INVESTIGATION INTO JAUNDICE IN FARMED CATFISH (*Pangasianodon hypophthalmus*, Sauvage) IN THE MEKONG DELTA, VIETNAM

THESIS SUBMITTED TO THE UNIVERSITY OF STIRLING FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

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March 2013

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Declaration

I hereby declare that the work and results presented in this thesis was conducted by me at the Institute of Aquaculture, University of Stirling, Scotland. The work presented in this thesis has not been previously submitted for any other degree or qualification. The literature consulted has been cited and where appropriate, collaborative assistance has been acknowledged.

Signature: _____

Signature of supervisor: _____

Date: _____

Acknowledgements

During the four years of work, many people contributed to the successful completion of this thesis. Here I would like to thank them all.

I would like to express my most sincere gratitude to Dr. Mags Crumlish, Prof. James Turnbull, and Prof. David Little who accepted me in their group. During these years, I could always count on their constant supervision, motivation and friendship, which were crucial at several moments. I am especially grateful for the special effort they made for the final corrections of this work.

I extend especial thanks to Prof. Le Thanh Hung and Dr. Ram C. Bhujel for successfully co-ordinating the ASIAN-LINK Project and for their part in funding the study, and similarly the University of Stirling for their role in financial support.

Special thanks to all my good friends in Stirling that helped me to find a home abroad. I also would like thank all my colleagues in the Institute for sharing great moments and memories with me.

During the period of data collection, I would like to express my gratitude to the Vietnam fish farmers for their willingness to be interviewed and also for spending time and effort in filling questionnaires. I am also grateful to all staff members of the Department of Fisheries in Vinh Long, Ben Tre, An Giang,

Dong Thap, and Can Tho for their moral support at the early stage of my research work.

I wish to express my sincere thanks to my parents, Luu Thanh Truong and Luong Kim Hoi, my sister, Luu Thi Xuan Mai for their continual encouragement, love, and prayers for my progress and success.

Abstract

Disease outbreaks continue to be a major problem in the sustainable development of the aquaculture industry. Clinical outbreaks can negatively impact on the welfare of the fish and the economic gain derived from this industry. Jaundice observed as a yellow colouration in the abdominal skin, sclera of the eyes and fin bases is a significant health problem affecting the Vietnamese freshwater catfish industry. This study was designed to investigate jaundice of farmed catfish, *Pangasianodon hypophthalmus* using several complementary approaches. These included clinical investigations and identification of potential aetiological agents as well as epidemiological analyses to identify farm-based risk factors for this economically devastating condition occurring in the catfish farms of the Mekong Delta.

The results of this survey demonstrated that the jaundice was not linked to a single geographical location as affected fish were found widely distributed throughout the five main production areas. Nor was any association found between any weight groupings, feed type or feeding regime applied in the affected farms. The highest prevalence occurred between June to October and fish mortalities ranged from 1 to 10% in the study sites. The duration of this condition was significantly correlated ($P < 0.05$) to mortality but not to total farm area, depth of pond, stocking density, or amount of water exchanged. The number of fish ponds affected was not as high in the large-scale farms compared to the small-scale farms.

The results from the clinical description study showed that the affected fish were suffering a form of jaundice or icterus. Histological examination revealed a number of serious pathologies in the affected fish. Spleenomegaly was associated with the loss of cell structure and connective tissue and the haematopoietic tissue had large areas of necrosis. In the liver, histological changes consisted of vasculitis and multifocal to diffuse hepatocellular necrosis. The presence of haemosiderin was observed in melano-macrophage centres in the spleen and kidney of jaundiced fish. No single pathogen was identified in the jaundiced fish.

Myxosporean infection was found in both apparently normal fish and jaundiced fish. However, there was a definite tendency for jaundiced fish to be more heavily infected. Histopathological examination found several changes that could not be ascribed to specific aetiological factors and presume that both groups (jaundiced alone and myxosporean-affected jaundiced fish) have similar lesions. The results of this study would suggest that the parasite identified as *M. pangasii* was not a primary pathogen associated with the haemolytic jaundice. Neither were the gills myxosporeans associated with the haemolytic jaundice and they may be considered more as a nuisance rather than as primary pathogens in farmed *P. hypophthalmus* in the Mekong Delta, Vietnam.

Univariate analysis of the whole dataset showed several variables were significantly associated with the haemolytic jaundice. However, none of the variables achieved lasting statistical relevance in multivariable models.

In conclusion, this study identified a haemolytic jaundice condition affecting farmed *P. hypophthalmus* in Vietnam, but no single aetiological agent or farm based risk factor was identified with this condition. Several priority areas for further work were identified and include a prospective, longitudinal cohort study to identify further the risk factors associated with the clinical jaundice condition.

Table of contents

List of Figures

List of Plates

List of Tables

Abbreviation list

µg	Microgramme
µl	Microlitre
µm	Micrometre
16S rDNA	Ribosomal DNA of the 16 subunit
API-20E	Analytab system of 20 biochemical test for Enterobacteriaceae
ASEAN	The Association of Southeast Asian Nations
BKD	Bacterial kidney disease
BNP	Bacillary Necrosis of Pangasius
BOD	Biochemical oxygen demand
bp	Base pair
COD	Chemical oxygen demand
DMAB-Rhodanine	Dimethyl-amino-benzylidene rhodanine
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
EASRD	Rural Development and Natural Resources East Asia and Pacific Region
EDTA	Ethylene Diamine Tetraacetic Acid
EU	European Union
FAO	Food and Agriculture Organization of the United Nation
FCR	Food Conversion Ratio
FE	Fisher's Exact
g	gram
GAP	Good Agriculture Practices
h	Hour
H&E	Haematoxylin and Eosin
Hb	Haemoglobin
Ht	Hematocrit
kg	Kilogramme
MARD	Ministry of Agriculture and Rural Development
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MgCl	Magnesium chloride
min	Minute
MOFI	Ministry of Fisheries
MRC	Mekong River Commission
N	Nitrogen
NCBI	National Centre for Biotechnology Information
NH_3-N	Nitrogen ammonia
No.	Number
NO_2^-	Nitrite
^oC	Degree Celsius
P	Phosphorus
PAD	Pangasius Aquaculture Dialogue

PCV	Packed Cell Volume
pers. comm.	Personal Communication
PO_4^{3-}	Phosphate
ppb	Parts per billion
q.s.p	Solution pour perfusion standard
RIA 2	Research Institute for Aquaculture in Vietnam No.2
rpm	Revolution per minute
SPSS	Statistical Package for Social Science
SQF	Safe Quality Food
TAN	Total ammonia nitrogen
TCCA90	Tri Chloro Isocyanuric Acid 90%
TCVN	Vietnamese standards
TN	Total nitrogen
TP	Total phosphorus
TSA	Tryptone soya agar
TSS	Total suspended solids
UCB	Unconjugated bilirubin
UK	United Kingdom
USA	United State of America
USD ($)	US Dollars
v/v	Volume/volume
VASEP	Vietnam Association of Seafood Exporters and Producers
VND	Vietnamese Dong
w/v	Mass/volume
WWF	World Wide Fund for Nature

General introduction

1.1. Overview of Pangasius production in the Mekong Delta

Two pangasius species comprise of the majority of catfish production in the Mekong Delta, Vietnam and these are *Pangasianodon hypophthalmus* (Plate 1-1) and *Pangasius bocourti* (Plate 1-2). Currently, *Pangasianodon hypophthalmus* has the largest contribution to commercial catfish production in Vietnam. *Pangasianodon hypophthalmus* has a higher export value than *P. bocourti*, mostly due to a higher production yield at harvest and a shorter production cycle. According to VASEP (2007), more than 95% of the total pangasius production belongs to the *P. hypophthalmus*, more commonly known as the striped freshwater catfish. *Pangasianodon hypophthalmus* is an omnivorous species, native to the Mekong Delta. It feeds on fish, crustaceans, and vegetable matter. This fish species has an accessory breathing-organ, which enables them to breathe atmospheric air and survive in what is regarded for aquaculture as poor water quality with low oxygen levels in high density culture systems (Hill and Hill, 1994; MRC, 2001).

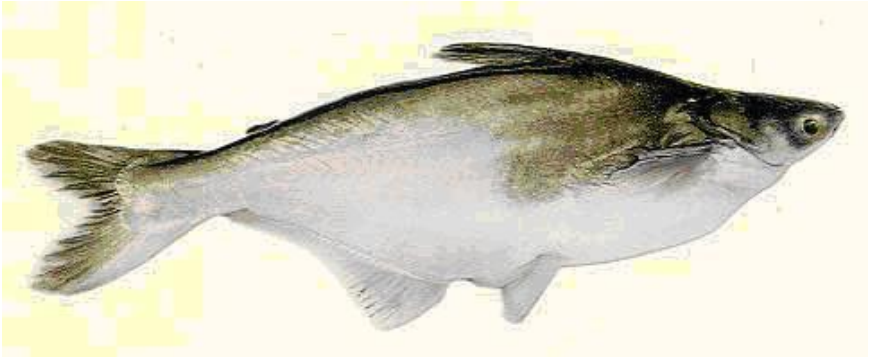

Plate 1-1 *Pangasianodon hypophthalmus* (*Source:* VASEP, 2012)

Plate 1-2 *Pangasius bocourti* (*Source*: VASEP, 2012)

1.1.1. Catfish production

Farming of pangasius fish started in the 1960's (Trong *et al*., 2002). Since 2000 in the Mekong Delta of Vietnam, the intensive rearing of *P. hypophthalmus* has grown rapidly due to the expansion of available international markets and increased consumer demand (Khoi, 2009). The farming area increased from 1,290 hectares in 1997 to 5,350 hectares in the year 2008, a 4-fold increase. Currently, the Vietnamese province, An Giang has 1,400 hectares of catfish culture that increased by 20.6% of that reported for 2007. A similar story has unfolded for other provinces producing catfish: Can Tho has 1,200 hectares (increased by 22.1%), Vinh Long has 450 hectares (increased by 13.2%) and Ben Tre has 360 hectares (increased by 500%) (World of Pangasius, 2008).

Between 2004 and 2006, the world growth of aquaculture averaged 6.1% in volume terms whereas Vietnamese aquatic production has grown by 17.6% in this period (FAO, 2009) where the main contribution for this growth in Vietnam was from farmed *Pangasius* spp. (Ministry of Fisheries-MOFI, 2008). According to the Ministry of Fisheries-MOFI (2009) the total aquaculture production of Vietnam had already increased to 2.2 million tonnes in 2009.

1.1.2. Culture systems and yield

Cages, ponds and net pens were the three common culture systems used in the Mekong Delta in 2005 (Hung *et al.*, 2007), however, the pond culture system is now the dominant production system. In fact the pond area for freshwater aquaculture has increased by 13% from 2003 to 2004. A corresponding decrease in cage culture systems has been observed with a 17% decline from 2,271 units to 1,871 units in the same year. The reasons for the reduction in cage farming are many-fold but reported to be due to fish losses through disease outbreaks in the rainy season producing wide-spread disease among cages which had a significant impact on fish production between 2002 and 2004. This reduction in fish production was thought to be primarily due to a lack of disease control in the cages, which were river based (Binh, 2008). Hence, the transition to earthen pond systems that have become more popular in Vietnamese catfish farming because it was considered as the most economical production method for these fish. With average pond sizes of 2,000m^2 (Wilkinson, 2008) and stocking density at about 60-80 fish per m^2 (Dzung, 2007), an average yearly yield stands at 50-80 metric tonnes/hectare after a growth period of 6-7 months. Besides the rapid increase in the *P. hypophthalmus* farming area, yield increase has been another important factor making the phenomenal growth of the total *P. hypophthalmus* output in the period 2000-2008 (Figure 1-1).

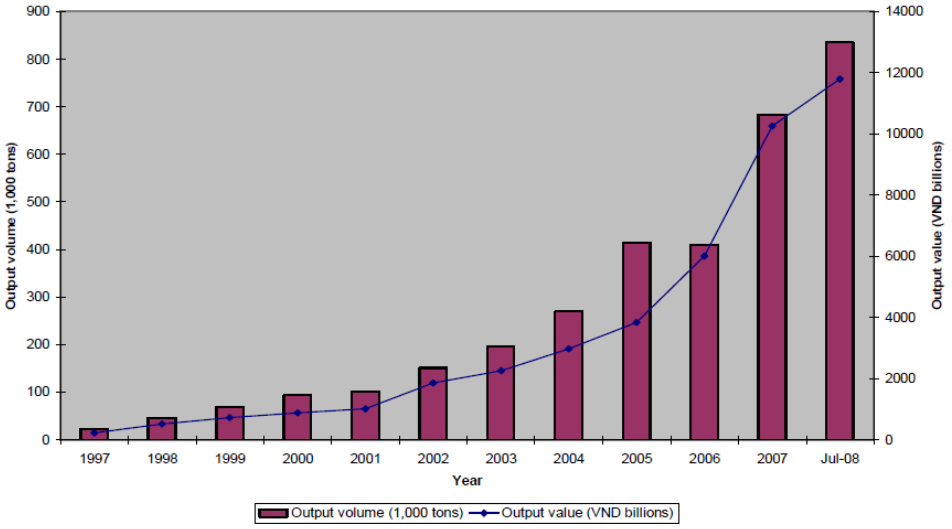

Figure 1-1 Pangasius production in Vietnam in the period 1997-2008
(Source: reproduced from VASEP 2008*)*

1.1.3. Pangasius export

According to Loc *et al*. (2009) approximately 91.3% of the total *P. hypophthalmus* production was processed and exported to foreign markets. The supply chain for the exported products is relatively simple (Figure 1-2). These products were readily sold on the well established markets such as the European Union and the United States, but were also accepted into emerging markets such as Russia and other ASEAN member countries. The exported catfish fillet production has increased 2-fold within 5 years from 5,000 tonnes in 1996 to 10,000 tonnes in 2001, of which 90% were from *P. hypophthalmus* (Tung *et al.*, 2004). The export value reached USD 1.15 billion in 2006, which was a 66.5% increase compared with previous years production (VASEP, 2006), to USD 1.453 billion in 2008 (Vietnam Economic Times, 2009), and to 1,141,000 tonnes in 2010. The high value gained from selling these products has contributed to the continued expansion of farming areas in Vietnam and

now supplying an even increasing global demand as these fish products are exported to 136 countries worldwide. The growth in earnings has been estimated at 1.4 billion USD for 2010 (Fisheries Directorate, 2010)

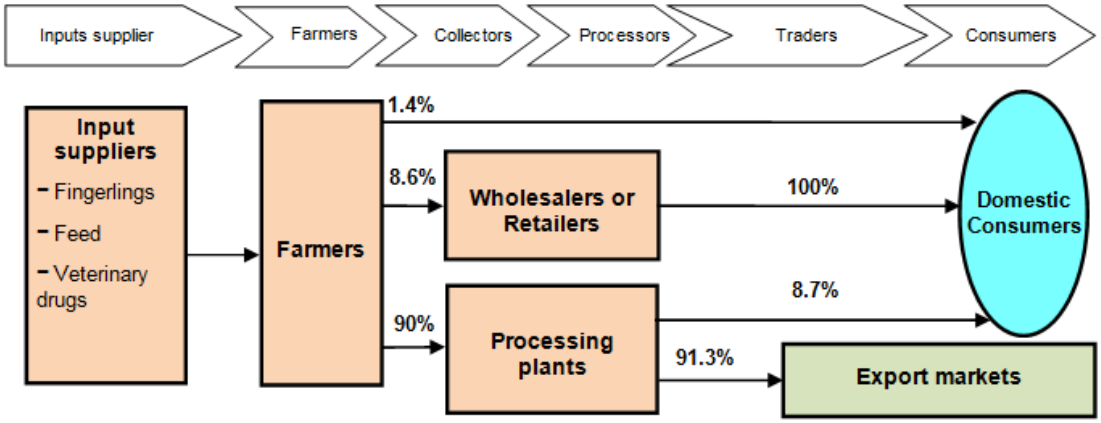

Figure 1-2 Supply chains of pangasius products
(Source: Adapted from Loc *et al*., 2009*)*

1.1.4. Feeding

In the pond culture system, the variety of feeds used ranged from home-made "traditional" pellets to commercial formulated pellets and more often than not a combination of these feed types have been used during the production cycle. Since 1998, formulated commercially available pelleted diets have been popular and in many commercial farming practises are the only feed type used (Hung *et al.*, 2007). The uptake of these pelleted diets has increased, as many farmers perceive that these types of diets help improve the quality of the fish flesh giving greater numbers of fish with the highly desired white meat at harvest. These diets are available from a wide range of commercial feed companies in Vietnam so it is not a single feed company supplying all *P. hypophthalmus* farming

communities. According to the survey conducted by Phan *et al.* (2009) feeding rate was from 1-18% body weight/day for commercial feed and from 1-10% body weight/day for home-made feed. These authors also reported that the average food conversion ratio (FCR) of fish was 1.69 (ranged 1.0-3.0) if fed with commercial feed and was 2.25 (ranged 1.3-3.0) if fed with home-made feed.

1.1.5. Water quality and pollution

Pangasianodon hypophthalmus farms usually do not include settling ponds in their layout. The function of a settling pond is to treat water before discharge to the aquatic environment. Whilst there have been some improvements in the water quality and treatment of discharge water, often the waste water from *P. hypophthalmus* culture has a bad smell and is dark or black in colour, especially, the waste in the bottom of the ponds which can only be removed by diving at the end of the production cycle. This mud/waste is taken out and goes straight into rivers (Binh, 2008). Aquaculture in the Mekong Delta results in a total of nearly 500 million cubic meters of mud and waste, which include more than 2 million tonnes per year from the pangasiid culture exclusively (Institute for Fisheries Economic and Planning, 2009). As a result of the expansion of *P. hypophthalmus* culture, the hazards of water pollution and disease outbreak are also considered to have increased. Therefore, the Mekong Delta is facing a serious problem regarding water pollution (Ministry of Natural Resources and Environment, 2008).

Water quality indicators are routinely tested and Giang *et al*. (2008) reported that the *P. hypophthalmus* culture environment has total nitrogen (TN), total

phosphorus (TP), Biochemical Oxygen Demand (BOD) and H_2S levels much higher than the accepted Vietnamese standards for surface water quality (TCVN 5942-1995). The main driver for this is thought to be the increased production and high stocking densities on the catfish farms. The higher the stocking density the higher the amount of feed given to the fish which will affect not only the water quality but also the organic load in the water.

Pham *et al.* (2010) demonstrated that the nutrient enrichment in wastewater from the production of one tonne of *P. hypophthalmus* was 201 kg BOD, 247 kg COD, 557 kg TSS, 37 kg TN, and 9 kg TP, while the sludge from *P. hypophthalmus* farms was 35 kg BOD, 59 kg COD, 217 kg TSS, 1.5 kg TN, and 0.8 kg TP. De Silva *et al*. (2010) calculated an export of 31,602 tonnes of N and 9,893 tonnes of P to be discharged from a 70 km^2 pond-based *P. hypophthalmus* production in the Mekong Delta in 2007 based on the median nutrient discharged levels for commercial feeds. Thus, Bosma *et al.* (2009) reported that during discharges of sludge from the ponds a very high nutrient load in waste-water and sludge may have the potential for causing localized pollution of the canal water during the dry season when the water level is low.

1.1.6. Impact of disease on the *P. hypophthalmus* farming industry

Infectious and non-infectious diseases of farmed *P. hypophthalmus* have been reported to occur throughout the production cycle from hatcheries to grow-out. Several diseases have been observed some of which are non-pathogen driven i.e. poor nutrition or low water quality but many pathogens including parasitic, fungal, and bacterial diseases are often reported during the culture period. Phan

et al. (2009) provided information on the relative importance of the most significant diseases affecting production in this industry. These included bacterial bacillary necrosis of pangasius (BNP); varied parasite infections; red spot/haemorrhagic symptoms and swollen eyes; white gills and liver; and yellow colouration. Some of the diseases and levels of reported occurrence in *P. hypophthalmus* are summarised in Table 1-1.

Table 1-1 List of common diseases of farmed *P. hypophthalmus* in the Mekong Delta

Name of disease	Aetiological agent or suspected agent	Pathogens identified	Outbreak occurrence
Bacillary Necrosis of Pangasius (BNP)	*Edwardsiella ictaluri*	Bacteria	- Weather change - Rainy season - Water pollution - Fingerling quality - Feed quality
Parasites	*Ichthyophthirius multifiliis,* myxosporean	Parasites	- Weather change - Some rain, some sunshine - High stocking density
Red spot disease	*Aeromonas hydrophila,* *A. sobria,* *A. caviae*	Bacteria	- Weather change - Fingerlings transportation - Water pollution

Source: Dung et al., 2008b; Vander Braak, 2007; NAFIQAVED, 2007; PAD, 2008

Bacillary necrosis of pangasius (Plate 1-3) is a serious and economically significant bacterial infection in the Mekong Delta (Crumlish *et al*., 2002), which can cause 50-90% mortality during a single outbreak (Dung *et al*., 2004). Although *P. hypophthalmus* of all sizes are susceptible to BNP, fingerling and juveniles are thought to be impacted more severely resulting in high mortality (Dung *et al.,* 2008a). Crumlish *et al.* (2002) reported conditions that favour occurance of BNP are high stocking densities, weather changes, environmental pollution, and moderate water temperature (22-28^{o}C). There is no commercial vaccine at present and so farmers are reliant on antibiotic treatments which can

be expensive and due to the rapid emergence of antibiotic resistant are becoming less effective (PAD, 2008).

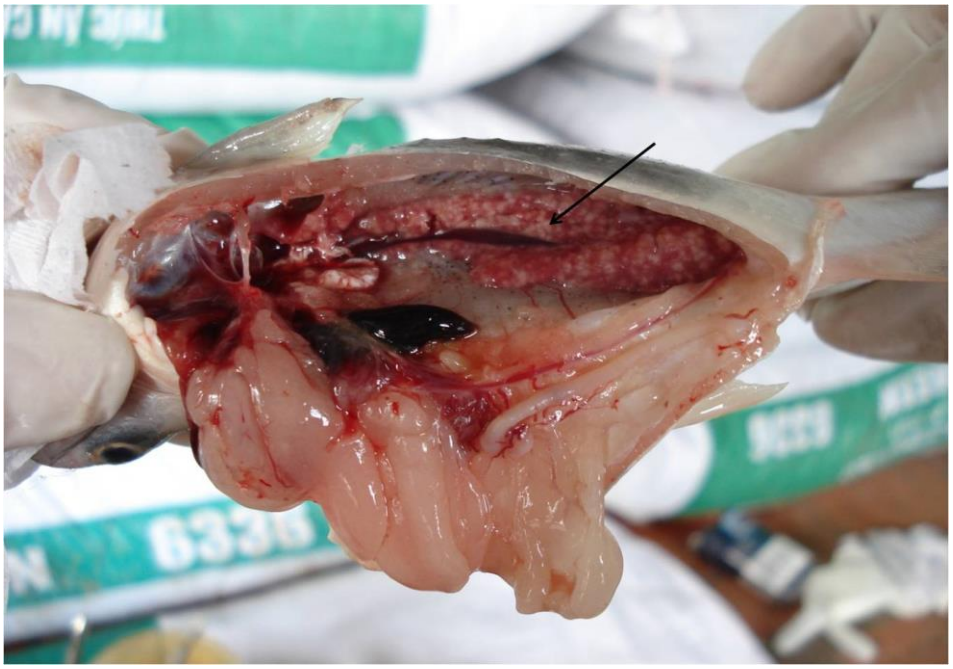

Plate 1-3 The clinical signs of BNP in *P. hypophthalmus*, arrow shows the numerous white spots on the kidney

Red spots/haemorrhagic symptoms (Plate 1-4) thought to be the common description given to bacterial septicaemia caused by a group of motile *Aeromonas* including *Aeromonas hydrophila, A. sobria, A. caviae* (Phuong *et al*. 2007; Dung *et al.*, 2008a). This disease was reported to cause a significant mortality in *P. hypophthalmus* farms (Ly *et al.*, 2009). Outbreak of this disease was reported to be associated with stress factors, including high stocking densities and environmental pollutants (PAD, 2008).

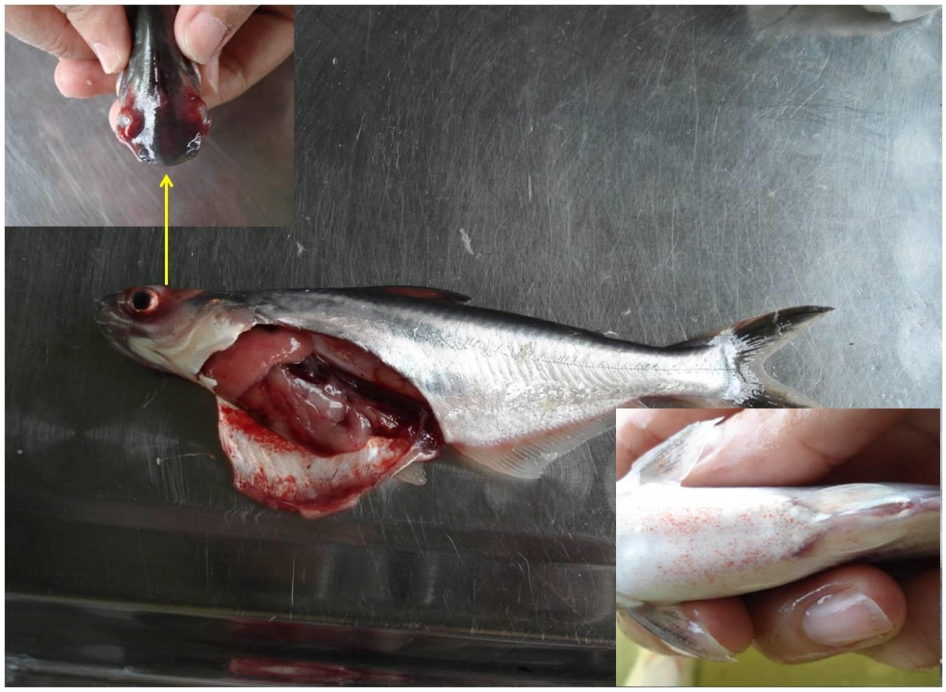

Plate 1-4 The clinical signs of red spot disease showing hemorrhages on the head, around the mouth and on the fins

Phan *et al*. (2009) reported fish losses due to pale (white) gills and liver disease (Plate 1-5) in farmed *P. hypophthalmus*. The occurrence of this disease was highest during the change from the dry to rainy seasons and during the flood season in the Mekong Delta. Diseased fish showed no external clinical signs, while the internal organs such as gills and liver were pale (Plate 1-5). The cause of pale gill, pale liver infection is still unknown, although Vietnamese researchers at RIA 2 have described young age classes of fish as being more sensitive where the mortality can be up to 70-80% (RIA 2, pers. comm., 2010).

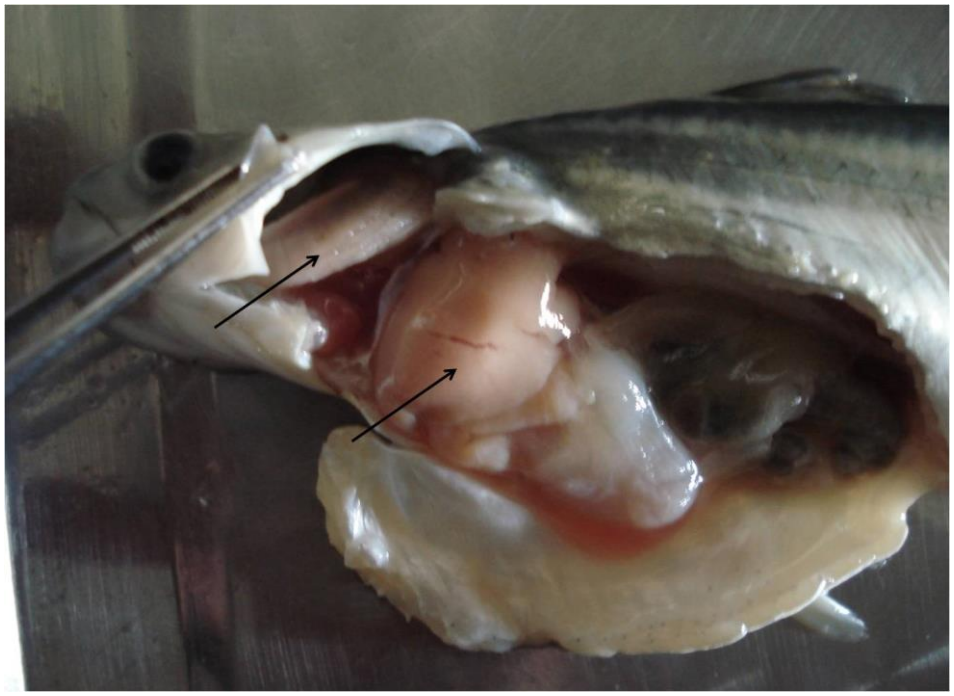

Plate 1-5 Fish presenting with pale (white) colouration of the gills and liver (arrows)

Other common diseases of *P. hypophthalmus* in the Mekong Delta are parasitic conditions, including monogenean gill parasites, ciliates (*Ichthyophthirius, Trichodina* and *Epistylis*) (Dung *et al.,* 2008a), myxosporean (*Myxobolus* and *Henneguya*), and round-worms (Nematodes).

Most monogeneans found on the gills of *P. hypophthamus* are described as dactylogyroids (Plate 1-6) (Hang *et al.*, 2008). *Pangasianodon hypophthalmus* seems to co-exist with its specific monogeneans in culture conditions. There were no records of ill effects or mortalities due to monogenean infection in fish with body weight more than 200g, even when numbers of the plasmodia are high on the fish gills. According to Paperna (1963a, 1963b) the larvae of *Dactylogyrus* either actively migrate to the gills after attaching to the skin of the

fish or become attached to the gills when washed over these tissues in swallowed water.

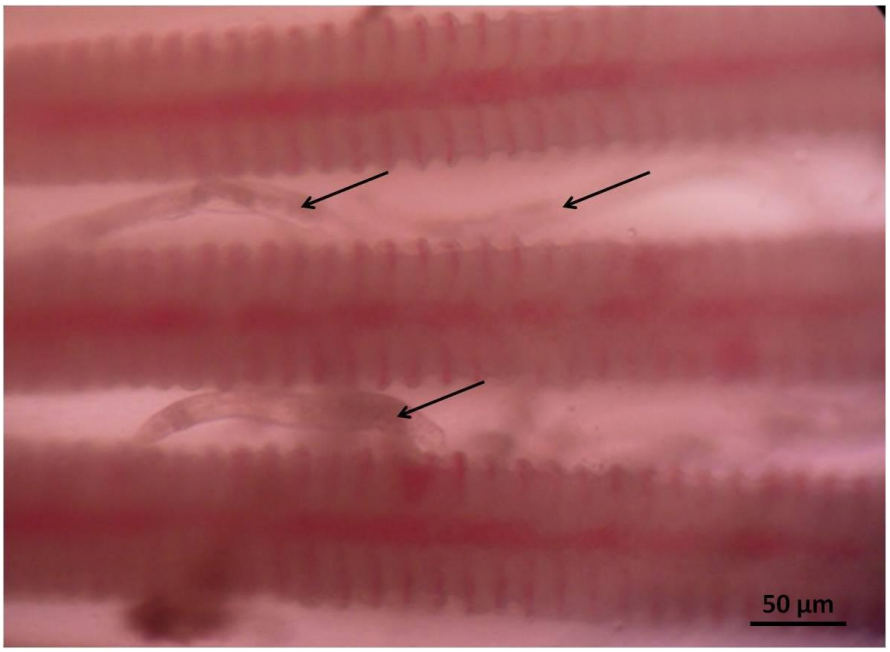

Plate 1-6 A gill wet mount of *P. hypophthalmus* showing presence of monogeneans (arrows)

Myxobolus, Hennegoides and *Henneguya* species belonging to the myxosporeans, are reported quite commonly on the gills of *P. hypophthalmus* farmed in the Mekong Delta, Vietnam (Ky and Te, 2000; Dung *et al.*, 2008a; Hang *et al.*, 2008). Until recently, five species of myxosporean including *Henneguya shariffi, H. berlandi, Hennegoides pangasii, Myxobolus baskai* and *H. malayensis* have been found parasitizing the gills of *P. hypophthalmus,* while *Myxobolus pangasii* was found in the spleen of this fish. According to Hang *et al.* (2008), the prevalence of *Myxobolus* sp found in cultured *P. hypophthalmus* was 80% and *P. hypophthalmus* were thought to infect all year round. This condition is usually benign; however, sometimes it can be associated with a high mortality, particularly in farmed fish. Currently, outbreaks of disease due to

myxosporean and microspora infections are being increasingly reported in farmed *P. hypophthalmus* from the Mekong Delta, Vietnam. Hang *et al.*, (2008) reported that heavy infections were associated with the unsightly milky oval shaped cysts (1-3 mm) within skeletal muscle and internal organs of farmed *P. hypophthalmus* (Plate 1-7), however, this was not usually associated with serious clinical infections and Hang *et al.* (2008) did not report on the pathogenicity of myxosporean and microsporan infections. Instead, the detrimental effects of these parasites produced economic losses mostly due to downgrading of fillets (Hang *et al.*, 2010).

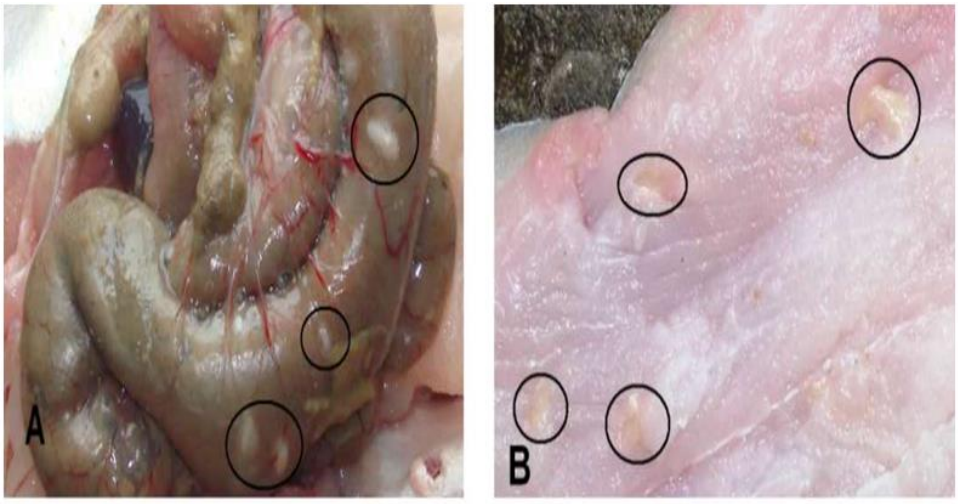

Plate 1-7 Myxosporean infection in *P. hypophthalmus* (A-Whitish cysts on intestine of affected fish; **B-** Whitish cysts within skeletal muscle of affected fish) **(circles)**
(*Source: Hang et al., 2008)*

The ciliated parasites *Ichthyophthirius*, more commonly known as white spot, are one of the most common diseases of freshwater fish. *Ichthyophthirius* appears to parasitise all freshwater fish (Edward, 1996; Harry, 2006), although scale-less fish, such as catfish and loaches are especially vulnerable (Harry, 2006). Most farmed fingerlings are infected by *I. multifiliis* (Plate 1-8) during the rainy season with a reported morbidity of 73.3% in *P. hypophthalmus* and

Clarias sp. and 100% in *Wailago dinema* (Te, 1998). The white spot trophont of the parasite forms a nodule in the skin or gill epithelium (Ewing and Kocan, 1986). Mortality can be as high as 100% (Meyer, 1974), and fish presenting heavy infections are not likely to survive, even if they are treated.

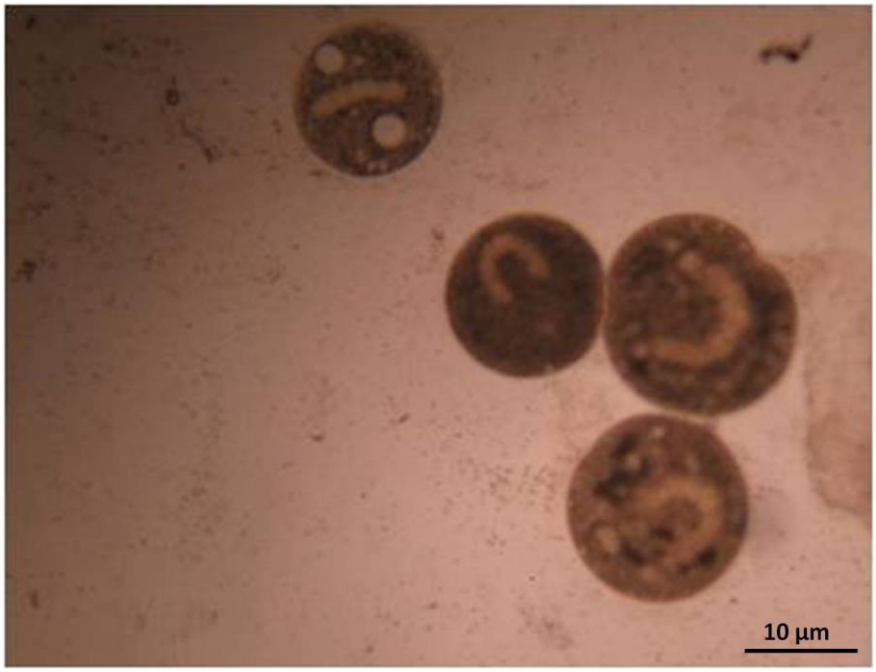

Plate 1-8 Wet mount of *I. multifiliis* from *P. hypophthalmus* skin

1.1.7. Antibiotic and chemical use

In aquaculture, the risk of bacterial infections among farmed fish is high. Therefore, large amounts of antibiotics are administered mixed in the feed for both disease prevention and disease treatment purposes in aquaculture (Sapkota *et al.,* 2008). Chemicals are used at all stages of the production for *P. hypophthalmus* (EASRD, 2006). Data from a previous survey showed that there were 29 products used in fish farming in the Mekong Delta, in which 17 products are a mixture of different group of antibiotics for prophylactic and therapeutic purposes (Phuong *et al.*, 2005). Oxytetracycline has been widely

used in aquaculture (Suzuki *et al.*, 2008), especially in the Mekong Delta, Vietnam (Dung *et al.*, 2008b). The wide and often indiscriminate use of antibacterial agents, especially through prophylactic treatments during the production cycles can quickly result in the development of increased antibiotic resistance and recurrent outbreaks necessitating further, costly treatments (Dung *et al.*, 2008b; Crumlish *et al.*, 2008), and this may have been the case with this industry. Additionally, over-use of some antibiotics can cause direct liver damage as shown in humans. Lloyd-Still *et al.* (1974) reported that, when used in large doses in man, antibiotics belonging to the tetracycline family (tetracycline, guamecycline, doxycycline and others) can cause jaundice.

1.2. The export demand for *P. hypophthalmus* industry in Vietnam

As with any farmed food industry the quality and safety of the final product is of the utmost concern and often in aquaculture there is a pre-conceived idea from the consumer as to what the end-product would look like. Therefore, the colour and size of the fish at harvest are crucial drivers for the sustainable marketing of the products and the continued demand from international consumers. The quality of the fillets at the time of harvest will determine the final price and export market for each animal sold, whether this is globally or locally in Vietnam.

In the fish processing plants, the main concern in relation to quality is the colour of the fillets where white demands the highest price compared with red to pink or dark yellow: the latter getting the lowest price at harvest (Plate 1-9). As a result yellow coloured fish are often considered as unmarketable due to the

presence of the yellowish colour as this is not highly favoured by the international consumers, particularly European and American consumers. The white and light pink meats are usually destined for the United States and European Union markets and provide the farmer with the highest price. Non-white fish flesh which includes the light yellow meat can still be exported but is often considered by the processing industry. However, the discoloured fillets can be sold to markets in Eastern Europe, Russia and the Ukraine as well as other Asian countries including Singapore and South Korea (VASEP, 2008).

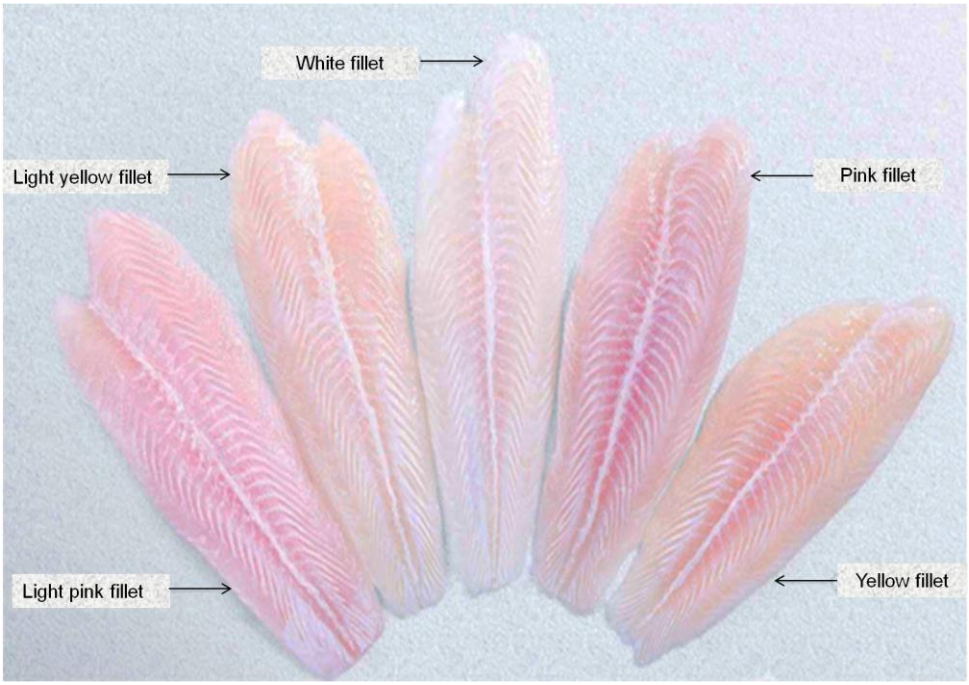

Plate 1-9 *Pangasianodon hypophthalmus* fillets representing five different grades of colour from white, via pink to yellow (*Source*: http://www.bianfishco.com)

The price paid by the importers for *P. hypophthalmus* fillets based on the colour were 3 to 3.2 USD kg^{-1}, 2.8 USD kg^{-1}, and 2 to 2.2 USD kg^{-1} for white, pink and light yellow fillets, respectively (Featherstone, 2010). Therefore it is not just the fish losses that affect the production sector but the actual profit gain by the fish

farmer as although the affected fish can still be sold, the farm-gate price of yellow coloured fish is often lower than their total production cost. Any fish with external signs including dark yellow colour will be automatically rejected at the pond-side or at the processing plants before they are unloaded from the boats. These rejected fish can only be sold for a much lower price (approx. 8,000 to 10,000 VND kg^{-1}) on the domestic markets and they are often sold not as a whole fish product but as part of a product including dried fish. These economic losses due to fillet downgrading or total rejection have led to severe problems for the economic sustainability production of *P. hypophthalmus*.

1.2.1. Jaundice in *P. hypophthalmus*

A disease problem, identified by yellow colouration in the skin of the abdominal areas, eyes and fins in *P. hypophthalmus* (Plate 1-10) has led to large numbers of fish losses in Vietnam. The affected fish were described as having pale gills, yellow ascitic fluid, enlarged spleen, kidney and gall bladder, and/or yellow flesh (Dung, 2006). According to Khoi *et al.* (2008), yellow colouration ranks fourth (17% of visited farm) after BNP (63.5%), parasites (45%) and red spot (42%) in the most common diseases negatively affecting *P. hypophthalmus* farming. This condition can cause mortality but more often results in morbidity. But the biggest impact of the condition is the significant financial loss when these fish are sold on the international markets and it is therefore a serious threat to the sustainable production of this sector. Yellow coloured fish are rejected outright or the fillets are downgraded by the processing plants at post-harvest, thus reduced value of affected fish means less income per production site and reduced re-investment.

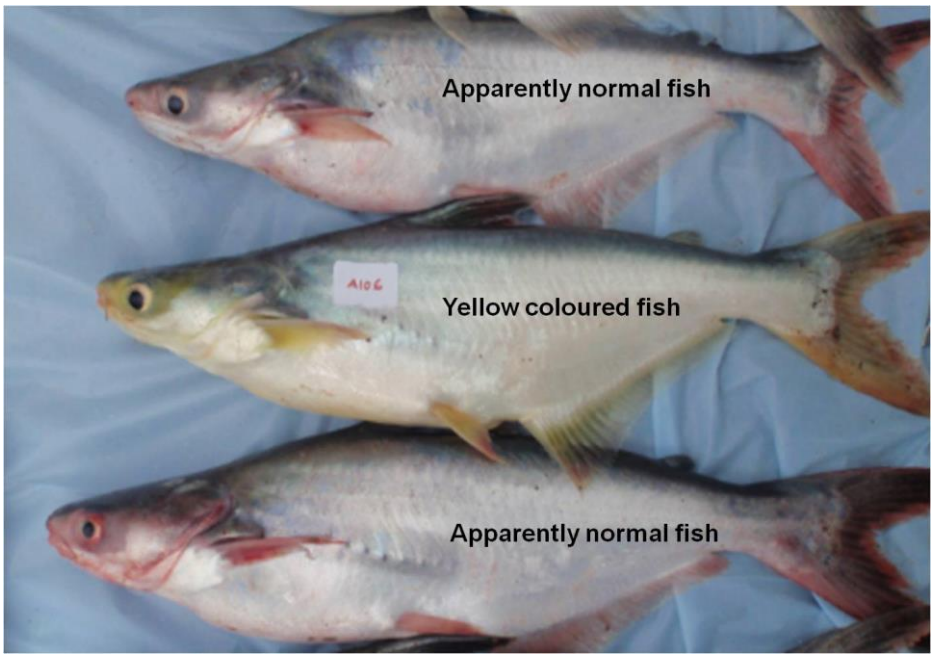

Plate 1-10 Gross clinical signs of *P. hypophthalmus* showing the yellow colouration in the skin of the abdominal areas, eyes and fins

The prevalence and cause of the yellow colouration in *P. hypophthalmus* has not yet been identified, but many factors have been suggested. These include suboptimal water quality, inadequate feed quality, and high stocking density or numerous aetiological agents (Vinh, 2006).

Hung *et al.* (2005) linked yellow colouration in *P. hypophthalmus* farms to the farm-made feed given to the fish which are usually composed of a mixture of rice bran, marine trash fish, soybean meal, fish meal, corn meal and vegetables. A positive correlation was found by Tin (2008) during an on-farm survey of *P. hypophthalmus* sites in Vietnam, where fish fed with a diet high in vegetable matter had a greater probability of yellow flesh. This issue was overcome by feeding commercial pellets in the final month of culture cycle (Hung *et al.*, 2007).

Environmental conditions have also been thought to be associated with the yellow colouration, where Binh (2008) suggested that *P. hypophthalmus* may become yellow in colouration if they are grown in stagnant ponds. This was thought to be linked to the eutrophic conditions and high densities of certain microalgae in the ponds. Wilkinson (2008) found that yellow flesh in *P. hypophthalmus* would be prevented or resolved if the fish were grown in deeper ponds (>4.5m) with a high level of water exchange on the order of 20-30% per day.

1.2.2. Jaundice in aquatic fish species

Yellow colouration has been reported as a pathological change in other species including yellowtail fish, *Seriola quinqueradiata* and eel, *Anguilla japonica* in Japan (Sakai *et al.*, 1988), hybrid catfish (*Clarias macrocephalus* x *Clarias gariepinus*) in Thailand (Tonguthai *et al.*, 1993), and hybrid catfish (*Clarias betrachus* x *Clarias fuscus*) in Taiwan (Chang *et al.*, 2008). All of these reported jaundice conditions were due to a variety of nutritional factors.

Previously, Ferguson (1989) reported that jaundice was not a commonly observed clinical feature in fish due to the type of bile pigment produced by the fish. Although the different bile pigments produced does vary between species (Cornelius, 1991). Accumulation of algal pigments such as zeaxanthis (Lee, 1987) and other dietary substances (Li *et al.*, 2007) can result in yellow colouration of internal and external tissues. These yellow pigments can be isolated and purified from the cyanobacterium *Microsystis aeruginosa* (Chen *et al.*, 2005). In yellowtail fish, jaundice was thought to be caused by lipid

peroxidation induced by the severe increase of reactive oxygen species in the animal tissue (Sakai *et al.*, 1990). The catfish *C. gariepinus* developed jaundice due to oxidative damage to lipids and vitamin E removal in the rancid chicken viscera used in the feed (Pearson *et al.*, 1994). Otto *et al.* (2000) reported that jaundice outbreaks may be associated with obstruction of the common bile duct by nematodes and inflammation of the bile ducts by extension from enteritis or by infection with trematodes. Infectious haemolytic anemia causes jaundice outbreaks in farmed marine coho salmon, *Oncorhynchus kisutch* (Walbaum) in Chile (Smith *et al.*, 2006). Yellow pigmentation syndrome in *Salmo salar* has been reported as a pollutant-induced haemolytic anemia and hyperbilirubinemia in Scotland (Croce and Stagg, 1997).

1.2.3. Classification of jaundice

Jaundice itself is not a disease; it is a symptom of a number of different diseases and disorders of the liver, gall bladder, and haemolytic blood disorders (Damjanov, 2000). Jaundice is classified into three categories by the point at which disruption to the normal functioning of the metabolism and excretion of bilirubin from the body occurs. The three categories are pre-hepatic/hemolytic, hepatic/hepatocellular, and post-hepatic/obstructive (Figure 1-3).

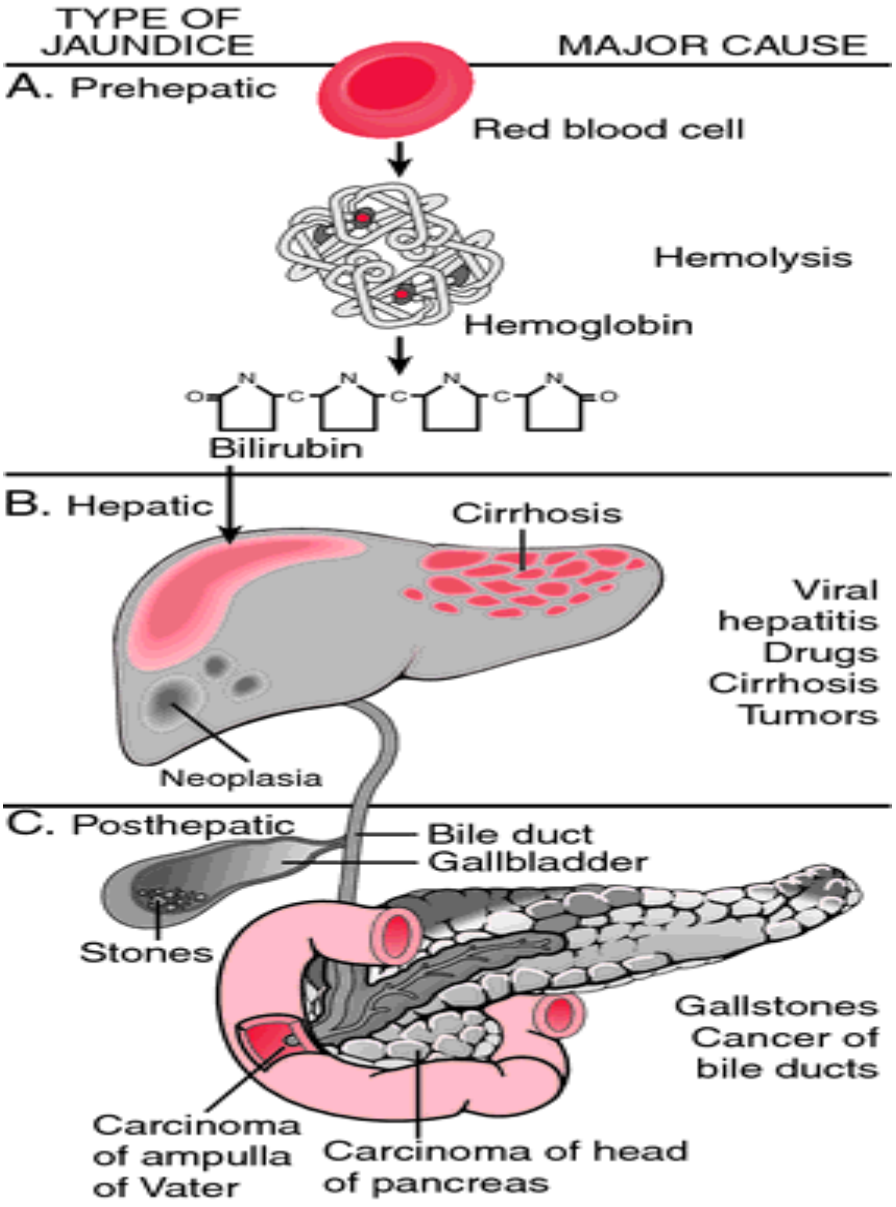

Figure 1-3 Types of jaundice. Jaundice may be attributable to pre-hepatic (A), hepatic (B) or post-thepatic (C) causes in human (Damjanov, 2000)

1.2.3.1. Pre-hepatic jaundice

Pre-hepatic jaundice is the result of conditions that cause the red blood cells to break down too rapidly (haemolysis). The increased production of bilirubin, leads to the increased production of urine-urobilinogen. Bilirubin is not usually found in the urine because unconjugated bilirubin is not water-soluble, so the

combination of increased urine-urobilinogen with no bilirubin in the urine is suggestive of a type of haemolytic jaundice.

1.2.3.2. Hepatocellular jaundice

Hepatocellular jaundice is usually caused by liver failure, hepatitis or artifacts acquired from chemicals/drugs. In this case, cell necrosis reduces the liver's ability to metabolize and excrete bilirubin leading to a build-up of unconjugated and conjugated bilirubin in the blood.

1.2.3.3. Post-hepatic jaundice

Post-hepatic jaundice has also been called obstructive jaundice and is caused by an interruption to the drainage of bile in the biliary system. A common cause is gallstones in the common bile duct, which create a blockage in the bile duct system. Also, a group of parasites know as "liver flukes'' can live in the common bile duct, causing obstructive jaundice (Khuroo *et al.,* 1990).

1.2.4. Bilirubin

Bilirubin is the pigment causing jaundice. Bilirubin is the end product of heme catabolism. The major source of heme (75-80%) is haemoglobin, from the breakdown of erythrocytes. Other heme sources include cytochromes, peroxidase, catalase, myglobin, and ineffective erythropoiesis (Crigler and Najjar, 1952; Shapiro, 2005).

The metabolism and excretion of bilirubin is shown in Figure 1-4. Indirect bilirubin or unconjugated bilirubin (UCB), a breakdown product of haemoglobin,

is water insoluble and neurotoxic. The first step in bilirubin production involves removal of iron and a protein moiety, followed by oxidative process catalyzed by the enzyme microsomal heme oxygenase and an equimolar amount of carbon monoxide and biliverdin are formed. This enzyme heme oxygenase is called the rate limiting enzyme in bilirubin metabolism. Biliverdin is then reduced to bilirubin by biliverdin reductase (Crawford *et al*., 1988). In step 2, extrahepatic bilirubin is then bound to albumin and sent to the liver. In step 3, UCB is conjugated in the liver by the hepatic enzyme UDP-glucuronosyl transferase to a water-soluble, non-toxic glucuronide, known clinically as conjugated bilirubin or direct bilirubin (Shapiro, 2003). In step 4, the water-soluble, conjugated bilirubin, is excreted into bile and then into the gut for elimination (Gourley, 2004). In step 5, conjugated bilirubin can however be deconjugated back to UCB in the gut so that the bilirubin is reabsorbed into circulation (Dennery *et al.,* 2001).

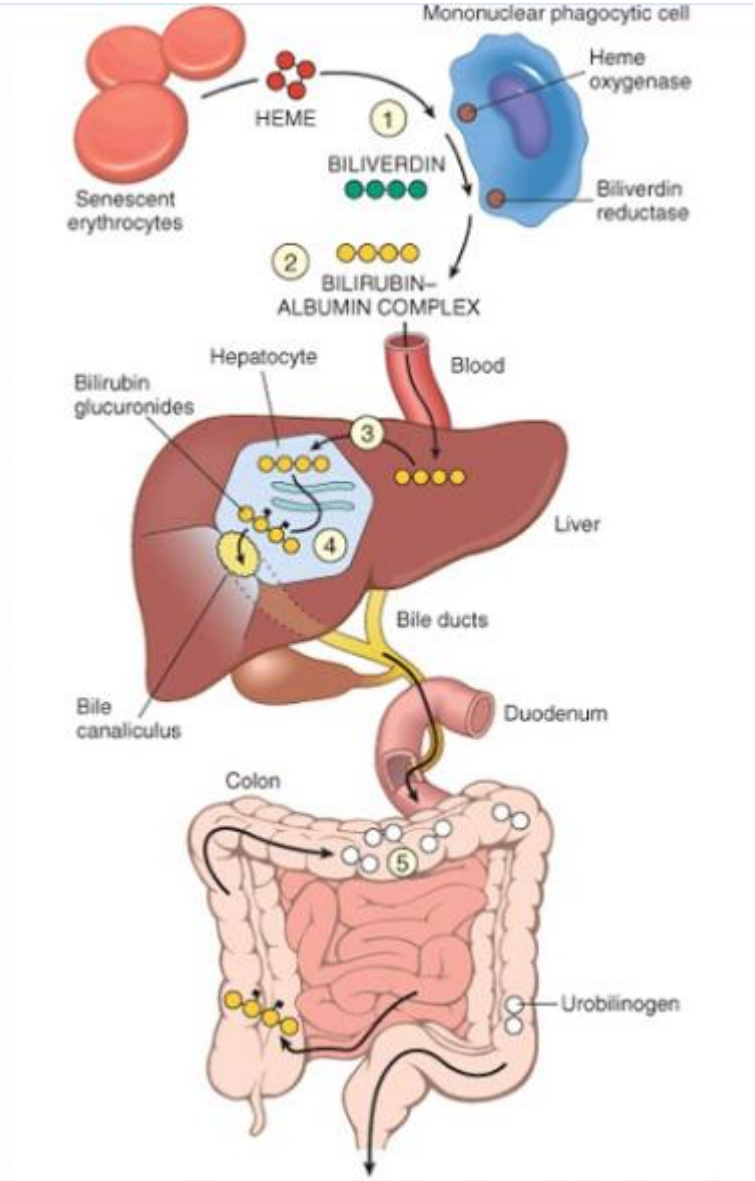

Figure 1-4 Physiological metabolism and excretion of bilirubin
(*Source: Kumar et al., 2009)*

Normally the liver cells absorb the bilirubin and secrete it along with other bile components. If the liver is diseased or if the flow of bile is obstructed or even if the destruction of erythrocytes is excessive, the bilirubin accumulates in the blood and eventually will produce jaundice. In mammals, haemoglobin is metabolized to form the green pigment biliverdin which is then reduced to form bilirubin. In direct contrast, the physiological roles of the bile pigments in fish are

still uncertain. Fish seem to have very complicated and species specific bile pigment compositions (Sakai, Tabata & Watanabe 1988). Therefore whilst jaundice reports in other farmed aquatic species may be useful they are of limited value when comparing between farmed fish species.

1.3. Research objectives

The overall study objective was to improve the existing knowledge on yellow colouration of farmed *P. hypophthalmus* in the Mekong Delta, Vietnam.

More specifically the study was designed to investigate this condition using several complementary approaches including clinical descriptions of the affected fish, identification of potential aetiological agents and epidemiological analyses to identify farm-based risk factors for this economically devastating condition in farmed *P. hypophthalmus*.

1.4. Outline of the thesis

The thesis is divided into six chapters. This chapter provides an overview of the development of the *P. hypophthalmus* industry and its current status in the Mekong Delta, Vietnam. Also described what is known about jaundice in pangasius at present.

The second chapter provides field based data on associations between farming management practices and outbreaks of jaundice in *P. hypophthalmus* produced in Vietnam

The third chapter examines the pathogenesis of yellow colouration using gross examination, histology and blood biochemistry of yellow coloured fish. It provides a clinical description of the jaundiced *P. hypophthalmus* farmed in Vietnam.

The fourth chapter identifies parasites occurring in jaundiced *P. hypophthalmus* in the Mekong Delta, Vietnam in order to investigate the role of the parasites observed within the jaundice condition.

The fifth chapter identifies risk factors at the farm level for the cross-sectional study. The tool chosen was a questionnaire-based cross-sectional survey.

Conclusions and discussions on the main findings are presented in Chapter 6. The detailed methodologies and research methods are presented and explained in each chapter.

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Current status of yellow colouration in farmed catfish (*Pangasianodon hypophthalmus*, Sauvage) from the Mekong Delta, Vietnam

2.1. Abstract

The yellow colouration has led to significant economic losses in the *Pangasianodon hypophthalmus* industry. A survey was performed to describe the current status of this condition in farmed *P. hypophthalmus*. All sites included in this survey produced fish in earthen ponds with the mean depth of 3.98 (± 0.93) m and were distributed throughout the main production areas of the Mekong Delta, Vietnam. The results of this survey demonstrated that yellow colouration was not linked to a single geographical location as affected fish were found distributed throughout the five sampling areas. The mean yield per culture period was 340.2 (± 101.2) tons ha^{-1} $crop^{-1}$. The results from this study clearly showed that yellow colouration was not associated with any weight grouping nor could any association be found between the onset of yellow colouration and the feed type or feeding regime applied. The highest prevalence of yellow colouration occurred between June to October. The fish losses caused by this condition ranged from 1 to 10% and the duration of the outbreak was significantly correlated ($P < 0.05$) to mortality but not to total farming area, pond depth, stocking density, or amount of water exchanged. The number of fish ponds affected by yellow colouration in large-scale farms was not more significant compared with occurrence in small-scale farms. In conclusion, the

present study provided valuable field based data on the relative associations between farming management practices and outbreaks of yellow colouration in *P. hypophthalmus* produced in Vietnam.

Key words: *Yellow colouration, Vietnamese catfish (Pangasianodon hypophthalmus), the Mekong Delta, farming practises.*

2.2. Introduction

Pangasianodon hypophthalmus (Sauvage) is more commonly known as striped catfish and is the most popular freshwater fish species farmed in Vietnam. The popularity of *P. hypophthalmus* in Vietnam is mostly due to a high yield and a shorter production cycle when compared with the other catfish species *P. bocourti*, which is also produced for the table market. The cultivation of *P. hypophthalmus* is more concentrated in the Mekong Delta of Vietnam, where the total fresh water area is approximately 641,350 ha (Phuong and Oanh, 2009). The export value reached USD 1.15 billion in 2006, which was a 66.5% increase compared with previous years production (Vietnam Association of Seafood Exporters and Producers-VASEP, 2006) to USD 1.453 billion in 2008 (Vietnam Economic Times, 2009). This is a viable food industry with a high value export commodity currently sold in approximately 80 countries worldwide (Phan *et al*., 2009).

As with many aquatic farming systems, fish losses through disease outbreaks are considered as one of the largest constraints to the sustainable future of *P.*

hypophthalmus farming. The cost of disease prevention and treatment is approximately 5% of the total production cost (Hung *et al*., 2005; Dung *et al*., 2008a, 2008b; Khoi *et al*., 2008) but this can vary depending on the production intensity. In 2006, Dung reported the presence of an increase in the number of farmed catfish presenting with a yellow colouration of the skin and the flesh, which was thought to be an infectious disease. Whilst this condition does not often lead to high mortalities it can produce high morbidity and significantly reduce the market value of the fish. This discolouration from white to yellow is considered as a serious threat to the catfish industry by many involved in the production network.

Several disease conditions have been described in farmed *P. hypophthalmus* in Vietnam and those of economic significance were primarily of bacterial origin but yellow discolouration was found in 28% of 89 visited farms (Phan *et al.,* 2009). Discolouration of the flesh in farmed fish species has been reported for other farmed fish species (Sakai *et al*., 1990; Tonguthai *et al*., 1993; Croce and Stagg, 1997; Smith *et al*., 2006). Studies on the gross and histological presentation of jaundiced hybrid *Clarias* spp. catfish (*C. macrocephalus* x *C. gariepinus*) have been reported from Thailand in 1992 (Tonguthai *et al*., 1993, Pearson *et al*., 1994) and Taiwan (*C. betrachus* x *C. fuscus*) in 2001 where over 90% of affected fish died (Chang *et al*., 2008). Yellow coloured Clarias catfish showed a yellow colouration all over the body. In these fish the spleen, kidney and gall bladder were usually enlarged and pale and the body cavity of fish was filled with a yellow ascitic fluid. Additionally, these fish had severe haemolysis

with abnormally high mean corpuscular volume (MCV) values. High mortalities associated with jaundice have been identified as a serious problem affecting production of non-catfish species including yellowtail fish, *Seriola quinqueradiata* and eel, *Anguilla japonica* in Japan (Sakai and Tabata, 1988).

The cause of the yellow colouration in *P. hypophthalmus* has not yet been identified, but many factors, including inadequate water, seed and feed quality have been proposed. Furthermore, numerous aetiological agents have also been suggested as the cause of this condition in farmed *P. hypophthalmus* (Vinh, 2006). This study presents a systematic description of the current status and prevalence of yellow colouration in farmed *P. hypophthalmus* produced in earthen ponds in the Mekong Delta of Vietnam.

2.3. Materials and methods

2.3.1. Study areas

Primary data from forty-six *P. hypophthalmus* grow-out farms located in five provinces (Dong Thap, Vinh Long, An Giang, Can Tho, and Ben Tre province) in the Mekong Delta ($8^{o}33'$–$10^{o}55'$N, $104^{o}30'$–$106^{o}50'$E) of Vietnam were obtained. The questionnaire surveys were conducted from 28th April to 20th August 2008 and 12th October to 10th November 2009.

The fish farms were selected from the fisheries profile records provided by the provincial fisheries departments, where a list of *P. hypophthalmus* farms in the region and their locations was obtained and those that could be visited within a

single day were included. Each farmer was contacted through phone calls prior to site visits to help with compliance. To facilitate data collection, questionnaire forms were completed with the farm owner or manager at the time of visit. This questionnaire was used to help identify the range of health problems on the farms included in the survey (Appendix 1). The questionnaires were pilot tested with a small number of *P. hypophthalmus* farms which were not then included in the larger survey.

Secondary data were collected from newspaper articles, organizations' reports and official documents to cross-check the primary information obtained through the interviews. A lot of the secondary information was from local Vietnamese sources produced in a wide range of formats.

2.3.2. Data Analysis

All the data gathered were coded and entered in both Statistical Package for Social Science version 16 (SPSS 16) and Microsoft Excel 2007 spreadsheets. The data were analysed using SPSS 16, the main descriptive statistical tools used being descriptive and cross tabulation which provided information on the background of yellow colouration in the Mekong Delta of Vietnam. Apart from the descriptive analyses of the data, the relationship between average yield (tons ha^{-1} $crop^{-1}$) and selected parameters such as stocking density (fish m^{-2}), depth of pond (m) during the survey were analysed using simple linear regression. The relationship between duration/length of yellow colouration outbreak (days) (i.e. how long the fish remained yellow) and other parameters

selected were also analysed as previously described. Microsoft Excel was used to produce charts and tables. Means with ± standard deviation (SD) and percentages (%) were calculated.

2.4. Results

The total number of *P. hypophthalmus* farms visited in each province was: Dong Thap (20 farms), Vinh Long (15 farms), An Giang (5 farms), Can Tho (4 farms) and Ben Tre (2 farms). The location of these farms is shown in Figure 2-1.

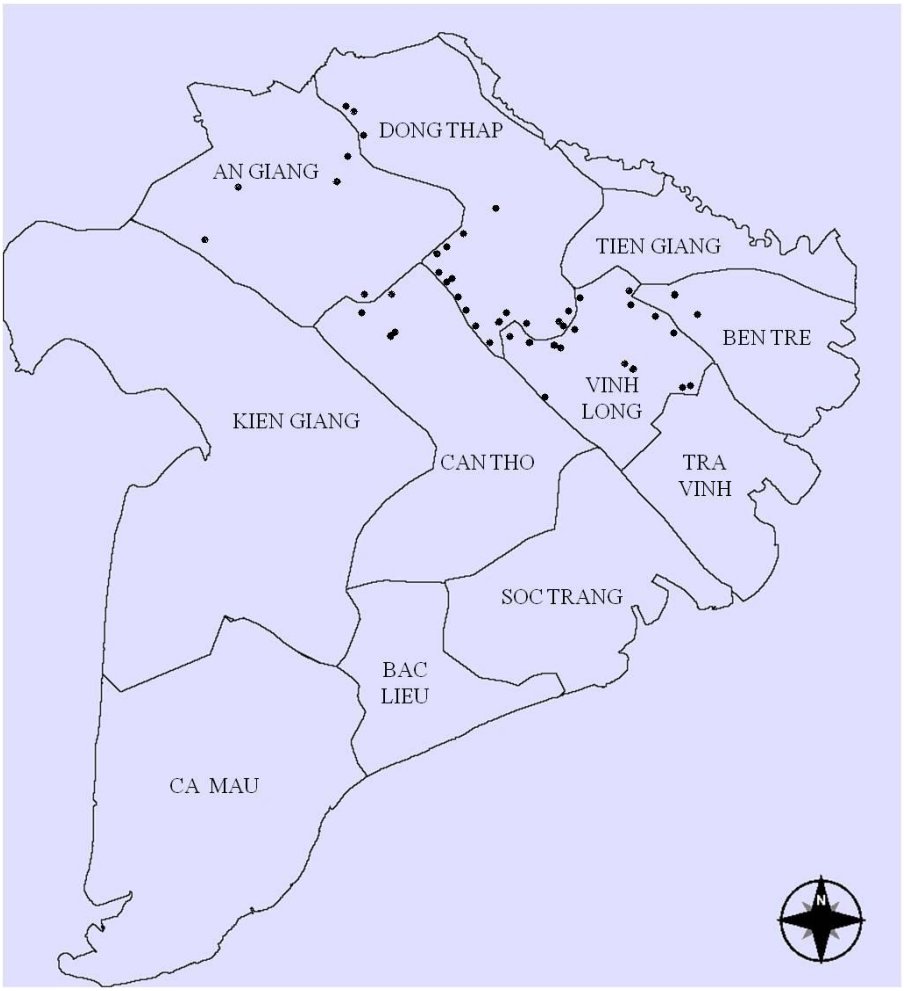

Figure 2-1 Map of farm location (•) in the various provinces, the Mekong Delta of Vietnam

2.4.1. Farm operation

All of these fish farms raised their stock in earthen ponds, often located near the bank of the river and islands for the convenience of water exchange and transportation. The ponds were designed rather simply without water storage or a reservoir. Approximately 2% of those interviewed produced *P. hypophthalmus* for less than 2 years, 85% for 2-5 years, and 13% for more than 5 years.

The two categories of land ownership found were as follows: 70% of total farms were owned outright and 30% of total farms were rented, where one farmer in Dong Thap province paid 50 million Vietnamese Dong (VND) (approx. USD 2,362) per year for a 14,000 m^2 pond with a five years contract to rent the land. There was a large variation in the total productive area per farm between the different farms visited. The maximum was 300,000 m^2 and the minimum was only 3,500 m^2. From the results of the survey the farms could be separated into three categories depending on their farm area. The first category comprised small-scale farms having less than 10,000 m^2 which represented about 30% of the total farms included. The second category was medium-scale farms which had a pond area between 10,000 and 50,000 m^2 (37% of total farms), and the third category was large-scale (considered as industrial) farms having pond area over 50,000 m^2 (33% of total farms). The large-scale farms could be owned and operated by a multi-owner corporation or by processing plants that produce whole chain of catfish production in one corporation. The number of fish ponds per farm ranged from 1-25. The characteristics of the 46 farms visited are summarised in Table 2-1.

Table 2-1 Characterisation of the production systems farming *P. hypophthalmus* (n = 46)

Farm Criteria	Mean (± Standard Deviation)	Min	Max
Total area of fish farm (hectare)			
Small-scale	0.63 (± 0.16)	0.35	0.90
Medium-scale	2.94 (± 1.30)	1.00	4.80
Large-scale	13.44 (± 6.84)	5.00	30.00
Size of pond (hectare)	0.71 (± 0.48)	0.10	3.00
Stocking density (fish per square metre)	47.4 (± 11.1)	25	85
Duration of culture (months crop^{-1})	6.90 (± 0.80)	5	8
Size of fingerling (cm)	2.1 (± 0.4)	1.2	2.5

The stocking density per farm ranged from 25 to 85 fish m^{-2} and the fingerling size (measured as the body height at the point where the dorsal fin meets the body) was from 1.2 to 2.5 cm at the time of stocking (Table 2-1). According to the farmers interviewed, fingerlings are one of the factors that have direct effect on the quality of fish produced at harvest and all farmers checked the appearance of the fingerlings prior to stocking which included screening the overall colour.

The “healthiest fish” were thought to be dark green on the dorsal side, silver on the ventral side, with clear stripes on the lateral sides. The fingerlings/fry were checked visually for active swimming response before stocking. Most farmers did these checks themselves, but one farmer used an external service. All farmers stated that the stocking density depended on the size and availability of the fingerling/fry at the time of stocking and the financial capacity of each farmer to purchase fingerlings. The price of fingerlings varied according to size and

season (Table 2-2). The farmers also described that the market price of the fingerlings fluctuated where the driving factors included number of *P. hypophthalmus* farms being stocked, the availability of fingerlings, and with the price of *P. hypophthalmus* exports at the time of stocking.

Table 2-2 The cost of catfish fingerlings

Size of fish fingerlings (cm)	Price (VND)
1.2	200 – 300
1.5	400
1.7	430 – 600
2	630 – 700
2.5	700

The survey findings indicated there were many sources of fingerling supplies within the study area, where the smaller-scale producers bought their stocks from either middlemen or traders who, in turn purchased their fish from different suppliers. No health certification was provided from these fingerling suppliers but the middlemen could provide different quantities of the fish depending on the amount required per farm. However, larger-scale producers mostly purchased their fish stocks from the Government hatcheries as these were health screened and certified as safe using a quality food standard that has provided support to improve the quality, safety and traceability of the product in the supply chain. This was only available to the larger producers as the Government hatcheries only sold larger volume of fingerlings on each occasion. The study findings also showed that the fingerlings used by 54% of the farmers originated from Dong Thap province, 20% from An Giang province, and 22% from other provinces. Only 4% of the farmers interviewed owned the hatchery or used self-propagating fingerlings. The farmers interviewed reported that the

fingerlings purchased from different suppliers were not kept together but were stocked in separate ponds.

It was the farmers' perception that to operate a good management practise for their *P. hypophthalmus* farm it required more than 2 workers to feed the fish, monitoring the ponds and perform water exchange on a timed interval. The large-scale farms always employed a graduated fisheries technician who had trained for simple diagnostic procedures and health management.

2.4.2. Pond and water management

The farm sites included in this study used the water directly from the Mekong River as their main water source. All of the farmers managed and prepared the pond following the same method before stocking: mechanical removal of the bottom sludge and liming of the ponds. Prior to stocking fish in the pond, all farmers used more than one method of water/pond treatment (Figure 2-2). In 26% of the farmers interviewed it was found that they used other chemicals to treat pond water including TCCA90 (Tri Chloro Isocyanuric Acid 90%), iodine, Yucca (vegetable extract to reduce NH_3) and $CuSO_4$ purchased from local suppliers.

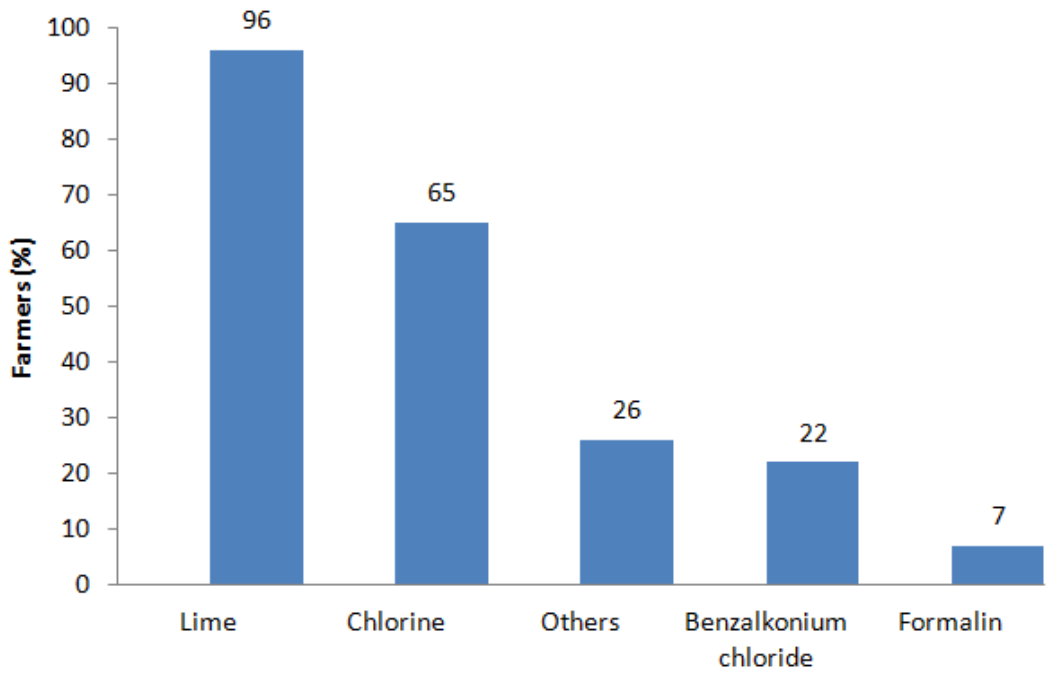

Figure 2-3 Percentage of fish farmers using the described chemicals as water treatments

The doses of chemicals loaded into the *P. hypophthalmus* ponds during culture period were lime at 1,000 kg ha^{-1} once every 10 days, chlorine at 15 kg ha^{-1} once every 15 days, benzalkonium chloride 5 l ha^{-1} once every 15 days, Tri Chloro Isocyanuric acid 90% at 8 kg ha^{-1}, and Yucca extract given at 20 kg ha^{-1}.

All farmers also exchanged water into the fish pond and removed sediment and faeces from the pond after harvest. The survey results showed that there was a mixture of water exchange methods applied. Tidal force, requiring no imputs from the producers if their ponds were next to a river and those not close to a main water source actively pumped out the water. Approximately 67% of farmers exchanged water once per day, 26% exchanged water twice per day and these farmers relied mainly on diurnal tidal fluctuations, and 7% exchanged

water depending on the water quality which checked regularly either by measurement or visual inspection and experience. The findings showed that there was no clear difference regarding frequency of water exchange between small and large-scale farming systems. The average percentage of water exchange level was estimated between 20-40% per time, irrespective of the scale of the farming system. In the present study, the waste water containing sludge from the ponds was released directly into the river or connecting channels twice per month and once per culture period without any additional treatment.

The present study found that 52% of farmers measured water quality during the production cycle using commercially available water quality test kits, while other farmers mainly manage pond water based on their visual observation and own experiences. For the 52% of farmers that used a kit measured pH (100%), total ammonia nitrogen (75%), temperature (17%), and dissolved oxygen (29%). There were several different periods of water measurement performed by the interviewed farmers (Table 2-3).

Table 2-3 The periods of water measurement in the recent study

Water measurement	Number of farmers	Percentage (%)
Daily	2	8.3
Weekly	11	45.8
Bi-weekly	2	8.3
Monthly	2	8.3
Based on the weather	7	29.2
Total	24	100

2.4.3. Feed type and feeding regime

Approximately 96% of farmers interviewed in this study fed their fish with commercial pellets, (Plate 2-1). While 2% of farmers used home-made pellets which they manufactured themselves during the culture period and another 2% of farmers used a combination of commercial pellets and home-made wet feed. In farms where both types of feed were used, according to the farmers opinion the fingerling did not feed well on home-made feed initially and so commercial pellets were often used in the first month of culture. This also helped them achieve faster growth of the fingerlings and during the last month, commercial feed was used to help achieve good quality flesh at harvest time. The results of this study showed that a wide range of local and international feed products were used on the farms, which were supplied by various feed companies.

During the culture period, fish were fed twice daily with three different crude protein levels of 28, 26 and 22% depending on the growth phase: fingerling stage to marketable size, respectively (Table 2-4). The protein of home-made wet feed was usually determined at approximately 18%. The feed conversion ratio for commercial pellets and home-made pellets ranged from 1.5 to 1.7 and 2.2, respectively. The fish were also fed with essential micronutrients in the form of vitamins and minerals which were mixed into the feed and in all cases vitamin C was added to the diet. The addition of the vitamins was an attempt to enhance disease resistance and up to 52% of farmers used other products for the same purpose. These contained vitamin complexes, enzymes and probiotics to increase feed digestibility. It was found that the most common

methods of feeding the fish was to spread the feed widely on the pond surface area to allow better access to the feed by all the fish in the pond. The present study found that commercial feeds were usually stored for 7-10 days at the fish farm in such a way as to permit air circulation at least 20 cm above the ground on dry wooden pallets, and protected from rain, sunshine and wind, while the home-made feeds were not stored for more than one day.

Plate 2-1 Type of feeds used in the visited farms. (A)-Commercial pellets**, (B)-** Home-made pellets

Table 2-4 Rate and frequency of feeding during *P. hypophthalmus* culture

Weight of fish (g)	Feeding rate (% body weight day^{-1})	Frequency (times day^{-1})	Crude protein of feed (%)
<200	4-3	3	28
200-500	3-2	2	26
>500	2-1	2	22

2.4.4. The productivity of *P. hypophthalmus* in pond culture systems

At harvest time, the farmers in this study pumped the water to drain the pond and then used seine nets to catch the fish. This seemed to be performed by groups of commercial harvesters. The average yield of *P. hypophthalmus* was 340.2 (± 101.2) tonnes ha^{-1} crop^{-1}, ranging from 110 to 610 tonnes ha^{-1} crop^{-1}.

The individual mean weight was from 900 to 1,400 g fish^{-1}. The average yield in the farms included in this study was not correlated to depth of pond and stocking density ($P > 0.05$).

The reasons given by the interviewed farmers for reduced productivity included disease outbreak (74%), water pollution (54%), poor fingerling quality (57%), and lack of knowledge and skills in fish health management (37%).

2.4.5. Disease status in farmed *P. hypophthalmus*

According to the results of the survey, most farms had experienced previous disease outbreaks which seemed to occur throughout the production cycle. In general a wide range of microbial organisms or disease descriptions were provided by those interviewed. All farmers interviewed had parasite infections at some point during the culture period, 98% reported fish losses due to bacillary necrosis of pangasius (BNP, bacterial infection), 89% observed yellow discolouration, 78% had red spot/haemorrhage symptoms and/or swollen eyes (these are often associated with opportunistic bacterial infections), and 17% reported fungus and 7% had other clinical signs including pale gill and liver. Farmers considered BNP as a major problem in *P. hypophthalmus* culture. They also considered that yellow colouration was a more severe problem during the culture periods.

2.4.6. Yellow colouration in *P. hypophthalmus* farming

In this study 89% of the fish farmers interviewed had observed outbreaks of yellow discolouration in their fish populations at some stage during the culture period. Most of these farmers were able to describe the common characteristics of yellow coloured fish, where yellow colouration in the skin of abdominal area, eyes and fins was the most common description provided. The present study found that a wide range of internal clinical signs were also associated with the presence of the yellow coloured fish (Table 2-5).

Table 2-5 Percentage of gross clinical signs of disease described by fish farmers in yellow coloured fish

	Description	Frequency (n = 41)	Percentage (%)
External	Yellow colouration in the skin of abdominal area, eyes and fins	41	100
	Pale gills	13	32
Internal	Yellow ascitic fluid	22	54
	Gallbladder is enlarged with dark blue bile	18	44
	Yellow fat	17	42
	Yellow flesh	16	39
	Common bile duct with nematodes	13	31
	Swollen liver, spleen or kidney	11	27
	No feed in stomach	10	24
	Yellow ascitic fluid which coagulates in air	6	15

None of the farmers interviewed were able to provide a single cause for this condition and instead a wide range of possibilities were suggested (Table 2-6). During a yellow colouration outbreak, there was only one farmer in Ben Tre province had sent a sample of the fish feed to the laboratory for detection of Aflatoxin where the results found that their feed contained Aflatoxin measured at 5-6 µg/kg by dry weight. The example of causes for yellow colouration according to the interviewed farmers is presented in Box 2-1, 2-2, and 2-3.

Table 2-6 Different causes for yellow colouration based on the opinion of interviewed farmers (n = 31)

Causes	Number of farmers	Percentage (%)
Nematode (Round worm)	20	66
Water quality	15	48
Feed quality	11	35
Pond conditions	2	6
After a treatment of bacillary necrosis of catfish (BNP)	1	3

Box 2-1 Example of yellow colouration due to feed quality problem according to farmers

Farmer: **NGUYEN VAN DAM**
Address: Cao Lanh-Dong Thap province
Total number of ponds: 09; Pond size: 0.5 ha; Depth of pond: 4 m
Stocking density: 60 fish/m^2; Size of fingerling: 2.5 cm
The yellow coloured fish/sampled fish: 15/15

Fish were fed by commercial floating pellet two times per day. In this farm, the farmer changed water daily using tidal exchange at approximately 40% per day. The yellow colouration outbreak was first observed on 25th July 2008 with the weight of fish more than 300g in nine ponds. The mortality of fish presenting with clinical signs of yellow colouration was 200 fish per day. During the outbreak, he frequently checked the DO, ammonia and pH of the pond water but these parameters were considered suitable for *P. hypophthalmus* feeding and growth.

Clinical signs examined by the researcher from Nong Lam University showed that fish exhibited a yellow colouration in the skin of the abdominal area and fin. Internally, they had pale gills, yellow ascitic fluid, enlarged liver, kidney and spleen, normal size of common bile duct. There was no evidence of the presence of roundworm (nematodes) in the common bile duct of yellow coloured fish, nor was there any myxosporean detected on the gills of yellow coloured fish.

Mr. Dam said that when the outbreak happened he discovered that the feed had a bad smell. To cope with this problem, the farmer used a new brand of feed. He mentioned that after changing the feed the severity of the yellow coloured fish decreased. For Mr. Dam's opinion, feed quality is an important factor that effects yellow colouration in *P. hypophthalmus*.

(Survey, 2008)

Box 2-2 Example of yellow colouration due to roundworm (nematodes) infection according to farmers

Farmer: **VU THI LU**
Address: Cao Lanh-Dong Thap province
Total number of ponds: 08; Pond size: 0.9-1.5 ha; Depth of pond: 5 m
Stocking density: 30 fish/m^2; Size of fingerling: 1.5 cm
The yellow coloured fish/sampled fish: 4/4

Fish were fed by commercial floating pellet two times per day. In this farm, the farmer changed water daily using tidal exchange at approximately 40% per day. During the first farm visit (11th May 2008) no abnormalities were observed grossly in the fish sampled except the common bile duct had roundworm (nematodes) infection with an incidence rate of 72%.

The yellow colouration outbreak happened at the second visit (29th May 2008) with the weight of fish more than 500g in two ponds. The mortality of fish with yellow colouration and clinical signs was less than 30 fish per day. The farmer frequently checked the water quality with a single pH test but found that this parameter was suitable for *P. hypophthalmus* feeding and growth.

Clinical signs examined by the researcher from Nong Lam University showed that fish exhibited a yellow colouration in the skin of the abdominal area and fin. Internally, they had pale gills, yellow ascitic fluid, enlarged liver, kidney and spleen, abnormal size of common bile duct (swollen common bile duct with presence of roundworm) in yellow coloured fish. There was no presence of myxosporeans on the gills of yellow coloured fish.

For Mrs. Lu's opinion, roundworm (nematodes) is an important factor that effects yellow colouration in *P. hypophthalmus*.

(Survey, 2008)

Box 2-3 Example of yellow colouration due to myxosporean infection according to farmers

Farmer: **DO HUU NGHIA**
Address: Cao Lanh-Dong Thap province
Total number of ponds: 01; Pond size: 0.64 ha; Depth of pond: 3.5 m
Stocking density: 40 fish/m^2; Size of fingerling: 2.5 cm
The yellow coloured fish/sampled fish: 7/10

Fish were fed by commercial floating pellet two times per day. In this farm, the farmer changed water daily using tidal exchange at approximately 40% per day. During the first visit (26th October 2009) the fish sampled for disease diagnosis appeared normal.

The yellow colouration outbreak happened at the second visit (4th November 2009) when the weight of yellow coloured fish was 500g. The mortality of fish with yellow colouration was less than 30 fish per day. The farmer frequently checked the water quality with a single pH test but found that this was suitable for *P. hypophthalmus* feeding and growth.

Clinical signs examined by the researcher from Nong Lam University showed that fish exhibited a yellow colouration in the skin of the abdominal area and fin. Internally, they had pale gills, yellow ascitic fluid, enlarged liver, kidney and spleen, normal size of common bile duct in yellow coloured fish. Myxosporean was found on the gills of yellow coloured fish with an incidence rate of 80%.

Mr. Nghia did not know what the cause of yellow colouration might be. The findings showed that myxosporean might be a factor that effects yellow colouration in *P. hypophthalmus* due to earlier myxosporean was not found in the fish affected by yellow colouration.

(Survey, 2009)

The survey data showed that a wide weight range of fish was affected by yellow discolouration (Figure 2-3). The highest prevalence was described in June to October corresponding with the flooding season in these areas, as well as increased rainfall and lower water temperatures (Figure 2-4, 2-5). When asked for the history of yellow colouration in the farm, 68% of the interviewed farmers reported that they had experienced this health problem in their fish during the previous crops in the year 2007 and 2008.

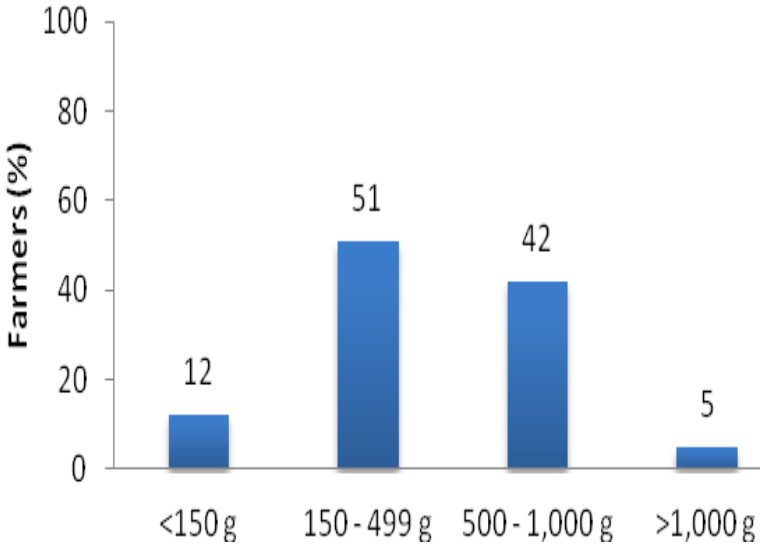

Figure 2-3 Weight range (g) of yellow coloured *Pangasianodon hypophthalmus* described from the visited farms

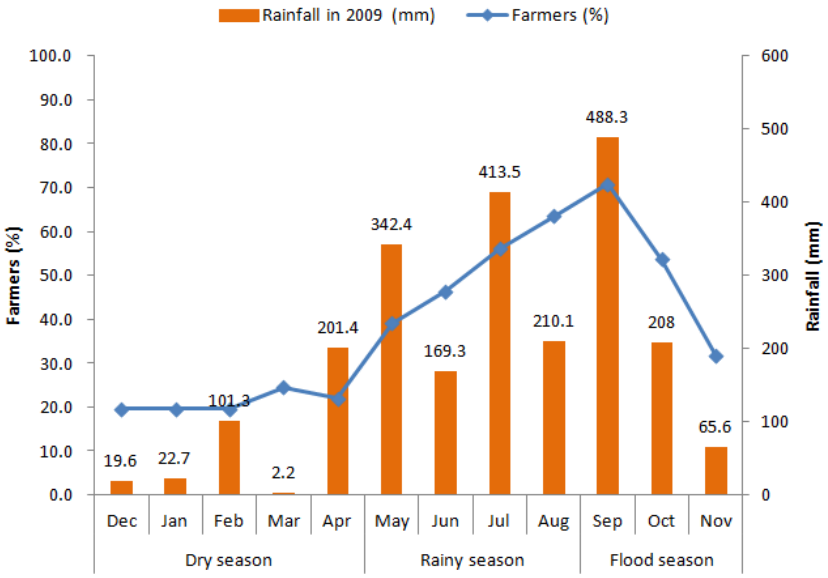

Figure 2-4 The report of yellow colouration in *P. hypophthalmus* farms with seasonal rainfall patterns
(*Source of rainfall data:* General Statistical Office of Vietnam 2009*)*

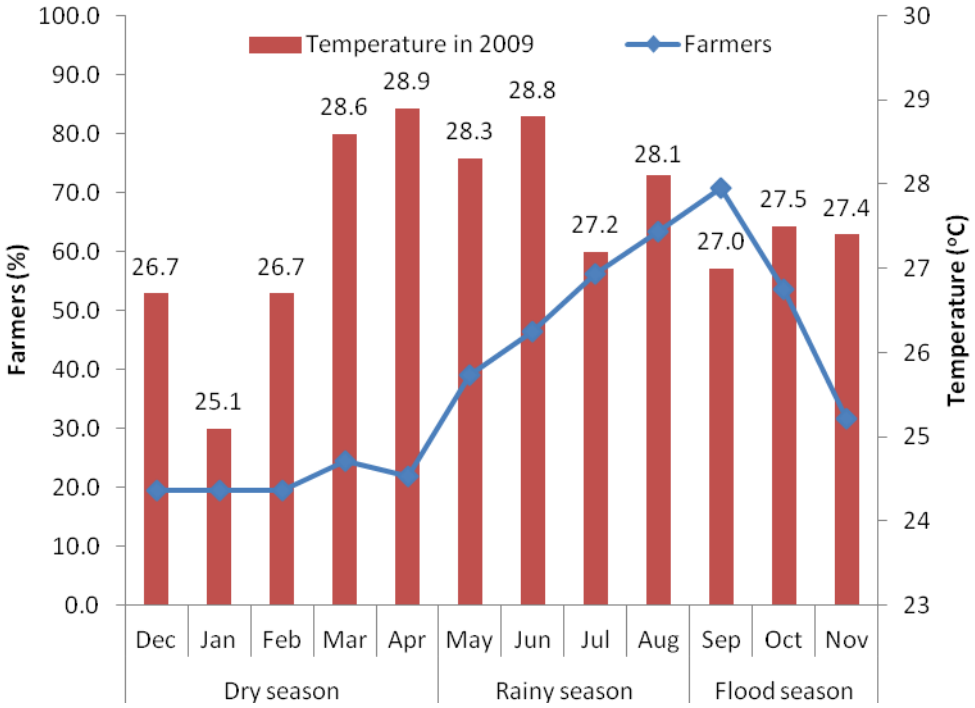

Figure 2-5 The report of yellow colouration in *P. hypophthalmus* farms corresponding with water temperature
(*Source of air temperature data:* General Statistical Office of Vietnam 2009*)*

The fish losses described from yellow colouration outbreaks in the interviewed farms ranged widely (Table 2-7). According to these farmers, fish showing clinical signs of the yellow colouration had low mortality ranging from 1 to 10%. The survey data showed that yellow colouration had caused mortality in 51% of visited farms (Plate 2-2), while 49% of visited farms had experienced a yellow colouration outbreak but no associated mortality. The number of ponds affected within a farm at any one time point ranged from 1 to 9 per farm (Figure 2-6). However, no significant difference ($P > 0.05$) was found between the number of affected ponds and the size of fish farm.

Table 2-7 Farmed fish losses reported as due to yellow colouration in *Pangasianodon hypophathalmus* farms

Number of yellow coloured fish (fish/pond/day)	Number of farm	Percentage (%)
<30	14	34
30 - <100	5	12
100 - <200	6	15
200 - 500	15	37
>500	1	2
Total	41	100

Plate 2-2 Mass mortality of farmed *P. hypophthalmus* showing external signs of yellow colouration in the Mekong Delta, Vietnam

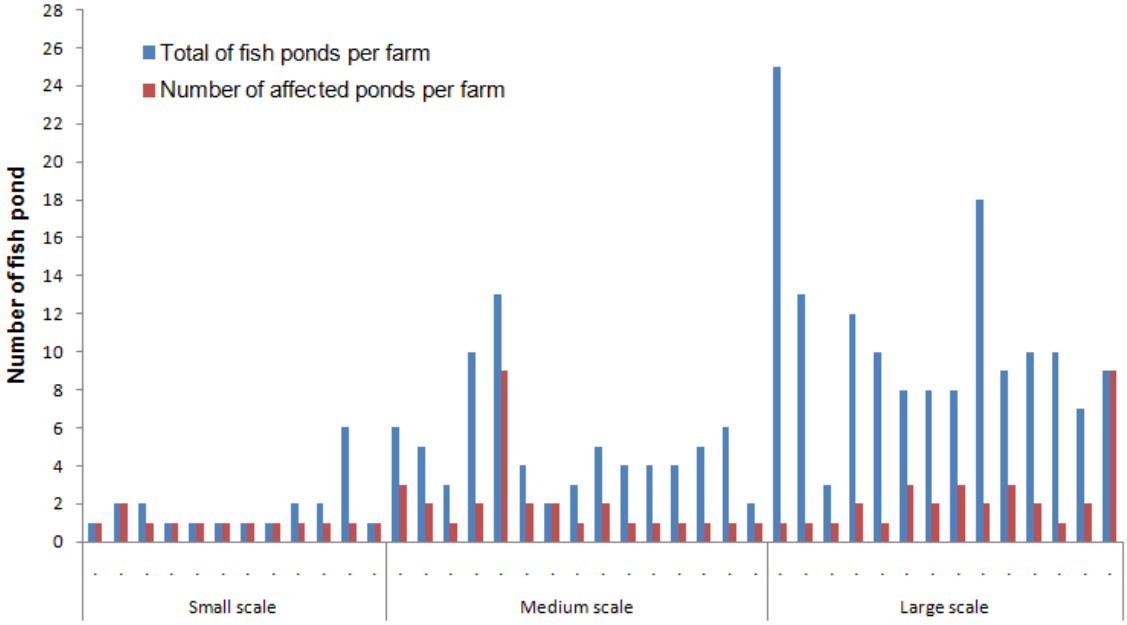

Figure 2-6 Number of ponds with yellow coloured fish on the farms visited

The present study showed that the duration of a yellow colouration outbreak in these farms which had no associated mortality was 6.5 (± 3.2) days whereas for farms experiencing mortalities it was 17.0 (± 11.6) days (Figure 2-7). There was a significant relationship between the duration of yellow colouration outbreak and mortality in the visited farms ($P < 0.05$).

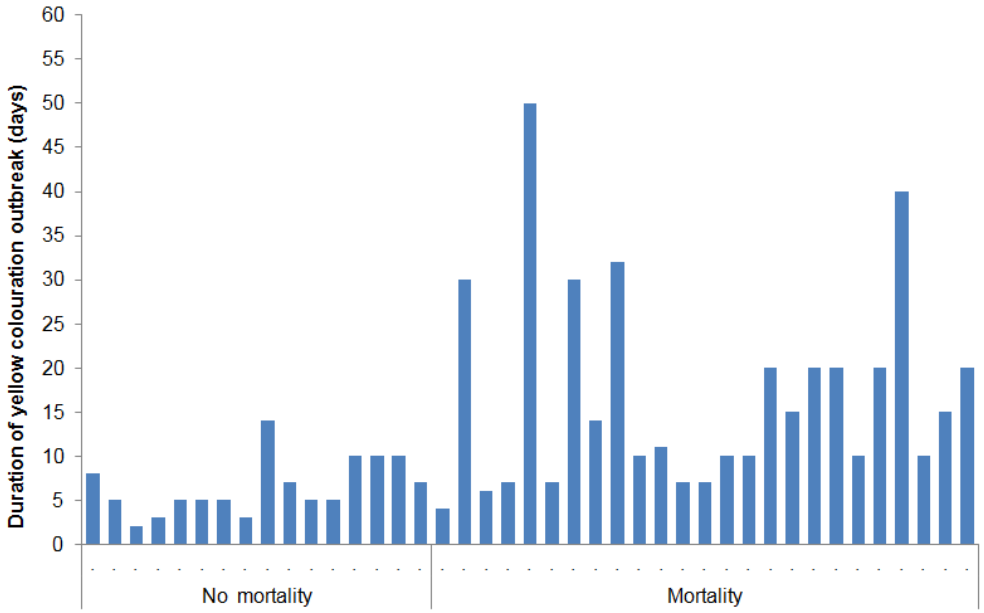

Figure 2-7 Duration of yellow colouration outbreak in the farms visited

The data were analysed further to explore the relationship between the duration of yellow colouration outbreak against a range of variables including total area of the fish farm, depth of the pond, stocking density, and amount of water exchanged (as a percentage of the total water volume). The findings showed that the duration of yellow colouration outbreak was not correlated ($P > 0.05$) to these parameters.

Once yellow colouration was present in the ponds, 81% of interviewed farmers reported that they applied some form of treatment, while 19% of farmers did nothing to solve this problem except removing the affected fish from the pond. When treating, the farmers used different approaches such as chemicals/veterinary drugs, water treatment (including exchanged water as well as treatments such as benzalkonium chloride (BKC), chlorine and zeolite (microporous, aluminosilicate minerals commonly used as commercial

adsorbents)), and a change in the feed company (Figure 2-8). The interviewed farmers thought roundworm infections were one of the causes of yellow colouration. Therefore, the farmers regularly treated their fish during the culture period with anthelmintic such as levamisole and ivermectin. Among chemical/veterinary drug treatments, 52% of farmers applied anthelmintic, 45% of farmers applied additional vitamin C, 33% applied medicine which contained 40% of sorbitol and other treatments often in multiple combinations.

The data collected during this survey also showed that one farmer used local herbal medicines to treat the *P. hypophthalmus* suffering from yellow colouration. This farmer had used three kinds of fresh herbs including Ox knee (*Achyranthes bidentata*), wild maracuja (*Passiflora foetida*), and bitter cumin (*Glinus oppositifolius*) in the feed for 6 consecutive days. It was this farmer's opinion that these are commonly used herbs in the treatment of jaundice, inflammation of the liver for humans and widely available in the local areas. The farmer boiled herbs in water until sufficiently extracted and mixed the pulped herbs with pellets before feeding.

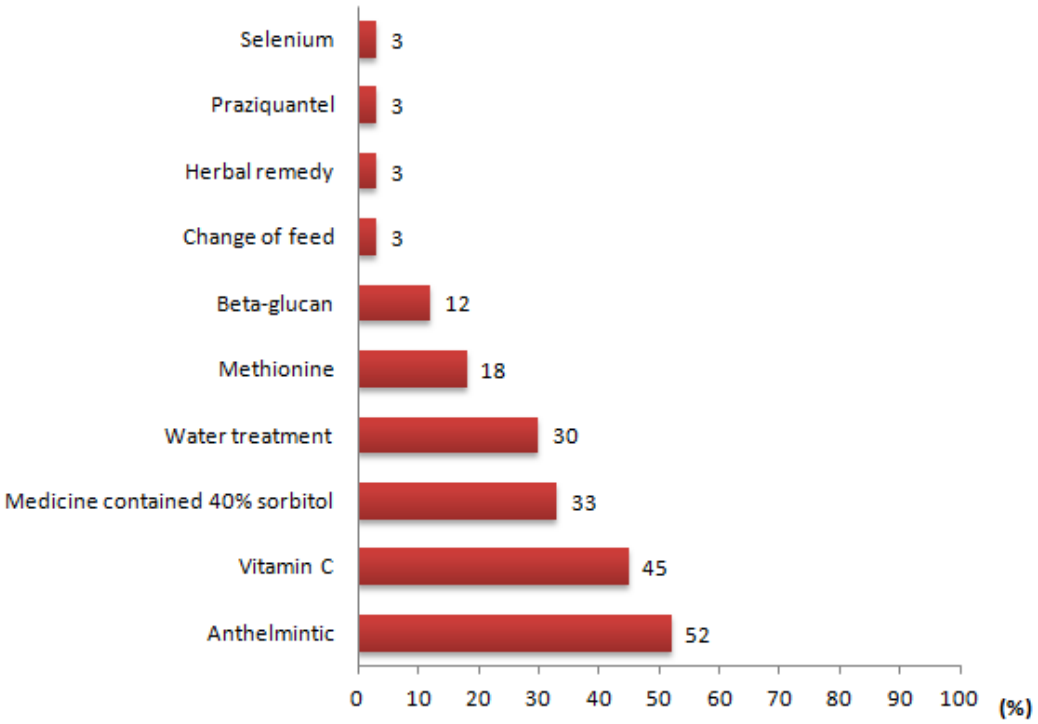

Figure 2-8 Frequency distribution of the varied "treatments"against yellow colouration

The results of this survey showed that when the disease condition occurred a single treatment or management regime was not universally applied (Figure 2-8). Instead fish farmers used a combination of their own experiences, advice from extension officers and/or chemical/veterinary salesmen, and university researchers for fish disease diagnosis, guidance and suggestions. However, no single effective treatment for yellow colouration of farmed *P. hypophthalmus* was found in this study.

2.5. Discussion

There is no doubt that earthen pond culture of *P. hypophthalmus* in Vietnam is the most popular production method covering approximately 3,458 ha in the Mekong Delta. This increase is consistent with the change from river-based

cages to predominantly pond culture systems (Hung *et al*., 2005). The reason for this change in production system was not always obvious, but pond culture may be considered a more economical model for these fish species. It was commonly found that farms using pond systems are relatively newer when compared with cage culture systems, in which the fish farmers frequently have over 14 years experience (Hung *et al*., 2007). The different degrees of fish farming experience may play a role in recognising abnormal from normal behaviour in the fish stocks. Farming experience is also crucial in the early detection of disease conditions in the farmed animals and in particular the emergence of new diseases/conditions affecting production, such as the development of yellow colouration in *P. hypophthalmus*. This is because fish husbandry and health management will vary depending on the experience of the fish farmers therefore influencing their ability to recognise clinical signs of disease associated with their fish stocks.

The results from the primary data collected in this study would suggest that a wider weight range of fish was affected than previously reported (Dung, 2006). However, in the study presented this more diverse weight range of affected fish was not linked nor confined to a single geographical location, thus supporting the high prevalence of this health condition in *P. hypophthalmus* farming systems in Vietnam. Since 2006, there has been more awareness of the yellow colouration in these farmed fish which might explain its occurrence in a wider weight range of fish as farmers in 2008 may be able to recognise this condition better compared with earlier years. Therefore it was difficult to define whether

these changes were due to the disease or an artefact from increased farmer awareness. Even though all farmers in the study were aware of this particular condition they still considered that they required more training from extension services and/or aquaculture research institutes to assist them in improved health management of their stocks.

A wide range of environmental and husbandry variables have been proposed as the cause of yellow discolouration in farmed *P. hypophthalmus*. Hung *et al*. (2007) described the occurrence of yellow *P. hypophthalmus* in farms using home-made wet feed. However, there was no dietary association at all in this study.

The association found by Hung *et al.* (2007) was probably a reflection of the change in the Vietnamese catfish industry as home-made wet feeds were common in *P. hypophthalmus* farming in 2005. Further studies on dietary influence in yellow colouration in farmed *P. hypophthalmus* showed a positive correlation with fish fed a diet high in vegetable matter (Tin, 2008). However, this was not supported by the findings of Hung *et al*. (2007) who suggested that the risk of yellow colouration could be reduced if the fish were not fed a diet with high vegetable matter during the culture period. Thus illustrating the conflicting results associated with nutrition and yellow discolouration in farmed *P. hypophthalmus*.

In Vietnam, many fish feed companies have been established and have produce commercial feeds since 1998 when the animal feed manufactures recognized the marketing opportunities. Commercial pelleted diets are increasingly being used in *P. hypophthalmus* and Vietnamese fish farmers have a much wider range of commercial feed products available to them compared with the United Kingdom or European aquaculture. It is not uncommon that the Vietnamese farmers will used products from many different feed suppliers throughout a single production cycle, which may all have the same basic ingredients but the individual amounts may vary. This “mix-and-match” strategy is predominantly ruled by feed costs but may also be a factor contributing to poorer husbandry practises. However, in this survey no association was found with yellow coloured fish and any single diet fed to the fish including home made or commercial pellets. Whilst a dietary influence cannot be ruled out completely, the lack of a strong association was expected. Fish farmers use varied sources of diets and often change their diet brands throughout the production cycle making a simple diet association within this condition improbable. Nevertheless, the results from this study suggested that no association could be found between the onset of yellow colouration and any feed type or feeding regime applied.

The average yields of *P. hypophthalmus* from the present study is consistent with those of the Sub-Institute for Fisheries Economics and Planning in Southern Vietnam (2009) and Phan *et al*. (2009), which reported the yield of 200-400 $tons^{-1}$ ha^{-1} $crop^{-1}$ and 70-850 $tons^{-1}$ ha^{-1} $crop^{-1}$, respectively. The

average stocking density found in the visited farms was higher compared with the studies carried out by Phuong *et al*. (2004), which reported that the average stocking density of *P. hypophthalmus* in the pond was 20.5 fish m^{-2}. However, Phan *et al*. (2009) have reported stocking density up to 125 fish m^{-2}. In this study the higher stocking density gave higher fish yield at harvest time which was in agreement with the findings reported by Phan *et al*. (2009) but in the present study no statistically significant relationship between fish yield and stocking density was found associated with the occurrence of the yellow fish condition.

No systematic water quality assessment was performed in this survey but it was the fish farmers' perceptions that some water quality parameters such as unionised ammonia (NH_3) could cause or at least be partly responsible for the yellow discoloured fish. Previous work had suggested that the flesh of *P. hypophthalmus* may become yellow in colour if they are grown in a stagnant pond due to the presence of blooms of blue-green algae, which can develop rapidly in a stagnant pond during hot and dry weather. However, the interviewed farmers in this study were aware that *P. hypophthalmus* ponds with high frequency and high volume exchange of water appeared to have reduced numbers of unhealthy fish and it was found that a variety of methods and sources of water exchange was practised by the *P. hypophthalmus* fish farmers. Wilkinson (2008) also found that yellow coloration could be prevented or would resolve if the pond was 4.5 metres deep with a high water exchange rate at 20-30% per day. Whilst no association was found in this study between water

quality parameters and the yellow colouration, there is no doubt that better water exchange would improve the general health and fitness of the farmed fish.

Whilst there is added value in investigating the seasonal effect reported by fish farmers, no association was found between the duration of yellow colouration and water depth or the amount of water exchange or even the frequency of exchanged water in the *P. hypophthalmus* ponds. In fact, the environmental conditions of the *P. hypophthalmus* ponds were largely influenced through water exchange using the river water which can be affected by waste products, including fish feed, sediment, pathogens from other local *P. hypophthalmus* farms to name only a few. Due to the fish quality control, environmental concerns, and the specific quality requirements for the European export market (i.e. detailed record-keeping, waste-water treatment ponds) the local authorities suggested that *P. hypophthalmus* farmers who construct a new farm must include the waste-water treatment system (MOFI, 2008). However, these requirements are difficult for small-scale farmers to comply with due to the high price of available land in the Mekong Delta (Khoi *et al*., 2008; Phan *et al*., 2009).

During the present study *P. hypophthalmus* farms did not have a waste-water management or treatment systems. At present no definitive water quality parameters have been produced for farmed *P. hypophthalmus*. Instead researchers have measured the water quality parameters in varied farming systems and used these as a guide. Giang *et al*. (2008) reported that the *P.*

hypophthalmus culture environment has total nitrogen (TN), total phosphorus (TP), Biochemical Oxygen Demand (BOD) and hydrogen sulphide (H_2S) levels much higher than the accepted Vietnamese standards for surface water quality (TCVN 5942-1995), as a result of the high stocking density. The intensification of *P. hypophthalmus* production systems has led to an increase in the use of feed applied to water for improved production and this has resulted in more waste being added to the environment from these farms in the form of uneaten feed and fish excretes. Whilst the nutrient enrichment loading in the wastewater and sludge from *P. hypophthalmus* farms may be high this level of discharge into the environment is usually smaller than the potential runoff from fertilizers used in rice field in the Mekong Delta (De Silva *et al*, 2010). Thus, Bosma *et al.* (2009) reported that during discharges of sludge from the ponds, a very high nutrient load in waste-water and sludge may have a potential for causing localized pollution of the canal water, especially when the ponds are located intensively along small canals during the dry season when the water level is low. It may be then that the fish ponds are at risk of contamination by rain water from the paddy fields where fertilizers or pesticides have been used via daily water exchange. It would certainly be of value to conduct a longitudinal study investigating the water quality parameters on the incidence of yellow colouration to fully explore the association. The water quality in the *P. hypophthalmus* farms is explored further in the clinical chemistry chapter (Chapter 3).

The farmers interviewed in this study considered that disease outbreaks occurred more frequently during the flooding season and it was too hard for

them to control disease outbreaks. These were outbreaks in general but according to the farmers, most of the yellow colouration problem mainly occurred during the rainy and flood seasons. During the rainy and flood seasons the water from the Mekong River changes due to a high volume discharge, resulting in high turbidity and pollution load (Ko and Lee, 2000). In the Mekong Delta, the mean annual rainfall is about 1,600 mm, of which more than 80% falls during the rainy season (Statistical office of Can Tho City, 2008), which compact the soil and increase runoff and soil erosion. According to Bash *et al*. (2001) changes in turbidity can have both a direct and indirect effect on fish by injuring their gills which can also provide an opportunity for primary and secondary pathogens to invade and establish. Research indicates that fine particles are mostly responsible for increases in plasma glucose, plasma cortisol, and blood sugar levels in fish species (Servizi and Martens, 1987; Redding *et al*., 1987; Servizi and Martens, 1992). These are all indicators of stress, and can impact physiological systems, cause reduce growth and decrease resistance of the host to disease (Svobodová *et al*., 1993). Increased disease and subsequent mortality of fresh water fish in the Mekong Delta are regularly observed during the flood season probably influenced by runoff from agriculture and urban waste water (Poisson, pers. comm., 2005). The findings from this survey study also confirmed that there was an increase in yellow colouration or disease in general during the flood season.

Vietnamese fish farmers were aware of many different disease conditions affecting their stocks. Yellow colouration was only one of these and it appeared

to occur with and without the presence of other diseases. Nevertheless, the data from this survey confirmed that yellow colouration on its own did not cause high mortality but contributed towards morbidity. As mentioned previously, the farmers in this study also reported that after completion of antibiotic treatment for Bacillary necrosis of catfish (BNP), the fish developed a yellow colouration. The development of *P. hypophthalmus* in recent years has been affected by the frequent occurrence of infections disease such as BNP caused by the bacterium identified as *E. ictaluri*. Bacillary Necrosis of catfish is a serious and economically significant bacterial infection in the Mekong Delta (Crumlish *et al*., 2002; Dung *et al*., 2004), which causes 50-90% mortality during a single outbreak (Dung *et al*., 2004). The present study did not investigate the relationship between BNP and yellow colouration. However, both BNP and antibiotic treatments can contribute towards complete liver failure in the affected fish, which can produce jaundice due to the impaired metabolism of bilirubin in the damaged liver cells (Polson and Lee, 2005). The perception associated with yellow coloured *P. hypophthalmus* in Vietnam is that this condition is occurring more frequently, but whether this is a true association or a reflection of raised awareness and improved experience of the fish farmer is unknown. However, it would appear that as a health problem, yellow discolouration alone does not significantly appear to cause high mortalities in the farms. It is often difficult to treat grumbling morbidity, particularly when the fish are kept at high stocking densities and so the presence of the yellow colouration would likely have enabled secondary infections to occur.

The interviewed farmers made clear that they have little knowledge about yellow colouration in their fish and because of this they tend to use multiple chemicals/drugs applied during the production cycle. The present study highlighted that farmers are intensively using such chemicals/drugs cocktails in *P. hypophthalmus* farming to prevent or in an attempt to treat yellow colouration in their fish stocks. It is impossible to list all the chemicals/drugs used by the fish farms as some of them are being used discreetly and often in isolated cases. However, the findings clearly showed that such chemical treatments are less effective because of limited farmers' knowledge and their lack of awareness of prudent practices. Thus, there is an urgent need to improve the farmers' knowledge about improving health management of their fish stocks and in particular, therapeutic management to reduce the incidence of yellow colouration. So, that any treatment can be applied in a cost effective manner to prevent and at the same time minimize any associated side effects including poor environmental or health hazards. It is recognised that giving advice on therapeutic treatments against yellow colouration is difficult at this time as no single variable or pathogen was associated with the condition, however, by improving the overall chemical/drug administration on these farms there would be an expected improvement of the overall health of the farmed fish population.

Vietnamese catfish farmers have to endure financial looses on their farms during the production cycle from yellow discolouration combined with other diseases. However, the effect of this condition extends further, past the production and into the harvest sector. As yellow coloured fish are often

considered unmarketable due to the yellowish presentation of the fish flesh. This is particularly a problem should the farmer wish to sell his product on the export market. At the current time, the colour and size of the fish at harvest are important for the price of fish and the export markets available for the products. Therefore it is not just the fish losses that affect the production sector but the actual profit gain by the fish farmer as fish being downgraded due to yellow discolouration and the fillets fetch lower price in the market even lower than their production cost. According to Phan *et al.* (2009) the average production cost per kg of fish was 14,200 VND (ranged from 11,000 VND to 17,000NVD). In recent years, pond-gate price paid by processors for live *P. hypophthalmus* based on the colour of the fillets were 16,800 VND kg^{-1}, 16,400 VND kg^{-1} and 15,200 VND kg^{-1} for white, pink and light yellow fillets, respectively (Featherstone, 2010). However, the fish with external signs in dark yellow colour will be rejected at the pond-side or at the processing plants before they are unloaded from the boats. These rejected fish can only be sold for a much lower price (approximately 8,000 to 10,000 VND kg^{-1} which reduced value by more than 50%) on the domestic markets or as dried fish product. These economic losses led to severe problems for the sustainable production of *P. hypophthalmus*.

The central findings of the present study indicated that yellow colouration is a disease condition which has a significant impact for the *P. hypophthalmus* farming industry. None of the previously suggested variables were confirmed in this study as true associations. A single pathogen could not be identified in

association with the yellow colouration in *P. hypophthalmus*. The present study strongly suggests that affected fish frequently suffer from other infections, and whether these fish are more susceptible to opportunistic pathogens due to the yellow discolouration is uncertain but likely. Further studies are required to monitor this and other disease conditions in the *P. hypophthalmus* production units in Vietnam, not only to find out the prevalence but to confirm the significance. This could be performed using longitudinal studies measuring a range of environmental, biological and husbandry parameters in several *P. hypophthalmus* farming systems over many production cycles.

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Clinical description of jaundice in farmed catfish (*Pangasianodon hypophthalmus*, Sauvage) in the Mekong Delta, Vietnam

3.1. Abstract

The presence of yellow colouration in the skin of the abdominal area, sclera of the eyes and fin bases is a serious health problem affecting the catfish aquaculture industry in Vietnam. The causes of this problem are unknown but outbreaks continue to reduce the capacity for sustainable production in Vietnam. The aim of the present study was to investigate and describe the clinical characteristics in affected fish in five provinces of the Mekong Delta. Externally, the fish sampled exhibited a yellow colouration in the skin of the abdominal area, sclera of the eyes and fin bases. Internally, they had pale gills (67%), yellow ascitic fluid (60%), yellow flesh (56%), enlarged liver, kidney, and spleen (54%), dark blue bile in the swollen gall bladder (48%), and swollen common bile duct (27%). The total erythrocyte counts, haematocrit, haemoglobin concentration, and mean corpuscular haemoglobin concentration (MCHC) were significantly reduced in affected fish compared with apparently normal fish, while mean corpuscular volume (MCV) was significantly increased ($p < 0.001$). The total, direct and indirect bilirubin of fish was significantly higher than that of apparently normal fish, indicating the yellow colouration was a form of jaundice or icterus. Histological examination revealed a number of serious pathologies in the affected fish. The study would suggest that the observed

yellow colouration in *P. hypophthalmus* is a form of jaundice but not associated with any single pathogen.

Key words: Jaundice, Vietnamese catfish (*Pangasianodon hypophthalmus*), the Mekong Delta.

3.2. Introduction

Since 2000, intensive farming of *Pangasianodon hypophthalmus* catfish in the Mekong Delta of Vietnam has become popular and grown exponentially. The Fisheries Directorate (2010) stated that catfish production amounted to 1,141,000 tonnes in 2010 and has contributed to the continued expansion of farming areas and increased exported products to 136 countries worldwide. Whilst catfish farming in the Mekong Delta appears profitable, disease outbreaks continue to affect growth and sustainable production.

Varied disease conditions have been reported to occur in these farmed fish species and they may be pathogen or non-pathogen driven. These fish suffer from parasite, fungal, and bacterial disease as well as those more likely to be associated through sub-optimal environmental conditions or inadequate husbandry practises. In 2006, Dung described a clinical condition affecting the farmed *P. hypophthalmus* where the fish had a yellow colouration in the skin of the abdominal areas, eyes and fins. Whilst this condition does not always result in mortality is can lead to significant morbidity in the fish stocks (Dung 2006). This condition had been observed in the farms at varied levels for several years (Crumlish pers. comm.). However, it is only over the last 6-7 years that the

impact of this condition has been investigated and this is mostly because yellow coloured fish are rejected or the fillets are downgraded by the processing plants at post-harvest, thus reduced value of affected fish means less income per production site.

The cause of the yellow colouration in *P. hypophthalmus* has not yet been identified, although many factors have been suggested. In a field based study performed (Chapter 2) no associations between farming management practises and outbreaks of yellow coloured *P. hypophthalmus* were found. The condition would appear to be widespread within all the production areas of the Mekong Delta, Vietnam and no obvious association between feed or water sources or fingerlings were found (Chapter 2). While the yellow colouration observed in farmed *P. hypophthalmus* has been referred to as jaundice this has not previously been confirmed. Jaundice is a clinical condition resulting from the accumulation of the bile pigments in the tissues but whether this is the cause of yellow colouration in these fish is currently undetermined.

Ferguson (1989) reported that jaundice was not a commonly observed clinical feature in fish possibly due to the type of bile pigment produced by the fish, although the different bile pigments produced does vary between species (Cornelius, 1991). The causes of fish jaundice have varied but included accumulation of algal pigments such as zeaxanthin (Lee, 1987) and other dietary substances (Li *et al.*, 2007). Jaundice has been reported in other farmed catfish species in Thailand (*Clarias gariepinus*) again there was a dietary association from oxidative damage to lipids and vitamin E removal in the rancid

chicken viscera used in the feed (Pearson *et al.*, 1994). In terrestrial animals jaundice outbreaks may be associated with obstruction of the common bile duct by nematodes (Otto *et al.,* 2000).

Therefore, it would appear that there are varied causes of jaundice in other fish species. Previous studies on *P. hypophthalmus* have focused on identification of specific pathogens or risk factors associated with the condition whereas a complete clinical description has not yet been produced. The aim of the current study was to provide a clinical description of the yellow coloured *P. hypophthalmus* farmed and selected from registered farms in Vietnam.

3.3. Materials and methods

Site visits were conducted from 28th April to 20th August 2008 and 12th October to 10th November 2009. All visited sites produced fish in earthen ponds and were distributed throughout the main production areas of Mekong Delta, Vietnam. A total of 34 *P. hypophthalmus* grow-out farms were visited and were located in Vinh Long, Can Tho, Ben Tre, Dong Thap, and An Giang provinces, the Mekong delta, Vietnam. The fish farms were selected randomly from the fisheries profile of the provincial fisheries departments, where a list of *P. hypophthalmus* farms in the region and their locations was obtained and those that could be visited and sampled within a day were approached. These farmers were contacted by phone prior to site visits to help with compliance.

3.3.1. Water quality parameters

Water temperature was measured using a thermometer, dissolved oxygen (DO), pH, and total ammonia-N were measured once between 9 am-2 pm using aquarium test-kits (Sera Heinsberg, Germany). The level of unionised ammonia (NH_3) was calculated from total ammonia-N using pH and temperature measurement following the manufacturer's instruction. In addition, the water quality parameters were checked from the farmer records.

3.3.2. Fish

All the yellow coloured fish in this study were identified by a case definition created from previous literature and based on the gross external presentation (Hung, 2004; Dung, 2006). The case definition was: ''Yellow colouration is a condition of *P. hypophthalmus* presented as gross external signs as yellow pigmentation in the skin of the abdominal area, sclera of the eyes, and fin bases".

At each site the sampled fish were collected from a single pond by the farm manager using a small cast net. These fish had been fed with a wide range of commercial pelleted diets, typically found in Vietnam. Prior to sampling, collected fish were examined for any gross clinical signs of disease. Then a standardised necropsy was performed and all information recorded. Samples were taken for haematological, bacteriological, parasitological and histopathological analyses.

3.3.3. Haematology

Blood samples were taken from the caudal vein using 1 ml sterile plastic syringe with a 23-guage needle (Vinahankook Medical Supplies Co. Ltd, Vietnam). Whole blood was collected from each fish into two different clean centrifuge tubes: one with EDTA (Ethylene Diamine Tetraacetic Acid) used for haematological investigations and another without anticoagulant was used to obtain serum for measurement of bilirubin concentration. Erythrocyte counts were performed with a Neubauer chamber using a stain solution (1:200, v/v; 38 g sodium citrate, 20 ml 37-40% formalin, 0.2 g toluidine blue, distilled water in q.s.p 1000 ml) (Oliveira-Junior *et al.*, 2009). Total leukocyte counts were performed with a Neubauer chamber after the blood was diluted (1:20, v/v) with Turk's solution (Blaxhall and Daisley, 1973). Hematocrit (Ht) was determined by microhematocrit centrifugation at 12,000 rpm for 5 minutes, and expressed in percentage packed cell volume (PCV). Haemoglobin concentration (Hb) was determined using diagnostic kits (Sigma Chemical Company Ltd, Poole, Dorset, UK). Mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentrations (MCHC) were calculated from Ht, Hb and erythrocyte values (Penev and Dukova-Peneva, 2007).

The blood without anticoagulant was left to clot overnight at 4^{o}C, and centrifuged at 3500 rpm for 10 min. Serum was separated from the blood clot with a micropipette into aliquots of 100 µl for bilirubin measurement. Total and direct bilirubin was measured by a photometric test (Diagnostic Systems, Holzheim, Germany) with the Jendrassik-Gróf method (Jendrassik-Gróf, 1938) following the manufacturer's instruction. Additional indirect bilirubin value was

calculated by subtracting the direct bilirubin value from the total (Cheesbrough, 2005).

3.3.4. Parasitology

After blood sampling the fish were examined for the presence of nematodes in the common bile duct. Each fish was dissected and the common bile duct was removed and placed into a sterile Petri dish containing 10ml saline solution 0.85% (w/v) and slit longitudinally to expose any nematodes. All nematodes discovered were washed in saline solution (0.85% w/v), fixed and preserved in 70% (v/v) ethanol and cleared with glycerine for light microscopy examination (Moravec *et al.*, 1997).

3.3.5. Bacteriology

Bacteriological samples were taken aseptically from the kidney, liver and spleen. The abdomen of the fish was opened and the internal organs gently moved to one side being careful not to rupture the gut. A sterile bacteriology loop was then carefully inserted into the liver, kidney or spleen of sampled fish and inoculated onto a Tryptose Soya Agar plate (TSA, Oxoid, Basingstoke, UK). These were sealed using Nesco film (Bando Chemical IND. Ltd. Nescofilm) and then incubated at 28^{o}C for 48 h. All plates were checked for colony growth after 24 h and primary bacterial identification tests performed. These included Gram staining, catalse, oxidase, and motility following the methods of Frerichs and Millar (1993). The biochemical profiles of the Gram-negative bacteria were performed using API 20E-identification kit (BioMérieux, France), following the manufacturer's instructions but modified for a temperature of incubation of 28^{o}C

(Crumlish *et al.,* 2002). Gram-positive cocci were identified presumptively using Chapman-Agar (BioMérieux) for *Staphylococcus* (Rollo *et al.*, 2006) and Pastorex strep agglutination test (Bio-Rad, Marnes-la-Coquette, France) for *Streptococcus* (Both *et al.*, 2005).

3.3.6. Histopathology

Samples of the liver, spleen, gills, kidney, skeletal muscle, and common bile duct were fixed in 10% (v/v) neutral buffered formalin solution for 24 h, transferred to 70% (v/v) ethanol and processed following routine methods (Chopra *et al.*, 2008). Five-micron thick sections were cut and stained with haematoxylin and eosin (H&E) and Perls' Prussian blue stain, which was used for examination of haemosiderin deposits (Perls, 1867). The sections were also stained with DMAB-Rhodanine (Di-methyl-amino-benzylidene rhodanine) for copper in the liver and Alcian blue pH 2.5 for goblet cells/mucous cells in the common bile duct.

3.3.7. Data Analysis

All data management and analyses were conducted in Microsoft Excel 2007^{TM} (Microsoft, USA), Statistical Package for Social Science version 16 (SPSS 16). Statistically significant differences were tested with non-parametric Mann-Whitney U-test for continuous variables and Fisher's Exact (FE) for dichotomous variables with 95% confidence limit interval as described by Sokal and Rohlf (1981). Further analysis was performed on the different clinical presentations seen exclusively in yellow coloured fish using Ward's linkage method (Hill and Lewicki, 2007).

3.4. Results

3.4.1. Water quality measurements

The water temperature (^{o}C) and dissolved oxygen was similar in all provinces (Table 3-1). However, the pH, total ammonia and unionised ammonia were more varied between farms and provinces.

Table 3-1 Range of water quality parameters measured in the fish ponds

Parameters	Location				
	An Giang	Dong Thap	Vinh Long	Ben Tre	Can Tho
			Mean (Range)		
Temperature (^{o}C)	29 (29)	29 (28-30)	29 (28-30)	29 (29)	29 (29)
Dissolved oxygen (mg l^{-1})	3.8 (3.5-4.0)	4.2 (3.5-5.0)	4.6 (3.5-6.0)	3.5 (3.5)	4.0 (4.0)
pH	6.8 (6.5-7.0)	7.0 (6.5-7.5)	7.2 (6.9-7.5)	6.8 (6.5-7)	6.5 (6.5)
Total ammonia-N (mg l^{-1})	3.0 (1.0-5.0)	2.1 (0.5-5.0)	1.5 (0.5-5.0)	1.5 (1.0-2.0)	5.0 (5.0)
Unionised ammonia (NH_3) (mg l^{-1})	0.018 (0.006-0.03)	0.014 (0.003-0.03)	0.015 (0.006-0.03)	0.008 (0.006-0.01)	0.03 (0.03)

3.4.2. Fish

A total of 357 *P. hyophthalmus* were sampled from 34 sites in the Mekong Delta. Of these, 202 were moribund and consistent with the case definition for yellow colouration. Table 3-2 shows total numbers of fish sampled and classification categories based on gross external clinical signs.

Table 3-2 Number of farms visited, fish sampled and gross clinical signs

Province	Number of farms	Number of fish sampled			
		Total	Apparently normal	Yellow coloured	Other clinical signs*
Vinh Long	12	122	22	67	33
Dong Thap	17	175	40	90	45
An Giang	2	32	1	28	3
Ben Tre	2	21	5	14	2
Can Tho	1	7	1	3	3
Total	34	357	69	202	86

**: with the clinical signs of bacillary necrosis Pangasius or red spots/haemorrhagic symptoms*

In the present study, mean weight of apparently normal fish was 421g (± 335; SD) and ranged from 25 to 1,700 g and mean weight of yellow coloured fish was 468g (± 274; SD), ranged from 60 to 1,800 g (Figure 3-1). Yellow coloured fish demonstrated abnormal swimming behaviour including crowding at the water surface and near the inflowing water. Gross external and internal abnormalities in the sampled fish are summarised in Table 3-3 and Plate 3-1. Several of the internal pathological changes were significantly more prevalent in moribund yellow coloured fish.

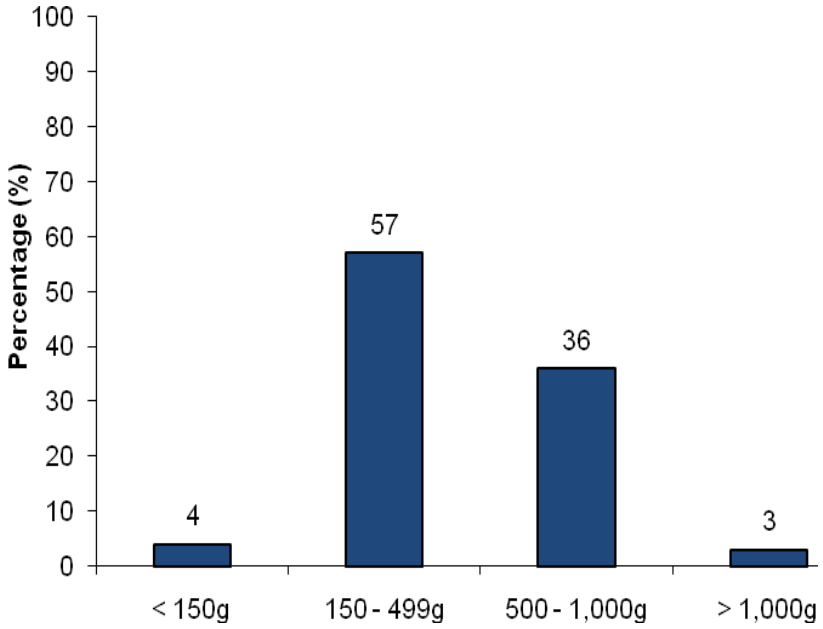
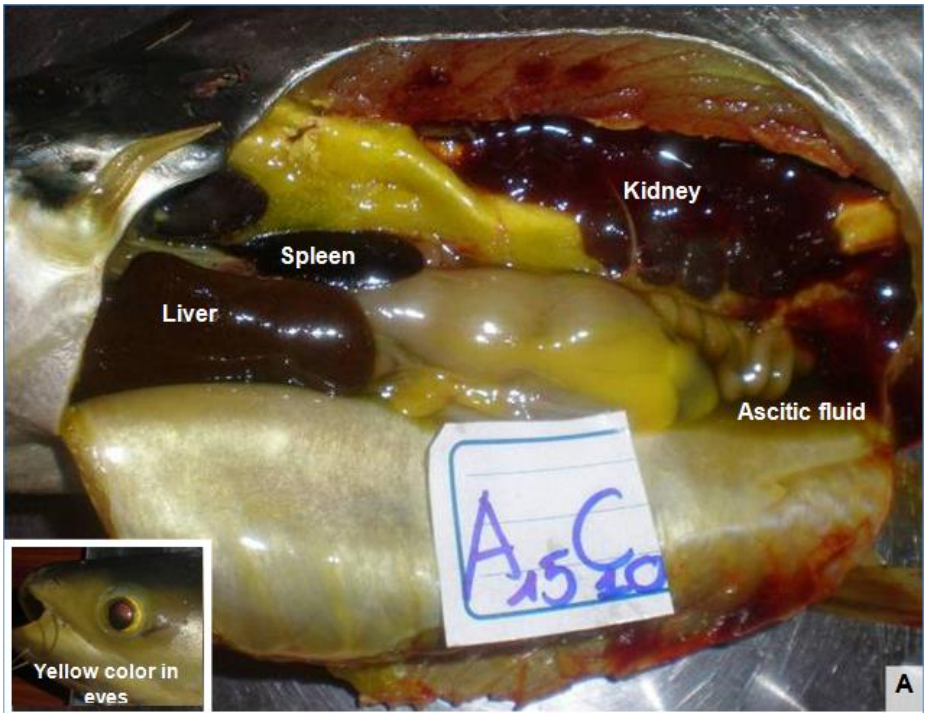


Figure 3-1 Weight range of yellow coloured *P. hypophthalmus* sampled expressed as % per category



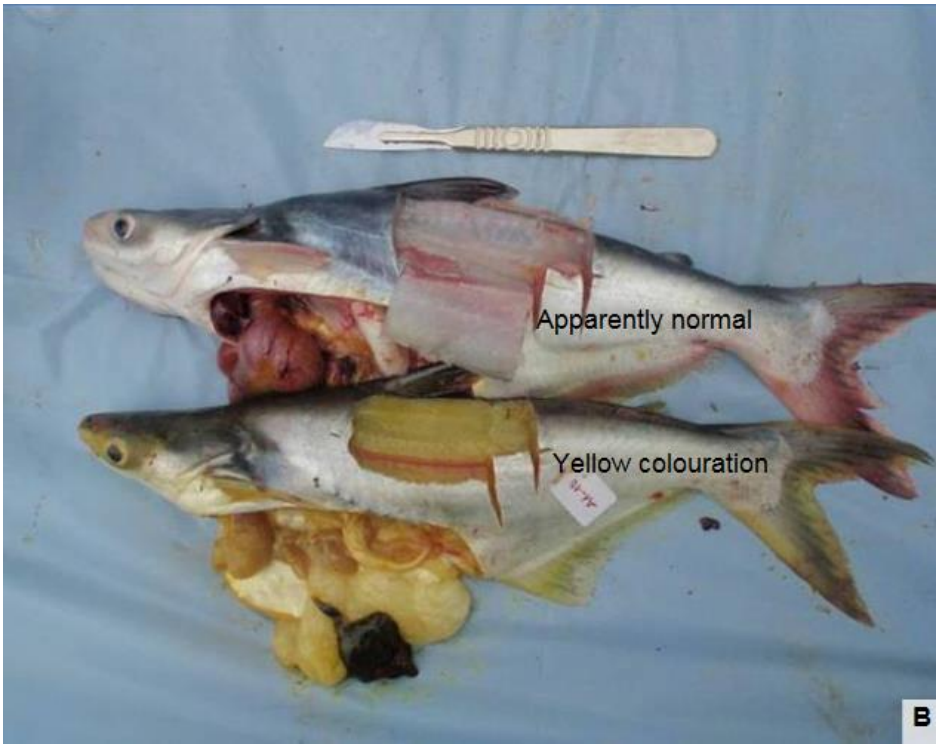


Plate 3-1 Gross pathology of *P. hypophthalmus* with yellow colouration (A-Internal gross signs in yellow coloured fish**, B-**Yellow flesh**)**

Table 3-3 Gross external and internal signs in all fish sampled

	Description	Frequency in sampled population (%)	Relative % yellow coloured fish*	OR (95% CI)	P-value (FE)
External	Yellow colouration in the skin of abdominal area, sclera of the eyes and fin bases	202 (57%)	100%	**	**
	Pale gills	175 (49%)	67%	6.1 (3.8-9.8)	< 0.001
Internal	Yellow ascitic fluid in abdomen	123 (34%)	60%	234.9 (32.2-1711.9)	< 0.001
	Yellow flesh	114 (32%)	56%	195.5 (26.8-1424.5)	< 0.001
	Swollen liver, kidney or spleen	134 (38%)	54%	6.5 (3.9-10.9)	< 0.001
	No feed in stomach	106 (30%)	50%	38.5 (13.7-107.9)	< 0.001
	Gall bladder is enlarged and dark blue	112 (31%)	48%	7.9 (4.4-14.1)	< 0.001
	Friable liver, kidney or spleen	109 (31%)	42%	3.97 (2.4-6.7)	< 0.001
	Yellow fat	77 (22%)	33%	6.4 (3.2-12.5)	< 0.001
	Pale liver	78 (22%)	27%	2.1 (1.2-3.6)	0.005
	Swollen common bile duct	104 (29%)	27%	0.8 (0.5-1.2)	0.15
	Any internal haemorrhage	116 (32%)	26%	0.5 (0.3-0.8)	0.003
	Pale kidney	48 (13%)	18%	2.6 (1.3-5.2)	0.004
	Yellow ascitic fluid become coagulated in air	12 (3%)	6%	N/A	< 0.001

** Relative to the total number of fish where each pathological change was observed. OR: odds ratio of the association with yellow coloured fish; 95% CI: 95% Confidence Interval; FE: Fisher's exact test; **: no analysis; N/A: no result.* $N_{total} = 357$

Hierarchical cluster analysis was used to analyse the possibility of different gross internal presentations associated with yellow colouration in *P. hypophthalmus*. It identified the presence of three distinct clusters identified as A-C, and within the population of yellow coloured fish sampled (Figure 3-2).

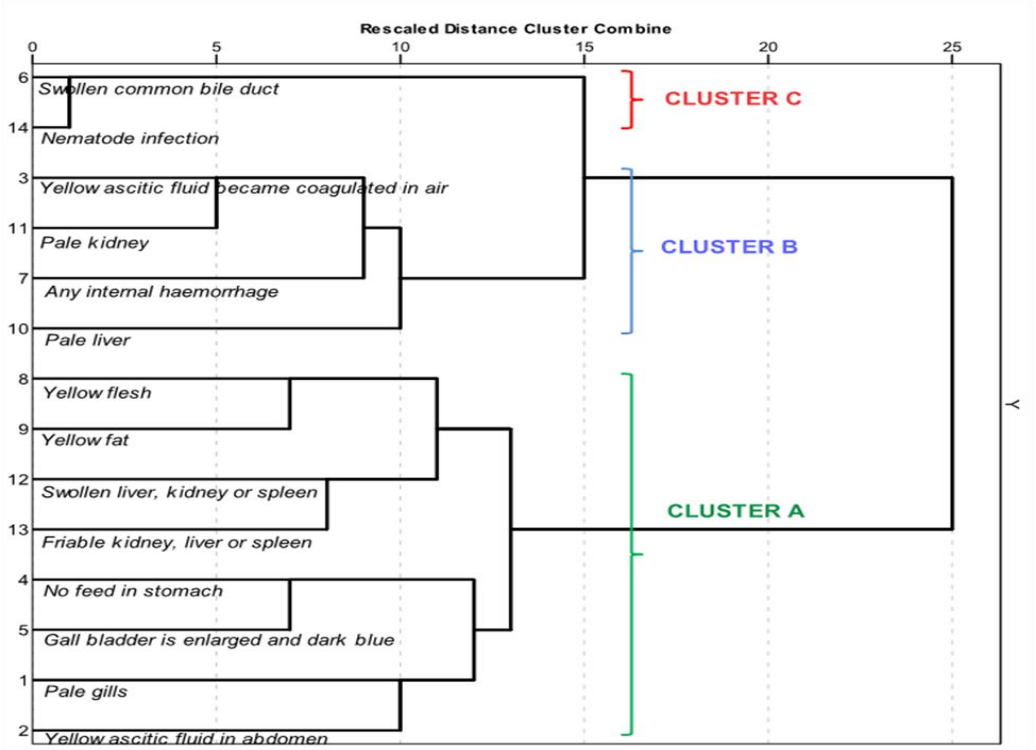


Figure 3-2 Cluster analysis of gross pathological signs in yellow coloured *P. hypophthalmus* (n = 202)

Gross pathological changes observed in the largest cluster A were found in 33-67% of yellow coloured fish, and significantly more frequently in the yellow coloured fish. These pathological changes are not commonly reported in other previous studies for other catfish diseases. While, cluster B and cluster C presentations included pathological changes that were infrequent in the yellow coloured fish. Cluster B and C revealed that these pathological signs can be found in other disease entities, including bacterial and parasitic conditions.

3.4.3. Haematology

Blood samples from affected fish varied in colour from yellow to orange to red (Plate 3-2).

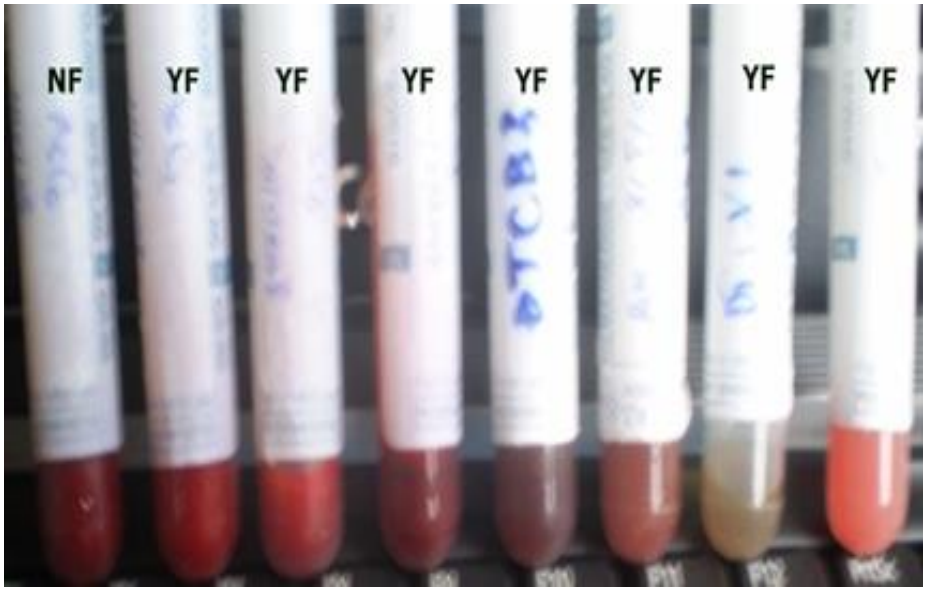


Plate 3-2 Blood of sampled fish (NF: Apparently normal**; YF:** Yellow coloured**)**

Due to constraints on time and budget only 165 out of 357 from apparently normal fish and yellow coloured fish were used for haematological parameter measurement (Table 3-4) and 75 out of 357 sampled fish were examined for bilirubin measurements (Table 3-5).

Table 3-4 Haematological values for sampled *P. hypophthalmus*

Analyte	Apparently normal (n = 52)		Yellow coloured (n = 113)	
	Mean (± SD)	Range	Mean (± SD)	Range
Erythrocyte (10^6/mm^3)	2.2 (± 0.6)^a	1.2-4.0	0.2 (± 0.4)^b	0.01-2.2
Leukocyte (10^3/mm^3)	15.9 (± 9.9)^a	5.6-52.8	26.8 (± 15.7)^b	4.8-81.2
Hematocrit (Ht., %)	30.5 (± 6.5)^a	19.0-45.5	6.7 (± 6.7)^b	1.0-38.0
Haemoglobin (Hb., g dl^{-1})	15.0 (± 4.1)^a	8.0-22.0	1.5 (± 3.2)^b	0.1-21.0
MCV (fl)	147.7 (± 34.4)^a	74.0-261.4	743.6 (± 1,386.6)^b	55.6-10,000
MCHC (g dl^{-1})	49.0 (± 7.8)^a	31.6-62.7	21.5 (± 16.0)^b	0.5-64.6

Note: Mean (± standard deviation (SD)). Mean values followed by different superscript letters in the rows are significantly different (P < 0.001)

As shown in Table 3-4, total erythrocyte counts, Ht value and Hb concentration, were significantly reduced (P < 0.001) in yellow coloured fish compared with apparently normal fish, indicating anaemia. This is shown in Plate 3.3.

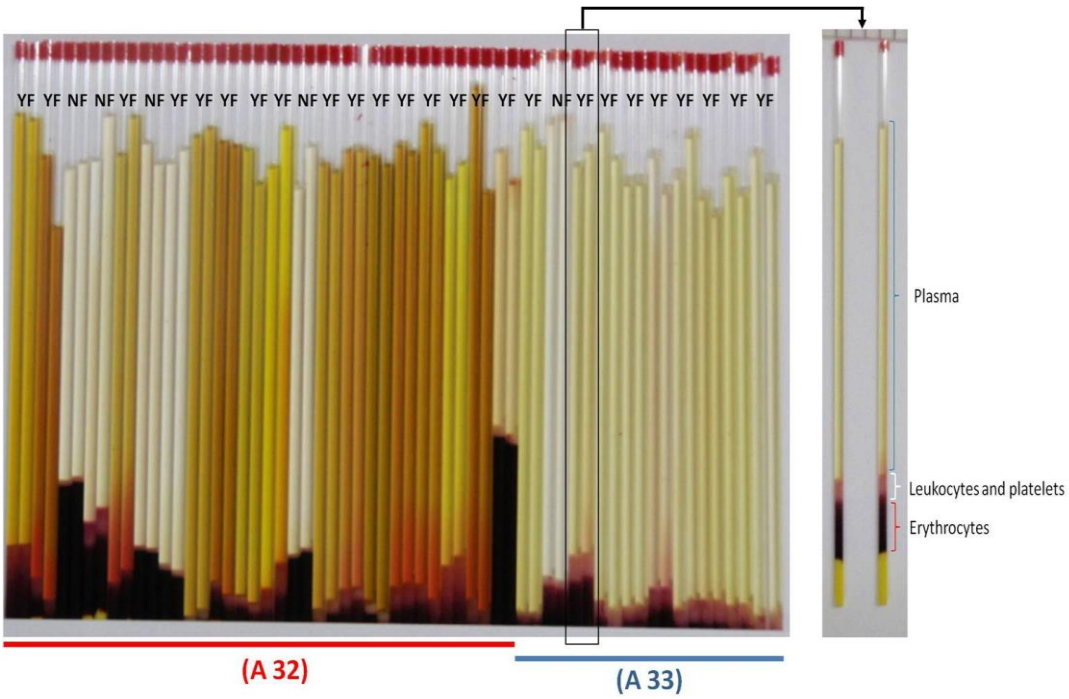


Plate 3-3 Microhaematocrit tubes with blood sampled from yellow coloured fish showing reduced packed cell volume (PCV)
(NF: Apparently normal; **YF**: Yellow coloured**; A 32:** the fish sampled at the 19th day of the yellow colouration outbreak; **A 33:** the fish sampled at the 2nd day of the yellow colouration outbreak)

The Mean Corpuscular Volume (MCV) was significantly increased in affected fish (P < 0.001) compared with apparently normal animals which indicated hypochromic macrocytic anaemia. Most of the immature erythrocytes, as well as fragmented erythrocytes were found in the circulating blood from severely affected fish. Red blood cells appear as “helmet” cells (keratocyte), “tear drop” cells (dacrocyte), and other irregular shapes when examined on the blood smears from yellow coloured fish (Plate 3-4). The total leukocyte counts of yellow coloured fish were significantly higher compared with apparently normal

fish 15.9 x 10^3/mm^3 (± 9.9; SD) (P < 0.001, Table 3-4). A similar increase was found in serum concentration of total, direct and indirect bilirubin (Table 3-5, P < 0.001).

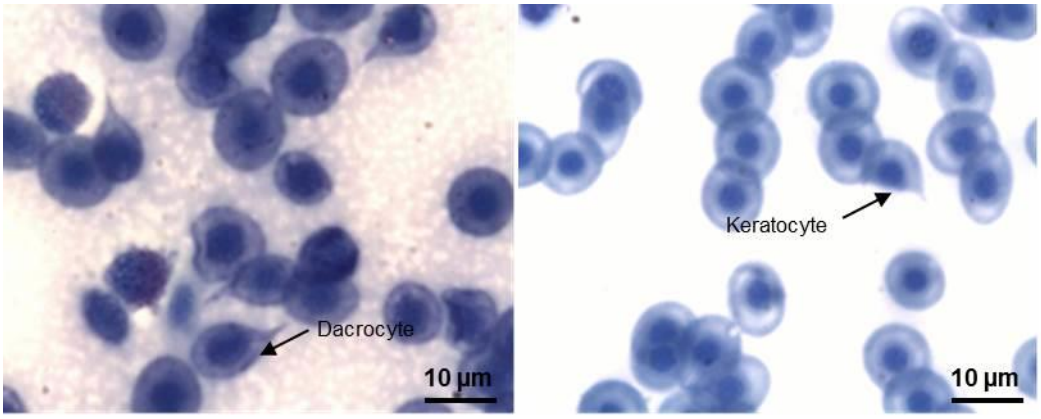


Plate 3-4 Giemsa-stained blood smears showing the irregular shapes of erythrocytes from yellow coloured fish (arrow)

Table 3-5 Bilirubin measurements for *P. hypophthalmus*

Analyte	Apparently normal (n = 13)		Yellow coloured (n = 62)	
	Mean (± SD)	Range	Mean (± SD)	Range
Total bilirubin (mg dl^{-1})	0.3 (± 0.2)[a]	0.08-1.00	4.1 (± 2.9)[b]	0.3-11.4
Direct bilirubin (mg dl^{-1})	0.1 (± 0.1)[a]	0.04-0.50	2.0 (± 1.9)[b]	0.01-10.08
Indirect bilirubin (mg dl^{-1})	0.2 (± 0.3)[a]	0.02-0.90	2.1 (± 1.5)[b]	0.01-7.40

Note: Mean (± standard deviation (SD)). Mean values followed by different superscript letters in the rows are significantly different (P < 0.001)

3.4.4. Parasitology

Nematodes were observed in the swollen common bile ducts (Plate 3-5) in both apparently normal and yellow coloured fish.

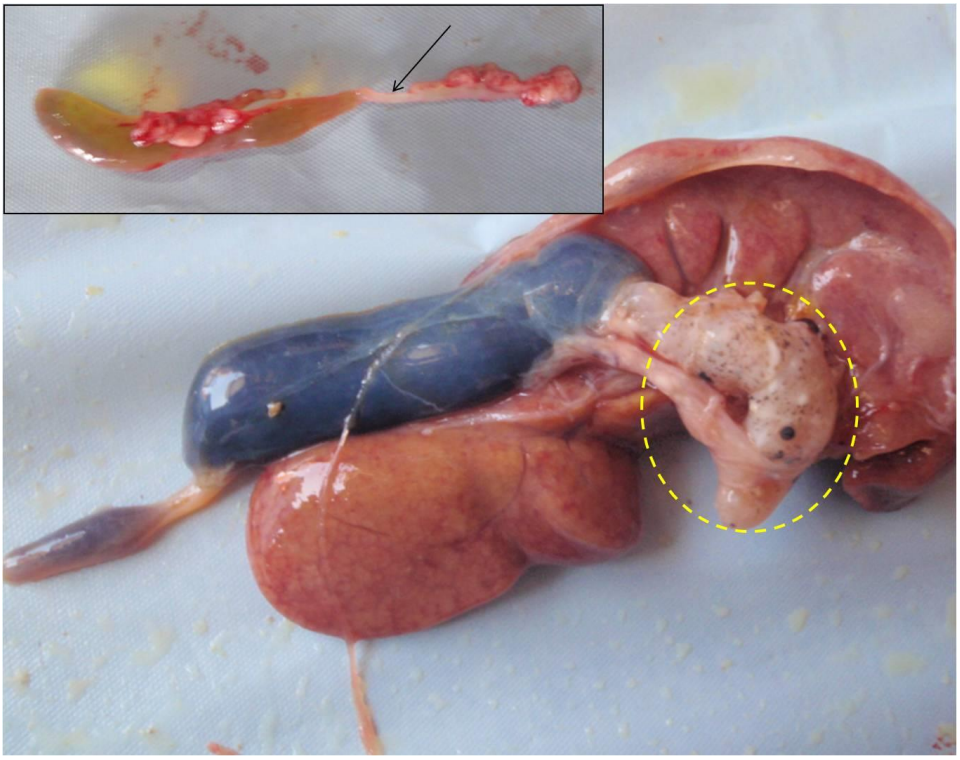


Plate 3-5 Swollen common bile duct found in the yellow coloured fish (broken circle) and the normal sized common bile duct in the apparently normal fish (arrow)

Prevalence of the nematodes in the common bile duct was 22% and 27% in apparently normal and yellow coloured fish respectively, and the nematodes were morphologically consistent with *Cucullanus* spp. The worms appeared round in cross-section and elongated, with a simple digestive tract (Plate 3-6A) and the head end was rounded. The oral opening was dorsoventrally elongated, surrounded by narrow membranous collarette with row of small teeth at its base (Plate 3-6B). The esophagus opened into the intestine through a valve (Plate 3-6C). The tail was conical and pointed (Plate 3-6D). The number of *Cucullanus* found in the common bile ducts of both yellow coloured and apparently normal fish ranged from 1 to 16.

Plate 3-6 Parasites were found from sampled *P. hypophthalmus*.
A-General view of *Cucullanus* sp; **B, C-**Photomicrograph of anterior end of *Cucullanus* sp to show buccal cavity and oesophagus; **D-**Photomicrograph of posterior end of *Cucullanus* sp

3.4.5. Bacteriology

From the apparently normal fish no more than 10 bacterial colonies were recovered from each fish from any organ and those were identified and considered as environmental contaminants. Bacterial growth was recovered from 19% of the yellow coloured fish but no single aquatic bacterial pathogen was consistently found in the yellow coloured fish. The primary identification results showed that 65% of the isolates recovered from affected fish were identified as Gram negative rods and 35% were Gram positive cocci (Table 3-6).

Table 3-6 Bacterial isolation from yellow coloured fish during the present study

Clinical signs	Number of isolates				
	E. ictaluri	*A. salmonicida* subsp. *salmonicida*	Staphylococcus spp	Un-identified	Total
Yellow coloured alone	2	1	6	8	17
Yellow coloured with bacillary necrosis	18	0	1	1	20
Yellow coloured with haemorrhage	0	0	1	0	1
Total	20	1	8	9	38

Further identification of the Gram negative rods found that 18% were *Edwardsiella ictaluri*, 9% were *Aeromonas salmonicida* subsp. *salmonicida* and the remaining 73% were not identified. The biochemical reaction of isolated *E. ictaluri* and *A. salmonicida* subsp. *salmonicida* are shown in Table 3-7. The Gram positive isolates were presumptively identified as *Staphylococcus* spp. as they were all white colonies on Chapman agar, catalase positive and oxidase negative (Barrow and Feltham, 1993). No further identification tests were performed on these isolates.

Table 3-7 Biochemical profiles from isolates recovered from yellow coloured *P. hypophthalmus* in API-20E test

Biochemical test	*E. ictaluri*		*A. salmonicida spp salmonicida*	
	No. +ve reaction	No. –ve reaction	No. +ve reaction	No. –ve reaction
Gram	0	13	0	1
Mobility	0	13	1	0
Oxidase	0	13	1	0
Catalase	13	0	0	1
Oxidation of glucose (OF-O)	13	0	0	1
Oxidation of glucose (OF-F)	13	0	1	0
Triple sugar Iron test (TSI)	Glucose fermentation only (K/A)		-	-
Beta-galactosidase (ONPG)	0	13	0	1
Arginine dihydroxydase	0	13	0	1
Lysine decarboxydase	13	0	0	1
Ornithine decarboxydase	13	0	0	1
Citrate	9	2	0	1
Na thiosulfate (H_2S)	0	13	0	1
Urease	0	13	0	1
Trytophane deaminese	0	13	0	1
Indole	0	13	0	1
Voges-Proskauer reaction	0	13	0	1
Gelatinase	8	5	1	0
Glucose fermentation	13	0	1	0
Mannitol fermentation	0	13	1	0
Inositol fermentation	0	13	0	1
Sorbitol fermentation	0	13	0	1
Rhamnose fermentation	0	13	0	1
Saccharose fermentation	0	13	0	1
Melibose fermentation	0	13	0	1
Amygdaline fermentation	0	13	0	1
Arabinose fermentation	0	13	0	1
Nitrogen dioxide (NO_2)	13	0	1	0
Nitrogen gas (N_2)	13	0	0	1
Macconkey	13	0	1	0
API profile index	00400400		000610455	

3.4.6. Histopathology

There was mixed and varied pathology in 69% of the yellow coloured fish sampled. The remaining 31% had no other associated clinical signs except yellow colouration. The sections were all negative for copper as tested by DMAB-Rhodanine stain.

The gills of 95% of the yellow coloured fish sampled had various pathologies including multifocal epithelial hyperplasia (Plate 3-7) and fusion at the base and

the tips of the lamellae with some lamellar aneurism (telangiectasis) (Plate 3-8). There was an increase in the numbers of goblet cells particularly on the tip of the lamellae (Plate 3-9). Separation of lamellar epithelium (Plate 3-10) and the presence of numerous blood cells in the secondary gill lamellae were observed.

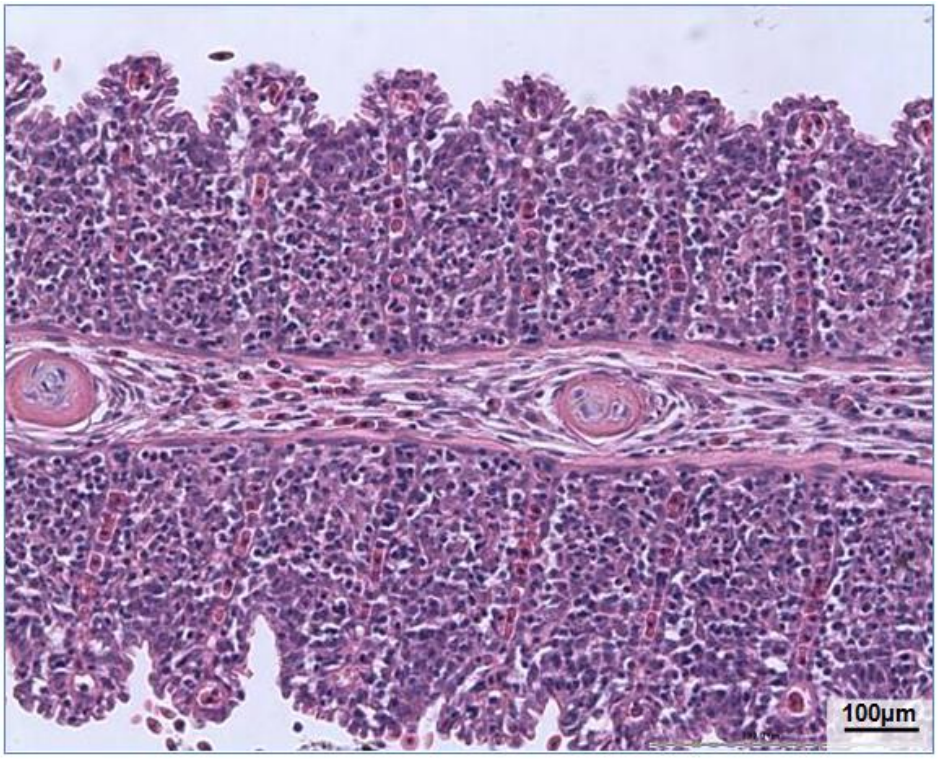


Plate 3-7 Gill lamellar epithelial hyperplasia in affected fish, associated with the presence of inflammatory cells (H&E)

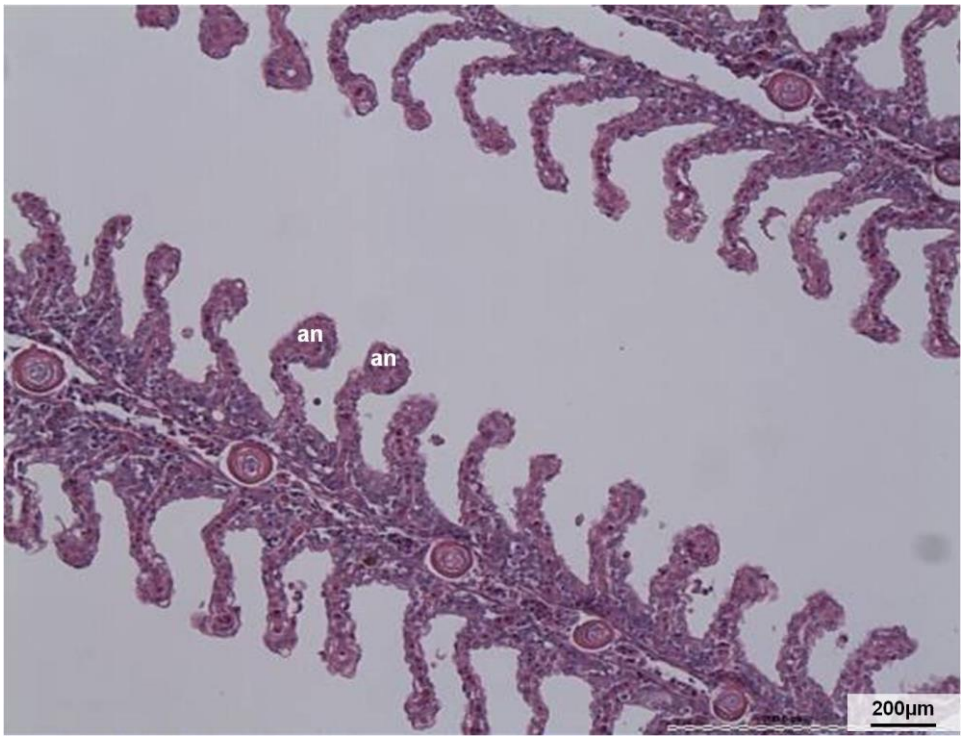


Plate 3-8 Lamellar aneurism (an) in yellow coloured fish (H&E)

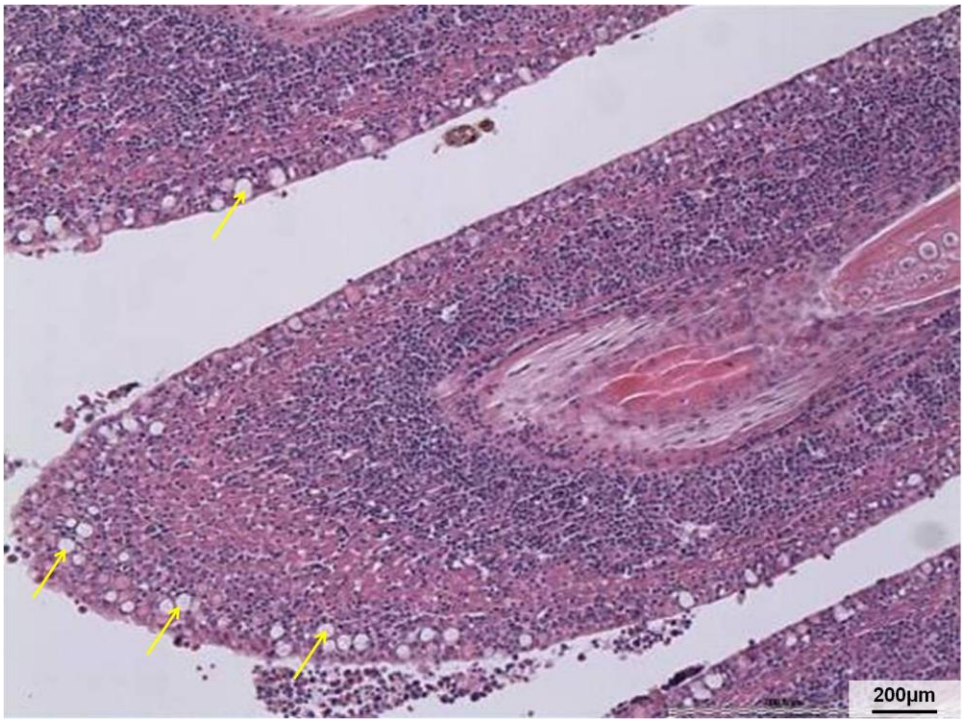


Plate 3-9 Increased numbers of goblet cell (arrows) are observed in yellow coloured fish (H&E)

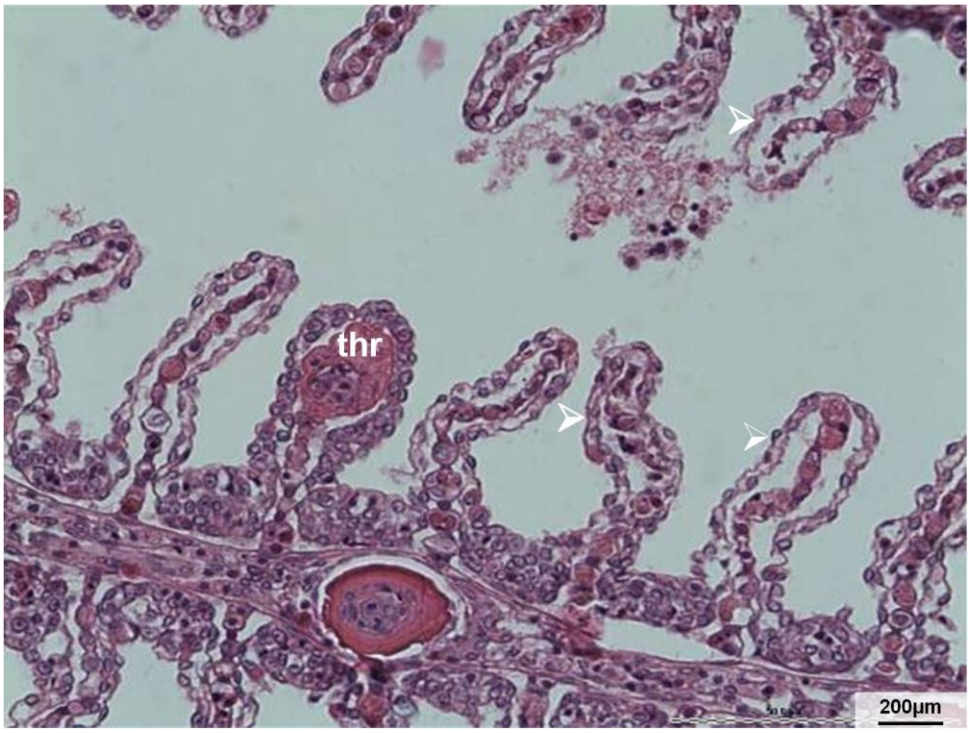


Plate 3-10 Separation of secondary lamellar epithelium (➢) from the pillar cells and thrombosis (thr) from yellow coloured fish (H&E)

There was also a strong association between gill pathology (such as epithelial hyperplasia or gill fusion or inflammation or necrosis) and jaundice in affected *P. hypophthalmus* (OR = 11.06, 95% CI = 4.12, 29.72, P < 0.0001). Monogenean and myxosporean parasites were observed on the gills of apparently normal and yellow coloured fish. The results showed that the highest number of monogenean and myxosporean were detected in the yellow coloured fish (see more detail in chapter 4).

In the common bile duct from apparently normal fish without nematode infection, the surface epithelium composed of single layer of uniform, tall columnar cells with basal nuclei (Plate 3-11A). The abnormal bile ducts were characterized by columnar cells without a clear brush border and by the presence of many goblet cells of varying sizes and shapes (Plate 3-11B).

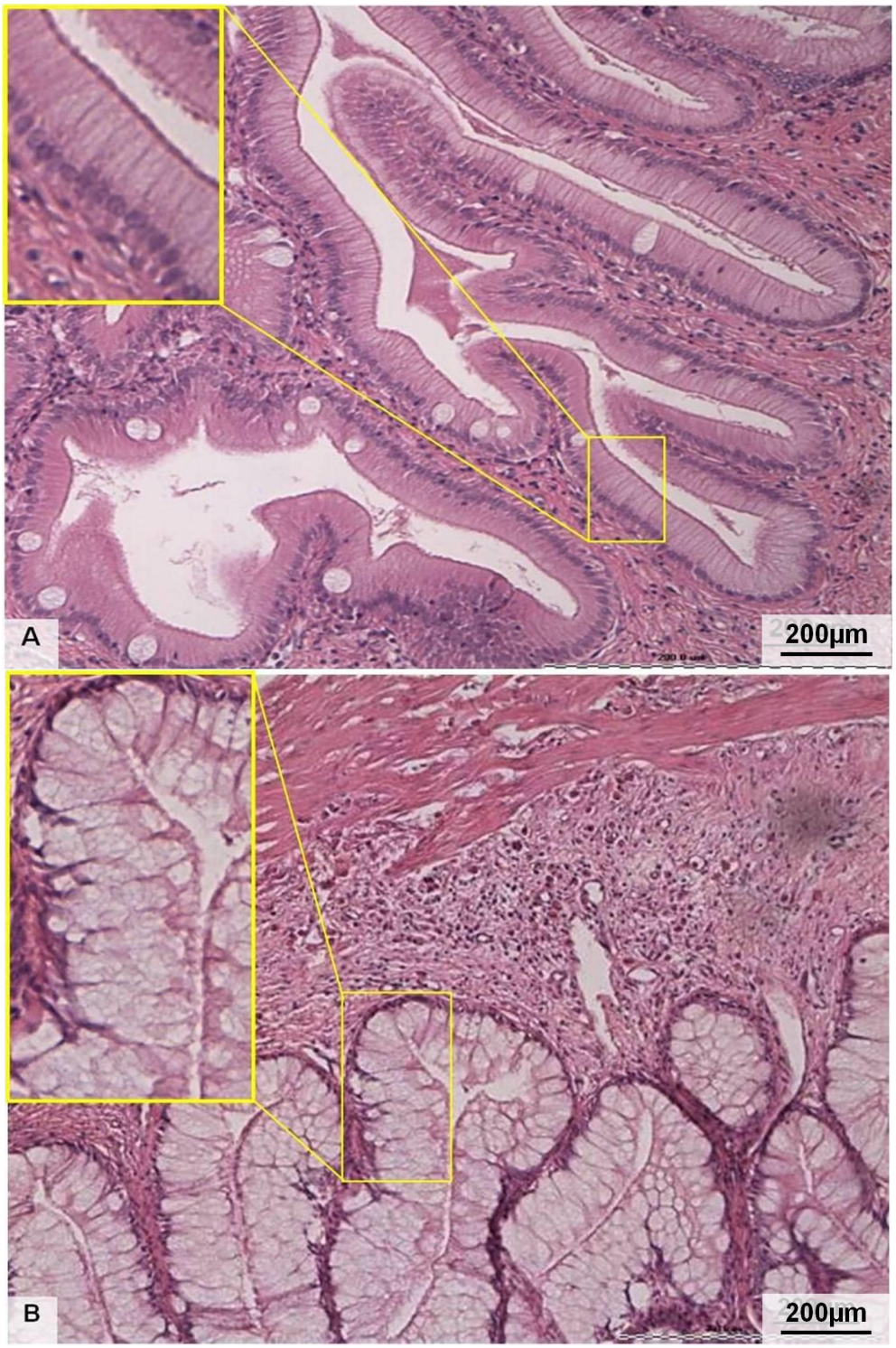


Plate 3-11 Hyperplasia of mucous cells (arrow) in the common bile ducts of *P. hypophthalmus* infected with nematodes (H&E).
A-Sections showing well-defined goblet cells and displaying well developed brush border; **B**-Common bile duct showed columnar cells without a clear brush border and presence of many goblet/mucous cells of varying sizes and shapes

In both yellow colouration and apparently normal fish with nematode infection, muscular hypertrophy was identified in the common bile duct and lumens were reduced or blocked with mucous cells (Plate 3-12B) and parasitic obstruction (Plate 3-13A). Additionally, the nematode-infected common bile duct of *P. hypophthalmus* showed severe damage within the whole thickness of its walls. Damage to connective tissue due to nematode larval migration resulted in large space around the larva and mixed inflammatory cell response (Plate 3-13B). However, the inflammation and hypertrophy of mucous cells was also observed in some cases where nematodes had not been found in the common bile duct.

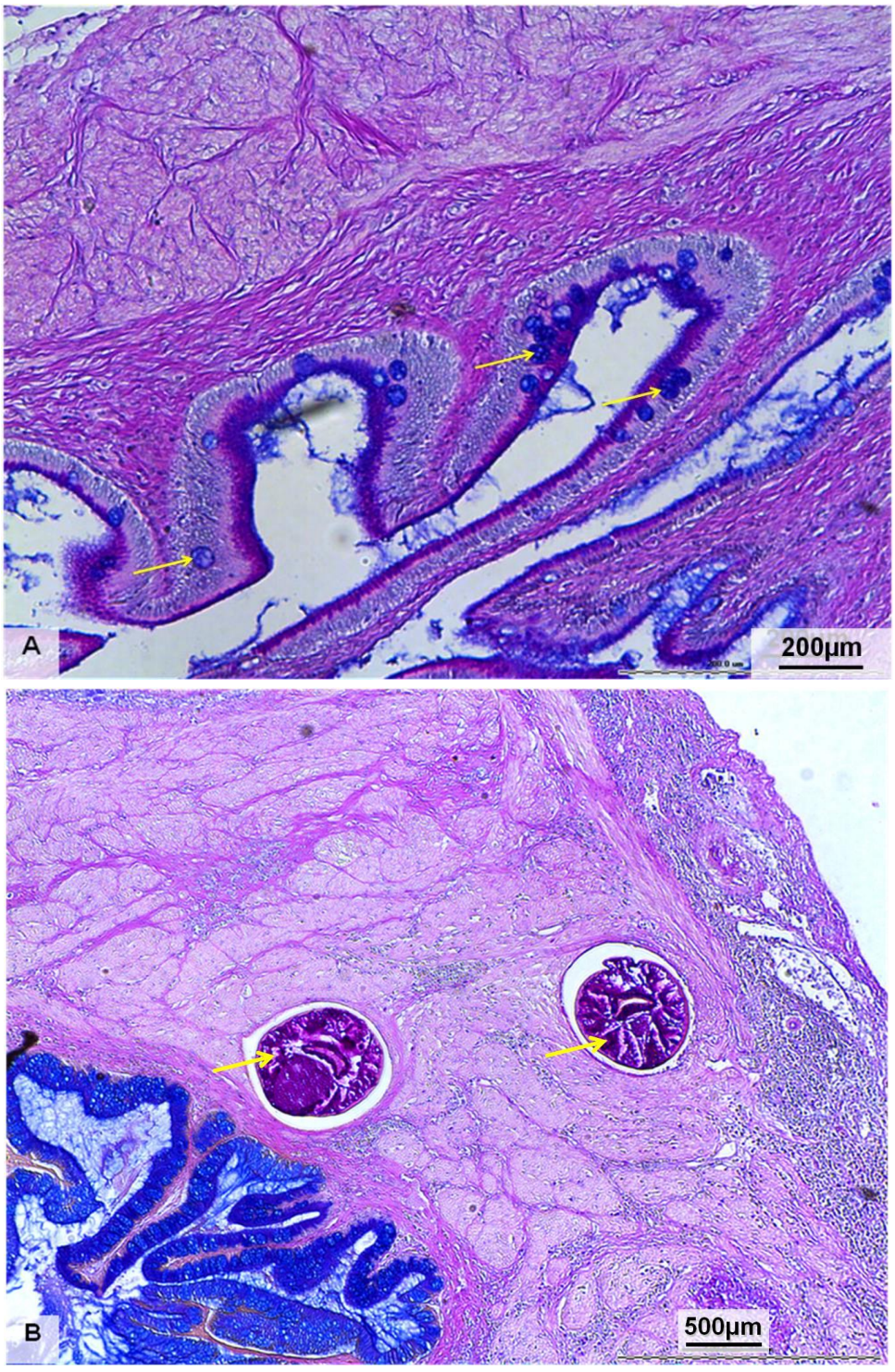


Plate 3-12 Sections of common bile ducts stained with Alcian blue pH 2.5.
A – Sections from normal size of common bile duct with few goblet cells (arrows)**;**
B – Sections of common bile ducts from *P. hypophthalmus* infected with nematodes (arrows). Common bile duct exhibits hyperplasia of the superficial epithelium and goblet cells (stained blue). Dense inflammatory cell infiltration and fibrosis were also present

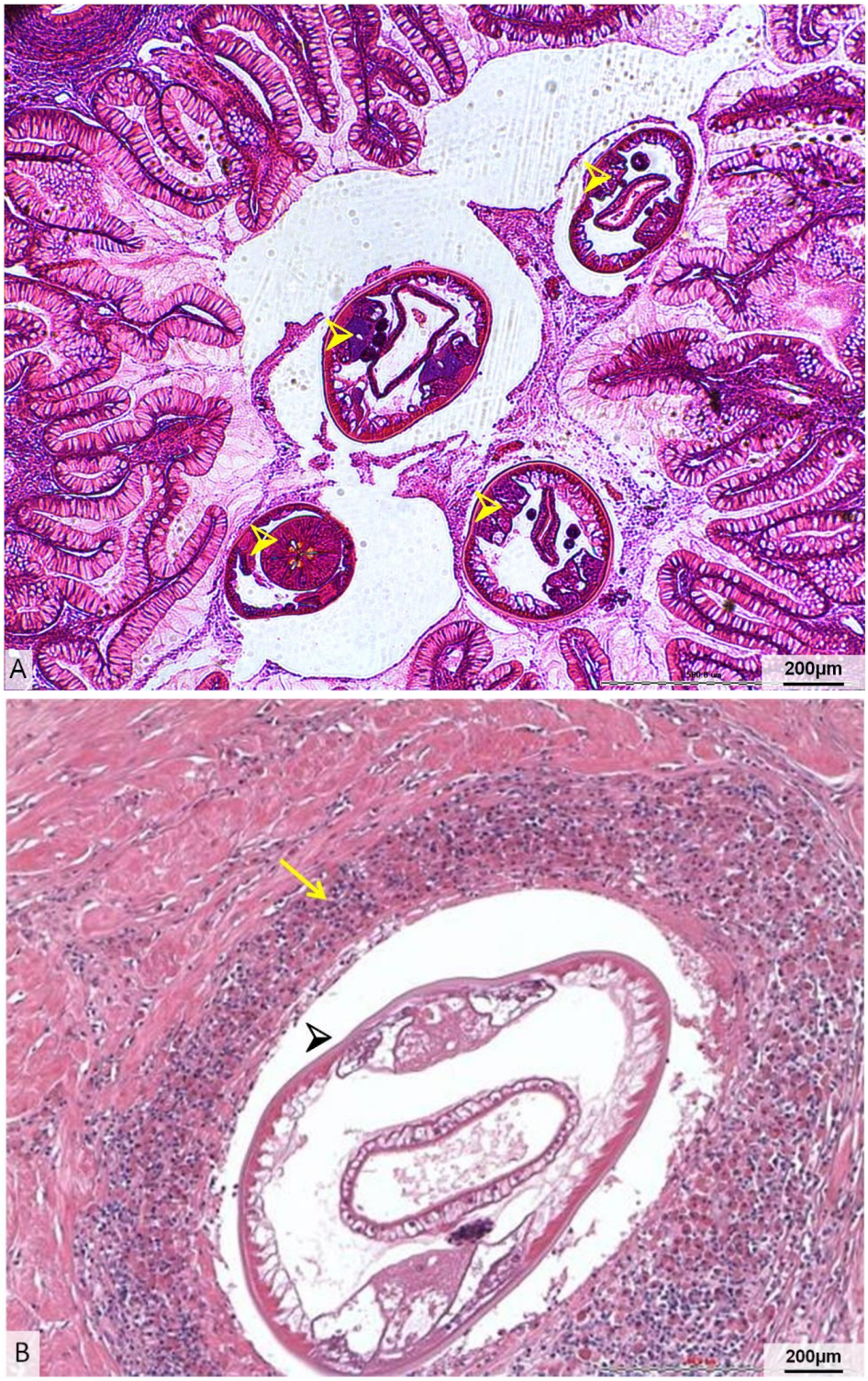


Plate 3-13 Nematodes infection in sampled fish (H&E).
A-Presence of nematodes (arrow head) in lumen of common bile duct; **B**-Infection of nematode (arrow head) were associated with the tissue damage, mixed cell inflammatory response (arrow) within wall of bile ducts;

In the liver sections from yellow coloured fish, histological changes consisted of vasculitis (Plate 3-14), and a marked multifocal to diffuse hepatocellular necrosis (Plate 3-15). Vasculitis was characterized by endothelial cell hypertrophy, presence of leukocytes within the wall of the blood vessels and fibrin thrombi within the lumen of affected blood vessels. Inflammatory response with a mixed cell infiltrated were observed especially in and around the bile ducts (Plate 3-16A), and the bile duct was focally surrounded by mild fibrosis (Plate 3-16B). Bile duct hyperplasia consisted of an increased number of variably-shaped bile ducts, when compared with the apparently normal tissue (Plate 3-17). The pathological changes were similar in all sections taken from yellow coloured fish with nematodes. These were mainly associated with the inflammatory reaction around the parasite (Plate 3-18). Ninety-four percent of the yellow coloured fish showed anaemia but no significant association was found between the anaemia and liver damage (OR = 2.5, P = 0.52).

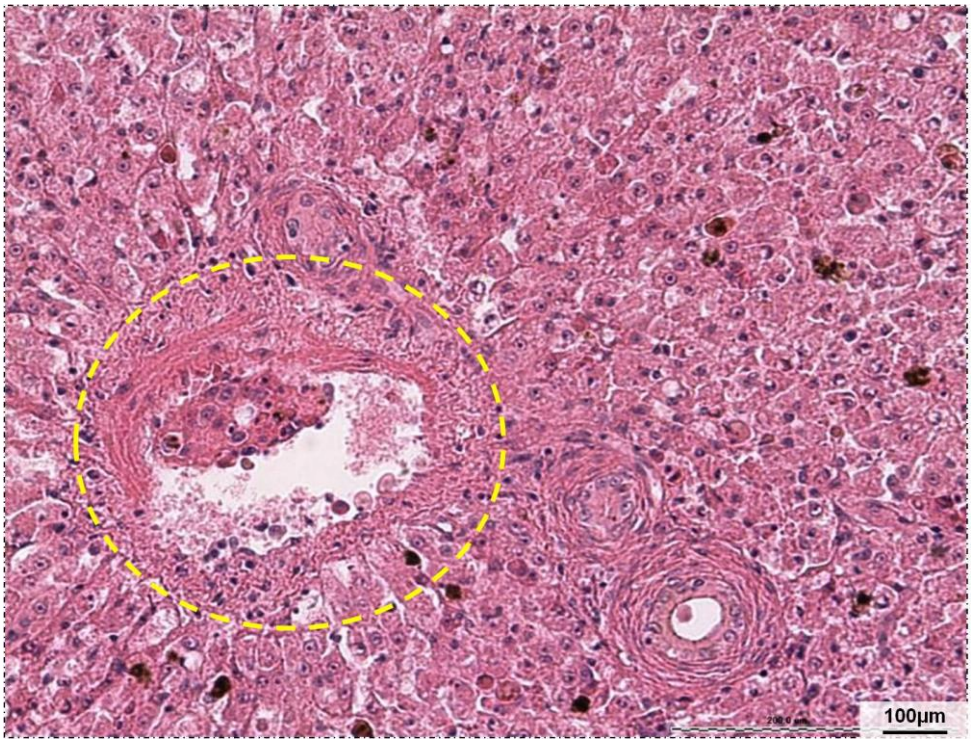


Plate 3-14 Blood vessels are obliterated by fibrin thrombi and their walls are infiltrated by inflammatory cells (vasculitis) (broken circle) from yellow coloured fish (H&E)

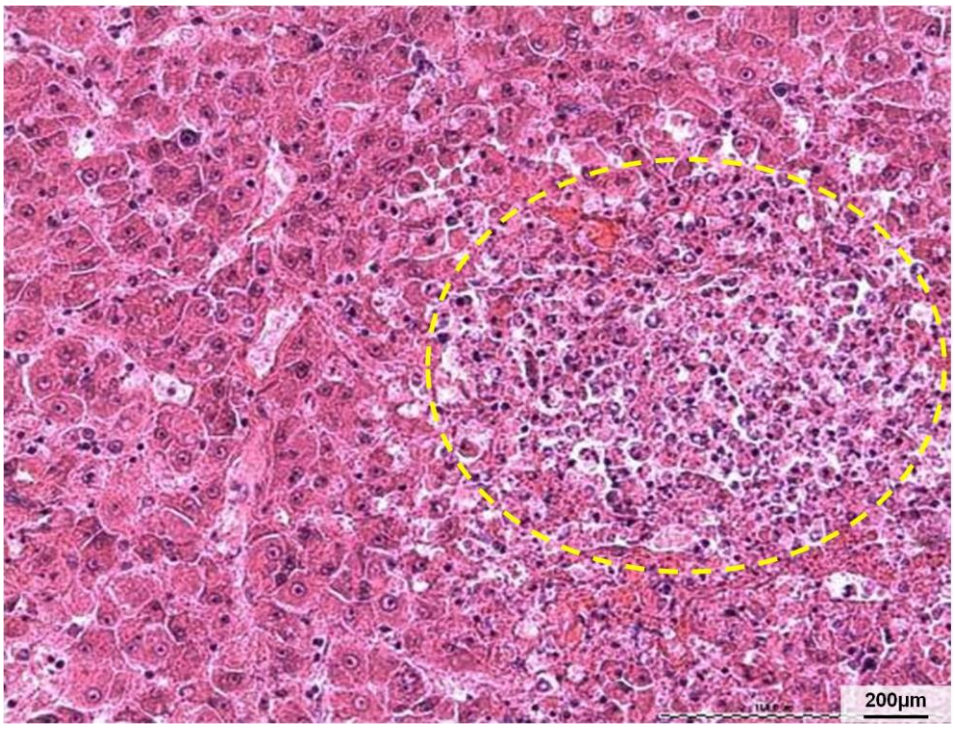


Plate 3-15 Liver from yellow coloured fish showing multifocal areas of necrosis (broken circle) with associated inflammation (H&E)

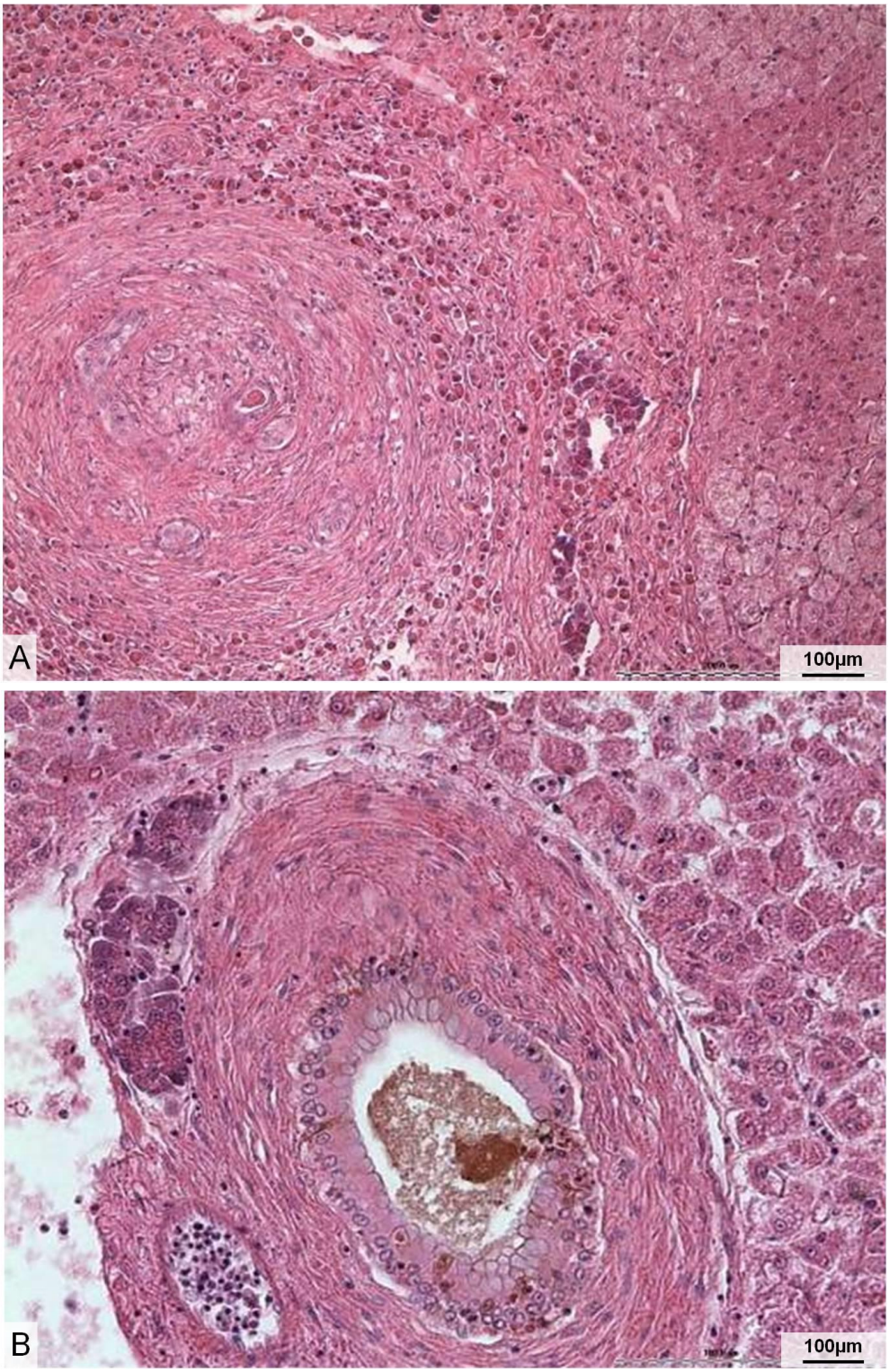


Plate 3-16 Bile duct showing severe increase in surrounding connective tissue, moderate influx of inflammatory cells, and abnormal bile duct epithelial cells from the yellow coloured fish (H&E)

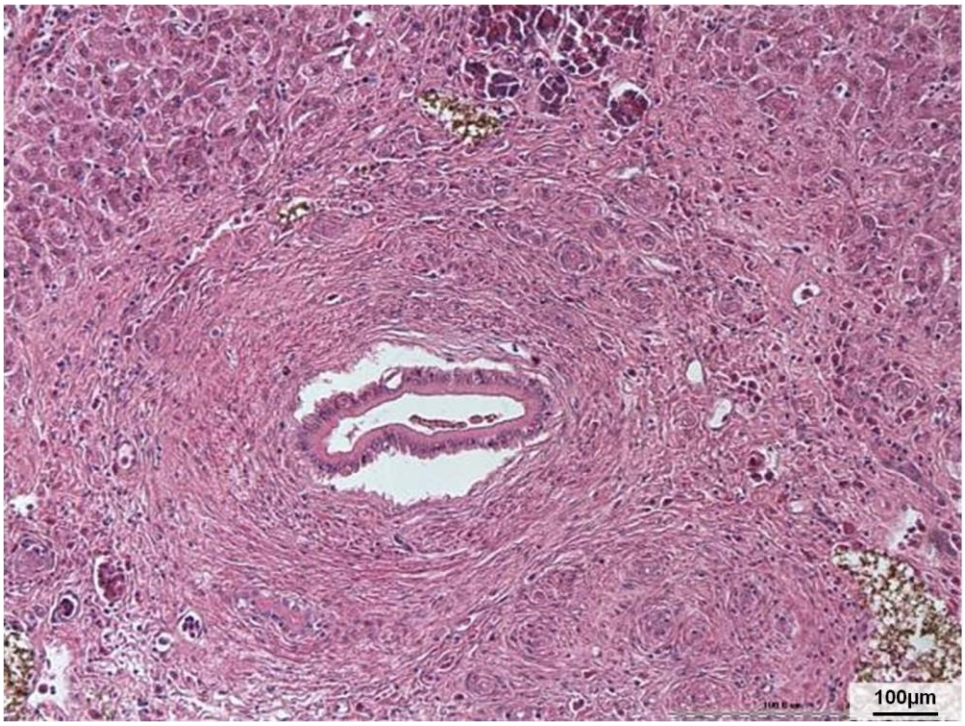


Plate 3-17 An area showing fibrosis surrounding a bile duct from a liver with marked increase in the number of bile ducts (H&E)

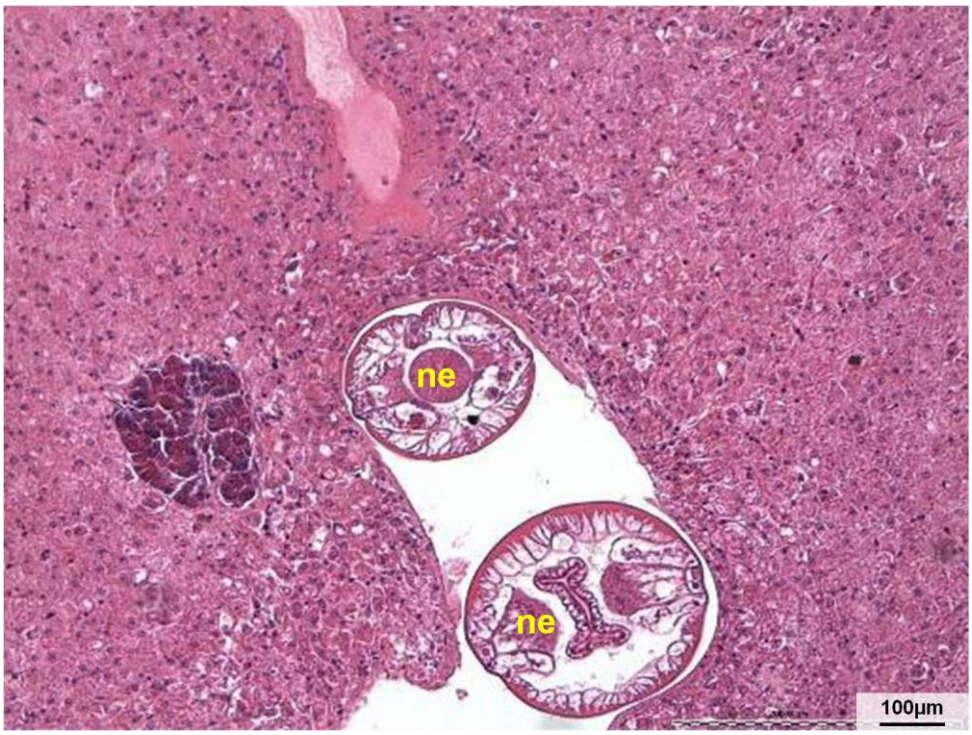


Plate 3-18 Liver from a yellow coloured *P. hypophthalmus* showing nematodes (ne) cut in cross section and the associated inflammation (H&E)

Fifty-four percent of yellow coloured fish exhibited splenomegaly. The splenic haematopoietic tissue was necrotic with an associated mixed cell inflammatory response (Plate 3-19). Increased numbers of melano-macrophage centres (MMCs) were observed in several sections (Plate 3-20) compared with no or few MMCs in apparently normal fish. In many tissues there was also fibrinoid vasculitis and formation of fibrinous thrombi in small blood vessels with multifocal infiltrates with low number of neutrophils, eosinophils, and scattered free brown pigment granules (Plate 3-21A, B). Damaged erythrocytes were found within the blood vessels.

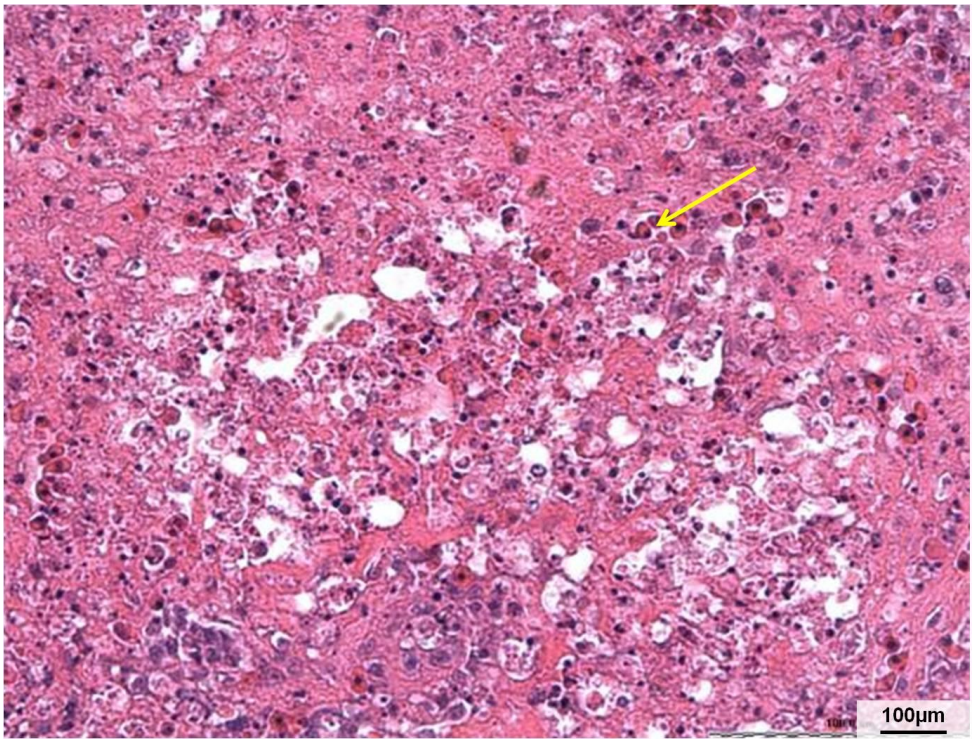


Plate 3-19 Splenic sections showing multifocal areas of necrosis (arrow) and associated inflammation from yellow coloured *P. hypophthalmus* (H&E)

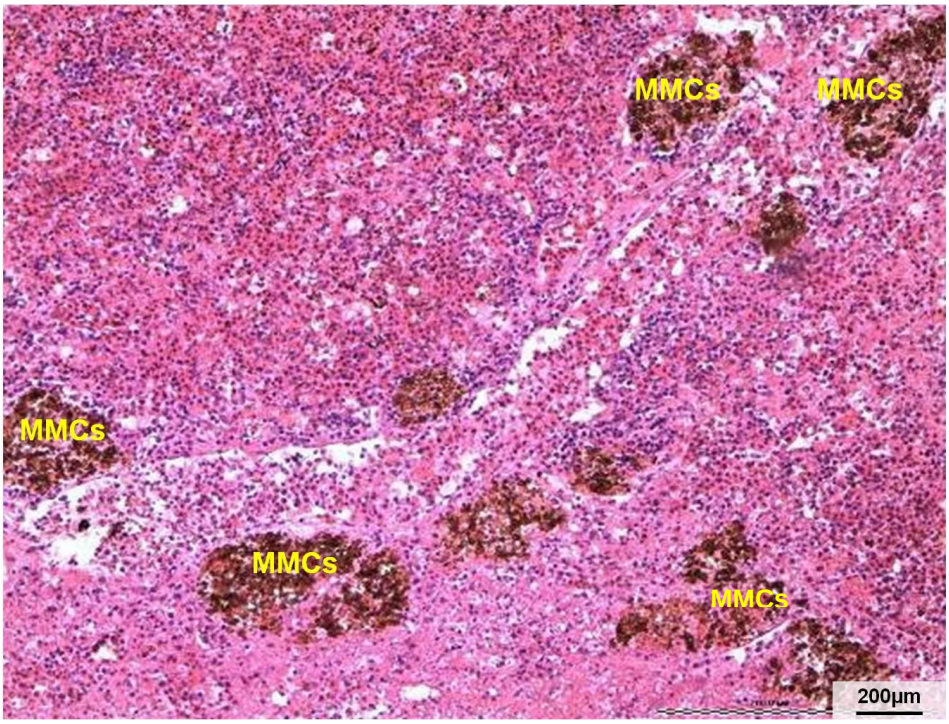


Plate 3-20 Splenic sections showing an increase of melano-macrophage centres (MMCs) from the fish with yellow colouration clinical signs (H&E)

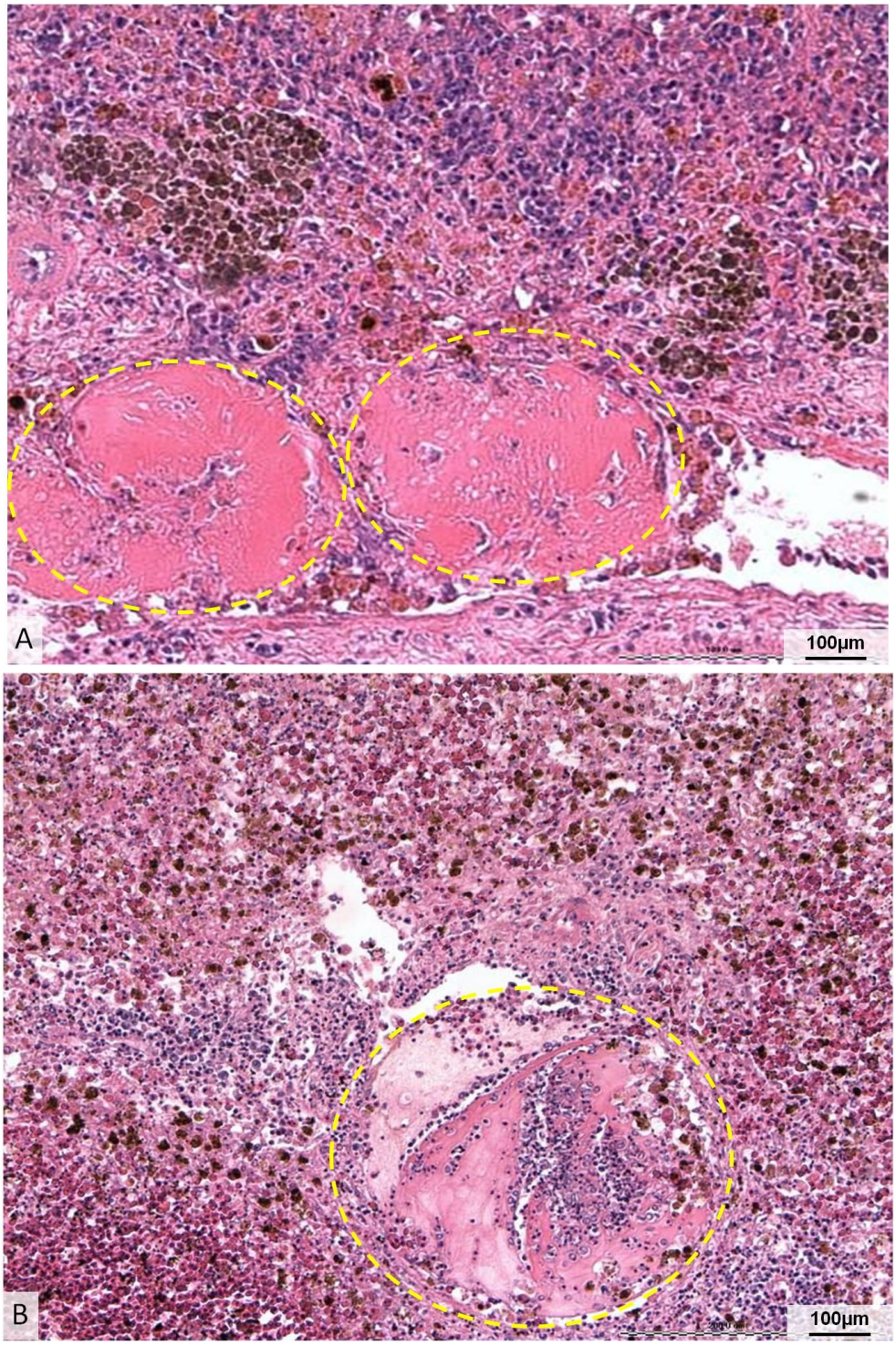


Plate 3-21 A, B-Sections showing presence of leukocytes within the wall and fibrin thrombi (broken circle) within the lumen of blood vessels in spleen from yellow coloured fish (H&E)

In the kidney, multifocal necrosis associated with inflammatory cell infiltration was only seen in the interstitium of yellow coloured fish (Plate 3-22). The

histological sections of kidney also showed thickening of basement membrane and marked atrophy of some glomerular cells showing large Bowman's spaces (Plate 3-23). Tubular changes were slight when compared with the glomerular damage, and were most obvious in 54% of those fish with severely damaged glomeruli. Hyaline droplets were present in the renal tubular epithelial cells, especially in the distal tubules (Plate 3-24) of 36% of yellow coloured fish, and deposits of eosinophilic material were present in the dilated tubular lamina. Tubular cell changes also included flattening of epithelial cells. The vasculitis was also found in renal sections of 48% of yellow coloured fish.

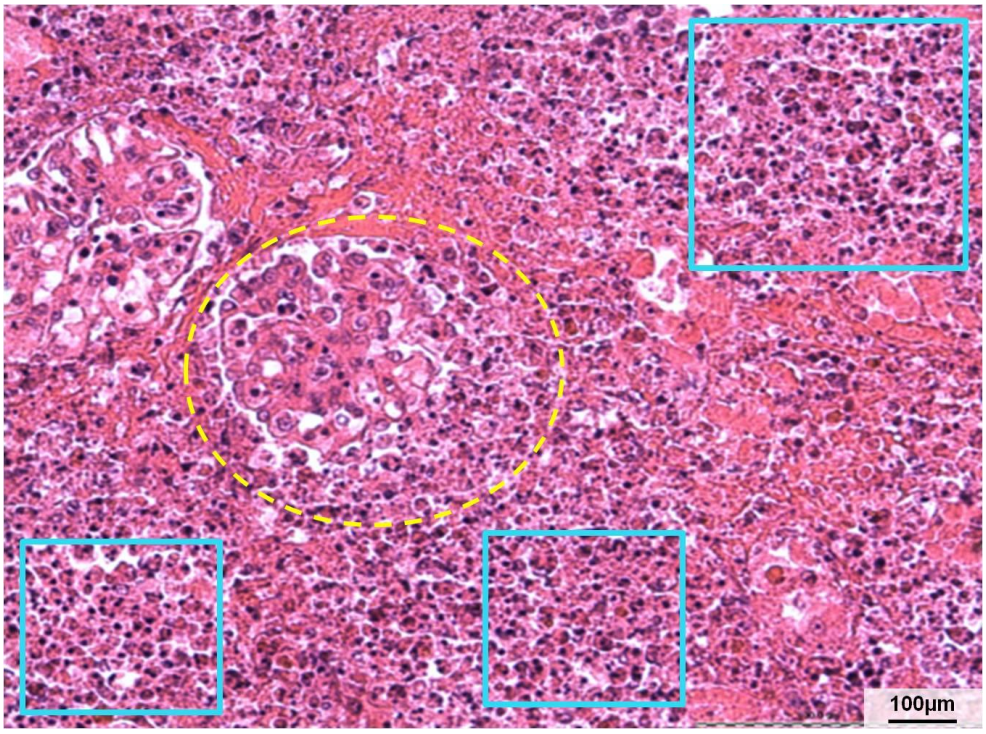


Plate 3-22 Multifocal necrosis associated with infiltration of inflammatory cells was evident in the interstitium (squares) and glomeruli (broken circle) from yellow coloured fish (H&E)

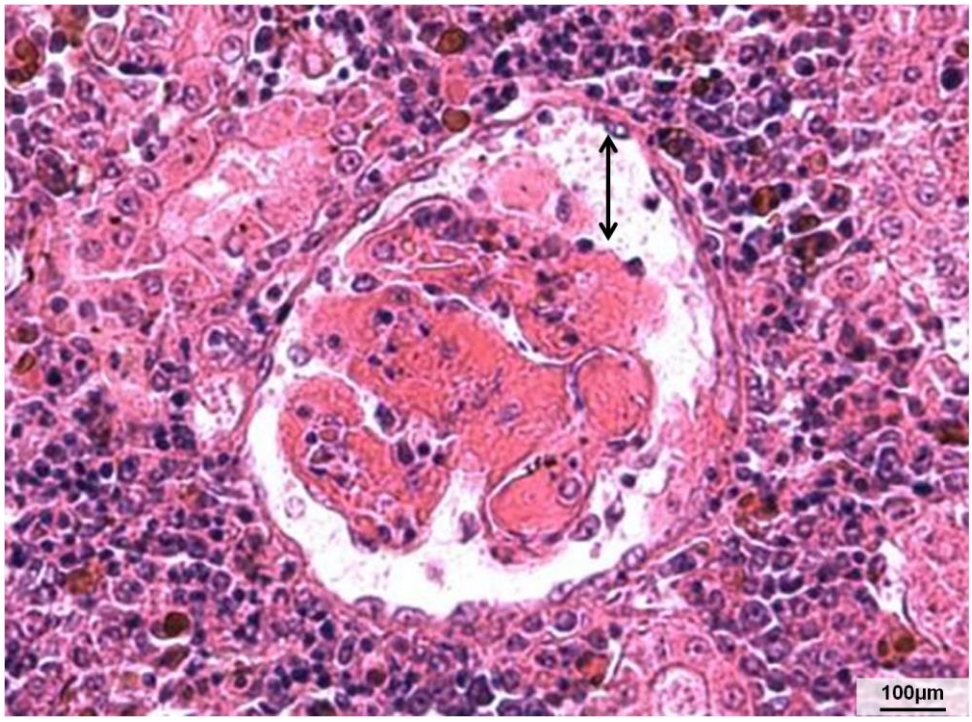


Plate 3-23 Section from yellow coloured fish showing glomeruli with dilatation of Bowman's space (two headed arrow), thickening of the basement membranes (H&E)

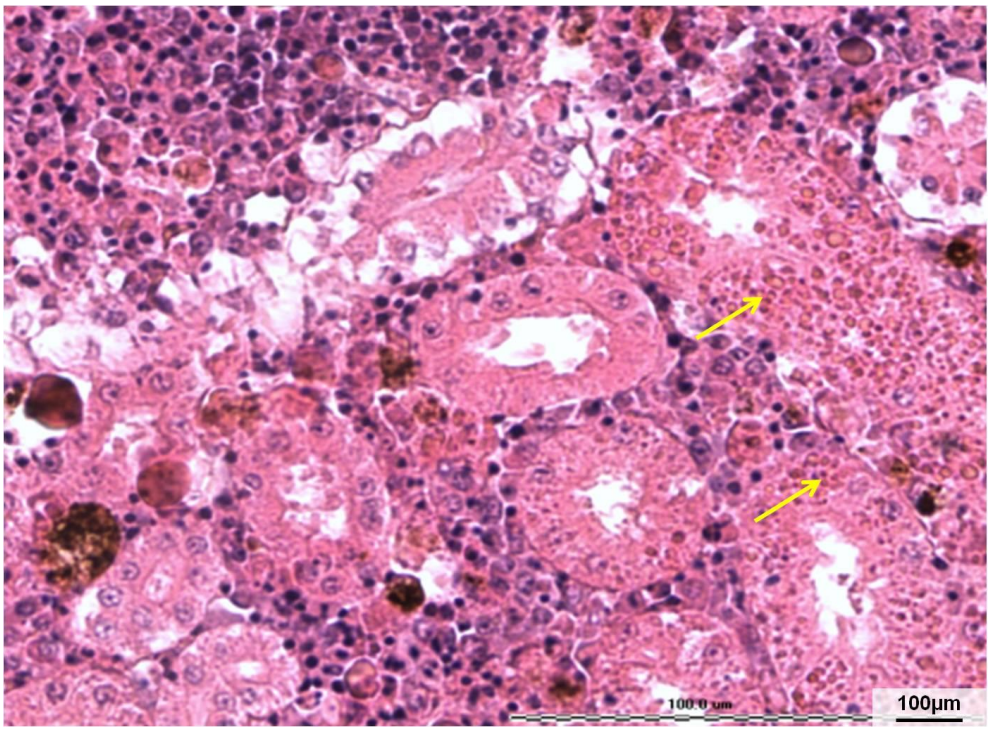


Plate 3-24 Renal tubular degeneration with hyaline droplets (arrows) in the renal tubular epithelial cells from yellow coloured fish (H&E)

Deposition of haemosiderin was not found in the melano-macrophage centres (MMCs) of the liver, kidney and spleen from apparently normal fish (Plate 3-25). However, the presence of haemosiderin was observed in varying amounts in the spleen and kidney of 78% of the yellow coloured fish examined, especially in the MMCs. By comparison, haemosiderin was rarely found in the liver of yellow coloured fish. Spleens showed marked haemosiderin accumulation associated with MMCs (Plate 3-26) and small deposits scattered throughout the tissue (Plate 3-27). Haemosiderin was also found in the tubular epithelial cells (Plate 3-28). The results indicated that severe haemolysis was occurring in the yellow coloured fish.

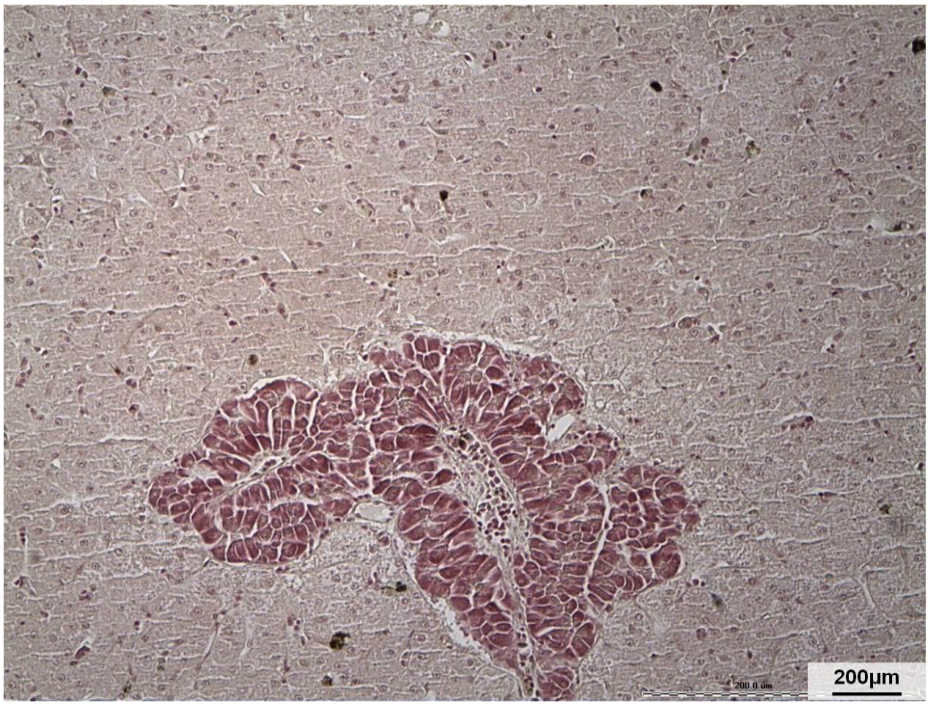


Plate 3-25 Spleen section from apparently normal *P. hypophthalmus* negative for haemosiderin

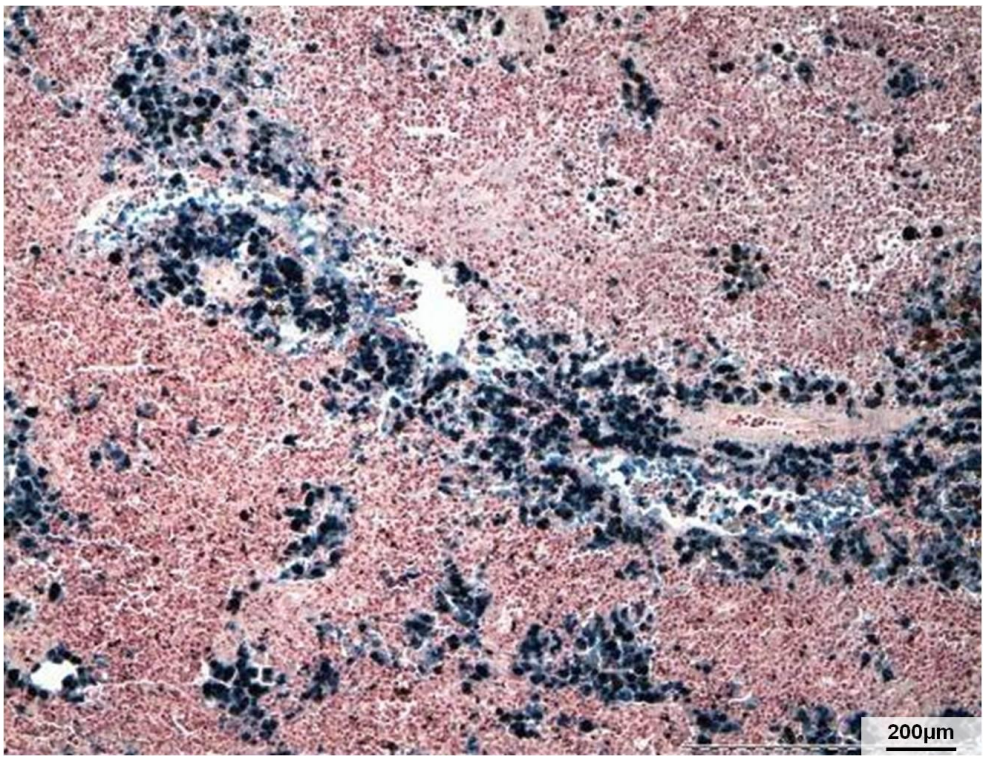


Plate 3-26 Spleen section from yellow coloured fish with haemosiderin deposits (blue colour) in the MMCs

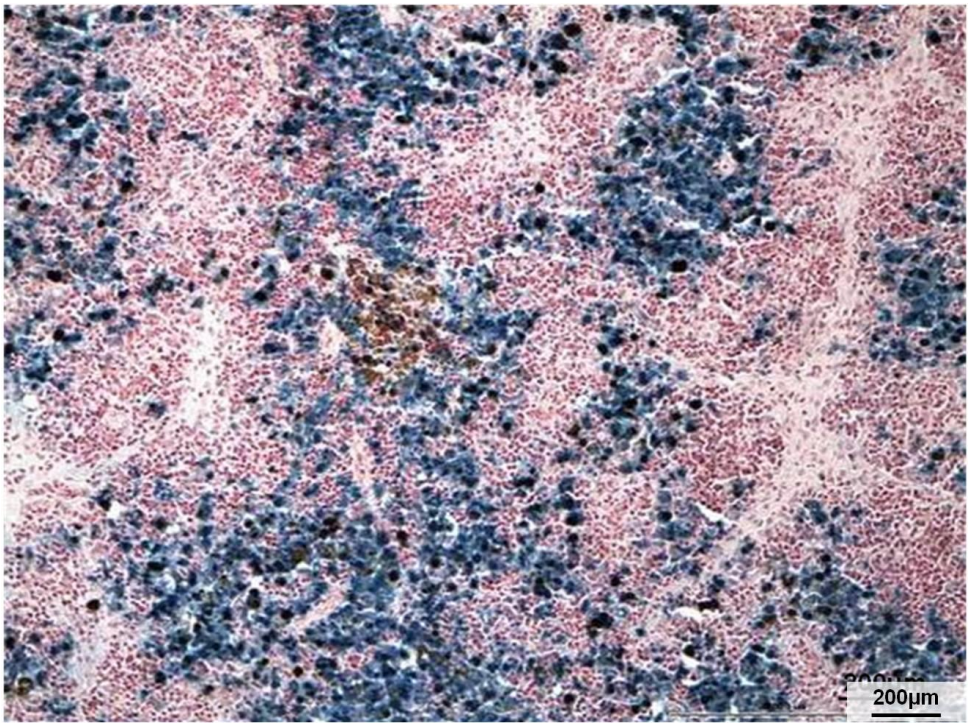


Plate 3-27 Deposits of haemosiderin were also diffusely spread throughout the pulp within single cells and in small accumulations of cells at the MMCs from yellow coloured fish

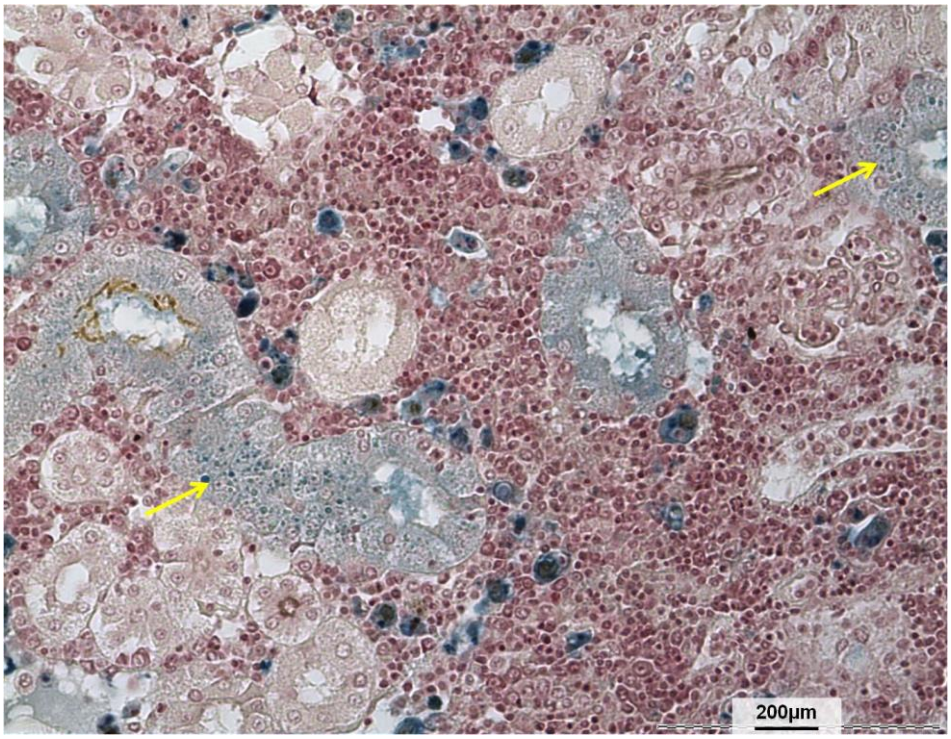


Plate 3-28 Kidney from a yellow coloured fish, haemosiderin was seen in macrophages of the haemopoietic tissues and in the tubular epithelial cells (arrow)

3.5. Discussion

This study has provided the first clinical description of yellow discolouration in farmed *P. hypophthalmus* produced in Vietnam. These results produced from this study are consistent with the description of clinical jaundice or icterus in other fish species and are the first time this condition has been described for farmed *P. hypophthalmus*. From here on, the word “jaundice”, will be used associated with yellow colouration in *P. hypophthalmus*.

The cluster analysis data performed in this study showed that the clinical signs associated with this condition belonged to cluster A and were more frequently associated with jaundice in *P. hypophthalmus*. Further analysis revealed that the gross signs included in the cluster A group were significantly associated

with moribund jaundiced fish, suggesting that this presentation reflects the final clinical stages of jaundice in *P. hypophthalmus*. These gross signs are similar to those of jaundice disease in other descriptions for hybrid *Clarias* spp. catfish (*C. macrocephalus* x *C. gariepinus*) in Thailand (Tonguthai *et al*., 1993, Pearson *et al*., 1994) and in hybrid catfish (*C. betrachus* x *C. fuscus*) in Taiwan in 2001 (Chang *et al*., 2008).

The discolourations of the serum samples taken from the jaundiced fish were very marked and are due to the presence of the bilirubin pigment or bile pigments. Bile pigments are yellow, green or orange in colour and are the breakdown products of normal haemoglobin (Pearson *et al*., 1994; Mehra *et al.*, 2009; Adeyemi *et al*., 2010). Haemoglobin is metabolized to form the green pigment biliverdin which is then reduced to form bilirubin in mammals. However, this is a more complicated and species specific process in fish (Sakai *et al*., 1988). Both indirect and direct bilirubin concentrations were elevated in the jaundiced fish examined in this study. This is not simply an indication of liver pathology but results from the failure to excrete both indirect bilirubin produced by the breakdown of red blood cells and direct and conjugated bilirubin produced by the liver. In mammals haemolytic anaemia resulted in increased indirect bilirubin (Molina and Reddy, 1999; Otto *et al*., 2000; Roche and Kobos, 2004); liver damage may also have reduced the capacity to conjugate the indirect bilirubin. The high level of direct or conjugated bilirubin detected in the jaundiced *P. hypophthalmus* may also indicate a blockage or failure to excrete bile pigments from the liver after conjugation. This can either be a failure in the bile ducts themselves or as a result of liver damage which may occlude the

small canaliculi, therefore blocking excretion (West *et al*., 1987; Guyton and Hall, 1996; Mehra *et al*., 2009). However, the cause of any blockage in the catfish examined was not clear.

In this study, the increased blood destruction observed in the jaundiced fish resulted in a haemolytic anaemia. Macrocytosis was observed only in the jaundiced fish in this study and may have been due to the increased proportion of immature erythrocytes with poor haemoglobin content as a direct consequence of the anaemia. According to Jones and Hunt (1983) macrocytic anaemia is most frequent following acute blood loss or acute haemolysis. The cause of this condition in farmed *P. hypophthalmus* is not obvious but impacts from suboptimal environmental conditions may exacerbate the condition.

In this study, jaundiced *P. hypophthamus* showed abnormal behaviour including crowding at the surface, near the inflowing water, which was also observed by Dung (2006). The dissolved oxygen level in the present study was low, but *P. hypophthalmus* is able to breathe air through the swim-bladder which allows the fish to live in water with low level of dissolved oxygen (Hill and Hill, 1994). The jaundiced fish were observed aggregated near the inflowing water, which may indicate respiratory problems. This may have been due to reduced oxygen transportation from the haemolysis and anaemia, which was severe enough to have induced the observed clinical behaviour. Similar signs, including anaemia and pale gills were seen in farmed hybrid catfish (Pearson *et al*., 1994) and Coho salmon in Chile (Smith *et al*., 2006) with yellowing of the base of the fins and on the abdomen. However, the changes in the gills observed in this study

were consistent with and more likely associated with the presence of the monogenean parasite. It may be that the jaundice and the gill infections resulted in lethargy in the affected fish leading to higher levels of gill parasites such as monogenean and myxosporean. Therefore it is unlikely that these are a single cause of the jaundice condition but, the condition itself will enable colonisation of parasites. This in turn will affect the fish behaviour and disease susceptibility.

In other fish species jaundice has been reported to have several causes, including obstruction of the common bile duct by nematodes and inflammation of the bile ducts by extension from enteritis or by infection with trematodes (Otto et al., 2000). Nematodes were found in the common bile duct of jaundiced *P. hypophthalmus*, but these were also present in the apparently normal fish. These findings suggest that the nematodes are unlikely to be the main cause of the problem.

The kidney of jaundiced fish had fibrinoid necrosis of the blood vessels and a markedly thickened basement membrane. The mechanism responsible for these pathological effects could not be easily identified but is considered to be similar to the Schwartzman reaction described in rabbits and later in rats and humans (Starzl *et al*., 1968). The generalized Schwartzman reaction is a pseudoallergic phenomenon resulting in a thrombohaemorrhagic response normally to a toxin. According to Nordstoga (1977), fibrinoid necrosis of blood vessels, especially the glomerular capillaries in rabbits may be caused by generalized Schwartzman reaction. Further immunofluorescence studies are needed to confirm an immune-complex mediated glomerulonephritis. Hyaline

degeneration was more numerous in the jaundiced fish compared with the apparently normal *P. hypophthalmus* as shown by hyaline droplets in the renal tubular epithelial cells. These “droplets” are absorbed haemoglobin proteins which have passed through the glomeruli (Croce and Stagg, 1997). However, these can also occur in the epithelial cells of renal tubules under normal conditions, where bright eosinophilic droplets within the proximal tubular epithelial cells can be found in normal fish (Takashima and Hibiya, 1995; Reimschuessel and Ferguson, 2006). Therefore, the accumulation of protein in renal tubule epithelium might not specific clinical sign to jaundiced fish.

Damaged erythrocytes are disposed of in the spleen (Aguis, 1979), which may result in increased blood haemoglobin levels. This in turn, is broken down into 2 separate components called heme and globin, which is then converted into amino acids. Destruction of the erythrocytes and high haemoglobin breakdown within the spleen allows cells to release and then to retain iron in the form of haemosiderin (Grover, 1968; Yu *et al*., 1971; Roberts, 1978; Agius, 1979; Pearson *et al*., 1994). According to Robertson and Newman (2006), splenomegaly is a common finding in haemolytic anaemia as intact and damaged erythrocytes fill the splenic sinusoids. In this study haemosiderin deposition was found in 80% of the jaundiced fish. The presence of haemosiderin is a good indicator of increased haemolysis (Takashima, 1982), which supports the findings in this study. In this study increased numbers of large individual MMC were observed in the liver, kidney and spleen of the jaundiced fish. Increased number of MMCs has been associated with haemolytic anaemia but can also increase due to destruction of surrounding

tissue as splenic necrosis which found in the jaundiced fish (Crosby, 1957; Roberts, 1975 and 1978; Fulp and McMillan, 1984; Press *et al*., 1996; Noga, 2006).

The vasculitis was also found in the hepatic vascular system. This is similar to the observation of Sakai *et al.* (1998) which found vasculitis in the liver of jaundiced yellowtail *S. quinqueradiata*. Ninety-four percent of the jaundiced fish with no other pathologies showed anaemia but no significant association was found between anaemia and liver damage. This may suggest that both liver pathology and haemolytic anaemia independently contribute to the jaundice. Additionally, the use DMAB-Rhodanine staining in this study is a technique that localizes copper in cells as bright orange to brown granules in the positive control sections. According to Ko and Lee (2000) the Mekong River water has not shown any signs of heavy metal/chemical contamination and is good for aquaculture purposes. However, in the aquaculture activities, the copper products are primary use to treat the algae but all copper formulations can be toxic to some species of fish at recommended application rates, especially if the water has less than 50 ppm of carbonate hardness (Liepolt and Weber, 1958; Lloyd, 1961). Copper does temporarily accumulate in fish, but more in the gills and the liver in rainbow trout (Handy, 1993), brown bullhead (Brungs *et al.,* 1973) and carp (Svobodova *et al.,* 1994) than in muscle tissue. The negative reactions from the liver sections of jaundiced *P. hypophthalmus* confirmed that there was no copper contamination in the supply water.

In general, it would appear that the yellow coloured *P. hypophthalmus* are suffering from a form of jaundice causing haemolytic anaemia. The presence of the gill parasites and the gall bladder nematodes did not appear to be associated with the condition as these were also found in the apparently normal fish. It would appear from the results of this clinical descriptive study that a single aetiological agent could not be found or identified in the jaundiced *P. hypophthalmus.* However, the jaundiced fish were susceptible to other infections and certainly the presence of the other identified pathogens would leave the fish weakened if not becoming moribund. The clinical case definition worked well and the cluster analyses supported these findings. So although it was disappointing not to be able to identify a single aetiological agent causing the jaundice condition, it is clear that this is a complicated condition affecting the fish at varied levels pathologically. No viral work was performed under this clinical investigation study as the original pathology did not support a viral aetiology. However, samples for viral screening should be included in future work on the jaundiced fish.

No farmers reported any spontaneous recovery of the jaundiced fish as they were usually removed from the pond environment as were dead fish. At present the impact of the jaundice condition is actually unknown and although work from this study now clarified the clinical picture of the affected fish in the culture system. The question for the future work is “Can the jaundiced fish recover if they are removed from their pond environment?” If the recovery experiment of jaundice were successful, it would help to understand the cause or the potential

risks of this condition on the farms and what preventative treatments can be implemented.

3.6. References

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Myxosporean infection associated with mortality in jaundiced farmed catfish (*Pangasianodon hypophthalmus*, Sauvage) in the Mekong Delta of Vietnam

4.1. Abstract

Fish losses due to pathogen and non-pathogenic causes continue to be a significant constraint to the development of the Vietnamese catfish (*Pangasianodon hypophthalmus*) industry in Vietnam. In this study the examination of gill parasites from jaundiced fish revealed a mixture of several parasites including *Myxobolus*, *Henneguya*, *Hennegoides* and monogeneans. The gill myxosporeans have not yet been identified to the species level whereas the *Myxobolus* species found in the spleen of the jaundiced fish was consistent with *Myxobolus pangasii.* No significant relationship was detected between the location of the fish farm and the infection level or mortality of jaundiced fish and infection level when measured as the number of myxosporean plasmodia per gill arch. Myxosporean infection in the gills was not associated with the haemolytic jaundice and may be considered as a nuisance rather than a primary pathogen in farmed *P. hypophthalmus* in the Mekong Delta, Vietnam.

Key words: Jaundice, myxosporean, *Myxobolus, Henneguya,* Vietnamese catfish (*Pangasianodon hypophthalmus*), the Mekong Delta.

4.2. Introduction

Jaundice is a serious health problem within the *P. hypophthalmus* aquaculture industry in Vietnam. This problem was first reported in the Mekong Delta (Hung, 2004) where affected fish appeared with a yellow discolouration of the skin and/or flesh as observed by the naked eyes. The results from a clinical pathology investigation (chapter 3) showed clearly that the affected fish presenting with this condition were suffering from a haemolytic anaemia resulting in jaundice. Although a single pathogen could not be associated with this condition, a wide range of microbial pathogens were detected in the affected fish, and these may contribute towards the morbidity and mortality reported in jaundiced catfish.

Vietnamese fish farmers have tried to ameliorate the jaundice condition in their farmed fish stocks through improved husbandry and fish health management but with limited success. This condition is wide spread within the *P. hypophthalmus* farming industry of Vietnam where fish mortalities associated with this condition occurred in 51% of farms visited during 2009 (chapter 2). One of the constraints to controlling this condition in the Vietnamese industry is the amount of conflicting information related to the cause of the condition, which remains undetermined. It may be that there is no single aetiological agent but instead this is a combination of varied assaults on fish health resulting in the jaundice. However, the frequent presence and detection of parasites has confused some of the pathology descriptions investigating the aetiology of jaundice in *P. hypophthalmus*.

Myxosporeans are commonly found in farmed fish and are described either as histozoic (i.e. in tissues) or coelozoic (i.e. within the urinary tract, gall bladder or bile duct) parasites affecting fish world-wide (Lom and Dyková, 1994). Myxosporean infections are reported frequently, and some species were reported to be potential pathogens of fish, which cause serious damage to economically important freshwater and marine fish species. Among these species, *Myxobolus cerebralis* has been reported as one of the most significant fish pathogens, causing whirling disease and this parasite acts as a primary pathogen in salmonids (El-Matbouli *et al.*, 1992; Hedrick *et al.,* 1998).

Many reports described the host reaction against myxosporeans (Table 4-1), but the view that most parasitic species cause little response is commonly held. According to Lom (1970), coelozoic myxosporeans cause little host reactions and have been considered the most harmless however, an inflammatory reaction consisting of leucocyte infiltration, desquamation and necrosis of epithelial cells in marine fish. Increased mucus secretion was found in the infection by myxosporeans parasitizing the gall bladder of fish (Fantham and Porter, 1912). Other studies investigating *Myxobolus* infections have described damage to the red blood cells and production of malignant anaemia in fish (Shiczakow, 1935; Kocylowski and Myazynski, 1963; and Chavda *et al.*, 2010). *Myxobolus* which has been found in the skin, on the fins and gills of many fish species and the spores may be released directly into the outside world after the cysts burst.

Table 4-1 Fish immune responses to Myxozoan parasites (Alvarez-Pellitero, 2008)

Parasites	Diseases	Fish host	Activity/factor
Ceratomyxa shasta	Ceratomyxosis	Salmonids	- Inflammation/cellular responses - Lysozyme - Protective responses/specific antibodies (Abs)
Enteromyxum leei	Enteromyxosis	*S. aurata, Diplodus puntazzo*	- Inflammation/cellular responses - Phagocytes action/oxidative mechanism - Eosinophilic granule cells (EGCs) - Complement - Peroxidase - Lysozyme - Antiproteases - Cytotoxic activity - Gene expression
E. scophthalmi	Enteromyxosis	*Psetta maxima*	- Carbohydrate terminals - Inflammation/cellular responses - Phagocytes action/oxidative mechanism - Complement - Lysozyme - Antiproteases - Protective responses/specific antibodies (Abs)
Myxobolus artus		*Cyprinus carpio*	- Specific antibodies (Abs)
M. celebralis	Whirling disease	*Oncorhynchus mykiss*	- Carbohydrate terminals - Gene expression - Specific antibodies (Abs)
M. cyprinid		*Leuciscus cephalus*	- Abundance of melano-macrophage centres (MMCs)
Sphaerospora dicentrarchi	Sphaerosporosis	*Dicentrarchus labrax*	- Phagocytes action/oxidative mechanism - Complement - Lysozyme - Antibody secreting cells
Tetracapsuloides bryosalmonae	Proliferative kidney disease (PKD)	*Oncorhynchus mykiss*	- Carbohydrate terminals - Inflammation/cellular responses - Phagocytes action/oxidative mechanism - Cytotoxic activity - Gene expression - Protective responses

Note: The name of the disease is indicated when it is recognized as pathologically important.

Severe damage to the gill has been observed by histological examinations during the myxobolasis of catla, common carp and gible carp (Dyková and Lom, 1988; Wang *et al.*, 2003). *Myxobolus* infection in cultured carp resulted in the presence of lesions, inflammation, congestion and hyperplasia of the gills

(Mohammad *et al.*, 2001). Post (1987) and Chavda *et al.* (2010) also stated that *Myxobolus* affected fish crowded at the surface, inlet or pond edges. Awal *et al.*, (2001) reported high mortality of fish due to myxosporidian infection in the gills of carp from nursery ponds in Bangladesh. Similar results were described in carp infected with *M. artus* (Yokoyama *et al.*, 1996) from Japan. All of which suggest compromised respiration or osmoregulation in the affected fish due to the presence of these gill parasites.

Ferguson *et al.*, (2001) reported the presence of *Myxobolus* and *Sphaerospara*-like myxosporean parasites within the glomerulus of *P. hypophthalmus*, considered as secondary invaders in BNP affected fish. The presence of *Myxobolus, Hennegoides* and *Henneguya* species belong to the myxozoa seem to be very common on gills of *P. hypophthalmus* in the Mekong Delta, Vietnam (Ky and Te, 2000; Dung *et al.*, 2008; Hang *et al.*, 2008). Until recently, five species of myxosporean including *Henneguya shariffi, H. berlandi, Hennegoides pangasii, Myxobolus baskai* and *H. malayensis* have been found parasitizing the gills of *P. hypophthalmus,* while *Myxobolus pangasii* was found only in the spleen of this fish. Out of the five new species infecting the gills of *P. hypophthalmus*, two species identified as *H. shariffi* and *H. berlandi* had typical epithelial location, two others (*H. pangasii, M. baskai*) developed in the blood vessels, and *H. malayensis* selected the cartilage as a typical location site (Molnár *et al.*, 2006a). According to Hang *et al.* (2008), the prevalence of *Myxobolus* sp found in cultured *P. hypophthalmus* was 80% and appeared to be present in these fish all year round. This condition is usually benign; however, sometimes it can be associated with a high mortality, particularly in farmed fish

(Hang *et al.,* 2008). Currently, outbreaks of disease due to myxosporean and microspora are increasing being reported in *P. hypophthalmus* farming in the Mekong Delta, Vietnam. Hang *et al.*, (2008) reported that heavy infections were associated with the unsightly milky oval shaped cysts which were 1-3 mm in size located within skeletal muscle of farmed fish. However, this was not usually associated with serious clinical signs and the authors could not give data on the pathogenicity of myxosporean and microspora infections. Instead, the detrimental effects of these parasites result in economic losses to the aquaculture industry due to downgrading of fillets (Hang *et al.*, 2010).

The aim of the present study was to identify myxosporeans occurring in jaundiced *P. hypophthalmus* in the Mekong Delta, Vietnam, and to try to determine the role of the parasites observed with the jaundice condition.

4.3. Materials and methods

4.3.1. Fish samples

Farm site visits were conducted from 12th October to 10th November 2009. All sites produced fish in earthen ponds and were distributed throughout the main production areas of Mekong Delta, Vietnam. A total of 22 *P. hypophthalmus* grow-out farms were visited and were located in Vinh Long, Can Tho, Ben Tre, Dong Thap, and An Giang provinces, the Mekong delta, Vietnam. The data collection and fish sampling refers to the methods used to collect data and fish already described in chapter 2 and 3.

4.3.2. Histopathology of fish samples

Numerous tissue samples (liver, spleen, gills, kidney, skeletal muscle, and common bile duct) were taken for histopathology as described in Chapter 3.

As part of this investigation, the intensity of myxosporean infection on respiratory problems was investigated based on the number of plasmodia observed in the gill histology sections. The first gill arch on the left side of each fish was examined and 15 mm of gill tissue was aseptically sliced and routinely processed for paraffin embedding. Tissue sections were cut at 5 µm thick, stained with Haematoxylin and Eosin (H&E) and the number of plasmodium per gill arch were enumerated under light microscopy (Olympus BX51, Watford, Hertfordshire, UK). During the examination signs of other parasites infection were also carefully checked. Pictures were taken and saved as digital images.

The presence or absence of the following histological features were included in the examination: mucous hypertrophy in the common bile duct, fibrinoid vasculitis, general necrosis, haemorrhage, lymphocytes infiltration, bile duct hyperplasia, degeneration and hyaline droplet formation within tubular epithelial cells, thickening of the glomerular basement membrane, fibrin thrombi and haemosiderin deposit.

4.3.3. Myxosporean detection by PCR and sequence identification

The PCR assay performed used formalin fixed wax embedded tissue samples from spleen, kidney, muscle, common bile duct and gills taken from fish with clinical signs of jaundice (n=111). These tissue samples had been pre-screened

and myxosporean parasites had been positively detected by light microscopy (Frasca *et al.*, 1999).

4.3.3.1. DNA extraction

Total DNA from formalin-fixed paraffin-embedded gill, spleen, kidney, muscle, liver, and common bile duct tissue samples were subsequently extracted using a DNeasy™ Tissue Kit (Qiagen™, Crawley, UK). The method used was as described by Crumlish *et al.* (2007). Briefly, the tissues were suspended in 125 µl enzymatic lysis buffer [20 mM Tris-Cl at pH 8, 2 mM ethylenedianetetraacetic acid (EDTA), 1.2% Triton X-100, 20mg lysozyme per ml buffer, and 20 µl protein K] and incubated at 56^{o}C overnight until complete lysis. DNA extraction was then conducted according to the kit manufacturer's instruction. The total DNA concentration of each sample was measured using a Nanodrop spectrophotometer (ND-1000, Labtech International, East Sussex, UK). All DNA samples were then store in aliquot at -20^{o}C until required.

4.3.3.2. Polymerase chain reaction (PCR) amplification

The parasite pre-screening observations in the affected tissues identified spores and organs of a *Myxobolus* species that was morphologically similar to *M. pangasii* spores (Molnár *et al.* 2006a). Specific-forward (5'-TTAGTTCGTGGAGTGATCTG-3') and specific-reverse (5'-TCTAAGGGCATCACAGACCTG-3') primer pairs were designed from the initial sequence reads of *M. pangasii*, deposited in GenBank (accession number FJ816270) to amplify a 370 base pair (bp) target in rDNA genes (Freeman pers.

comm.). A single step PCR assay was performed using the PCR conditions described by Freeman *et al.* (2008) with some modification.

The PCRs were carried out in a volume of 25 µl comprising approximately 200 ng of extracted total DNA mixed with 1.25 Units of Klear Taq DNA polymerase (KBioscience, US) and the related 1X PCR buffer with a final $MgCl_2$ concentration of 1.5 mM, 0.2 mM dNTPs, and oligonucleotide primers (10 pmol of each) in MilliQ water. The reaction mixtures were subjected to an initial denaturation step for 95^{o}C for 15 min allowed the appropriate activation of the Klear Taq DNA polymerase which then would ensure a reliable amplification in a DNA thermocycle (Biometra T gradient^{TM}, Goettingen, Germany), followed by 35 cycles of 95^{o}C for 50s, 56^{o}C for 60s, 72^{o}C for 60s, and was finished with terminal extension at 72^{o}C for 7 min, then rested at 4^{o}C. The PCR products were electrophoresed on 1.5% agarose gel (Invitrogen UK) stained with 1 µl ethidium bromide to visualise under ultraviolet light (UV). A DNA molecular weight marker (1 Kb plus DNA Ladder; Invitrogen^{TM}, Crisbad, CA 92008, USA) was used to determine the size of the PCR products where the expected product was a 370 bp molecular weight. The negative control (no DNA) consisted of a PCR mixture with molecular grade water and another with genomic *P. hypophthalmus* DNA (i.e. negative control DNA) from the spleen tissue of normal fish without the presence of *Myxobolus*. The limiting factor was that PCR assay was performed without a positive control.

4.3.3.3. Sequencing

A total of 8 samples was purified using a QIA quick Kit (Qiagen, Crawley, West Sussex, UK) following the manufacturer's protocol. DNA sequencing was performed in forward and reverse directions with the specific-forward (5'-TTAGTTCGTGGAGTGATCTG-3') and specific-reverse (5'-TCTAAGGGCATCACAGACCTG-3') primer pair using a BigDyeTM Terminator v30 cycles sequencing ready reaction kit and an ABI 310 automatic gene sequencer (Applied Biosystems Inc. Foster City, CA). The consensus sequences obtained were aligned to create a partial small subunit (SSU) rDNA sequence of the parasite, which was compared and contrasted with the SSU rRNA gene sequence of *M. pangasii* available in the GenBank database using the Basic Local Alignment Search Tool (BLAST).

4.3.4. Data Analysis

MS Excel was used to store all the data, and to produce charts and tables. Categorical variables were presented as frequencies (N, %). Where data sets were unable to be transformed to a suitable form, non-parametric analysis was carried out using Mann-Whitney U-test or Kruskal-Wallis test on multiple groups with 95% confidence limit interval. Histological data are presented as a cross-tabulation for presence or absence of histological changes, differences in proportions of categorical variables between groups was evaluated with a Chi-square test. The relationship between number of myxosporean plasmodia per gill arch and weight of fish (g) or stocking density (fish m^{-2}) were analysed using simple linear regression.

4.4. Results

4.4.1. Fish sampling

The present study showed that all the 22 interviewed farmers had experienced parasite problems in their fish stocks and this was always in combination with other diseases. The dominant parasite was a monogenean where 91% of famers had found it in their fish stocks, followed by nematodes (52%), myxosporean (30%) and other parasites including *Ichthyophthirius* and *Trichodina* (11%). According to the interviewed farmers, fish appear to co-exit with the monogenean parasites present in the culture system and a heavy infection was found only during the rainy season or periods of cooler weather. It was their opinion that mortality due to monogean and nematode parasites had never occurred in adult fish in their farms. The farmers always sent their moribund fish to mobile laboratories often established by the chemicals/veterinary drugs companies for the diagnosis of parasitic diseases. The present study also found that in the large-scale fish farms, the technicians were able to check for the parasite by using a light microscope. The parasitic checking was performed if there are external signs of disease such as white spots and abnormal behaviour.

Parasitic disease management practices varied between farms and were dependant on the farmer's previous experience. Greater variability was observed regarding treatment regimes, suggesting the absence of an established or standardised strategy across the farms included in this sample. The medicines or chemicals used for parasitic therapy included: Benzalkonium chloride (15 L ha^{-1}), formalin (20 L ha^{-1}), $KMnO_4$ (5 L ha^{-1}), $CuSO_4$ (4 kg ha^{-1}),

iodine (10 L ha^{-1}), and different medicines to treat external parasites including FIBA (10 L ha^{-1}, extracted with methanol from seeds of *Combratum quadrangulare,* Kurz), praziquantel (2 kg ha^{-1}), and ivermectin 90% (1.7 mg kg^{-1} of fish). There was no difference in the chemical used for parasite treatment compared with prevention during the culture period.

4.4.2. Histopathology of fish samples

4.4.2.1. Parasite detection

Among the fish sampled for parasite examination, jaundice was the dominating external clinical sign comprising 51% of the total number of fish analyzed. Myxosporean plasmodium was detected on the gills, in the spleen, kidney, muscle (Plate 4-1), and common bile duct tissues belonging to apparently normal and jaundiced fish, where myxosporean infection was recorded in 18 of the 22 visited sites.

The findings showed that 79 out of 111 jaundiced fish had plasmodia located on the gills. The presences of myxosporean plasmodia in other organs of jaundiced fish are given in Table 4-2, where the highest infection (71% of jaundiced fish) corresponded to gill type and the lowest (5% of jaundiced fish) to kidney type. Myxosporean infections were not detected in livers of any of the jaundiced fish examined.

Table 4-2 Percentage of organs with myxosporeans parasite detected from jaundiced fish (n = 111)

Organ	Number of infected fish	Percentage (%)
Gills	79	71
Muscle	15	14
Common bile duct wall	13	12
Spleen	21	19
Liver	0	0
Kidney	5	5

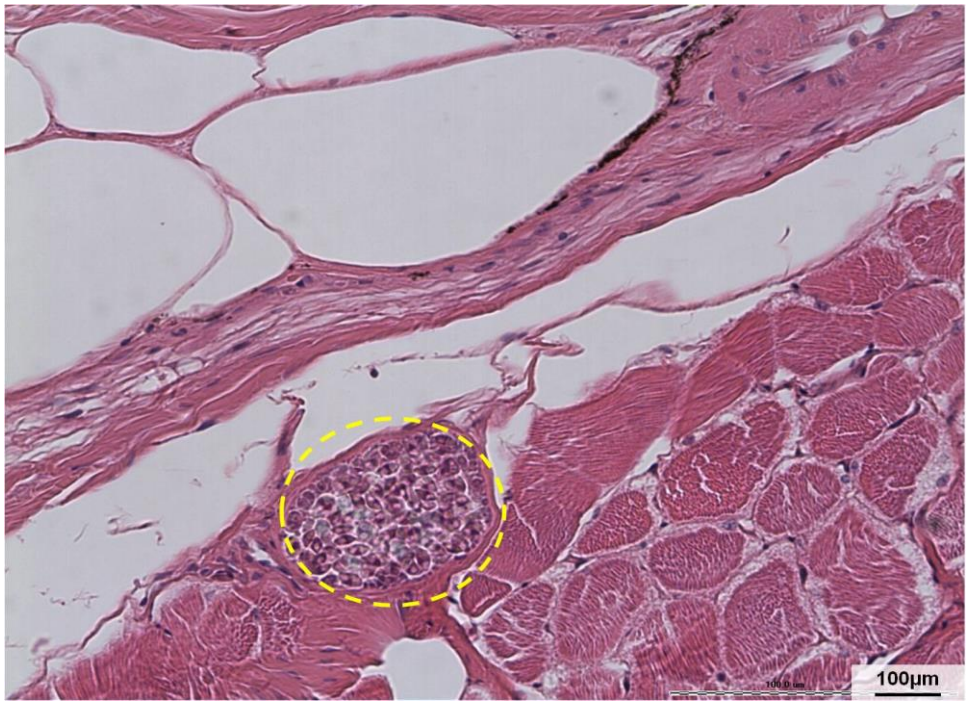

Plate 4-1 Muscle section showing the myxosporean plasmodia (broken circle) from jaundiced *P. hypophthalmus* (H&E)

Table 4-3 lists the sampling location and the percentage of fish per site infected with gill parasites in *P. hypophthalmus*. The significant highest number of myxosporean plasmodia was found in the jaundiced fish, following by affected fish without jaundice and apparently normal fish (Table 4-4, $P < 0.05$).

Table 4-3 Myxosporea prevalence in *P. hypophthalmus* sampled

Provinces	No. of farm	N_{Total} (%)[a]	Apparently normal fish		Jaundiced fish		Affected fish without jaundice*	
			N_f (%)[b]	N_{Myxo} (%)[c]	N_f (%)[b]	N_{Myxo} (%)[c]	N_f (%)[b]	N_{Myxo} (%)[c]
Dong Thap	10	105 (48.4)	32 (61.5)	5 (16)	46 (41.4)	35 (76)	27 (50.0)	12 (44)
Vinh Long	8	60 (27.6)	15 (28.9)	2 (13)	26 (23.4)	20 (77)	19 (35.1)	9 (47)
Can Tho	1	7 (3.2)	1 (1.9)	0 (0)	3 (2.7)	0 (0)	3 (5.6)	0 (0)
An Giang	2	32 (14.8)	1 (1.9)	1 (100)	28 (25.3)	16 (57)	3 (5.6)	1 (33)
Ben Tre	1	13 (6.0)	3 (5.8)	0 (0)	8 (7.2)	8 (100)	2 (3.7)	1 (50)
Total	22	217 (100)	52 (100)	8 (15)	111 (100)	79 (71)	54 (100)	23 (43)

** With the clinical signs of bacillary necrosis or haemorrhage*

[a]N_{Total} is total of sampled fish, %: percentage of sampled fish in each province

[b]N_f is number of fish; %: percentage of fish in each province

[c]N_{Myxo} is number of myxosporea infected fish; %: prevalence of myxosporea in each province (Prevalence was calculated as the ratio of the number of individuals containing at least 1 visible plasmodia to the total number of fish examined: N_{Myxo}/N_f)

Table 4-4 The number of plasmodia observed on the gill arch of sampled *P. hypophthalmus*

Clinical signs	Number of plasmodia per histological slide		
	Mean (± SD)	Min	Max
Apparently normal (n=8)	2.0 (± 1.0)	1	3
Jaundiced (n=79)	5.0 (± 7.0)	1	35
Affected fish without jaundice* (n=23)	2.0 (± 2.0)	1	7

** With the clinical signs of bacillary necrosis or haemorrhage*

The findings shown in Table 4-3 show the wide distribution of the parasites throughout the *P. hypophthalmus* farms. The highest average number of myxosporean plasmodia observed on the gills was found in the fish sampled from Ben Tre and Dong Thap provinces, especially Dong Thap province (Figure 4-1). In these fish the gill arch carried up to 35 plasmodia, but this was not significantly different from animals in other locations ($P > 0.05$). Although the numbers of gill plasmodia were higher in the jaundiced fish and these had high mortalities (ranged from 120-600 dead fish per day) no statistically significant relationship was detected between mortality of jaundiced fish and the level of myxosporean infection (Figure 4-2, $P = 0.092$).

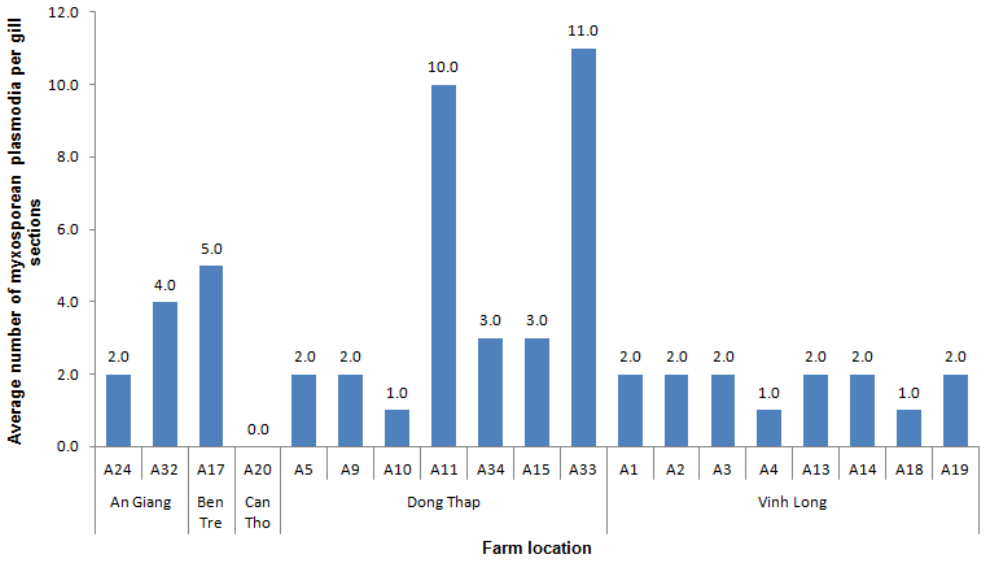

Figure 4-1 Average number of myxosporean plasmodia on gills of *P. hypophthalmus* for different geographical locations.

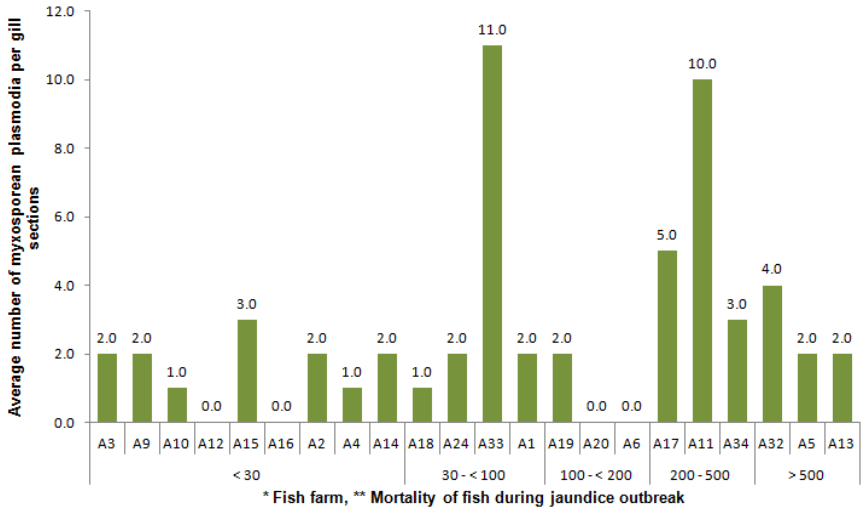

Figure 4-2 Average number of myxosporean plasmodia on gills of *P. hypophthalmus* in farm for different of mortality of jaundiced fish

The data were analysed further to explore the relationship between the number of myxosporean plasmodia per gill arch against the weight of fish (g) and stocking density (fish m^{-2}). The findings showed that the number of myxosporean plasmodia per gill arch was not correlated ($P > 0.05$) to both weight and stocking density.

Morphologically, the gill myxospores found correspond to the genera *Myxobolus, Hennegoides* and *Henneguya*. The *Myxobolus*-like spores were found from 11 blood smears of sampled fish, 8 of which were from jaundiced fish and 3 of which were from affected fish without jaundice (Plate 4-2). The gills of sampled fish were parasitized by *Myxobolus*-like (Plate 4-3), *Henneguya*-like (Plate 4-4), and *Hennegoides*-like species (Plate 4-5). *Myxobolus*-like plasmodia were found in the gill capillaries. *Hennegoides*-like plasmodia were found within the gill arteries and cartilage.

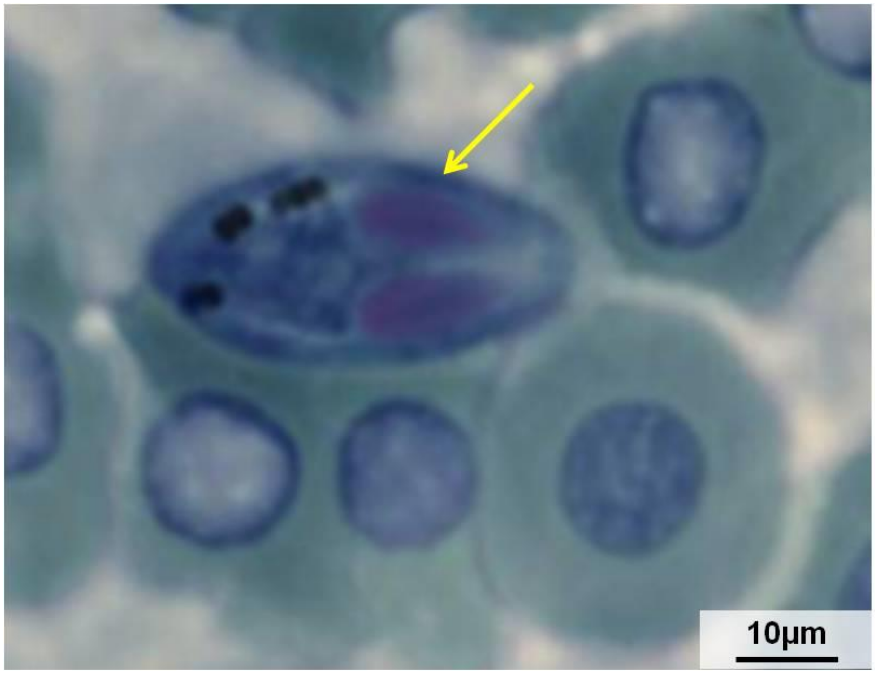

Plate 4-2 *Myxobolus*-like spore (arrow) in Giemsa stained blood smear from jaundiced *P. hypophthalmus*

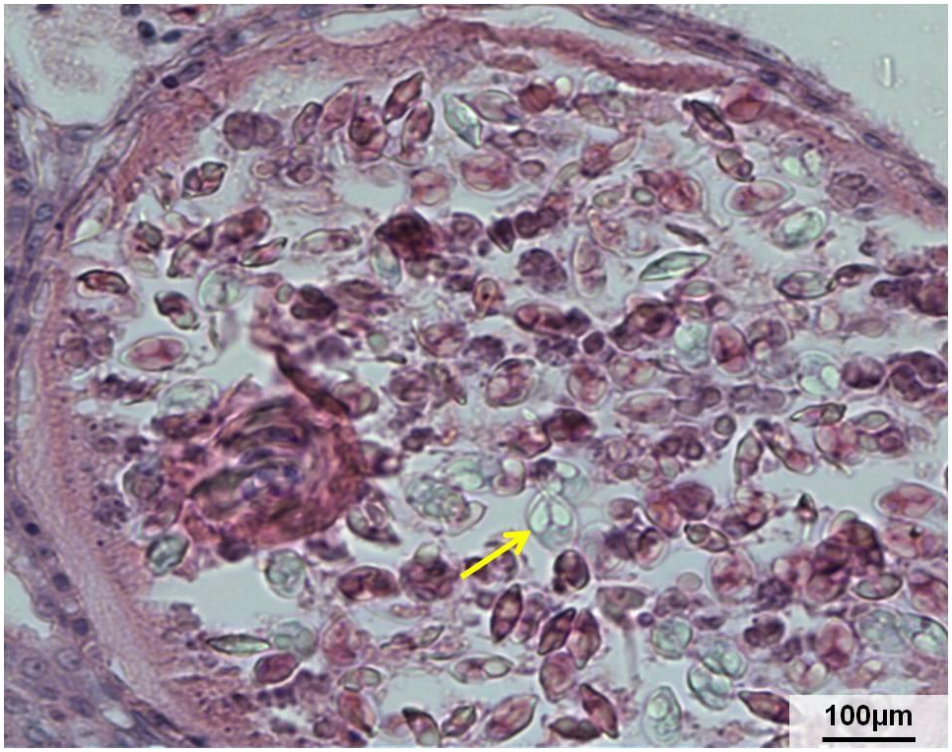

Plate 4-3 *Myxobolus*-like plasmodia with mature spores (arrow) were found on gills of jaundiced fish (H&E)

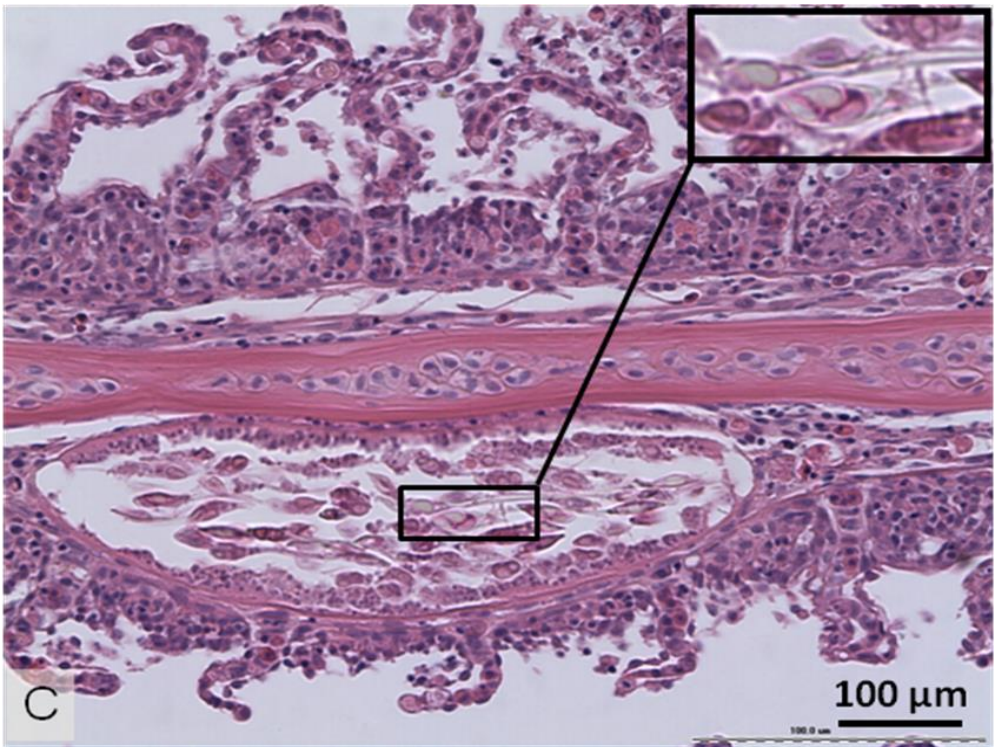

Plate 4-4 *Henneguya*-like plasmodia with mature spores were found on gills of jaundiced fish (H&E)

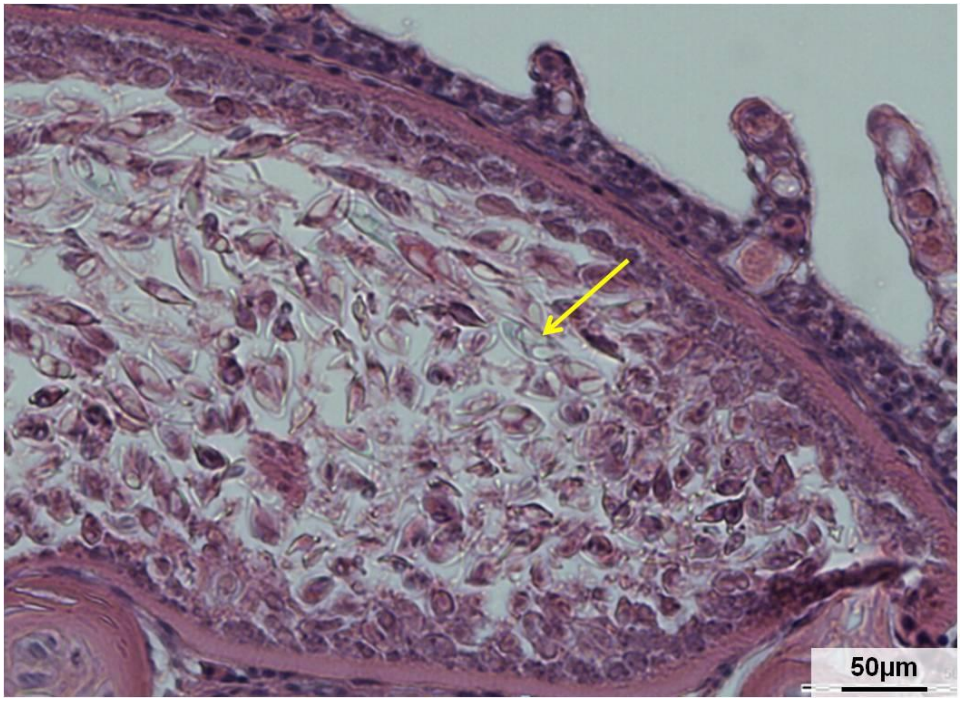

Plate 4-5 *Hennegoides*-like plasmodia were found on the gills of jaundiced *P. hypophthalmus* (H&E)

Plasmodia with mature spores were found in the spleen of the twenty-one jaundiced fish. Round whitish cysts were visible with a milky substance containing large amounts of mature spores. Myxosporean in the spleen of sampled fish are typically pear shaped organism, the spores' present pyriform and elongated morphology. The spore wall is thick at the anterior part and the polar capsule is elongated but equal in size (Plate 4-6). The spores appeared to belong to the genus *Myxobolus* (family Myxobolidae).

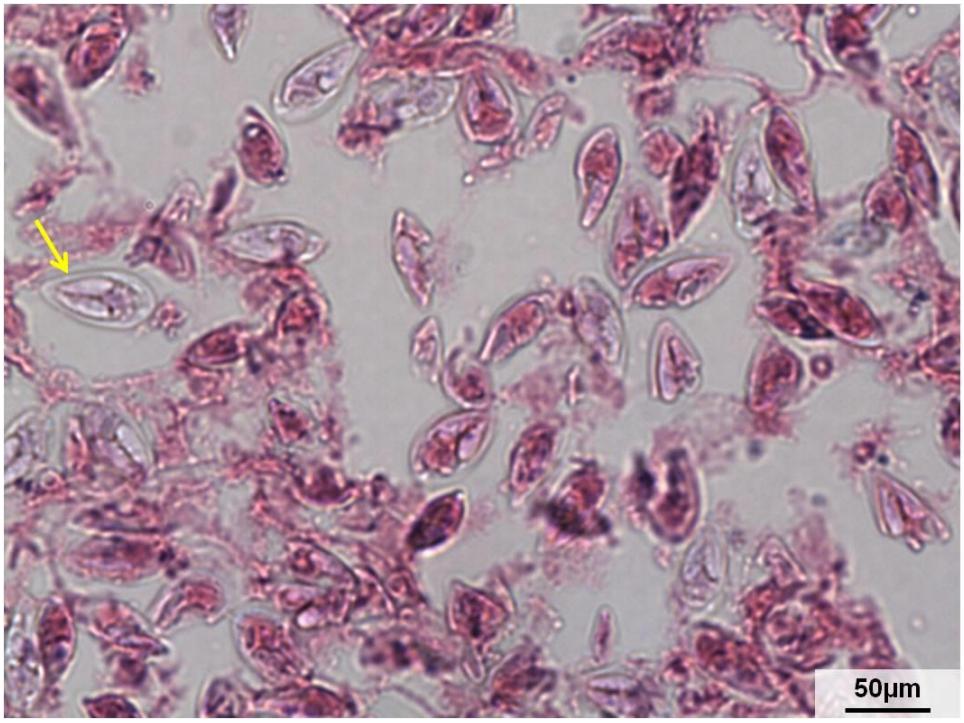

Plate 4-6 *Myxobolus*-like spores from a large plasmodia in the spleen of jaundiced *P. hypophthalmus*

In addition to myxosporean infections, parasites belonging to other groups such as *Trichodina* (Plate 4-7) and monogeneans were also found (Plate 4-8). The average prevalence of monogenean parasites ranged from 3-10% in apparently normal fish ranged from 22-32% in affected fish but without jaundice, and from 39-73% in jaundiced fish. The results showed that the average number of

monogenean per gill arch was 2.0 (± 1.0), but this ranged from 1 to 3 parasites per gill arch, 5.0 (± 5.0), ranged from 1 to 30 parasites, and 3.0 (± 2.0), ranged from 1 to 7 parasite in apparently normal, jaundiced and affected fish without jaundice, respectively.

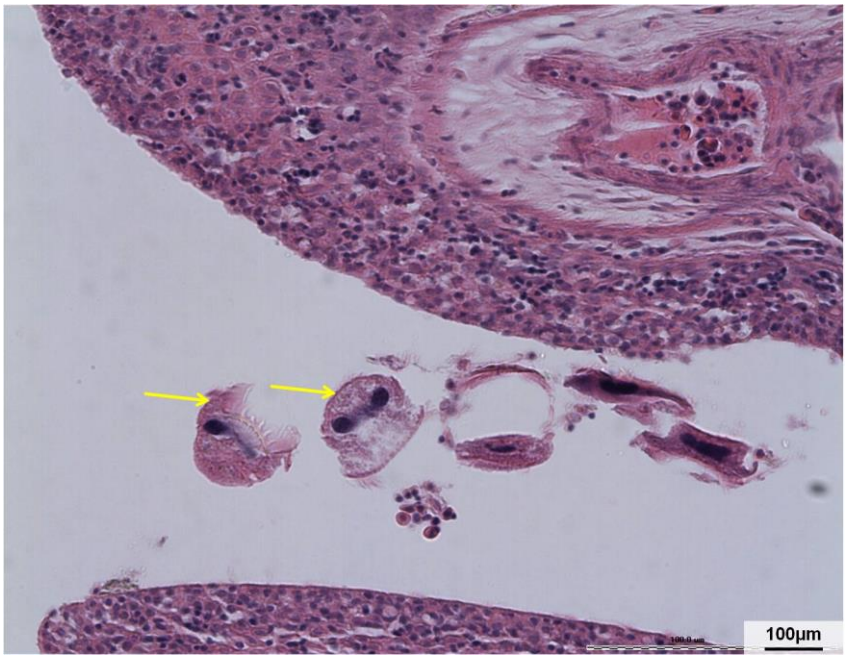

Plate 4-7 *Trichodina* (arrows) were found on the gills of the fish sampled (H&E)

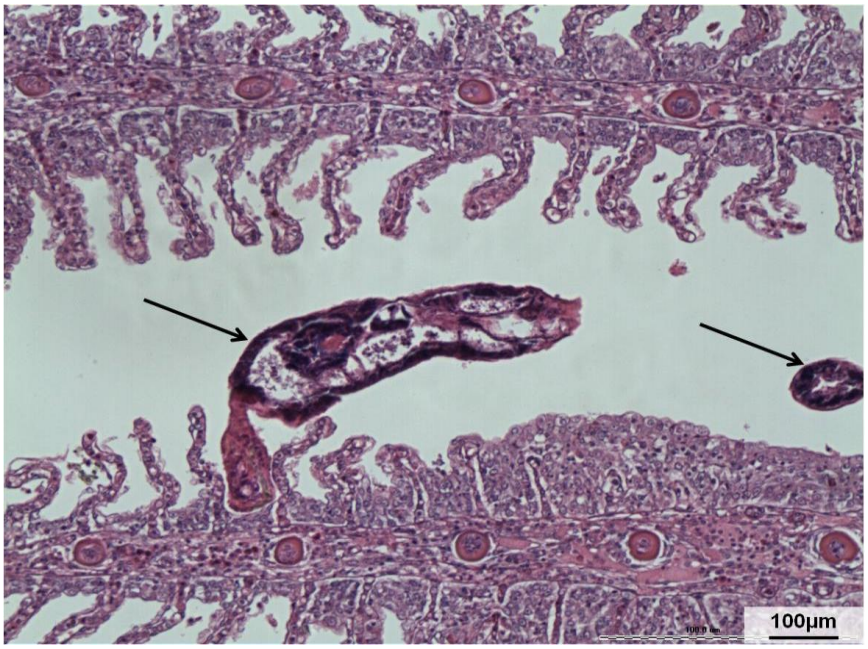

Plate 4-8 Monogeneans (arrows) were found on the gills of the fish sampled (H&E)

4.4.2.2. Histopathological changes

The fish sampled from the apparently normal and the jaundiced group were then divided into four groups by the presence of myxosporean as observed grossly and microscopically. Group A is apparently normal (n=44), group B is myxosporean-affected apparently normal (n=8), group C is jaundiced alone (n=32), and group D is myxosporean-affected jaundiced fish (n=79).

There were no histological changes in the apparently normal fish (group A). The histological changes from liver, kidney and spleen of fish in groups B, C and D are presented in Table 4-5.

Table 4-5 Histological changes of sampled *P. hypophthalmus* cross-tabulation

Histological changes	Group B (n=8)	Group C (n=32)	Group D (n=79)	Chi-square	P-value
Mucous hypertrophy in the common bile duct	0^{a}	13^{a}	57^{b}	22.42	<0.0001
Fibrinoid vasculitis	3^{a}	29^{b}	76^{b}	33.65	<0.0001
Necrosis	1^{a}	30^{b}	65^{b}	28.45	<0.001
Haemorrhages	0^{a}	3^{a}	16^{a}	3.73	0.15
Lymphocytes infiltration	4^{a}	22^{a}	53^{a}	1.12	0.57
Bile duct hyperplasia	0^{a}	12^{a}	30^{a}	4.75	0.09
Degeneration of tubular	1^{a}	21^{b}	39^{ab}	7.5	0.02
Thickening of the glomerular basement membrane	1^{a}	17^{ab}	57^{b}	13.56	<0.001
Hyaline droplet within tubular epithelial cells	1^{a}	10^{a}	36^{a}	4.78	0.09
Fibrin thrombi	1^{a}	11^{a}	17^{a}	2.61	0.27
Haemosiderin deposit	0^{a}	28^{b}	69^{b}	39.65	<0.0001

Note: Values followed by different superscript letters in the rows are significantly different

The heavy myxosporean plasmodial infection associated with inflammatory changes and mononuclear cell infiltration on the gills is shown in Plate 4-9. These parasites were observed attached to both primary and secondary gill lamellae. In gills infected with the myxosporean parasite swelling and fusion of the epithelium (Plate 4-10), hyperplasia in the base and tips of secondary lamellae (Plate 4-10, 4-11). Blood cells were also found in the secondary gill lamellae (Plate 4-11, 4-12). Gill filaments were also damaged as a separation of

secondary lamellar epithelium from the pillar cells (Plate 4-13). These histological changes were also found in jaundiced alone fish.

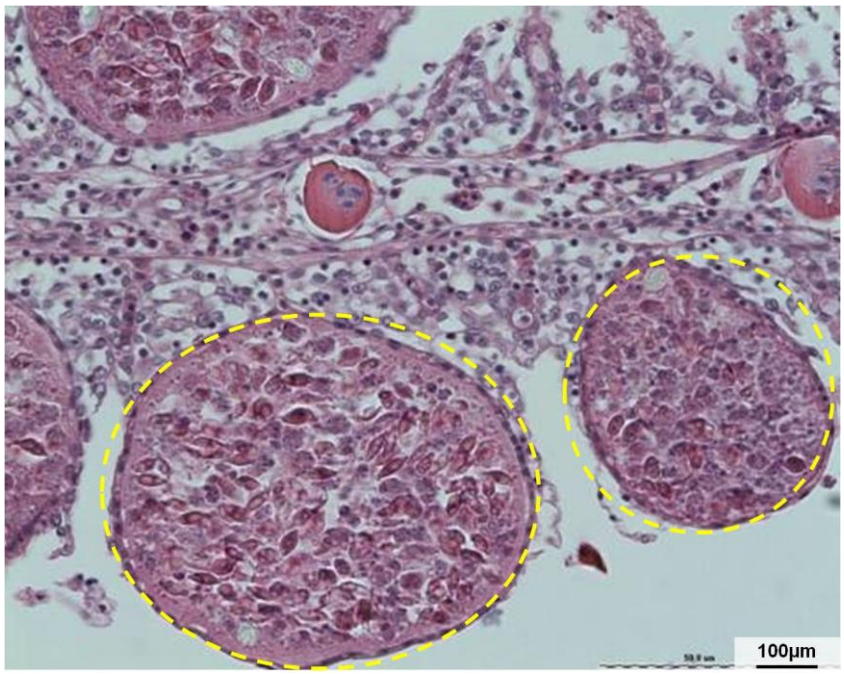

Plate 4-9 Myxosporidian plasmodia (broken circles) with mature spores is present at the base of the gill lamellae in secondary lamellae from jaundiced fish (H&E)

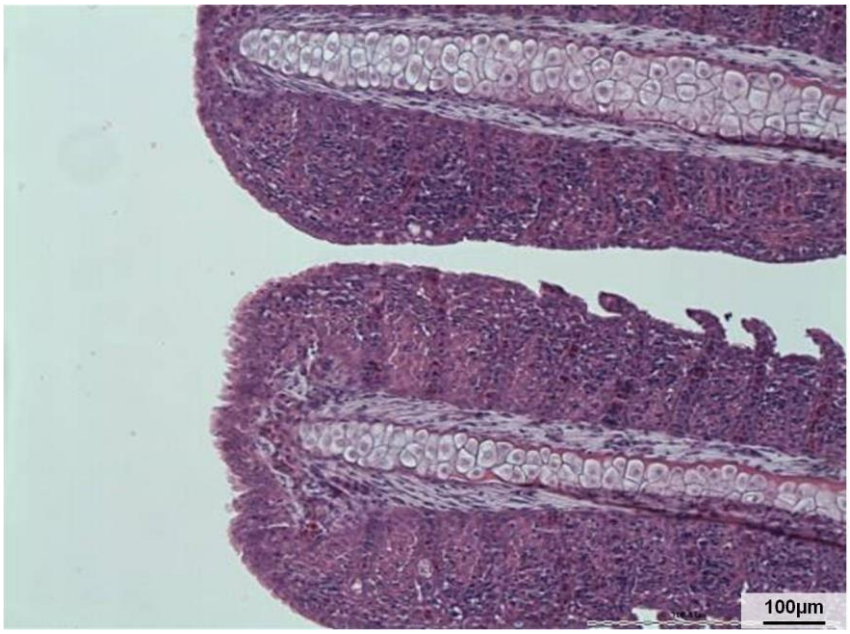

Plate 4-10 Section showing the gills lamellar epithelial hyperplasia from myxosporean-affected apparently normal fish (H&E)

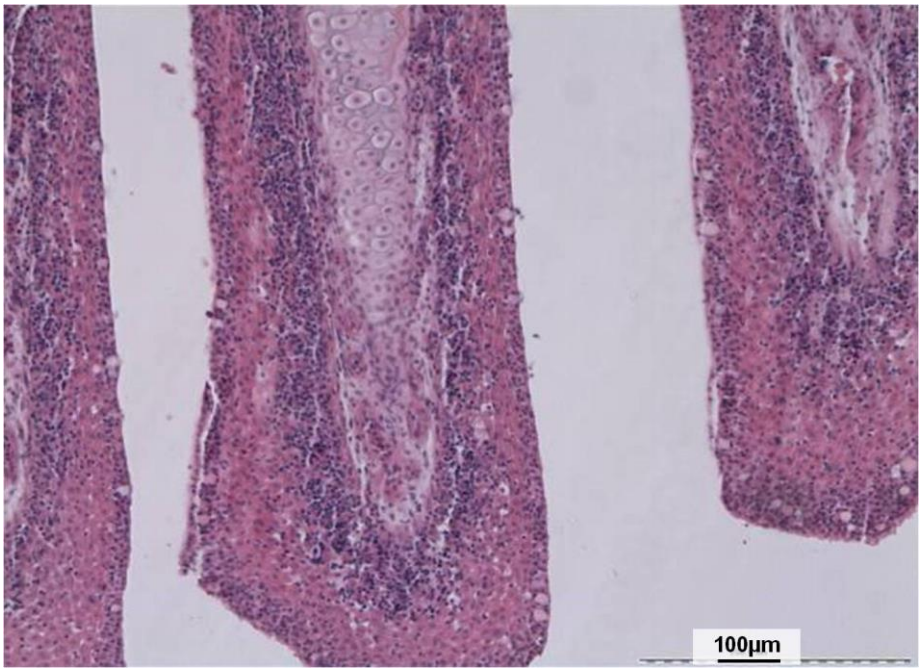

Plate 4-11 Lamellar epithelial hyperplasia causing the characteristic clubbing of filaments from myxosporean-affected jaundiced fish (H&E)

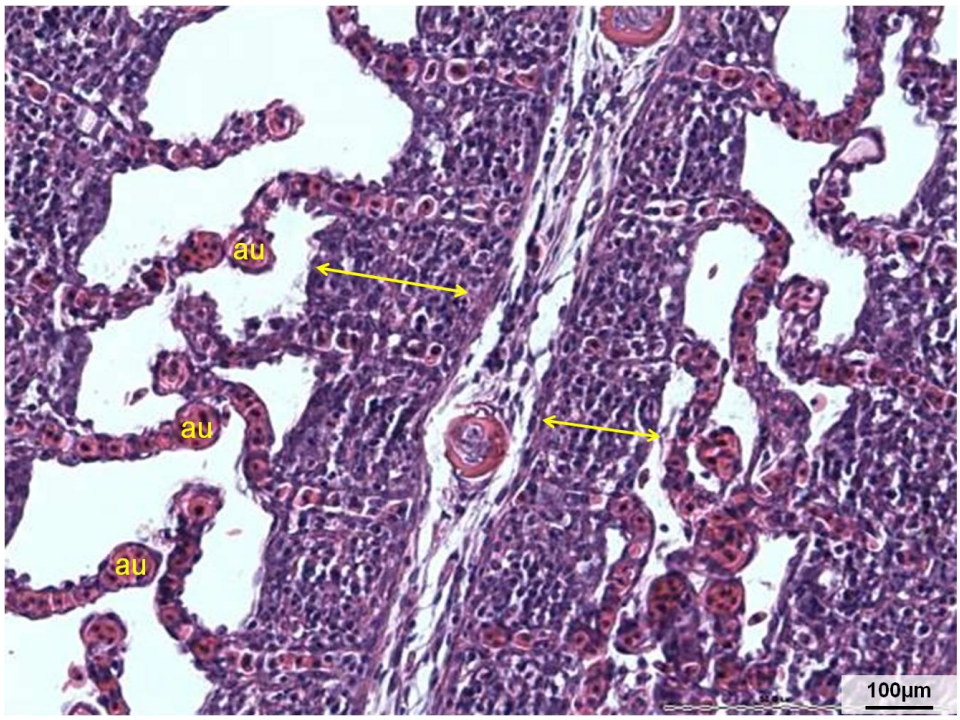

Plate 4-12 Gill section showing hyperplasia of the epithelium (two headed arrows) and lamellar aneurism (au) from myxosporean-affected apparently normal fish (H&E)

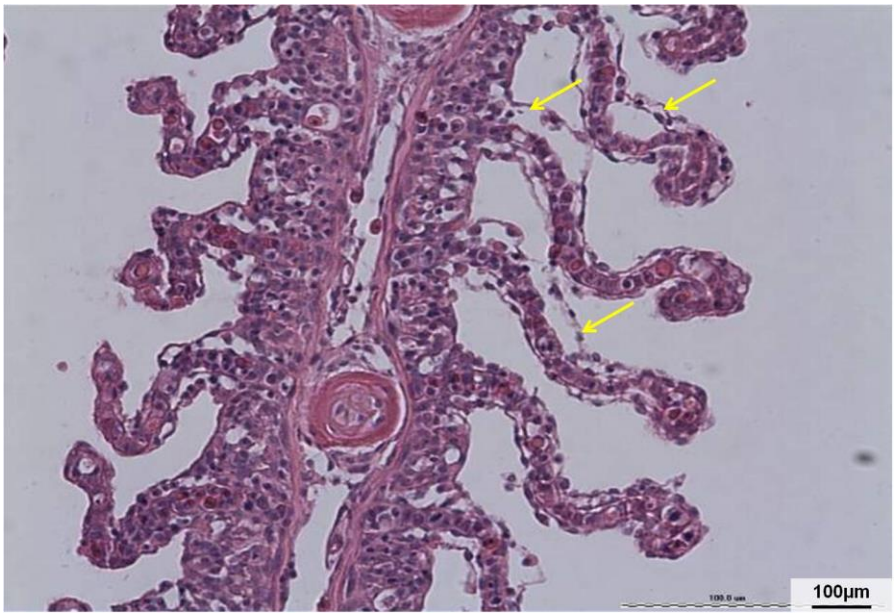

Plate 4-13 Gills structure with epithelial cell proliferation at the base of secondary lamellar and separation of secondary lamellar epithelium from the pillar cells (arrows) from myxosporean-affected jaundiced fish (H&E)

In chapter 3 found that the histological changes in the liver associated with the jaundice included necrosis, presence of leukocytes within the wall of blood vessel and fibrin thrombi within the lumen of affected vessels (vasculitis). The presence of these histological changes were 91% and 95% in the liver of jaundiced only fish and myxosporean-affected jaundiced fish, respectively (Plate 4-14). These were statistically different compared with the liver of myxosporean-affected apparently normal fish was 36% (χ^2 = 33.65, P < 0.0001). Multifocal inflammatory reaction with lymphocytes, monocytes, and neutrophils especially around portal bile ducts were found in myxosporean-affected fish. Additionally, bile ducts were surrounded by mild fibrosis in the myxosporean-affected jaundiced fish. In these cases, infiltrating wall were observed in some part of the bile duct, the most common thickening of the wall

as a result of involvement may be caused by epithelial hyperplasia and goblet cell metaplasia (Plate 4-15).

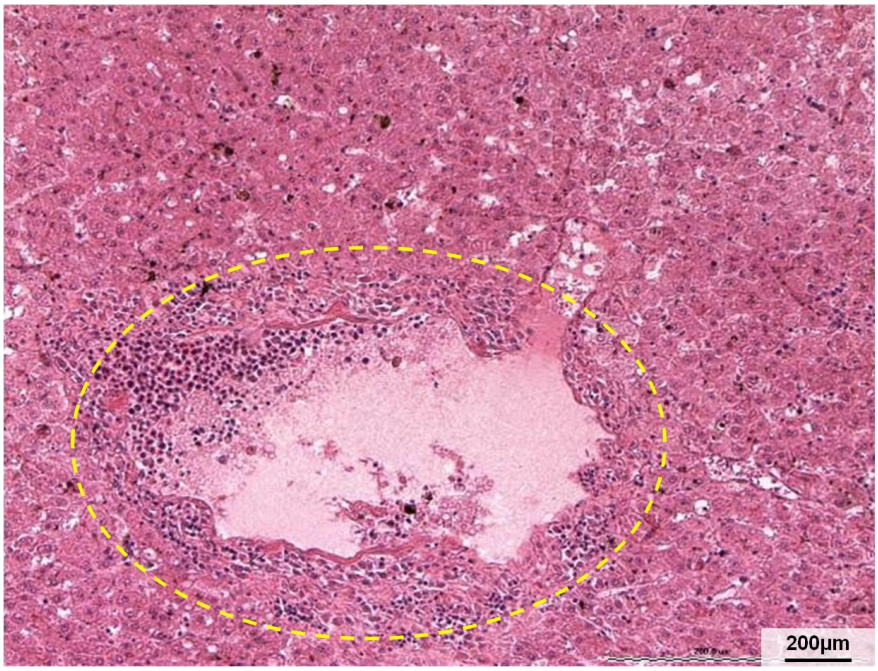

Plate 4-14 Sections of the liver from sampled *P. hypophthalmus* showing fibrinoid necrosis, inflammation of vessel walls (broken circle) (H&E)

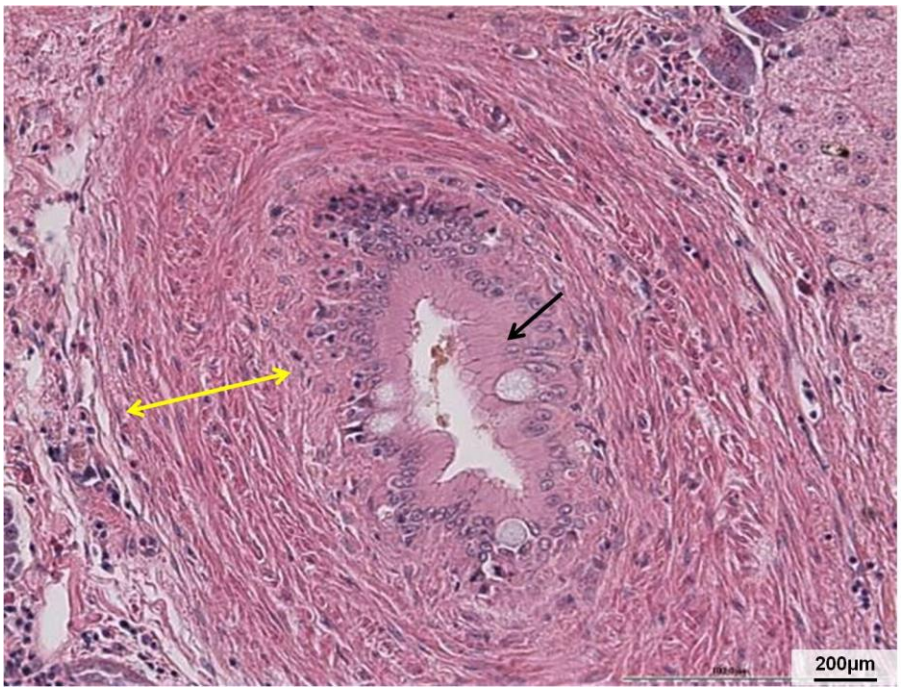

Plate 4-15 Section of bile ducts showing severely increase in surrounding connective tissue (yellow two headed arrow) and abnormal bile duct epithelial cells (arrow) in myxosporean-affected jaundiced fish (H&E)

In the spleen of myxosporean-affected jaundiced fish, plasmodia were found in the serosa covering the spleen. The plasmodia were large, circular or oval in shape and surrounded by a layer of connective tissue, while others were occasionally found close to the blood vessels and spores were often observed surrounded by melanomacrophages (Plate 4-14, 4-15) showing the attachment of the melano-macrophages to large myxposporean spores and transporting them into melano-macrophage centres. Inflammatory cells including lymphocytes, macrophages and neutrophils were the most common cell types observed in the spleen of myxosporean-affected fish and in the layer surrounding the plasmodium (Plate 4-16). The results showed that there was an increase of melano-macrophage centres in the myxosporean-affected jaundiced fish (Plate 4-17). In some cases, myxospores spread among the inflammatory cells (Plate 4-18). However, myxobolus species found in the location of the spleen in myxosporean-affected apparently normal fish (group B) did not appear to induce any serious host response, the spore being concentrated in the MMCs.

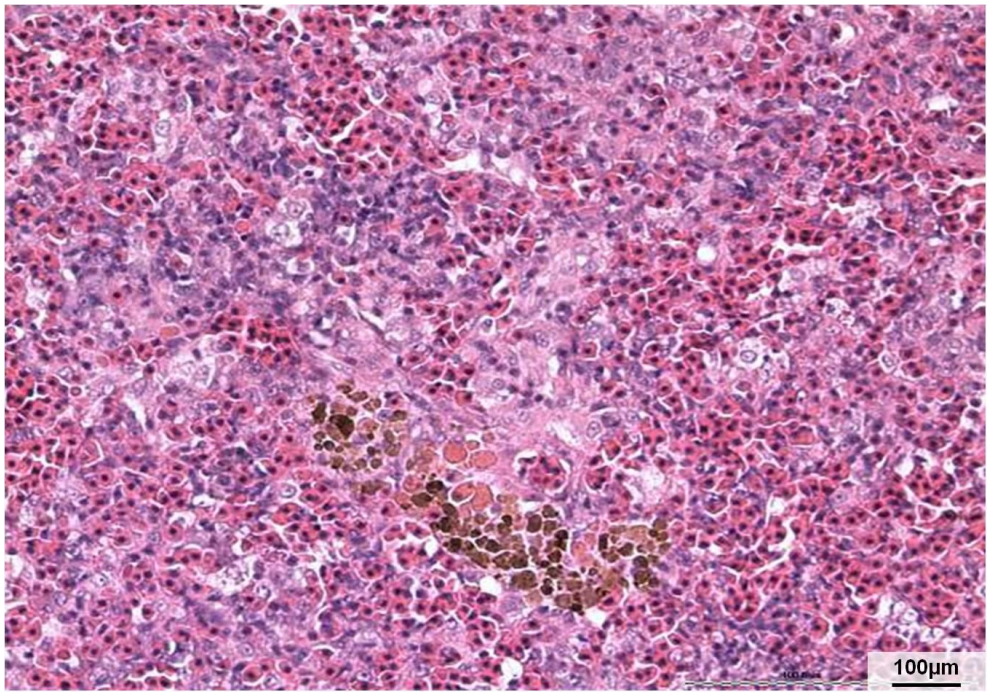

Plate 4-16 Histological changes in spleen showing the presence of inflammatory cells in spleen section from myxosporean-affected fish (H&E)

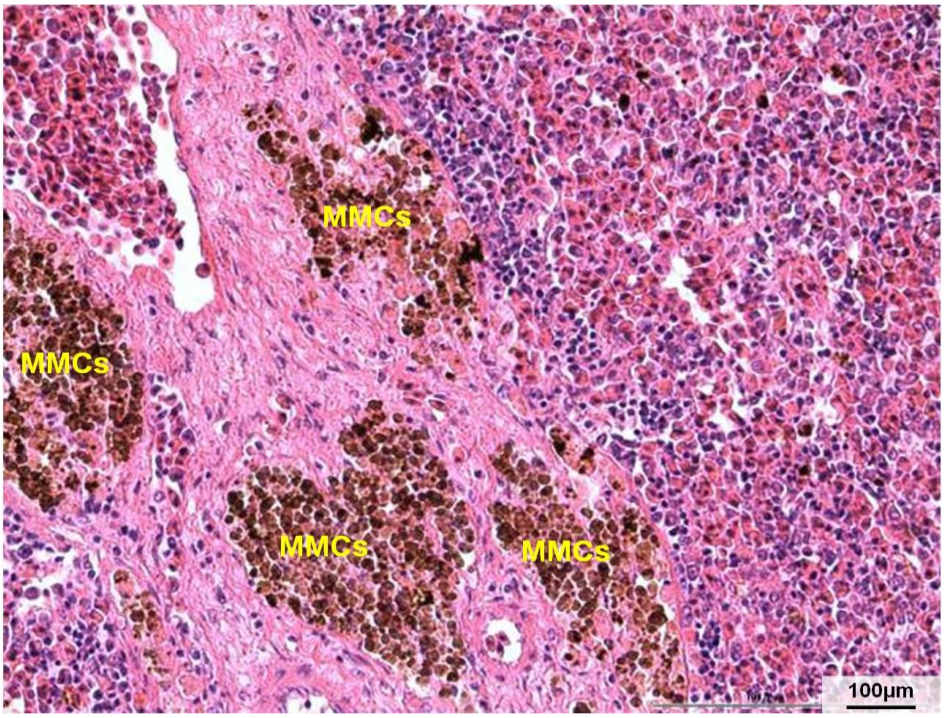

Plate 4-17 Spleen section showing an increase of melano-macrophages centres (MMCs) from myxosporean-affected jaundiced fish (H&E)

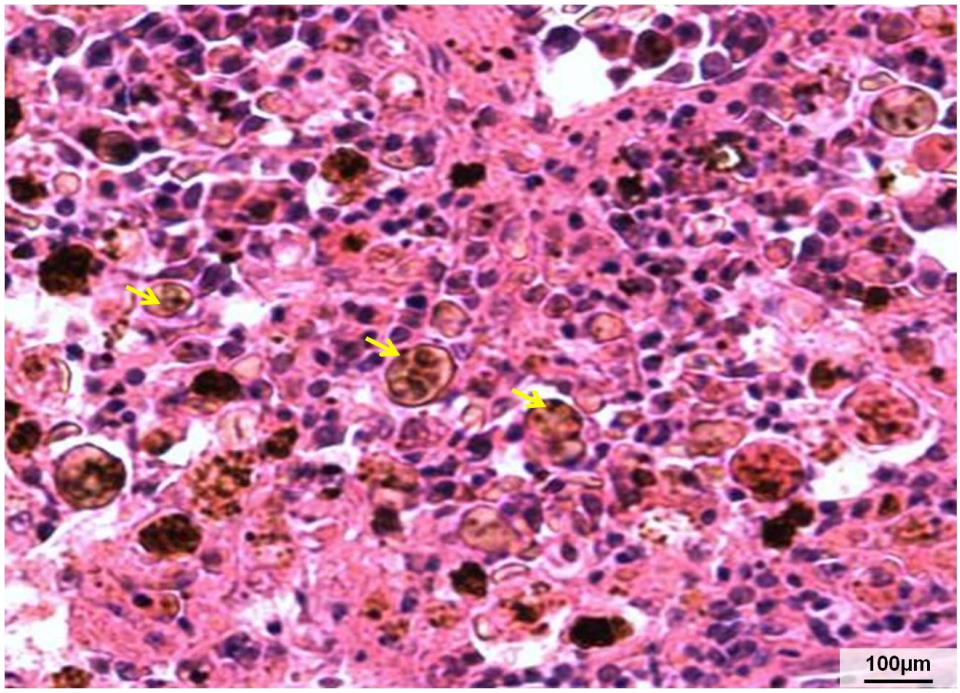

Plate 4-18 Spleen section with myxospores (arrows) spread among the inflammatory cells (H&E)

The histological findings showed that the normal architecture of the kidney was changed and replaced by multifocal necrosis to diffuse areas in the jaundiced alone fish.

The renal sections showed that the glomerular basement membrane is diffusely thickened (Plate 4-19) only in one case (13%) from myxosporean-affected apparently normal fish, while it accounted for 53% and 71% in the jaundiced alone fish group and the myxosporean-affected jaundiced fish, respectively (χ^2 = 13.56, P < 0.0001). By comparison with the glomerular damages, tubular change were light, and were most obvious in those fish with severely damaged glomeruli in jaundiced alone fish and myxosporean-affected jaundiced fish (χ^2 = 7.5, P = 0.02).

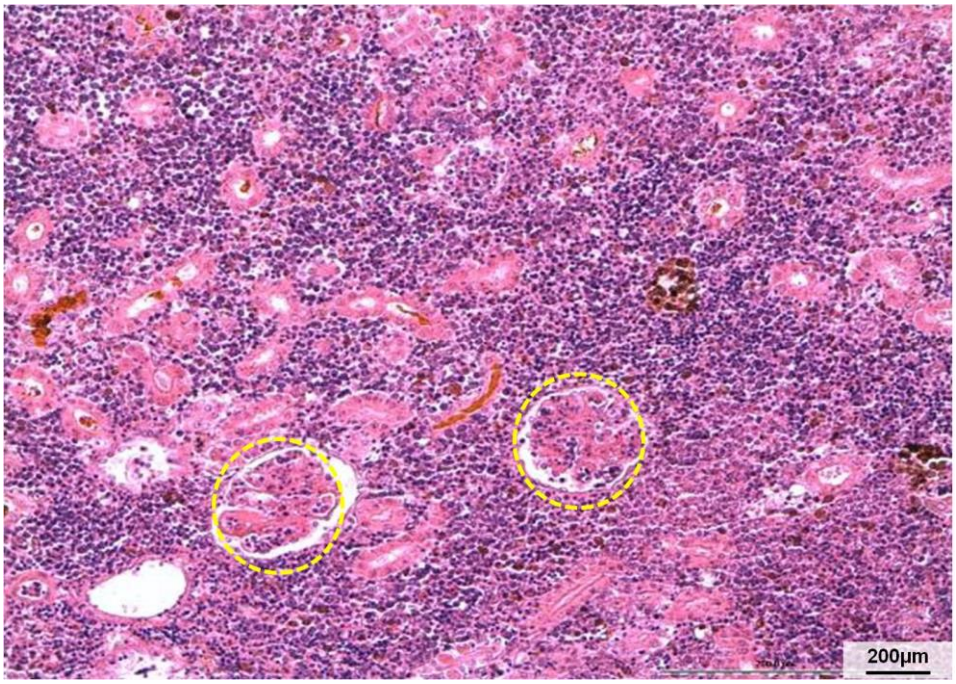

Plate 4-19 Glomeruli showing thickening of the basement membrane from myxosporean-affected jaundiced fish (broken circles) (H&E)

Kidney sections revealed a marked atrophy of some glomeruli cells showing large Bownman's space in myxosporean-affected jaundiced fish. Similar to those described in fish with jaundiced alone (group C). Additionally, hyaline degeneration was found in the renal tubular epithelial cells from myxosporean-affected jaundiced fish (Plate 4-20). The size of the granules was not uniform, but varied in size. Similar findings were observed in myxosporean-affected apparently normal fish and jaundiced alone fish (χ^2 = 4.78, P = 0.09). In a high number of myxosporean gill plasmodia cases, the spores were also observed in the blood vessels from renal sections (Plate 4-21).

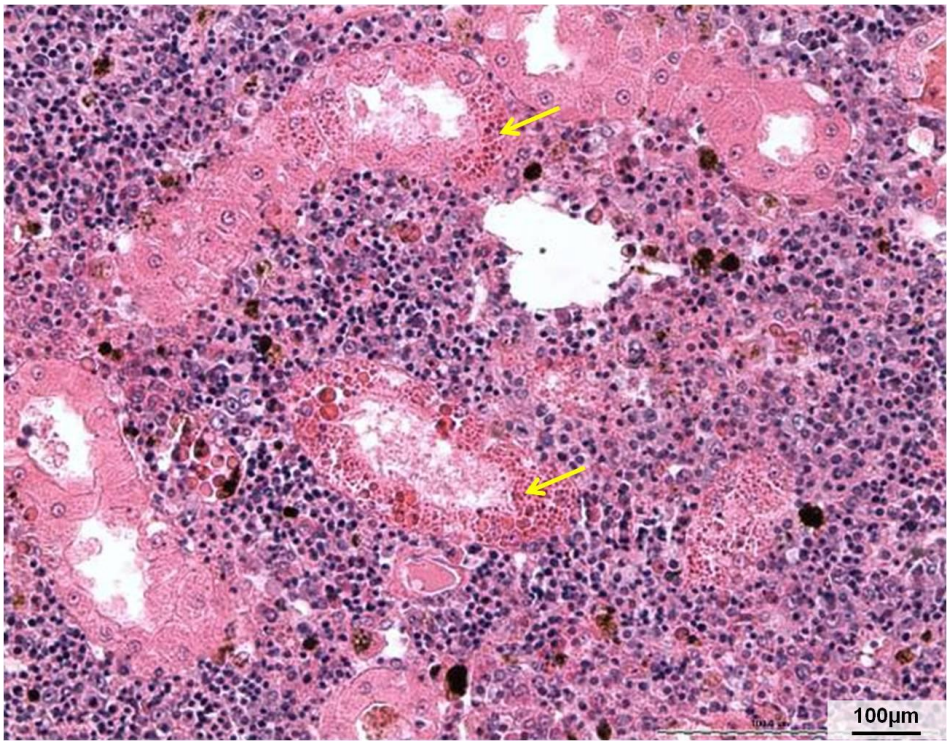

Plate 4-20 Renal tubular degeneration with hyaline droplets (arrows) in the renal tubular epithelial cells from myxosporean-affected jaundiced fish (H&E)

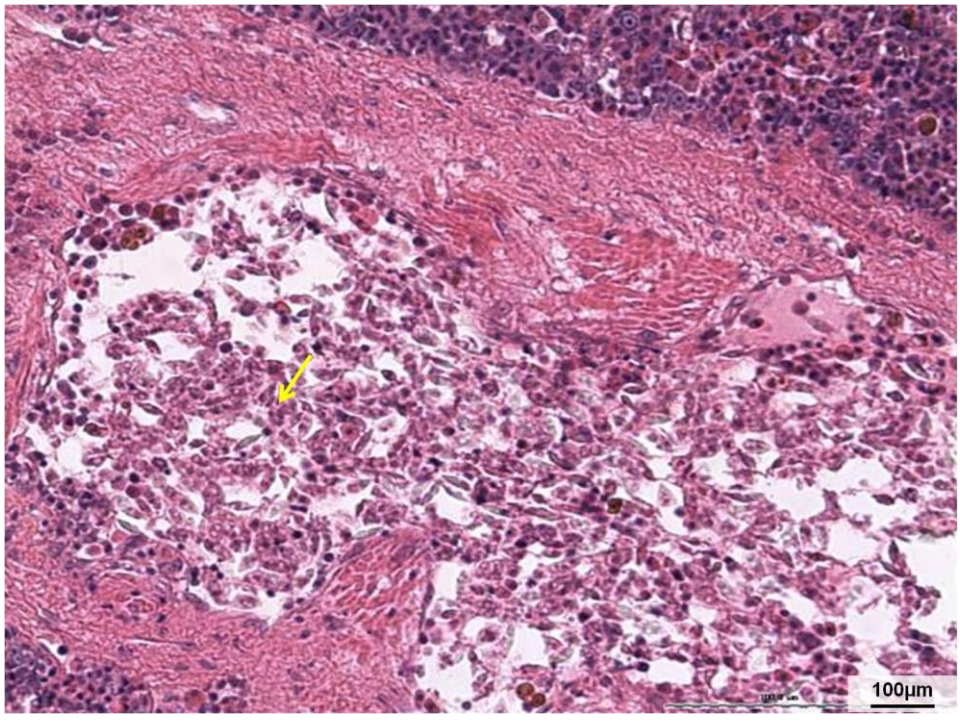

Plate 4-21 Section of kidney showing the presence of myxosorean spores (arrow) within vessel from myxosporean-affected jaundiced fish (H&E)

Deposition of haemosiderin was only observed with varying amounts in the spleen and kidney and significantly increased in jaundiced alone fish and myxosporean-affected jaundiced fish compared with those in apparently normal fish (χ^2 = 39.65, P < 0.0001), especially in the melano-macrophage centres

4.4.3. Myxosporean detection by PCR and sequence identification

4.4.3.1. Polymerase chain reaction (PCR) amplification

The results showed that 100% of the tissue samples from myxosporean-affected jaundiced spleen were positive by PCR. The positive PCR gave bands at the expected molecular weight (370bp) (Plate 4-22). No amplification products were observed in any of the gill, kidney, muscle, common bile duct tissues or the negative control samples.

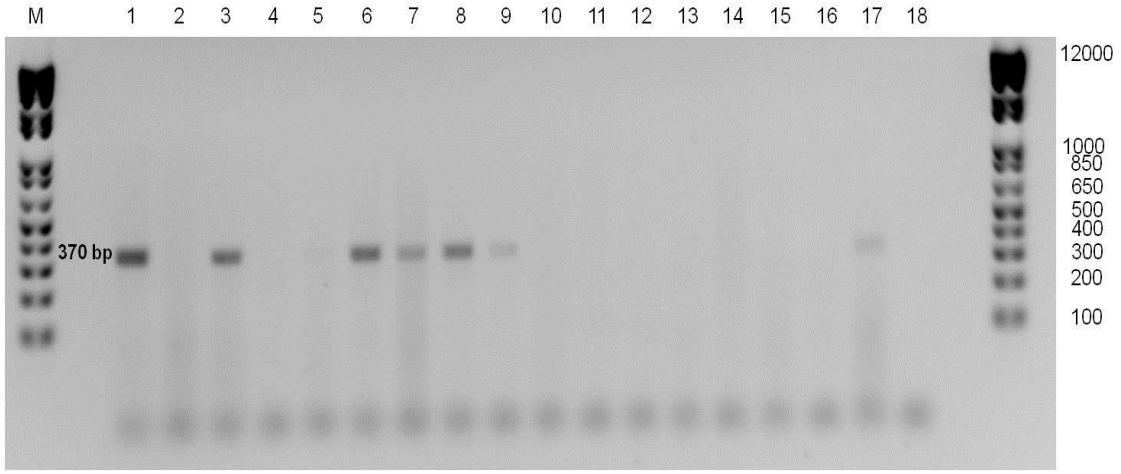

Plate 4-22 Photograph of an agarose gel containing ethidium bromide-stained *Myxobolus* amplicon.

Lane M: the molecular weight marker contains 1 Kb plus DNA ladder; **Lanes 1, 3, 5, 6, 7, 8, 9, and 17**: DNA from myxosporean-affected spleen tissues; **Lane 2, 4:** DNA from myxosporean-affected kidney tissues; **Lane 10:** *P. hypophthalmus* genomic DNA from uninfected fish (i.e. negative control DNA); **Lanes 11 to 16:** DNA from myxosporean-affected gill tissues; and **Lane 18** is negative control (No DNA).

4.4.3.2. Sequencing

A 99% match was obtained for the myxosporean-affected jaundiced spleen samples taken in this study to *Myxobolus pangasii* (GenBank TM accession number FJ816270.1). Although other matches were also obtained the percentage homology was rather low at 96% for *Myxobolus hakyi* (GenBank TM accession number FJ816269.1), and so these were not included in further analyses.

4.5. Discussion

In the present study, parasites belonging to genera *Myxobolus, Hennegoides, Henneguya* and monogenean were found on the gill samples. The farmers interviewed noted a seasonal incidence of heavy infestation of parasites on *P. hypophthalmus* farms during the rainy season or in cooler weather. The ubiquitous presence of these parasites meant that farmers provided a high dose of varied chemicals to try to treat or prevent the parasite infestations. Although the fish farmers were aware of the presence of parasites in their fish stocks they appeared to lack basic knowledge on monitoring, selection and application of chemical/veterinary drugs.

Among the myxosporea Butschi 1881, *Myxobolus* and *Henneguya* genera together represent almost half of the described diversity of Myxozoa found in farmed fish species, with about 800 and 200 described parasite species, respectively (Eiras *et al.*, 2005). Infections of the myxosporeans identified as *Henneguya, Myxobolus,* and *Hennegoides* have been reported in cultured freshwater fish from the Mekong Delta, Vietnam (Ky and Te, 2000; Dung *et al.*,

2008; Hang *et al.*, 2008). Hence it was not surprising to find the presence of these parasites in the farmed *P. hypophthalmus* sampled in this study. Furthermore, 91% of the farmers interviewed reported the presence of monogenean parasites in the gills of *P. hypophthalmus*.

Most monogeneans found on the gills of *P. hypophthamus* are described as dactylogyroids (Hang *et al.*, 2008). *Pangasianodon hypophthalmus* seems to co-exist with their specific monogeneans in the farming conditions without significant adverse effects to their health. There were no records of ill effects or mortalities due to monogenean infection in fish with body weights more than 200g, even when numbers of plasmodia were high on the fish gills. According to Paperna (1963a, 1963b) the larvae of *Dactylogyrus* either actively migrate to the gills after attaching to the skin of the fish or become attached to the gills when washed over these tissues. Hoole *et al.* (2001) said that the parasite infection causes hyperplasia of the epithelium and deformation of the gill lamellae. In fry and fingerlings of *P. hypophthalmus* this cellular damage can be particularly problematic and results in gross respiratory failure. However, monogeneans can also causes focal cellular damage at their attachment sites, thus increasing the risk of secondary opportunistic pathogens infections (Paperna, 1963b).

It is unfortunate that the co-occurrence of myxosporean on the gills from the genera *Myxobolus, Henneguya, Hennegoides* which was clearly observed microscopically during this study, did not provide high quality DNA samples. This may have been an artefact of using formalin fixed and wax embedded

tissue sections or it may be that the amount of parasite DNA was very low and mixed with large amounts of host DNA. According to Eszterbauer (2004), to avoid contamination by other myxosporeans, it is important to collect samples very carefully, and to use only separated cysts for molecular biological examination. Some authors reported that PCR is more problematic with DNA extracted from fixed tissues, as the fixative and fixation process lower the quantity and quality of specific DNA extracts (Schader and Halanych, 2003; Bucklin and Allen, 2004). It would be useful to collect fresh gill samples for PCR and compare them with the fixed tissue presented in this study. However, better success was found when using the myxosporean DNA recovered and amplified from spleens where the large individual cysts were traced and easily collected.

The myxosporean species infecting the spleens of jaundiced *P. hypophthalmus*, cultured in the Mekong Delta of Vietnam, was consistent morphologically and molecularly with *M. pangasii*. The examination of gill, liver, muscle, and common bile duct did not show the presence of *M. pangasii,* possibly due to the specific and possibly preferred location of *M. pangasii* within the fish. According to Molnár *et al.* (2006a) *M. pangasii* is a little known myxosporean parasite that seems to prefer splenic tissues and was described for the first time by Molnár *et al*., (2006a) in *P. hypophthalmus* from Malaysia. However, the life cycle of *M. pangasii* like that of most myxosporeans remains unknown. In spite of its common occurrence, little is known about the morphology of the infective stage and the initial site of infection in the host. Transmission occurs naturally when susceptible fish are exposed to water containing the infectious unit which remains undescribed. Further challenge studies in the laboratory are needed to

elucidate the transmission of this myxosporean. The ability to manipulate infection in the laboratory would result in scientific information on the relationship between myxosporean and jaundice syndrome.

The prevalence of the gill myxosporean was generally high in all of the fish sampled during the present study, which again was unsurprising. Furthermore, there did not appear to be a significant association with the presence of the parasite and other affected clinical conditions expressed in the fish. However, although statistically not significant, the parasite was found at higher numbers and/or higher amounts of tissue infected per fish from the group suffering from the haemolytic jaundice. The findings are similar to those observed in a survey conducted by Hang (2010). This author reported that fish with heavy infections of myxosporean was always associated with clinical signs of other diseases (e.g. bacillary necrosis of catfish, hemorrhages symptom, and jaundice). It may be that the jaundiced fish are immuno-compromised or weakened in some way as they are coping with the jaundice, hence making them more susceptible to parasitism. Feist and Longshaw (2006) stated that most myxozoans, even if they cause acute disease, do not kill their host unless secondary stressors and/or reduced immunocompetence have a significant effect. In other words, they are not the primary cause of mortalities. Therefore, these parasite infections may be considered more of nuisance as they may exacerbate fish losses, especially if a higher infection intensity of the parasite is associated with poor environmental or biological conditions, which are common in these fish farms.

Myxosporean infections were not detected in the liver of any sampled fish but instead the highest infection of myxosporean was found in the gills, which might be due to the suitable habitat for myxosporean. The intensive proliferation of mononuclear cells, observed in gills of infected *P. hypophthalmus* was considered as a normal immune reaction of fish to an injury/assault and was directed against the parasite infection. Piper *et al.* (1982) suggest that *Henneguya* is the most common pathogen found in the freshwater channel catfish, in which produces infection in the gill, skin and internal organs. Most members of the myxosporean class are typically organ specific, and infect only certain target organs (Molnár, 1994; Noga, 1996). Severe damage to the gill has been reported by histological examination during the myxobolasis of catla, common carp and gible carp (Dyková and Lom, 1988; Wang *et al.,* 2003).

Myxobolus infection in cultured carp resulting in lesions, inflammatory congestion and hyperplasia in the gills are histological changes described by several authors (Mohammad *et al.*, 2001). The jaundiced fish in these affected ponds were found aggregated near the inflowing water and this may be due to the presence of the heavy parasite load on the gills, which compromises respiration, hence the need to distribute themselves near the highest oxygen inlet. Rukyani (1990) stated that dense infestation of *Myxobolus* resulted in pathological damage to the gill structure due to the development of plasmodia affecting respiratory function; fish were swimming near the surface with distended opercula. Molnár *et al.* (2006a) reported that members of the genus *Henneguya* Thelohan are the best known myxosporean parasites of catfishes. The gill is the major respiration organ, the primary site of nitrogenous waste

excretion and plays an important role in ionic balance (Noga, 2000). Hence any alteration of the gill will impact on the fish health and welfare in the farm. Species from genus *Henneguya* have a different manner of interaction with fish gill structures, resulting in different presentation of the disease (Dyková and Lom, 1978; Martins *et al.*, 1997; Molnár *et al.*, 2006a, 2006b). Feist and Longshaw (2008) also mentioned that the pathogenicity of parasites in the gills depends on the intensity of infection and that the number of plasmodia influences the respiratory function. Myxosporean-affected jaundiced fish in this study exhibited clinical signs in agreement with Post (1987) and Chavda *et al.* (2010) for myxosporidiosis outbreaks.

The mechanism of migration and replication of myxosporeans within Vietnamese freshwater fish have not yet been fully elucidated. The presence of myxosporeans in the blood vessels may indicate how it moves towards the target organs via the circulatory system (Kent and Hedrick, 1985; Molnár, 1994). An increase in the abundance of melano-macrophage density in the splenic pulp can be caused by pathological events such as protozoan infection, haemolytic anaemia of various aetiology, or iron overload (Crosby, 1957; Roberts 1975, 1978). According to Dyková (1984), melano-macrophages can attach to large myxosporean spores and transport them to melano-macrophage centres where the spores can accumulate in these centres.

The results indicated that severe haemolysis was occurring in the jaundiced *P. hypophthalmus.* The present study found the parasite in the blood vessels and this was higher in the jaundiced fish compared with the apparently normal fish.

In the vascular system, the vessels were inflamed, swollen and produced nodules so that blood flow was reduced. The tissues which receive blood from those vessels can be damaged and contributed towards the overall anaemia and possible necrosis. *Myxobolus* infection may also cause haemolytic anaemia, which is characterized by reduced haemoglobin concentration, haematocrit and erythrocyte number or damage of the red blood cells in sampled fish. These supposed and accepted by Shiczakow (1935), Kocylowski and Myazynski (1963), Schulman (1966), and Chavda *et al.*, (2010). However, given the distribution of these parasites in many of the fish with and without clinical signs of disease it is unlikely that they are the true cause of the haemolytic anemia in the farmed *P. hypophthalmus*.

The present study showed that in the case of splenic infection with *M. pangasii* alone did not show any serious damages and host response. The evidence from this study would support that *M. pangasii* was not a primary pathogen associated with a single clinical condition and was not the cause of the haemolytic jaundice in the farmed *P. hypophthalmus*. It might exacerbate the condition in already affected fish and it might leave them compromised for other primary pathogens but the presence of these parasites was not associated with the haemolytic jaundice and may be considered as a nuisance rather than a primary pathogen in farmed *P. hypophthalmus* sampled in Vietnam.

4.6. References

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Identification of risk factors for jaundice of farmed catfish (*Pangasianodon hypophthalmus*, Sauvage) in the Mekong Delta, Vietnam

5.1. Abstract

A health condition identified as jaundice occurring in farmed *P. hypophthalmus* raised in the Mekong Delta has increased in recent years. A cross-sectional study of 46 *P. hypophthalmus* farms was conducted to establish the extent and severity of this condition and identify farm-level risk factors. An interview-based study was conducted from 2008 to 2009 using a semi-structured questionnaire and logistic regression analyses were performed to identify potential risk factors. It was found that jaundice was present in 89% of the study sites from 2007 to 2009. The total number of affected sites had increased annually with prevalence values in the sample ranging from 48% during 2007 to 82% during 2009. The jaundice condition in *P. hypophthalmus* can increase morbidity and mortalities on the farms but significantly affects the industry through increased downgrading and/or rejection of fish due to reduced fillet quality by processors. None of the farm-level variables investigated with this condition achieved lasting statistical relevance in multivariable models.

Key words: Jaundice, Vietnamese catfish (*Pangasianodon hypophthalmus*), risk factor, the Mekong Delta.

5.2. Introduction

Pangasius catfish farming is an important contributor to the Vietnamese economy, through global exports of a wide range of food products sold internationally. Vietnamese Pangasius exports provided a potential earning capacity estimated at 1.43 billion USD in 2010 (VASEP, 2010). More than 90% of total pangasius production from Vietnam was processed and exported to 136 countries worldwide in 2010 (Fisheries Directorate, 2010), and the continued demand for these products explains much of the expansion of this now intensive farming practise.

Since the initial establishment of this aquatic industry, improved culture systems, provision of well managed hatcheries, and commercial feeds have all contributed significantly to the valuable growth in Vietnamese pangasius production. These high quality food products are raised in many provinces in the Mekong Delta of Vietnam but primarily found in An Giang, Dong Thap, CanTho and Vinh Long. The fast growth of the *Pangasianodon hypophthalmus* industry, however, has led to production losses through mortality caused by disease outbreaks and environmental degradation in almost all regions of the Mekong Delta, Vietnam. A jaundice condition, identified grossly by yellow colouration in the skin of the abdominal area, eyes and fins has led to large numbers of fish losses in Vietnam (Dung, 2006). According to Khoi *et al.* (2008), jaundice ranks fourth after bacillary necrosis of catfish, haemorrhagic, and parasitic diseases.

The jaundice condition is not always associated with high mortality but often with morbidity and other infections (Chapter 2). However the significant impact of this health condition is related to the number of farms affected by downgrading and/or rejection of their fish at harvest time. Fillet colour is part of the grading scheme used by the processing plants in Vietnam to determine the fish fillet quality and related price for the product. Therefore, downgrading of the final product by the processors often results in significant financial loss to the farmers and has a negative economical impact on *P. hypophthalmus* industry in the Mekong Delta of Vietnam. Recently, the jaundice condition has been reported more frequently and the continued increase in jaundice cases in this area was identified as a significant problem to the development of this aquatic food sector.

The cause and progression of the jaundice in *P. hypophthalmus* is not well understood, but several on-farm factors have been suggested. Jaundice in *P. hypophthalmus* may be induced by a variety of factors including suboptimal water quality, inadequate feed quality, and high stocking density or presence of numerous pathological agents (Hung *et al.*, 2005; Vinh, 2006; Binh, 2008; Tin 2008). Clinical diagnostic investigations showed that no single infectious agent was found associated with this disease (Chapter 3). Similar clinical findings have been reported for other Clarias catfish species in Thailand (Tonguthai *et al.*, 1993); and in Taiwan (Chang *et al.*, 2008), but this condition has not yet been fully understood for farmed Pangasius catfish.

Previous studies investigating jaundice in Clarias species found several causes including feeding fish with rancid chicken viscera (Pearson *et al.*, 1994), lipid peroxidation induced by the severe increase of reaction oxygen species in the animal tissue (Sakai *et al.*, 1990), a pollutant-induced haemolytic anaemia and hyperbilirubinemia (Croce and Stagg, 1997), obstruction of the common bile duct by *nematodes* and inflammation of the bile ducts by extension from enteritis or by infection with trematodes (Otto *et al.*, 2000), and infectious haemolytic anaemia (Smith *et al.*, 2006). Therefore it would appear that clinical jaundice in fish is not a simple process and many factors may affect the different fish species and production systems.

The aim of the current study was to examine the farm-level risk factors for jaundice condition in the *P. hypophthalmus* production systems in Vietnam using a cross sectional study. Cross-sectional studies are often called "observational studies" that make investigations about the relationship between the disease and other variables of the population of interest, allowing large numbers of potential risk factors to be screened (Del-Pozo *et al.,* 2009).

5.3. Materials and methods

5.3.1. Sampling sites

This cross-sectional study of *P. hypophthalmus* catfish farms was conducted from 28th April to 20th August 2008 and 12th October to 10th November 2009, with data collected between 2007 and 2009. All sites produced fish in earthen ponds and were distributed throughout the main production areas of Mekong Delta (8^{o}33$^{'}$–10^{o}55$^{'}$N, 104^{o}30$^{'}$–106^{o}50'E), Vietnam. Forty-six *P. hypophthalmus*

grow-out farms distributed in Vinh Long, Can Tho, Ben Tre, Dong Thap, and An Giang provinces, Vietnam were selected from the fisheries profile of provincial fisheries department. The data collection and fish sampling was described in chapters 2 and 3. In this study the unit of observation was the whole farm, and the risk factors were those associated with jaundice positive farms. Variables were categorized as shown in Table 5-1.

Table 5-1 List of variables used during jaundice cross-sectional study in *P. hypophthalmus* from 2007 – 2009

Category	Variables	Variable descriptions
Outcome	Presence of jaundice from 2007 to 2009	Yes/ No
	Number of jaundiced fish	(<30 fish)/ (30-<100 fish)/ (100-<200 fish)/ (200-500 fish)/ (>500 fish)
Farm management	Farm location	Vinh Long/ Dong Thap/ Can Tho/ Ben Tre/ An Giang
	Farm scale	Small/ Medium/ Large
	Fish farming experience (years)	Continuous, min, max, mean
	Culture cycle (months/crop)	Continuous, min, max, mean
	Depth of pond (m)	Continuous, min, max, mean
	Pond preparation	Yes/ No
	Water exchange	Yes/ No
	Frequency of water exchange	Everyday/ Depend on water quality
	Amount of water exchange	20%/ 30%/ 40%/ 50%
	Water treatment	Yes/ No
	Type of feed	Home-made pellets/ Commercial pellets/ Combination
	Number of fish farm within local area	(1-5 farms)/ (6-10 farms)/ (>10 farms)
Environment (Seasonality of jaundice outbreak)	Dry season	Yes/ No
	Rainy season	Yes/ No
	Flood season	Yes/ No
Fish variables	Source of fingerling	An Giang province/ Dong Thap province/ Self-propagating/ Others
	Stocking density (fish/m^2)	Continuous, min, max, mean
	Size at stocking (cm)*	Continuous, min, max, mean
	Mean production (tonnes/ha/crop)	Continuous, min, max, mean
	Size of jaundiced fish on the days of the outbreak	(<150g)/ (150-499g)/ (500-1,000g)/ (>1,000g)
General disease history	Bacillary necrosis of catfish (BNP)	Yes/ No
	Haemorrhagic symptoms	Yes/ No
	Swollen eyes	Yes/ No
	Parasite	Yes/ No
	Fungus	Yes/ No
Parasite infection history	Monogenean	Yes/ No
	Nematode (round worm)	Yes/ No
	Myxosporean	Yes/ No
	Others	Yes/ No

*Note: * Measured as the body height at the point where the dorsal fin meets the body.*

5.3.2. Questionnaire design and validation

Detailed on-farm surveys were conducted in each site using a single questionnaire grouped into five sections. Each section included questions on farmer background, farm profile, pond and water management, feed and feeding management, and diseases. Both closed and open questions were allowed in the questionnaire with open questions having a limited number of options (Oppenheim, 1992; Thrusfield, 2006). Open questions were used to obtain information about pond area, stocking density and yield. The information required for the survey was obtained from literature searches on jaundice and information available on the usual management procedures of *P. hypophthalmus* sites. These questionnaires were pilot tested with a small number of *P. hypophthalmus* farms which were not then included in the larger survey.

Secondary data was collected from newspaper articles, organizations' reports and official documents to cross-check the primary information obtained through the interviews.

5.3.3. Data Analysis

All the data management and analysis were conducted using Statistical Package for Social Science version 16 (SPSS Inc.), Microsoft Excel 2007™ (Microsoft, USA) and EpiInfo™ (CDC, Atlanta, USA). The identification of risk factors was based in statistical association with the dichotomous outcome problem a case of jaundice in 2007-2009 = Yes/No. This defined as: ''A case of jaundice is a *P. hypophthalmus* producing site that had yellow coloured fish

reported by farmer on fish samples from one or more of its productions units at any moment between 2007 and 2009". Jaundiced fish were identified according to a case definition created from previous literature and based on the gross presentation (Hung, 2004; Dung, 2006). The case definition was: ''Jaundice is a condition of *P. hypophthalmus* present with any gross external signs as yellow pigmentation in the skin of the abdominal area, sclera of the eyes, and fin bases"

The analysis of the data was firstly descriptive, which provided information on the background of jaundice in the Mekong Delta of Vietnam. Initially, two-by-two tables were used to evaluate confounding between potential farm level risk factors. The data was not normal and univariable analysis used Fisher's Exact (FE) for dichotomous variables and non-parametric Kruskal-Wallis (KW) tests for continuous variables, with results with $P < 0.05$ considered as statistically significant. To construct the multivariables analysis, all variables were categorized and incorporated into univariable logistic regression to identify independent variables for jaundice. Variables with a Wald P-value ≤ 0.25 were selected for multivariable analysis. Continuous factors were first screened to ensure the assumption of logit of the continuous variables with dependent variable was done by taking the natural log of the explanatory variable and incorporating the cross-product of this and the untransformed data as an interaction term within the model. Continuous data that did not meet this assumption were included as dichotomous variables (i.e. high/low) only.

Variables included in the models were also examined for multicolinearity using Pearson correlation and variables presenting significant correlation ($P < 0.05$) were not included in the same model. Stepwise regression analysis using forward and backward were carried out and the best model was determined by a significant reduction in the -2*log likelihood statistic. Logistic regression model fit was assessed using Hosmer-Lemeshow test and all possible interaction terms were tested (Dohoo *et al.,* 2003; Thrusfield, 2006).

5.4. Results

5.4.1 Study response and interviewed sites

In the study area, the ponds were built without water storage or a reservoir. Most farmers had 2-5 years' experience raising *P. hypophthalmus* (85%), 13% had more than 5 years, and 2% less than 2 years. Of the 46 farms included, 30% were small-scale farms having less than 10,000 m^2 of pond area, 37% were medium-scale farms having a pond area between 10,000 and 50,000 m^2, and 33% were large-scale farms having pond area over 50,000 m^2.

Source of fingerling supply was varied according to the interviewed farmers. Approximately 54% of farmers used the fingerlings originating from Dong Thap province, 20% from An Giang province, and 22% from other provinces. Only 4% of the farmers interviewed owned the hatchery or used self-propagating fingerlings.

The main water source used for all *P. hypophthalmus* farming operations was directly from the Mekong Delta River. Prior to stocking, the farmers used more

than one method of water/pond treatment including TCCA90 (Tri Chloro Isocyanuric 90%), iodine, Yucca (vegetable extract to reduce NH_3) and $CuSO_4$ purchased from local suppliers.

To reduce sediment and faecal build up in the pond during the culture period, 67% of farmers exchanged water once per day, 26% exchanged water twice per day where these farmers relied mainly on diurnal tidal fluctuations, and 7% exchanged water depending on the water quality on their farm which they checked regularly either by measurement or visually. Exchanging pond water was at a rate of 20-40% per time, irrespective of the scale of the farming system.

Commercial feed pellets were the most popular food source used on the *P. hypophthalmus* farms visited, only 2% of farm used home-made pellets which the manufactured themselves and another 2% used home-made wet feeds. The farmers interviewed used several feed brands and all of them had changed brand, formula or pellet size during the production cycle. Therefore, the feeding information was not sufficiently reliable to statistical analysis.

5.4.2 Impact and distribution of jaundice condition in farmed Vietnamese *P. hypophthalmus*

Jaundice had been observed at least once during the production history of *P. hypophthalmus* culture and was reported in 89% of interviewed sites (Table 5-2).

Table 5-2 Regional distribution of observed jaundice condition in farmed Vietnamese *P. hypophthalmus*

Region	Jaundice present (%)	Jaundice absent (%)	Total
Dong Thap	14 (93%)	1 (7%)	15
Vinh Long	16 (80%)	4 (20%)	20
Can Tho	4 (100%)	0 (0%)	4
Ben Tre	2 (100%)	0 (0%)	2
An Giang	5 (100%)	0 (0%)	5
Total	41	5	46

An increasing trend in percentage of jaundice positive cases was observed from the data (Figure 5-1). About 51% of the interviewed farmers perceived fish mortality as a big problem associated with jaundice outbreaks, but 49% of interviewed farmers still perceived the main problem as economic losses due to the downgrading and/or rejection of fish by processors.

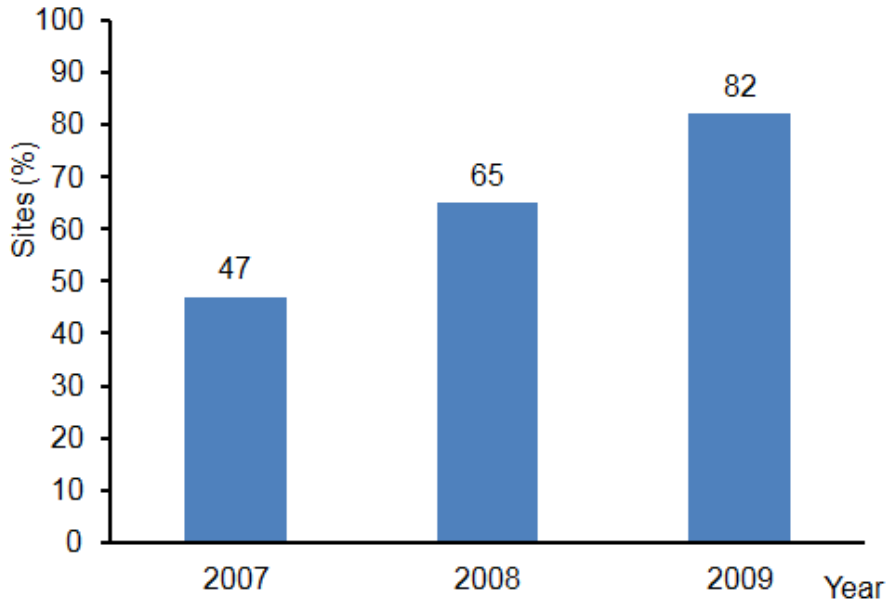

Figure 5-1 Percentage of jaundice positive cases per year in farmed *P. hypophthalmus* sites, Vietnam (n = 46)

The total number of affected ponds per farm varied greatly and ranged from 1 to 9 ponds per farm interviewed. The affected farms stated that the occurrence of

jaundice was highest between June and October, which corresponded to the flooding season with increased rainfall and lower water temperature.

5.4.3 Determination of risk factors

Using the cut off P ≤ 0.25 value, univariable analysis reduced the numbers of potential candidate variables for consideration in the multivariable model (i.e. risk factor) from 47 to 10, of which 8 were dichotomous and 2 were continuous (Table 5-3). However, the univariable analysis identified only 5 variables statistically associated with jaundice outbreak. These were the amount of water exchange at 30% and 40% per day, size of fish at range 500-1,000g, seasonality of the condition outbreak during flood season, and the stocking density.

Table 5-3 Risk factors identified through univariable logistic regression (n = 46)

Dichotomous variable (Variable type)	Crude Odds Ratio	95% Confidence interval (T)	P-value (FE)
Farm location_Dong Thap province	6.3	0.6 – 61.1	0.15
Amount of water exchange (30% per day)	0.8	0.7 – 1.0	0.05*
Amount of water exchange (40% per day)	12.4	1.2 – 124.2	0.03*
Seasonality of jaundice outbreak_rainy season	0.9	0.7 – 1.1	0.10
Seasonality of jaundice outbreak_flood season	0.8	0.6 – 1.1	0.05*
Size of jaundiced fish_(150 – 499g)	0.9	0.8 – 1.0	0.21
Size of jaundiced fish_(500 – 1,000g)	0.6	0.3 – 1.2	0.01*
Nematode (Roundworm) infection	0.8	0.7 – 1.0	0.18

Note: (T) Taylor series, (FE) Fisher's exact

Continuous variable (Measure unit)	Median (mean) Jaundice cases	Median (mean) non-jaundice cases	P-value (KW)
Fish farming experience (years)	4.0 (4.2)	2.0 (3.8)	0.21
Stocking density (fish m^{-2})	50 (47)	40 (38)	0.03*

Note: (KW) is Kruskal-Wallis analysis

Although correlation analysis revealed significant multicolinearity between several variables (Table 5-4) these were not statically significant in a multivariable model (P > 0.05).

Table 5-4 Intercorrelation between selected variables to be offered to the multivariable logistic regression model (P-value for Pearson correlation)

Variables	a	b	c	d	e	f	g	h	i
a-Farm location_Dong Thap province	--								
b-Amount of water exchange (30% per day)	0.01	--							
c-Amount of water exchange (40% per day)	0.01	0.00	--						
d-Nematode (Roundworm) infection	0.15	0.98	0.15	--					
e-Size of jaundiced fish_(150 – 499g)	0.71	0.65	0.35	0.68	--				
f-Size of jaundiced fish_(500 – 1,000g)	0.07	0.68	0.53	0.53	0.12	--			
g-Seasonality of jaundice outbreak_rainy season	0.46	0.24	0.44	0.76	0.02	0.71	--		
h-Seasonality of jaundice outbreak_flood season	0.89	0.94	0.54	0.65	0.001	0.04	0.85	--	
i-Stocking density (fish m^{-2})	0.19	0.89	0.74	0.82	0.79	0.89	0.06	0.60	--
j-Fish farming experience (years)	0.08	0.15	0.01	0.57	0.89	0.15	0.24	0.94	0.56

Note: Headers a-j: Letters are used to abbreviate the table headers and correspond to each variable n the first column.

5.5. Discussion

Jaundice is a relatively newly reported condition described in the *P. hypophthalmus* catfish farms in Vietnam (Dung 2006). From the data presented in chapter 3, we now understand that these fish are suffering from a clinical condition described as a haemolytic anaemia resulting in the jaundice presentation. Anecdotal and in-country reports have described increased occurrence within the farms and the data presented in this study would support an increased prevalence over time. Whether this is a true representation of the condition or more of a reflection to raised awareness of the problem by farmers, thereby increased recognition and reporting is still to be confirmed. One of the issues within this health condition in the catfish farms is that jaundice alone causes morbidity rather than mortality. Therefore, the farmers may not always readily recognise the extent of the jaundice in their stocks until just before harvest. At pre-harvest a sample of the fish population will be selected and visually inspected for fish quality by the processers. Flesh colour is a proxy-indicator used to determine the quality and market price of the fish stocks, all of which is determined by the processing plants. If the stock is downgraded or rejected this is a significant economic impact on the fish farmer and the

domestic market as many of the down graded products can still be sold locally but at a much reduced price. Understanding when the fish populations become affected is crucial to implement any health management strategy to reduce the prevalence and spread of this condition.

It was certainly apparent from the secondary and primary data sources that this condition was widespread found in all of the main catfish producing areas of the Mekong delta. No association could be found between this condition and any single geographical area. Nor was any association found between the different types of farms (size) and jaundice outbreak. Although the farms included in this study were representative of the varied levels in the industry the studied sites constitute only a small proportion of the total number of sites in operation in Can Tho, Ben Tre, and An Giang provinces. In this study the limiting factor was that biological samples were collected and processed at each farm together with primary data collected through questionnaires and interviews. This meant that a smaller number of farms could be visited practically. So although the data would support that no association was found between jaundice and farm site, it is recognised that further work should be performed to include a larger data set to confirm this with certainty.

The large variation in average fish weights at the time of a jaundice outbreak was a result of the wide range in the timing of disease occurrence throughout the production cycle. None of the feed types or fingerling sources was associated with jaundice, suggesting that jaundice outbreaks did not have a simple common source linearly associated with the condition. It is not

unreasonable to consider that the trigger for this condition may in fact be something common such as fingerling source or feed type as many farms suffer at the same time. However, this was not supported by the data presented.

The univariable analysis did suggest that higher stocking densities were significantly associated with jaundice cases, but this was not supported in the final model. It may be that in the more intensive culture systems with a highest stocking densities there would be more persistent stress and physical injury/assaults on the fish stocks during the production cycle. These either individually or in combination can impair the physiological and immune responses of the fish. Furthermore the higher stocking densities simply means that there are more individuals and so the quantity of fish movements within intensive sites is likely to be higher, increasing the probability of disease introduction and spread (Bucke, 1980; Ashley, 2007).

The results from this study showed that farms exchanging 30% and 40% water per day were significantly associated with jaundice in the univariable analysis, but further analysis suggested that a case of jaundice due to the amount of water exchange was unlikely. In fact, the environmental conditions of the *P. hypophthalmus* ponds were largely influenced through water exchange using the river water which can be affected by waste products, including fish feed, sediment, pathogens from other local *P. hypophthalmus* farms. Bosma *et al.* (2009) reported that during discharges of sludge from the ponds, a very high nutrient load in waste-water and sludge may have a potential for causing localized pollution of the canal water, especially when the ponds are located

intensively along small canals during the dry season when the water level is low.

A significant association between seasonality and jaundice outbreak was found in the univariable analysis. This was during the rainy season which was also suggested as the peak time for this condition by the fish farmers. During the rainy season there is an increase in the amount of water as well as a lower water temperature which given that fish are poikilothermic animals the water temperature alone is bound to effect their normal behaviour. If prolonged this may cause a mild stress response and leave them weakened and susceptible to secondary infections. Furthermore, the increased amount of water present will also mean that there may be more water discharged through run-off into the farming systems either directly from the surrounding land or indirectly through water exchange. This might also affect the fish themselves due to the presence of varied environmental conditions but also through the presence of other microorganisms in the water.

Whilst the analyses performed in this study was unable to establish a true cause and effect or identify any significant relationships, certain aspects of the fish farming management and environment was highlighted for further consideration. Although these factors were not associated with the outcome (presence of jaundice problem) in the multivariable analysis, they still point out their possible role and impact in the presentation of jaundice in *P. hypophthalmus* production cycle. Further work is required to investigate all of these variables in much more depth, however, one of the key aspects found

was that no single risk factor was identified with the condition. This in itself was expected but was the first documented study investigating farm level risk factors on this condition in *P. hypophthalmus*. Increased sample sizes would definitely benefit the associations that were highlighted, in particular the water sources/seasonality. It would appear that this condition does not "spread" throughout the farming environments or between ponds on the same farms like an infection but is more suggestive of a contamination. The obvious sources would be the nutrition and the water. Whilst farmers pay a lot of attention to the diet of the fish, they have limited control on the water quality aspects of the farms and even less on the water sources available to them. It is not unreasonable to suggest from the data presented and the seasonal reports from the fish farmers that jaundice condition in these fish may be multi-factorial. Future research in this area should concentrate on looking at recovery of the jaundiced fish when placed in a "clean" or "regulated" water sources i.e. an aquarium to investigate spontaneous recovery. This would significantly help our understanding of the true influence of the aquatic environment in this condition.

Further research will be required to clarify the influence of risk factors identified on the presentation of jaundice, including transmission and epidemiology studies at the productive pond level.

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General discussion

6.1. Introduction

This study investigated a jaundice condition occurring in Pangasius production sector located in the Mekong Delta and did not deal with other related issues such as quality assessment of fillets, the export Pangasius value chain, or aquaculture resource management. The study used both primary data collected through field studies and secondary data from a wide range of sources. The field research was conducted mainly from 28th April to 20th August 2008 and 12th October to 10th November 2009. The fish farms were selected from the fisheries profile provided by the Vietnamese provincial fisheries department in the Mekong Delta and those that could be visited and sampled within a day were approached.

The objective of this study was to enhance the current understanding of a particular health condition affecting farmed *P. hypophthalmus* from Vietnam. The affected fish presented with a yellow colouration on the body and fins which was reported to occur in a wide range of catfish production systems distributed throughout the Mekong Delta, Vietnam. This condition was first described in these fish by Dung (2006) but the aetiology and the clinical presentation had not previously been investigated nor described fully.

6.2. The major research findings and conclusions

The results from this study clearly showed that the affected catfish were suffering from a type of jaundice, resulting in a haemolytic anaemia.

Chapter 3 found that jaundiced catfish presented grossly with yellow pigmentation of the skin, sclera of the eyes, and fin bases. Additionally the internal clinical signs in the *P. hypophthalmus* were similar to those previously described for jaundice in hybrid *Clarias* spp. catfish in Thailand (Tonguthai *et al*., 1993) and in hybrid catfish in Taiwan (Chang *et al*., 2008). The clinical chemistry results provided in this study were important as these clearly showed the extent of the blood destruction observed in the jaundiced fish, which resulted in a haemolytic anaemia (Chapter 3). Macrocytosis was observed only in the jaundiced fish which was likely due to the acute blood loss or acute haemolysis (Jones and Hunt, 1983). The high level of direct or conjugated bilirubin detected in the jaundiced *P. hypophthalmus* was indicative of a blockage or failure to excrete bile pigments from the liver after conjugation. This can either be a failure in the bile ducts themselves or as a result of liver damage which may occlude the small canaliculi, therefore blocking excretion (West *et al*., 1987; Guyton and Hall, 1996; Mehra *et al*., 2009).

The impairment of the bile ducts in the jaundiced *P. hypophthalmus* was further supported by the pathology results where a thickening of the common bile duct wall was clearly observed in these fish. Otto *et al.* (2000) described a similar pathology where the obstruction of the common bile duct was due to nematodes. This resulted in an inflammation of the bile ducts by extension from

enteritis or by infection with the trematodes (Otto *et al.,* 2000). Nematodes were observed in the gall bladder of jaundiced *P. hypophthalmus* sampled in this study and as reported by others (Otto *et al.,* 2000) may act as a potential cause of the jaundiced condition observed in the Vietnamese catfish. Therefore, it was not unreasonable that the presence of the nematodes observed in the *P. hypophthalmus* investigated in this study may have contributed towards the observed bile duct pathology, but they were also located in the apparently normal fish. Hence it would be unlikely that these nematodes would be the primary or single cause of the jaundice condition observed in farmed *P. hypophthalmus*. In the fish sampled in this study, inflammation and mucous cell hyperplasia was also found in the common bile duct of jaundiced fish without the presence of nematodes. Therefore the acute inflammatory changes in the gallbladder may progress to the common bile duct and through the lymphatic channel into the liver producing jaundice without blocking the common bile duct. This was also observed and reported by Walter (1937). It would appear that the farmed Vietnamese catfish affected by the haemolytic anaemia was not due to the presence of the nematodes.

Once it was clear that clinically, the yellow presentation was a jaundice condition in the *P. hypophthalmus,* further investigation of the hepatic vascular system showed vasculitis. This was similar to descriptions of jaundiced yellowtail *Seriola quinqueradiata* (Sakai *et al.,* 1998). Ultimately the clinical approach taken to describe clearly the jaundice condition in farmed *P. hypophthalmus* was in agreement with jaundice condition described for other farmed fish species (Tonguthai *et al*., 1993; Chang *et al*., 2008). However, a

single pathogen could not be identified in the jaundiced *P. hypophthalmus.* But instead the results from the presented study would suggest that the affected *P. hypophthalmus* frequently suffered from other infections. The presence of the other pathogens or disease conditions caused confusion but this finding was not so surprising, as the jaundiced catfish will certainly be immunocompromised and susceptible to any pathogens in the aquatic environment. These animals are kept in freshwater farming conditions with high bacterial loads in the aquatic environment hence, secondary infections would be quite common. However, it is recognised that the general lack of a diagnostic approach combined with the detection of other pathogens in the jaundiced catfish has contributed towards the confusion in aetiology of this condition when previously reported. In this study a clinical diagnostic approach was taken to provide confidence that a single aetiological agent was not the cause of the condition.

No viral work was performed in this study, as the pathology did not support a viral aetiology. However, for completion sake, future studies should include viral samples as part of a wider health screening study. This could be conducted by the Vietnamese researchers and Government laboratories under a passive surveillance scheme to support the health and welfare of animals in this production sector. Farmed *P. hypophthalmus* suffer from bacterial and parasite infections (Phuong *et al.,* 2007) but that does not mean that these fish are not susceptible to viruses. Whilst it is interesting to observe however, that no natural viral diseases have been reported or detected in farmed *P. hypophthalmus,* this does not mean they are not susceptible and needs to be clarified. Aquarium based studies could be perform under experimental

conditions to investigate their susceptibility to viruses independent of the jaundice conditions.

Several factors including the presence of parasites as the cause of jaundice in the *P. hypophthalmus* were reported by farmers and other researchers. In this study gill parasites were found in the sampled fish which belonged to different genera *Myxobolus, Hennegoides, Henneguya* and also monogeneans (Chapters 3 and 4). The contribution of the gill parasites to the jaundice may need further exploration, however, in this study, the monogenean parasites were very common. Nearly all of the farmers reported monogenans in their freshwater fish stocks and so the catfish would appear to be able to co-exist with these parasites with no obvious ill-effects. Unless there is a secondary condition such as the jaundice.

All of the above gill parasites are reported to occur in freshwater farming environments (Lom and Dyková, 1994; Ky and Te, 2000; Dung *et al.*, 2008; Hang *et al.*, 2008) and are often described as providing an opportunity for secondary invaders such as bacteria or fungi (Paperna, 1963). These gill parasites do not necessarily cause a significant health threat to the catfish stocks but can lead to increased disease susceptibility or at least perhaps in the case of the farmed *P. hypophthalmus* contribute to the mortalities associated with the jaundiced fish. However, the ubiquitous presence both in the environment and on the fish sampled in this study would not support that they are the primary cause of the jaundice condition (Chapters 2, 3, and 4). A myxosporean species was found infecting the spleen of jaundiced *P.*

hypophthalmus, cultured in the Mekong Delta of Vietnam, and was morphologically and molecularly consistent with *Myxobolus pangasii* (Molnár *et al.*, 2006). The clinical evidence from this study would support that *M. pangasii* was not a primary pathogen associated with a single condition and was not the cause of the haemolytic jaundice in the farmed *P. hypophthalmus*.

It is accepted that the presence of this parasite might exacerbate the jaundice condition in already affected fish and may be considered as a nuisance rather than a primary pathogen in farmed *P. hypophthalmus* sampled in Vietnam. Therefore, in summary although bacteria and parasites were recovered from the jaundiced fish none of the data would support that these are the primary cause of the jaundice condition (Chapters 3 and 4). It would be to the benefit of the fish farmer to reduce the incidence of the bacterial and parasitic load in their stocks but this is unlikely to reduce the haemolytic jaundice.

The catfish farming industry in Vietnam has changed dramatically since 1999. At this time the farmers were able to artificially reproduce the fish (Cacot, 1999) and there was a ready supply of seed rather than the seasonal reliance on wild seed for the farms. This combined with the available market outlets for the fish products were the main drivers for the rapid intensification of the production system. Initially fish were raised in both ponds and river-based cages but in year 2003-2004 the production system changed to predominantly earthen ponds. The jaundice condition has only been observed and reported in the earthen based pond systems with confidence and Dung (2006) described the jaundiced fish at approximately 500g. However, the results from the present

study indicated that the weight range of this condition was now much wider. While this was certainly the case from the questionnaire data gathered in this study, the reason for this increase in weight range was not clear. As it may indeed be that jaundice in Vietnamese *P. hypophthalmus* does affect a wider weight range of fish, but it may also be a reflection of raised awareness and improved experience of the fish farmer to recognise this condition.

The market demand for the final fish product depends on the actual market outlet and the weight of the fish at harvest is predicted by the market price per kg (Khoi *et al.,* 2008). The jaundice condition may only have been reported at 500g previously because that was the harvest weight in the farming practises at that time. It is only at the pre-harvest stage that the farmers inspect the fish closely to ensure that they are ready for transportation to the processing plants. So the weight diversity observed in the data collected in the current study may also be a reflection of the change in the harvest weight. As the market demands change this will affected the production strategies which may mean that fish remain on the farms for longer. Further work is required to fully evaluate the weight or size range in natural jaundice condition of Vietnamese catfish.

Given that this condition is widespread in the main catfish producing areas it was sensible to investigate the farming practises and to look for risk factors associated with this condition at the farm level. In the present study, fingerlings, stocking density, feed and feeding practises as well as water management were all evaluated. No single source of fry, feeding, stocking density or water

management practises were associated with the jaundice condition (Chapters 2 and 5).

Chapters 2 and 5 in this study it was found that all interviewed farmers understood the benefit of having a waste-water treatment pond as part of their farm management strategy. Equally they considered that it was practically impossible to apply this within their production sites, as they could not afford the costs. During the culture cycle, *P. hypophthalmus* farmers must exchange 30 to 50 percent of the pond water daily. The capacity needed for a sediment pond to be used as a waste-water treatment is approximately a third of the pond production area. According to VASEP (2007), the successful development of *P. hypophthalmus* culture has been a significant factor contributing towards the rising land prices in the Mekong Delta. Between 2002 and 2007, the price of land for *P. hypophthalmus* farming increased approximately three to five times. As mentioned previously, most farmers lack the capital to purchase more land for waste-water treatment systems. Equally they are unable to "sacrifice" a proportion of the farming area for a sediment/treatment pond. Therefore, the high land price has led to an increase of river pollution and disease transmission (Khoi *et al.*, 2008; Phan *et al.*, 2009).

All fish farmers are aware of the importance of high quality water for their fish stocks. In the present study most farmers used varied sources and methods to optimise the water quality in their farms (Chapter 2). Building a settling pond was one of the immediate solutions suggested by the local government but only 10.2% of the farmers interviewed were able to comply (Khoi *et al.,* 2008). In

addition, to treat wastewater effectively, it has to be kept in the pond for many hours in order to decompose waste and pollutants before being released into the environment. However, this is not compatible with the frequent water exchanges practices, therefore it was the farmers perception that this measure alone was not suitable with current farming practises not effective in treating waste-water. To reduce the potential spread of unwanted pathogens/material in the discharged water, the farming practises would need to change dramatically.

Given that this is a jaundice condition then an association with feed quality or practises is a reasonable assumption. One of the perceptions prior to undertaking the study was possible toxins from contaminated feed. In the wet season, fish feed may be affected by aflatoxin (Caguan *et al.,* 2004) which is a toxic compound produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. The presence of the toxin is due to the high moisture content and improperly stored feeds (Caguan *et al.,* 2004). Ottingeer and Kaattari (2000) showed that aflatoxins depressed the immune response making fish more susceptibility to disease. Sahoo and Mukherjee (2001) reported that there was an impaired immune function in Indian major carp (*Labeo rohita*) after exposure to aflatoxin B1 in the feed at dose as low as 1.25 mg/kg body weight. Furthermore, the study by Cagauan *et al.* (2004) found that the yellowing body surface of tilapia was indeed the result of aflatoxin contamination of fishfeed. Although no specific toxin work was undertaken in the present study, the data would not suggest that this is the cause of the jaundice condition in fish. The nutritional capacity on the farms is crucial for the optimal growth of the animals and a large proportion of the farm running costs are taken up by feed costs. In

this study, it was found that farmers are aware of the importance of properly stored feed and they mostly buy commercial feed pellets which they only stored on their farms for relatively short periods of time. Thus reducing the ability of the toxin production. Furthermore no association could be found with any feed type or source and the jaundice condition. Nevertheless, the study on feed contamination by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* must be included in the future studies. This possibility is likely to be challenging because the identification of mycotoxins as the cause of disease outbreak or mortality can be difficult. In addition to the delayed effects of aflatoxicosis, diagnosis of the condition can be confused because of its similarity with other disease signs.

The internal clinical signs in the cluster analysis (Chapter 3) such as yellow flesh; yellow fat; swollen liver, kidney, and spleen; friable kidney, liver or spleen; no feed in stomach; enlarged gall bladder contained dark blue bile; pale gills; and yellow ascetic fluid in abdomen were more frequently associated with moribund jaundiced fish. The data suggested that these clinical presentations within a single cluster reflect the final clinical stages of jaundice in *P. hypophthalmus*. This was useful data in producing a clinical case definition of the jaundiced catfish.

In general no bacterial or parasitic agents could be found associated with jaundice and any of the farm level risk factors investigated in this study (Chapters 3 and 5). The retrospective study conducted was designed to have as large a sample size as possible and to be representative of the farming

practises undertaken in the Mekong Delta at the time (Chapter 5). It is recognized that the study sites selected constitute only a small proportion of the total number of sites in operation in the Mekong Delta. However, the condition was found in all of the farming systems which were categorized into small-scale (pond area per farm less than 10,000 m^2), medium-scale (between 10,000 and 50,000 m^2), and large-scale (over 50,000 m^2). Supporting that this is a wide spread condition affecting all farming practises throughout the 4 main catfish production provinces in Vietnam. Although farm level factors in chapter 5 were not associated with the outcome (presence of jaundice problem) in the multivariable analysis, they highlighted that such risk factors can have an impact and may still play an important role in the presentation of jaundice in *P. hypophthalmus* production cycle. To clarify this fully it is suggested that a prospective, longitudinal cohort study should be designed to include sampling throughout the entire production cycle, ensuring an accurate determination of exposure status for both case and the control sites. This would really tease out the farm level factors involved in this condition such a study has been successful in determining the farm level factors associated with the white spot syndrome in shrimp (Corsin *et al.,* 2005).

Development of antibiotic resistance in aquatic pathogens is rapid (Cabello, 2006) particularly in Asian countries including Vietnam (Crumlish *et al.*, 2008). This is often due to the lack of a therapeutic approach where farmers provide antibiotics and other chemicals prophylactically. Whilst work on the antibiotic resistance against bacterial pathogens affecting *P. hypophthalmus* is on-going the effect of the multi-drug use on the health status of these fish in less well

understood. Although chemical residue levels are tested in the fish prior to harvest these are focusing more on their potential impact on human health and not the cumulative effect on the fish health. Field studies to assess the effects of antibiotic treatment on liver function of fish during the culture cycle are necessary because some antibiotics can cause a danger of toxicity to the fish, frequently causing liver, kidney, or other organ damage that may or may not be reversible. In aquaculture, risk of bacterial infections among farmed fish is high mainly due to high density of fish. Therefore, large amounts of antibiotics are used in fish feed for both disease prevention and disease treatment purposes in aquaculture (Sapkota *et al.,* 2008). Oxytetracycline has been widely used in aquaculture (Suzuki *et al.*, 2008), especially in the Mekong Delta, Vietnam (Dung *et al.*, 2008). In humans, when used in larger doses of antibiotics in the Tetracycline family (tetracycline, guamecycline, doxycycline and others) can cause jaundice (Lloyd-Still *et al.,* 1974). Given that a single aetiological agent was not detected nor that any of the farm level risk factors could be associated with the condition, future studies should investigate the effect of antibiotics on this condition.

At present the true impact of the jaundice condition in this freshwater farming sector is actually unknown. Although work from this study now clarifies the clinical picture of the affected fish further work is required to investigate the spontaneous recovery of the jaundiced fish. There were no farmers' reports or experimental evidence available on any spontaneous recovery of the jaundiced fish. This is mostly because the jaundice condition itself does not cause high mortalities and farmers are only alerted to clinical conditions if there are high

daily mortalities. It is only when there is a clinical disease outbreak or pre-harvest fish quality screening that the extent of the jaundice condition is recognised. If the recovery experiment of jaundiced fish was successful, it would be possible to provide farmers with management solutions to help alleviate or solve this problem. But more importantly, it would help clarify the potential risks of this condition on the farms and what preventative treatments can be implemented.

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Appendix 1 (Chapter 2)

Survey questionnaire for the study of "Yellow colouration" of farmed Pangasius spp in the Mekong Delta (Vietnam)

Responsible: □ Owner □ Manager □ Owner/Operator

2. How long have you been operating your farm?

□ 1 – 2 years □ 3 – 4 years □ 5 – 6 years □ > 7 years

3. Land ownership:

Farmer code: Province: ……

Interviewer name: …… Date of interview: ……

A. Farmer background

1. Farmer name: …… Sex: …….. Age: …….

Address: ……

Tel: ……..

Location of the farm: ……

……

□ Owner □ Rent if rent, how much……VND per year

B. Farm profile

1. Type of culture system:

□ Pond □ Cage □ Pen

2. Total area of your farm: …… (Hectare), used for:

□ Fish pond: …… Ponds ……hectare/pond

□ Effluent treatment: ……. Ponds ……hectare/pond

□ Others: ……

3. Depth of ponds: …… (m)

4. What is the source of water supply for your farm?

□ River □ Irrigation canal □ Others

5. Source of stocks: ……..

6. Size of stocks: …… Price of stocks: …….. (VND)

7. At what density have you stocked fish into the pond? …… (Fish/sqr.m)

8. What was the average yield of Tra catfish production in your farm?

……ton/ha/crop

9. How many crops do you produce annually? ……crops

10. How long do you culture the Tra catfish per cycle? ……months/cycle

11. How many fish farm within local area?

□ 1 – 5 farms

□ 6 – 10 farms

□ > 10 farms

C. Pond and water management

1. Do you do pond preparation after each cultured cycle?

□ Yes □ No

If yes, how do you prepare the ponds?

Kinds of pond preparation	How long or how often?
Pond drying	…….
Mechanical removal of mud	…….
Others	…….

2. Do you treat the ponds with chemical?

□ Yes □ No

If yes, how do you apply chemical in pond preparation per cycle?

Kinds of chemical	Rate and frequency of application	Costs/kg (VND)
Lime	…….	……

BKC (is Benzalkonium chloride)	…….	……
Chlorine	…….	……
Formalin	…….	……
Others	…….	……

3. Do you do water exchange during the culture period?

□ Yes □ No

4. How often do you add/change water into the pond? .....

and how many percentage? ……..

5. Do you measure the water quality in fish pond?

□ Yes □ No

If yes, what parameters do you measure?

□ Alkalinity

□ pH

□ Temperature

□ Dissolved oxygen

□ Others …….

If no, please give the reason why …….

6. How often do you measure the water quality?

□ Daily

□ Weekly

□ Bi-weekly

□ Monthly

□ Others …….

D. Feed and feeding management

1. What is the type of fish feed used during the cycle?

□ Home-made feed

□ Commercial pellets

□ Both (home-made and pellets)

2. What is protein level of the feed you provide to the fish? …….

3. What is the source of feeding table being use for feeding rate?

□ Feed company

□ Fishery government

□ Farmer's modification

□ Others ……

4. What is the price of pellets? …… VND/kg

5. What are other feed supplements do you use? ……..

6. Where is the feed stored on the farm? ……..

7. Is there any factors affecting the growth rate?

□ Type of feed

□ Frequency and rate of feeding

□ Water quality

□ Pond cleanliness

□ Stocking density

8. What are the factors that contribute to high survival rate?

□ Species

□ Size

□ Sources (local or imported?)

□ Condition of the fingerling

□ Disease prevention

□ Water quality

□ Handling techniques

□ Seasonality of supply

E. Disease problems

1. Do you observe any disease problem with your fish?

□ Yes □ No

If yes, what disease problem have you encountered?

□ Parasite

□ Bacillary Necrosis of Pangasius

□ Red spot

□ Swollen eyes

□ Fungus

□ Others ……

2. What types of parasite do you observe on your fish, during the period?

□ Monogenean

□ Nematodes

□ Others ……

3. How do you treat the parasite in your pond, during culture period?

Chemical: …… kg or L/pond

…… kg or L/pond

…… kg or L/pond

4. What treatment has been most successful to eradicate parasite, during culture period?

Treatment: ……..

5. Do you apply any chemicals to control disease during flooding seasons?

□ Yes □ No

If yes, which chemicals: …… kg or L/pond

…… kg or L/pond

6. Usually how many times do fishery extension workers visit to advice?

□ Never

□ Once a week

□ Once a month

□ Once in 3 months

□ Once a year

7. Have any fish in your ponds ever shown signs of yellow skin syndrome?

□ Yes □ No

If yes, do you know why the fish got infected? (From your answer)

□ Water quality

□ Spread from other fish species

□ Caused by pond conditions

□ Feed quality

□ Others ……

8. Identification of "yellow skin" syndrome (from your answer)

□ Yellow colouration in the skin

□ Pale gills

□ Yellow ascitic fluid in abdomen

□ Yellow ascitic fluid become coagulated in air

□ Spleen, kidney and liver are often enlarged

□ Body fat is yellow in colour

□ Yellow flesh

□ No feed in the digestive system

□ Gall bladder enlarged and dark blue

□ Bile duct is enlarged with nematodes

9. Describe the condition:

How many fish were affected? ……

What species? …….

What size of fish? ……

10. The seasonality of yellow skin syndrome outbreak?

Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec

11. How did you rid of the yellow skin syndrome?

……..

……..

12. From your experience, how have the fish responded to the therapy and/or management changes?

□ Increase □ Decrease □ No change

Please give the reasons (why, from your answer): ……

……

13. Do you have any management problems or difficulties in Tra catfish culture since operation commenced?

□ Lack of skilled manpower

□ Lack of knowledge

□ Loss/damage equipment

□ High cost of fingerling

□ Low survival rate

□ Others (specify): ……

If yes, how do you prevent or overcome them (please elaborate): ……

……