Diseases of Asian seabass (or barramundi), *Lates calcarifer* Bloch

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Declaration

I declare that this thesis is an account of my research and contains work which has not been previously submitted for a degree at any tertiary education institution. Contributions by co-authors have been duly acknowledged.

Susan Gibson–Kueh

Acknowledgements

Preface

Chapter 1 serves as a brief introduction to husbandry practices and diseases previously reported in cultured *Lates calcarifer*. It also includes a section on the interactions between the host, environment and pathogens which need to be considered in the investigation and managing of disease outbreaks. Chapters 2 to 4 are based on published papers while Chapter 5 is a manuscript intended for publication in a scientific journal. There has been a need to adapt the chapters based on published papers to integrate them into a thesis. Chapter 6 discusses management strategies in relation to specific diseases in *L. calcarifer* at the htachery, nursery and growout levels. Citations style is in keeping with that used in Journal of Fish Diseases. Citations with more than 2 authors are quoted in full when it appears in text within each chapter for the first time, and thereafter only as first author & *et al.*

Abstract

Other than the study by Griffiths (2009) on gill diseases, there has been no comprehensive study and report on the major diseases of Asian seabass (or barramundi) *Lates calcarifer* Bloch. It is a food fish species of growing importance in Asia and Australia. This study investigates some of the major diseases encountered in the various stages of the culture of *L. calcarifer,* at the histopathological, ultrastructural and molecular levels. Culture practices can have significant impacts on fish health. Disease outbreaks are influenced by factors involving the host, environment and pathogen. Current knowledge on diseases of *L. calcarifer*, and these factors which may influence disease outbreaks are discussed in Chapter 1.

This is the first report of an intestinal *Eimeria* infection in *L. calcarifer*. The *Eimeria* infection was associated with severe pathology and significant mortality in the absence of other pathogens. It was detected in diseased L. calcarifer in all five nurseries in Ca Mau, Vietnam. Although these were small scale nurseries which stocked an average of 3000 to 5000 fish at any one time, a mortality rate of up to 30% was reported and is the cause of significant economic losses for these nurseries. Moderate to heavy *Eimeria* infestation were observed in greater than 80% of diseased fish examined. This high rate of *Eimeria* infestation is suspected to be linked to the low daily water exchange rates practised in these nurseries. However, the examination of only diseased fish does not allow the determination of prevalence. A systemic iridovirus infection was concurrently observed in some of the fishes but was not consistently present when compared to the *Eimeria* infection. Molecular analysis showed that the *Eimeria* of *L. calcarifer* from Vietnam formed clades with the *Eimeria* detected in

L. calcarifer cultured in Australia, but clustered separately from other known *Eimeria species*. Although *Cryptosporidium* was detected in these *L. calcarifer* tissues, it could not be demonstrated histologically or ultrastructurally, suggesting a low grade infestation or perhaps an environmental contaminant in fish tissues tested. *In situ* hybridization using labeled PCR products showed that labeled DNA probes generated from 18S PCR products could not be used to distinguish between closely related genera such as *Cryptosporidium* and *Eimeria*. Future investigation to determine the origin, transmission and risk factors associated with this *Eimeria* infestation in *L. calcarifer* are needed.

'Scale drop syndrome' is a novel disease first reported in *L. calcarifer* in Penang, Malaysia in 1992. Cases with similar gross and clinical presentations were observed in Singapore in 2002, 2006 and 2009. Affected fish have loose scales, which dropped off easily when handled. The disease was initially observed in 100-300g fish, and later in larger fish up to 5kg bodyweight. Cumulative mortalities of 40 to 50% were reported by farms, posing significant economic losses of larger more valuable fish. This investigation forms the first pathological description of 'scale drop syndrome' (SDS) in *L. calcarifer*. To aid recognition of new cases for study, a case definition was developed for 'scale drop syndrome' in *L. calcarifer* as a systemic vasculitis associated with tissue necrosis in all major organs including the skin, with apparent targeting of cells of epithelial origin. Attempts to isolate or detect the causative agent(s) by cell culture, PCR and immunohistochemistry have proven unsuccessful. Further studies to elucidate the definitive aetiology, isolate the causal agent(s) and reproduce the disease will help better understanding and control of SDS.

Although systemic iridoviral disease has been previously reported in many freshwater and marine fish species, this study forms the first report of this disease in *L. calcarifer*. Systemic iridoviral disease was observed in 5 to 20g *L. calcarifer* usually 2 to 3 weeks post-transfer into sea cages at two farms. Inclusion bodies suggestive of a systemic iridovirus infection were observed in clinically healthy *L. calcarifer* from the land-based nursery of one of these two farm; the presence of an iridovirus infection was supported by positive PCR results using Red Sea bream iridovirus (RSIV) primer 1. The presence of inclusions was not accompanied by any tissue necrosis in these clinically healthy fish. This finding suggested that the systemic iridovirus infection occurred before stocking at sea, and did not originate from wild fish or older fish in adjacent sea cages as initially suspected by this farm. Immunohistochemistry on tissues of clinical cases of systemic iridovirus gave positive results using the Red Sea bream iridovirus monoclonal antibody (RSIV M10), although intensity varied between tissues, possibly related to varying exposure of different tissues to fixation chemicals. Inclusion bodies in clinically healthy fish from the same farm did not show positive reaction with RSIV M10. This may be due to a lack of antigenic expression by the viral infected cells at this early stage of infection.

Viral nervous necrosis (VNN) is a serious disease of hatchery reared *L. calcarifer* fry in this study. Mortalities of 50 to 100% were reported in 3wo fry. VNN can be difficult to diagnose in older fry, where it can be associated with few vacuolations or an absence of viral inclusions

'Pot belly disease' (PBD) was previously reported in *L. calcarifer* fry less than 1g, in association with an intracellular coccobacillus infection and mortalities of 80 to 100%. In this study, PBD was observed in 120g *L. calcarifer*

at two sea cage farms, in association with significant granulomatous enteritis. The extent of the granulomatous enteritis is likely to have an effect on affected fish. It was observed concurrently with systemic iridoviral disease at one farm and nocardiosis at another farm. Diagnosis by histopathology and the lack of other confirmatory tests for PBD may result in underdiagnosis of this disease. The epidemiology of PBD needs further study to establish origin and modes of transmission, to facilitate better disease control.

Diseases associated with infections by ubiquitous bacteria such as *Vibrio*, *Tenacibaculum* were commonly observed in *L. calcarifer* post-handling. Tenacibaculosis and vibriosis often occurred concurrently with other diseases such as streptococcosis, systemic iridviral disease or PBD. Streptococcosis can affect fish up to 3kg bodyweight, resulting in significant mortalities greater than 40 to 50%. Like SDS, because streptococcosis can affect up to market size fish, they can cause considerable economic losses. Although vaccines against Streptococcosis are available, conflicting views are held on the efficacy of *Streptococcus* vaccines by various research groups. Overall, the South-east Asian *L. calcarifer* farms which practiced vaccination against *Streptococcus iniae* reported a reduction of mortality, especially in fish greater than 1 to 1.5kg bodyweight.

Nocardiosis has been reported as an emerging disease in marine food fish species caused by acid fast filamentous branching bacterium. Although nocardiosis was observed histopathologically in *L. calcarifer* at two sea cage farms, the numbers of samples examined were small and no other tests were attempted due to lack of suitable samples. More intensive and extensive study is needed to determine the significance of nocardiosis in *L. calcarifer*. Chronic

granulomatous enteritis was not uncommon in the cases submitted to the Fish Health Laboratory in Perth. Although the peritonitis was associated with heavy bacteria infection, it is unclear if these are secondary invaders. Schipps, Bosmans & Humphreys (2009) reported that *Vibrio harveyi* and *Photobacterium damsela damsela* vaccinations appeared to be not efficacious, suggesting that these bacteria were not the primary cause of the disease.

It is well recognized that disease outbreaks in farmed fish are influenced by the interaction between host, the environment and pathogens. While serious diseases are often reported in association with specific aquatic pathogens, not much is known about the risk factors which trigger fish disease outbreaks. Disease outbreaks often occur after stressful events such as net transfers, recent handling or poor water quality. In fact, diseases are often caused by ubiquitous pathogens that are commonly present in the culture environment. Although further research is necessary to gather more information to improve diagnosis and management of specific diseases, general health management strategies can be applied at the various stages in the culture of *L. calcarifer* to minimize disease outbreaks. This is discussed for *L. calcarifer* in Chapter 6.

Observations of types of disease agents may be influenced by site conditions or the types of tests or materials examined. For example, some parasites may be more prevalent in certain sites where intermediate hosts abound, or loosely attached ectoparasites may be lost unless wet mount microscopic examinations of fresh tissues were carried out. The study of emerging diseases such as scale drop syndrome (SDS) or pot belly disease (PBD) in *L. calcarifer* has been hampered by lack of confirmatory diagnostic tools and inadequate knowledge on critical epidemiological factors such as mode of

transmission or potential reservoirs. While ideally identification and isolation of the causal agent will help fulfil Koch's postulates, it may be possible to improve the understanding of disease via cohabitation or infectivity trials using tissue homogenates from diseased fish when pure isolates are not available. There is a need to conduct research to not only establish a definitive aetiology, but also to identify risk factors to facilitate successful disease control. The successful management of disease in aquaculture does not lie in any one strategy but an integrated management of all risks encountered during the culture cycle against disease occurrence or incursions.

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List of abbreviations

AP   alkaline phosphatase

DIG   dioxygenin

DO   dissolved oxygen

FFPE   formalin fixed paraffin embedded

IHC   immunohistochemistry

ISH   *in-situ* hybridization

PBD   pot belly disease (or big belly)

ppm   Parts per million

ppt   Parts per thousand

RSIV   Red Sea bream iridovirus

SDS   scale drop syndrome

SSC   saline sodium citrate

TBS   tris-buffered saline

VNN   viral nervous necrosis

Chapter 1   Introduction

This is a description of the diseases of Asian seabass (or barramundi) *Lates calcarifer* Bloch from various farms in Southeast Asia. It includes description of diseases based on histological materials obtained from the Aquatic Animal Health Laboratory, AgriFood & Veterinary Authority of Singapore and the Fish Health Laboratory, Department of Fisheries, Western Australia. Chapter 1 puts in perspective the importance of *L. calcarifer* as an aquaculture food fish species. Culture practices can have significant impacts on fish health at the farm level. Hence, the different production phases of *L. calcarifer* are outlined in brief to explain the background against which diseases may occur. In addition, sections on the host, environmental and pathogen factors that may influence disease outbreaks and hence are important considerations in conducting disease investigations are discussed. This chapter also provides an overview of the diseases or disease agents which have been previously reported, to which this study expands the repertoire of known diseases of *L. calcarifer*.

1.1   Culture of Asian seabass (or barramundi), Lates calcarifer Bloch

1.1.1   Importance as an aquaculture food fish species

Asian seabass (or barramundi) *Lates calcarifer* Bloch is a cultured food fish species of rapidly growing importance in Asia and Australia. It is a warm water fish species, and can be cultured in areas where the winter water temperature remains above 25°C. Being a euryhaline fish species, it is tolerant of a wide range of salinities from freshwater to full strength seawater. The culture of *L. calcarifer* is divided into specialized operations in hatcheries, nurseries and grow-out farms (Barlow 1998; Rimmer 2006).

Farms are generally small to medium scale though some larger grow out farms have reported an annual production of 300-400 metric tones, with projections of up to 1000 metric tonnes in 2011 (Queensland Department of Primary Industries and Fisheries 2008; Marine Produce Australia 2010; Forristall 2010). The latest global production of *L. calcarifer* was reported as 49,299 metric tones (FAO 2006). Thailand and Indonesia are currently the largest producers of cultured *L. calcarifer* at 15,700 and 4,417 tonnes, respectively (Kongkeo, Wayne, Murdjani, Bunliptanon & Chien 2010).   The culture of *L.* calcarifer is well established in Australia and Malaysia, and there is growing interest in *L. calcarifer* farming in Singapore, Vietnam and India. The aquaculture production of *L. calcarifer* at 3,300 metric tonnes has exceeded wild fisheries at 1,500 metric tonnes in Australia (Queensland Department of Primary Industries and Fisheries 2008).

1.1.2   Production of juveniles of *L. calcarifer*

The spawning of *L. calcarifer* in captivity was pioneered more than 40 years ago in Thailand in 1971 (Tattanong & Maneewongsa 1988). The production of *L. calcarifer* is well established with the hatchery and nursery phase carried out in separate operations to grow-out facilities. Broodstock gonadal maturation and larvae rearing need to be carried out in saline water (Tattanong & Maneewongsa 1988; Barlow 1998). Some hatcheries maintain their own broodstock while others buy in fertilized eggs to hatch. The hatchery stage is very specialized and labour intensive due to the need for live feed production. Eggs hatch in less than a day. Fry need to be fed live feed such as rotifers when 2 do and artemia starting at 14 do. Larvae are weaned onto an artificial feed starting at 20 do. Earlier introduction of a special weaning microdiet diet at 8 do has almost eradicated

mortality associated with failure to wean, and advanced weaning to 16 to 20 do versus the conventional 30 do. The early nursery stages (20-35mm bodylength) need to be graded every three to four days while larger fish (50-100mm bodylength) need to be graded weekly to control cannibalism (Schipp, Bosmans & Humphrey 2007).

1.1.3   Grow-out of *L. calcarifer*

In Southeast Asia, *L. calcarifer* are traditionally grown out in small scale farms with an average of 30 sea cages, in 5 to 8m or 10 to12m deep waters in sheltered coastal seas (Anil, Santhosh, Jasmine, Saleela, George, Kingsly, Unnikrishnan, Rao & Rao 2010; Joseph, Joseph, Ignatius, Rao, Sobhana, Prema & Varghese 2010). Culture of *L. calcarifer* in brackish water ponds occurs in Thailand, Malaysia and Vietnam. Sea-cages approximately 3 to 5m long by 3 to 5m wide and 2 to 3m deep are each stocked with 1000 to 3000 fish fingerlings for grow-out. Average stocking density is typically kept less than 15-16 kg fish per cubic metre water throughout the culture period (Gibson-Kueh S., personal observations). Some of the larger farms in Southeast Asia and Australia grow out *L. calcarifer* in circular steel cages that measure 12, 18 or 28m diameter and 10m deep. These circular steel cages are stocked with 50,000 to 100,000 fish fingerlings. The fish fingerlings are stocked into the smaller 12 or 18m cages, and transferred into the larger 28m diameter cages as the fish grow (Appendix 1. Questionaire - Farm A). One marine cage farm exists in Western Australia while most of the *L. calcarifer* culture in Australia occurs in fresh or saltwater ponds in Queensland and Northern Territory. The fish are reared in cages suspended by a solid frame in these ponds. Up to 4 cages measuring 3m x 2m, and up to 1.5m deep are suspended in one pond (Australian Barramundi Farmers Association 2008;

Queensland Department of Primary Industries and Fisheries 2009; Marine Produce Australia 2010).

Mariculture of *L. calcarifer* involves the stocking of 5 to 20g fish sourced from nurseries into sea cages or ponds. Farmed *L. calcarifer* can grow up to 350g in six months and 2 kg in 2 years (Rimmer 2006). In Queensland, Australia, *L. calcarifer* are grown to 500g bodyweight in net cages suspended in freshwater ponds. Farms in Asia generally produce 700-800g fish for the fresh fish markets. There is a trend for farms in both Asia and Australia to produce fish for the lucrative fillet market with the production of 3 to 5kg fish (Rimmer 2006; Queensland Department of Primary Industries and Fisheries 2008; Forristall 2010; Yeow 2010).

Fish are fed mainly commercial fish pellets. In smaller farms in Asia, feeding may be supplemented with by-catches of wild marine fish termed trash (or bait) fish. Farms are moving away from the feeding of trash fish to pellets, partly due to inadequate supply and better awareness of potential sources of disease. The feeding of trash fish gives very poor food conversion ratios from 6:1 to 10:1 as compared to 1.2:1 to 2:1 for commercial pelleted diets (Schipp *et al.* 2007).

1.2 Disease outbreaks are influenced by interactions between host, aquatic environment and pathogen

Disease outbreaks in aquaculture are influenced by interactions between the host, the aquatic environment and pathogens. Although not exhaustive, the sections below introduce some of the important factors which need to be taken into account when conducting fish disease investigations. These factors should also be taken into consideration to ensure successful disease management in

farmed fish. The increasing replacement of fish meals by alternative plant protein sources can cause diseases as a result of mineral, amino acid and fatty acid deficiencies (Oliva-Teles 2012). Hence adequate nutrition is an important consideration in disease investigations, and some cases reported in *L. calcarifer* are mentioned in Section 1.5. The review by Glencross (2006) serves as a good guide to nutritional needs of *L. calcarifer*.

1.2.1 Host factors

Fish are poikilothermic animals that thermoconform to enviromental temperature, which influences their metabolism, growth and immunity. Stress in fish is defined as a state when the adaptive responses to maintain homeostasis are exhausted. This can lead to disease caused by normally benign organisms present in the culture environment. Hence it is important that any microbiological findings in diseased fish must be interpreted in the light of recent background information or history on husbandry (Roberts & Rodger 2012).

Fish are the first animal phyla to possess both the innate and adaptive immune response. The adaptive immune system of fish is known to mature much later in marine than freshwater fish species (Magnadottir 2010). Hence both the species and age of fish determine the ability of fish to respond to disease. Although the adaptive immune system in fish is responsible for induction of lasting immunity in vaccinations, the innate system is necessary to prime the adaptive immune system during an antigenic response. Stressors such as transport, handling and high stocking density are known to suppress the immune system and hence disease resistance in fish. Constant challenge from increased bacterial or high organic loads can deplete the immune system of fish,

leaving them more prone to infections by ubiquitous and normally benign organisms present in the culture environment. For example, concurrent ectoparasitisism by *Trichodina species* have been found to increase losses from *Streptococcus* infections in channel catfish, *Ictalurus punctatus* Rafinesque (Evans, Klesius, Pasnik & Shoemaker 2007).

Host responses in fish can be acute or chronic inflammatory responses, and are often accompanied by tissue degeneration and necrosis. These host responses themselves contribute towards the clinical signs observed, so that disease management must not only take into consideration the elimination of the causative agent(s) but also supportive therapy to enhance tissue healing. For example, external bacterial or parasitic infections may result in damage to gills and skin. The gill is an important organ in gas exchange and waste excretion. The skin is vital in providing a barrier to support homeostasis and osmoregulation. The severity of tissue damage, intrinsic ability of affected organs to regenerate and acute versus chronic tissue responses must be taken into account in disease management protocols (Roberts & Rodger 2012). Good aeration to ensure optimal oxygenation and increased water exchanges to keep nitrogenous and organic wastes low are beneficial in disease management. Dropping salinity in seawater or increasing salinity in freshwater tolerant fish species closer to that of the osmolarity of body fluids will help osmoregulation. Dehydration from osmoregulatory dysfunction in marine fish may present with lethargy, anorexia and darkened bodies (Greenwell, Sherrill & Clayton 2003). Avoiding unnecessary handling procedures in inappetant sick fish will help conserve energy for vital processes such as oxygen uptake, osmoregulation and tissue repair (Tseng & Hwang 2008).

1.2.2 Environment

Aquaculture may be carried out in inland ponds, tanks or in sea cages, involving static, through-flow or closed recirculation systems, and be semi-intensive or intensive. Static or recirculation systems with low water exchange rates may result in the build up of nitrogenous wastes and potential disease agents. Intensive aquaculture with high biomass needs good aeration to maintain optimal dissolved oxygen (DO) levels and high water exchange rates to keep nitrogenous waste levels low (Shepherd 1993). Net management and removal of mortalities take up a large proportion of labour in sea cages. Net management ensure adequate water flow through sea cages while removal of mortalities minimizes disease spread and serves as a record for reliable assessment of stock numbers. Accurate records of stock number are important for feed management (Grant 1993). Although fish biomass may impact disease spread in a particular area, it has been shown via epidemiological modelling that disease spread is favoured by multiple small farms more than a few large farms in one area (Salama & Murray 2011).

Poor water quality can predispose fish to serious disease outbreaks. Although many water quality parameters can affect fish health, the dissolved oxygen (DO) level ranks among the most important. DO levels must be kept above 4-5ppm, and even at optimal levels of 8ppm is several thousand times less than in atmospheric air. High temperatures are associated with lower DO, which may exacerbate severity of disease. Parasitic infestations resulting in severe mortality have been associated with high temperature, presumably by a corresponding drop in DO and increased pathogen proliferation rates (Khan 2012). Rapid diurnal fluctuations of parameters such as temperature, DO and pH

are stressful to fish (Roberts 2012). Fish stocking density, microbial loads and water exchange rates in aquaculture systems can affect DO levels. Ammonia can be toxic to fish even at low levels. Ammonia and other organic wastes may be derived from fish wastes fed high protein diets or uneaten feed, resulting in increased bacterial loads which can overwhelm the immune system of fish (Olafsen 2001). Ammonia levels must be kept in check by good water exchanges or use of biological filters, andn even at sublethal levels can compromise the health of fish. High stocking densities are commonly practiced in aquaculture to maximize returns (Southworth, Stone & Engle 2006). High stocking density increases opportunity for pathogens to contact host and also host to host transmissions, hence affecting the dynamics which may lead to disease outbreaks (Krkosek 2010). Although management procedures maybe in place in intensive aquaculture, it is equally important to routinely monitor both water quality and fish health to allow early intervention before disease occurs.

1.2.3 Pathogens

Ubiquitous organisms can cause disease in the stressed fish host. Parasites may have direct life cycles, being able transmit to another fish host or they may require intermediate hosts for transmission. Protozoans may proliferate by binary fission while metazoan parasites such as nematodes and trematodes produce eggs or live young. Modes of proliferation affect the rate at which parasites increase in numbers in a fish population and cause disease. Parasites may be able to persist in the environment as resistant cysts, making them difficult to eradicate (Wootten 2012). Protozoans can rapidly reach large numbers in aquaculture systems with a high stocking density and hence increased accessibility to fish hosts. Severe disease is attributed to some

protozoans such as *Ichthyophthirius multifiliis* and *Ichthyohodo necator* more so than others such as *Trichodina* or *Epistylis* (Rintamaki-Kinnunen & Valtonen 1997). Recent genetic studies suggested that *I. necator* isolates are comprised of several species. This knowledge can be used to conduct studies on the likely pathogenicity of different *Ichthyobodo species* (Callahan, Litaker & Noga 2005). For years, amoebic gill disease (AGD) was thought to be due to an opportunistic amoeba infestation during the warmer summer months. Each infected fish and even dead fish can carry thousands of amoebae, posing an important source of AGD transmission (Munday, Zilberg & Findlay 2001). However, it was only with the recent identification and isolation of *Neoparamoeba perurans* as the specific aetiological agent of AGD that the disease could be reproduced experimentally. These findings will allow improved understanding of the epidemiology and pathogenesis of AGD (Crosbie, Bridle, Cadoret & Nowak 2012). Besides protozoans, monogeneans have been the dominant parasites of cultured fish. The high density of a single species of fish in many aquaculture facilities suits transmission of parasites that do not need intermediate hosts (Mladineo 2005).

There are many bacterial and viral pathogens reported to cause serious disease in cultured fin fish. Most fish bacteria reported to cause serious disease in cultured fish are commonly present in the environment. *Vibrio* and *Aeromonas species* are amongst the most commonly reported fish bacterial pathogens, usually in association with poor water quality or after handlng. Strict intracellular bacteria belonging to chlamydiaceae, rickettsiaceae and francisellaceae have been reported as emerging fish pathogens. These intracellular bacteria require specialised culture techniques which may not be readily available in some diagnostic laboratories (Roberts 2012). Intracellular

bacteria possess mechanisms to escape the host immune system, and are not affected by many common antibiotics. Ubiquitous bacteria such as *Flavobacterium spp.*, *Aeromonas spp.* and *Vibrio spp.* could be reisolated from water up to 4 months after inoculation, while *Aeromonas salmonicida*, which is considered an obligate fish bacterium, could not be recovered from water 14d after inoculation. Infected or dead fish would therefore be more important sources of transmission for obligate bacterial pathogens (Femandez , Rodriguez & Nieto 1992).

Water current is less effective than aerosols for long range transmission of viral pathogens and wild reservoir fish species generally occur at lower densities. Hence, it would logical to assume that diseased or carrier fish within aquaculture facilities are more important potential sources of viral disease agents. However, some viral diseases such as infectious haematopoietic necrosis, viral haemorrhagic septicaemia, infectious salmon anemia and epizootic haematopoietic necrosis were shown to have originally spread from wild to farmed fish. Systemic iridoviruses such as the Red Sea bream iridovirus and megalocytiviruses such as infectious spleen and kidney necrosis virus have been both reported in greater than 30 species of cultured marine fish in Asia. Although recent molecular work has resulted in the placement of these systemic viruses into specific groups, more research is needed to ascertain epidemiological factors such as host range, potential reservoirs and modes of transmission necessary for successful disease management (Walker & Winton 2010). Infectious pancreatic necrosis (IPN) caused by aquabirnaviruses was previously reported as a serious disease of salmonid fry but has been recently recognised also as serious disease of post smolts in sea cages. The emergence of IPN in older

fish may be due to changes in characteristics of the virus, the fish host or the environment. The most important concern is the persistence of the IPNV in subclinical carrier fish that survive disease outbreaks. Aquabirnaviruses are increasingly detected in non-salmonid species although their exact contribution to disease is unclear (Crane & Hyatt 2011). Viral nervous necrosis caused by nodaviruses has been reported in more than 40 species of marine and freshwater fish world-wide. Recent research on nodaviruses has shown that several sub-genotpes appeared to be restricted to certain locations associated with climatic conditions (Crane & Hyatt 2011). Viruses have been increasingly detected due to the availability and application of cell culture and molecular techniques. This will facilitate the better understanding of the epidemiology of different virus strains within families detected in various fish species, and aid future disease management.

1.3   Infectious diseases with a major impact on the culture of *L. calcarifer*

1.3.1 Viral diseases

Significant diseases reported as limiting the culture of *L. calcarifer* include viral nervous necrosis (VNN). VNN is usually observed in 15-18 do *L. calcarifer* fry, often resulting in mortality of 50-100%. Fry affected with VNN exhibit lethargy, anorexia, pale discolouration and abnormal swimming in a corkscrew or darting fashion. A dietary deficiency of unsaturated fatty acids and high levels of undissociated ammonia were initially suspected as possible causes for the vacuolative encephalopathy and retinopathy, until a picorna-like virus was identified in brain and eye tissues of affected fry   (Glazebrook, Heasman & de Beer 1990; Munday, Langdon, Hyatt & Humphrey 1992). The viral causative agent of VNN was eventually assigned to the family Nodaviridae based on

morphological and biochemical properties (Mori, Nakai, Muroga, Arimoto, Mushiake & Furusawa 1992). Nearly two decades after VNN was first reported, it remains a significant cause of mortality in hatcheries in the Philippines, India and Malaysia where the culture of *L. calcarifer* is gaining popularity (Maeno, De La Pena & Cruz-Lacierda 2004; Parameswaran, Rajesh Kumar, Ishaq Ahmed & Sahul Hameed 2008; Ransangan & Mannin 2010).

1.3.2 Bacterial diseases

Streptococcosis due to *Streptococcus iniae* is a serious disease in cultured warm water fish species including *L. calcarifer*. In younger fish, streptococcosis may present with minimal clinical signs and severe mortality up to 70%. In subacute cases, affected fish display bilateral exopthalmos, darkened bodies and reddening of skin at the base of the fins and on the ventral abdomen. Fish up to 1.5 and 3 kg bodyweight can be affected (Creeper & Buller 2006; http://www.thefishsite.com/articles/1086/diseases-of-farmed-barramundi-in-asia, assessed 18 Dec 2012). In subacute cases, cumulative mortality can be up to 50% over several weeks. Large numbers of gram positive cocci in pairs or chains are often observed free within blood vessels or within histiocytes of major organs such as the spleen, kidney, liver and brain (Bromage, Thomas & Owens 1999; Creeper & Buller 2006; Humphrey, Benedict & Small 2010). Griffiths (2009) reported the importance of *S. iniae* as a serious pathogen in two farms in South Australia.

Flavobacterium (previously *Cytophaga* or *Flexibacter*) and *Tenacibaculum* species are ubiquitous gram negative filamentous bacteria with gliding motility, in the freshwater and marine environment, respectively. Flavobacteriosis and tenacibaculosis are serious disease of cultured finfish worldwide in the

freshwater and marine environment, respectively. Ulcerative cutaneous lesions are observed on body surfaces of the head, mouth, fins, flanks and gills. Mats of filamentous bacteria may or may not be present on these cutaneous ulcerations. Outbreaks of disease have been reported following stressful events such as handling, high stocking density or poor water quality (Carson, Schmidtke & Munday 1993; Avendano-Herrera, Toranzo & Magarinos 2006; Labrie, Ng, Komar & Sheehan 2007). Tenacibaculosis can occur concurrently with vibriosis, and both produce similar external cutaneous ulcerations (Handlinger, Soltani & Percival 1997). Tenacibaculosis was reported as most significant in 1-100g *L. calcarifer* in Southeast Asia. Cumulative losses may reach 50-60% (http://www.thefishsite.com/articles/1086/diseases-of-farmed-barramundi-in-asia, assessed 18 Dec 2012). Bacterial septicaemias attributed to opportunistic pathogens such as *Aeromonas species* in freshwater, and *Vibrio species* and *Photobacterium damselae* subsp. *damselae* in sea water have been reprted to cause serious mortalities in cultured *L. calcarifer* (Ruangpan 1988; Sreevetana 1992; Azad, Thirunavukkarasu, Kailasam & Rajan 2004: Kanchanopas-Barnette, Labella, Alonso, Manchado, Castro & Borrego 2009).

Pot-belly disease was reported in *L. calcarifer* fry from 3 weeks old, with mortality of up to 80%. Affected fish were darkened and emaciated, with abdominal distensions. Multifocal to coalescing granulomatous enteritis with presence of clusters of large gram negative coccobacilli were observed histopathologically. The disease often extended systemically with granulomatous lesions in peritoneum, spleen and kidney in association with these large coccobacilli. Although attempts at culture were unsuccessful, these coccobacilli

reacted positively with Immunohistochemistry using a polyclonal antibody against *Edwardsiella ictaluri* (Gibson-Kueh, Crumlish & Ferguson 2004a).

1.3.3 Monogenean parasites

Neobenedenia melleni infestation was associated with large losses of 200,000 fish at a sea cage farm in Queensland, Australia. These are large monogenean parasites that measure 2230-4190 by 997-1560 μm, and are armed with hooks which can cause significant damage to the host during their feeding activity (Deveney, Chisholm & Whittington 2001). *Neobenedenia* was considered the most serious ectoparasite in *L. calcarifer* in sea cages in Southeast Asia. Juvenile *L. calcarifer* were infested with Neobenedenia in both cases: 124-174mm fish in Queensland and 15-100g fish in Southeast Asia. Affected fish quickly become inappetant, lethargic, and develop exophthalmos, skin and body rot resulting in cumulative mortality of 30-40% (http://www.thefishsite.com/articles/1086/diseases-of-farmed-barramundi-in-asia, assessed 18 Dec 2012).

1.3.4 Protozoan parasites with a direct life cycle

Protozoan parasites can cause significant disease and mortality in closed systems such as tanks or ponds which, with high stocking density, provide ideal conditions for their rapid proliferation. Heavy infestations of protozoan parasites result in irritation and tissue responses such as hyperplasia, oedema, hydropic degeneration and necrosis in skin and gills. *Cryptocaryon irritans* referred to as 'marine Ich' or 'marine white spot', and its freshwater counterpart *Ichthyophthirius multifiliis* both burrow under the epithelium of fish, resulting in damage to skin when the parasite matures, and exit the host to form very

resistant cysts in the pond or tank bottom. Each cyst produces up to 200 infective stages, which results in a greater exponential increase in parasite numbers compared to other protozoan parasites. The infective stages of both *C. irritans* and *I. multifiliis* must find hosts within 12-24 h or perish, and hence fallowing of tanks and ponds in between batches of fish can be useful in managing these parasites (Colorni & Burgess 1997; Matthews 2005). Extensive damage by heavy protozoan parasites will affect the ability of skin to function effectively as an osmotic barrier, resulting in osmoregulatory dysfunction such as water influx in freshwater or dehydration in sea water, both of which can be fatal to fish host. *Ichthyophthirius multifiliis* was associated with 50% mortality in a batch of *L. calcarifer* undering a purging process but not in the other fish in the main tank at one farm in South Australia, suggesting a stress related disease outbreak. Although the increase in mucus and epithelial hyperplasia on gills may be caused directly by invading *I. mulitfiliis*, predisposition by poor water quality or overcrowding in the purging tanks cannot be ruled out (Griffiths 2009). *Ichthyophthirius multifiliis* infections are often associated with presence of grossly visible white spots on skin and gills. The absence of grossly visible white spots in this case reported by Griffiths (2009) suggests an acute infection, and presence of smaller invading or developing parasites embedded in skin and gill epithelium. Other external protozoan parasites reported in *L. calcarifer* included *Ichthyobodo* and *Trichodina* (Ruangpan 1988; Leong 1997). Low grade *Icthyobodo* infestations were reported in all fish examined from disease outbreaks at three land-based farms in South Australia, together with concurrent epitheliocyctis and streptococcosis or filamentous bacteria on gills (Griffiths 2009). The precise contribution of *Icthyobodo* infestations to disease in these

cases were uncertain, disease outbreak was post-handling in one farm, dissolved oxygen level was reported to frequently drop below 4ppm at another farm, and high stocking density exceeding 80kg fish/m^3 water was practiced at the third farm. A blood borne *Trypanosoma sp.* has been associated with severe mortalities in the Northern Territory (Schipp *et al.* 2007). *Trypanosoma* infestations were also observed in diseased *L. calcarifer* in sea cages in Singapore, in association with concurrent parasitic or bacterial infections, although their exact contribution towards disease needs further investigation (Chee D., personal communications).

1.4   Other infectious diseases observed in cultured *L. calcarifer*

Although the diseases described in this section have been observed in *L. calcarifer*, their impacts are either unknown or they are not associated with significant losses under culture conditions.

Lymphocystis was one of the first viral diseases described in cultured *L. calcarifer*. It is caused by an iridovirus that can result in extensive white verrucous lesions on the skin and fins that can contribute to carcass rejection. Although mentioned in several previous reports, this disease is not currently a major problem in cultured *L. calcarifer* (Ruangpan 1988; Schipp *et al.* 2007; Gibson-Kueh S., personal observations). It is likely that better husbandry practices have resulted in fewer external skin injuries, which can predispose fish to lymphocystis. Epitheliocystis has been reported in 8- and 12-week old *L. calcarifer* with no clinical disease in sea-cages in Queensland (Anderson & Prior 1992). Similarly, the extent of the pathological effect by epitheliocystis on gills of *L. calcarifer* in sea cages surveyed by Griffiths (2009) is mostly mild. Epizootic ulcerative syndrome (EUS) has been reported as serious epizootics in wild fish

including *L. calcarifer* in fresh and brackish water systems in Australia. However, EUS has not been seen in *L. calcarifer* in marine environments or farms (Schipp *et al.* 2007).

Although myxosporean parasites with with *Henneguya* type spores were found in gills of all fish examined from a *L. calcarifer* sea cage farm located in a tidal river sea cage farm, these fish were clinically healthy with minimal gill pathology (Griffiths 2009). An extensive study carried out in a *L. calcarifer* sea-cage farm in Indonesia showed high prevalence of single host parasites, notably the diplectanid monogeneans. Prevalence of these diplectanid parasites was high irrespective of seasons, but no mention was made of any pathology or other ill effects (Ruckert, Palm & Klimpel 2008). Diplectanid monogeneans infesting gills have also been reported in *L. calcarifer* cultured in China (Wu, Li, Zhu & Xie 2005; Yang, Kritsky, Sun, Zhang, Shi & Agrawal 2006). These reports were based mainly on morphological and molecular descriptions, and few included history, pathology or assessment of impacts on fish. Diplectanid parasites armed with multiple hooks are relatively large parasites measuring close to 500µm, and could potentially cause significant tissue damage in infected fish. Low grade dactylogyrid infestation in cage cultured *L. calcarifer* in Lake Argyle was associated with mild to moderate gill hyperplasia, although its precise contribution to disease outbreaks was unknown (Griffiths 2009).

Blood flukes or sanguinicolid infestations were reported to cause minimal pathological changes in *L. calcarifer* stocked in sea-cages off Malaysia (Herbert, Shaharom & Anderson 1995). Sanguinicolid eggs lodged in gills or other organs can cause severe reactions, and may result in clinical disease in heavy infestations with release of large numbers of eggs into the circulation.

Lernanthropus infestation of gills can cause anemia, but other than the report by Ruangpan (1988), a recent report was limited to morphological descriptions (Ho & Kim 2004).

1.5   Non-infectious diseases reported in *L. calcarifer*

One of the major hurdles in the early culture of *L. calcarifer* was cannibalism during larviculture and the initial grow-out stages. Cannibalism has been quite successfully managed by regular grading of fish (see 1.1.2; Questionaire Farm A Appendix 2). Although the level of larval deformities has been reduced with essential fatty acid enrichment of live feed, it still occurs to a significant level in many hatcheries (Schipp *et al.* 2007). Several studies have shown that dietary Vitamin C prevents spinal and jaw deformities. Current commercial diets are generally adequately supplemented with Vitamin C levels although this can be altered by feed storage conditions. However, that larval deformity continues to be observed in larval stage *L. calcarifer* suggests that this is likely to be multifactorial (Fraser, Anderson & de Nys 2004; Fraser & De Nys 2005; Fraser & De Nys 2011). Additionally, apoptosis has been observed in gills and kidneys of fish fed diets deficient in Vitamin C (Phromkunthong, Boonyaratpalin & Starch 1997).

Weaning is a stressful period during larviculture, and failure of a significant percentage of fry to wean onto an artificial diet can result in mortality of these fry from starvation once live feed is withdrawn. One of the major breakthroughs in *L. calcarifer* larviculture is the use of microdiets. Microdiet can be introduced as early as 8 days post hatch, allowing full weaning to formulated diet at day 16-20 versus day 35. This not only dramatically reduces the amount of artemia that needs to be fed, but reduces mortality as a result of failure of fry

to wean onto an artificial diet (Schipp *et al.* 2007). Artemia is an expensive feed

commodity in the culture of fin fish fry.

Recently, myoskeletal abnormalities have been reported in juvenile *L.

calcarifer* with the use of saline ground water at old mining sites, in association

with potassium deficiency (Partridge & Creeper 2004). Such cases of skeletal

myopathy were observed to respond to potassium supplementation in water but

not in the diet (Partridge G.J., personal communication). Vitamin E deficiency

was implicated in *L. calcarifer* with myopathy (Bowater & Burren 2007). Stephen

& Ingram (2006) reported gill necrosis and mortality due to aluminium toxicity

in *L. calcarifer* kept in ponds with low pH.

1.6   Aims of present study

Other than the study by Griffiths (2009) on gill diseases, there has been no

comprehensive study and report on the major diseases of Asian seabass (or

barramundi) *Lates calcarifer* Bloch, a food fish species of growing importance in

Asia and Australia. This study aims to investigate the diseases encountered in the

various phases of the culture of *L. calcarifer*. It hopes to improve the recognition

of novel or emerging diseases and to increase knowledge on diseases previously

reported in the culture of *L. calcarifer*, and set the platform for future research.

Chapter 2   An intestinal *Eimeria* infection in juvenile Asian seabass, *Lates*

***calcarifer* Bloch cultured in Vietnam – a first report**

Based on an adaptation of the published article: Gibson-Kueh S., Thuy N.T.N., Elliot A., Jones J.B., Nicholls P.K. & Thompson R.C.A. (2011) An intestinal *Eimeria* infection in juvenile Asian seabass (*Lates calcarifer*) cultured in Vietnam - a first report. *Veterinary Parasitology* **181**, 106-112.

ABSTRACT

This is the first report of an intestinal *Eimeria* infection in Asian seabass, *Lates calcarifer* Bloch at the histopathological and ultrastructural levels. The *Eimeria* infection was often associated with severe pathology and significant mortality in the absence of other pathogens. This showed that it is an important disease of juvenile *L. calcarifer* in small scale nurseries in Vietnam. Heavy infection and high prevalence of the *Eimeria* infection are suspected to be linked to the low daily water exchange rates practised in these nurseries. A systemic iridovirus infection was concurrently observed in some of the fishes but was not consistently present when compared to the *Eimeria* infection.

2.1 Introduction

Piscine apicomplexan parasites exhibiting epicytoplasmic development on host cells may belong to the genera *Cryptosporidium, Eimeria, Epieimeria* or *Goussia Cryptosporidium* is typically epicytoplasmic while *Eimeria* and *Goussia* may be either epicytoplasmic or intracytoplasmic parasites (Davies & Ball 1993; Paperna 1995; Paperna & Vilenkin 1996; Alvarez-Pellitero & Sitja-Bobadilla 2002; Alvarez-Pellitero, Quiroga, Sitja-Bobadilla, Redondo, Palenzuela, Padros, Vazquez & Nieto 2004; Molnar 2006). There have been two reports of *Cryptospordium*, but none to date of *Eimeria* in *L. calcarifer* (Glazebrook & Campbell 1987; Gabor, Srivastava, Titmarsh, Dennis, Gabor & Landos 2011). A review of numerous reports on histopathology and ultrastructure of piscine *Eimeria* or *Goussia* showed that it was difficult to distinguish between these two groups of parasites in host tissues based on morphology (Molnar & Baska 1986; Landsberg & Paperna 1987; Molnar 1989; Lukes & Dykova 1990; Szekely & Molnar 1992; Landsberg 1993; Benajiba, Marques, Lom & Bouix 1994; Costa & MacKenzie 1994: Alvarez-Pellitero, Palenzuela & Sitja-Bobadilla 1997; Baska 1997). Recent work based on molecular analysis appeared useful in the specific identification as well as phylogenetic placement of *Cryptosporidium* in fish (Ryan, O'Hara & Xiao 2004; Murphy, Bradway, Walsh, Sanders & Snekvik 2009; Palenzuela, Alvarez-Pellitero & Sitjà-Bobadilla 2010; Reid, Lymbery, Ng, Tweedle & Ryan 2010; Zanguee, Lymbery, Lau, Suzuki, Yang, Ng & Ryan 2010).

The classification of apicomplexan parasites belonging to the genus *Eimeria* or *Goussia* depended largely on the morphology of sporulated oocysts by light and electron microscopy. Oocysts of *Eimeria* and *Epieimeria* have four dizoic sporocysts each with a stieda body or polar plug. In *Goussia*, oocysts are

characterized by four dizoic sporocysts each with a suture line. *Cryptosporidium* oocysts have four naked sporozoites (Davies & Ball 1993; Molnar 2006). Key distinguishing features of oocysts were often not easily observed. Parasites with indistinct suture lines and the absence of stieda bodies in their sporocysts were often ascribed to the genus *Goussia* (Upton, Reduker, Current & Duszynski 1984; Molnar & Ogawa 2000; Molnar, Avenant-Oldewage & Szekely 2004).

Meronts, gamonts and oocysts of piscine *Cryptosporidium* with a size range of 3 to 5µm are much smaller than corresponding stages of *Eimeria* and *Goussia*, in the size range of 5 to 20µm. The presence of invaginating feeder organelles at the attachment juncture of *Cryptosporidium* distinguishes it from the other genera (Valigurova, Jirku, Koudela, Gelnar, Modry & Slapeta 2008). The attachment organelles of epicytoplasmic *Eimeria* and *Goussia* vary ultrastructurally from monopodial to multiple finger-like attachment organelles (Paperna 1991; Benajiba *et al*. 1994; Alvarez-Pellitero *et al*. 1997; Lukes 1992; Lukes & Stary 1992).

This is the first report of an intestinal *Eimeria* infection in juveniles of *L. calcarifer*, at the histopathological and ultrastructural levels. The *Eimeria* infection was often associated with severe pathology even in the absence of other significant pathogens, and is therefore a significant disease of *L. calcarifer* in small scale nurseries in Ca Mau, Vietnam.

2.2 Materials and methods

2.2.1 Background information on samples examined

The emerging *L. calcarifer* industry in Vietnam depends on the grow-out of juvenile fish from small scale nurseries, from which the samples in this study were taken. Diseased juvenile *L. calcarifer* 2.5 to 7cm in body length were

sampled from a total of five nurseries in Ca Mau, Vietnam. It is estimated that these nurseries stock 3000 to 5000 fish at any one time. These fish samples were collected by field officers from the Minh Hai Sub-Institute for Fisheries Research, Ca Mau, Vietnam in Jan to Mar 2008, Mar and Dec 2009, and Nov to Dec 2010, as part of their active disease surveillance programs. Fixed tissue samples were sent to Murdoch University, Australia for this study. A total of 181 fish were processed for examination by histopathology and 10 fish for transmission electron microscopy. Alcohol fixed oocysts obtained from discharged waste water from culture tanks were also examined.

2.2.2   Light microscopy (LM)

Tissues were fixed in 10% phosphate buffered formalin for at least 24 h, dehydrated in an ethanol-xylene series and embedded in paraffin blocks. Formalin fixed bony tissues were decalcified in 5% nitric acid overnight prior to dehydration and embedded in paraffin blocks. 5µm tissue sections were dewaxed in xylene, rehydrated in an ethanol series and stained by haematoxylin & eosin (H&E) or Giemsa stain.

2.2.3   Transmission electron microscopy (TEM)

Tissues were fixed in 5% glutaraldehyde in phosphate-buffered saline (PBS) at 4°C overnight, washed in several changes of PBS and post-fixed in Dalton's chrome osmic acid for1 h at 4°C. Fixed tissues were dehydrated through a graded ethanol series to propylene oxide followed by immersion in a 60:40 solution of propylene oxide/epoxy resin for 1 h, pure epoxy resin on a rotator overnight and baked in an oven at 60°C for 24 h. Ultra-thin sections were stained with uranyl acetate and lead citrate for viewing on a Philips CM100 Bio TEM.

2.3. Results

2.3.1 Field observations made on *L. calcarifer* nurseries sampled in this study

The *L. calcarifer* nurseries in Ca Mau, Vietnam were mainly small scale with less than five ½- to 1-tonne tanks. These nurseries obtained their fry from hatcheries in Vung Tau or Khanh Hoa Province in Vietnam, or as imported fry from Thailand. Fiber glass or cement tanks were mainly used as holding facilities with static or closed water recirculation systems. Partial daily water exchange rates less than 20-30% were practised. In earthen ponds which were less commonly used, the fry were kept in nets suspended in the water column. Salinity of rearing water ranged from 15 to 25 parts per thousand (ppt). Stocking density varied from 280 to 350 fish/m³ water. Fish were fed commercial feed pellets supplemented with coarsely chopped trash fish. The trash fish fed consisted of wild fish caught from the sea.

Nurseries stocked 1 to 3 cm *L. calcarifer* fry obtained from hatcheries, and grown to 7 to 9 cm body length fish to sell to grow-out farmers. Nursery reared 2.5 to 7.0 cm body length *L. calcarifer* juveniles were reported to suffer low grade clinical disease soon after stocking, with a cumulative mortality of up to 30% of stocked fish. Clinical signs and lesions included fish hanging at water surface, inappetance, lethargy, darkened bodies, tail rot and scale loss.

2.3.2 Histopathology

An *Eimeria* infection was observed in greater than 60% of diseased *L. calcarifer* sampled from each of the five nurseries, often as early as the first week post stocking. The fish sampled in this study were kept in cement or fiber glass tanks, or ponds, and in salinities of 15 or 25 ppt. Fish were found to be infected with

Eimeria in both tanks and ponds, and irrespective of salinities of rearing water. Cytoplasmic inclusion bodies suggestive of systemic iridoviral infection were observed histopathologically in approximately 20% of diseased *L. calcarifer* with a concurrent *Eimeria* infestation. Low grade to heavy gill trichodinid infestation was sometimes observed but not associated with any significant pathological changes.

The primary infection site of the *L. calcarifer Eimeria* was the small intestine. Both merogony and gamogony were epicytoplasmic and occurred simultaneously (Fig. 1). Infection levels varied from light to heavy, often with obliteration of the microvillous brush border. Meronts were much smaller than gamonts, and had merozoites arranged in rosettes or in parallel (Figs 1 & 2a). Intracytoplasmic meronts or unusually large meronts with at least 18 merozoites were occasionally observed (Fig. 2b). Macrogamonts with foamy cytoplasm due to the presence of amylopectin granules often outnumbered microgamonts. Microgamonts were smaller than macrogamonts and had peripherally arranged nuclei (Figs 1 & 2c). Mature microgamonts had numerous flagellated microgametes (Fig. 2c). Meronts measured 4.8 x 3.5 μm (n=5), macrogamonts 13.1 x 7.6 μm (n=10) and microgamonts 8.1 x 6.0 μm (n=5). Table 1 shows a summary of the size range of meronts, gamonts and oocysts.

Sporulated oocysts were very rarely observed in histological tissue sections, in fact in only 1 out of 181 fish examined, and measured 18.5 x 12.3 μm (n = 5). These oocysts in faecal materials within the intestinal lumen had four pairs of sporozoites held loosely within a thin membranous oocyst wall (Fig. 3a). Both unsporulated and sporulated oocysts were readily observed in faeces collected from tank bottom and in waste water from rearing tanks by wet mount

microscopic examinations (Figs 3b & c). Nomarski interference microscopy on alcohol fixed sporulated oocysts showed the absence of Stieda bodies and suture lines. Alcohol fixed sporulated oocysts measured 36.6 x 22.8 µm (n=5). A residual body was present in oocysts, and each pair of sporozoites was held together by a thin sporocyst membrane (Fig. 3c).

Squamous to cuboidal intestinal epithelium and low grade to severe mononuclear inflammatory infiltrates in the lamina propria were frequently observed in association with the *Eimeria* infection. The inflammation was sometimes extended into the mucosal epithelium. There were often focal to extensive areas of intestinal mucosal degeneration, necrosis and sloughed necrotic cells in intestinal lumen (Fig. 4). Extra-intestinal parasite stages were not commonly observed, except for two macrogamonts in renal tubules from 1 fish. Other abnormalities observed included dermatitis in caudal peduncle, renal glomerular degeneration, moderately reactive spleens with white pulps depleted of leucocytes, and reduced levels of hepatic glycogen stores.

Table 1 Size range of various stages of *Eimeria* in *L. calcarifer*

Stage	Size range	Mean size
Histology tissue sections		
meronts	4.6-5.6 x 3.1-5.2 µm	4.8 x 3.5 µm (n=5)
macrogamonts	8.0-14.4 x 4.8-9.6 µm	13.1 x 7.6 µm (n=10)
microgamonts	7.2-10.0 x 5.6-8.3 µm	8.1 x 6.0 µm (n=5)
Sporulated oocysts	17.9-20.0 x 12.1-14.8 µm	18.5 x 12.3 µm (n = 5)
Alcohol fixed samples from tank water discharge		
Sporulated occysts	34.2-38.2 x 15.8-23.4 µm	36.6 x 22.8 µm (n=5)

Figure 1 *Eimeria* infection on the brush border of intestinal mucosa in juvenile *Lates calcarifer* from Vietnam and increased mononuclear infiltrate (Inf) in lamina propria. Meronts (Me) with merozoites arranged in rosettes, macrogamonts (Ma) with foamy cytoplasm, microgamonts (Mi) with peripherally arranged nuclei and more intensely basophilic trophozoites (T) (H&E).

Figure 2 (a) Meronts (Me) with merozoites arranged in parallel and merozoites (z) apparently still within parasitophorus envelopes (Giemsa). (b) An unusually large meront (arrow) with at least 18 merozoites (H&E). (c) Microgamonts (*) with peripherally arranged nuclei and microgametes (arrows) (Giemsa)

Figure 3 (a) Sporulating oocysts (*) had four sporocysts. Sporulated oocysts (arrows) in faecal materials within intestinal lumen had four pairs of sporozoites and a thin membranous wall (H&E). (b) Unsporulated oocyst observed in discharged tank waste water. (c) Sporulated oocyst from waste water with oocyst residual body (R) and sporozoites in pairs, bounded by thin sporocyst membrane. Sporocysts (S) had no stieda bodies or suture lines (*Nomarski interference microscopy*).

Figure 4 The intestinal epithelium was denuded (arrows) in some areas with corresponding intense inflammation (Inf) in the lamina propria, and a significant amount of sloughed cellular debris (D) in the intestinal lumen (Giemsa).

2.3.3 Ultrastructural observations by TEM

Parasitic stages were observed within complete parasitophorous envelopes at extracytoplasmic positions on the microvillous brush border of intestinal epithelium. Shortening or loss of microvilli and necrosis of affected intestinal epithelium were often observed. Both meronts and gamonts had finger-like attachment organelles that extended into host cells but were limited to the extracytoplasmic cellular boundaries (Figs 5, 7a, 7b & 8). A large number of rodlet cells were often seen in association with these parasitic infections, and sometimes within blood vessels in the intestine (Fig. 6).

Meronts had up to eight merozoites, each with apical complexes in various stages of formation (Figs 7a & b). Macrogamonts had abundant amylopectin granules (Fig. 8) while microgamonts had flagellated microgametes with a large residual body (Fig. 5).
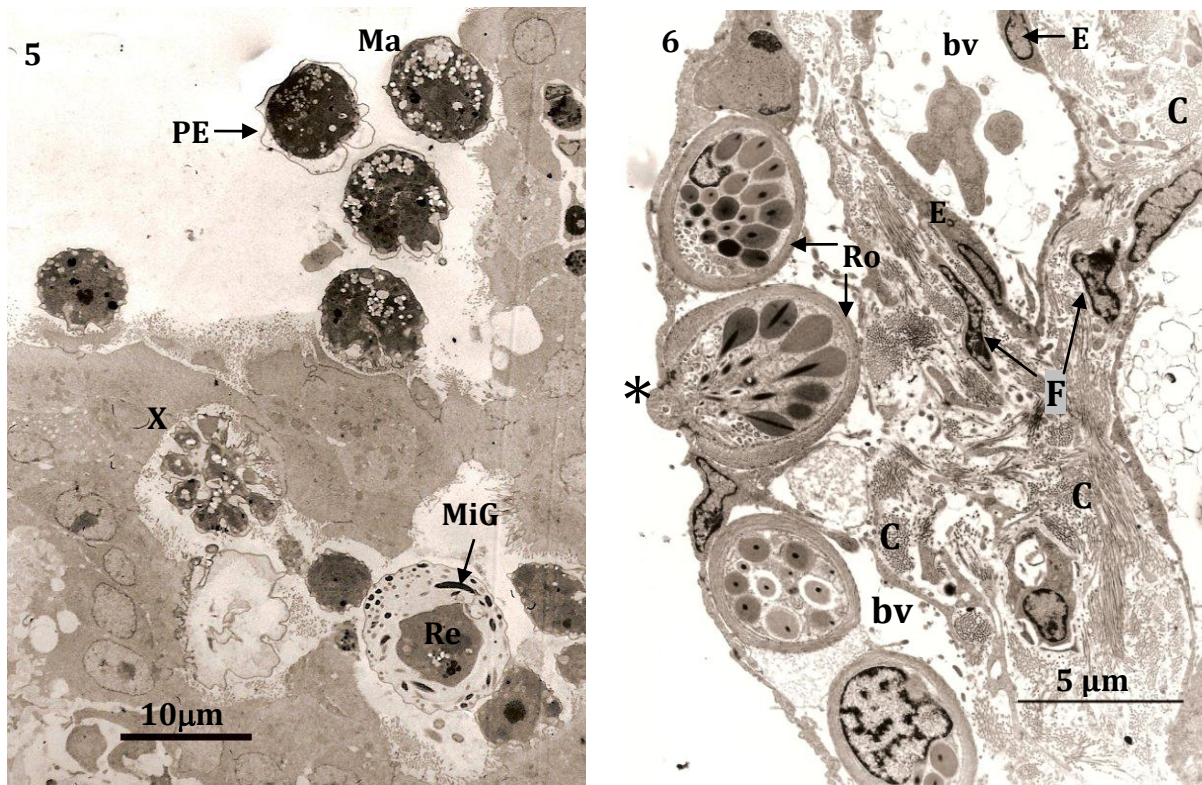
Figure 5 Macrogamonts (Ma) with amylopectin granules and microgamonts with microgametes (MiG) at the microvillous brush border of intestines. 'X' was presumably a microgamont from which microgametes had been released, thus giving a crenate appearance. Parasitophorous envelopes (PE), residual body (Re) in microgamont.

Figure 6 Rodlet cells (Ro) often associated with parasitism in fish were observed within blood vessels (bv) in intestines. One of the rodlet cells appeared to be in the process of exiting the blood vessel (*). Vascular endothelial cells (E), fibroblasts (F) that produced the collagen (C) of blood vessel wall.
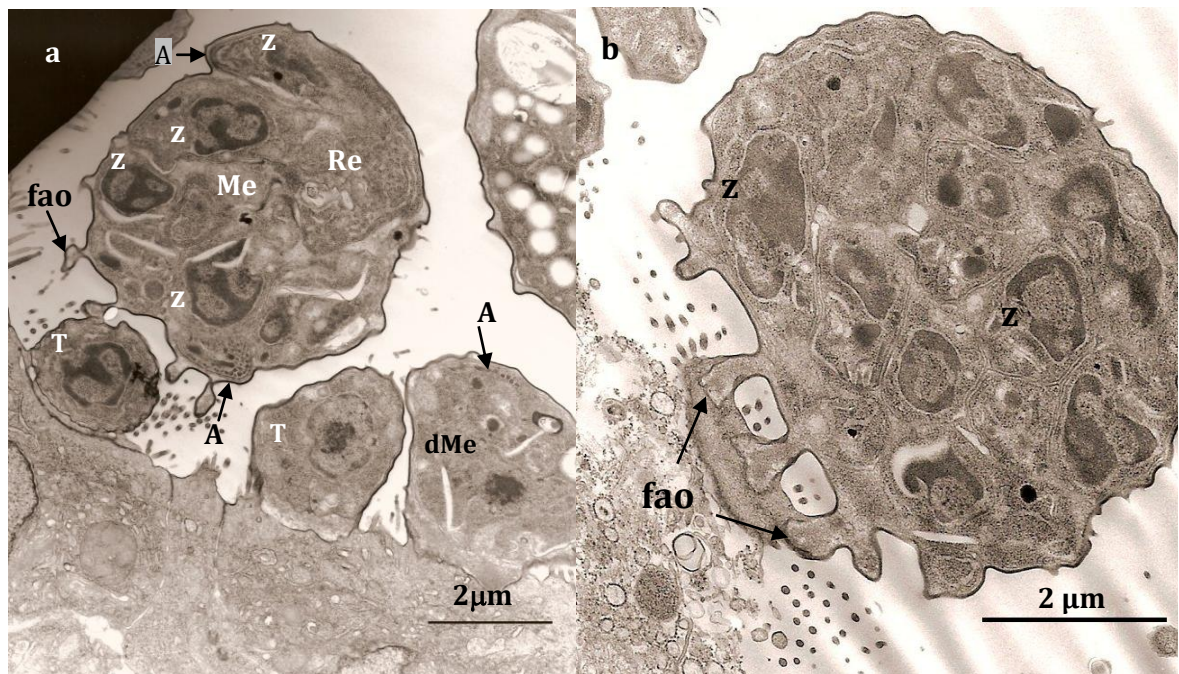
Figure 7(a) Meront (Me) with finger-like attachment organelles (fao) and residual body (Re). Merozoites (z) had apical complexes (A) at various stages of formation. Trophozoites (T) and developing meront (dMe) in epicytoplasmic position. (b) Meront with at least 8 merozoites (z) and finger-like attachment organelles (fao).
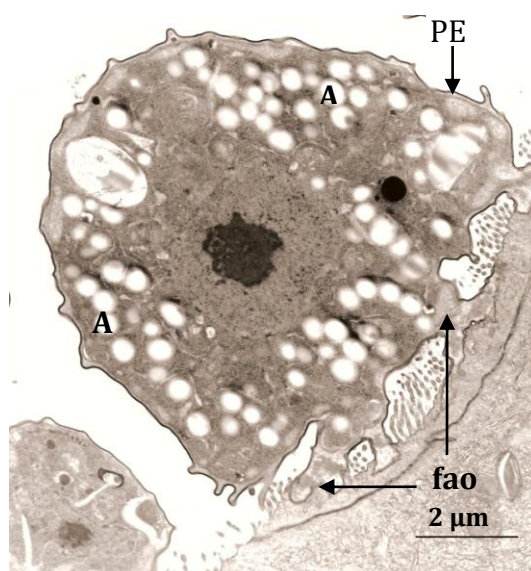
Figure 8 Macrogamont with abundant amylopectin (A) granules and finger-like attachment organelles (fao) extended into host cell but limited to the epicytoplasmic boundary. parasitophorus envelope (PE).

2.4 Discussion

This is the first report of a natural *Eimeria* infection in *L. calcarifer*. The mortality of 30% was based on what was reported by these nurseries. A total of 181 fish were processed for examination by histopathology. The *Eimeria* infection was observed in nearly all fish sampled over the period samples were taken in 2008 to 2010. However, an estimation of prevalence was not possible as only diseased fish were examined. The *Eimeria* infection was associated with significant intestinal necrosis and inflammation. Cumulative losses of 30% of an average stock of 3000 to 5000 fish would constitute a significant loss for these small scale nurseries.

While it does not supply all the answers, consistent findings of the *Eimeria* infection in association with severe pathology showed that it is a significant disease under nursery culture conditions in Ca Mau, one which will need to be managed. Significant pathology was frequently reported in fish with apicomplexan infections (Benajiba *et al*. 1994; Morrison, Leger & Morrison 1993; Jendrysek, Steinhagen, Drommer & Korting 1994; Hemmer, Steinhagen, Drommer & Korting 1998; Molnar 2006; Gjurcevic, Kozaric, Bambir, Petrinec, Kuzir, Gudan & Bazdaric 2008). A systemic iridoviral disease was concurrently observed but was not consistently present when compared to the *Eimeria* infection in diseased fish. Nonetheless, iridovirus is a serious pathogen which can co-contribute to losses during the culture cycle (Gibson-Kueh, Netto, Ngoh-Lim, Chang, Ho, Qin, Chua, Ng & Ferguson 2003).

The *L. calcarifer Eimeria* did not possess the feeder organelles typical of *Cryptosporidium* but had finger-like attachment organelles very similar to epicytoplasmic species of *Eimeria* and *Goussia*. Although the sporocysts in

oocysts examined in this study lack Steida bodies, this was also the case in some previously described piscine *Eimeria* (Upton *et al.* 1984; Landsberg & Paperna 1987; Paperna 1995; Molnar 2006). Therefore, we will refer to the *L. calcarifer* apicomplexan parasite in this study as *Eimeria*. Molecular studies conducted on the *L. calcarifer* apicomplexan parasite, presented in Chapter 3, further support its identity as *Eimeria* based on the morphology reported here. Since sporulated oocysts were rarely observed in tissue sections of *L. calcarifer* with an *Eimeria* infection, it is presumed that sporulation was mainly exogenous. Both unsporulated and sporulated oocysts were readily observed in faecal materials collected from tank bottoms. The *L. calcarifer Eimeria* oocysts in histological tissue sections were almost half the size of alcohol fixed oocysts obtained from waste water, likely due to the dehydration process used in histology. Shrinkage due to fixation was suggested as a possible cause for differences in oocyst size observed in fresh and fixed materials in a study of eimeriids in littleneck clams, *Protothaca staminea* (Desser & Bower 1997). There is also the possibility that more than one *species* of *Eimeria* were involved.

A study on *Goussia carpelli* in common carp, *Cyprinus carpio* suggested the correlation of infection rates to stress and immunosupression (Steinhagen, Hespe, Ellmer & Korting 1998). The diseased fish examined in this study were sampled during the initial post-stocking period when the fish were still recovering from transport and acclimatization stress. Depletion of splenic white pulp of leucocytes in diseased *L. calcarifer* examined in this study can be expected to have an impact on immunity, and may explain the heavy *Eimeria* infection often observed.

The origin of the *Eimeria* infection in *L. calcarifer* from nurseries in Ca Mau is unknown, and warrants further study. Intestinal coccidiosis was observed to occur in cyprinids and cichlids, with losses occurring in 2 to 3 weeks shortly after hatch (Paperna 1996). The feeding of trash (or bait) fish is a possible source of infection, and future studies can examine trash fish for *Eimeria* or look at *L. calcarifer* from nurseries that do not feed trash fish. Direct transmission of *Eimeria* via ingestion of sporulated oocysts of *Eimeria vanasi* was reported by Kim and Paperna (1992) although others such as Fournie and Overstreet (1983) reported obligate intermediate hosts for another piscine coccidia, *Calyptospora funduli* (Paperna 1996).

The stocking of fish in static or closed recirculation aquaculture systems with relatively low daily water exchange rates (20-30%) may cause infective stages to build-up and explain *Eimeria* infection in greater than 80% of all sick fish examined. Large scale *L. calcarifer* hatcheries and nurseries in Indonesia, Singapore and Australia practised very high water exchange rates of 100 to 300% an hour (Schipp *et al.* 2007; Appendix 3.2). Whether the *Eimeria* infection will persist in older fish as a chronic infection or were present in fish before being stocked in nurseries in Vietnam remains to be elucidated and is vital information for its effective management. Experimental challenge or co-habitation trials will complement what has been learnt from examination of the naturally infected fish in this study.

There are currently no treatment options. The sequestering of these epicytoplasmic parasites in parasitophorus envelopes away from the intestinal lumen and host cell cytoplasm makes it resistant to currently available therapeutic drugs (Sterling 2000). Recent research revealed that addition of

proteins produced by *Cryptosporidium* competitively inhibited their attachment to intestinal epithelium (Tzipori and Ward 2002). A similar approach could be applied for this epicytoplasmic *Eimeria*.

Chapter 3 **The molecular characterization of an *Eimeria* and *Cryptosporidium* detected in Asian seabass (*Lates calcarifer*) cultured in Vietnam**

Based on an adaptation of the published article: Gibson-Kueh S., Yang R., Thuy N.T.N., Jones J.B., Nicholls P.K. & Ryan U. (2011). The molecular characterization of an *Eimeria* and *Cryptosporidium* detected in Asian seabass (*Lates calcarifer*) cultured in Vietnam. *Veterinary Parasitology* **181**, 91-96

ABSTRACT

An intestinal *Eimeria* was previously reported as a significant pathogen of Asian seabass (*Lates calcarifer*) in nurseries in Vietnam. In the present study, both *Eimeria* and *Cryptosporidium* were detected by sequence analyses of fragments of the 18S rRNA gene amplified from these Vietnamese *L. calcarifer* tissues. Based on these analyses, the *Eimeria* from the Vietnamese *L. calcarifer* formed clades with the *Eimeria* detected in *L. calcarifer* tissues from Australia, but clustered separately from other known *Eimeria* and *Goussia species*. The *Cryptosporidium* detected in *L. calcarifer* from Vietnam clustered closest with *C. parvum* and *C. hominis*. *In situ* hybridization using DIG-labeled DNA probes generated from 18S PCR products on the Vietnamese *L. calcarifer* wax block tissues showed that this method could not be used to distinguish between *Eimeria* and *Cryptosporidium,* due to the conserved nature of the 18S locus. A previously published study on the morphology of parasite developmental stages and oocysts in the Vietnamese *L. calcarifer* tissues showed only an intestinal *Eimeria* infection. The *Cryptosporidium* could be present at very low levels undetectable by microscopy in intestines or, being ubiquitous, was a possible

contaminant from feed or water. While molecular analysis is a very useful tool in

the study of disease and identification of aetiological agents, this study reiterates

the importance of demonstrating organisms *in situ* in tissues.

3.1 Introduction

The exact identity of an apicomplexan parasite of *Lates calcarifer* was initially unclear based on morphology, and hence molecular analysis for both *Eimeria* and *Cryptosporidium* was undertaken. A great variety of coccidian parasites belonging to the genera *Eimeria* or *Epieimeria* have been reported in fish but these are based solely on morphological decriptions (Upton, Reduker, Current & Duszynski 1984; Paperna 1991; Landsberg 1993; Sitja-Bobadilla, Palenzuela & Alvarez-Pellitero 1996). Genetic sequences for piscine-derived *Eimeria* are not available. An intestinal *Eimeria* was previously reported as a significant pathogen of *L. calcarifer* in nurseries in Vietnam (Gibson-Kueh, Thuy, Elliot, Jones, Nicholls & Thompson 2011a).

   Cryptosporidium is an apicomplexan parasite that is now recognized as being more closely related to gregarines than to coccidia (Barta & Thompson 2006). There are two well recognised species of *Cryptosporidium* in fish: *Cryptosporidium molnari* in gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), and *Cryptosporidium scophthalmi* in turbot (*Psetta maxima, syn. Scophthalmus maximus*) (Alvarez-Pellitero & Sitja-Bobadilla 2002; Alvarez-Pellitero, Quiroga, Sitja-Bobadilla, Redondo, Palenzuela, Padros, Vazquez & Nieto 2004). Other novel *Cryptosporidium species* have been reported in aquarium fish such as angelfish and guppies (Ryan, O'Hara & Xiao 2004; Murphy, Bradway, Walsh, Sanders & Snekvik 2009; Zanguee, Lymbery, Lau, Suzuki, Yang, Ng & Ryan 2010), and in wild fish including mullet and whiting (Reid, Lymbery, Ng, Tweedle & Ryan 2010). There are two reports of *Cryptosporidium* infections in Asian seabass (or barramundi), *Lates calcarifer* (Glazebrook & Campbell 1987;

Gabor, Srivastava, Titmarsh, Dennis, Gabor & Landos 2011). Genetic sequences are however only available for *C. molnari* (GenBank accession number HM243547) (Palenzuela, Alvarez-Pellitero & Sitja-Bobadilla 2010). A total of 9 additional species/genotypes have been identified in fish using molecular tools: piscine genotype 1 from a guppy (*Poecilia reticulata*) (Ryan *et al.* 2004), piscine genotype 2 from a freshwater angelfish (*Pterophyllum scalare*) (Murphy *et al.* 2009), piscine genotype 3 from sea mullets (*Mugil cephalus*) (Reid *et al.* 2010), piscine genotypes 4-6 from ornamental fish (Zanguee *et al.* 2010) and *C. parvum*, *C. xiaoi* and pig genotype II in whiting (*Sillago vittata*) (Reid *et al.* 2010).

In the present study, analysis of a partial 18S rRNA gene sequences, detected in tissues of these nursery reared *L. calcarifer* from Vietnam, identified novel species of *Eimeria* and *Cryptosporidium*. These are the first piscine-derived *Eimeria* partial 18S gene sequences to be published.

3.2 Materials and methods

3.2.1 General

Fixed tissues from 211 fish were originally sampled from five nurseries in Ca Mau, Vietnam between 2008 and 2010. Of these samples, 181 fish were previously examined by histopathology while 10 fish were examined by electron microcopy, and found to be infected by an intestinal *Eimeria*. Intestinal tissues from 10 formalin fixed fish sampled in 2008, and 10 alcohol fixed fish sampled in 2010, both from Vietnam were processed for DNA extraction. Polymerase chain reaction (PCR) amplification of 18S rRNA gene was performed on DNA extracted from both formalin and alcohol fixed Vietnamese *L. calcarifer* tissues. Included in

this study were wax block tissues from one case of cultured barramundi (also *Lates calcarifer*) from Western Australia (WA).

3.2.2 18S Polymerase Chain Reaction (PCR) and sequencing

DNA was extracted from formalin and alcohol fixed tissues of *L. calcarifer* from Vietnam and wax block tissues of *L. calcarifer* from Australia, using a Qiagen DNeasy tissue kit (Qiagen, Germany). Nested PCR of a fragment of the 18S locus of *Cryptosporidium* was conducted as previously described (Ryan, Xiao, Read, Zhou, Lal & Pavlasek 2003). For *Eimeria*, a hemi-nested PCR was used. The primary amplification was conducted using the primers EIF1 5'- GCT TGT CTC AAA GAT TAA GCC (Power, Richter, Emery, Hufschmid & Gillings 2009) and EIR3 5' – ATG CAT ACT CAA AAG ATT ACC (this study). Diluted amplicons (1:10) of the primary PCR were used as template for a secondary amplification using the primers EIF3 5'- CTA TGG CTA ATA CAT GCG CAA TC (this study) and EIR3. The PCR was performed in a 25 μl reaction mixture that contained approximately 15 ng of DNA, 1 x PCR buffer (FisherBiotech Perth, Western Australia), 0.2 mM deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, 5% (wt/vol) dimethyl sulfoxide, 0.2 μmol of each primer and 1 U of Tth+ DNA polymerase (FisherBiotech Perth, Western Australia). Reactions were initially denatured at 94°C for 3 min. and then subjected to 45 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 90 sec. The final extension was 72°C for 7 min. The reaction mixture and cycling program were identical for both the primary and secondary PCRs but the cycle number was lowered to 35 for the primary PCR. An *Eimeria* isolate from Western Grey Kangaroo designated WGK2533 (GenBank Accession number JF419349)

and a *Cryptosporidium* isolate from mullet (Reid *et al.* 2010) was used as positive controls. Sterile distilled water served as negative controls in both PCR.

PCR products were purified using QIAquick PCR purification spin columns (Qiagen, Germany), and sequenced using an ABI Prism Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California). All commercial kits were used according to the manufacturer's instructions unless otherwise specified. PCR products were sequenced in both directions and analyzed using SeqEd version 1.0.3 (Applied Biosystems). For all DNA extracts from fish tissue samples that were positive at the 18S locus for *Cryptosporidium* by PCR, attempts were also made to amplify the actin locus as previously described (Ng, Pavlasek & Ryan 2006).

3.2.3 Phylogenetic analysis

Nucleotide sequences were analyzed using Chromas lite version 2.0 (http://www.technelysium.com.au) and aligned with reference genotypes from GenBank using Clustal W (http://www.clustalw.genome.jp). Phylogenetic trees were constructed using additional isolates from Genbank. Distance estimation was conducted using TREECON (Van de Peer & De Wachter 1994), based on evolutionary distances calculated with the Tamura-Nei model and grouped using Neighbour-Joining. Parsimony analyses were conducted using MEGA version 3.1 (MEGA3.1: Molecular Evolutionary Genetics Analyses software, Arizona State University, Tempe, Arizona, USA). Bootstrap analyses were conducted using 1,000 replicates to assess the reliability of inferred tree topologies. Maximum Likelihood (ML) analyses were conducted using the program PhyML (Dereeper, Guignon, Blanc, Audic, Buffet, Chevenet, Dufayard, Guindon, Lefort, Lescot,

Claverie & Gascuel 2008) and the reliability of the inferred trees was assessed by the approximate likelihood ratio test (aLRT) (Anisimova & Gascuel 2006).

3.2.4 *In situ* hybridization using dioxygenin labeled 18S PCR products

The *in-situ* hybridization (ISH) steps were adapted from published literature (Bearham, Spiers, Raidal, Jones & Nicholls 2007a; Bennett, Woolford, O'Hara, Warren & Nicholls 2008). QIAquick PCR purification spin column (Qiagen, Germany) purified products from both *Eimeria* and *Cryptosporidium* 18S PCR described above were used to generate dioxygenin (DIG) labeled DNA probes using DIG-nick translation mix (Roche, Germany) according to the manufacturer's instructions. The DIG-nick translation kit is expected to produce a cocktail of DIG-labeled DNA probes ranging between 200 to 500bp from the PCR products. DIG labeled DNA probes made from 1 μg of PCR products were used to make up 1 ml of DNA probe mixture that contained 50% formamide, 10% dextran sulphate and 2x saline sodium citrate (SSC) buffer. The DNA probe mixture was stored at 4°C until required. A negative control DIG-labeled DNA probe generated from 18S PCR products to detect *Trypanosoma irwini* was kindly provided by Linda McInnes, School of Veterinary and Biomedical Sciences, Murdoch University. All incubations were at room temperature unless otherwise stated.

Formalin fixed paraffin embedded (FFPE) 5μm tissue sections on silanised slides were deparaffinized in two changes of xylene and rehydrated through an ethanol series to tap water. Tissue sections in Tris EDTA buffer (pH 9) bath were subjected to 'reheat' for 4 minutes and 'low heat' function for 4 minutes in a domestic microwave (Kambrook Model KER-686LE), followed by

cooling in running tap water. Tissue sections were incubated in tris-buffered saline (TBS) 0.05% Tween 20 for 5 minutes before tapping off. DIG labeled DNA probe mixture (30-50µl) were added to each tissue section which were then cover-slipped, and incubated at 95°C for 15 minutes. The hybridization step was carried out at 42°C overnight (approximately 16-20 hours) in a moist chamber. Cover-slips were removed and tissue sections washed for several seconds with 2x SSC buffer containing 0.05% Tween 20. A blocking solution (0.1% bovine serum albumin in TBS 0.05% Tween 20) was added to tissue sections for 10 minutes before tapping off. Alkaline phosphatase (AP) conjugated anti-dioxygenin (anti-DIG) (Roche, Germany) antibody diluted 1:600 in blocking solution was added to each tissue section for a 60-minute incubation. The AP conjugated anti-DIG was rinsed off followed by a 5-minute bath in TBS 0.05% Tween 20. Liquid Permanent Red (Dako, USA) with 300 µg/ml levamisole to block endogenous alkaline phosphatase was added to each slide for 20 minutes, and rinsed off with tap water. Tissue sections were counterstained with haematoxylin for 30 seconds and rinsed in tap water followed by a few short dips in Scott's solution. Slides were flicked dry prior to cover-slipping with Faramount Mounting Media (Dako, USA) for visualization on an Olympus BX51 fluorescent microscope using the U-MWIBA2 filter.

3.3 Results

3.3.1 Sequence and phylogenetic analysis of *Eimeria*

Partial 18S rRNA gene sequences were obtained from two alcohol fixed Vietnamese *L. calcarifer* tissue samples (VTASB1 and VTASB2) and two from Australian *L. calcarifer* wax block tissue samples (AUBara1 and AUBara2). The

partial 18S gene sequences VTASB2, AUBara1 and AUBara2 measured 1475bp, while VTBSB1 measured 1468bp. Neighbour-joining, parsimony and ML analysis of the 18S rRNA partial sequences from these four samples and a range of *Eimeria, Goussia* and other species obtained from GenBank produced similar results and showed that these isolates grouped separately from known *Eimeria* and *Goussia* species (Fig. 9 - NJ tree shown). VTASB1 & 2 and AUBarra1 & 2 shared 99.93% - 98.1% similarity to each other and formed a distinct clade, but shared only 92.8- 88.7% similarity with all other species.

The unique partial 18S rRNA gene sequences of the Vietnamese *L. calcarifer* and Australian *L. calcarifer Eimeria* genotypes have been deposited in the GenBank database under accession numbers JF261140 (VTASB1), JF261139 (VTASB2), JF261138 (AUBarra1) and JF261137 (AUBarra2), respectively.

3.3.2 Sequence and phylogenetic analysis of *Cryptosporidium*

Partial 18S rRNA gene sequences were obtained from two formalin fixed Vietnamese *L. calcarifer* tissue samples (H08384 and H08391). These sequences obtained were 518 and 519bp, respectively. Neighbour-joining, parsimony and ML analysis of the 18S rRNA sequences from these two samples and a range of *Cryptosporidium* species and genotypes obtained from GenBank produced similar results and showed that these DNA sequences grouped most closely with *C. parvum* and *C. hominis* (Fig. 10 - NJ tree shown). H08384 and H08391 had 3 single nucleotide polymorphisms (SNPs) from each other, and between 7 and 12 SNPs from *C. parvum* KSU isolate type B (GenBank accession no. AF308600). H08384 and H08391 had between 11-17 SNPs from *C. parvum* (GQ121019) and

between 14-17 SNPs from *C. hominis.* H08384 and H08391 shared 98.5% genetic

similarity with each other, 97-98% similarity with the *C. parvum* KSU type B
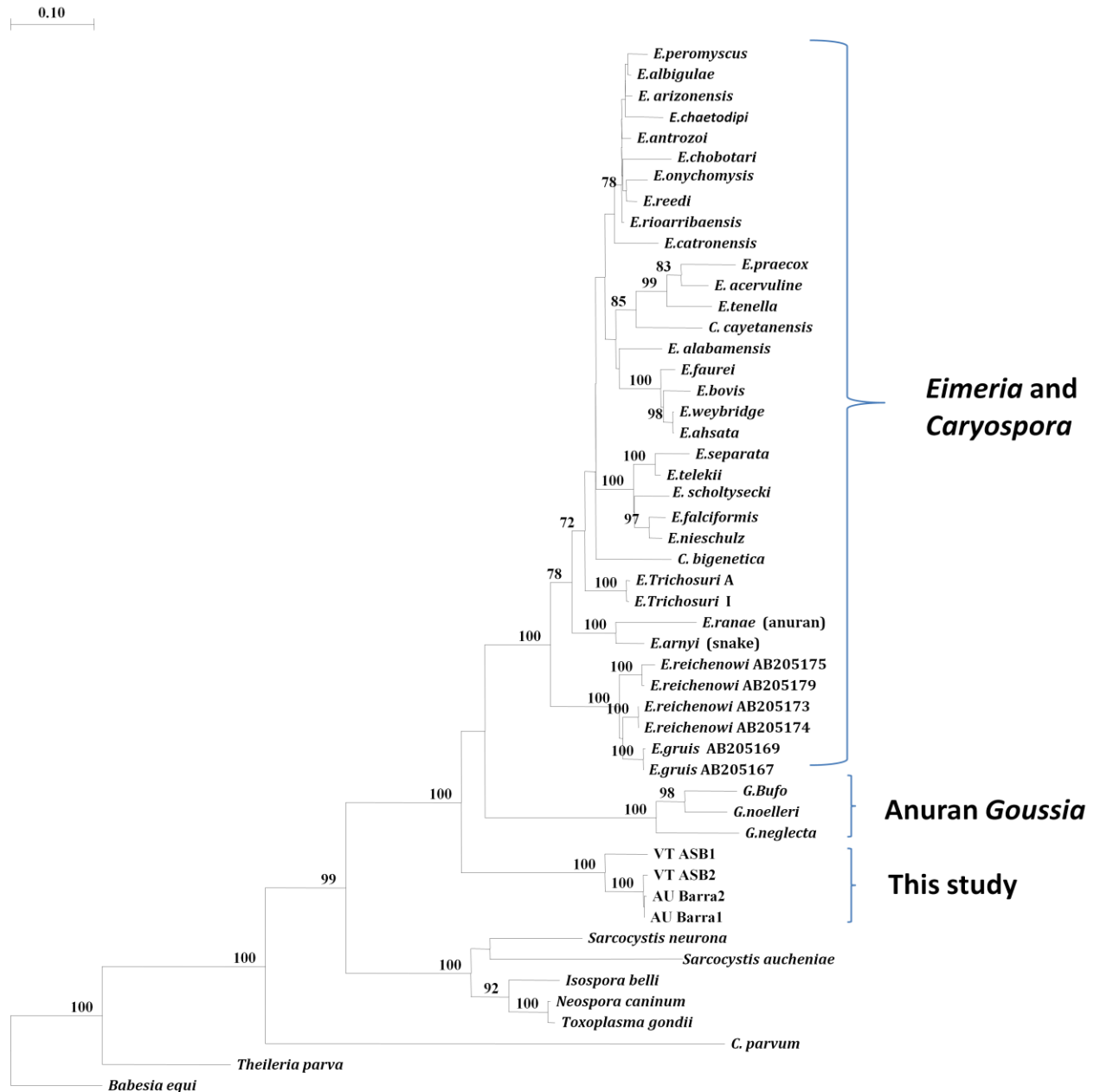
Figure 9 Phylogenetic tree of *Eimeria* detected in *L. calcarifer* from Vietnam and Australia, with GenBank database accession numbers JF261140 (VTASB1), JF261139 (VTASB2), and JF261138 (AUBarra1) and JF261137 (AUBarra2), respectively.
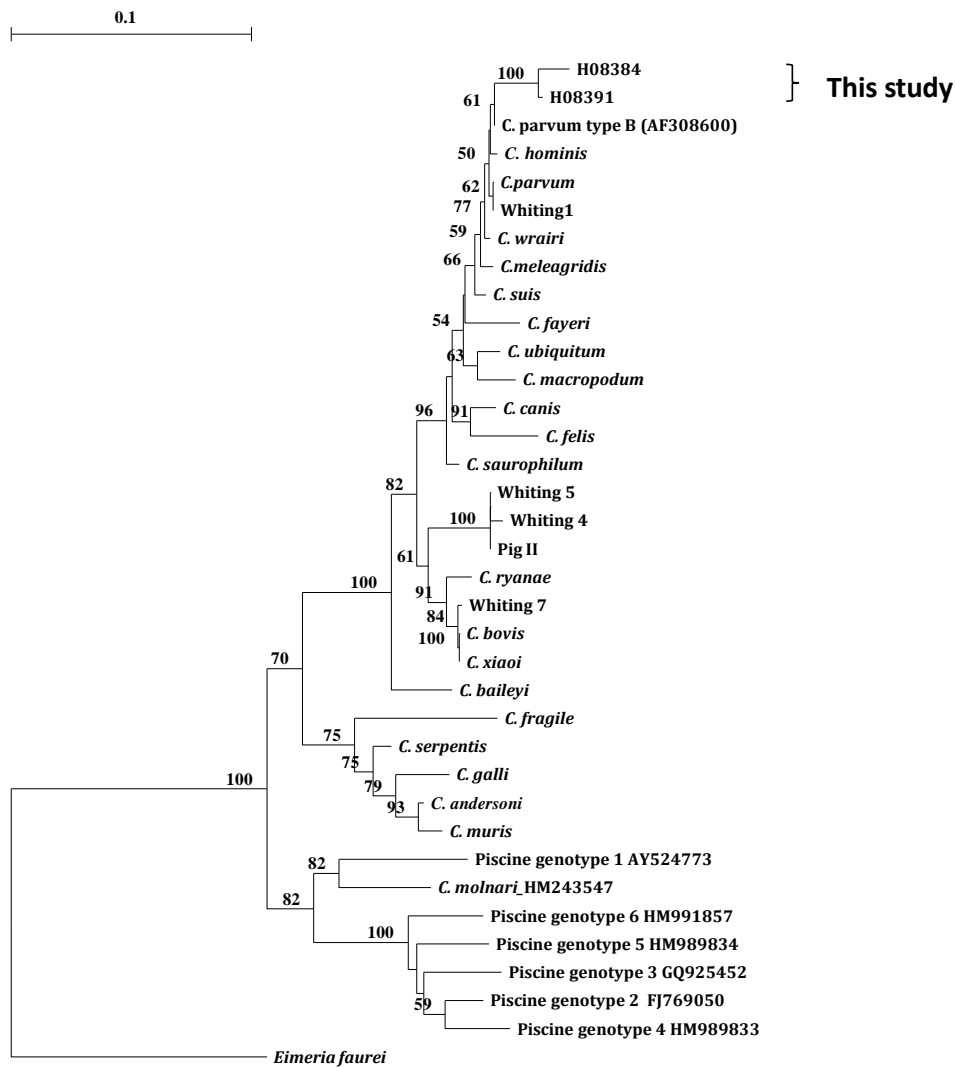
Figure 10 Phylogenetic tree of *Cryptosporidium* detected in *L. calcarifer* from Vietnam, with GenBank database accession numbers JF285332 (H08384) and JF285333 (H08391).

isolate, and 96-97.5% similarity with *C. parvum* and *C. hominis*. Unfortunately all attempts to amplify at the actin locus were unsuccessful.

The unique partial 18S rRNA sequences of the *Cryptosporidium* genotypes detected in Vietnamese *L. calcarifer* have been deposited in the GenBank database under accession numbers JF285332 (H08384) and JF285333 (H08391).

3.3.3 In-situ hybridization (ISH)

The epicytoplasmic intestinal *Eimeria* in tissue sections of *L. calcarifer* from Vietnam showed positive red fluorescence in ISH carried out using DIG-labeled *Eimeria* 18S PCR products (Fig. 11). Negative controls using DIG-labeled 18S PCR products generated to detect *Trypanosoma irwini* showed negative fluorescence (inset of Fig. 11). Low to moderate level red fluorescence was observed on the same organisms in tissue sections using DIG-labeled *Cryptosporidium* 18S PCR products (Fig. 12).
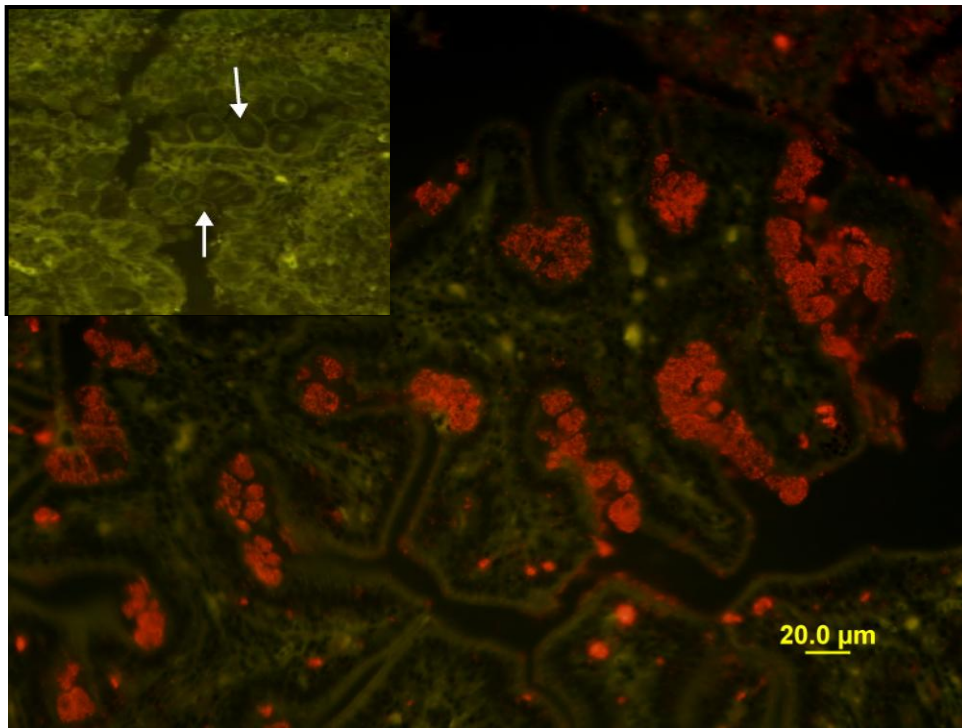
Figure 11 Epicytoplasmic Asian seabass *Eimeria* in intestines showed positive red fluorescence in ISH using DIG-labeled *Eimeria* 18S PCR products and Permanent Red (Dako). Inset (to same scale) shows no fluorescence in ISH using negative control DIG-labeled *Trypanosoma* 18S PCR products.
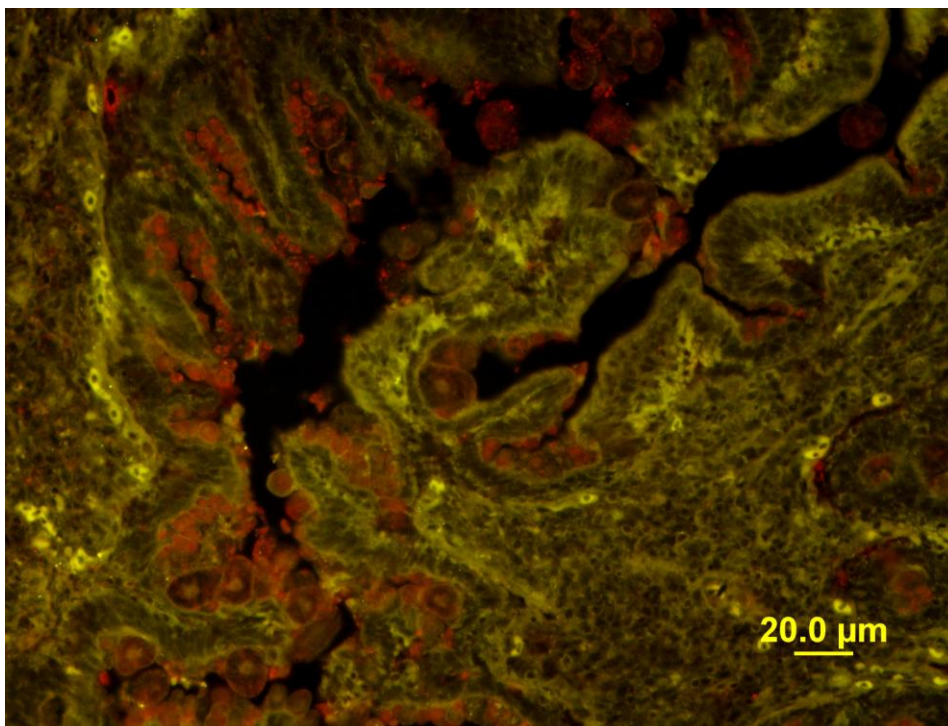
Figure 12 Epicytoplasmic Asian seabass *Eimeria* in intestines showed mild to moderate red fluorescence in ISH using DIG-labeled *Crytopsoridium* 18S PCR products.

3.4 Discussion

Vietnamese and Australian *L. calcarifer* are the same fish species, but are from distinct geographically separated populations. Analysis of *Eimeria* from both Vietnamese and Australian *L. calcarifer* revealed that they are unique and form a clade basal to the rest of Eimeriidae, which is clearly distinct from previously described clades including anuran *Eimeria* and *Goussia* (Jirku, Jirku, Obornik, Lukes & Modry 2009a; Jirku, Jirku, Obornik, Lukes & Modry 2009b). They represent the first published sequences of piscine *Eimeria* and are most probably conspecific due to their high sequence similarity.

Phylogenetic analysis of partial 18S sequences revealed that the *Eimeria* sequences were genetically very distinct and likely to be a separate species. In order to confirm the species status of the novel *Eimeria*, sequence and phylogenetic analysis at another locus such as the mitochrondial cytochrome oxidase gene (COI) is required. Previous studies have reported heterozygous alleles in *Eimeria species* at the 18S locus (Hill, Richter & Power 2012). Studies comparing the analysis of the 18S and COI genes indicate the latter has higher resolving power for *Eimeria species*, especially with respect to recent speciation studies (Ogedengbe, Hanner & Barta 2011). COI has become the target gene for the Barcode of Life project that aims to use the marker for rapid identification of animals, including parasites (Ratnasingham & Hebert 2007). One drawback of using this gene in the context of piscine studies is the paucity of *Eimeria species* sequences while available for hosts such as poultry, rodents and more recently marsupials (Hill *et al*. 2012). Analyzing the isolates at multiple loci will provide a more in-depth analysis of the species status and evolution of piscine-derived *Eimeria*.

Cryptosporidium is known to have four type A and one type B ribososmal units which differ genetically (Le Blancq, Khramtsov, Zamani, Upton & Wu 1997). Analysis of the two *Cryptosporidium* partial 18S rRNA sequences detected in Vietnamese *L. calcarifer* in the present study revealed that they clustered closest with *C. parvum* type B. Analysis at a second locus is therefore essential to determine the true identity and phylogenetic placement of this *Cryptosporidium*. Unfortunately all attempts to amplify at the actin locus as described by Ng *et al.* (2006) were unsuccessful.

Maximum likelihood (ML) is considered the most accurate method in molecular phylogenetics, based on analysis of the specific DNA sequence using alignment and an evolutionary model. Although neighbour-joining (NJ) or maximum parsimony (MP) may produce the correct topology, they may not give good estimates of branch length (Sleator 2011). However, when the extent of sequence divergence is approximately 5% and greater than 1,000 nucleotides are used, all three (NJ, MP and ML) methods show essentially the same efficiency in obtaining the correct topology and in estimating branch lengths (Tateno , Naoko & Neij 1994). The *Eimeria* 18S gene sequences measured 1475bp and 1468bp, and when compared with other GenBank genotypes, sequence differences were greater than 5%. The *Crytopospridium* 18S gene sequences of L. calcarifer measured 418 and 419bp, and when compared with other GenBank genotypes, sequence differences was less than 5% with some isolates such as *C. parvum*. However, neighbour-joining, parsimony and maximum-likelihood analysis of the 18S rRNA sequences compared with species and genotypes

obtained from GenBank produced similar results for both the *Eimeria* and *Cryptosporidium* detected in *L. calcarifer*.

Previous studies in fish for which morphological and genetic data are available have identified piscine-derived *Cryptosporidium* species as genetically very distinct. For example, *C. molnari*, and piscine genotype 1 and 2, which were all identified in the stomach, clustered separately in a clade basal to other gastric *Cryptosporidium species* (Ryan *et al.* 2004; Murphy *et al.* 2009; Palenzuela *et al.* 2010). While *C. scophthalmi* has been identified in turbot intestines (Alvarez-Pellitero *et al.*, 2004), no molecular data are available for comparison. In addition, morphological but not molecular descriptions have been reported in the intestinal villi of *L. calcarifer* (Glazebrook & Campbell 1987; Gabor *et al.* 2011) and carp, *Cyprinus carpio* (Pavlasek 1983). Although both *Eimeria* and *Cryptosporidium* were detected at the molecular level in the *L. calcarifer* tissues from Vietnam, only *Eimeria* could be identified at the light and electron microscopic levels (Gibson-Kueh *et al.* 2011a). This could possibly be due to a low rate of infection by *Cryptosporidium*. Being ubiquitous organisms, *Cryptosporidium* might be a contaminant in the *L. calcarifer* intestines from feed or culture water, and was not a true colonizing infection. This was further supported by the clustering of this *Cryptosporidium* closest to mammalian genotypes (Fig. 10).

Positive fluorescence was obtained for Vietnamese *L. calcarifer Eimeria* in tissues by ISH using DIG-labeled DNA probes generated from *Eimeria* and *Cryptosporidium* but not the negative control (*Trypanosoma irwini)* 18S PCR products. The fluorescence was more intense with ISH using the DIG-labeled

Eimeria as compared with *Cryptosporidium* PCR products (Figs 11 & 12). The DIG-nick translation mix (Roche, Germany) results in the production of a cocktail of DNA probes of lengths varying between 200-500bp. Polynucleotide probes are useful for detecting a wide range of species within a phylum, but will not be as species specific as fixed length oligonucleotide probes (Bearham, Spiers, Raidal, Jones & Nicholls 2007b). However, this study showed that ISH using DIG labeled 18S PCR generated products could not be used to distinguish between *Eimeria* and *Cryptosporidium* due to the conserved nature of the 18S locus.

Future studies on piscine *Eimeria* and *Cryptosporidium* should include both molecular and ultrastructural characterizations. Descriptions of natural infections at the histopathological and ultrastructural levels are inadequate for the identification of these parasites, which undergo complex life cycles. While molecular analysis is a very useful tool in the identification of aetiological agents in disease, this study reiterates the importance of demonstrating organisms *in situ* in tissues, particularly for ubiquitous organisms which can easily be environmental contaminants in fish tissues. Demonstratration of presence of associated tissue pathology is very important to determine the significance of such parasite infestations, as are studies to assess the significance of risk factors for expression of clinical disease.

Chapter 4   The pathology of 'scale drop syndrome' in Asian seabass, *Lates calcarifer* Bloch

Abstract

This is the first pathological description of 'scale-drop syndrome' (SDS) in Asian seabass, *Lates calcarifer* Bloch. Cumulative mortality was estimated at 40-50%. The vasculitis in all major organs including the skin and associated tissue necrosis was distinctive. The dermis overlying scale beds was often necrotic and associated with scale loss. Necrosis of splenic ellipsoids, renal glomeruli and *choroid rete* glands of eye were further hallmarks of a disease with systemic vascular involvement. The brain was not spared vascular damage, and the resulting multifocal encephalomalacia probably accounts for the spiral swimming behaviour in some affected fish. Other lesions included accentuated hepatic lobulation and gastric gland necrosis. Nuclear chromatin margination and karyolysis in hepatocytes, renal tubular epithelium, and gastric and intestinal epithelium suggest specific targeting of cells. Basophilic cytoplasmic inclusions were present in spleen, kidney, liver, heart and *choroid rete* but they were not prominent. Using transmission electron microscopy, two morphological forms of virions were observed: single- and double-enveloped hexagonal virions. Based on size and morphology, these virions resemble iridovirus or herpesvirus.

The cause of SDS is unknown, but the pathological changes, especially the vasculitis, suggest an infectious aetiology, possibly viral.

4.1 Introduction

'Scale-drop syndrome' (SDS) was first reported in Asian seabass (or barramundi) *Lates calcarifer* Bloch by farmers in Penang, Malaysia in 1992. It manifested as a disease associated with loose scales, which dropped off easily when fish are handled. 'Scale drop sundrome' was initially observed in 200 to 300g *L. calcarifer* in Penang. Affected fish were lethargic, swam sluggishly at the water surface and typically died 3 to 4 days after onset of clinical signs. In 1998 to 2000, SDS in Penang was reported to affect larger as well as smaller fish than earlier cases. Larger 700 to 800g fish with SDS appeared to survive longer after initial onset of clinical signs. The impact of SDS was great enough that farmers in Penang farm *L. calcarifer* only in earthen ponds since 2002. SDS appeared to be less of a problem in fish kept in ponds (Leong TS, personal communication). The author only managed to obtain this information from Dr T.S. Leong after the publication of the paper on which this chapter is based. There is an MSc and corresponding PhD thesis by the same student at the University Sains Malaysia (USM) on SDS in *L. calcarifer*. It is unfortunate that attempts to obtain a copy of both theses were unsuccessful.

Cases with similar gross lesions and clinical presentations were observed in 100-300g *L. calcarifer* in sea cages in Singapore in 2002, 2006 and 2009. The disease was initially believed to be caused by *Tenacibaculum maritimum*, a serious bacterial pathogen of cultured marine food fish and usually associated with dermal ulcerations (Avendano-Herrera, Toranzo & Magarinos 2006).

This is the first published description of the clinical presentation and pathology of SDS in *L. calcarifer*. While the precise aetiology of SDS remains to be elucidated, this report provides the case definition for this serious emerging

disease in an economically important marine food fish, to facilitate better recognition, future epidemiological studies and disease control.

4.2   Materials and methods

4.2.1   Background

Fish with typical 'scale drop' lesions were submitted for diagnostic purposes to the Aquatic Animal Health Laboratory, AgriFood & Veterinary Authority of Singapore in 2002, 2006 and 2009.   These materials were subsequently examined at Murdoch University mainly by light and electron microscopy. 50 fish were examined by light microscopy and 10 fish by transmission electron microscopy.

4.2.2   Light microscopy (LM)

Tissues were processed for light microscopy as outlined in 2.2.2. Tissue sections were stained by haematoxylin & eosin (H&E) stain.

4.2.3   Transmission electron microscopy (TEM)

Tissues were processed for electron microscopy as outlined in 2.2.3. Ultra-thin sections were stained with uranyl acetate and lead citrate for viewing on a Philips CM100 Bio TEM.

4.2.4   Immunohistochemistry

Immunohistochemistry was carried out on paraffin block tissues using the Red Sea bream iridovirus (RSIV) monoclonal antibody M10 as outlined in 5.2.4. The RSIV M10 was kindly supplied by Dr Jun Kurita, National Research Institute of Aquaculture, Fisheries Research Agency, Mie, Japan (Nakajima & Sorimachi 1995; Nakajima, Maeno, Fukudome, Fukuda, Tanaka, Matsuoka & Sorimachi

1995). *L. calcarifer* tissues from farm A in Indonesia (Chapter 5) diagnosed histopathologically with a systemic iridoviral disease was found to produce positive fluorescence in immunohistochemistry using the monoclonal antibody RSIV M10, and served as positive control in testing *L. calcarifer* tissues with SDS.

4.2.5 PCR and tissue culture

Polymerase chain reaction (PCR) using RSIV primer set 1 which targets a large range of iridoviruses (Kurita, Nakajima, Hirono & Aoki 1998), and viral isolation on ATCC gruntfin, *Haemulon sciurus* Shaw (GF) and Asian seabass, *Lates calcarifer* Bloch (SB) cells (Chong, Ngoh & Ng 1987) were carried out by the Aquatic Animal Health Laboratory, AgriFood & Veterinary Authority of Singapore.

4.3 Results

4.3.1 History, clinical & gross observations

'Scale drop syndrome' (SDS) was observed in 100-300g fish more than 3 to 4 months post-stocking in cages off the coast of Singapore. Fish were almost always described as eating well with no signs of disease until onset of clinical 'scale drop syndrome'. The disease appeared to progress within a few index cages and then 'spread' onto surrounding cages stocked with *L. calcarifer*. The disease did not affect other fish species stocked in the same farm. Based on reports by farms, daily mortality of up to 1-2% was observed in affected cages over a period of more than 3-4 weeks, with average cumulative losses of 40-50% of stocked fish. Severely affected fish stopped schooling, and occasionally showed abnormal neurological behaviour characterized by spiral swimming. Gross observations included darkened bodies, scale loss over extensive areas

with loss of skin colour (Fig. 13), tail/fin erosion, pallor of gills, focal to extensive areas of hepatic lipidosis (Fig. 14), petechial to ecchymotic haemorrhage in the liver, kidney and spleen, splenomegaly or atrophied shrunken spleen, and renomegaly. Although low to moderate levels of parasites such as trichodinids, monogeneans and myxosporeans were observed by wet mount microscopic examinations in gills of some fish, they were not consistently present in affected fish and therefore were considered opportunistic or incidental infestations.

Figure 13 Darkened skin and loss of scales (arrows) over extensive caudal half of body with loss of colour in Asian seabass, *Lates calcarifer* Bloch with 'scale drop syndrome'.
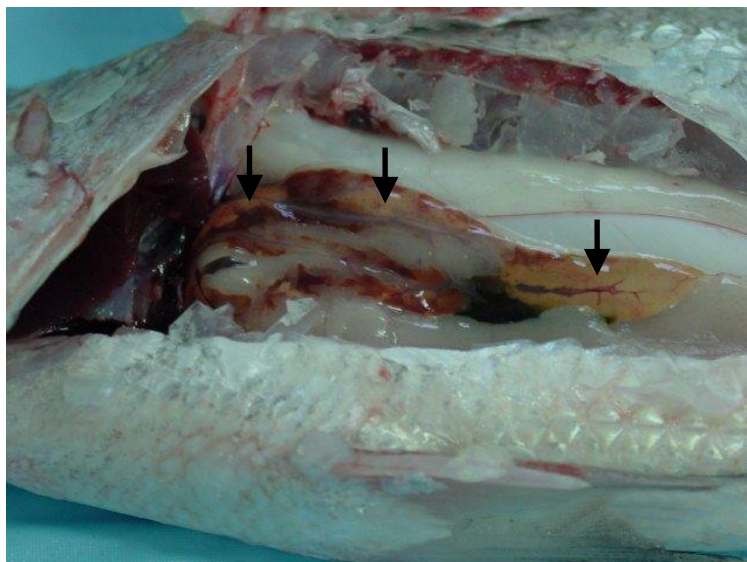
Figure 14 Foci of fatty liver (arrows) in *L. calcarifer* with 'scale drop syndrome'.

4.3.2 Histopathology

The most distinctive histopathological feature of SDS was the vasculitis in all major organs and associated tissue degeneration, haemorrhage and necrosis of varying severity (Figs 15a-c & 16a-d). In some cases, thrombosis was evident (Fig. 16d). Marked inflammatory endothelial reactions in major blood vessels of organs such as the gills, liver, kidney and spleen, resulted in reduced luminal diameter (Fig. 17a).

The dermis overlying scale beds was often necrotic (Fig. 15b), corresponding to areas with loss of scales and skin colour (Fig. 13). Extensive gill epithelial necrosis also occurred. Ellipsoidal necrosis in spleen with multifocal to more extensive coalescing areas of infarction and haemorrhage, renal glomerular and tubular necrosis, necrosis of coronary blood vessels and *choroid rete* glands of eye were further hallmarks of a disease with widespread systemic vascular damage (Figs 15c, 16a-d, 18a-b & 19a). The brain was not spared the perivascular inflammation (Fig. 17b) with associated multifocal encephalomalacia, neuronophagia and gliosis. Other observations included accentuated hepatic lobulation, likely stemming from increased cell loss or karyolysis, and distinct multifocal necrosis of gastric glands (Figs 20a-b & 21). Nuclei with marginated chromatin were often present in spleen, kidney, liver, stomach and intestines (Fig. 20b).

Inclusion bodies in spleen, kidney, liver, heart and *choroid rete* were not as prominent in SDS as those seen in typical systemic iridoviral disease with megalocytosis, but they were present nonetheless. Inclusions were basophilic and had a hyaline appearance. They were observed within shrunken necrotic glomeruli, in renal interstitia, spleen and in cardiac endothelium (Figs 18a-b, 19b

& d). Inclusion bodies were often observed in a perivascular location, and in the

heart were readily distinguished from hypertrophied endocardium (Figs 19b-d).
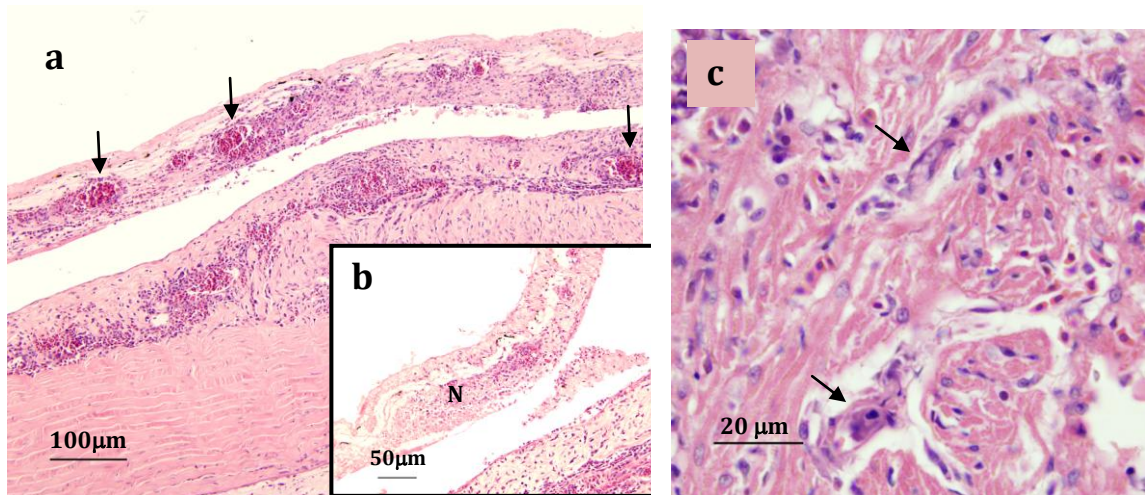
Figure 15 (a) Scale loss was associated with intense dermal perivasculitis (arrows) in scale beds. (b) Necrotic scale bed (N), seen grossly in fig. 12 as areas of skin with loss of colour and scales. (c) Ventricle showing necrosis of coronary blood vessels (arrows) in compact myocardium. (H&E)
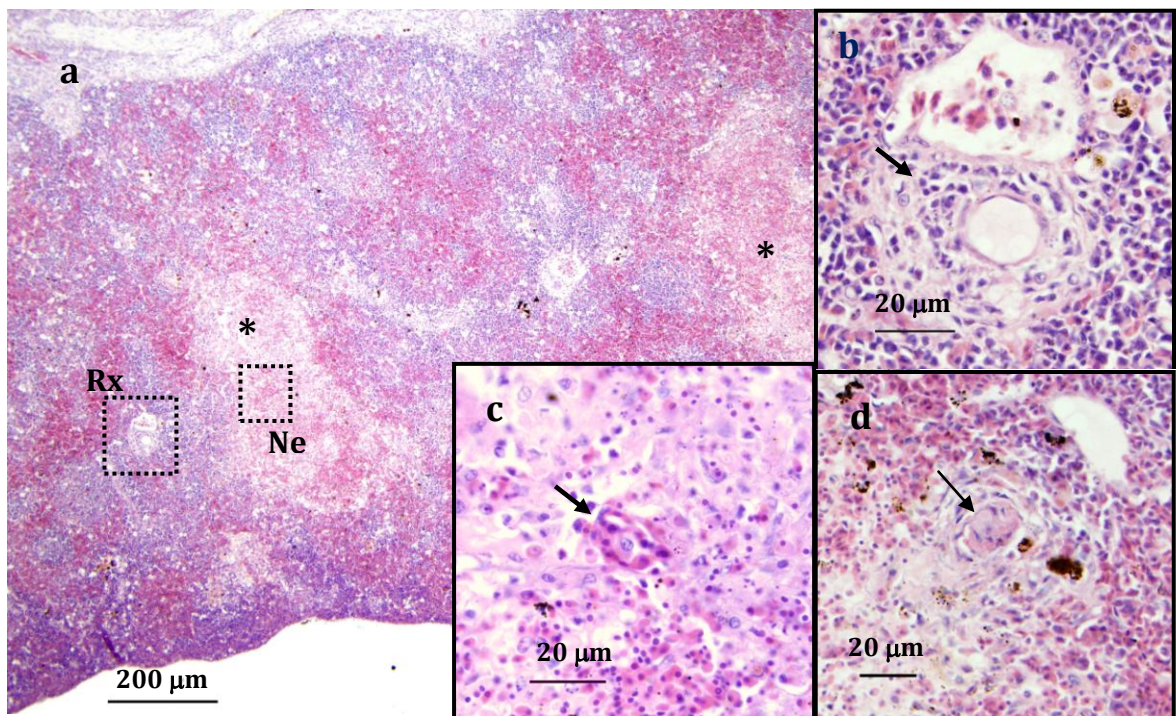
Figure 16 (a) Multifocal areas of splenic necrosis (*) were associated with reactive (RxE) and necrotic ellipsoids (NeE), shown at higher magnification in Figs. b and c (arrows) respectively. (d) Thrombus formation (arrow) in an ellipsoid. (H&E)
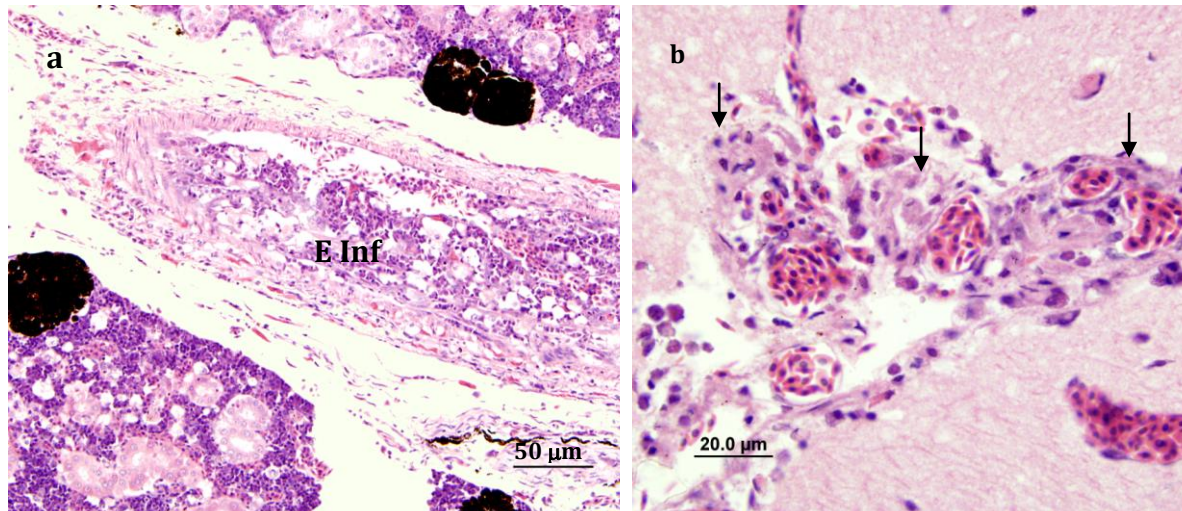
Figure 17 (a) Severe endothelial inflammation (E Inf) in renal artery. Such a lesion would have compromised blood supply to the kidney and produced severe renal necrosis if not for its portal blood supply. (b) Brain with pronounced perivascular inflammation (arrows). (H&E)
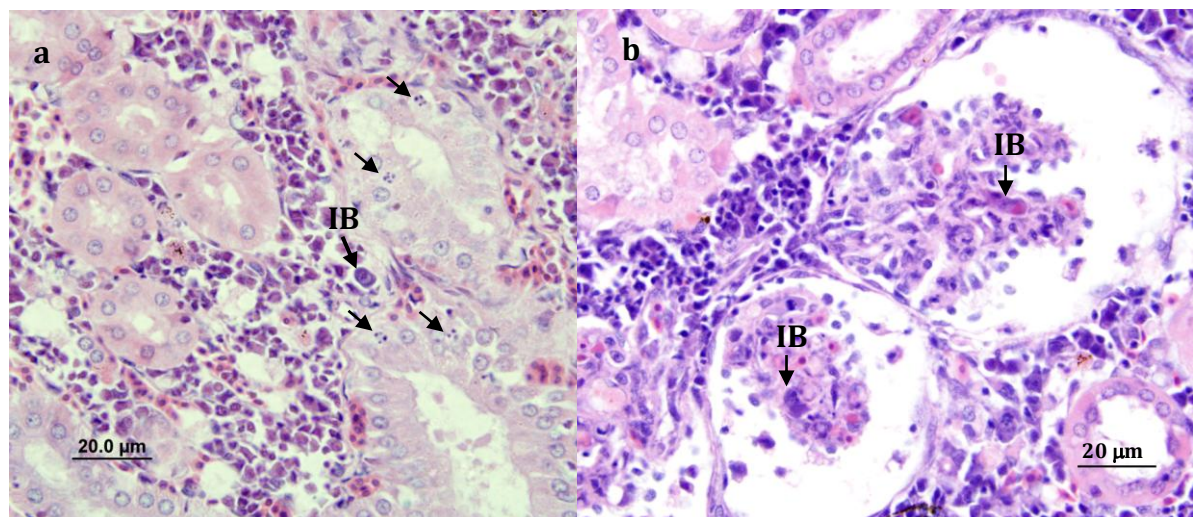
Figure 18 (a) Cytoplasmic inclusion bodies (IB) were often present in renal interstitium of *L. calcarifer* with SDS. The IB can be hard to distinguish due to the presence of haematopoietic cells. Karyorrhexis (arrows) in renal tubules may be secondary to vasculitis or due to specific targeting of epithelial cells. (b) IB with a basophilic hyaline appearance in degenerated glomeruli. These IB are suggestive of a systemic iridovirus infection (Gibson-Kueh *et al*., 2003), which are now endemic in many *L. calcarifer* farms in South East Asia, so that they may be an incidental finding of a common pathogen concurrently with other diseases. (H&E)
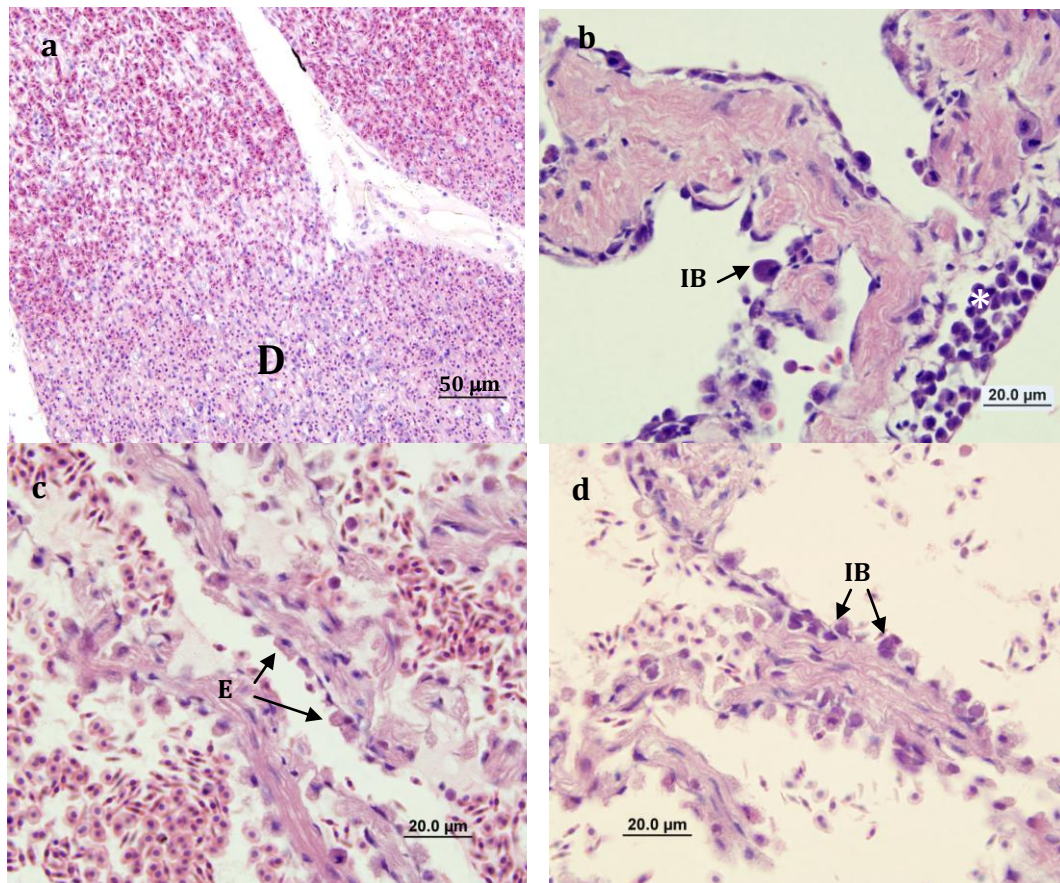
Figure 19 (a) Hydropic change (D) in *choroid rete* of eye. (b) The heart was often a good place to look for viral inclusion bodies without the 'clutter' of haematopoietic tissues. Enlarged basophilic inclusion bodies (IB) can be seen bulging from the endocardium. There was a subacute to chronic pericarditis (*). (c) Hypertrophied myocardial endothelium (E) needs to be differentiated from (d) basophilic inclusion bodies (IBs) located on endothelium. The presence of the IB typical of systemic iridoviral disease must be taken into context of a disease that can be endemic, and may occur concurrently with other diseases. (H&E)
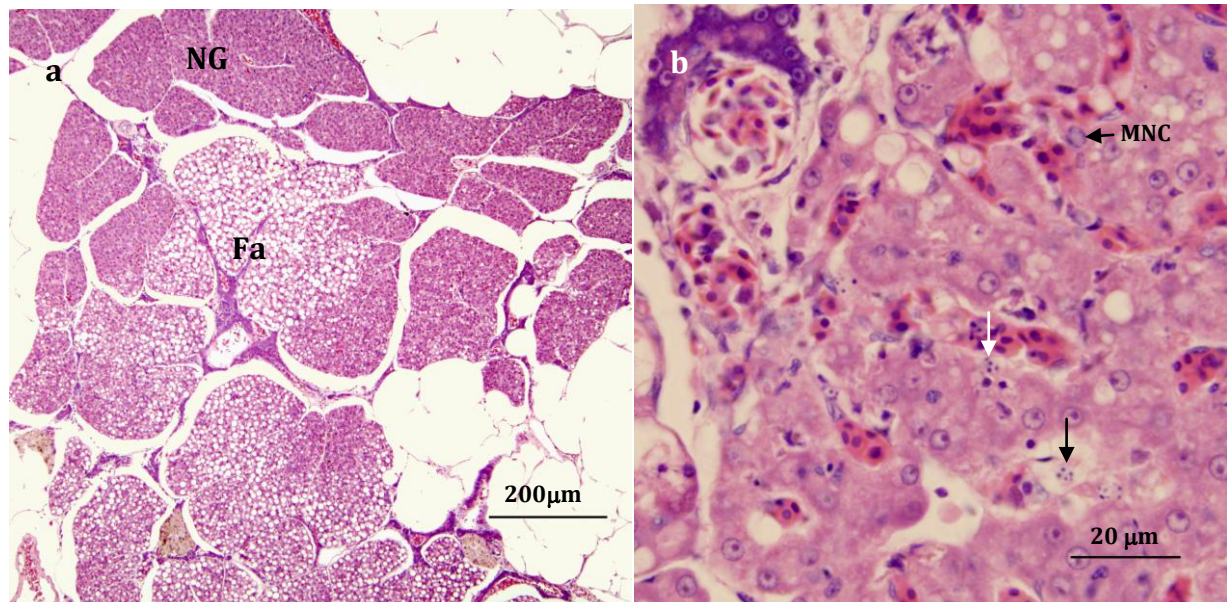
Figure 20 (a) Prominent hepatic lobulation in *L. calcarifer* with SDS. This may be attributed to increased cell death. Fatty liver or hepatic lipidosis (Fa) typified by vacuolation can be focal to extensive involving whole segments of livers, as seen grossly in fig. 13. Liver with reduced glycogen reserves stained more intensely basophilic (NG). (b) Diffuse karyorrhexis (arrows) and nuclear chromatin margination (MNC) in liver. (H&E)
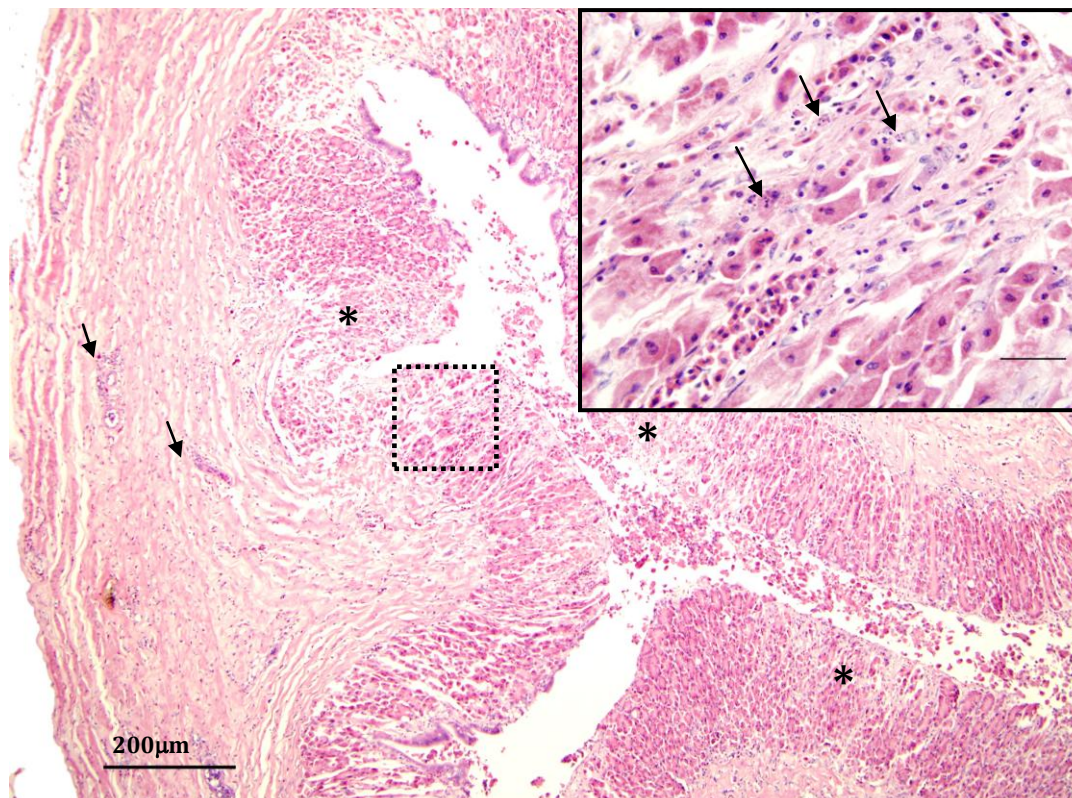
Figure 21 Multifocal areas of gastric glands necrosis (*) associated with perivasculitis (arrows) in *L. calcarifer* with SDS. Demarcated area is shown at higher magnification in inset: paler areas of gastric gland necrosis and karyolysis (arrows) in gastric mucosa. (H&E)

4.3.3   Transmission electron microscopy

Transmission electron microscopic changes were dominated by tissue degeneration and necrosis. Although the identity of the necrotic cells was unclear, renal haematopoietic cells were remarkably unaffected. Necrotic cells often had marginated nuclear chromatin or the presence of electron dense lamellae (Fig. 22). Enveloped hexagonal virions measuring 188 to 269 nm (n=8) were observed in spleen and kidney (Fig. 23a). Where virions were observed together with ribosomes, they were presumed to be located within remains of cytoplasm (Figs 23a & b). The double enveloped virions in the kidney appeared to have budded from the cytoplasm, as evidenced by the absence of ribosomes (Fig. 22 inset). Much smaller hexagonal virions with an average diameter of 133nm (n=2) and electron-lucent cores were also occasionally observed (Fig. 23b).

4.3.4   Immunohistochemistry, PCR and viral isolation

Fluorescent immunohistochemistry on paraffin block tissues using the Red Sea bream iridovirus (RSIV) monoclonal antibody M10 (Nakajima & Sorimachi 1995; Nakajima *et al.* 1995) gave negative results. *Lates calcarifer* tissues with a systemic iridoviral disease served as positive control (Fig. 25).

Polymerase chain reaction (PCR) using RSIV primer set 1 which targets a large range of iridoviruses (Kurita *et al.* 1998) also gave negative results. Attempts at viral isolation on ATCC gruntfin, *Haemulon sciurus* Shaw (GF) and Asian seabass, *Lates calcarifer* Bloch (SB) cells (Chong *et al.* 1987) were not successful (Wang Y.H., unpublished data).
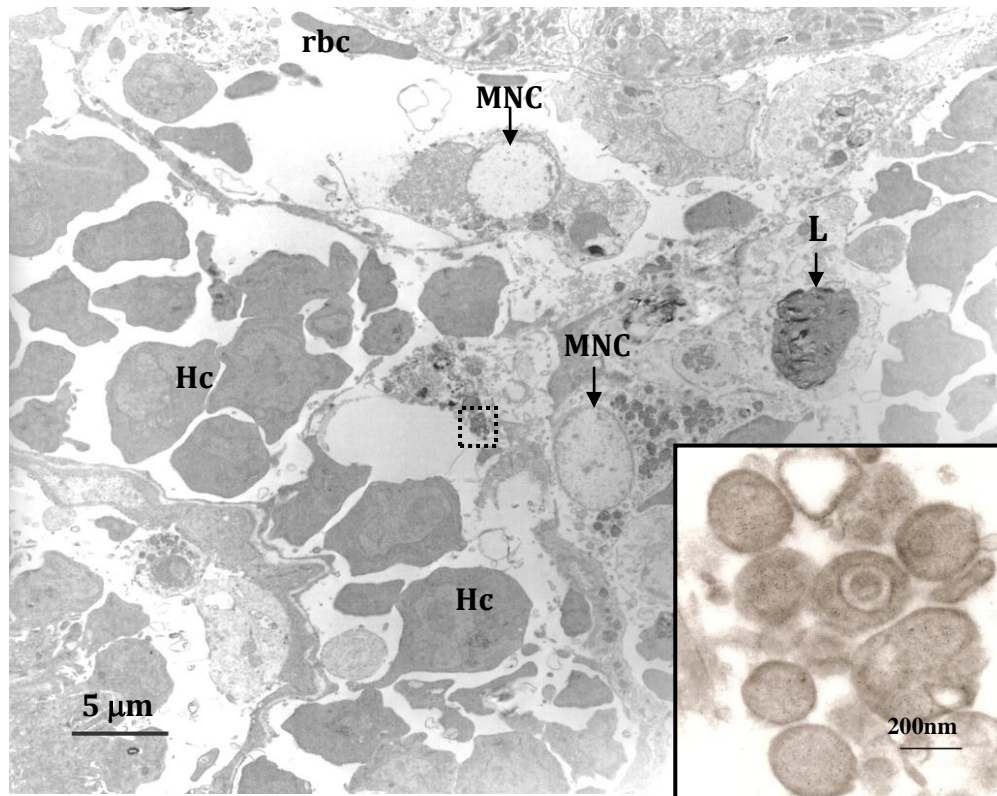
Figure 22 *L. calcarifer* with SDS have remarkably normal renal haematopoietic cells (Hc). Necrotic cells have marginated chromatin (MNC) or presence of very electron dense lamellae (L) in nucleus. Red blood cells (rbc) in blood vessel. Demarcated area is shown at higher magnification in inset: double enveloped hexagonal virions with ribosomes absent, presumably as virions have budded out from cytoplasm of cell.
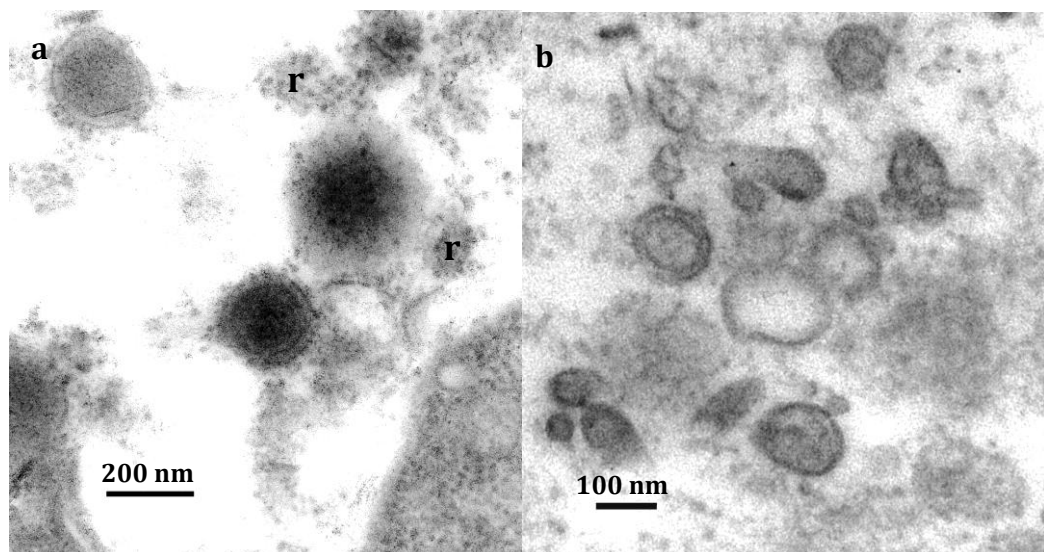
Figure 23 (a)Enveloped hexagonal virions in spleen in remains of cytoplasmic ribosomes (r), with a size range of 188 to 269 nm (n=8). (b) Smaller enveloped hexagonal virions measuring 133nm (n=2) in cytoplasmic remnants in kidney. These virions have electron-lucent nucleocapsids.

4.4   Discussion

This first pathological description of scale-drop syndrome (SDS) provides a case definition for future work, including epidemiological studies. The cause of SDS is not known, but it appears to be infectious. Moreover, the histopathological changes and the presence of enveloped hexagonal virions in the absence of other consistent causal agents support the possibility of a viral aetiology.

The lesions seen in SDS differed in several ways from other systemic iridoviral diseases (Reddacliff & Whittington 1996; Gibson-Kueh, Netto, Ngoh-Lim, Chang, Ho, Qin, Chua, Ng & Ferguson 2003). In systemic iridoviral disease, inclusion bodies were shown to be viral infected fibroblasts located under vascular endothelium, which eventually rupture to release virions into the circulation, and resulting in vascular damage and necrosis in spleen and kidney. This may be accompanied by limited to extensive necrosis of parenchyma in organs such as the spleen or just the glomerulus in the kidney (Gibson-Kueh et al. 2003). In other iridoviral disease such as epizootic haematopoietic necrosis, the main lesion observed was necrosis of haematopoietic tissues in spleen and kidney (Reddacliff & Whittington 1996). In contrast, the pattern of tissue necrosis in SDS suggested that infarction was linked directly to the vasculitis that was such a prominent feature overall. Vascular compromise in the skin would be expected to affect the health of scale beds and lead to scale loss, one of the main clinical features of SDS. The extensive necrosis of the dermis (Fig. 13 & 15b) where an estimated 80% of lymph, almost four times the volume of circulating blood is located, would in itself be fatal (Ferguson 2006). The karyorrhexis in liver, kidney, spleen, stomach and intestines suggest a disease associated with specific target cells, possibly of epithelial origin.

There was no histopathological evidence for an overt bacterial infection including *Tenacibaculum*. The vasculitis of SDS may, however, be the result of an immune hypersensitivity reaction to bacterial antigens such as that suggested for strawberry disease in salmonids (Ferguson, Girons, Rizgalla, LaPatra, Branson, McKenzie, Davies, Collins, Diab & Crumlish 2006).

Based on size and morphology, some of the observed virions resembled iridovirus (Fig. 23a) but fluorescent immunohistochemistry on paraffin block tissues using the Red Sea bream iridovirus (RSIV) monoclonal antibody M10 (Nakajima & Sorimachi 1995; Nakajima *et al.* 1995) gave negative results. Polymerase chain reaction (PCR) using RSIV primer set 1 which targets a large range of iridoviruses (Kurita *et al.* 1998) also gave negative results. Attempts at viral isolation on ATCC gruntfin, *Haemulon sciurus* Shaw (GF) and Asian seabass, *Lates calcarifer* Bloch (SB) cells (Chong *et al.* 1987) were not successful (Wang Y.H., unpublished data). There are many explanations why virus isolation may be unsuccessful. These include the choice of inappropriate cell lines, or possibly the low numbers of virions present in tissues, even those with significant pathological changes. The enveloped nature of virions may also explain the difficulty in culturing a potentially fragile virus. While it is very tempting to implicate the virus-like particles in SDS, it is important to remember that they were relatively hard to find. Moreover, as presented in Chapter 5, systemic iridoviral disease is now endemic in some *L. calcarifer* farms and their presence in fish with SDS could be an incidental finding of a common pathogen.

The vasculitis and associated necrosis that were the hallmarks of SDS are not typical of iridoviral disease, but they were seen in herpesvirus infections, as were cells with marginated nuclear chromatin in target organs such as liver,

spleen, kidney and gastrointestinal   tracts (Wolf, Herman & Carlson 1972; Plumb & Gaines 1975; Plumb, Gaines, Mora & Bradley 1975; Hedrick, Gilad, Yun, Spangenburg, Marty, Nordhausen, Kebus, Bercovier & Eldar 2000; Garver, Al-Hussinee, Hawley, Schroeder, Edes, LePage, Contador, Russell, Lord, Stevenson, Souter, Wright & Lumsden 2010). Although Cowdry type A inclusions were not observed in SDS, this was also the case in herpesviral disease of pilchards (Jones, Hyatt, Hine, Whittington, Griffin & Bax 1997). The electron-dense lamellae observed in the nucleus in Fig. 22 resemble those described in cell culture of channel catfish herpevirus (CCV). The extensive release of CCV into supernatant as early as 4 hours post-inoculation into cell culture may explain the intense vasculitis often observed in herpesviral disease (Wolf & Darlington 1971).

Assuming that SDS is indeed infectious, what was its source in these *L. calcarifer*? It is possible that the fish were infected after stocking in sea cages. As there are often multi-age groups of *L. calcarifer* in the vicinity of affected cages or farms, it is likely that the infection originated from older carrier fish. Carrier fish have been implicated in a recent review on iridoviruses causing epizootic haematopoietic necrosis and it has long been suspected in channel catfish virus disease (CCVD) (Plumb 1986; Whittington, Becker & Dennis, 2010). If infection was present in fish from early on, it is peculiar that the disease onset occurred well after the initial stressful period post-stocking when most viral diseases usually occur, *e.g.* 'sleepy grouper disease' (SGD) or other systemic iridoviral disease (Chua, Ng, Ng, Loo & Wee 1994; Gibson-Kueh, Ngoh-Lim, Netto, Kurita, Nakajima & Ng 2004b). There is a possibility that the susceptibility of fish to SDS was inversely related to age, with implications of maternal antibody protection as observed in CCVD (Hanson, Rudis & Petrie-Hanson 2004).

With the increasing culture of *L. calcarifer*, SDS is expected to occur more frequently. Since the publication of SDS by Gibson-Kueh *et al.* (2012), the size range of *L. calcarifer* affected by SDS has been extended to 3 to 5kg fish. SDS affects larger more valuable fish and will result in greater economic losses. The case definition for SDS is proposed as a systemic vasculitis in Asian seabass, *L. calcarifer* Bloch, associated with tissue necrosis in all major organs including the skin, with apparent targeting of cells of epithelial origin. The inclusion bodies observed were thought to be due to an underlying systemic iridoviral infection, although this needs further investigation. Further study of this disease to elucidate its definitive aetiology via the use of degenerate PCRs designed to detect large DNA viruses (Hanson, Rudis, Vasquez-Lee & Montgomery 2006) and attempts to isolate the agent via use of more cell lines are underway. Cohabitation of naïve fish with *L. calcarifer* with overt clinical signs of SDS, or subjecting clinically healthy *L. calcarifer* from farms with history of SDS to external stressors under laboratory conditions to see if SDS can be reproduced, may indeed help us understand this disease better.

Chapter 5   Other viral and bacterial diseases observed in cultured Asian seabass (or barramundi), *Lates calcarifer* Bloch

Abstract

This is a first report of systemic iridoviral disease in Asian seabass (or barramundi) *Lates calcarifer*. The iridoviral disease was observed in 5 to 20g *L. calcarifer* two to three weeks post-transfer into sea-cages at two farms. Clinically healthy *L. calcarifer* fry had histopathological lesions indicative of systemic iridovirus infection and were tested positive by PCR using Red Sea bream iridovirus (RSIV) primer 1. This suggested that the iridovirus infection occurred before stocking in sea cages, and did not originate from wild fish or older fish in adjacent cages. Viral nervous necrosis (VNN) remained a significant disease of *L. calcarifer* fry. Lower mortality and less severe vacuolative encelopathy and retinopathy were observed in older *L. calcarifer* fry with VNN. Pot belly disease (PBD) was previously reported in 3-week-old *L. calcarifer* fry. In this study, extensive granulomatous enteritis associated with intracellular coccobacilli similar to PBD in fry was observed in 120g *L. calcarifer* in sea-cages. Nocardiosis is an emerging disease of cultured marine food fish. This is a first description of nocardiosis in *L. calcarifer* in sea cages. Streptococcosis, tenacibaculosis and vibriosis were often observed concurrently with systemic iridoviral disease and PBD.

5.1   Introduction

Systemic iridoviral disease has been previously reported in many marine fish species (Chua, Ng, Ng, Loo & Wee 1994; Matsuoka, Inouye & Nakajima 1996; Chou, Hsu & Peng 1998; Nakajima, Inouye & Sorimachi 1998; Gibson-Kueh, Ngoh-Lim, Netto, Kurita, Nakajima & Ng 2004b). Histologically, iridovirus infections are recognized by the presence of large numbers of basophilic hypertrophied cells in all major organs (Gibson-Kueh, Netto, Ngoh-Lim, Chang, Ho, Qin, Chua, Ng & Ferguson 2003). Hence systemic iridovirus has been recently termed megalocytivirus (Mahardika, Haryanti, Muzaki & Miyazaki 2008).

Viral nervous necrosis (VNN) is a serious disease of cultured marine fish including Asian seabass (or barramundi) *Lates calcarifer* fry. VNN produced very distinctive histopathology described as vacuolative encephalopathy and retinopathy (Glazebrook, Heasman & de Beer 1990; Munday, Langdon, Hyatt & Humphrey 1992; Maeno, De La Pena & Cruz-Lacierda 2004; Parameswaran, Rajesh Kumar, Ishaq Ahmed & Sahul Hameed 2008). Both systemic iridoviral disease and VNN can be confirmed via PCR (Chang, Ngoh & Kueh 1997; Kurita, Nakajima, Hirono & Aoki 1998; Gibson-Kueh *et al.* 2004b).

'Pot belly disease' (PBD) was previously reported in *L. calcarifer* fry with mortalities of 80 to 100%. 'Pot belly disease' is characterized by perforating granulomatous enteritis and peritonitis in association with large intracellular gram negative coccobacilli (Gibson-Kueh, Crumlish & Ferguson 2004a). All attempts to isolate the bacterium associated with PBD have been unsuccessful. Molecular methods to test for PBD are not available, and diagnosis is solely by histopathology.

Streptococcosis is a serious bacterial disease of farmed marine finfish including *L. calcarifer*, and can cause severe mortality in fish up to 3kg bodyweight (Creeper & Buller 2006; Labrie, Komar & Sheehan 2007; Wendover 2009). Outbreaks of streptococcosis have been associated with stormy weather conditions on several occasions and in some cases are linked to increased river discharges (Siung-Chang & Lum-Kong 2001; Creeper & Buller 2006; Humphrey, Benedict & Small 2010).

Although vibriosis and tenacibaculosis can cause serious mortality in cultured *L. calcarifer*, they are generally considered as opportunistic diseases in stressed fish (Ruangpan 1988; Azad, Thirunavukkarasu, Kailasam & Rajan 2004; Avendano-Herrera *et al.* 2006; Labrie *et al.* 2007; Humphrey *et al.* 2010).

Nocardiosis is an emerging disease in cultured marine finfish. *Nocardia* can be recognized histologically as gram positive, acid fast with Fite Faraco stain, filamentous and often branching bacteria in association with systemic granulomatous necrotic lesions. Culture techniques and PCR have been established for the detection of *Nocardia* (Kudo, Hatai & Seino 1988; Labrie, Ng, Tan, Komar, Ho & Grisez 2008).

This is a first report of systemic iridoviral disease and nocardiosis in *L. calcarifer.* Pot belly disease was found to affect older *L. calcarifer* than previously reported. Viral and bacterial diseases reported in this study can cause significant mortality in cultured *L. calcarifer*.

5.2   Materials and methods

5.2.1 Background

Table 2 is a summary of the general information on Farms A to D including location, proximity to other farms, general husbandry practices and number of

years each farm has been in operation. Appendix 1.1 is a questionaire completed by Farm A and appendix 1.2 by Farm B, on husbandry practices.

Materials examined included fixed *L. calcarifer* tissues sent to Murdoch University, Perth by Farm A in Indonesia between 2007 and 2010. The range of diseases encountered in Farm A is well documented. Farm A has a history of systemic iridoviral disease in 10-20g *L. calcarifer* fingerlings two to three weeks post transfer into sea cages. As the farm was comtemplating using an iridovirus vaccine, the purpose of this study was to determine if the systemic iridovirus infection occurred before transfer to sea cages. *Lates calcarifer* tissues were examined by histopathology at Murdoch University and corresponding samples sent to Merck Aquatic Animal Health Laboratory in Singapore for testing by PCR.

All samples examined at Murdoch University were either formalin fixed or alcohol fixed *L. calcarifer* tissues, and presumptive diagnoses are based mainly on histopathological evidence. PCR, immunohistochemistry and *in-situ* hybridization was carried out only on selected *L. calcarifer* tissues from Farm A with histopathological evidence of a systemic iridoviral disease.

Histology slides from *L. calcarifer* cases submitted to Aquatic Animal Health Laboratory (AAHL), Agri-Food & Veterinary Authority of Singapore in Singapore from 1993 to 2006, and Fish Health Laboratory, WA Department of Fisheries from 2003 to 2008 were also examined. Reference to specific bacteria species are based on results available in case records from routine culture or PCR.

5.2.2   Light microscopy (LM)

Tissues were processed for light microscopy as outlined in 2.2.2. Tissue sections were stained by haematoxylin & eosin (H&E) and Fite Faraco, a modified Ziehl Neelsen stain.

5.2.3   Transmission electron microscopy (TEM)

Tissues were processed for electron microscopy as outlined in 2.2.3. Ultra-thin sections were stained with uranyl acetate and lead citrate for viewing on a Philips CM100 Bio TEM.

Table 2 Summary of general information on Farm A to D

	Location	Years in operation	Fish species cultured	
Farm A	island farm in Indonesia with the nearest fish farm 10km away	10	Mainly *L. calcarifer*	produces fry from its own broodstock in a land based hatchery. Nursery fish are routinely vaccinated with the Intervet Streptococcus vaccine at 10g bodyweight. 20-40g fish are stocked into 12m or 18m diameter circular cages that are 10m deep, or square cages.
				50,000 to 100,000 fish are stocked into circular cages 12 or 18 m in diameter, and 10m deep. Estimated >500,000 fish on farm at any one time.
Farm B	island farm in Indonesia with the nearest fish farm 200-300m away	5	Mainly *L. calcarifer*	buys in 30 do L. calcarifer fry to grow to 2-8g fish before stocking into sea cages. The fish are stocked mainly into 640 wooden cages 3.25 x 3.25 x 2.5m. The farm vaccinates fish against streptococcosis.
				1000-3000 fish are stocked per cage. Estimated >500,000 fish on farm at any one time.
Farm C	sea cage farm located in close proximity to at least 20 other farms in a narrow strait off Singapore.	>10	Stocks multiple fish species besides L. calcarifer	small scale farm with less than 50 wooden cages each measuring approximately 3 x 3 x 2.5m, and buys in *L. calcarifer* fingerlings for stocking directly into sea cages
				1000-3000 fish are stocked per cage. Estimated >20,000 fish on farm at any one time.
Farm D	sea cage farm located a 45-minute boat ride from the next nearest fish farms in Malaysia.	3	Mainly *L. calcarifer*	small scale farm with less than 50 wooden cages each measuring approximately 3 x 3 x 2.5m, and buys in *L. calcarifer* fingerlings for stocking directly into sea cages
				1000-3000 fish are stocked per cage. Estimated >50,000 fish on farm at any one time.

5.2.4   Immunohistochemistry using RSIV M10

Formalin fixed paraffin embedded (FFPE) *L. calcarifer* tissue sections of selected cases from Farm A with histopathological signs of a systemic iridoviral disease (Gibson-Kueh *et al.* 2003) were tested by immunohistochemistry (IHC) using a monoclonal antibody against the Red Sea bream iridovirus (RSIV M10) (Nakajima & Sorimachi, 1995). The RSIV M10 was kindly supplied by Dr Jun Kurita, National Research Institute of Aquaculture, Fisheries Research Agency, Mie, Japan. The immunohistochemistry method after Adams and de Mateo (1994) with modifications as described below was used. Negative control is carried out with substitution of the RSIV M10 with blocking solution.

FFPE 5µm tissue sections on silanised slides were deparaffinized in two changes of xylene and rehydrated through an ethanol series to tap water. Antigen retrieval was performed by placing rehydrated tissue sections in Tris EDTA buffer (pH 9) bath in a domestic microwave (Kambrook Model KER-686LE) and subjected to 'reheat' for 4 minutes and 'low heat' function for 4 minutes, followed by cooling in running tap water.

A blocking step was carried out by adding 0.1% bovine serum albumin in Tris buffered saline (TBS) (pH 7) with 0.05% Tween 20 directly to tissue sections for a 10-minute incubation. After the blocking solution was tapped off, the primary antibody RSIV M10 diluted 1/10 in blocking solution was added to each tissue section for a 30-minute incubation. FFPE tissues incubated with blocking solution instead of RSIV M10 served as the negative control. The primary antibody was rinsed off, followed by addition of TBS (pH7) with 0.05% Tween 20 to tissue sections for a 5-minute incubation.

Polyclonal rabbit anti-mouse immunoglobulin conjugated with alkaline phosphatase (Dako, US) diluted 1:50 in blocking solution was added to tissue sections for a 30-minute incubation, followed by rinsing in TBS (pH7) with 0.05% Tween 20 added to each slide for 5-minute incubation. Liquid permanent red (Dako, US) with levamisole (300µg/ml) to block endogenous alkaline phosphatase activity was added to tissue sections for a 20- minute incubation, and rinsed off with tap water. Tissue sections were counterstained with haematoxylin for 1 minute, rinsed in tap water and flicked dry prior to cover-slipping with Farmount mounting media (Dako, US).

5.2.5 Polymerase chain reaction & i*n situ* hybridization

Red Sea bream iridovirus (RSIV) PCR as published by Kurita *et al.* (1998) on *L. calcarifer* tissues from Farm A was carried out by the Merck Aquatic Animal Health Laboratory in Singapore. RSIV PCR was carried out at Murdoch University to generate dioxigenin labeled DNA probes from PCR products for *in situ* hybridization (ISH) on formalin fixed paraffin embedded (FFPE) tissues. DNA was extracted from alcohol fixed *L. calcarifer* tissues from Farm A with histopathological evidence of systemic iridoviral disease, using a QIAamp DNA FFPE Tissue kit (Qiagen, Germany), according to the manufacturer's instructions.

Briefly, PCR reaction mixes made up using TopTaq Master Mix (Qiagen, US) according to the manufacturer's instructions, containing 0.2 µM of RSIV primer set 1 and 1 µl of extracted DNA were incubated in an Eppendorf Mastercycler under PCR conditions as described by Kurita *et al.*, 1998. After amplification, 10 µl of PCR products from each reaction tube was analyzed in a 1 % agarose gel in 0.5x TBE buffer with SybrSafe, and visualized under UV illumination.

RSIV PCR products obtained from fish tissues from Farm A were purified using QIAquick PCR purification spin columns (Qiagen, Germany) and labeled with dioxigenin (DIG) using DIG-nick translation mix (Roche, Germany), according to the manufacturer's instructions. The DIG-nick translation kit results in the production of a mixture of DIG labeled DNA probes between 200 to 500bp in size from purified PCR products. *In situ* hybridization was carried out using these DIG-labeled DNA probes on FFPE *L. calcarifer* tissues with overt clinical and histopathological evidence of a systemic iridoviral disease from the same Farm A. PCR products were also purified using QIAquick PCR purification spin columns (Qiagen, Germany), and send to a commercial company for sequencing.

DIG labeled DNA probes made from 1 μg of PCR products were used to make up 1 ml of DNA probe mixture that contained 50% formamide, 10% dextran sulphate and 2x Saline Sodium Citrate (SSC) buffer. The DNA probe mixture was stored at 4°C until required. A negative control DIG labeled DNA probe generated from PCR products to detect a papilloma virus of bandicoot was kindly provided by Mark Bennett, School of Veterinary and Biomedical Sciences, Murdoch University (Bennett, Woolford, O'Hara, Warren & Nicholls 2008).

The ISH steps are carried out as described in Section 3.2.4 for the detection of *Eimeria* in *L. calcarifer* tissues except for the use of DIG labeled RSIV PCR product as the DNA probe.

5.3   Diseases observed in *L. calcarifer* tissues examined

Table 3 is a summary of the diseases observed in *L. calcarifer* from Farms A to D, and cases submitted to the Aquatic Animal Health Laboratory from 1993 to 2003. Table 4 is a summary of the diseases observed in *L. calcarifer* cases

submitted to the Fish Health Laboratory, WA Department of Fisheries from 2002 to 2008. Viral followed by bacterial diseases of *L. calcarifer* will be described.

5.3.1 Systemic iridoviral disease

Systemic iridoviral disease was observed histopathologically in 5 to 10g *L. calcarifer* 2 to 3 weeks after transfer into sea cages at Farm A and C (Table 3). Affected fish showed darkened bodies and no other clinical signs. Mortality in batches of affected fish was reported as 95% in 5 to 6g fish and 75% in larger 10g fish in sea-cages in Farm A. Farm A has an estimated greater than 500,000 fish in sea cages at any one time, and at least 100,000 to 200,000 fish will fall into the age group affected by systemic iridoviral disease. The most consistent gross abnormalities were pale gills and splenomegaly. Systemic iridovirus infection was diagnosed histopathologically in clinically healthy nursery 0.2-1.2 g *L.calcarifer* from Farm A (Table 3). This was supported by positive PCR results carried out by the Merck Aquatic Animal Health Laboratory in Singapore using the RSIV primer 1 (unpublished data).

Inclusion bodies suggestive of systemic iridovirus infection were observed in 5 to 10g sea-cage *L.calcarifer* with overt clinical signs as well as in clinically healthy 0.2 to 1.2 g nursery *L. calcarifer* in Farm A. Inclusion bodies in the clinically healthy fish were associated with no observable tissue necrosis (Fig. 24a), although these tested positive by RSIV PCR carried out by the Merck Aquatic Animal Health Laboratory in Singapore. Tissue necrosis was observed in multiple ogans including the spleen and kidney in clinically diseased 5 to 10g fish with systemic iridovirus infections (Fig. 24b). This suggests a lag period between viral infection and onset of clinical signs, and possible triggering of clinical disease by handling stress associated with transfer of fish into sea cages.

Tissues of clinically diseased *L. calcarifer* from Farm A with histopathological evidence of a systemic iridovirus infection reacted positively with Red Sea Bream iridovirus (RSIV) monoclonal antibody M10 by fluorescent IHC (Fig. 25). Negative control tissue with substitution of the RSIV M10 by blocking solution showed no fluorescence (Fig. 25 inset). Sections of intestines, spleen, liver and kidney with inclusion bodies gave varying degrees of positive reaction with the RSIV M10 IHC, with the most intense results observed in heart tissue (Fig. 25). Immunohistochemistry using RSIV M10 on tissues with inclusion bodies but from clinically healthy *L. calcarifer* gave negative results. It is possible that the antigen against which the monoclonal RSIV M10 is not yet expressed in these early viral inclusions in subclinical fish.

RSIV PCR on DNA extracted from alcohol fixed *L. calcarifer* tissues from Farm A with histopathological evidence of systemic iridoviral disease produced the expected 570bp product (Fig. 26). *In situ* hybridization using DIG labeled PCR products generated using RSIV primer 1 on tissues with histopathological signs of a systemic iridovirus infective did not yield any reactions, despite use of optimization steps such as microwave treatment in Tris EDTA buffer (pH 9). To show that the PCR products were generated from iridoviral DNA, the DNA sequence of the purified PCR products carried out using RSIV primer 1 was analysed using the blast function on GenBank, and found to be 99% homologous to aligned sequences of three iridovirus isolates: an infectious spleen and kidney necrosis virus (AF371960), iridovirus of barramundi (FJ222554) and grouper iridovirus (AY059399) (unpublished data).
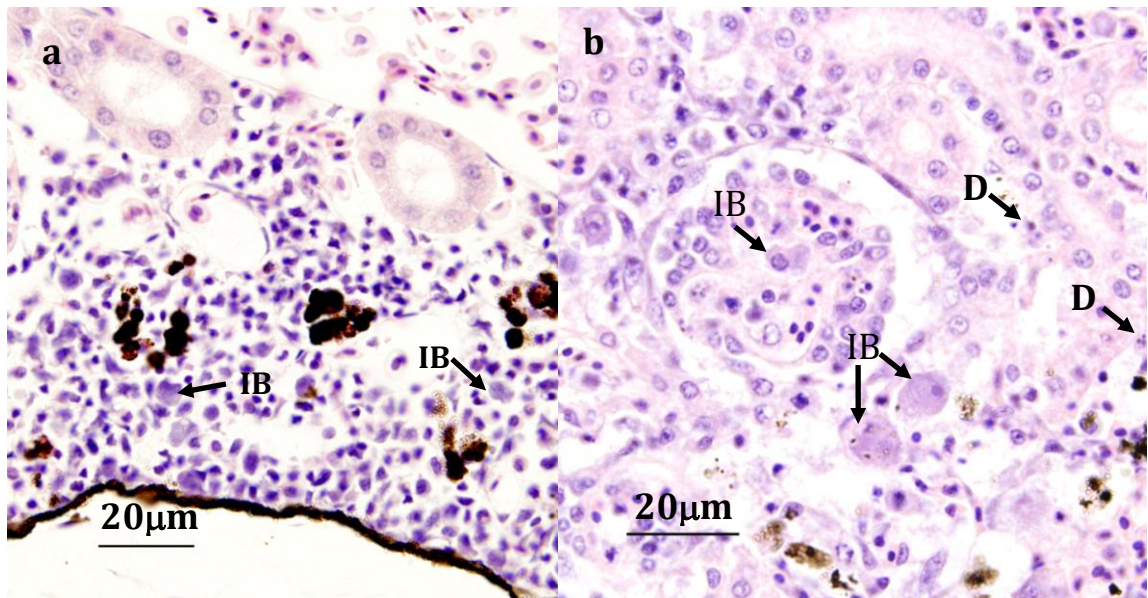
Figure 24 Systemic iridovirus infections in (a) clinically healthy 0.2g *L. calcarifer*, and (b) clinically diseased 4g *L. calcarifer*. Tissue degeneration with hydropic changes and cell deaths (D) were more evident in the clinically diseased 4g fish in b. Inclusion bodies (IB) could be observed in both fish.
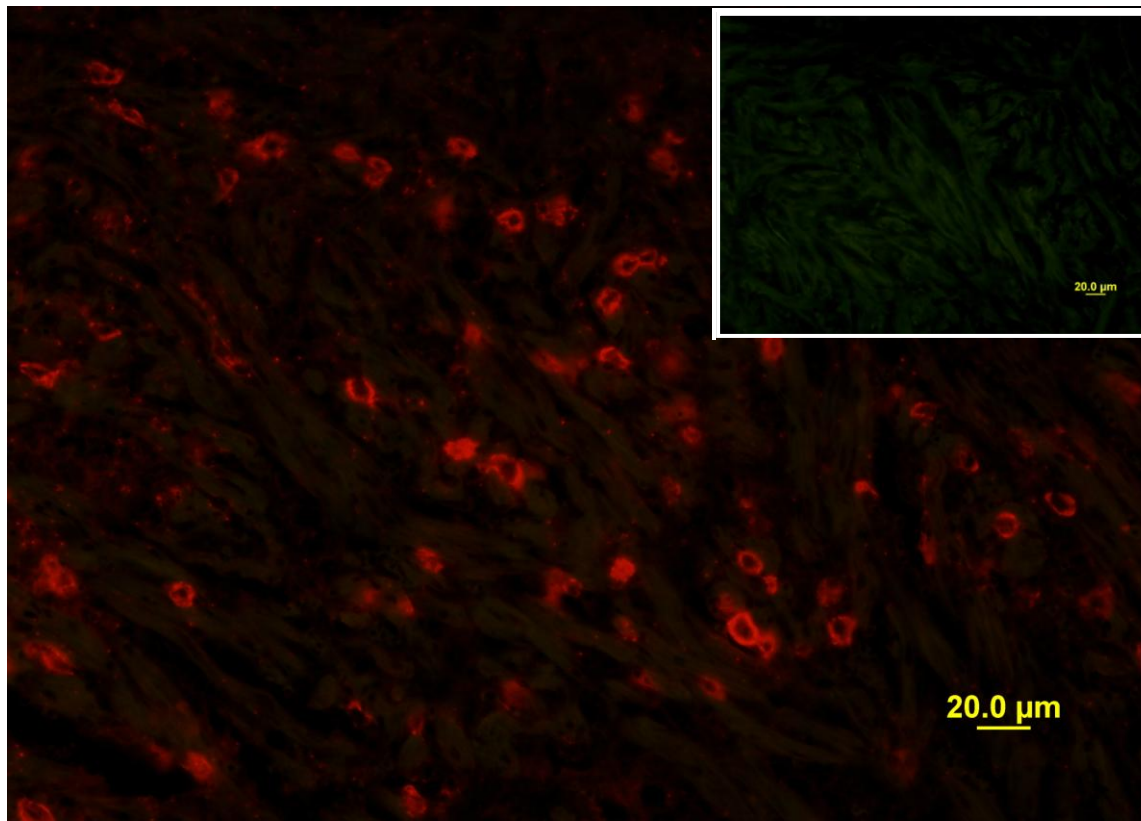
Figure 25 Cytoplasmic inclusion bodies in cardiac tissue with positive red fluorescence in immunohistochemistry using RSIV M10 and Permanent Red (Dako). Inset shows no fluorescence in negative control.

Figure 26 PCR on alcohol fixed *L. calcarifer* tissues from diseased fish with histopathological signs of a systemic iridovirus infection showed an expected 570bp product (Kurita *et al.* 1998). Lane 1 & 11: 100bp ladder, Lane 2: negative control ultrapure water, Lanes 3-5: Fish 1 (triplicates), Lanes 6-8: Fish 2 (triplicates), Lanes 9-10: Fish 3 (duplicates)

5.3.2   Viral nervous necrosis (VNN)

Viral nervous necrosis (VNN) was diagnosed histopathologically in 2 to 3 week-old *L. calcarifer* with mortalities of up to 100% at Farm A. Mortality was reported as less than 50% in older fry with VNN at Farm A. Clinical signs observed included inappetance and pale fish, with or without abnormal neurological signs (Table 3).

Marked vacuolation and abundant basophilic inclusion bodies were observed histopathologically in the grey matter of the brain and in the retina of less than 3-week old fry affected by VNN (Figs 27a & b). In two cases of VNN submitted to the Fish Health Laboratory in Perth, in fry older than 3 weeks of age (Table 4), the vacuolation in brain and eye was less marked and inclusion bodies present in smaller numbers or not at all (Fig. 27c). Vacuolation was confined to the grey matter of the brain and spinal cord. TEM showed specific targeting of nerve cells by VNNV (Fig. 27d).
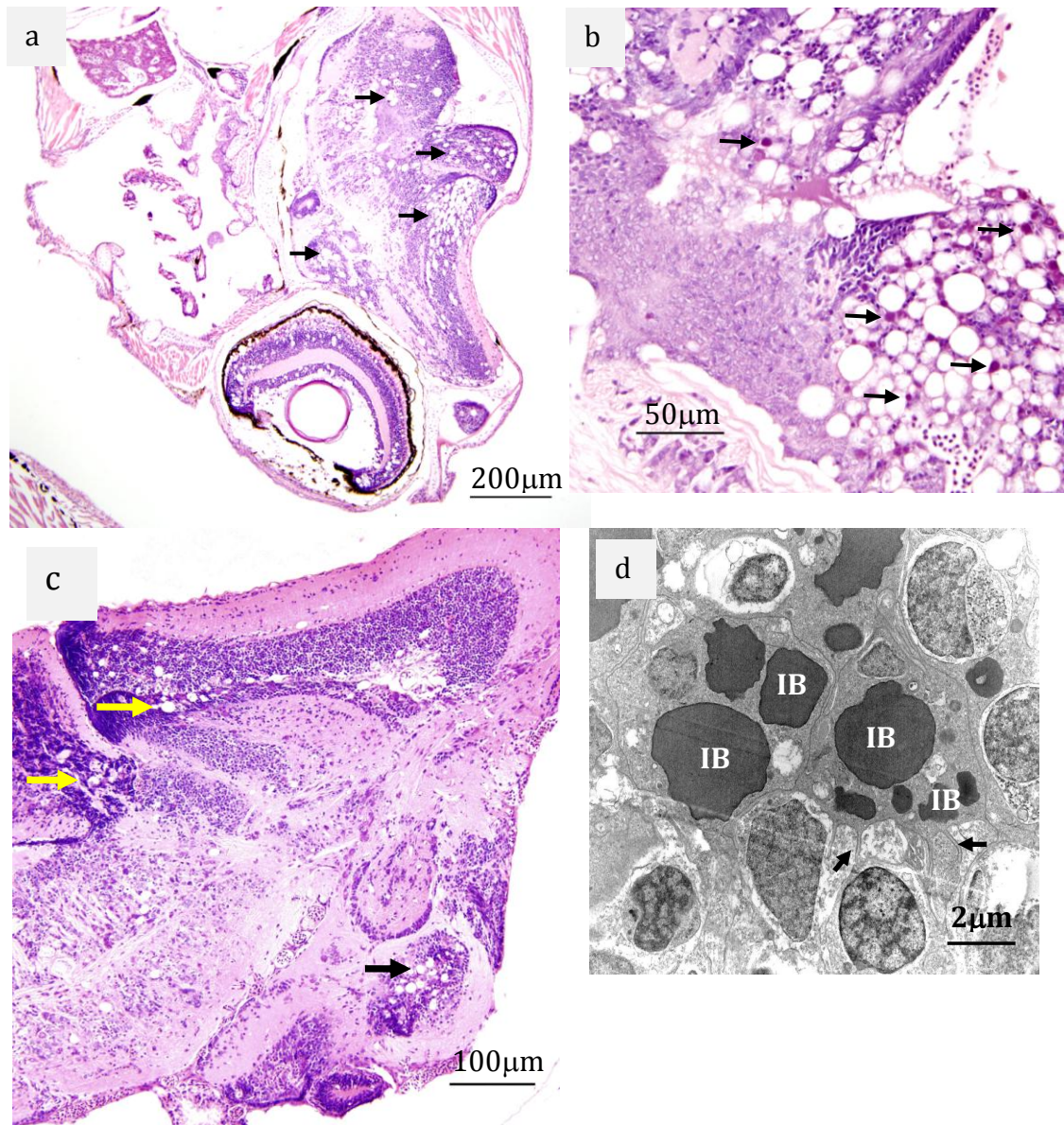
Figure 27 (a, b & d) Viral encephalopathy and retinopathy or viral nervous necrosis (VNN) in 12 do *L. calcarifer* fry from Farm A (a) Severe vacuolation (arrows) in the brain and eye. (b) Vacuoles were often associated with basophilic inclusion bodies (arrows). (c) VNN in 22 do fry submitted to the Fish Health Laboratory in Perth, with mild vacuolation (arrows) and no inclusion bodies. (H&E) (d) Nerve cells with dendritic processes (arrows) and electron dense inclusions (IB) from VNNV infection (TEM). This explains the location of vacuolation in grey matter of the brain where the cell bodies of neurons are located.

5.3.3 'Pot Belly Disease' (PBD)

Mortality of 90 to 95% was reported in *L. calcarifer* fry at Farm A, in association with 'pot belly disease' (PBD) (Table 3). PBD was also observed in older *L. calcarifer* examined in this study: 1 to 5g *L. calcarifer* in the land-based nursery of Farm A, and up to 120g *L. calcarifer* at two sea cage Farms B and D.

While the granulomatous enteritis tended to be less severe in older fish, it affected significant portions of the intestine in affected fish (Figs 28a & b). Clusters of large coccobacilli were observed in both groups of older fish (Figs 28a & b insets). Remnants of peritonitis with and without the presence of coccobacilli were observed in some fish. PBD often occurred concurrently with other diseases such as systemic iridoviral disease in *L. calcarifer* in sea cages (Table 3). The contribution of PBD to percentage mortality in grow-out cages in not known, as it is often complicated by other concurrent diseases.

5.3.4 Streptococcosis

Streptococcosis was observed in 4.5 to 15g, 100 to 500g and up to 3kg *L. calcarifer* with cumulative mortality greater than 40 to 50% (Tables 3 & 4). In fish smaller than 15g, non-specific clinical signs such as darkened bodies, lethargy and anorexia were observed. In 100 to 200g fish, additional clinical signs such as exophthalmos, cloudy eyes and abnormal neurological signs were observed (Table 3). Onset of mortality tended to be more acute, occurring over several days in smaller fish while larger fish exhibited low grade mortality over several weeks. Large numbers of gram positive, coccoid-shaped bacteria in pairs or chains were present within tissues and blood vessels in all organs including the brain (Fig. 29a) and intracellularly within phagocytic cells in spleen, kidney

and cardiac endothelium (Fig. 29b). Severe multi-organ multifocal to coalescing

necrosis may be observed.
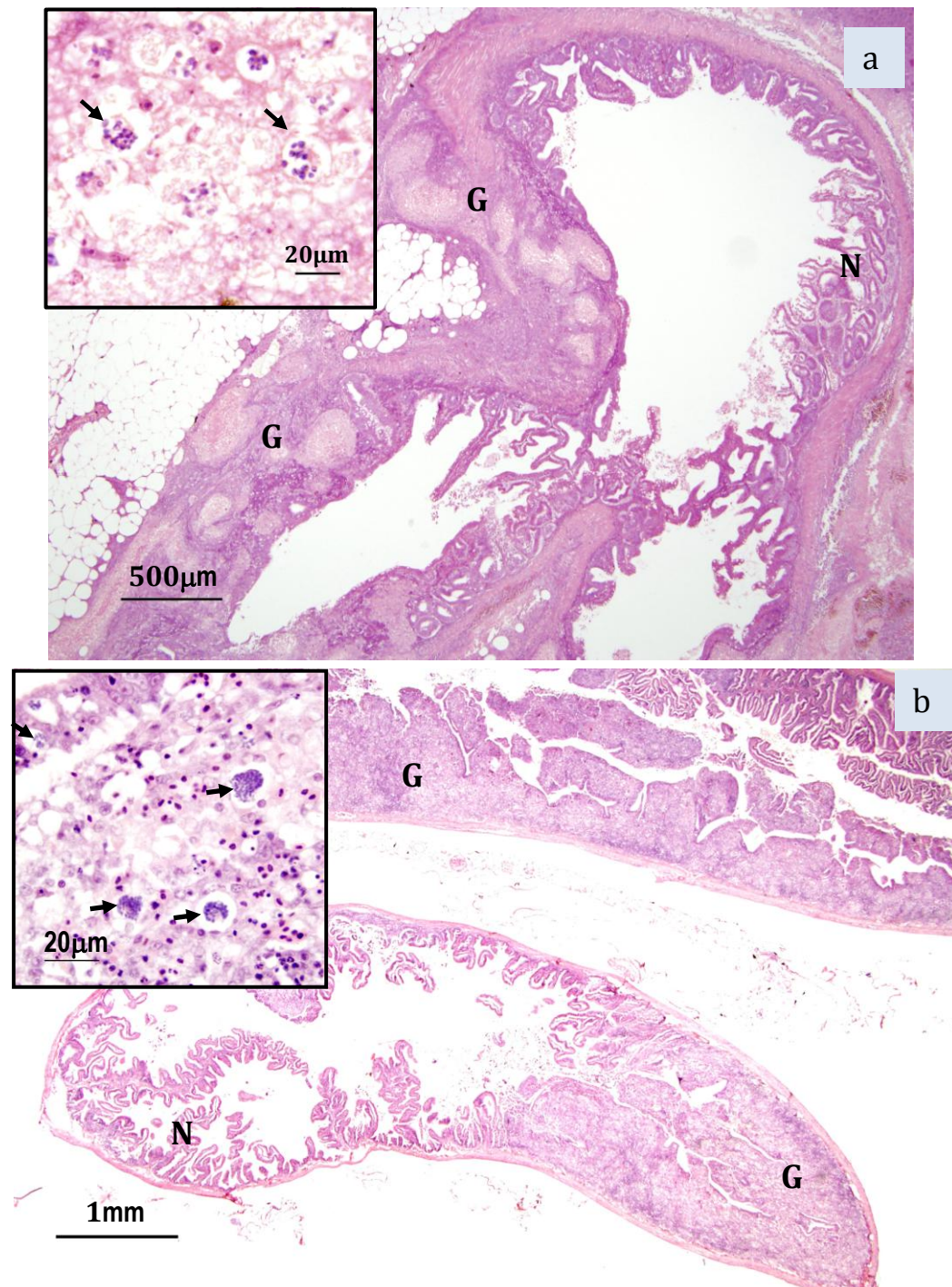
Figure 28 (a) Pot belly disease (PBD) in nursery-reared 1.8g *L. calcarifer* with granulomatous enteritis (G) as well as unaffected parts of intestines (N). (b) PBD in 80-120g fish in sea cages. Although associated pathology was less marked in these older fish than in younger fry, the granulomatous enteritis has affected significant parts of intestine. Insets: Higher magnification with clusters of large coccobacilli (arrows). (H&E)

5.3.5   Vibriosis and tenacibaculosis

Vibriosis and tenacibaculosis often occurred post handling in *L. calcarifer* less than 25g (Tables 3 & 4). Mats of filamentous bacteria were observed histopathologically on skin, mouth, eye and gill in association with severe tissue necrosis in tenacibaculosis (Fig. 29c). Tenacibaculosis was often observed concurrently with other diseases such as systemic iridoviral disease, PBD, streptococcosis or vibriosis (Table 3). Filamentous and/or rod shaped bacteria were frequently observed concurrently on necrotic skin and gill lesions. Based on case records of materials examined, the most common rod shaped bacteria isolated were *Vibrio species*. Vibriosis may progress systemically resulting in multifocal to extensive necrosis of spleen, kidney and liver, whereas tenacibaculosis typically presents as external infection of skin, gill, eye and mouth.
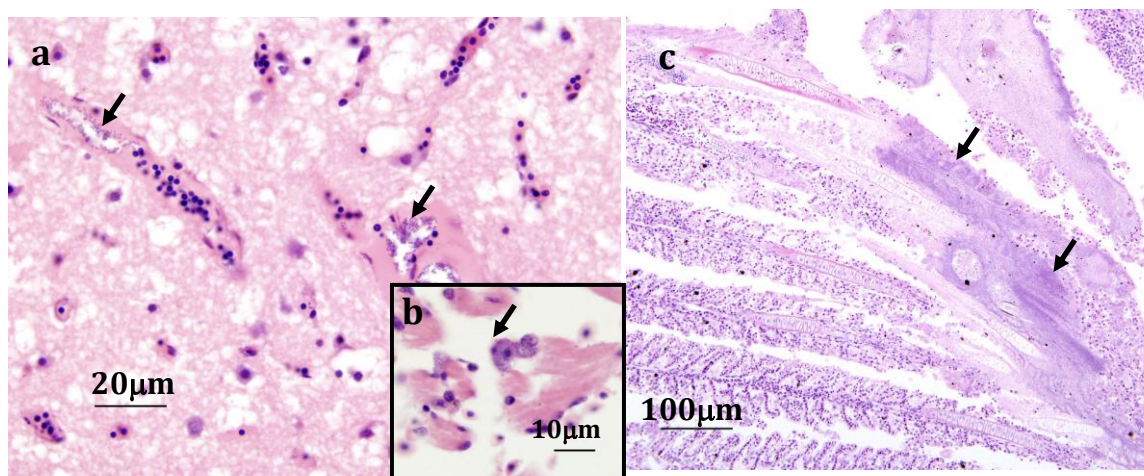
Figure 29 (a) Coccoid-shaped bacteria, presumably *Streptococcus species* were observed within blood vessels (arrows) in various organs including the brain, and (b) within phagocytic endothelium lining the heart (arrows). (c) Clusters of filamentous rods (arrows), presumably *Tenacibaculum maritimum* associated with gill necrosis.

5.3.5 Nocardiosis

Nocardiosis was observed in 50 to 120g *L. calcarifer* at two sea-cage farms: Farm

B in Indonesia and Farm D in Malaysia (Table 3). Moderate to severe

granulomatous lesions were observed in multiple organs including the spleen,

kidney, liver, intestines, heart, eye and gills. Filamentous rods stained positively

with Fite Faraco, a modified Ziehl Neelsen stain, were observed in some of these

granulomatous lesions (Fig. 30 inset). Filamentous bacteria were present in

larger numbers in the *choroid rete* of the eye rather than other organs, in one

severely affected fish from Farm B (Fig. 30). In both these two cases of

nocardiosis in *L. calcarifer* in sea-cage farms, it occurred concurrently with PBD.

Both Farms B and D also culture pompano, *Trachinotus blochii*, in which

nocardiosis was frequently observed.
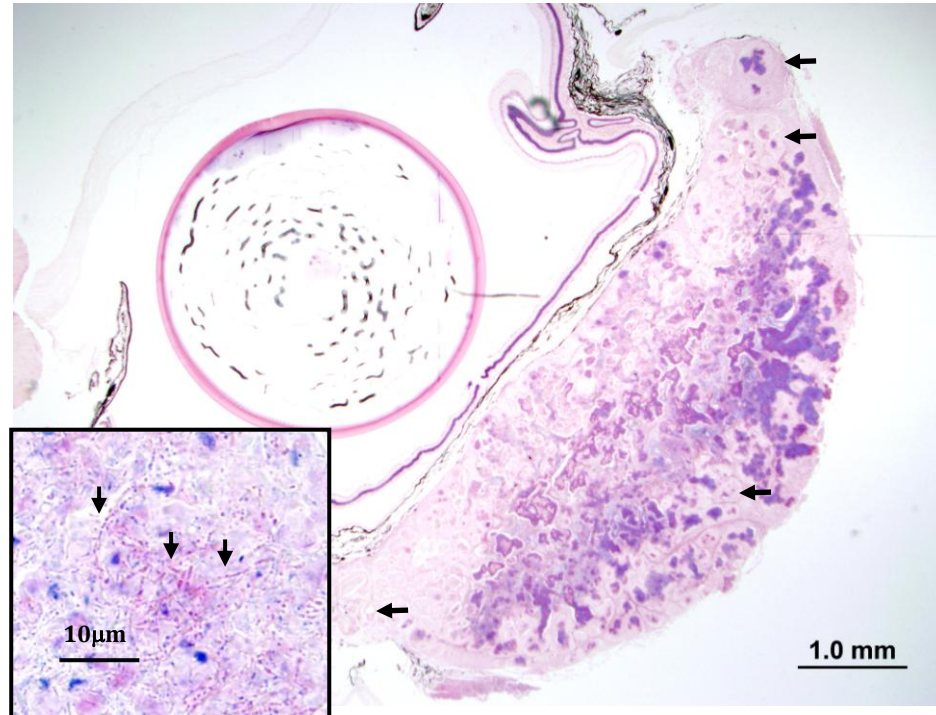
Figure 30 Severe granulomatous response (arrows) was observed in the
choroid rete of the eye of this severely affected 50g *L. calcarifer* from Farm B.
The branching filamentous rods stained positively with Fite Faraco (arrows),
a modified Ziehl Neelsen stain (inset). Granulomatous lesions were also
observed in the intestines, liver, gills, heart, kidney and spleen of this fish
although with few to no filamentous bacteria (not shown). (H&E)

5.3.6   Chronic peritonitis

Moderate to severe chronic peritonitis was observed in both clinically healthy as well as diseased fish samples submitted to the Fish Health Laboratory in Perth (Table 4). Low grade mortalities less than 1% occured 2-4 weeks post stocking. Affected fish have abdominal swelling and blood stained ascitic fluid. According to the case history, mortality was associated with increased water temperatures and feeding rates.

Adequate glycogen stores in the liver and the presence of food in gastrointestinal tracts were observed in clinically healthy fish with chronic peritonitis. Severe granulomatous peritonitis was associated with the presence of mainly gram negative rods, and the occasional gram positive cocci and coccobacilli. Pericarditis may be observed. The presence of ingested materials associated with early granuloma formation in the peritoneal cavity suggested gastrointestinal perforation. Bacterial culture was generally insignificant. The aetiology of chronic peritonitis is uncertain.

Table 3 Diseases observed in *L. calcarifer* cases from Farms A to D in South East Asia, and cases submitted to AAHL

Size /no. examined	Clinical History	Diagnosis based on histopathological observations
Hatchery Farm A		
2-4 wo fry x60	Inappetance and pale fish, with or without abnormal nervous signs. 50-100% mortality reported.	Viral nervous necrosis (Munday *et al.* 2002)
>3wo fry	Skinny, darkened bodies, off feed, not schooling. 90-95% mortality reported.	Pot belly disease (Gibson-Kueh *et al.*, 2004a)
Land-based nursery, **Farm A**		
0.2 to 1.2g (x67 fish)	Clinically healthy	Systemic iridovirus infection (Gibson-Kueh *et al.*, 2003). The presence of inclusion bodies was associated with minimal tissue necrosis or inflammation. Observations correlated with positive PCR results using RSIV primer 1 (Kurita *et al.*, 1998).
1 to 5g (x20 fish)	Skinny, darkened bodies, off feed, not schooling. Enlarged abdomens.	Pot belly disease. Concurrent systemic iridoviral infection.
4.5 to 15g (x15 fish)	Darkened bodies, lethargy, anorexia	Streptococcosis with abundant gram positive cocci in all tissues including blood.
Sea cages		
Farm A, C 5 to 10g x14 fish	Recently stocked. Darkened bodies, not schooling, swimming near surface, pale gills & enlarged spleens. Cumulative mortality 75 to 95% over 3 weeks.	Clinical systemic iridoviral disease associated with tissue necrosis. Secondary bacterial dermatitis and branchitis associated with rod shaped or filamentous bacteria, presumably *Vibrio species* or *Tenacibaculum maritimum*. Concurrent pot belly disease at Farm A.
Farm B, D 50 to120g x4 fish	Moribund fish with pale gills, systemic granuloma	Nocardiosis with acid fast filamentous bacteria and concurrent pot belly disease
7 to 30g x10 fish	Recently stocked, bodyrot	Dermatitis, branchitis and conjunctivitis associated with rod shaped bacteria presumably *Vibrio sp.*
100-200g x30 fish	Darkened bodies, lethargy, anorexia May have exophthalmos, cloudy eyes, exhibit abnormal neurological signs – spinning 0.5-2% daily mortality, cumulative exceeding 40-50% over 2-3 weeks.	Streptococcosis with abundant gram positive cocci in all tissues including blood. Bacteria isolates were tested as *Streptococcus iniae* by PCR (Zlotkin, Hershko & Eldar 1998) by AAHL. Multi-organ inflammation and necrosis.

Table 4 Diseases observed in *L. calcarifer* cases received at the Fish Health Laboratory, WA Department of Fisheries 2002-2008

Size /no. examined	Clinical History	Diagnosis based on histopathological observations
Land-based nursery >3 wo fry x60 2 cases	Abnormal swimming behavior. High mortality	Viral nervous necrosis
Land-based nursery (marine) 0.8g x7 fish 1 case	Stocked as 0.15g fry from a local hatchery, with no significant post transfer losses. 60% mortality overnight followed by low grade mortality.	Tenacibaculosis. Severe oral epidermal ulcerations on mandible and necrotic gills were observed in association with filamentous rods. Mucoid cell metaplasia observed in gills. Other observations include presence of ingesta in stomach and adequate hepatic glycogen stores, supporting an acute disease onset.
Land-based recirculation tanks (marine) 5g x 40 fish 6 cases Sea-cages >10g x6 fish 1 case	Low grade Mortalities (<1%) 2-4 weeks post stocking. Abdominal swelling and blood stained ascitic fluid. Affected fish often still have food in gastrointestinal tracts. One case history noted that mortality was associated with increased water temperatures and feeding rates.	Chronic peritonitis, uncertain aetiology. May present as chronic serositis of abdominal organs to severe granulomatous peritonitis with presence of mainly gram negative rods, and few gram positive cocci and coccobacilli in both clinically healthy as well as diseased fish. Pericarditis observed. Adequate glycogen stores in liver. The presence of ingested materials associated with early granuloma formation in peritoneal cavity suggested gastrointestinal perforation. Bacterial culture generally insignificant.
Sea-cages 200g x2 fish 1 case	>50% mortality over 3 days, peaking on day 2. Affected fish had pale gills, finrot and white skin patches.	Streptococcosis. Severe bacteriaemia with abundant coccoid shaped bacteria in spleen and kidney in association with severe necrosis. Acute branchitis. Bacteria isolated were identified as *Streptococcus iniae*.
Freshwater cages 350 to 500g x6 fish 1 case	Index case in 2.5 to 3kg fish, with cumulative mortality of 50%. Disease was subsequently observed in 350-500g fish in vicinity of the larger ones. Affected fish were lethargic and had exophthalmia, empty gastrointestinal tracts and enlarged spleens.	Streptococcosis. The publication by Creeper & Buller 2006 contains the full case details. Bacteria isolated were identified as *S. iniae* by biochemical methods. Affected fish had severe bacteraemia with abundant coccoid shaped bacteria in spleen and kidney, in association with severe necrosis. One fish had severe necrotizing branchitis with mats of filamentous bacteria, presumably *Flavobacterium columnare*.
Sea cages 5 to 10g x 11 fish 1 case	Recently graded and develop redness in eyes and base of operculum the next day.	Tenacibaculosis and Vibriosis. Necrosis of tissue on head/mandible and gills associated with filamentous bacteria in three fish, presumably *Tenacibaculum maritimum*. Rest of fish had dermatitis associated with rod shape bacteria, presumably *Vibrio species*. Case records showed that *Vibrio species* was isolated from gills while bacteria were not isolated from internal organs.

5.4   Discussion

Although systemic iridoviral disease has been previously reported in many marine fish species, this study forms the first report of this disease in *L. calcarifer*. Our findings of systemic iridoviral infection in clinically healthy fish in the land-based nursery of Farm A level suggested that the infection occurred before stocking into sea cages, and did not originate from wild fish or older fish in adjacent sea cages. The possibility of a systemic iridovirus infection in clinically healthy fish reiterates the importance of routinely screening fish for subclinical infections, and will impact any vaccination programs.

Attempts to perform *in situ* hybridizations (ISH) using DIG-labeled RSIV PCR products on FFPE *L. calcarifer* tissues with histopathological signs of systemic iridoviral disease from Farm A gave negative results. PCR products were generated from and tested on tissues of fish with clinical systemic iridoviral disease from farm A. It is suspected that ISH may have been affected by the nitric acid decalcification process used on *L. calcarifer* tissues, which may result in denatured DNA (Kucukodaci, Haholu, Sucullu & Baloglu 2012). Although immunohistochemistry gave positive results using the RSIV M10, the intensity of the antibody reaction varied between tissues such as intestines, liver, spleen and kidney with abundant inclusion bodies, and was most intense in heart tissues. This may be related to the prolonged exposure of different tissues in specimens fixed as whole fish to histoprocessing chemicals and masking of antigen (Webster, Miller, DuSold & Ramos-Vara 2010).

Viral nervous necrosis can be difficult to diagnose histopathologically in cases where it is associated with few vacuolations and an absence of inclusion bodies. In such cases, diagnosis should be verified by PCR (Chang *et al.* 1997).

In a previous publication of PBD, the causative agent was identified as a large gram negative coccobacillus, tested positive by immunohistochemistry using polyclonal antibodies to *Edwardsiella ictaluri* (Gibson-Kueh et al. 2004a). Culture and PCR detection methods for the bacteria causative agent for pot belly disease have been established by Global Aquatic Animal Health laboratory, Merck Animal Health Singapore, although this has not been published and hence is not available for use at diagnostic laboratories (http://www.thefishsite.com/articles/1086/diseases-of-farmed-barramundi-in-asia, accessed 18 Dec 2012). Pot belly disease' (PBD) which was originally described only in *L. calcarifer* fry were observed in 120g fish in sea cages in this study. These observations showed that PBD can occur in much older fish than previously reported. Granulomatous enteritis affecting significant parts of intestines are likely to have an effect on affected fish. The epidemiology of PBD in all phases of the culture cycle of *L. calcarifer* needs further study, to facilitate better disease control.

External lesions caused by *Vibrio* and *Tenacibaculum* cannot be distinguished grossly, and usually present as ulcerations on the skin of body, head or mouth areas or as necrosis of the gills . While the presence of rod-shaped bacteria can signify a *Vibrio* infection and filamentous rods indicate a *Tenacibaculum* infection, the results are best verified by bacteria culture from freshly dead fish when possible. When bacterial cultures cannot be performed within 6 hours on freshly dead fish by the laboratory, primary culture plates performed at the farm level will produce optimal results (Buller 2004).

Streptococcosis is best recognized by the presence of gram positive cocci in pairs or chains within blood vessels, and often associated with mortality in the

absence of other clinical signs particularly in juvenile *L. calcarifer* less than 15g bodyweight. In such cases, the quickest method of diagnosis is the examination of gram stained blood or tissue smears.

The diagnosis of nocardiosis was presumptive, based on histopathology and positive reaction with Fite Faraco, a modified acid fast stain. Although there is a possibility that acid fast organisms may be *Mycobacterium*, the acid fast bacterium detected in this case was very long and filamentous rather than the rod shape typical for *Mycobacterium*. Confirmation by isolation and further identification of the bacterium by PCR are always recommended to avoid misdiagnosis (Chinabut 1999). However, in this study, only histological materials were available for examinations. Although in-situ hybridization could be carried out with labeled DNA probes generated from PCR products, this was not carried due to prior experience with poor success of ISH on decalcified tissues, the only materials available from these cases. Further studies are necessary to establish if nocardiosis in farmed *L. calcarifer* occurred due to a spill over of pathogens from the presence of a highly susceptible fish host such as pompano, *Trachinotus blochii* (Power & Mitchell 2004; Labrie *et al.* 2008).

Chronic granulomatous peritonitis was not uncommon in the cases submitted to the Fish Health Laboratory in Perth. Anecdotal evidence suggested that chronic granulomatous peritonitis can be managed by feeding rates. Although the peritonitis was associated with abundant bacteria identified as *Vibrio harveyi* and *Photobacterium damsela* subspecies *damselae*, the exact aetiology is unclear and warrants future study. This may explain why attempts at vaccinations with *Vibrio harveyi* and *Photobacterium damsela damsela* have not been efficacious at controlling the disease (Schipp, Bosmans & Humphrey 2007).

Live fish challenge trials using filtered and non-filtered tissue homogenates to try reproduce the disease may be useful in at least ruling out an infectious cause. Examination of clinically healthy fish from farms with a history of chronic peritonitis may be useful in greater understanding of this disease.

Chapter 6   Disease management and future research strategies

It is well recognized that disease outbreaks in farmed fish are influenced by the interaction between host, the environment and pathogens. While serious diseases are often reported in association with specific aquatic pathogens, not much is known about the risk factors which trigger fish disease outbreaks. Implementation of management strategies based on assessment of risk factors in shrimp farms has resulted in increased production and reduced incidence of disease outbreaks (Corsin, Turnbull, Hao, Mohan, Phi, Phuoc, Tinh & Morgan 2001; Subasinghe 2005). Disease outbreaks often occur after stressful events such as net transfers, recent handling or poor water quality (Magnadottir 2010). In fact, diseases are often caused by ubiquitous pathogens that are commonly present in the culture environment, such as the bacteria *Vibrio species* or the parasite *Trichodina species* (Leong 1997: Tendencia 2002; Ruckert, Palm & Klimpel 2008). The health and hence immune status of the fish host are influenced by nutrition, culture conditions and husbandry practices (Oliva-Teles 2012). Although further research is necessary to gather more information to improve diagnosis and management of specific diseases, general health management strategies can be applied at the various stages in the culture of *L. calcarifer* to minimize disease outbreaks.

6.1 Hatchery

Vertical transfer and horizontal transmission have both been implicated as important modes of disease spread in outbreaks of viral nervous necrosis (VNN) (Azad*,* Jithendran, Shekkar, Thirunavukkarasa & de la Pena 2006). Reducing stocking densities (< 10 fry/L) has been found to lower the incidence of

outbreaks of viral nervous necrosis (VNN) (Munday, Kwang & Moody 2002; Azad *et al.* 2006). Hatcheries reported fewer incidences of pot belly disease (PBD) with a lower stocking density and stringent disinfection of incoming water supply (Gibson-Kueh S., personal observations). Although the importance of vertical or horizontal transmissions is unknown in PBD, fewer incidences of disease outbreaks with lower stocking densities may be related to reduced horizontal transmission.   Although not proven for PBD, water has been implicated as a potential source of VNNV (Hick, Schipp, Bosmans, Humphrey & Whittington 2011). Regular daily removal of dead fish from rearing tanks will help reduce the load of potentially infectious materials and hence minimize disease transmission (Manin & Ransangan 2011). An all-in-all-out stocking management in hatcheries will allow adequate disinfection in between batches of fry (Hick *et al.* 2011).

Weaning fry from live feed to an artificial pellet is a stressful period during the hatchery phase. Any stressful events can lower the immunity of fry, and predispose them to disease (Magnadottir 2010). Changes in feed offered to fry should be carried out gradually and carefully monitored to ensure that they are feeding well. Microdiets have been reported to improve the weaning process from live to an inert feed and reduce weaning induced mortality (Schipp, Bosman & Humphrey 2007).

Some studies have supported the use of immunostimulants to boost the innate immunity and hence disease resistance of young fry (Bricknell & Dalmo 2005). Probiotics have been reported to promote a healthy gut environment and out-compete potentially pathogenic bacteria, and are now incorporated into some commercial fish diets (Irianto & Austin 2002; Vine, Leukes & Kaiser 2006;

Gomez, Geovanny, Balcázar, & Shen 2007; Zhou, Buentello & Gatlin 2010). This has become quite popular in hatcheries, with some farms even putting probiotics into the culture water (Gibson-Kueh S, personal observations). *Tenacibaculum maritimum* were found to remain viable longer in sterile than natural seawater, suggesting that natural microbial population in natural seawater may play a part in keeping this bacterium in check (Avendaño-Herrera, Irgang, Magariños, Romalde & Toranzo 2006).

Some farms have reported good results treating PBD with antibiotics with intracellular effects such as florfenicol. Diseased fry often show few clinical signs or abnormalities, so that it is difficult to determine the exact cause of disease. Hence, treatment needs to be preceded by proper laboratory disease diagnosis for optimal disease control and to prevent emergence of antibiotic resistance.

Experimental trials have proved vaccination to be efficacious for controlling VNN (Husgar, Grotmol, Hjeltnes, Rødseth & Biering 2001; Thiery, Cozien, Cabon, Lamour, Baud & Schneemann 2006; Pakingking Jr., Seron, de la Pena, Yamashita & Nakai 2009; Kai, Su, Tai & Chi 2010). However, occurrence of VNN in fry less than 3 wo makes a vaccination program not viable in *L. calcarifer*. The identification of VNNV infection in broodstock based on available tests is currently unreliable (Huang, Tan, Chang, Munday, Mathew, Ngoh & Kwang 2001; Munday *et al.* 2002).

6.2 Nursery

Most of the good husbandry practices discussed under the hatchery phase will also be beneficial at the nursery phase. In addition, cannibalism is a serious problem in the culture of *L. calcarifer* at nursery and early growout phases, due to rapid growth and

resulting large disparity in fish size. This can result in severe losses directly, or indirectly, and can cause skin damage on the head and tail that may predispose to bacterial infections. The early nursery stages (20-35mm bodylength) need to be graded every three to four days while larger fish (50-100mm bodylength) need to be graded weekly to reduce disparity in size (Schipp *et al.* 2007). The grading procedure itself can result in external injuries that can be predisposed to secondary bacterial infection such as tenacibaculosis and vibriosis. As such, the grading procedure should be carried out with well designed systems.

In heavily stocked tanks with rapidly growing fish, the oxygen level can easily drop below 4ppm and organic load may build up. Trichodiniasis in eels, *Anguilla anguilla* in recirculation systems was found to be positively correlated to high organic loads (Madsen, Buchmann & Mellergaard 2000). Low daily water exchange rates were suspected to be one of the reasons for severe intestinal *Eimeria* infestations in *L. calcarifer* in small scale nurseries in Vietnam, likely from build up of infective parasite stages (Gibson-Kueh, Thuy, Elliot, Jones, Nicholls & Thompson 2011). Higher water exchanges ensure optimal DO levels and prevent build-up of organic matter and bacteria, which can predispose to disease outbreaks (Wedekind, Gessner, Vazquez, Maerki & Steiner 2010).

Streptococcosis and tenacibaculosis were associated with higher mortality in warmer conditions with less dissolved oxygen (Wakabayashi 1991; Soltani, Munday & Burke 1995; Handlinger, Soltani & Percival 1997; Ferguson, St John, Roach, Willoughby, Parker & Ryan 2000; Creeper & Buller 2006; Speare & Ferguson 2006; Bromage & Owens 2009). Management of diseases with pronounced effects on blood oxygen should include avoiding any stressful handling procedures, ensuring good aeration and withholding of feed until

mortality has abated. High mortality is often experienced even with antibiotic therapy in *Streptococcus* outbreaks at both the nursery and growut stages. A monovalent inactivated vaccine (Norvax® Strep *Si*) marketed by Intervet can be used as an immersion or an injectable (http://aqua.merck-animal-health.com/products/norvax-strep-si/productdetails_127_112317.aspx accessed 13 Dec 2012). Bath vaccinations may be an option for streptococcosis in fish from 3g bodyweight. This is usually followed by intraperitoneal vaccinations in fish greater than 20g, to extend protection throughout the grow-out period. However, vaccinations may not cross protect against different biotypes of Streptococcus and hence proper diagnosis is critical in developing a successful vaccination program (Wendover, Aguirre, Zanolo & Cericarto 2011). Commercial vaccines against tenacibaculosis are currently not available.

It is important to screen nursery fish for any underlying infections which may become clinical with transport, transfer to sea or any handling procedures including grading. Systemic iridoviral infections can be present in clinically healthy 0.2 to 1.2g *L. calcarifer* well before clinical disease is observed. Although vaccination against systemic iridovirus has been shown to be efficacious, this early preclinical infection may impact the success of vaccination programs (Nakajima, Maeno., Honda, Yokoyama, Tooriyama & Manabe 1999; Mahardika, Haryanti, Muzaki & Miyazaki 2008) A new commercial iridovirus vaccine (AQUAVAC® IridoV) has been released by Intervet in Singapore (http://www.fishupdate.com/news/fullstory.php/aid/18164/AQUAVAC_AE_IridoV_receives_first_market_authorisation_in_Singapore.html accessed 13 Dec 2012). It is vital to know the health status of fish before deciding on any treatment or control programs.

6.3 Grow-out

Fish should ideally be vaccinated against significant diseases that can occur on each farm before stocking into sea cages. Treatment of fish in sea cages can be difficult. Bath treatments are laborious and stressful to fish. Fish are often not eating by the time disease is noticed so that oral medications are impossible. Removal of mortality is very important in disease outbreaks, critical to reduce the exposure of other fish to infectious materials. Failure of removal of dead fish also attracts large predators and results in net damage.

Although many diseases such as vibriosis and tenacibaculosis are precipitated by transfers or grading, these husbandry procedures cannot be avoided. It is important that farm personnel understand basic fish health, so that these procedures can be carried out with minimal stress to fish. Handling time can be reduced with proper planning and the right equipment, and the conditions kept optimal with appropriate aeration, good water exchanges and anaesthetics. Cannibalism can be quite a problem in *L. calcarifer* in early grow-out phase, so that grading should be continued until fish are at least 300g bodyweight. Large size disparity will result in cannibalism and direct mortality or predisposition to opportunistic bacterial or parasitic parthogens.

Outbreaks of streptococcosis in *L. calcarifer* in Lake Argyle in Western Australia and Northern Territory in Australia were associated with extreme weather conditions and increased suspended solids (Creeper & Buller 2006; Humphrey *et al.* 2010). Suspended solids have been documented to act as physical carriers of infectious agents (Siung-Chang & Lum-Kong 2001; Simon, Grossart, Schweitser & Ploug 2002; Lyons, Smolowitz, Uhlinger, Gast & Ward

2005). Locating future sea cage sites well away from discharges from major rivers may reduce outbreaks of streptococcosis.

Parasitism or immunosuppression have been shown to reduce the efficacy of *Streptococcus iniae* vaccination in fish (Martins, Shoemaker, Xu & Klesius 2011; Eldar, Horovitcz & Bercovier 1997). The efficacy of vaccination should be supported by concurrent parasite control programs and husbandry practices that minimize stress to fish. Although vaccines are available, conflicting views are held on the efficacy of *Streptococcus* vaccines by various research groups (Eldar, Shapiro, Bejerano & Bercovier 1995; Bachrach, Zlotkin, Hurvitz, Evans & Eldar 2001; Agnew & Barnes 2007). Overall, the farms in Southeast Asia which practiced vaccination against *S. iniae* experienced benefit in the reduction of mortality in *L. calcarifer* (http://www.thefishsite.com/articles/299/duration-of-immunity-of-norvax-strep-si-under-normal-farming-conditions accessed 18 Dec 2012; Wendover N., personal communications).

Anecdotal evidence from established farms in Southeast Asia and Australia has shown that the stocking of 30 to 40g *L. calcarifer* in sea-cages is associated with a higher percentage survival. The adaptive immune system is known to develop much later in marine fish species than their freshwater counterparts (Magnadottir 2010). Age related susceptibility to disease has been suggested by studies of disease outbreaks in farmed *L. calcarifer* in Asia further supporting the stocking of older more immune competent fish into sea cages (Tan, Komar & Enright 2006).

As there are often multi-age groups of *L. calcarifer* each farm site, older carrier fish can be a source of infectious agents. Carrier fish have been implicated in a recent review on iridoviruses causing epizootic haematopoietic necrosis and

it has long been suspected in channel catfish virus disease (CCVD) (Plumb 1986; Whittington, Becker & Dennis, 2010). There should be some thought given to managing sites as single year classes to facilitate disease control in the future.

6.4 Conclusion & future research

Although low to moderate levels of parasites such as trichodinids, monogeneans and myxosporeans (presumably *Henneguya* based on spore morphology) were observed by wet mount microscopic examinations in gills of some fish with scale drop sundrome, they were not consistently present in affected fish and therefore were considered opportunistic or incidental infestations. Disease outbreaks due to myxosporean parasites are known to be affected by the abundance of oligochaete intermediate hosts in rivers (Kaeser & Sharpe 2008). Occurrence of parasites can be quite site specific, as Griffiths (2009) found that although epitheliocystis was found at a seacage and in wild fish in a river, it was not observed in *L. calcarifer* in pond culture. It was noted that the sea cages were near river systems and the ponds had water drawn from a river. A survey of parasite infestations in grey mullet, *Liza aurata* in the Mediterraneans suggested that helminth loads was determined both by local environmental conditions and conduciveness to parasite transmissions (Miguez-Lozano, Pardo-Carranza, Blasco-Costa & Balbuena 2012). Only land based nurseries in Vietnam were included in this study, and maybe the reason why only low grade to heavy gill trichodinid infestation was sometimes observed (2.3.2 para 1) but not other parasites. Direct microscopic examinations of wet mounts of gills and skin scrapings may be the best method to detect parasites which are not firmly attached to tissues such as *Trichodina species*, which may drop off from fixed

tissues processed for histology. Most of the results in this study are based on histological materials rather then wet mount examinations as in the study by Griffiths (2009), and may explain the difference in incidences of some types of loosely attached parasites observed.

The study of emerging diseases such as scale drop syndrome (SDS) or pot belly disease (PBD) in *L. calcarifer* has been hampered by lack of confirmatory diagnostic tools and inadequate knowledge on critical epidemiological factors such as mode of transmission or potential reservoirs. The aetiological agent has not been identified or isolated in SDS, and it can currently only be recognized by the pathological lesions observed in diseased fish. Koch postulates outline the importance of demonstrating the causal agent in diseased tissues, isolating the organism in pure form for use in replicating the disease and re-isolating the organism from experimental animals to proove causality in a specific disease (Walker, LeVine & Jucker 2006). Along the same lines, demonstrating and trying to isolate a causal agent would be the next steps in future research on SDS. While amoebic gill disease (AGD) was described 21 years ago, Koch's postulates were fulifilled only recently when the aetiological agent was identified as *Neoparameoba perurans* and successfully cultured *in-vitro* (Crosbie, Bridle, Cadoret & Nowak 2012). While ideally identification and isolation of the causal agent will help fulfil Koch's postulates, it may be possible to improve the understanding of disease via cohabitation or infectivity trials using tissue homogenates from diseased fish when pure isolates are not available. The case definition of scale drop syndrome (SDS) established in this study will help identify cases in *L. calcarifer* in which identification and isolation of the aetiological agent can be attempted. SDS has been reported in fish several

months after stocking in sea cages, and whether the disease is spread from older

carrier fish on site or is a subclinical disease needs further study. The reservoir

of the PBD bacterium and how the organism is transmitted to fry, and its

persistence in the environment needs to be established. This study showed that

PBD can occur in much older *L. calcarifer* than initially reported in fry. It is

possible that the PBD bacterium can persist in adult fish, some of which may be

used as broodstocks. The epidemiology of PBD in all phases of the culture cycle

of *L. calcarifer* needs further study, to facilitate better disease control.

Examination of apparently healthy as well as diseased fish upon stocking and

serial follow-up examinations during the nursery phase will complement what

has been learnt from examination of the *L. calcarifer* naturally infected with

Eimeria in nurseries in Vietnam.

There is a need to extend knowledge of disease beyond mere detection of

the causal agent, as disease onset is also influenced by both the health status of

host and the environmental conditions. There is a need to conduct research to

not only establish a definitive aetiology, but also to identify risk factors to

facilitate successful disease control. The successful management of disease in

aquaculture does not lie in any one strategy but an integrated management of all

risks encountered during the culture cycle against disease occurrence or

incursions. Ferguson, 2006 in the Preface of his book Systemic Pathology of Fish

(2nd Ed.) laments the over-dependence on the detection of disease agents by the

myriad of molecular diagnostic tools available today, without understanding the

disease process and the impacts on their hosts.

It is not easy to establish disease risk factors and understand the

epidemiology of disease at the farm level. Diagnosis has often to depend on

examination of fixed tissue samples as farms are often located in remote areas. Farm staff turnover is often high, and loss of staff trained in fish health makes even appropriate sample submission a big challenge. Government laboratories offer mainly diagnostic services, and research on novel diseases is often carried out at universities located far away from farms. Culture and PCR detection methods for the bacterial causative agent for pot belly disease have been established by Global Aquatic Animal Health laboratory, Merck Animal Health Singapore, although this has not been published and hence is not available for use at diagnostic laboratories (http://www.thefishsite.com/articles/1086/diseases-of-farmed-barramundi-in-asia, assessed 18 Dec 2012). These drawbacks make progress on establishing epidemiology of emerging fish diseases slow at best.

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Appendix 1.1 – Response to questionaire from Farm A

Farm name   :   Farm A (sea-cage farm with own broodstock, hatchery and nursery)
Location   :   Island A off Indonesia
Tel   :   *-withheld-*
Email   :   *-withheld-*
Objective of questionnaire: Collect information on the main diseases observed in Asian seabass/barramundi production
Date questionnaire filled up: 10 Dec 2008
Number of years farm in operation: 10 years on pilot and commercial scale.
Nearest fish farm(s) in km (if any):   floating cages for Pomfret 10km away
Fish species farmed other than barramundi: *Epinephelus fuscoguttatus, Lutjanus argentimaculatus, Rachycentrum canadum* on pilot
                          scale

Water source: Sea water
Salinity of water used: Full salinity 34ppt
Number of tanks/ponds/cages on farm: *Information not supplied*
Number of Asian seabass on farm currently of each age group: All from larvae to market size (3kg) and captive broodstocks
Size of ponds or cages in metres: Cages 12 and 18 meters diameter, and 10 meter depth.
Stocking density of fry in hatchery: Mesocosm technique 10-30/liter
Age & size of fry in hatchery: All sizes from day 0 to day 30
Feed type & rates in hatchery: Rotifer artemia weaning feed and pellet
Stocking density of fry in nursery: 2-5 kg/m3
Age & size of fry in nursery: From day 30 to 10-20g
Feed types & rates in nursery: Pellet of different sizes; Feeding rates from 10% to 3%
Age & size of fry or fingerlings at stocking in growout facilities: 10-30g all vaccinated by injection against Streptococcus
Initial stocking density at grow-out: 50.000-100-000 in 12 meter circular cages 10.000 to 20.000 in square cages
Time from stocking in growout to first grading: Graded prior to stocking. Fish from one cage may be split into different cages as fish
                          grows

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Frequency of grading:　　　　　No grading from 100g to 3kg
Frequency of net changes:　　　5-8 months
Water exchange rate in tank/pond:　　100% per hour at nursery stage
Temperature range of culture water:　28-29°C all over the year
If water is recirculated, how is water managed or treated?　　　Through flow water system
Type of vaccination used if any:　　Routine Streptococcus vaccination
When do diseases occur in the culture cycle?　　Larvae, juveniles and adults
　　　　　　　　　　　　　　　　　　　VNN, Iridovirus, Big belly (synonymous with pot belly), *Tenacibaculum maritimum*,
Describe the main diseases observed?　　*Benedenia*, *Streptococcus*
What are the main disease agents known to occur on farm, as diagnosed by laboratory?　　As above
What percentage losses are associated with the main diseases observed?　　100% (VNN) 75% (irido) variable on others.
Are the following disease conditions experienced on farm? Yes/No　　Please indicate percentage affected or associated losses experienced?
Swimbladder hyperinflation:　　Associated with VNN and Big Belly
Scale loss:　Not frequent and occurring after rough current or rough handling
Enlarged Abdomen:　　　　Big belly disease
Unexplained low grade mortality:　　Not observed
Sudden overnight mortality with unknown cause :　　Not observed

If not applicable, please write NA. If unknown, please indicate.

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Appendix 1.2 - Response to questionaire from Farm B

Farm name   : Farm B (seacage grow-out farm with own nursery, buys in fry)
Location   : Island B off Indonesia
Tel   : *-withheld-*
Email   : *-withheld-*
Objective of questionnaire: Collect information on the main diseases observed in Asian seabass/barramundi production
Date questionnaire filled up: 02 May 2008
Number of years farm in operation: 4-5 years
Nearest fish farm(s) in km (if any): Nearest fish farms 200 to 300m away
Fish species farmed other than barramundi: Crimson snapper, pompano, tiger grouper
Water source: Sea water
Salinity of water used: Full salinity 30-34ppt
Number of tanks/ponds/cages on farm: 640 wooden framed and 4 steel cages
Number of Asian seabass on farm currently of each age group: 1.2 million fish as on 2008
Size of ponds or cages in metres: Wooden cages 3.25 x 3.25 x 2.5m
Stocking density of fry in hatchery: NA
Age & size of fry in hatchery: NA
Feed type & rates in hatchery: NA
Stocking density of fry in nursery: 2-3kg biomass/metric tonne
Age & size of fry in nursery: From day 30 to 2 to 8g
Feed types & rates in nursery: Pellet of different sizes as fish grows
Age & size of fry or fingerlings at stocking in growout facilities: 2 to 8g fish
Initial stocking density at grow-out: *Information not supplied*
Time from stocking in growout to first grading: *Information not supplied*

Frequency of grading: *Information not supplied*
Frequency of net changes: Every week for small fish to every 2 weeks for larger fish in nets with larger mesh size
Water exchange rate in tank/pond: 100% per hour at nursery stage
Temperature range of culture water: 28-30°C all over the year
If water is recirculated, how is water managed or treated? NA
Type of vaccination used if any: Routine Streptococcus vaccination via intraperitonal route
When do diseases occur in the culture cycle?   Within first month of stocking at sea
Describe the main disease signs observed?   Scale loss, gulping at water surface and darkened fish
What are the main disease agents known to occur on farm, as diagnosed by laboratory?   *Tenacibaculum maritimum* & *Streptococcus sp.*
What percentage losses are associated with the main diseases observed?   10-20% mortality
Are the following disease conditions experienced on farm? Yes/No   Please indicate percentage affected or associated losses experienced?
Swimbladder hyperinflation: Not observed
Scale loss: 10% mortality
Enlarged Abdomen: Less than 1%
Unexplained low grade mortality: Not observed
Sudden overnight mortality with unknown cause :   Not observed

If not applicable, please write NA. If unknown, please indicate.

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