. At the end of the 14 day

experimental period, 50% of the surviving fish in all treatment groups and the controls were sampled as described above.

Kidney, spleen, eyes, brain, liver, intestine, gills, heart and muscle obtained from dead or the moribund fish, 50% of the survivors and 50% of the control fish were fixed in 10% (v/v) neutral buffered formalin and processed using standard protocols for histopathology (Roberts, 2001).

4.4 Results

The study presented performed in critical condition due to unable to control at high water temperature by heating system as the ambient water was too low at that time.

4.4.1 Study of the effect of different weights

4.4.1.1 Mortality

The cumulative percentage of daily mortalities during the experiment is provided in Figure 4.1. Mortalities were first observed in the 10, 15 and 25 g fish groups on day 5. The initial mortalities in fish of the 30 and 40 g groups started on day 7. It was noted that the mortalities started to reduce up to 4 days in all treatment groups (Figure 4.1). There were only 5 weight groups included 10, 15, 25, 30 and 40 g fish with final percentage mortalities recorded at 9, 20, 22,

7 and 33%, respectively. No mortality was observed in the 20 g, 35 g fish and the control group, when observed over a 14 day period.

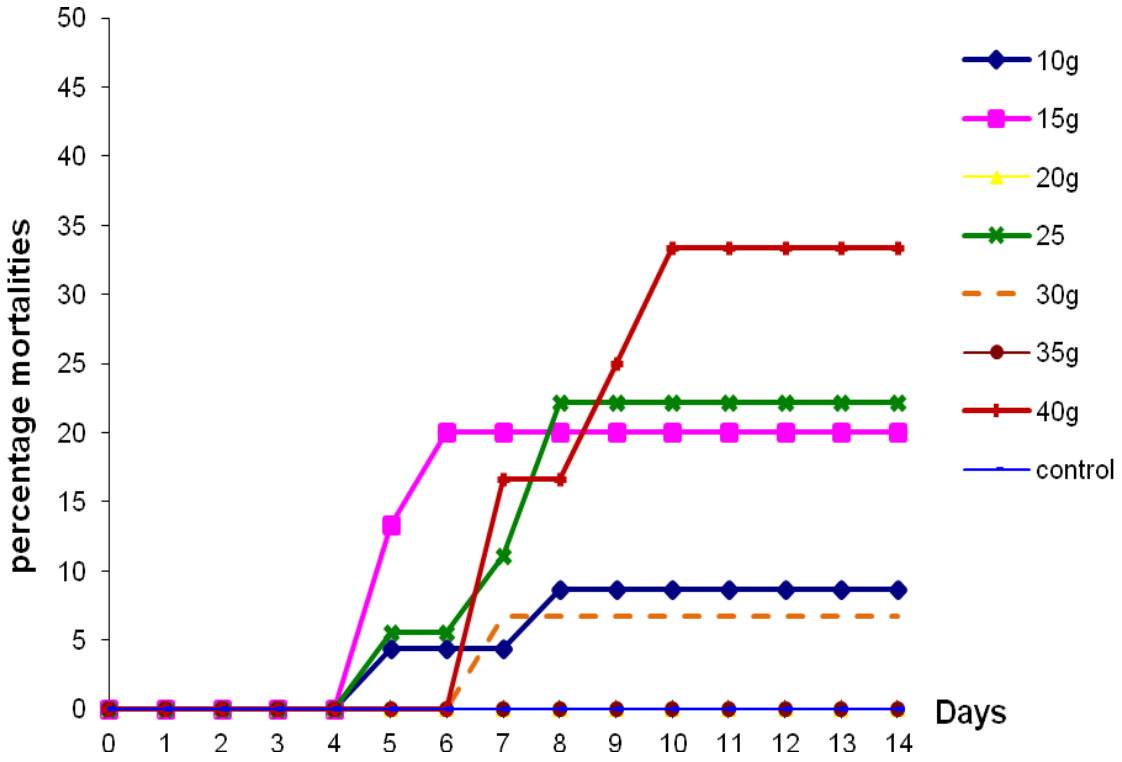

Figure 4.1 Cumulative percentage daily mortalities in the tanks of different sized fish following i.p. injection with *Streptococcus agalactiae*.

The percentage of fish mortality in all treatment groups varied from 0 to 33% between groups but the association with weight was weak (R^2 = 0.02) as shown in Figure 4.2.

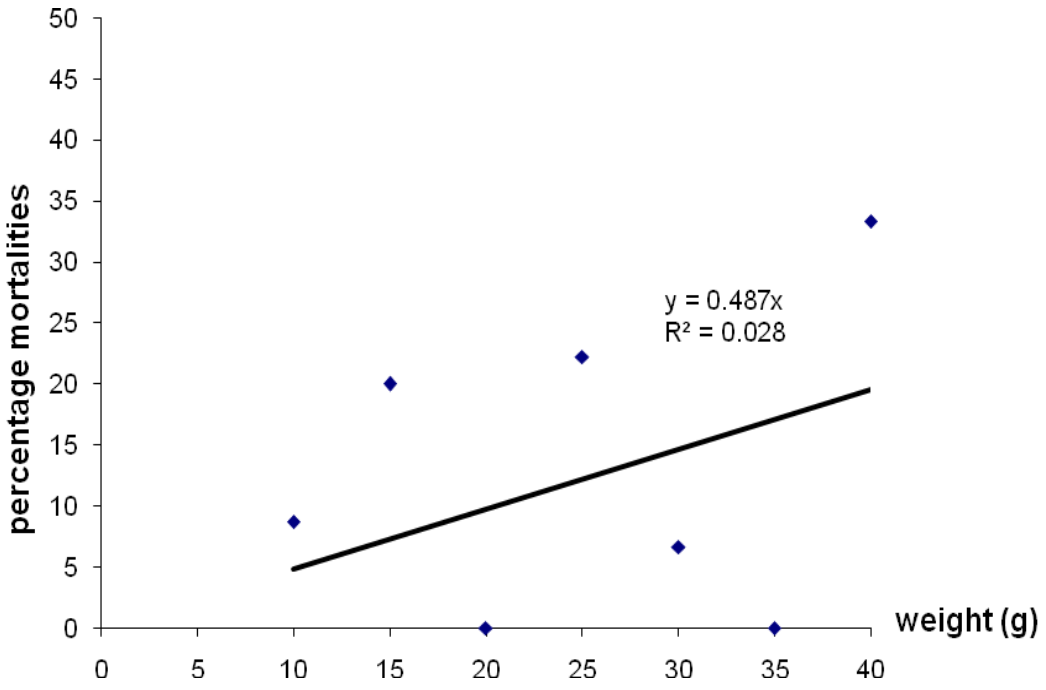

Figure 4.2 A correlation graph showing a weak relationship between cumulative percentage daily mortality and fish weight following a trial whereby differently sized fish were injected intra-peritoneally with *Streptococcus agalactiae*.

4.4.1.2 Clinical signs and macroscopic findings

Affected fish in the 10, 15, 25, 30 and 40 g fish group showed moderate clinical signs including lethargy, anorexia, erratic swimming/spinning or remaining stationary at the bottom of the tank between days 3 and 5 post-exposure. Internal gross lesions including the presence of splenomegaly (Figure 4.3) were observed in the dead and moribund fish of these groups while in the 20 and 35 g fish group only mild clinical signs were observed. None of the control fish showed any clinical signs of disease.

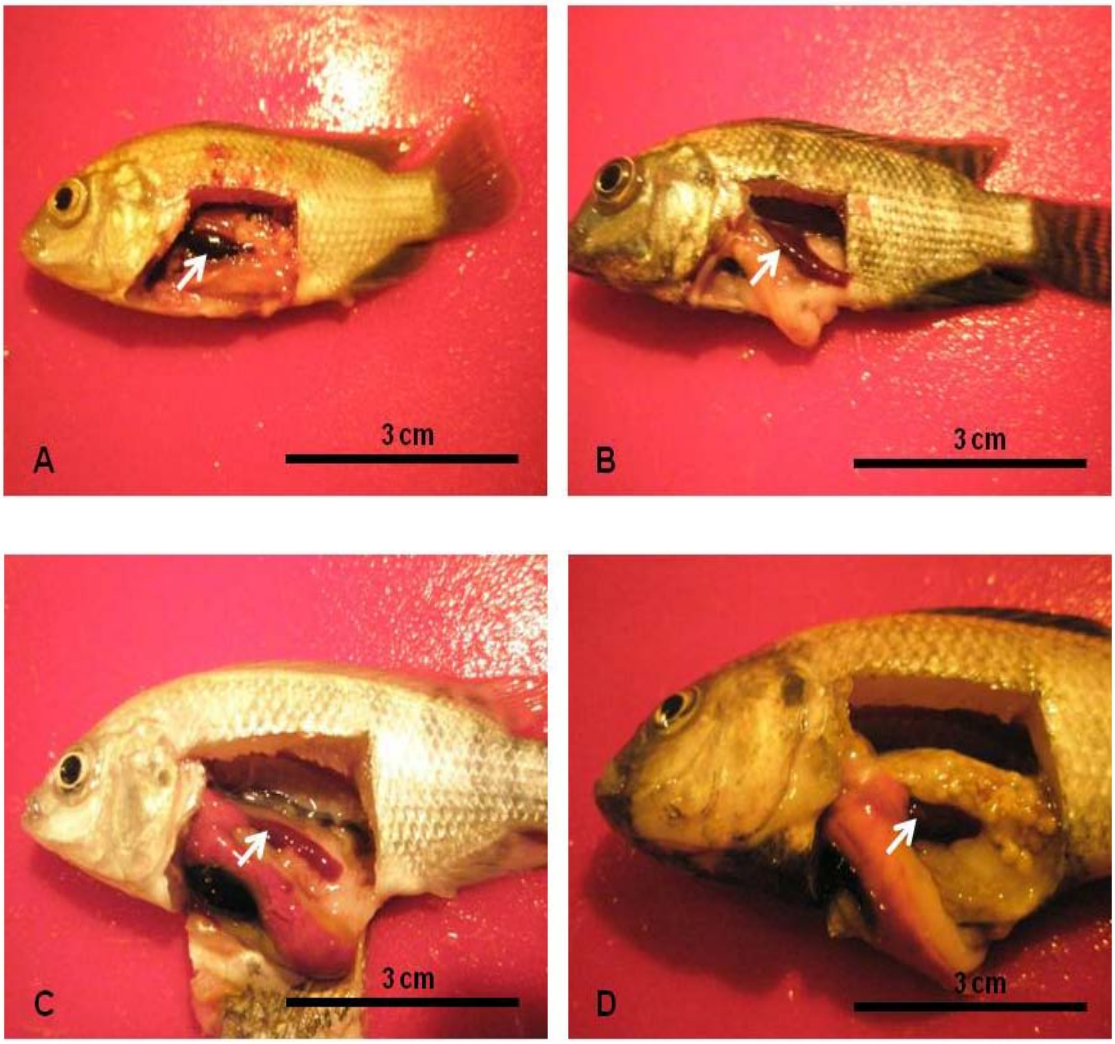

Figure 4.3 Fish receiving on intra-peritoneal injection of *Streptococcus agalactiae* showed splenomegaly (arrows). A, dead 10 g fish on Day 5; B, moribund 15 g fish on Day 5; C, moribund 25 g fish on Day 5; D, moribund 40 g fish on Day 7.

4.4.1.3 Bacterial identification and PCR assay

Bacteria were recovered from the kidney, spleen, eye and brain of all (i.e. 100%) of the freshly dead and moribund fish from the 10, 15, 25, 30 and 40 g groups of fish. No bacteria were recovered from any of the surviving fish in any of the treatment groups or in the control group of fish that were sampled.

All bacterial colonies contained Gram-positive cocci, which were non-motile, oxidase negative, showing β-haemolysis, and were positive for Lancefield serogroup B and positive only for Voges–Proskauer, hippurate hydrolysis, alkaline phosphatase, leucine arylamidase, arginine dihydrolase, ribose and trehalose as tested in the API 20 STREP system. Isolates were identified through primary and biochemical tests as *S. agalactiae* were then confirmed by PCR assay. The results showed that all the samples were similar to the *S. agalactiae* type strain NCIMB 701348 and all gave a positive band at the correct molecular weight of 270 bp using the STRA-AgI/STRA-AgII primer set (Figure 4.4). No bands were visible for the negative control samples.

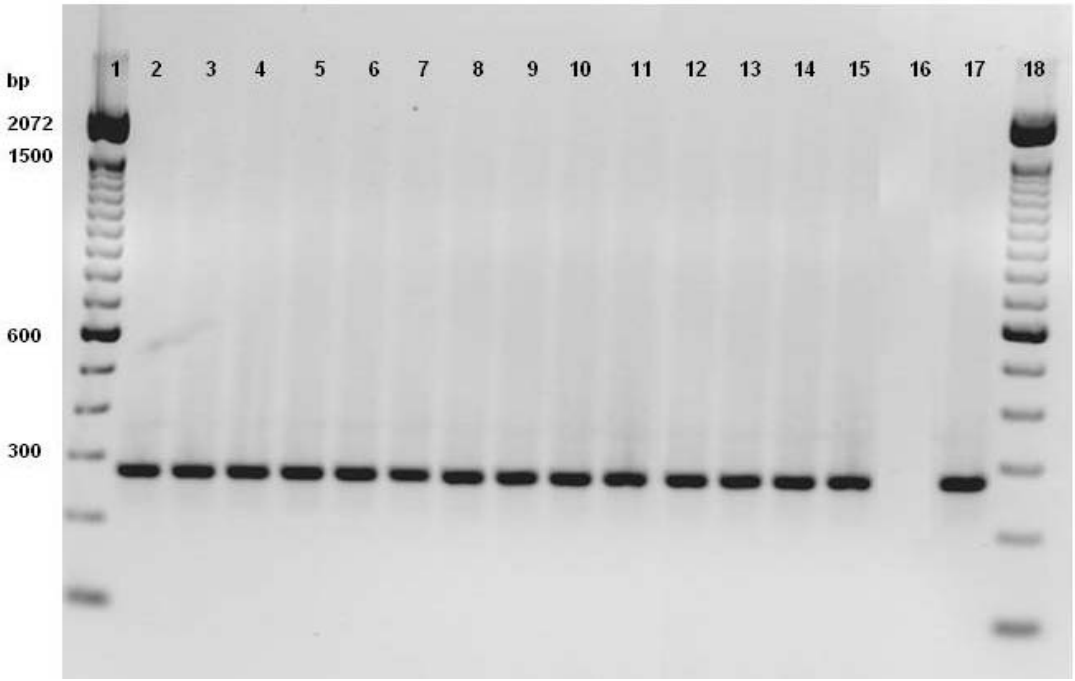

Figure 4.4 PCR amplification of samples from the dead or moribund fish from each treatment group. Lanes 1 & 18, 100 bp DNA ladder; Lanes 2-3, the 10 g fish; Lanes 4-6, the 15 g fish; Lanes 7-10, the 25 g fish; Lane 11, the 30 g fish; Lanes 12-15, the 40 g fish; Lane 16, negative control (no DNA); Lane 17, positive control *Streptococcus agalactiae* type strain NCIMB 701348.

4.4.1.4 Histopathology

The pathological changes observed in the affected 10, 15, 25, 30 and 40 g fish presenting with gross lesions of streptococcosis were located mainly in the spleen, brain and eyes. Common histopathological changes included moderate to severe, diffuse, necrotic, inflammatory lesions involving lymphocytes and macrophages with visible cocci bacteria in these tissues from fish infected during the experiment (Figure 4.5-4.8). No pathology was observed in the 20 g and 35 g treated fish or in the control group.

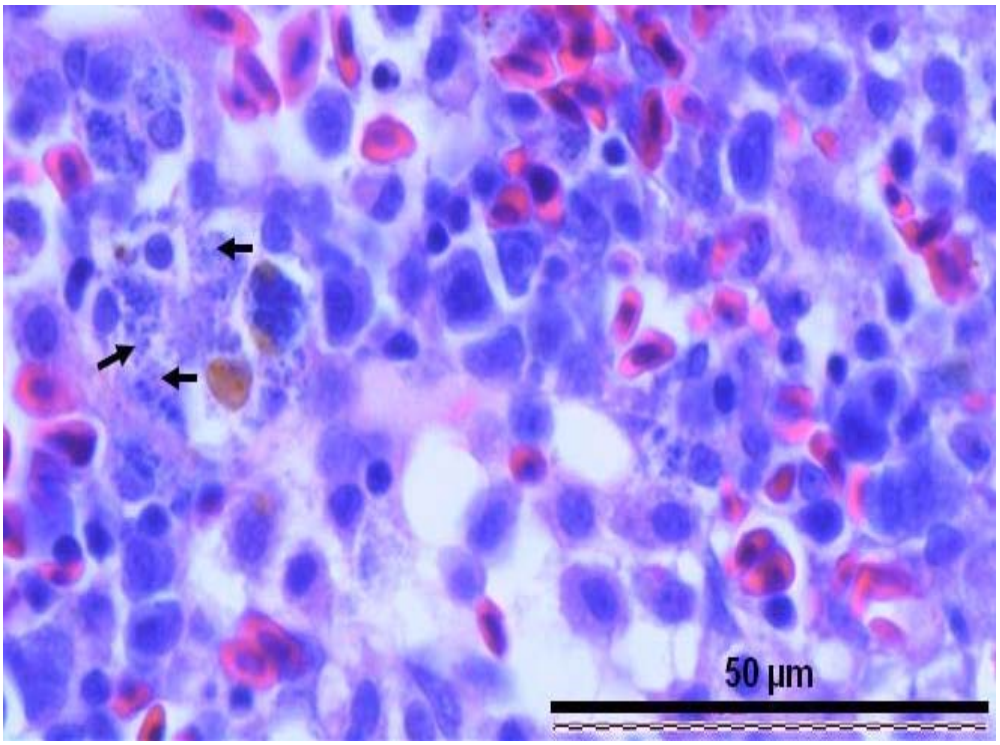

Figure 4.5 Spleen from a moribund fish of 25 g fish group at day 5 with tissue necrosis and bacteria clearly visible within macrophages (arrows).

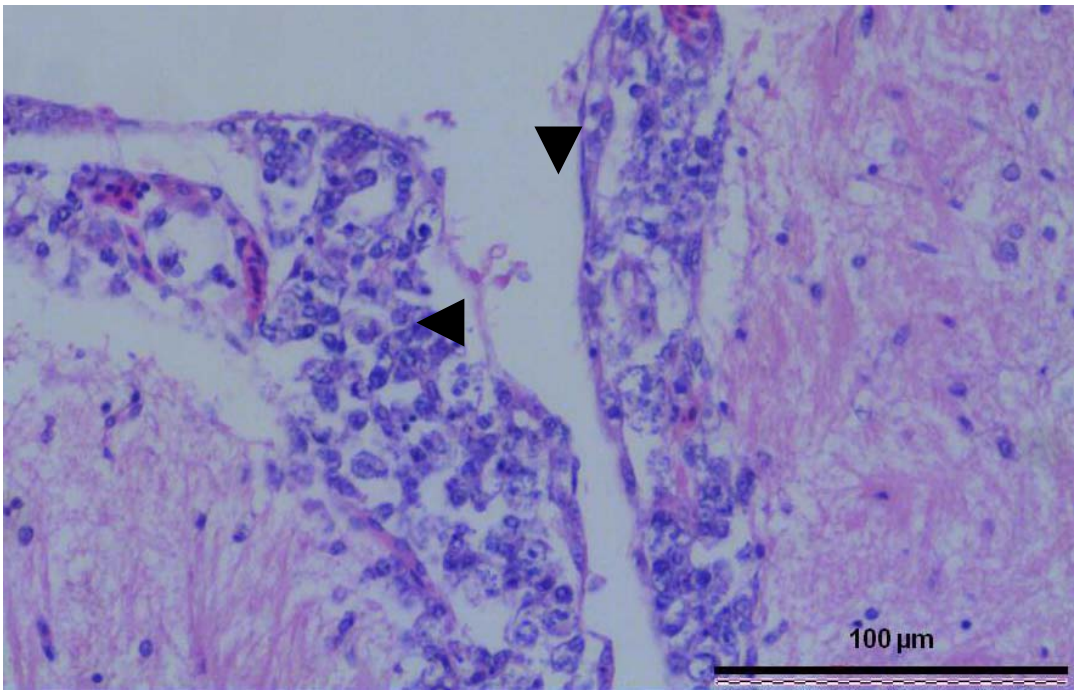

Figure 4.6 Brain of a moribund fish of the 15 g fish group at day 5 with severe meningo-encephalitis. Meninges are thickened with an infiltration of numerous macrophages and lymphocytes (arrowheads).

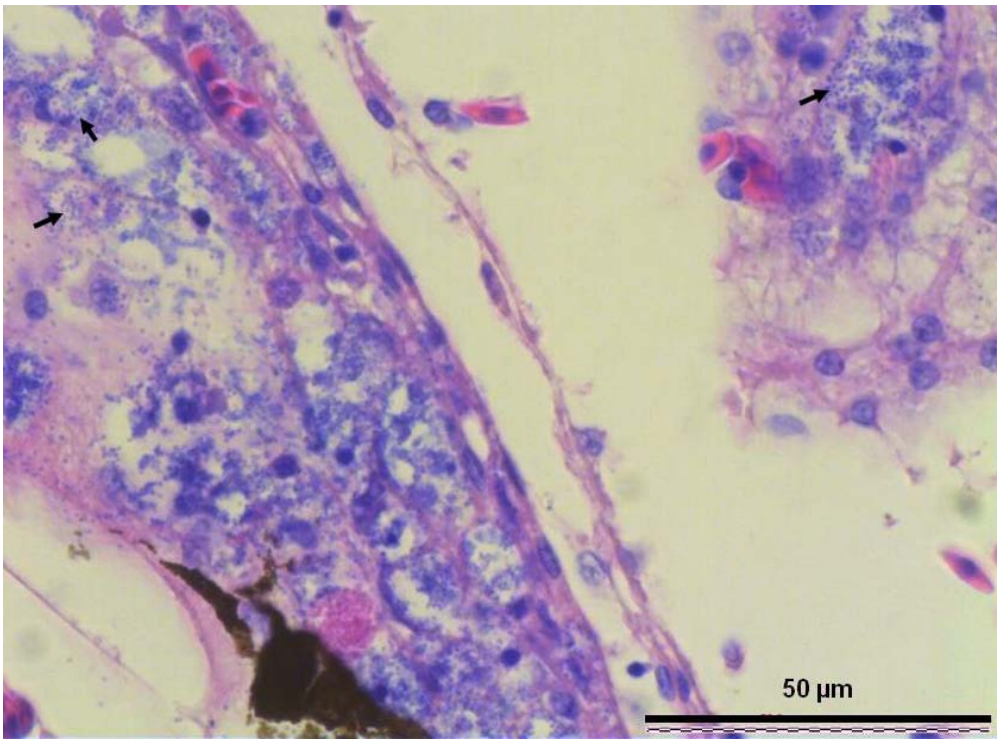

Figure 4.7 Bacteria (arrows) were widely distributed within the meningeal tissue from a moribund fish of 25 g fish group at day 5.

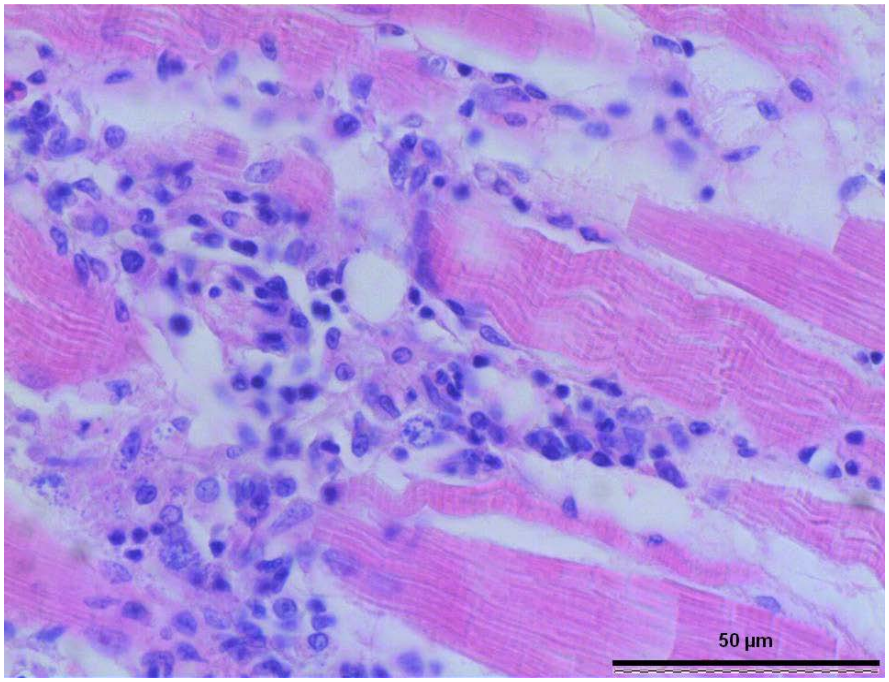

Figure 4.8 Eye of a moribund fish of 40g fish group at day 7. An inflammatory lesion surrounded by numerous macrophages and lymphocytes in the periorbital tissues and the muscles.

4.4.2 Study of different ages

The cumulative percentage daily mortalities during this experiment are provided in Figure 4.10. The total percentage mortalities in the 4 month old fish group was 24%, which was higher than the 4% mortality in the 8 month old fish group when observed over a 14 day period (Figure 4.10). The mortalities were first observed in the 4 month old fish group on day 3 post-bacterial exposure whereas the mortalities in the 8 month old fish group started on day 5. No mortality was observed in any of the control groups during the study period.

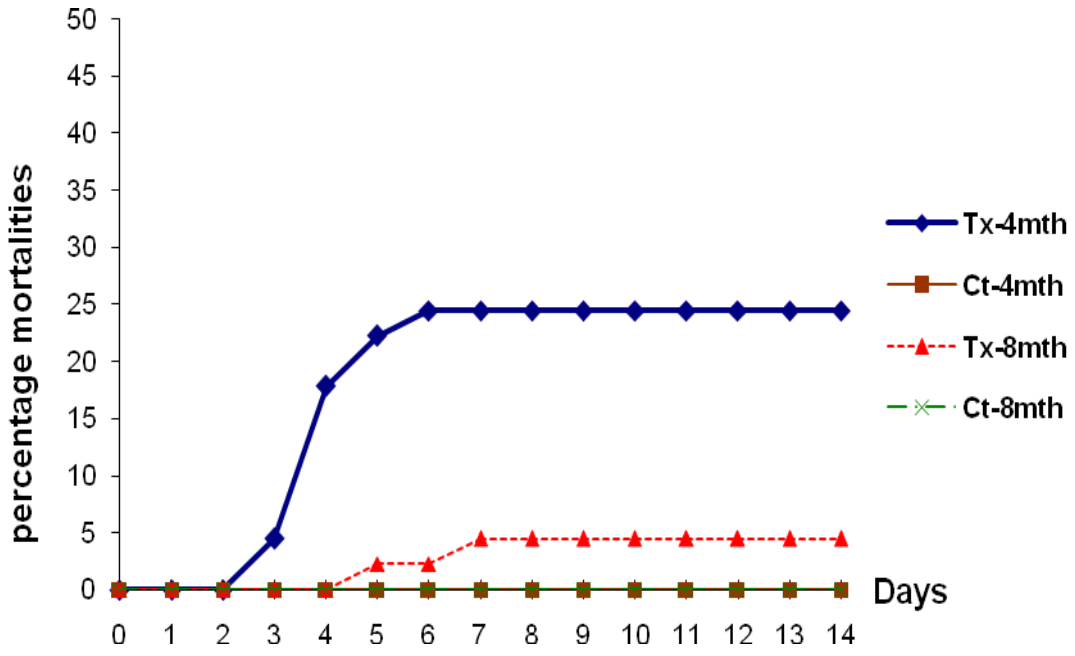

Figure 4.9 Cumulative percentage daily mortalities in the tanks of different aged fish following i.p. injection with *Streptococcus agalactiae*.

All affected fish presented with lethargy, anorexia and erratic swimming/spinning. Internal gross lesions, splenomegaly was observed in the dead and moribund fish. Pure bacterial cultures were recovered from the kidney, spleen, eye and brain of all (i.e. 100%) of the fresh dead and moribund fish. None of the control fish showed clinical signs of disease. Based on the standard conventional methods, API 20 Strep system and Lancefield grouping results, these isolates were identified as group B *S. agalactiae*.

4.5 Discussion

This study investigated the relationship between tilapia fish weight or age (the cut-off point chosen at 20 g fish) and uptake and establishment of a bacterial infection due to *S. agalactiae* through experimental bacterial challenge. The results of this study found that infected fish showed clinical signs, lesions and pathological changes similar to those of natural infection as described in Chapter 3 (Salvador et al., 2005; Zamri-Saad et al., 2010; Abuselina et al., 2011; Ye et al., 2011). However, the results from this study found no apparent association between fish weight and mortality. The mortality data from this study in fish of the same age but different weight varied between the weight groups and showed that the bacteria were able to cause infection in a wide range of fish weights. This was in agreement with the results described by Suanyuk et al. (2008). However, the results of the study present were also in contradiction to the findings of Hernández et al. (2009) and Jiménez et al. (2011), who reported that tilapia less than 20 g in weight were not susceptible to *S. agalactiae* infection. The reason for the reported weight related difference

in susceptibility of tilapia to streptococcal infections may be linked to the high stocking density, handling and intensive culture conditions. In the current study of differently aged fish but those that were the same weight, higher total mortality was seen in the younger fish (4 months) compared with the 8 month fish. This would suggest an association between the age of fish and *S. agalactiae* infection in Nile tilapia, under these experimental conditions. This difference could be due to the development of the immune response in the two different ages of fish (Evans et al., 2004a). The immunological response the fish at these two categories was not evaluated in this study. However, future work should be performed to determine the immune response to *S. agalactiae* infection of different age ranges of fish leading to disease susceptibility.

Additionally, the differing establishment of the bacterial disease may vary according to natural and experimental *S. agalactiae* infections. The difference in fish species, bacterial strain and concentration, routes of infection, water quality, and variation in management, environment conditions and other factors associated with co-infections (Eldar et al., 1999b; Evans et al., 2000; McNulty et al., 2003; Austin and Austin, 2007; Xu et al., 2007, 2009) may all affect the uptake of the pathogen. The findings of earlier studies by Siti-Zahrah et al. (2008), Suanyuk et al. (2008), Hernández et al. (2009) Mian et al. (2009) and Zamri-Saad et al. (2010) showed that when Nile or red tilapia in different weight and / or age groups were randomly sampled during the periodic natural outbreaks of *S. agalactiae* infection in the farm. Moreover, the farms were typical for the region in that they were comprised of earth ponds and floating

cages located in lakes or rivers. Sample collection and prevalence data records showed that a large variation in the weight of fish affect the mortality. In contrast, the fish in both studies presented were kept under experimental conditions at a high stocking density using a bacterial strain and concentration by i.p. injection. In addition, the fish in this study were known to be infected because they had been intraperitoneally inoculated, meaning that the natural defence barriers which the microorganism must usually transverse on its route to systemic distribution would thus have been avoided (Jiménez et al., 2011). This fact may explain the difference in susceptibility to infection and the reproducibility of the typical clinical signs and lesions caused by *S. agalactiae* in tilapia.

The onset of mortality in these studies was slower than that seen in the previous studies in presented Chapter 3. Also the mortality was lower than the previously reported 50% mortality at 216 h in Chapter 3 using the same bacterial isolate and concentration. The reason for this could be that a different fish population was used that had a different weight ranges i.e. 10-40 g at 8 months old or an average weight of 5 ± 1 g at 4 and 8 months old, while the fish in the previous study were an average 20 ± 5 g in weight at 6 months old. In addition, water temperatures used during the current challenge study was slightly lower (25 ^{o}C) compared to the 27 ^{o}C used in the previous studies in Chapter 3. There is a strong association between water temperatures and increased mortality of streptococcal infections in tilapia (Agnew and Barnes, 2007; Siti-Zahrah et al., 2008; Bromage and Owens, 2009; Filho et al., 2009;

Mian et al., 2009; Rodkhum et al., 2011). In particular, Rodkhum et al. (2011) demonstrated that Nile tilapia had no subsequent mortality when exposed to *S. agalactiae* at 25 ^{o}C via an experimental water immersion route. However, the fish in this study did present with clinical signs, lesions and that the fish did die and that bacteria were recovered in this study, the water temperature alone might not be the only factor responsible for fish susceptibility to this disease. To investigate this further challenge studies could be performed to determine the effect of weight and / or age range at different water temperatures.

The results of this study would support that tilapia can become infected over a wider weight range than previously reported in the published literature. In addition, the age of fish appears to be associated with their susceptibility to *S. agalactiae* infection in Nile tilapia. This work also demonstrated that the experimental challenge model was reproducible in producing mortalities, clinical signs, lesions and pathological changes associated with *S. agalactiae* which were similar to those described in natural streptococcal infections. Further work is required to investigate the variation of individual fish responses, farming conditions and water temperatures on the establishment of the infection at varied weights and / or age ranges to obtain further knowledge on streptococcosis in Nile tilapia, in order to make it possible to control and eradicate the disease in fish farms.

Chapter 5 - The effect of coping style on susceptibility to experimental *Streptococcus agalactiae* infection in Nile tilapia, *Oreochromis niloticus*

5.1 Abstract

Individual animals differ in their physiological responses to challenge or show different behavioural responses, often referred to as coping styles. An experimental study was performed, to investigate the correlation between different coping styles and susceptibility to *Streptococcus agalactiae* infection in Nile tilapia (*Oreochromis niloticus*). Fish were screened and scored depending on their risk-taking behavioural responses to a range of different environmental conditions. This was repeated up to 3 times. Individual differences in behavioural responses were evident but only consistent across behavioural trials for some individuals. A selection of fish with consistent responses across the trials was exposed to the 6×10^7 CFU/ml of *S. agalactiae* by intraperitoneal (i.p.) injection and their disease susceptibility determined. No difference was seen in the aggression, body colour changes and ventilation frequency between the different fish categories. However, there was a relationship between different coping styles and *S. agalactiae* infection, with shy fish experiencing higher mortality rates than bold fish suggesting that the bold fish might be less susceptible to infection than shy fish.

5.2 Introduction

Individual animals differ in their behavioural responses to a variety of challenges or situations, such as feeding, mating and aggression. A tendency to respond in a certain manner has been referred to as a coping style, behavioural syndrome or even personality (Korte et al., 2005; Koolhaas et al., 2007). There are many ways to describe the various behavioural adaptations in a variety of situations and they have often been described in simplistic terms such as “bold” or “shy” although these tend to be the extremes of a spectrum of behaviour and do not include the various aspects of response. For example, in many situations it is not known whether if bold correlates with aggression. If the individual is described as “bold” or proactive, they tend to have an adrenaline-based response and be more prone to take risks or put themselves in danger. They would typically respond with a fright and flight reaction and may be more aggressive. If the individual is described as “shy” or reactive, they tend to have a cortisol-based response. They are typically risk-avoiders who are more sensitive to danger, typically showing a passive as freeze and hide or avoidance behaviour in situations perceived as dangerous (Sih et al., 2004; Huntingford et al., 2010).

Identifying bold or shy fish is not easy but recently risk-taking has been used in a number of different behavioural screening tests, including; foraging under predation risk (Bell, 2005), response to novel objects (Frost et al., 2007), exploration of novel environments (Huntingford et al., 2010), time to resume feeding in a novel environment (Ruiz-Gomez et al., 2008). However, the

behaviour displayed by these individuals is not always static and may vary across situations and over time, therefore some individuals may be considered as flexible in their behavioural response (Sih et al., 2004; Bell, 2007; Ruiz-Gomez et al., 2011).

In some animal populations social interaction can be a potential stressor. This can be a particular stressor for fish populations where they form social hierarchies. If subordinate or non-aggressive fish cannot escape certain threatening behaviour of dominant or aggressive individuals, then this can cause a stress response in the subordinate individuals often resulting in chronic or repeated stress. All forms of stress, if it repeated, chronic or mismanaged can result in physiological alterations including immune suppression and increased disease susceptibility to pathogens (Peters et al., 1988, 1991). In a recent study, MacKenzie et al. (2009) demonstrated that such differences in coping styles have an influence on gene expression associated with metabolic, stress and immune responses in individual common carp (*Cyprinus carpio*). This result also showed significant differences and opposite responses to bacterial lipopolysaccharide (LPS), as an inflammatory challenge between bold and timid fish. In addition, a study by Huntingford et al. (2010) showed that the plasma lactate and glucose concentrations and expression of the cortisol receptor gene were significantly higher in risk-avoiding than in risk-taking fish. Therefore, individual variability in behavioural response to challenge seems to be related to differences in the physiological status. This might lead to increase in possible disease susceptibility in fish with different coping styles.

Tilapia are commercially important fish farmed worldwide, supplying the international food chain. These fish species are well known to be aggressive, but little is known about the effect of individual coping styles. In this study, response to a novel environment was used to determine the risk-taking phenotype in Nile tilapia, to develop methodologies to check for individual consistency and to investigate their susceptibility to the pathogenic bacterium *S. agalactiae* on different coping styles in Nile tilapia. All fish experiements had been done under a Home Office Project Licence number 60/3949.

5.3 Materials and methods

5.3.1 Fish

One-hundred twenty five Nile tilapia*, Oreochromis niloticus* with a weight range of 20-50 g, from a single population from the same breeding pair were obtained from the Tropical Aquarium, Institute of Aquaculture, Stirling, UK. Prior to the experiments these fish had been reared together in the same tank for 7-9 months under normal conditions in a 500L fibreglass tank maintained with continuous flow-through water, a 12 h light: 12 h dark cycle and water temperature at 28 ± 2 ^{o}C. Aeration was supplied through an air stone to each tank and fish were fed with a commercial diet (Skretting Trout Standard Expanded) twice daily.

5.3.2 The test compartment

All behavioural studies were performed in the Tropical Aquarium, Institute of Aquaculture, Stirling, UK in a 162 L fibreglass tank 180 × 45 × 30 cm (length × width × height) in dimension with a water depth of 20 cm (Figure 5.1) with a warm-water circulating system and water temperature at 28 ± 2 ^{o}C. The tank consisted of 2 areas: a covered or sheltered area and an open or exposed area. At one end of the tank there was an enclosed, darkened settling area (50 cm in length), separated with a removable transparent plastic partition or double opaque / transparent plastic partitions (with and without opening; 5 cm in width and 8 cm in height formed an exit into the open area of the tank), once opened, it permitted the fish to see the novel environment. The novel environment consisted of the open area, with a Petri dish containing commercial pellet food in the middle of the tank or groups of 5 fish as an attractant.

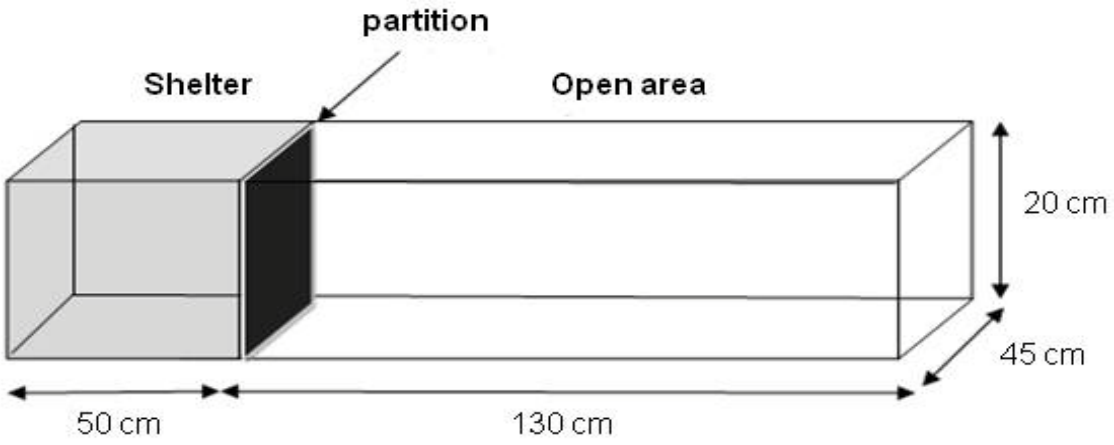

Figure 5.1 Schematic diagram of the screening tank used in a novel environment test. The shelter area was separated from the open area by a single or double removable plastic partition. In the open area a dish of food or a group of 5 fish served as the “attractant”.

5.3.3 Screening for risk-taking behaviour

All the behavioural experiments in this study are listed in Table 5.1. Food was withheld for 24 h prior to fish being moved to the screening tank to ensure that during the test all the fish were hungry and they had to make the decision to leave a safe area in order to eat. The fish were allowed to settle in the shelter for 5 min, after which the partition was gently opened, giving the fish the option to explore a novel, potentially dangerous environment.

Table 5.1 Behavioural screening tests of individual/groups of tilapia in a novel environment containing a range of attactants.

Experiment Number	Fish	Attractant	Condition in a tank with the partition	Total number of fish	Section
1	In group	food	a removable partition	30	5.3.3.1
2	Individual	food	a removable partition	10	5.3.3.2
3	Individual	fish	a removable partition	25	5.3.3.3
4	Individual	fish & food	a removable partition & a partition with opening	20	5.3.3.4
5	Individual	food & fish	a partition with opening	20	5.3.3.5

5.3.3.1 Screening a group of fish in a tank with a removable partition using food as an attractant

A total of 30 fish were used. Ten fish at a time were placed into the shelter separated by a transparent plastic partition from the open area containing food. The behaviour of the fish was then observed after removing the partition (Figure 5.2). The fish were housed together between trials. This process was repeated twice within a 24 h period.

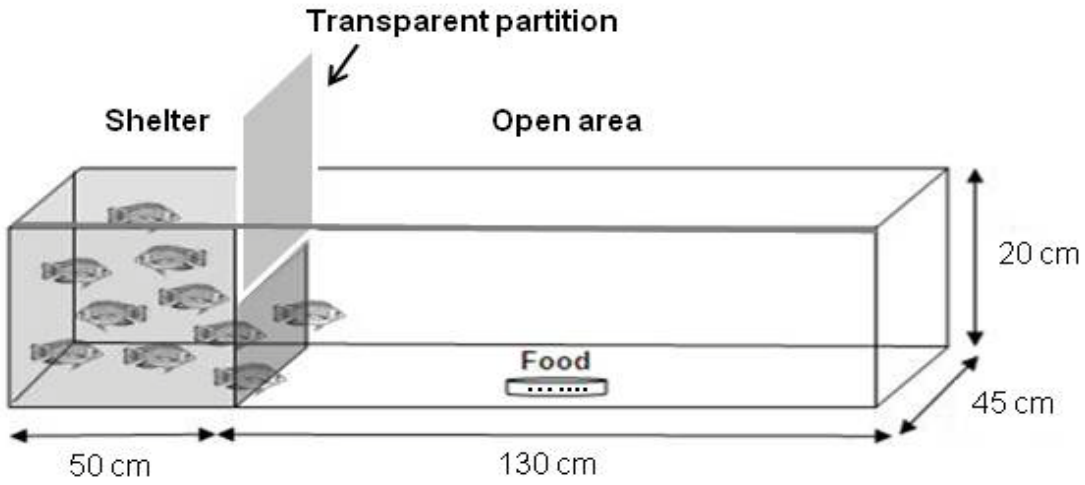

Figure 5.2 Schematic diagram of the screening tank with a removable transparent partition used in a novel environment test. Fish were observed in the portion of the tank containing the food by removing the partition.

5.3.3.2 Screening of individual fish in a tank with a removable partition using food as an attractant

Ten fish were used for this trial. Each fish was placed into the shelter separated by a transparent plastic partition from the open area containing the food. The behaviour was then observed after removing the partition (Figure 5.3). When not being tested, the fish were housed together between trials and this was repeated twice within 24 h.

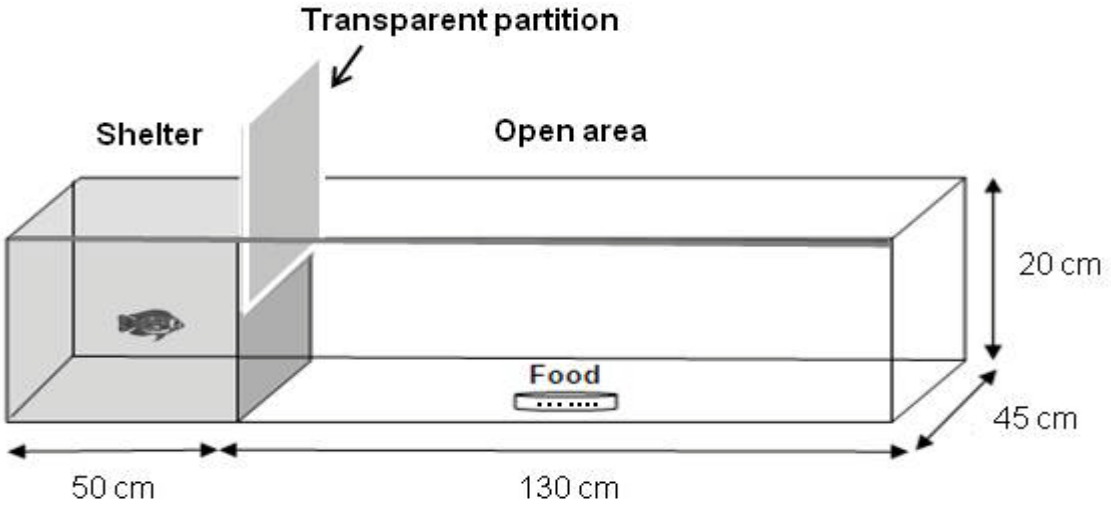

Figure 5.3 Schematic diagram of the screening tank with a removable transparent partition used in a novel environment test. Individual fish were observed in the tank containing food by removing the partition.

5.3.3.3 Screening of individual fish in a tank with a removable partition using groups of fish as an attractant

Twenty-five fish were used for this trial. Each fish was placed individually into the shelter separated by a transparent plastic partition from the open area containing a group of 5 fish. The groups of fish were moved to the end of the open area behind a transparent partition and the partition between the shelter and the open area removed and the behaviour observed and recorded (Figure 5.4). The fish were housed together between trials. This process was repeated twice with an interval of 7 days.

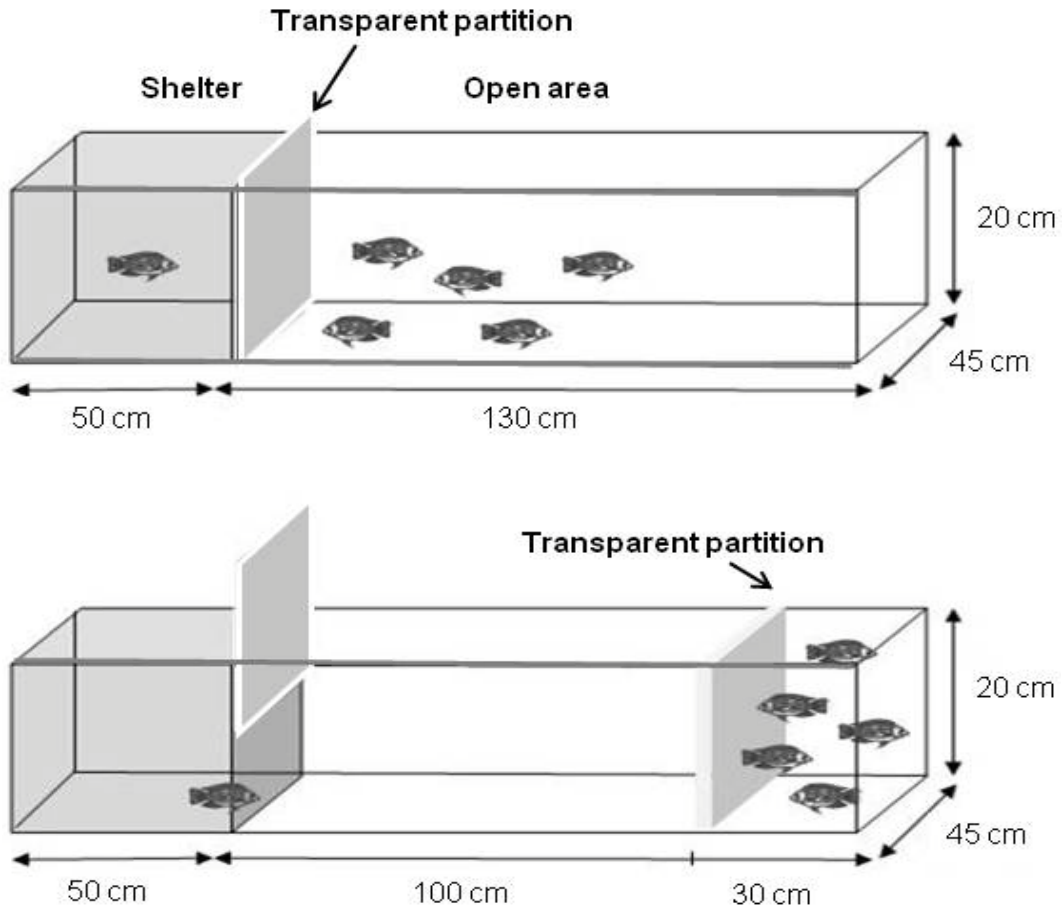

Figure 5.4 Schematic diagram of the screening tank with a removable transparent partition used in a novel environment test. Individual fish were observed in a tank containing groups of fish by removing the partition.

5.3.3.4 Screening of individual fish in a tank with a removable partition using groups of fish as an attractant and a partition with an opening using food as an attractant

A total of 20 fish were screened individually with the groups of fish and this was repeated three times with an interval of 7 days as described previously in 5.3.3.3 (Figure 5.4). Then the fish were re-used and screened by placing each fish into the shelter partitioned by double opaque plastic partitions with and without openings to the area containing the food. Their behaviour was then observed by removing the solid partition revealing the opening (Figure 5.5). The fish were housed together between trials. This process was repeated three times with an interval of 7 days.

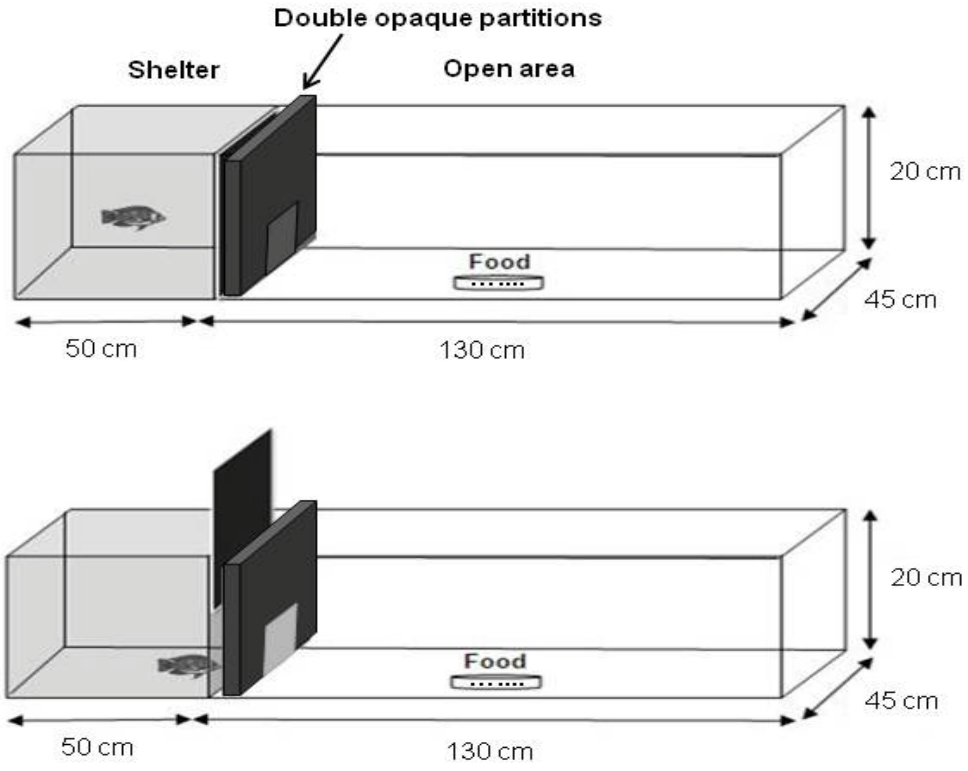

Figure 5.5 Schematic diagram of the screening tank with double opaque partitions used in a novel environment test. Individual fish were observed in the tank containing food by removing the solid partition.

5.3.3.5 Screening of individual fish in a tank with a partition with an opening using food and groups of fish as attractants

A total 20 fish were screened individually with the food and this was repeated three times with an interval of 7 days as described previously in 5.3.3.4 (Figure 5.5). Then the fish were re-used and screened by placing each fish into the shelter partitioned by double transparent plastic partitions with and without opening to the area containing the groups of fish. Their behaviour was then observed by removing the solid partition revealing the opening (Figure 5.6). Fish were housed together between trials. This process was repeated three times with an interval of 7 days.

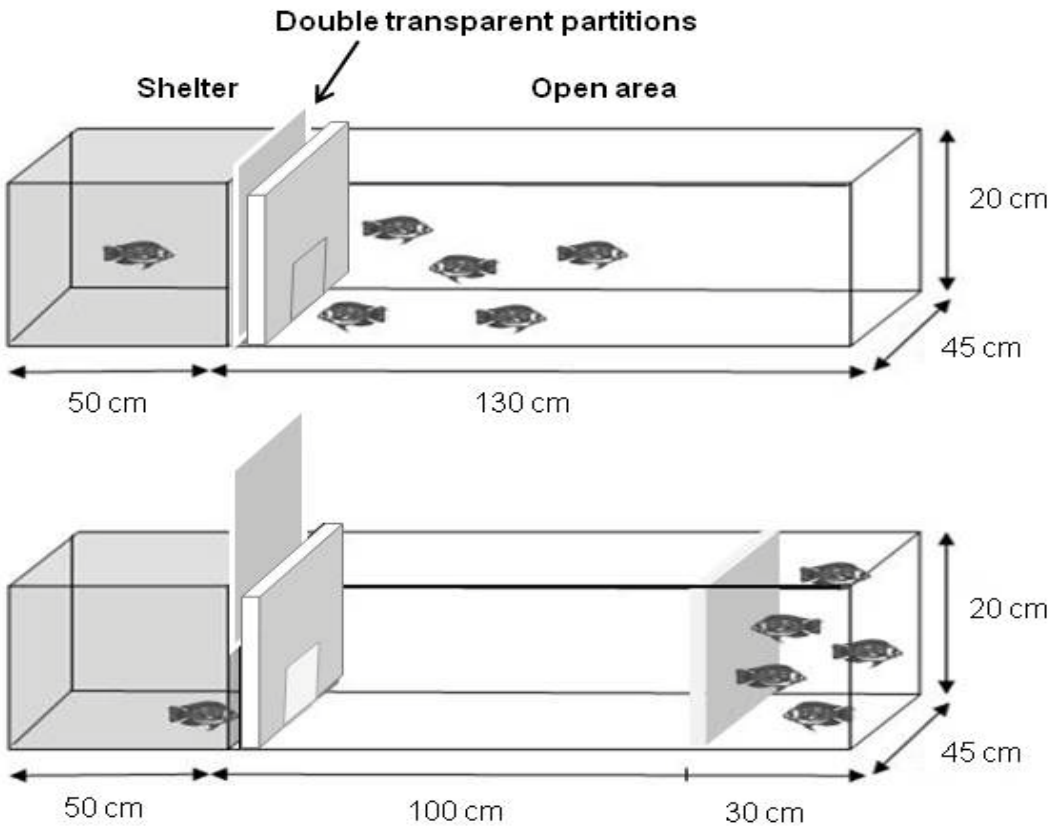

Figure 5.6 Schematic diagram of the screening tank with double transparent partitions used in a novel environment test. Individual fish were observed in the tank containing groups of fish by removing the solid partition.

5.3.3.6 Behavioural observation

Each fish was observed for its behavioural response, starting immediately after the partition was gently lifted and removed from the tank. The time for the fish to leave the shelter area was recorded, up to a maximum of 10 min. A set of criteria were then developed to distinguish between risk-taking phenotypes; very bold, bold, intermediate and shy fish which were categorised according to their emergence sequence, but with the time limits (Table 5.2). The fish were individually marked using Panjet with alcian blue dye to identify the individuals within the behavioural groups. Then they were placed back into a single holding tank.

Table 5.2 Criteria used to distinguish between the risk-taking phenotypes in individual fish.

Risk-taking phenotypes	Criteria
Very bold	The fish was close to the partition, then entered the open area immediately
Bold	The fish was around the shelter and sometimes close to the partition, then entered the open area within 4 minutes
Intermediate	The fish was around the shelter and sometimes came in and out, then entered the open area between 4-10 minutes
Shy	The fish was at the edge away from the partition and remained in the shelter for up to 10 minutes

Once the fish had been classified they were then selected for a subsequent experimental bacterial challenge study.

5.3.4 Social interactions

Ten randomly chosen pairs of fish were used for the individual screening when testing aggression. Each pair of fish was removed from the stock tank, placed into the test tank and their behaviour observed for 20 min. Behavioural responses that were looked for included aggressive behaviour, fighting, battling for rank and body colour change. In addition, the ventilation frequency of the individual fish was determined by counting the number of opercular movement min^{-1} for 15 min. Then they were placed back into a single holding tank. This process was repeated three times with an interval of 24 h. At the end of the experiment, the behaviour of each fish was determined as dominant / aggressive or subordinate / non-aggressive fish and marked accordingly, as previously described. Then they were placed back into the single holding tank.

5.3.5 Pilot experimental challenge study

Following behavioural screening of the tilapia, an experimental challenge study was performed in the Aquatic Research Facility (ARF), Stirling in a 10 L plastic tank with continuous flow-through water at 0.38 L/minute, a 12 h light: 12 h dark cycle and water temperature at 27 ^{o}C. Aeration was supplied through an air stone to each tank and fish were fed with a commercial diet (Skretting Trout Nutra 25) to apparent satiation once daily.

A group of twenty fish determined from the behavioural study (5.3.3.3) as being either “bold” (n=10) or “shy” (n=10) received an i.p. injection of 0.1 ml of 6×10^7 CFU/ml of the 3rd passage of *S. agalacti*ae isolate number 1. These fish were held at a stocking density of 45 g/L. The bacterial passage, bacterial challenge inoculums and bacterial identification works were described previously in Chapters 2 and 3.

All fish were monitored daily for 14 days post challenge and checked for mortality and clinical signs of disease. Any fresh dead or moribund fish were removed and observed the gross lesions both externally and internally. Then the kidney, spleen, eye and brain were aseptically sampled for recovery of *S. agalactiae* on TSA (Oxoid, U.K.). Bacterial identification was performed as previously described in Chapter 2. At the end of the 14 day experimental period, all surviving fish were sampled as described above.

5.4 Results

5.4.1 Screening for risk-taking behaviour

All the results in this study were summarized and are listed in Table 5.3.

Table 5.3 Summary of the results from all behavioural screening tests of tilapia in a novel environment.

Experiment number	Fish	Attractant	Condition in a tank with the partition	Screening	Coping styles				Total number of fish	Section
					Very bold	Bold	Intermediate	Shy		
1	In group	food	a removable partition	All fish had emerged from the shelter within 5 min.					30	5.4.1.1
2	Individual	food	a removable partition	All fish remained in the shelter until 10 min.					10	5.4.1.2
3	Individual	fish	a removable partition	1st	5	5	3	12	25	5.4.1.3
				2nd	1	12	4	8		
4	Individual	fish	a removable partition	1st	5	5	5	5	20	5.4.1.4
				2nd	0	10	4	6		
				3rd	0	17	0	3		
		food	a partition with opening	1st	0	5	2	13		
				2nd	0	8	2	10		
				3rd	0	8	3	9		
5	Individual	food	a partition with opening	1st	0	7	5	8	20	5.4.1.5
				2nd	0	10	4	6		
				3rd	0	7	1	12		
		fish		1st	0	5	3	12		
				2nd	0	10	4	6		
				3rd	0	12	2	6		

5.4.1.1 Screening a group of fish in a tank with a removable partition using food as an attractant

When the partition was removed in the tank, the first 2 fish had emerged from the settling area within 2 min then the remaining 8 fish followed them. After a period of 5 min, all 10 fish had emerged from the "safe" area during this period.

5.4.1.2 Screening of individual fish in a tank with a removable partition using food as an attractant

After the partition was removed, all 10 fish remained in the shelter over the experimental period of 10 min.

5.4.1.3 Screening of individual fish in a tank with a removable partition using groups of fish as an attractant

This procedure showed considerable variability in emergence time, with the fastest fish emerging immediately and the slowest of those taking up to 9 min before leaving the "safe" area. There was an individual difference in number of fish between the 2 screening times (Table 5.4)

Table 5.4 Number of fish from individual screening in a tank with a removable partition using groups of fish as an attractant.

Screening	Coping styles				Total
	Very bold	Bold	Intermediate	Shy	
1st	5	5	3	12	25
2nd	1	12	4	8	25

Fish were classified as bold or shy if they appeared to consistently fall either side of intermediate and did not move more than one category between the two screening times. There were 10 bold, 10 shy and 5 flexible fish (Table 5.5).

Table 5.5 Categorisation by individual fish screening in a tank with a removable partition using groups of fish as an attractant and eventual classification

Fish number	Screening		Classified
	1st	2nd	
1	Very bold	Very bold	BOLD
2	Very bold	Bold	BOLD
3	Very bold	Bold	BOLD
4	Very bold	Intermediate	FLEXIBLE
5	Very bold	Shy	FLEXIBLE
6	Bold	Bold	BOLD
7	Bold	Bold	BOLD
8	Bold	Bold	BOLD
9	Bold	Bold	BOLD
10	Bold	Bold	BOLD
11	Intermediate	Bold	BOLD
12	Intermediate	Bold	BOLD
13	Intermediate	Shy	SHY
14	Shy	Shy	SHY
15	Shy	Shy	SHY
16	Shy	Shy	SHY
17	Shy	Shy	SHY
18	Shy	Shy	SHY
19	Shy	Shy	SHY
20	Shy	Bold	FLEXIBLE
21	Shy	Bold	FLEXIBLE
22	Shy	Bold	FLEXIBLE
23	Shy	Intermediate	SHY
24	Shy	Intermediate	SHY
25	Shy	Intermediate	SHY

5.4.1.4 Screening of individual fish in a tank with a removable partition using groups of fish as an attractant and a partition with an opening using food as an attractant

Two procedures showed considerable variability in their emergence times. Only 1st time of screening using groups of fish as attractant had the fish emerged immediately, classified as very bold. There was an individual difference in the number of fish between the 3 screening times of the two trials. The results of the two trials are presented in Table 5.6.

Table 5.6 Number of fish from individual screening in a tank with a removable partition using groups of fish as an attractant and a partition with an opening using food as an attractant.

Screening with	Coping styles				Total
	Very bold	Bold	Intermediate	Shy	
Fish group					
1st	5	5	5	5	20
2nd	0	10	4	6	20
3rd	0	17	0	3	20
Food					
1st	0	5	2	13	20
2nd	0	8	2	10	20
3rd	0	8	3	9	20

Fish were classified as bold, shy or intermediate if they appeared to consistently fell into the three screening times. Any other result was considered flexible. There were 2 bold, 1 shy and 17 flexible fish screened with groups of fish while the 3 bold, 6 shy and 11 flexible fish screened with food as an attractant (Table 5.7). Comparison between the two trials, both screening tests produced the same result in 12/20 (1 bold, 1 shy and 10 flexible fish). Whilst,

two fish were classified as flexible using other fish but bold with food as an attractant. In 5 fish, the other fish classified them as flexible and the food as shy. One fish was bold with other fish and flexible with food as attractant (Table 5.7).

Table 5.7 Categorisation by individual fish screening in a tank with a removable partition using groups of fish as an attractant and a partition with an opening using food as an attractant and eventual classification.

Fish number	Screening with fish group			Classified	Screening with food			Classified
	1st	2nd	3rd		1st	2nd	3rd	
1	VB	I	B	FLEXIBLE	B	B	I	FLEXIBLE
2	VB	I	B	FLEXIBLE	S	S	S	SHY
3	VB	S	B	FLEXIBLE	S	S	B	FLEXIBLE
4	VB	S	S	FLEXIBLE	S	S	S	SHY
5	VB	S	S	FLEXIBLE	S	S	S	SHY
6	B	B	B	BOLD	B	B	B	BOLD
7	B	B	B	BOLD	I	S	B	FLEXIBLE
8	B	I	B	FLEXIBLE	S	I	S	FLEXIBLE
9	B	I	B	FLEXIBLE	S	S	S	SHY
10	B	S	B	FLEXIBLE	B	B	B	BOLD
11	I	B	B	FLEXIBLE	S	S	B	FLEXIBLE
12	I	B	B	FLEXIBLE	S	B	S	FLEXIBLE
13	I	B	B	FLEXIBLE	B	B	I	FLEXIBLE
14	I	B	B	FLEXIBLE	B	B	B	BOLD
15	I	B	B	FLEXIBLE	S	S	S	SHY
16	S	B	B	FLEXIBLE	S	B	B	FLEXIBLE
17	S	B	B	FLEXIBLE	S	I	B	FLEXIBLE
18	S	B	B	FLEXIBLE	S	S	I	FLEXIBLE
19	S	S	B	FLEXIBLE	I	B	S	FLEXIBLE
20	S	S	S	SHY	S	S	S	SHY

Identification: VB, Very bold; B, Bold; I, Intermediate; S, Shy

5.4.1.5 Screening of individual fish in a tank with a partition with an opening using food and groups of fish as attractants

These two procedures showed considerable variability in the emergence time. In addition, there were no fish that emerged immediately after screening with both food and groups of fish as attractants. Individual differences in the number of fish between the 3 screening times were found and the results of the two trials are recorded on Table 5.8.

Table 5.8 Number of fish from individual screening in a tank with a partition with an opening using food and groups of fish as attractants.

Screening with	Coping styles				Total
	Very bold	Bold	Intermediate	Shy	
Food					
1st	0	7	5	8	20
2nd	0	10	4	6	20
3rd	0	7	1	12	20
Fish group					
1st	0	5	3	12	20
2nd	0	10	4	6	20
3rd	0	12	2	6	20

Fish were classified as bold, shy or intermediate if they responded consistently in all three screening times. Any other result was considered flexible. There were 5 bold, 1 intermediate, 6 shy and 8 flexible fish screened with both food and groups of fish as attractant but it was not consistent in the same fish in both screening tests (Table 5.9). Comparison between the two trials, both screening tests produced the same result in 9/20 (2 bold, 3 shy and 4 flexible). Whilst, two fish were classified as bold using food and shy with other fish as attractant. In 4

fish, the food was classified as 3 shy and 1 bold while using the other fish as flexible. Four fish were classified as flexible by the food but 2 bold, 1 intermediate and 1 shy by the other fish as attractant. One fish was intermediate with food but bold with other fish as attractant (Table 5.9).

Table 5.9 Categorisation by individual fish screening in a tank with a partition with an opening using food and groups of fish as attractants and eventual classification.

Fish number	Screening with food			Classified	Screening with fish group			Classified
	1st	2nd	3rd		1st	2nd	3rd	
1	B	B	B	BOLD	S	S	S	SHY
2	B	B	B	BOLD	B	B	B	BOLD
3	B	B	B	BOLD	B	B	B	BOLD
4	B	B	B	BOLD	S	I	B	FLEXIBLE
5	B	B	B	BOLD	S	S	S	SHY
6	B	I	S	FLEXIBLE	B	B	B	BOLD
7	B	I	S	FLEXIBLE	S	B	B	FLEXIBLE
8	I	I	I	INTERMEDIATE	B	B	B	BOLD
9	I	B	S	FLEXIBLE	I	B	B	FLEXIBLE
10	I	I	S	FLEXIBLE	S	S	S	SHY
11	I	B	S	FLEXIBLE	S	B	B	FLEXIBLE
12	I	B	S	FLEXIBLE	I	I	I	INTERMEDIATE
13	S	B	B	FLEXIBLE	S	I	I	FLEXIBLE
14	S	B	B	FLEXIBLE	B	B	B	BOLD
15	S	S	S	SHY	I	B	B	FLEXIBLE
16	S	S	S	SHY	S	I	B	FLEXIBLE
17	S	S	S	SHY	S	S	S	SHY
18	S	S	S	SHY	S	S	S	SHY
19	S	S	S	SHY	S	S	S	SHY
20	S	S	S	SHY	S	B	B	FLEXIBLE

Identification: B, Bold; I, Intermediate; S, Shy

5.4.2 Social interaction

Aggression to establish social dominance began during the 5-15 min experimental period. The conflicts consisted of threatening postures, frontal attacks and biting. The attacks were more or less violent and occurred at varying intervals of time. The subordinate fish when attacked by the dominant fish tried to defend themselves either by counterattacks, to escape attacks or to hide in the corner of the tank. During that time the rank order was maintained, thus each contained one dominant and one subordinate individual. After repeating 3 times, some of the individuals were consistently dominant or subordinate at the end of trials. In addition, none of the fish changed their body colour. The ventilation frequency of both dominant and subordinated fish increased immediately after placing them into the tank then it began to slow down and returned to the resting rate within 6-9 min.

Neither of these tests identified any significant differences between the fish as there was no difference observed in the display of aggression, change in body colour or respiration rate between those fish that had been selected as dominant and subordinate.

5.4.3 Pilot experimental challenge study

Cumulative percentage daily mortalities during the experiment are provided in Figure 5.7. Total percentage mortalities were 50% in the shy fish and 10% in the bold fish group, when observed over a 14 day period. The mortalities were first observed in the shy fish group on day 3 post-bacterial exposure whereas the mortalities in the bold fish group started on day 5 (Figure 5.7).

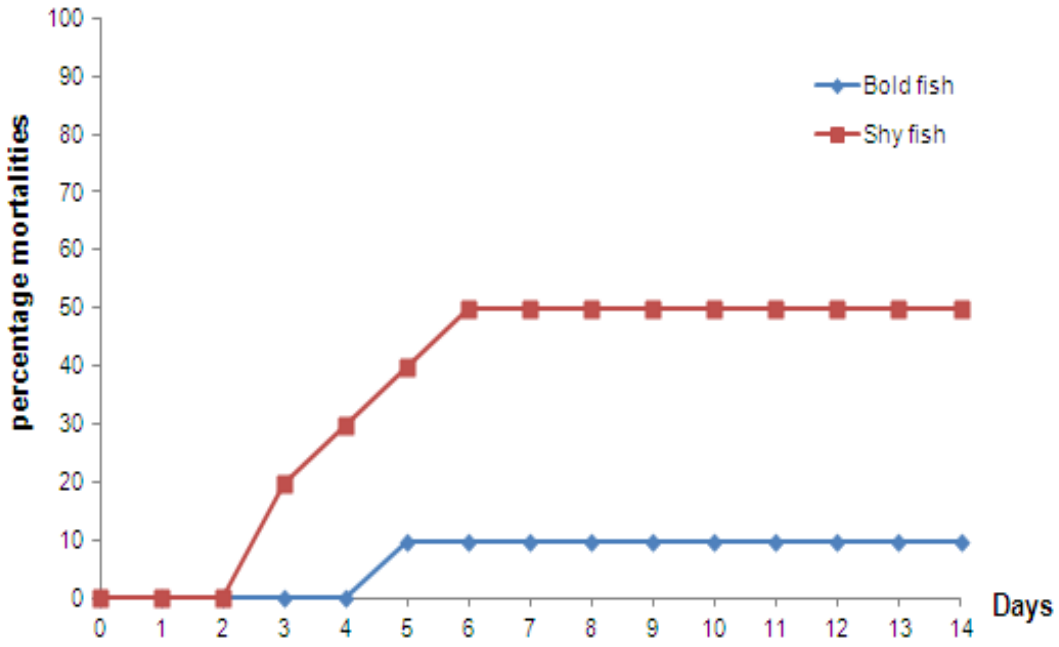

Figure 5.7 Cumulative percentage daily mortalities on different coping styles following i.p. injection with *Streptococcus agalactiae*.

All affected fish showed lethargy, anorexia and erratic swimming/spinning. Internal gross lesions included splenomegaly in the dead and moribund fish. Pure bacterial cultures were recovered from the kidney, spleen, eye and brain of all (i.e. 100%) of the fresh dead and the moribund fish. Based on the standard conventional methods, API 20 Strep system and Lancefield grouping results, these isolates were identified as group B *S. agalactiae*.

5.5 Discussion

The aim of this study was to examine the hypothesis that coping style explains some of the inter-individual variability in response to disease challenge. A number of experiments were conducted to assess the behaviour of the tilapia with variable success, however, one small challenge study was conducted and this demonstrated a difference between fish classified as bold or shy.

Coping styles refer to variability in individual animal behavioural responses. These traits may arise through a growth-mortality trade-off (Stamps, 2007; Biro and Stamps, 2008). According to this view, proactive (often referred to as bold) animals show both physiological and behavioural adaptations for efficient growth, including in terms of behaviour, a tendency to take a risks while foraging and to fight over food. Coping style could be described as the underlying persistent tendency or strategy adopted by the animal. In humans, such a strategy might be referred to as a personality (Korte et al., 2005). Most animals show some flexibility in response despite this underlying coping style and also the animal's coping style can be permanently changed by social or environmental conditions (Sih et al., 2004; Huntingford et al., 2010).

The approach adopted here to assess coping style was a screening tank exposing the fish to a relatively safe area and a relatively threatening area which also contained an attractant. Thus the tank presented a trade off between an attractant and a potential threat. This approach has been used in several

fish species but has required species specific modifications (Huntingford et al., 2010). In this study, a tank with a removable partition was used to screen behavioural traits in individual tilapia. A number of modifications were made in the tank and the attractant over this series of experiments in an attempt to more reliably differentiate fish with differing coping styles.

Two main attractants were used, food and conspecifics. Both have been successfully used as attractants in the past (Ward et al., 2004; Bell, 2005; Petrie and Ryer, 2006; McCormick and Larson, 2008; MacKenzie et al., 2009; Huntingford et al., 2010; Ruiz-Gomez et al., 2011), however, both attractants have their limitations. Many species of fish seek out the presence of conspecifics and this behaviour has been observed in tilapia (Martins et al., 2012). However, it is difficult to time emergence from a safe area to an exposed area with conspecifics since rapid emergence could either indicate a bold or risk taking strategy or a panic response to being isolated. Food might be thought of as a universal attractant, however, as cold-blooded animals, fish do not have the same drive to eat as warm-blooded animals. They can survive for much longer without food and naturally undergo periods of anorexia. In order to produce a robust assessment of coping style, both feed and conspecifics were used repeatedly.

In experiment 1 with groups of fish and food as an attractant, the fish appeared to follow each other rather than express individual differences in behaviour. The subsequent tests were changed to individual fish screening. In experiment 2 with individual fish and food as an attractant, the attractant was not adequate to overcome the threat and no fish emerged. Subsequently, in experiment 3 with individual fish and groups of fish as an attractant, the attractant potentially divided fish into categories on basis of emergence time, with some fish emerging immediately and others taking up to 9 min to emerge. However, the immediate emergence was not observed again when using the tank with the partition with an opening in experiments 4 and 5. In experiments 4 and 5 where individual fish were assessed using two different attractants, one using other fish as an attractant and one using food as an attractant. Moreover, the tank with a relatively small exit was made for screening risk-taking in order to test for the reliability of the influence of the attractant on their behaviour. Both tests gave different results and the fish were not consistent after screening up to 3 times with those methods. The eventual outcome of this series of behavioural screening trials was that the majority of the fish appeared to show a flexible approach with only a small proportion appearing to be consistently bold-risk takers or shy-risk avoiders. This may either be a weakness of this screening system or may reflect the nature of coping styles in tilapia. Additionally, differing coping styles may vary according to whether fish are from wild, farmed or laboratory populations.

Other methods that have been used in other species were also used in an attempt to assess coping style these include, respiratory rate following a stressful event, aggressive behaviour and colouration. Peters et al. (1988) found that the subordinate rainbow trout (*Oncorhynchus mykiss*) showed enhanced ventilation frequency when compared with dominant fish. Aggressive interactions between two fish involve chasing, rapid circling and biting which lead inevitably to one opponent retreating and ceasing to retaliate in response to the aggression of the other fish (Øverli et al., 1999). Aggressive dominant fish have a more proactive type of behavioural response, whereas non-aggressive subordinates are more adaptive and flexible, responding only when necessary (Koolhaas et al., 1999). The association between aggression and bold or risk taking coping style is not always clear but in many cases bold or risk taking fish are also aggressive (Huntingford, 1976; Bell and Stamps, 2004; Sih et al., 2004; Bell, 2005). Finally, skin discolouration has been found to be correlated to social status. In salmonid fish, skin darkening has been suggested to signal social subordination (Höglund et al., 2002). None of these measures showed any significant variability in this study and therefore were not considered useful measures of coping style in tilapia. However, the studies of Volpato et al. (2003) and Vera Cruz and Brown (2007) suggested that the eye colour pattern in Nile tilapia is associated with social interaction. After the aggressive encounter, subordinate fish had a darker eye colour pattern than dominant fish. Therefore, this could be used to evaluate the relationship of social status with eye colour pattern in tilapia.

There is a growing body of evidence that coping styles are not simply a superficial behavioural response but reflect a profound difference from the level of gene expression including differences in immune response (MacKenzie et al., 2009). In previous studies, Walters and Plumb (1980), Angelidis et al. (1987) and Peters et al. (1988) showed that the pathogens spread to more organs and were found in greater numbers in subordinate fish than in dominant fish. This might be related to the high expression of the cortisol receptor genes in risk-avoiding fish. This gene is known to be related to plasma cortisol levels, hence it is involved in the stress response and suppression of the immune system results in a reduced survival in fish (Peters et al., 1991; Øverli et al., 2005; MacKenzie et al., 2009; Huntingford et al., 2010).

In this study, there was a difference observed in the disease susceptibility of the tilapia to *S. agalactiae* with higher mortalities in the fish classified as “shy” compared with those classified as “bold”. These results must be viewed as preliminary since the challenge was a small pilot study and there are still reservations about the nature of coping styles in tilapia and our ability to detect them. The experiment could not be repeated due to lack of time and resources; however, repetition of this work on a larger scale will be necessary before drawing any firm conclusions. At this stage, it is still possible to speculate that coping style may have an effect not only on the susceptibility of individuals but also on infectious disease epidemics in populations. For example, shy and susceptible individuals could act as index cases where more resistant individuals might resist the challenge. Susceptible individuals could also change

the basic reproductive rate of the infection allowing more rapid or more severe epidemics to develop. There is the further possibility that coping styles can be manipulated or influences through selective breeding or husbandry manipulations (Tanck et al., 2001, 2002; Huntingford and Adams, 2005).

The priorities for future work should be to conduct a further study using a larger number of fish with a proper screening test. Replicate tanks containing bold and shy fish that were randomly allocated using a random block design could be set up using the following treatments; 100% shy, 50% shy 50% bold and 100% bold. A subsequent bacterial challenge could then determine the responses of bold versus shy fish, and the impact of the proportion of fish in each tank, i.e. are bold fish more at risk in a tank with shy fish or on their own and are shy fish more at risk in a tank with bold fish or on their own?.

This study has gone some way to identifying coping styles in tilapia and has provided tantalising evidence that they may be related to disease susceptibility. It is therefore of interest to see whether disease prevention and control in fish farm can be explained by selection for a risk-taking phenotypes. If behavioural and physiological variability are indeed organized into coping styles in tilapia, it might be possible to use behaviour as a proxy for physiology in mass screening. This is important in the development of husbandry systems that ensure a range of coping styles among fish.

Chapter 6 - General discussion

6.1 Summary

This study investigated a variety of factors including fish weight, age, and coping style in fish and their effect on experimental *S. agalactiae* infection in Nile tilapia. This study was developed as a result of data from natural outbreaks of *S. agalactiae* infection in farmed tilapia, which suggested that larvae and juvenile or fish weighing less than 20 g were not susceptible to this disease (Hernández et al., 2009; Jiménez et al., 2011). In addition, the weight and / or age factors influencing susceptibility to streptococcal infections had not been adequately investigated in Nile tilapia. In an attempt to try to elucidate the pathogenesis of *S. agalactiae* infection in tilapia, individual differences in the coping styles and susceptibility of fish were also examined. Behavioural syndromes or coping strategies are topics receiving increasing attention in aquaculture. Previous studies have demonstrated that individual fish in the same population show different coping styles, which is associated with cortisol levels and gene expression in response to the inflammatory challenge (MacKenzie et al., 2009; Huntingford et al., 2010). However, implications of coping styles in bacterial infections for example *S. agalactiae* infections in Nile tilapia have not been previously reported. The results of this study suggest that fish age (or growth rate) and coping style, but not the fish weight influences the development of the experimental *S. agalactiae* infection in Nile tilapia.

This research provides basic knowledge and understanding of the mechanisms of *S. agalactiae* infection in tilapia and may lead to mitigation measures reducing losses affecting many farms worldwide.

6.2 Discussion

In general, the cause of a disease is complicated and depends on many factors such as the pathogen, host, environment and management. The disease will occur only when the host is susceptible to a virulent pathogen in a suitable environment (Snieszko, 1974). A variety of factors have to be manipulated to establish a reproducible experimental challenge that produces similar disease characteristics to the natural infection in fish. The key factors include bacterial strain or the virulence of the isolate, bacterial concentration, route of infection, fish species, individual fish response and the environment in the challenge system.

6.2.1 Bacteria

Streptococcus agalactiae has similar phenotypic characteristics to other Gram-positive cocci including *Streptococcus iniae*, *Lactococcus* sp. and *Enterococcus* sp., leading to mis-diagnosis (Kusuda et al., 1991; Buller, 2004). Specific identification of *S. agalactiae* has to be achieved prior to any studies. In Chapter 2, a range of bacterial isolates recovered from natural disease outbreaks in farmed tilapia of different geographic origins were conclusively identified as group B *S. agalactiae* using a range of tests. Considerable

variations were observed in haemolysis and biochemical profile between the *S. agalactiae* isolates. Further serotyping and genotyping of *S. agalactiae* could provide additional information on the relationship between the strains. Serotyping could use capsular typing antisera and multiplex PCR assay while genomic DNA might be analysed by ribotyping, random amplified polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and multilocus enzyme electrophoresis (MLEE) (Brochet et al., 2006; Evans et al., 2008; Olivares-Fuster et al., 2008; Suanyuk et al., 2008; Pereira et al., 2010; Ye, et al., 2011). An understanding of strain differences and serotype would be an important step towards development of vaccines against *S. agalactiae* in tilapia. Study of more *S. agalactiae* isolates from different geographical locations from fish and mammals as well as human isolates may provide a better understanding of the epidemiology of these bacteria in humans and animals (Olivares-Fuster et al., 2008; Suanyuk et al., 2008; Evans et al., 2009).

Prior to performing the experimental challenge studies, it was necessary to develop of a challenge model for *S. agalactiae* in Nile tilapia. The model was used in subsequent experimental work. In this study, immersion was the route initially selected as it is likely to be one of the natural routes of transmission. The infectivity in such a model would indicate the capacity of the bacteria to evade the host external defences (Mian et al., 2009). Several attempts were made including increased bacterial concentration, exposure time, stocking density, immersing fish in washed bacteria or in bacterial and growth media and

the use of static water systems. However, all permutations failed to induce any significant morbidity and mortality. A similar failure to infect fish by immersion was reported by Abuseliana et al. (2011). In a study by Mian et al. (2009), Brazilian isolates were used in the immersion trial at 1.12×10^6 CFU/ml for 15 minutes producing a mortality rate of 40% in Nile tilapia. Immersion studies of Malaysian and Thai isolates at 1×10^5 CFU/ml in 42 h and 1.06×10^8 CFU/ml in 15 minute exposures, produced mortality rates of up to 58% in red tilapia and 60% in Nile tilapia, respectively (Ng et al., 2009; Rodkhum et al., 2011). The variation of tilapia susceptibility to *S agalactiae* in the various studies including the current study may be due to the bacterial strain, its virulence or characteristics of the fish population. There are various other exposure routes that could mimic aspects of natural infections including through water, oral, cohabitation, gill and nare inoculation. Currently, successfully experimental challenge studies by immersion, cohabitation and gill inoculation with *S. agalactiae* infection have been reported in tilapia (Mian et al., 2009; Ng et al., 2009; Rodkhum et al., 2011).

Due to the failure of the immersion model and the lack of time, an injection challenge was used. Thi was an artificial infection route, which by-passed some of the natural defence barriers and aspects of the host innate immune response (Jiménez et al., 2011). Therefore, it would be preferable to use the natural infection routes of this pathogen since this would more closely replicate transmission in culture systems (Robinson and Meyer, 1966). This would be more likely to provide an understanding of natural *S.agalactiae* infections in Nile

tilapia. In all experimental challenge studies (Chapters 3 to 5), the *S. agalactiae* isolate chosen was pathogenic to fish and successful infected Nile tilapia via an experimental i.p. challenge model. In this model, mortality was consistently close to 50% within 14 days after bacterial challenge. There were differences between the onset of clinical signs and accumulated mortality rates from challenges reported by Evans et al. (2004a), Pasnik et al. (2005), Evans et al. (2009), Pretto-Giordano et al. (2010a) and Ye et al. (2011). These could be due to bacterial strain or virulence, bacterial concentration and / or environmental conditions.

6.2.2 Fish

Many reported studies have described individual variability in disease susceptibility in fish associated with weight and / or age (Muzquiz et al., 1999; Agnew and Barnes, 2007; Siti-Zahrah et al., 2008; Suanyuk et al., 2008; Hernández et al., 2009; Mian et al., 2009; Zamri-Saad et al., 2010), fish species (Chang and Plumb, 1996b; Yuasa et al., 1999; Evans et al., 2000), genetic variation and immune response (Sarder et al., 2001). Similarly, the different coping styles in individual fish may influence the fish and their susceptibility to infection (Peters et al., 1988; MacKenzie et al., 2009; Huntingford et al., 2010). However, the current study was focused only on weight, age and coping style in fish in order to determine whether these factors could affect experimental *S. agalactiae* infection in Nile tilapia.

In Chapter 4, the hypotheses that weight or age of fish was associated with susceptibility to *S. agalactiae* in Nile tilapia were examined. There was no association between fish weight and susceptibility to *S. agalactiae* in fish as the bacterium was able to cause mortality in fish with a wide range of weights. The result of this study was in contrast to previous reports that found that tilapia weighing less than 20g were not susceptible to *S. agalactiae* infection (Hernández et al., 2009; Jiménez et al., 2011). This supported the hypothesis that tilapia can become infected over a wide range of weights, and similar results as were described by Suanyuk et al. (2008). In contrast, fish of different ages were shown to be associated with susceptibility to *S. agalactiae* infection in Nile tilapia. This could relate to the capacity of fish of different ages to generate a protective antibody-mediated immune response (Evans et al., 2004a).

A study by MacKenzie et al. (2009) demonstrated that fish with different coping styles which have been described as “bold” or “shy” in common carp (*Cyprinus carpio*) differed in baseline gene expression in specific tissues and also showed dramatically different individual responses to the experimental inflammatory challenge with bacterial lipopolysaccharide (LPS). Plasma lactate, glucose, cortisol levels and expression of the cortisol receptor gene were significantly higher in shy or risk-avoiding individuals than in bold or risk-taking fish (Huntingford et al., 2010). Subordinate fish show elevated plasma glucose levels, increased leucocyte volume, higher ventilation frequency and have pathogens spreading to more organs and occurring in greater numbers than

those in dominant fish. This seems to be related to differences in physiological status of individuals such as stress response and suppressed immune responses leading to disease susceptibility (Koolhaas et al., 1999). The results from Chapter 5 showed that there was a relationship between different coping styles and susceptibility to *S. agalactiae* infection in Nile tilapia, with shy fish more susceptible than bold fish. However, a further study should be performed using a larger number of fish with an improved screening test. In addition, the work could be expanded to examine different coping styles and their relationship to gene expression and immune response.

6.2.3 Environment and management

An increase water temperature, high stocking density, poor environment and management conditions causes stress in fish, resulting in a decrease in the ability of their immune competence and increased vulnerability to pathogens (Shoemaker et al., 2000; Austin and Austin, 2007; Bromage and Owens, 2009). In all experimental challenge studies by i.p. injection (Chapters 3 to 5), the virulence of the *S. agalactiae* isolate tested showed positive correlation with water temperature at high stocking density. High mortality was recorded in infected fish reared at 27 ^{o}C whilst low mortality was noted in a water temperature of 25 ^{o}C. Similar findings were reported by Siti-Zahrah et al. (2008), Mian et al. (2009) and Rodkhum et al. (2011) who suggested that high water temperatures (≥27^{o} C) influenced the occurrence of *S. agalactiae* infection and increased mortality in tilapia. Thus, high stock density, intensive

husbandry and high water temperature appear to be factors predisposing fish to disease outbreaks.

The condition of the i.p. experimental challenge model for *S. agalactiae* (Chapters 3 to 5) including water flow rate, water temperature and stocking density were decided based on previous studies which successfully induced streptococcal infections in tilapia (Shoemaker et al., 2000; Filho et al., 2009; Pretto-Giordano et al., 2010a; Abuseliana et al., 2011). The conditions used in this study were considered suitable since all affected fish showed disease signs and death while no clinical signs were observed in any of the control fish.

6.3 Further work

This study has identified the factors associated with experimental *S. agalactiae* infection in Nile tilapia. With this data, it is possible to generate further work including:

6.3.1 Aetiological studies

The molecular serotyping and genotyping of *S. agalactiae* isolates are necessary for epidemiological studies. These could assess the genetic diversity or relationship between geography and the host-pathogen interaction involved in *S. agalactiae* infection in Nile tilapia and their relationships with strains from humans and mammals. In addition, this could also be used to design

programme of future prevention and control strategies such as the development of a generic vaccine to provide cross-protection against multiple strains of *S. agalactiae* in cultured fish.

6.3.2 Experimental studies

Immersion exposure is considered to be more natural. Although the attempts made in the current study were unsuccessful, this exposure route would result in a more natural *S. agalactiae* infection in Nile tilapia. It would be possible to understand the different pathways of pathogen transmission in order to optimise disease control strategies in Nile tilapia. Additionally, further challenge studies could be performed to determine the host response to *S. agalactiae* of different age ranges of fish and further work on the association between coping style and susceptibility.

6.3.3 Field studies

Although several aspects of farm conditions have been associated with *S. agalactiae* infection in Nile tilapia, further field based studies could identify farm level risk factors which might be the basis of interventions to reduce the incidence or severity of *S.agalactiae* infections on commencial farms.

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