USE OF HISTOPATHOLOGY FOR DISEASE SURVEILLANCE IN JUVENILE BARRAMUNDI *Lates calcarifer* (Bloch, 1790)

By

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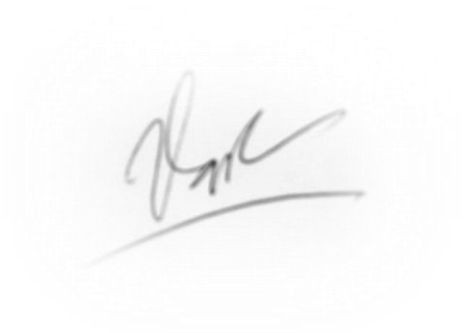
DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.



Hong Lan Pham **Date** **7th of July 2022**

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ABSTRACT

Asian sea bass (*Lates calcarifer* (Bloch, 1790)), also known as barramundi, is an important aquaculture species worldwide, especially in Asia. Barramundi are susceptible to bacterial, parasitic and viral infections. Disease surveillance plays a critical part in early diagnosis of fish disease and abnormalities thus reducing economic losses in aquaculture. Numerous diagnostic methods are used for early diagnosis including histopathology and molecular techniques. Histopathology is broadly used as a primary and efficient method for detection of fish pathology at first. Ideally, histology combined with other methods such as traditional bacteriology, virology, biochemical tests will provide more accurate results. The aim of this study was to conduct disease surveillance in juvenile barramundi at a local South Australia hatchery. Thirty juvenile barramundi were collected every month for regular examination for nine months. Fish were then analyzed by routine histology (H&E staining) for abnormalities. Histologically, there were 18 fish from 270 examined fish, which demonstrated pathological features of Betanodavirus infection but these all showed negative results when examined using the IHC technique. Epitheliocystis was evident in the gills with a high prevalence (118/270) while monogenean Diplectanidae sp. appeared at lower prevalence (36/270). Other abnormal structures found in various organs of sampled fish remain unknown and further research is required to identify these.

This thesis had several original findings:

Clinical symptoms such as skin darkening, lethargy, swimming in a swirling pattern and the appearance of vacuolation in the brain and central nervous system tissues were observed in barramundi fingerlings at a South Australian hatchery. These symptoms normally indicate viral nervous necrosis (VNN) disease however, no positive IHC confirmation of Betanodavirus was observed.

Epitheliocystis and parasitic infections with high prevalence were periodically recorded (every 2 months) without telangiectasis and vice versa.

Unknown pathological agents were observed in fish hatchlings during this study but these were unable to be identified as it was beyond the scope of this project. Nonetheless, this finding may open a new era for further barramundi disease surveillance research.

In conclusion, early diagnosis during regular disease surveillance in aquaculture is very useful. It reduces economic losses through stopping diseases from spreading, thus preventing potential outbreaks. Also, it helps to monitor for other abnormalities that could happen such that quick responses can be enacted. For instance, pathogens detected in this study appeared periodically and were highly associated with each other. This information was quickly reported to the local hatchery and was helpful for their disease management.

LIST OF ABBREVIATIONS

AAHL: Australian Animal Health Laboratory, CSIRO, Geelong

BFNNV: Barfin Flounder Nervous Necrosis Virus

CPE: Cytopathic Effect

CMC: Cell- Mediated Cytotoxic

DNA: Deoxyribonucleic Acid

ELISA: Enzyme Linked Immunosorbent Assay

FAO: Food and Agriculture Organization

FISH: Fluorescence *In Situ* Hybridization

IFAT: Indirect Fluorescent Antibody Test

Ig: Immunoglobulin

IHC: Immunohistochemistry

H&E: Haematoxylin and Eosin

PCR: Polymerase Chain Reaction

PC2: Physical Containment Level 2

PGD: Proliferative Gill Disease

QTL: Quantitative Trait Loci

RBC: Red Blood Cell

RGNNV: Red Spotted Grouper Nervous Necrosis Virus

RNA: Ribonucleic Acid

RPS: Relative Percent Survival

RT-PCR: Reverse transcriptase PCR

SJNNV: Striped Jack Nervous Necrosis Virus

TCID: Tissue Culture Infective Dose

TEM: Transmission Electron Microscope

TNV: Turbot Nodavirus

TPNNV: Tiger Puffer Nervous Necrosis Virus

TROs: Total Residual Oxidants

VLPs: Virus-like Particles

VNN: Viral Nervous Necrosis

VHS: Viral Haemorrhagic Septicaemia

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CHAPTER 1: LITERATURE REVIEW - SIGNIFICANT DISEASES OF JUVENILE BARRAMUNDI: A REVIEW OF THEIR CAUSATIVE AGENTS, DIAGNOSTIC METHODS AND MANAGEMENT

1.1. Overview

Barramundi is an important aquaculture species in the Asia-Pacific region, including Australia. The species has been commercially fished and has a well-established place in the market. In Thailand, aquaculture of barramundi started in 1970s and the industry grew quickly throughout Southeast Asia (Yue et al., 2009). Australia is now experiencing the development of large-scale barramundi farming as it is classified as a high-value species with high potential for commercial growth via research and development. Indeed, the annual 5668 tonnes production of Australian farmed barramundi is currently valued at $53.6 million The industry is growing production by 14% each year, with a national production reach of 20,000 tonnes by 2025 (Department of Agriculture, Fisheries and Forestry (DAFF), ABARES, 2018).

Barramundi are susceptible to many parasitic, viral bacterial and fungal infections (Hutson, 2014) especially when stress occurs. For instance, *Streptococcus iniae*, a Gram-positive bacterium, is reported to be responsible for serious losses in Australia, Israel and America in both fresh and marine water fish (Bromage & Owens, 2002). In terms of biosafety, handling of *S. iniae* diseased fish can cause infection in humans, known as mad fish disease (Hastein et al., 2005). Outbreaks of *S. iniae* in aquacultured barramundi have occurred in South Australia, Northern Territory and Western Australia (Creeper & Buller 2006). Creeper and Buller (2006) also observed via a case report another bacterial infection in barramundi, known as epitheliocystis. Histopathology is commonly used to diagnose this infection together with other molecular techniques such as polymerase chain reaction (PCR). In addition, parasitic diseases caused by protozoa and metazoa as well as diseases related to nutrient deficiency are of concern during barramundi culture.

Barramundi are also known to be prone to Betanodavirus. The first diagnosed case of VNN in Australia was in the late 1980s in hatchery-reared barramundi (Glazebrook et al., 1990). Within Australia, this disease has been reported in most states, except Victoria in both freshwater and marine finfish species (Munday et al., 2002, Moody et al. 2009 and Department of Agriculture and Water Resources 2017). The disease affects at least 50 marine fish species worldwide and is believed to be one of the most dangerous viral diseases affecting larvae and juvenile marine fish (Grove et al., 2003, Doan et al., 2017). Betanodavirus has been reported as the causal agent of VNN disease and infections can cause severe economic losses due to the rapid and significant mortalities which can reach 100% (Kuo et al., 2011, Fenner et al., 2006, Hodneland et al., 2011 and Ciulli et al., 2006).

Betanodavirus has been found in fish with or without clinical signs and has been detected naturally in fish or after experimental infection (Nguyen et al., 1996, Tanaka et al., 2004 and Gomez et al., 2006). In terms of transmission, this disease can be spread both horizontally and vertically (Hick &Whittington, 2010, Kuo et al., 2011). There currently is no practical treatment for VNN. Fenner (2006) stated that an understanding of Betanodavirus transmission route/s would help to prevent Betanodavirus infection. Indeed, it is essential to understand the pathogenesis of Betanodavirus as well as the organs which it targets during the infectious stage of VNN disease.

Surveillance and monitoring of diseases have played an important role in aquaculture. It can reduce economic losses as the spread of fish diseases might be prevented and isolated at the early stage of the infection allowing, notifiable diseases to be controlled and well managed by quickly responding to the abnormalities that have been detected using data from surveillance and monitoring (Hastein et al., 2001, Reschova et al., 2008). For

example, early diagnosis of disease allows for broodstock control which is the best form of management (Ciulli et al., 2006). In the case of viral infection, discarding all the fish from an affected farm and disinfecting the site before restocking is the most appropriate form of control.

Examination methods can vary broadly from molecular methods to histopathological methods, depending on the particular pathogenic agents that are being investigated. However, histopathology is commonly used as a primary and efficient method for detecting pathological changes. Histology stays effective as a general means for conducting initial fish health evaluations because it delivers detailed insights into pathological changes and allows for diagnosis of a broad variety of pathogens such as virus, bacteria and parasites (Feist & Longshaw, 2008, Kent et al., 1998). Most common parasites can be easily detected by histology (Gardiner et al., 1998, Brunol et al., 2006). Ferguson et al. (2011) have stated that histology is the most practical method for detecting a variety of parasites however, it is not a primary tool for identifying parasites. However, for Betanodavirus detection, it is stated that if clinical signs are noticed then observation of vacuolation in the retina and nervous system via haematoxylin and eosin (H&E) staining tissues is sufficient for VNN diagnosis, especially in larvae or fry. But, if there is any uncertainty, transmission electron microscopy (TEM), an immunohistochemistry (IHC) test or an indirect fluorescent antibody (IFA) test can be used to confirm the existence of the Betanodavirus in the tissue sections (ANZSDP, 2014). In addition, "the gold standard" that is recommended by the World Organisation for Animal Health (OIE) is to diagnose Betanodavirus infection via either cell culture or immunostaining methods as described in the Manual of Diagnostic Tests for Aquatic Animals 2019 (World Organisation for Animal Health, OIE). IHC is recommended as the most effective method for fixed samples. Many studies examining VNN disease utilise

H&E staining as the primary detection method prior to other analysis such as IHC, fluorescence *in situ* hybridization (FISH), enzyme linked immunosorbent assay (ELISA) or PCR (Gomez et al., 2004, Tanaka et al., 2004 and Fenner et al., 2006).

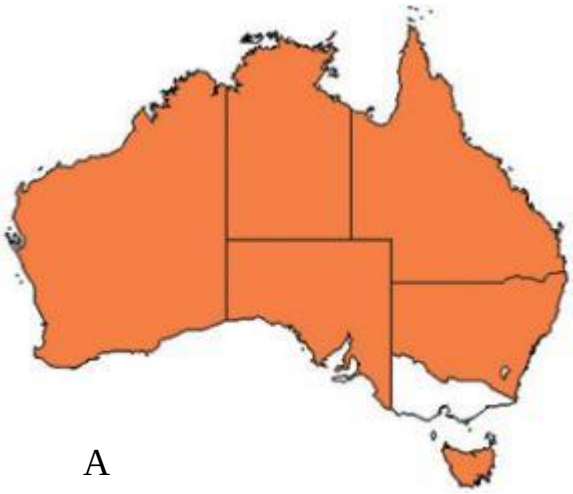
In this literature review, Betanodavirus and its main aspects such as genome, transmission route, method of detection, pathogenesis and potential management will be reviewed. In addition, a review of other pathogens including a variety of parasites, emerging diseases such as scale drop disease, big belly syndrome and epithelial conditions that may affect barramundi is provided.

1.2. Introduction of VNN

Marine resources have substantial economic value to Australia's economy. According to a report from Department of Agriculture, Fisheries and Forestry (DAFF), the gross value of fisheries and aquaculture production in 2017-2018 was $3.18 billion, in which aquaculture accounted for $1.42 billion (ABARES 2018). Barramundi is a high-value species and there is much potential for research and development to improve its commercial importance such as focus on selective breeding programs that can enhance flavor or the proportion of meat. There is steady growth in the production of barramundi in Australia because it is a valuable species for commercial aquaculture and recreational fishing. VNN, otherwise known as viral encephalopathy and retinopathy (VER), is a disease of significance that can affect barramundi production (Thiery et al., 2004, Gomez et al., 2008). The causative agent, Betanodavirus, is a common pathogen in marine fish and has been detected in fish in Australia and many Asian- Pacific countries (**Figure 1-1**). More importantly VNN disease has been implicated in mass mortalities in fish (Yoshikoshi et al., 1990). Betanodavirus infection damages the host because it specifically infects nerve cells, causing cellular vacuolation and neuronal degradation in the retina and central nervous system. This infection can result in high mortality in fingerling and juvenile infected fish as well as adults (Tanaka et al., 2004).

Betanodavirus transmission routes are both vertical and horizontal. Pakingking et al. (2009) stated that the main path of transmission for Betanodaviruses seems to be vertical, and this is shown by the very early incidence of clinical disease in juvenile fish. However, in a recent OIE Manual of Diagnostic Test for Aquatic Animals (OIE 2019), horizontal transmission was described as the most common mechanism for disease spread. While further knowledge of Betanodavirus transmission route may help to prevent VNN disease

spread by the identification and elimination of potential risks such as infected fish, contaminated rearing equipment, food or water this research is beyond the scope of this thesis.



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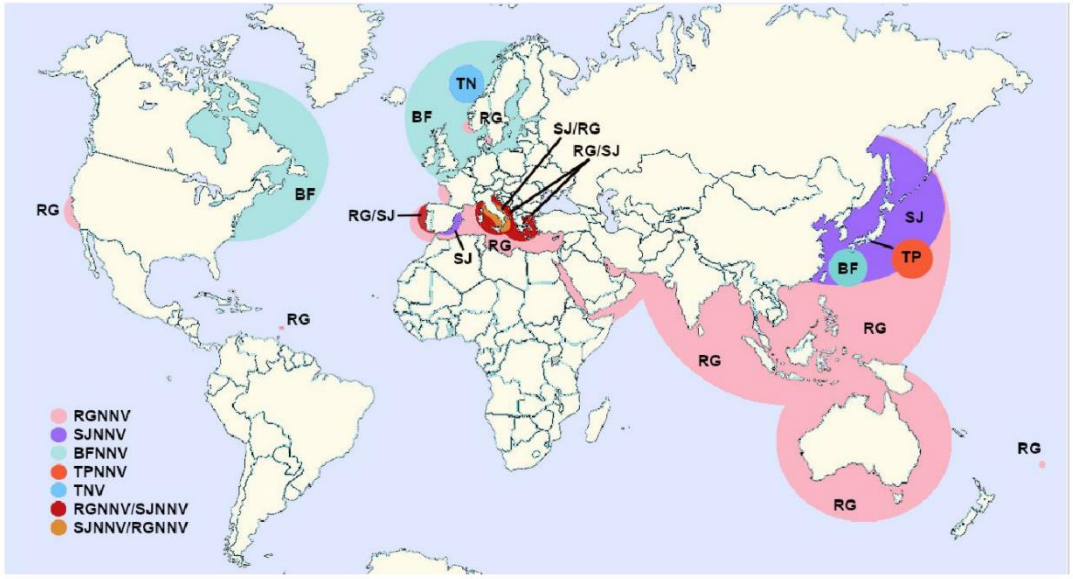

Figure 1-1 Presence of VNN in Australia and Asian-Pacific region. A: The orange areas show the states where VNN was reported in New South Wales, Northern Territory, Queensland, South Australia, Tasmania and Western Australia (this image is from Aquatic animals' diseases significant to Australia: Identification field guide 4th Edition). **B**: Distribution of Nervous necrosis virus (NNV) genotypes. Adapted from SMART (Servier Medical Art), licensed under a Creative Common Attribution 3.0 Generic License. **http://smart.servier.com/**.

1.3. Betanodavirus background

In-depth information regarding Betanodavirus classification, genome, host, transmission and detection methods is covered in this part. Moreover, its disease (VNN) and possible control and treatment are also discussed below.

1.3.1. Classification

Alphanodavirus and Betanodavirus are two genera that belong to the Nodaviridae family. Betanodaviruses are piscine-specific and Alphanodaviruses infect insects. The family name originated from Nodamura virus, that was first noticed in mosquitoes near the Japanese village of Nodamura. Nodaviruses replicate in cells from highly diverse organisms, including mammals, nematodes and plants (Knipe et al., 2007). Recently, a new genus *Gammanodavirus* of *Nodaviridae* family has been proposed thank to the isolation of nodavirus from other invertebrate host such as crustaceans (Bandin & Souto, 2020).

1.3.2. Genome structure

Betanodavirus is a small, non-enveloped, spherical, 25-30 nm in diameter rRNA virus (Gomez et al., 2008, Gomez et al., 2009). Its genome is composed of two positive-sense single- strands of RNA. The first and largest segment (RNA1) is 3.1 kb long and encodes a RNA polymerase of approximately 100 kDa that is called protein A, whereas the middle one, (RNA2), is 1.4 kb long and encodes the 42 kDa capsid protein (**Figure 1-2**) (Johnson et al., 2001, Okinaka & Nakai, 2008, Olveira et al., 2009, Chen et al., 2009).

(This image has been removed due to copyright restriction. Available online from: Johnson, K. N., Johnson, K. L., Dasgupta, R., Gratsch, T. & Ball, L. A. 2001. Comparisons among the larger genome segments of six nodaviruses and their encoded RNA replicases. *Journal of General Virology,* 82, 1855-1866).

Figure 1-2: Nodavirus genome organization and assembly. Positive-sense genomic RNA 1 encodes the RNA dependent RNA polymerase (protein A) and RNA 2 encodes the capsid precursor protein (protein α). RNA 1 also generates subgenomic RNA3, which encodes protein B2, a suppressor of host RNA silencing. Assembled from capsid precursor α, provirions undergo a maturation cleavage, producing capsid proteins β and γ. Infectious virions contain RNA 1 and 2 in equimolar ratios. Figure is from Johnson et al. (2001).

Johnson et al. (2001) found that there is sequence conservation amongst nodavirus RNA1 sequences and their encoded polypeptides. Moreover, the RNA dependent RNA polymerase (protein A) of Betanodavirus is quite similar to that of poliovirus. Amino acids found at the centre of the Betanodavirus polymerase protein domain can be modelled onto the crystal structure of the poliovirus polymerase protein. The capacity of Betanodavirus to infect various kinds of fish is determined by the coat protein and its capsid protein, which is encoded by RNA2, and a minor number of amino acid substitutions to the coat protein can significantly affect its ability to infect different animal species (Thiery et al., 2004, Chen et al., 2015).

1.3.3. Types of Betanodavirus

Original strains were named after the species in which they were first discovered and initially they were called strains but are not now since sequencing data obtained to be

different genotypes. So far, there are four genotypes of Betanodavirus have been identified and approved: SJNNV, RGNNV, BFNNV and TPNNV (Bandin & Souto, 2020). The SJNNV genotype is commonly seen in fish in Japanese waters such as sea bass in the Iberian Peninsula and gilthead sea bream. However, the BFNNV genotype only infects cold-water fish species while the RGNNV genotype has the widest host range as it can cause diseases in a large number of warm-water fish species. RGNNV and BFNNV are close in amino acid sequence and are serologically indistinguishable when using the alkaline phosphatase-conjugated goat anti-rabbit secondary (Bio-Rad, Hercules, Calif.) and antibody anti-SJNNV rabbit polyclonal antibody (Iwamoto et al., 2004) for detection. In addition, the RGNNV genotypic variant derived from warm-water fish has been found to proliferate well in cells cultured at high temperatures in comparison to the other three genotypes (Pakingking et al., 2009, Chen et al., 2014). TPNNV is thought to infect only one specific host. In terms of analysis, Okinaka & Nakai (2008) stated that most of the genotypes isolated belong to SJNNV however recent public papers have suggested RGNNV is the most frequently reported (Bandin & Souto, 2020). It is also concluded that isolates mainly correlate with geographical origin rather than host species (Thiery et al., 2004). Betanodavirus genotypes are classified based on similarities in their partial RNA2 sequences (T4 region) that have been isolated from different fish species. Examples of Betanodavirus isolates include tiger puffer nervous necrosis virus (TPNNV), striped jack nervous necrosis virus (SJNNV), red spotted grouper nervous necrosis virus (RGNNV) and barfin flounder nervous necrosis virus (BFNNV) (**Table 1-1**). An isolate that has emerged more recently is from turbot *Scopthalmus maximus,* which is known as the turbot nodavirus (TNV) (Fenner et al., 2006, Olveira et al., 2009 and Scapigliati et al., 2010). To be more specific, there are three additional genotypes have been proposed, turbot nodavirus (TNV), Atlantic cod nervous necrosis virus (ACNNV) and Korean

shellfish nervous necrosis virus (KSNNV) (Bandin & Souto, 2020, Kim et al., 2019). However, TNV has been widely accepted as the fifth genotypes.

A different classification scheme of betanodaviruses was proposed by Thiéry et al. **(**2004**)** which refers to betanodavirus genotypes as numbers (I, II, III and IV, as for RGNNV, BFNNV, TPNNV and SJNNV, respectively) and creates subgroups (a, b, c) within the genotypes**.** For example, in Australia, Moody et al. (2009) reported that Betanodavirus infections around Australia appear to be due to two distinct subtypes of the RGNNV genotype. Variability in the coat protein T4 region, indicates that subtype Ia was involved in outbreaks in Tasmania, Northern Territory and Queensland, whilst outbreaks in South Australia and New South Wales were linked to a new subtype Ic. The two subtypes Ic and Ia share 85.8 to 87.9% nucleotide identity between them, however a higher nucleotide identity (96.2 to 99.7%) is observed amongst isolates of the same subtype.

Table 1-1 Accession numbers for gene sequences for viral coat and capsid proteins from various Betanodavirus isolates (Cherif et al., 2009)

Isolate	Species	Source	Year of isolation	GenBank accession number
Sjori	Striped Jack (*Pseudocaranx dentex*)	Japan	1991	D30814
RG91Tok	Red Spotted Grouper (*Epinephelus akaara*)	Japan	1991	D38636
TP93Kag	Tiger Puffer (*Takifugu rubrips*)	Japan	1995	D38637
BF93Hok	Barfin Flounder (*Verasper moseri*)	Japan	1993	D38635
TNV	Turbot (*Scophthalmus maximus*)	Norway	2004	AJ608266
Y235	Seabass (*Dicentrarchus labrax*)	France	1991	AJ698105
DIEV	Seabass (*Dicentrarchus labrax*)	Greece	1996	Y08700
DIEV	Seabass (*Dicentrarchus labrax*)	France	2001	U39876
EA-105102IL	White Grouper (*Epinephelus aeneus*)	Israel	2004	AY284967
BB155	Seabass (*Dicentrarchus labrax*)	Tunisia	2004	AJ698106
V82	Seabass (*Dicentrarchus labrax*)	Tunisia	2004	AJ698109

1.3.4. Host and transmission

There is also a variety of host species that have been reported to be infected with Betanodavirus. Since the first report, the number of Betanodavirus infected fish species has reached 177 and they are mainly marine fish (Moody et al., 2009, Bandin & Souto, 2020). Betanodavirus can be found in both farmed fish and wild fish. Gomez et al. (2008) was also able to isolate the RGNNV strain from aquarium fish species and invertebrates.

In terms of virus transmission, both horizontal and vertical transmissions have been reported in different fish species. Horizontal transmission is known as the most common cause of viral spread, especially through the water (farmed and wild fish) and it is hard to control (Doan et al., 2017). Furthermore, viruses can spread via a variety of ways, contact between healthy fish and diseased larvae, healthy fish and contaminated facilities related to symptomatic fish, interaction of healthy fish with asymptomatic carriers (Doan et al., 2017, Bandin & Souto, 2020). It is also reported that Betanodavirus can spread from one fish species to another (interspecies) for example, between European sea bass and gilthead sea bream, between barramundi and brown marbled grouper (Castric et al., 2001, Manin & Ransangan, 2011).

1.3.5. Viral pathogenesis in Betanodavirus infected fish

1.3.5.1. Gross clinical signs

Disease symptoms in infected fish include abnormal swimming, darkened pigmentation, reduced feeding and lethargy. Infected fish show whirling swimming, darkened colour and hyperinflated swim bladders (Mori et al., 1992, Badin & Souto, 2020). They tend to swim in a darting, twisting way. Some fish go down to the bottom then float to the surface again. Infected juvenile and adult fish can present with a swollen belly (**Figure 1-3**). Moreover, infected fish can also present with a loss of appetite, lethargy and pale coloration (Johansen et al., 2002, Mladineo, 2003 and Nopadon et al., 2009). Internal disease symptoms include intestines filled with greenish to brownish fluid, pale livers, and empty digestive tracts (Thiery et al., 2004, Fenner et al., 2006).

A B

Figure 1-3: Images of infected fish A. *Epinephelus coioides* brood stock with bloated belly associated with VNN infection. B. *E. coioides* larvae showing bloated belly (this image is from the Australian Government Department of Agriculture, Fisheries and Forestry 2007)

1.3.5.2.Pathogenesis

Tanaka et al. (2004) believed that it is vital to determine the portal of entry and original multiplication site of Betanodavirus to provide insights into how to manage the problem in aquaculture. Tanaka et al. (2004) assumed that Betanodavirus firstly infects the fish

skin and is then transmitted to the epidermal structures. It can then be passed on to the brain and the retina from the nerves of the skin. Previous work has also indicated that the port of entry is via nervous tissue. Grotmol et al. (1999) and Mladineo (2003) both reported that the spinal ganglia and spinal cord were the primary sites for Betanodavirus infection. Nguyen et al. (1996) revealed via immunolabelling nerve cells that Betanodavirus particles were first observed in the spinal cord then in the brain and after that in the retina of SJNNV infected fish during a natural outbreak. Yet in the same study, when fish were experimentally challenged, the initial immunolabelling occurred in the spinal cord and medulla oblongata. This report also states that the epithelium can be the portal of entry for Betanodavirus, especially, skin and intestine epithelium whereas, olfactory and nasal epithelium did not show any signs of viral infection (Nguyen et al., 1996). Overall, the portal of Betanodavirus entry is still under debate and requires further investigation.

(This image has been removed due to copyright restriction. Available online from: Mladineo, I. 2003. The immunohistochemical study of nodavirus changes in larval, juvenile and adult sea bass tissue. *Journal of Applied Ichthyology,* 19, 366-370).

Figure 1-4: IHC images illustrating Betanodavirus positivity in Seabass (*Dicentrarchus labrax* L). **A**. Positive immunohistochemistry in olfactory lobes of seabass larvae (arrow). **B**. Immunocomplex aggregation in the brain tissue with high magnification (100X). The arrow shows the distribution of viruses in the cell. **C**. Positive immunohistochemistry reactions in brain tissue of an adult sea bass. The arrow points to a higher virus concentration in the molecular layer. **D**. Positive immunohistochemical reaction in endothelium of a liver vessel (arrow) Images are taken from Mladineo (2003).

Regarding histological changes, necrosis, vacuoles and intracytoplasmic inclusion bodies are often reported when analyzing histological structures of nervous tissues, retina and spinal cord infected with Betanodavirus (Tanaka et al., 2004, Thierry et al., 1999). When

infections of the olfactory lobe with Betanodavirus in sevenband grouper occur, infected cells are observed as small, round nerve cells lacking dendrites. Betanodavirus infected cells contain cellular debris and macrophages infiltrating these lesions. Infected cells can also be seen in the medulla oblongata. Those cells are necrotized and vacuolated in the granule cell layer of the olfactory lobe. Vacuolated cells in some cases form an extensive area that is called a spongy (Mladineo, 2003, Tanaka et al., 2004). All in all, vacuolation is a common histological change that has been reported in most VNN published papers. Therefore, it is important to search for the appearance of vacuolation in fish tissues when using histology to detect Betanodavirus infection.

1.3.6. Methods of detection

There are several techniques that can be applied for Betanodavirus detection including histopathology, cell culture and polymerase chain reaction (PCR) (Tanaka et al., 2004, Nopadon et al., 2009 and Hellberg et al., 2010). Yet, each method has its advantage and disadvantage (**Table 1-5).** PCR tests are a very useful detection method and are applied in a wide range of diseases. The use of molecular or serological techniques has gained importance over recent years for non-lethal purposes (such as testing on brookstock). However, PCR also has its problems as it is expensive and inefficient. Field-based detection of VNN requires the collection and extraction of good quality RNA from fish tissue in order to detect the virus via PCR (Fenner et al., 2006). ELISA, IHC and IFAT are three immunological based methods that have also been utilised (Totland et al., 1999, Fenner et al., 2006). Cell culture is a method for virological analysis and production which has been used in a large number of VNN research papers such as Iwamoto et al. (2001),

Coeurdacier et al. (2003), Chi et al. (2005) and Yamashita et al. (2009). Although in recent years, the number of cell lines reported to be susceptible to NNV infection has expanded significantly, most isolations are still used SNN-1 or E-11 cells. However, this procedure is time-consuming and indicates low sensitivity, which results in false negatives, particularly when examining fish with a low viral load. Moreover, a successful viral isolation is only completed when brain tissues are used, thus this method requires killing the fish (Bandin & Souto, 2020). Serological analysis, mostly an enzyme linked immunosorbent assay (ELISA), has been used for NNV detection with different protocols for fish antibodies and viral antigen detection. However, it has its disadvantages such as low reproducibility and high background optical density. Recently, a sandwich ELISA using immobilized fish sera has recently been reported and the last modification of the ELISA technique reported for NNV detection is an enzyme linked apta-sorbent assay (ELASA) procedure. Also, nanobased technologies have been reported for VNN detection at present such as nanoparticles (NP), carbon nanotubes (CNT), dendrimers, and quantum dots (QDs). These techniques can identify nucleic acids, proteins and viral particles as well as antibodies in fish. Unfortunately, they are not widely used and practical in aquaculture (Bandin & Souto, 2020).

The sections below will review those methods that have been used in the past by groups analysing Betanodavirus infections in fish.

1.3.6.1. Visual detection of Betanodavirus

1.3.6.1.1. Clinical signs

The first indication of fish disease is often through the observation of behavioural or gross visual signs. Diseased fish are normally physically examined where any abnormal behaviour and external lesions are seen and gills in particular are examined (Noga, 2000). Moreover, colour change is an indicator of a sick fish. Besides that, there are some

common gross and behavioural signs for identifying sick fish for instance they are often found near the water inlet or near the water surface. They have the tendency to congregate together and separate from the healthier stock. Other clinical signs are abdominal swelling and eyes lesions. Skeletal deformities are also symptoms of sick fish and can easily be identified through gross observations (Noga, 2000).

Fish with Betanodavirus infections have abnormal behaviour, abrasions and haemorrhage from the skin and fins (Tanaka et al., 2004). Dead fish have open mouths and operculae, and expanded swim bladders (Tanaka et al., 2004). Azad et al. (2006) observed that infected larvae have darkened pigmentation and are lethargic. Hellberg et al. (2010) also reported abnormal coloration and whirling swimming patterns for fish infected with Betanodavirus (**Figure 1-4**).

1.3.6.1.2. Microscopic techniques

Histopathology describes the microscopic examination of tissue with the intention of studying the manifestations of diseases (Wheater, 1991). It is presented by studying a section (slide) of tissue using light microscope or electron microscope. Histopathology allows a researcher to observe or differentially classify microscopic structures which are commonly enhanced via the utilization of specific histological stains (**Table 1-2**). Both gross anatomy and histopathology have been used in viral detection.

a. Light microscopy

Light microscopy uses visible light as well as a system of lenses to enlarge images of small samples. There are two types of light microscopy in use including single lens microscopy and compound microscopy. Light microscopy is widely utilized in histopathology in combination with different laboratory stains (**Table 1-2**, **Figure 1-5, Figure 1-6**). Regarding betanodavirus detection, H&E staining then analysing under a

light microscope is mostly applied among researchers (Tanaka et al., 2004, Nguyen et al., 1996, Toffan et al., 2017).

Table 1-2: Common laboratory stains used in light microscopy observation (from Ross & Pawlina, 2006)

Stain	*Common use*	*Nucleus*	*Cytoplasm*	*Red blood cell (RBC)*	*Collagen fibers*	*Specifically stains*
Haematoxylin	General staining when paired with eosin (i.e. H&E)	Blue	N/A	N/A	N/A	Nucleic acids—blue ER (endoplasmic reticulum)—blue
Eosin	General staining when paired with haematoxylin (i.e. H&E)	N/A	Pink	Orange/red	Pink	Elastic fibers—pink Collagen fibers—pink Reticular fibers—pink
Toluidine blue	General staining	Blue	Blue	Blue	Blue	Mast cells granules—purple
Masson's trichrome stain	Connective tissue	Black	Red/pink	Red	Blue/green	Cartilage—blue/green Muscle fibers—red
Mallory's trichrome stain	Connective tissue	Red	Pale red	Orange	Deep blue	Keratin—orange Cartilage—blue Bone matrix—deep blue Muscle fibers—red
Weigert's elastic stain	Elastic fibers	Blue/black	N/A	N/A	N/A	Elastic fibers—blue/black
Heidenhain's AZAN trichrome stain	Distinguishing cells from extracellular components	Red/purple	Pink	Red	Blue	Muscle fibers—red Cartilage—blue Bone matrix—blue
Silver stain	Reticular fibers, nerve fibers, fungi	N/A	N/A	N/A	N/A	Reticular fibers—brown/black Nerve fibers—brown/black
Wright's stain	Blood cells	Bluish/purple	Bluish/gray	Red/pink	N/A	Neutrophil granules—purple/pink Eosinophil granules—bright red/orange Basophil granules—deep purple/violet Platelet granules—red/purple
Orcein stain	Elastic fibres	Deep blue [or crazy red]	N/A	Bright red	Pink	Elastic fibers—dark brown Mast cells granules—purple Smooth muscle—light blue
Periodic acid-Schiff stain (PAS)	Basement membrane, localizing carbohydrates	Blue	N/A	N/A	Pink	Glycogen and other carbohydrates—magenta

(This image has been removed due to copyright restriction. Available online from: Naveen Kumar, S., Hassan, M., Mahmoud, M., Al-Ansari, A. & Al-Shwared, W. 2017. Betanodavirus infection in reared marine fishes along the Arabian Gulf. *Aquaculture International*, 25.4, 1543-554).

Figure 1-5: Histopathological examination (H&E stain) of eye of diseased sobaity seabream *Sparidentex hasta* showing a severe vacuolation (v) of inner granular layer of the retina (igl) and oedema of nerve fibres. b Vacuolation (v) of outer granular layer (ogl) of the retina. c Sub-epithelial haemorrhage (h) and congestion (c) in the pigmented epithelium layer of the retina (pel). d Severe congestion (c) of blood vessels in the periorbital tissue (pot). e Vacuolation and oedema (o) of optic nerve (on) and mononuclear cells infiltration (mi). f Congestion (c) of the blood vessels in periorbital region with focal aggregation of melanophores (m). (Naveen Kumar et al., (2017)).

b. Electron microscopy

Work on electron microscopes began in the 1930s and by 1940s electron microscopes had been developed all over the world. In the 1980s it was one of the fastest growing methods used by investigators in histopathology. The electron microscope has higher resolving power than light microscopes and reveals sub-microscopic structures that were previously invisible (Kennedy, 1972). Regarding Betanodavirus detection, Azad et al. (2006) have used an electron microscope to detect viral particles (VP) in epidermal layers of larvae seabass (**Figure 1-6**).

(This image has been removed due to copyright restriction. Available online from: Azad, I. S., Shekhar, M. S., Thirunavukkarasu, A. R. & Jithendran, K. P. 2006. Viral nerve necrosis in hatchery-produced fry of Asian seabass *Lates calcarifer*: sequential microscopic analysis of histopathology. *Diseases of Aquatic Organisms,* 73, 123-130).

Figure 1-6: Betanodavirus in dermal musculature of sea bass larvae detected by electron microscopy. Viral particles (VP) in epidermal layers of larvae and myofibrils of the dermal musculature (arrows), electron density of viral particles was 28-32 nm in diameter are shown (Figure is from Azad et al., 2006).

1.3.6.2. Antibody techniques

In fixed tissue, immunological detection of Betanodavirus, such as IFAT and IHC apply antibodies produced against viral antigens to bind to the virus. Then the antibodies attached to viral antigens in situ are identified by antibodies conjugated with a fluorescent marker or an enzyme which can be seen in fluorescence microscopy or bright field, respectively. Antibody based detections are commonly used after a histological stain to confirm a Betanodavirus positive case (OIE, 2003).

A few IHC protocols have been reported for the detection of VNN in various fish species (**Figure 1-7**) (Nguyen et al., 1996, Johansen et al., 2002 and Toffan et al., 2017). A IHC test that uses a polyclonal sheep anti-serum to a recombinant NNV capsid protein was developed. This antibody detects virus antigen in association with lesions in tissue sections, using peroxidase-labelled or fluorescein-conjugated secondary antibodies (Moody et al. 2004). This test should be used according to the procedure and reagents described in the ANZSDP (Betanodavirus infections of finfish, Moody & Crane 2014).

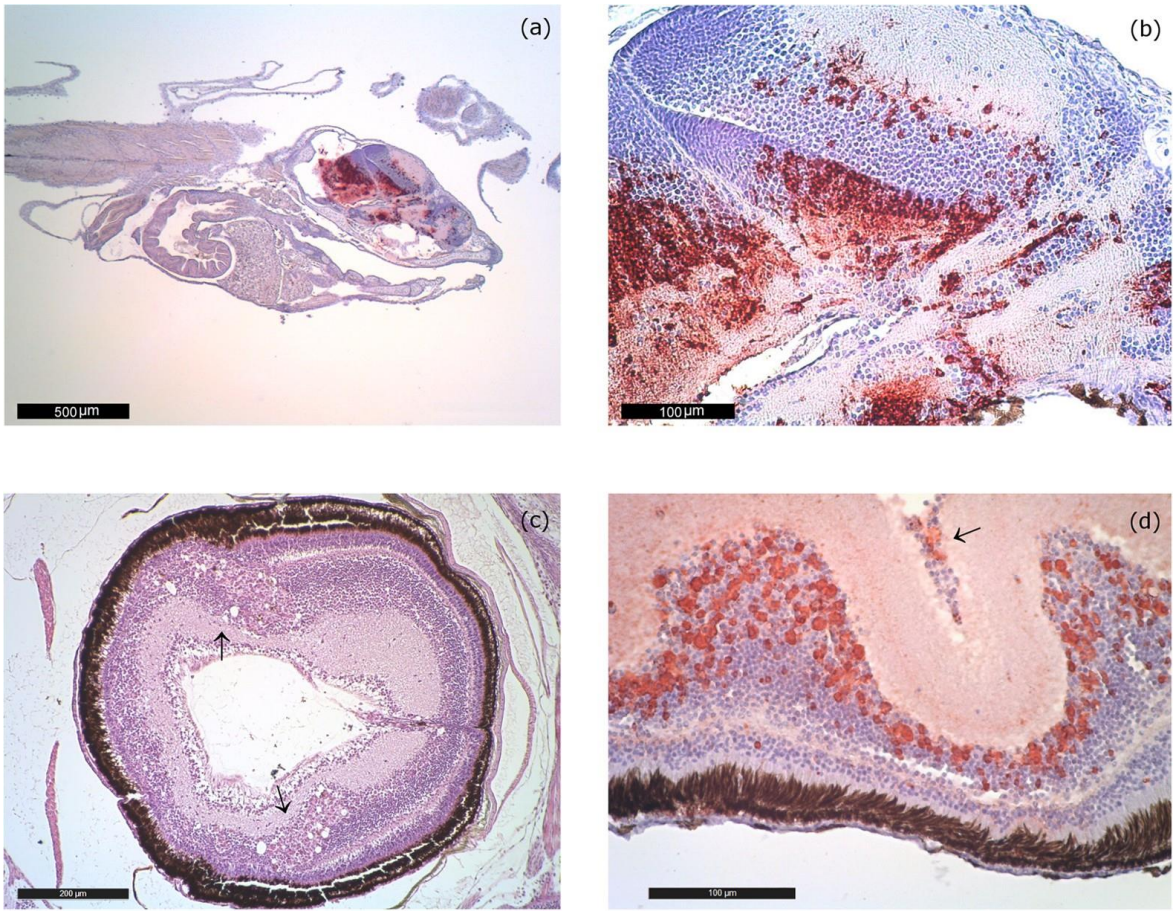

Figure 1-7: Acute lesions of sea bream larvae caused by Betanodavirus

(**a**) IHC Sea bream larvae of 16 days of age from farm 1 (2014–15). Bright red immuno precipitates are visible in the telencephalon, mesencephalon, diencephalon (hypothalamus) and cerebellum. IHC labelling is generally higher in larvae and post larvae than in juveniles. 40 magnification. (**b**) IHC of 16-day-old larvae from farm 1 (2014–15). Massive immuno precipitates in the telencephalon, mesencephalon, diencephalon (hypothalamus). Remarkably, no vacuolization is noticeable. 250 magnification. (**c**) Sea bream post larvae of 45 days of age from farm 1 (2014–15). Vacuolation in the inner nuclear layer of the retina (arrow). H&E 100 magnification. (**d**) IHC of a 55-day-old seabream eye collected in farm 1 (2014–15). Immuno precipitates are evident in the inner nuclear layer and the ganglion cell layer (arrow) of the retina. 250 magnification. Images are from Toffan et al. (2017).

1.3.6.3. PCR (Polymerase chain reaction) for viral detection

One of the most effective techniques to detect Betanodavirus is via isolation in permissive cell culture (E11 or striped snakehead cells SSN-1) followed by molecular (reverse transcriptase-PCR (RT-PCR), nested RT-PCR, real time or quantitative RT-PCR) or immunological identification using immunohistochemistry such as IFAT or ELISA (Nunez-Ortiz et al., 2016). But cell culture is expensive, time consuming, involves

technical expertise, and several Betanodavirus strains are not simple to identify as they are difficult to culture and/or they do not induce clear cytopathic effects. Hence, in recent years, PCR, especially real-time RT-PCR, has been increasingly used for Betanodavirus detection (Munday et al., 2002, Shetty et al., 2012 and Doan et al., 2017). Real time or quantitative RT-PCR assays have been designed, aiming at one or both genomic segments of Betanodavirus (RNA1 and RNA2). Moreover, a real time process linked with high resolution melting (HRM) has been recommended for VNN diagnosis. Genotyping quantitative RT-PCR has also been utilized as a non-lethal diagnostic approach, testing gills, blood, ovarian tissues, sperm and caudal fin samples from live fish (Doan et al., 2017, Bandin & Souto, 2020). These non- lethal approaches are highly effective surveillance tools when samples are frequently tested and are of high value (such as broodstock).

Nishizawa et al. (1994) established the first conventional RT-PCR to detect Betanodavirus however this proved to be suboptimal due to the variability observed in the RNA target region (Thiery et al., 1999). Since then, some PCR-based techniques (RT-qPCR, RT-PCR and nested PCR have been reported in various fish species for Betanodavirus detection (Thiery et al., 1999, Nopadon et al., 2009, Hick &Whittington, 2010, Lopez-Jimena et al., 2010 and Volpe et al., 2020).

However, the indiscriminate use of PCR can result in deceptive conclusions about disease attribution if applied without validation of test accuracy or careful interpretation of results with other data (virological, epidemiological, pathological, clinical).

Each method has its negative and positive aspects. Table **1-3** below generally summarises the advantages and disadvantages of the viral detection methods described above.

Table 1-3: Comparison among methods of Betanodavirus detection

Technique	*Samples*	*Advantages*	*Disadvantages*	*References*
Electron microscopy	Blood, biopsy, lethal nonlethal	Pathological changes Viral particle visual	Viral location in tissue	Mori et al. 1992; Grotmol et al.1997
Light microscopy	Nonlethal, biopsy, blood	Pathological changes, Long term storage	Cannot visualize viral particles	Toffan et al., 2017; Volpe et al., 2020
PCR	Blood, sperm and ovarian Nonlethal lethal	High sensitivity Quick result Control of contamination	Cannot investigate the pathogenicity or epidemiology	Nopadon et al, 2009; Hick and Whittington (2010)
Antibody techniques	Blood Nonlethal Lethal Biopsy	Easy observation High specificity High sensitivity	Expensive High technology Limited in speed and strain identification	Toffan et al., 2017
Cell culture	Biopsy Lethal Nonlethal	High specificity High sensitivity	High technology Limited in speed and strain identification	Volpe et al., 2020

1.3.7. Management and control of VNN disease

To date VNN disease has no effective treatment, therefore prevention is still the best way to limit VNN disease. Techniques that can be used to prevent infection include strict husbandry management in the hatchery phase, screening of broodstock for VNN using appropriate methods, improving water quality and avoiding stress factors related to water parameters (Doan et al., 2017). Reducing larval stocking density will also reduce viral transmission (Shetty et al., 2012). Therapeutics aimed at the immune system have been proposed as treatments for VNN, however to date strategies for reducing VNN disease have focused on prevention by the implementation of strict biosecurity practices. In addition vaccination has been proposed as a method for prevention of VNN in fish culture. Vaccination research in fish has delivered conflicting results ranging from very efficent to insignificant outcomes as a method of fish disease control (Bandin & Souto, 2020). All the methods of management and control are further discussed below.

1.3.7.1. Vaccination

Vaccines such as inactivated virus vaccines, attenuated live virus vaccines and recombinant vaccines are commonly applied to limit viral diseases in fish such as VNN disease (Liu et al., 2006). Vaccine delivery methods consist of oral, intramuscular and intraperitoneal injection as well as bath vaccination. Bathing fish in a vaccine has chiefly been applied for inactivated vaccines (Bandin & Souto, 2020).

Bath immunization using nano-encapsulated foramalin-inactivated Betanodavirus vaccine is reported as an effective method to defend grouper larvae against VNN (Kai & Chi, 2008). The vaccine is inactivated by 0.1-0.2% formalin after 7 days incubation at 24^{o}C and its efficent dose is 10^{6} Tissue Culture Infective Dose (TCID) 50/mL with 20 minutes bath immersion. The highest protection from this vaccine occurred 30 days post-vaccination and protection lasted for a further three months in grouper larvae (*E.coioides*)

(Kai & Chi, 2008). In another example, Lin et al. (2007) utilised an oral vaccine for grouper larvae (*E. coioides*) containing *Artemia*-encapsulated recombinant *E. coli* expressing the Betanodavirus capsid protein gene to protect against VNN. Moreover, Kai et al. (2010) reported that when grouper broodfish where intramuscularly injected with adjuvanted VNN vaccine it reduced risk of vertical transmission of VNN for up to 17 months post vaccination. A final example of a successful vaccine strategy was that two doses of 10 µg Virus-like Particles (VLPs) stimulated a great and specific antibody response against Betanodavirus in dragon grouper at three weeks and was able to protect fish from viral infection (Liu et al., 2006). More recent research has proposed using DNA vaccines as an alternative to inactivated recombinant proteins and virus. Testing of DNA and recombinant Betanodavirus vaccines in halibut and turbot demonstrated that the DNA vaccine provided no protection whilst the recombinant protein vaccine was effective with 50-67% Relative Percent Survival (RPS) (Sommerset et al., 2005). A live Betanodavirus vaccine was demonstrated to protect sevenband grouper against VNN (Nishizawa et al., 2011). Fish exposed to Betanodavirus at 17°C (at which the virus was not infective), had a drastically greater survival rate following a subsequent exposure at a higher 26°C compared to those that had not been challenged previously with the virus. Recent work such as Valero et al. (2016) studied vaccination and immune responses of seabass against betanodavirus using a chitosan-encapsulated DNA vaccine delivered via intramuscular injection method. The vaccine succeeded in up-regulating the expression of genes involved in cell-mediated cytotoxicity and the inflammatory interferon pathway. However, this method was unsuccessful at inducing NNV-specific antibodies. Falco et al. (2021) showed that the RGNNV isolate was fully inactivated by 3 mJ/cm^2 UV-C irradiation and 24 h 0.2% formalin treatment. They proposed promising NNV-inactivation procedures for potential vaccine entrants. Therefore, vaccination procedures are effective, feasible and rapid response strategies for VNN control in aquaculture. While

vaccination is promising, it is very costly and time-consuming to apply in large scale fish farming especially the most susceptible larval stage.

1.3.7.2. Disinfection of water supplies and wastewater

Arimoto et al. (1996) showed that Betanodaviruses can survive chloroform, ether and formalin treatment. It is able to survive pH levels from 2 to 11 and up to 24 hours with no reduction in infectivity.

However, chemical disinfectants such as calcium hypochloride, sodium hypochlorite, benzalkonium chloride, iodine and chloroquine or additional chemical (ozone ammonium, chloride) or physical actions (ultra-violet, light heat) are able to completely destroy the virus (Bandin & Souto, 2020). For example, chemical deactivation can be attained with sodium hypochlorite (50 mg/L for 10 min), ethanol at 60% and ozone (3.0 mg/L for 6-7 minutes) (Buchan et al., 2006, Arimoto et al., 1996). Thus, it is essential to use disinfected water in aquaculture and that can be achieved by using treatment systems that often make use of high efficiency sand filters and UV light or ozonisation.

1.3.7.3. Temperature

The efficacy of high water temperature in controling Betanodavirus infection in humpback grouper (*Cromileptes altivelis)* fingerlings was reported by Yuasa et al. (2007) who indicated that high water temperature inhibits betadnodavirus proliferation in fish. Setting rearing water temperature at 31^{o}C can lower the transmission of Betanodaviruses and 35^{o}C water can inhibit the viral infection. However, this high temperature needs to be restricted to only a few days as it if continued it is a risk to fish welfare. Thus temperature treatment is not desirable as it can lead to fish death.

1.3.7.4. Selective breeding for Betanodavirus resistance

Current research suggests that resistance to virus related disease at moderate to high levels is heritable in nearly all fish breeds, suggesting viral disease resistance can be enhanced significantly via selective breeding of cultivated fish. Up to now, heritability for Betanodavirus resistance has been observed, but only in Atlantic cod (Doan et al., 2017). Regarding Betanodavirus resistance in cod, five genomewide significant QTLs have been identified that account for 68% of the phenotypic variance for resistance. These were discovered using 161 microsatellite markers in Atlantic cod (Baranski et al., 2010). Another assessment with a 12K SNP array detected both the location of three of these QTLs and a high proportion of variance was explained using these genomic markers (Yu et al., 2014). In 2016, research by Liu et al. using a QTL study examining 149 microsatellites covering 24 linkage groups found that VNN resistance in Asian sea bass is dominated by many loci. Also, in that study, separate QTLs were identified for survival time and VNN disease resistance, and some were identified for both resistance and survival time (Liu et al., 2016).

In summary selective breeding has been proven to be an efficient solution to establish hardy aquaculture populations for some diseases. New genomics-based techniques in the foreseeable future will provide a means to make selective breeding for disease resistance even more achievable (Doan et al., 2017).

1.3.7.5. Hygiene and sanitation of equipment in aquaculture

Another method for protection against betanovirus outbreaks is to pay particular attention to hygeine and sanitation of all equipment used during aquacultutre including raceways and tanks. Betanodavirus can be easily deactivated by using chemical disinfectants such as sodium hypochlorite, iodine, calcium hypochloride, chloroquine and benzalkonium chloride or by other chemical (ammonium chloride, ozone) or physical treatments (ultra-

violet light, heat) (Adachi et al., 2007). For example, before and after use these should be sterilized with a chlorine disinfectant in addition to other equipment (net, brush) utilised on farms. If possible it is useful to ultilize ozonated or chlorine or electrolyzed seawater containing 0.5mg/L of Total Residual Oxidants (TROs) for 30 minutes for sterilisation (Lee & O'Bryen, 2003). After the disinfection process is completed, all equipment should be used separatedly. Ideally each tank should have different equipment (Lee & O'Bryen, 2003) and these interventions will help to reduce the risk of horizontal transmissions.

In conclusion, in terms of management and control of VNN a variety of methods have already been used but there is still room for improvement within all these methods.

1.4. Parasitic infections in barramundi

Barramundi is a prospective host to numerous different species of parasites (Hutson, 2014) that can cause major impacts or mortalities to both wild and caged fish stocks. Hence, fish health surveillance with accurate identification of parasites is crucial so that a build-up of parasite numbers can be prohibited. Information about parasite transmission and possible intermediate hosts is important to decide on the most suitable management to lessen economic losses in aquaculture. In this section, taxonomy information of some common parasites infecting barramundi will be covered.

The parasite fauna of cultured *L. calcarifer* comprises a total of about 90 known species (Rückert et al., 2008). That study also found parasites that infest barramundi in Indonesia belonged to the Protozoa, Myxozoa, Digenea, Monogenea, Cestoda, Nematoda and Acanthocephala.

Lernanthropus latis (Copepoda: Lernanthropidae) is a main parasite danger to the sustained aquaculture of barramundi. There are three free living stages and five parasitic

stages in the life cycle of *L. latis*. Morphologically, mature parasites varied from the original description by the existence of small setae on the legs, the parabasal flagellum and caudal rami as well as minor incongruences (Kua et al., 2012). Although *L. latis* shows extensive environmental tolerance, freshwater can be applied as an efficient management approach to alter the life cycle in fish farming (Brazenor & Hutson, 2013).

(This image has been removed due to copyright restriction. Available online from: Brazenor, A., & Hutson, K. 2013. Effect of temperature and salinity on egg hatching and description of the life cycle of *Lernanthropus latis* (Copepoda: Lernanthropidae) infecting Barramundi, *Lates calcarifer*. *Parasitology International,* 62.5, 437-47).

Figure 1-8: Dorsal view of *L. latis* nauplius I in egg membrane, scale=80μm. Image is taken from Brazenor & Hutson (2013).

Khalid et al. (2014) isolated parasitic crustaceans of *L. latis* from *L. calcarifer* its host, in barramundi collected from a cage culture farm in Setiu Wetland, Therengganu. Parasitic *L. latis* takes a 483h time to complete a life cycle at 30ºC.

More than 200 *Henneguya* species were described in the past and a new checklist of 43 new *Henneguya* species was reported with a variety of hosts (Eiras & Adriano, 2012). Whilst most *Henneguya* species do not initiate obvious disease in their hosts, some of them are economically crucial pathogens. Borkhanuddin et al (2019) reported three

species of *Henneguya* in cage farmed barramundi in Malaysia. Characterization of these infections applying myxospore morphology, tissue tropism and morphometry and 18S rDNA sequencing supported the description of those three new species as: *Henneguya voronini* n. sp., *Henneguya setiuensis* n. sp. and *Henneguya calcarifer* n. sp. In those three species, myxospores had typical *Henneguya* morphology, with an oval spore body, two polar capsules in the plane of the suture, two caudal appendages and smooth valve cell surfaces (Borkhanuddin et al., 2019).

Blood borne *Trypanosoma* sp. has been linked with severe barramundi deaths in the Northern Territory (Schipp et al., 2007). Other external protozoan parasites found in *L. calcarifer* include *Trichodina* sp. and *Ichthyobodo* sp. (Gibson-Kueh, 2012). In South Australia, low prevalence *Icthyobodo* sp. infestations were found in all samples studied from outbreaks at three farms, along with streptococcosis or filamentous bacteria on gills and epitheliocystis (Griffiths, 2009).

The genus *Chilodonella* includes numerous free-living ciliate varieties, but only two (*Chilodonella piscicola* and *Chilodonella hexasticha*) are described to be opportunistic parasites in freshwater fish. Three known species have been isolated from barramundi (*C. acuta, C. hexasticha* and *C. uncinata*). Bastos Gomes et al. (2017) used staining to detect these three species of parasites. They then examined the parasites for morphological description as well as completed comparative phylogenetic analyses utilizing the mitochondrial small subunit (mtSSU) rDNA marker to classify them into three species. More recently, Bastos Gomes et al. (2019) reported *C. hexasticha* in barramundi. This research described the bacterial composition related with the large quantity of a ciliated protozoan parasite, *C. hexasticha*, in freshwater ponds and the gills of barramundi *L. calcarifer*. The authors suggest co-surveillance of the multiple microbial communities

present in aquaculture systems will allow for a better understanding of their demographics and interactions and will contribute to improved animal health and biosecurity management.

Yang et al. (2006) reported four species of the Monogenoidea (*Laticola lingaoensis*, *Laticola latesi*, *Laticola paralatesi* and *Diplectanum penangi*) present in the gills of *L. calcarifer*. Later, Chotnipat et al. (2015) reported using a combined molecular and morphological investigation, proof that *L. paralatesi* is the dominant or single, species of diplectanid parasitising *L. calcarifer* in tropical Australia. Most species of Diplectanidae are distinguished from other skin or gill affecting monogeneans by a distinctive structure, named the lamellodisc or squamodisc, which is a disc-like organ with several rows of sclerotised rodlet structures placed in a U like-shape (Chotnipat et al., 2015). Squamodiscs usually appear on both dorsal and ventral surfaces and operate primarily like a friction pad, avoiding dislodgement of parasites by gill movements. The haptoral anchors, that are comprised of sclerotised proteins, assist in attachment by infiltrating deep into the connective tissue and basal membrane of gill lamellae (Chotnipat et al., 2015). The feeding activity of diplectanids can terminate epithelial cell layers, decreasing the amount of chloride cells on gills, triggering over-production of mucus which eventually weakens the respiratory performance of infected fish (Chotnipat et al., 2015).

In North Queensland, *Neobenedenia* sp. (Monogenea: Capsalidae) infected farmed barramundi were also reported in research by Brazenor & Hutson (2015). Putri et al. (2020) also examined *Rhadinorhynchus bicircumspinis* infections in barramundi (*L. calcarifer*) from floating net cages and ponds in Situbondo, Indonesia waters. Their work showed that the endoparasite *R. bicircumspinis* was found in the digestive tract of fish at a prevalence of 10% and 40% in pond and floating net cages, respectively. Gabor et al.

(2011) reported two cases of gastrointestinal cryptosporidiosis outbreak with high mortality in juvenile barramundi (*L. calcarifer*). They used histological methods following by electron microscopy to identify *Cryptosporidium*-like organisms within the distal stomach and proximal small intestine of fish (Gabor et al., 2011).

Cryptocaryon irritans was reported in barramundi in the early 1990s. In Australia, Bryant et al. (1999) infected barramundis with *C. irritans* to detect antibodies against the disease. Cervera et al. (2020) described *C. irritans* as a ciliate protozoan that causes marine white spot disease and is the most distressing parasitic disease in both mariculture and in ornamental fish, commonly occurring when temperatures are between 20–30 °C. *C. irritans* shows very low host specificity and can infect multiple fish species (Colorni & Burgess, 1997). During its life cycle, the theront- the infective stage, infects the fish epithelial layer and develops to a trophont stage. This stage feeds on tissue debris and body fluids then matures and leaves the host, becoming tomont cells which is the external stage. *C. irritans* invades fish epithelium of the skin, gills, and eyes, changing their physiological functions (Li et al., (2021). The main indications of cryptocaryoniasis include the formation of whitish blisters (spots) on the skin and eyes, respiratory distress, skin discoloration and anorexia due to excessive mucus secretion (Cervera et al., 2020).

1.5. Epitheliocystis

Epitheliocystis is widespread in numerous fish species that alters both the skin and gill epithelium. It was known as an intracellular contaminant with Gram-negative bacteria, causing hypertrophy of infected cells (Meijer et al., 2006). However, it is now recognized as being more complex. Environmental circumstances that approach or go beyond animals' physiological acceptances (for example salinity and temperature) are also believed to impact disease development and progression (Blandford et al., 2018). Infection can lead to respiratory distress and death, especially in cultured and juvenile fish.

Fish mortalities that are associated with epitheliocystis infections have been registered in at least 90 species of wild and farmed fish and this includes both freshwater and marine fish (including barramundi) (Nowak & LaPatra, 2006, Stride et al., 2013 and Blandford et al., 2018). Gill infestations can be correlated with epithelial hyperplasia, degeneration and necrosis, together with haemorrhage (Nowak & LaPatra, 2006). The explanatory term epitheliocystis is obtained from the presence of epithelial lesions that appear secondary to infection. A typical developmental cycle comprises a reticulate body, infective elementary body, and intermediate body (round cell) (Nylund & Isdal, 1998).

Infected cells include coccobacillary or coccoid bodies that finish a pleomorphic developmental cycle with certain morphological characteristics reliant on the stage of intracellular development. Most stages of epitheliocystis life cycle were present in the gill structure of examined fish indicating they had long term exposure to the epitheliocystis infection (Paperna & Dematos, 1984). More specifically, gradual enlargement of infected epithelial cells consequently ends in the creation of spherical cysts that are defined by an eosinophilic hyaline capsule evidently consisting of retained remnants of the infected cell cytoplasm and membrane (Paperna & Dematos, 1984). Stressful living conditions or exposure to sewage can increase the infection and mobility rate in fish. The disease cannot be identified by routine microbiology approaches, due to the bacteria not being culturable. That is why, if histology is not utilized in analysis, the disease can remain undetected (Nowak & LaPatra, 2006). Draghi et al. (2007) also agreed histology can be used primarily to confirm the disease. In recent years, molecular techniques are widely applied to detect epitheliocystis as well.

1.6. Scale drop disease

Since 1992 outbreaks of an illness occurred where the main symptom was loss of scales in farmed barramundi in Asia–Pacific, thus this disease was referred to as scale drop

syndrome (SDS) (de Groof et al., 2015). External lesions, observed in both naturally and experimentally infected barramundi, usually include fin rot, skin haemorrhage and scale loss, accompanied by extensive skin and muscle inflammation, whereas internally, multifocal necrosis often occurred in liver, spleen and kidney (de Groof et al., 2015). The virus was isolated and propagated in cell culture, where it showed a cytopathogenic effect in infected Asian seabass kidney and brain cells. Electron microscopy revealed icosahedral virions of about 140 nm, characteristic for the *Iridoviridae.* For disease diagnosis, published and patented single PCR and qPCR protocols (de Groof et al., 2015) were developed for scale drop disease but these are not yet fully validated. De Groof et al. (2015) also prepared vaccines from binary ethyleneimine inactivated virus, as well as from *E. coli* produced major capsid protein which provided efficacious protection against scale drop disease. Kayansamruaj et al. (2020) used metagenomic analysis based on next-generation sequencing to examine a sample collected from infected barramundi showing the notable signs of scale drop disease (SDD) to confirm the disease. Meanwhile, a newly developed semi-nested PCR (snPCR) method for detection of the virus causing scale drop disease from field samples was reported by Charoenwai et al. (2019). This protocol could detect down to 100 viral copies/μL template and was 100-fold more sensitive than single step PCR.

According to Gibson- Kueh et al. (2012)'s report, darkened bodies, scale loss over extensive areas with loss of skin colour, tail/fin erosion, pallor of gills, focal to extensive areas of hepatic lipidosis, petechial to ecchymotic haemorrhage in liver, kidney and spleen, splenomegaly or atrophied shrunken spleen and renomegaly are typical gross signs of scale drop disease. Pathologically, the most distinctive histopathological feature of SDS was the vasculitis in all major organs. The dermis overlying scale beds was often necrotic, corresponding to areas with loss of scales and skin colour. Extensive gill

epithelial necrosis was also observed. Ellipsoidal necrosis in spleen with multifocal to more extensive coalescing areas of infarction and haemorrhages and associated tissue degeneration, haemorrhage and necrosis of varying severity (Gibson- Kueh et al., 2012). Effective prophylactic and therapeutic measures against scale drop disease virus (SDDV) are not available (Charoenwai et al., 2019). Coinfection of SDDV and *L. calcarifer* herpes virus (LCHV) were detected in all fish examined in a commercial farm in Singapore using PCR but sick fish had higher viral load compared to healthy fish (Domingos et al., 2021).

1.7. Big belly syndrome

"Big belly" disease is a chronic, granulomatous bacterial enteritis and peritonitis, first reported in 3- to 4-week-old Asian seabass or barramundi, *L. calcarifer* (Bloch) fry in the 1990s. Affected fry are emaciated and have a swollen abdomen, thus the condition is referred to as 'big belly' syndrome. Gibson-Kueh et al . (2021) describe extensive histopathological changes in stomach tissues including chronic granulomatous enteritis, in association with intralesional, intestinal perforation as well as peritonitis and clusters of large coccobacilli scattered throughout intestinal mucosal epithelium, lamina propria and peritoneum (Gibson-Kueh et al., 2021). "Big belly" appears to be relatively host - specific to *L. calcarifer,* except for a rare case of pathology resembling "big belly" disease in red snappers, *Lutjanus* species (Gibson-Kueh et al., 2021). The causative agents was strongly suggested to be a novel *Vibrio* species based on evidence gained using in situ hybridization (ISH) and 16sRNA PCR.

1.8. Conclusions

Barramundi is a fish species that is economically important to the future of aquaculture in Australia and the Asia-Pacific region. As with any commercially farmed species it is important to monitor the condition of the livestock and prevent the outbreak of disease. This literature review of barramundi diseases demonstrates the numerous infections that

are a risk to barramundi health and survival. Among those infections, VNN is a disease with high mortality which can dramatically decrease the economical profit of barramundi farming. The known pathogenic agents reviewed vary from viruses to bacteria and parasites, which can all be detected by a variety of techniques such as IHC, routine histopathology and PCR.

Histopathology clearly plays an important role as an effective tool for detection of a broad range of pathogens. The advantage of histopathological techniques as a means of efficiently identifying diseases such as epitheliocystis and big belly syndrome is also stated in this literature review. It can detect abnormal pathology at early stage of the infection therefore it is useful and suitable for surveillance purposes, especially for novel pathogen investigation and can lead to the use of other methods such as PCR to identify the actual pathogen. In terms of Betanodavirus detection, H&E staining is mentioned as a common initial method for use in barramundi or routine fish disease surveillance to detect abnormalities

1.9. The aims of the thesis

This thesis was undertaken in order to: (a) use histopathology as the primary screening approach for observing the presence of pathogens in juvenile barramundi at a South Australia hatchery and (b) to investigate if VNN could be detected in fish with histopathological features of Betanodavirus infection using a gold standard immunochemistry method.

CHAPTER 2: METHODS

2.1. Experimental design

2.1.1. Location of site for fish collection

Fish for this study were obtained from a hatchery facility at West Beach about 10km west of Adelaide city, South Australia (South Australian Research & Development Institute (SARDI)). This hatchery facility was chosen as the collection site because VNN was reported to have occurred at this site previously in 2004 (Moody et al., 2009). At this hatchery, the water supply (**Figure 2-1**) is collected up to 1.5 km offshore and stored in various tanks with salinity from 30-35ppt. The system is constructed to produce a limit of 1500 litres of seawater per minute (around 1.8 million litres per day). Primary treatment of water consists of three 50 kL settlement tanks and automated primary sieves before sand purification then keep in two 100kL tanks. Distribution to indoor and outdoor tanks is through sand filters, after that the water can be additionally filtered and/or UV sterilised as required. Wastewater is treated by sand filtration, UV sterilisation and chlorination before releasing. The whole system can be operated in recirculation mode if necessary.

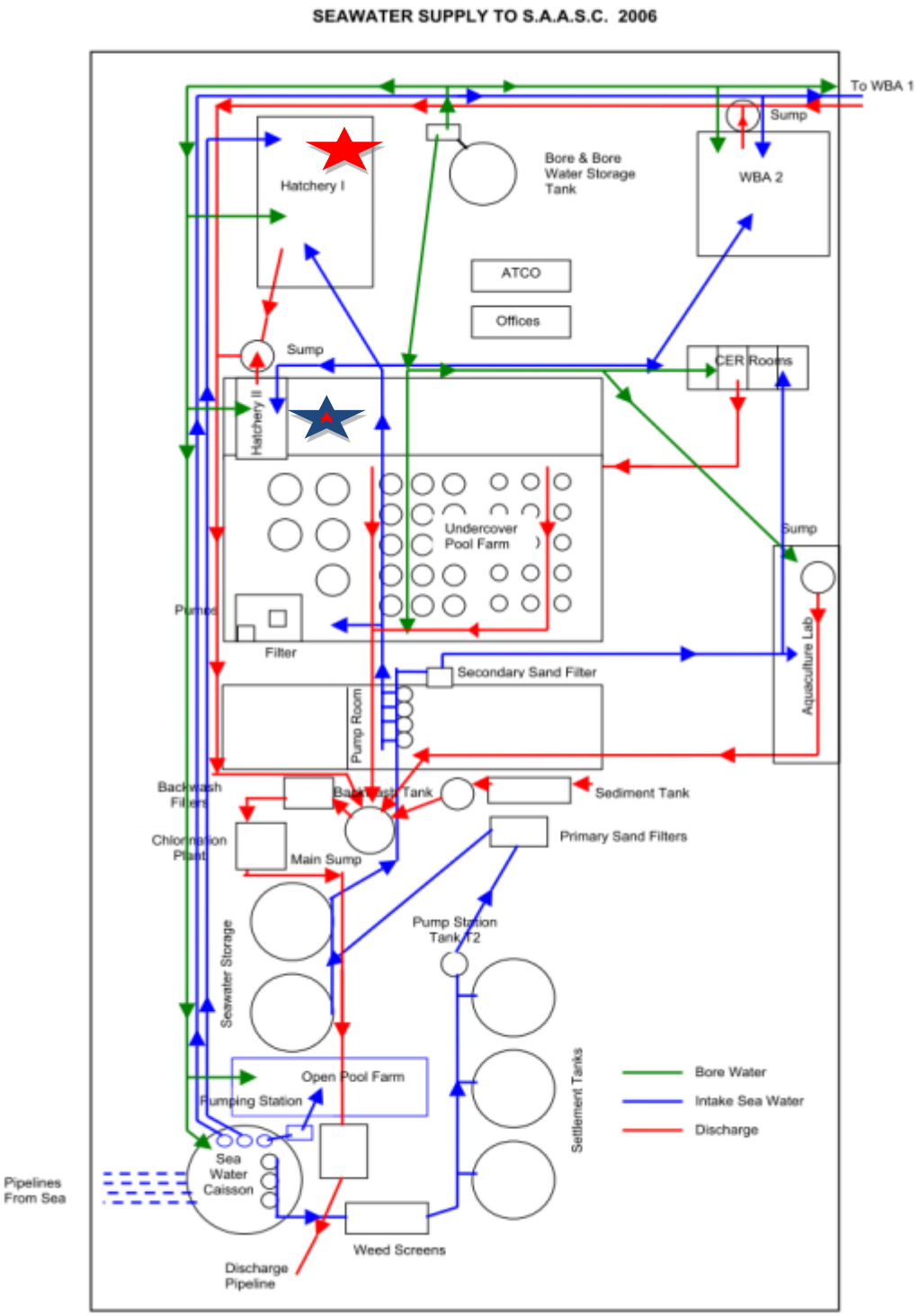

Figure 2-1: Diagram of water supply at West Beach farm: Samples were collected from hatchery I (red star) which has been run separately from hatchery II (blue star). The two hatcheries use the same water supply. (http://www.sardi.sa.gov.au/aquaculture/aquatic_sciences/sa_aquatic_sciences_centre/sa_aquatic_sciences_centre).

2.1.2. Material and frequency of fish collecting

All fish were collected and handled following a Standard Operating Procedure (SOP) which is managed by Flinders University. Animal ethics approval (E350) for using fish in this study was granted by the Flinders University Ethics Committee. The sample fish were collected nine times during a period of 11 months between September 2011 and August 2012. On average there was approximately one collection per month except for in January and February during the peak of summer. Each time, 30 juvenile barramundi were collected at random from the site for Betanodavirus examination. In total 270 samples were collected for each assessment in this project (**Table 2-1**). Fingerling fish were deliberately chosen around one month of age for collection. All samples were collected randomly but we biased towards fish which displayed abnormal behaviour. Before fixation, fish were euthanised with Aqui-S (40mL/1000L) as per the Flinders University SOP.

In addition, four VNN positive and two VNN negative paraffin blocks of 5 cm long barramundi were supplied by Nick Moody from ACDP (Australian Centre for Disease Preparedness) formerly known as AAHL (Australian Animal Health Laboratory, CSIRO, Geelong) and used as controls for routine histology and IHC tests.

Table 2-1: Fish collection information

Date	Label	Size(cm)	Gross signs
27/09/2011	1-30	3-5cm	Healthy Normal behavior
9/11/2011	31-60	3-5cm	Healthy Balanced swimming pattern
8/12/2011	61-90	2-5cm	Healthy No sign of abnormalities
02/03/2012	91-120	2-4cm	Healthy
22/03/2012	121-150	3-5cm	Unhealthy Darkened skin Some with up-side down swimming pattern
18/04/2012	151-180	3-5cm	Healthy
16/05/2012	181-210	4-5cm	Darkened skin Abnormal swimming pattern
20/07/2012	211-240	3-4cm	Healthy
11/08/2012	240-270	3-5cm	Healthy

2.2. Method of detection

2.2.1. Histopathology

2.2.1.1. Sample preparation

Whole juvenile fish were fixed in 10% neutral buffered formalin and stored in separate bottles for 24 hours (h) prior to histological techniques. The fish were then transferred to decalcification liquid (**Appendix 6-1)** for a further 24 h, after which their fins and excess internal fluid were removed, then samples were washed under running tap water for at least four hrs (Ferguson, 2006).

2.2.1.2. Routine histology (H&E staining)

Decalcified samples were processed and embedded with paraffin, sectioned (5µm thick) and stained with H&E following the protocol of the histology lab at Flinders Medical Centre (**Appendix 6-1**). Tissues were examined using an Olympus BX40 light microscope, with magnifications (10X, 20X, 40X and 100X) and a Nikon Eclipse Ti with a Nikon DS-Fi1C camera for microphotography.

2.2.2. IHC

The Australia and New Zealand Standard Diagnostic Procedure for Betanodavirus Infections of Finfish (ANZSDP, 2008, http://www.agriculture.gov.au/animal/health/laboratories/procedures/anzsdp/Betanodavirus1) protocol was applied to processed sections (**Appendix 6-2**) with modification of the primary antibody Sheep®α-NNV rCP polyclonal antibody being diluted to 1/500 instead of 1/1000 (obtained from the Queensland Government Department of Agriculture, Fisheries and Forestry, Biosecurity Sciences Laboratory (BSL), Brisbane, Queensland). Commercial Rabbit®α-Sheep IgG [H+L] HRP (Sigma Aldrich, USA) was used for detection of positive tissues following the protocol listed in **Appendix 6-2**. Stained tissues were examined by light microscopy as described above. Paraffin blocks containing specimens from four Betanodavirus positive and two negative fish were obtained from the CSIRO's Animal Health Laboratories, at Geelong, in Victoria and examined for use as positive and negative controls, respectively. The Betanodavirus status of these fish was determined in these laboratories using the IHC method described here. These samples were from larval barramundi and were about 5cm long and in the same age range as the specimens (3-5cm) collected in this thesis.

2.3. Histopathological changes analysis

2.3.1. Betanodavirus examination

Examination and assessment of H&E stained samples (**Table 2-2**) shows various levels of histological changes and how those levels were described. The descriptions are based on knowledge about Betanodavirus stated in the literature review of this thesis (Tanaka et al., 2004, Nguyen et al., 1996), Mladineo, 2003). Mostly sampled barramundi were examined for vacuoles in the brain, retina and the spinal cord.

2.3.2. Parasite examination

Parasitic infections observed in this project were scored with three levels following Ferguson (2006) and Lightner (1996). Observations were made on a whole slide. It was level one when the histological change occurred in less than 30% of an organ or tissue examined, level two was scored when histological change occurred in 30% to 60% of an organ or tissue examined and level three was scored when the histological change occurred in more than 60% of an organ or tissue examined. The level of severity shows how much damage parasitic infections can make in terms of histological changes in the fish tissues.

Table 2-2: Ranking table of histological changes

Level of changes	Definition	1	2	3
Retina	Swollen cells (hypertrophy)	<30 %	30 %-60 %	>60 %
	Vacuoles	Mild	Moderate	Marked
	Clogging	Mild	Moderate	Marked
Brain	Swollen cells	<30 %	30 %-60 %	>60 %
	Vacuoles	Mild	Moderate	Marked
	Clogging	Mild	Moderate	Marked
Spinal cord	Swollen cells	<30 %	30 %-60 %	>60 %
	Vacuoles	Mild	Moderate	Marked
	Clogging	Mild	Moderate	Marked
Gill	Hyperplasia	Isolated cells	Random distribution	Marked
	Vacuoles	Mild	Moderate	Marked
	Melanin granules	Mild	Moderate	Marked
	Haemorrhage	Mild	Moderate	Marked
Kidney	Renal Tubule	Enlargement	Fibrosis	Destruction
	Corpuscle	Enlargement	Fibrosis	Destruction
	Haemorrhage	Mild	Moderate	Marked
	Melanomacrophage centres	Isolated	Random distribution	Marked
Liver	Vacuoles	Mild	Moderate	Marked
	Haemorrhage	Mild	Moderate	Distribution
	Melanomacrophage centres	Isolated	Random distribution	Marked
	Hepatic Vein	Swelling	Vacuolation	Destruction
	Hepatic Artery	Swelling	Severe swelling	Destruction
Spleen	Vacuoles	Mild	Moderate	Marked
	Haemorrhage	Mild	Moderate	Marked
	Melanomacrophage centres	Isolated	Random distribution	Marked

2.4. Data analysis

Excel 2010 was used for data analysis in this project. In terms of histological changes in various barramundi tissues, coding was used as 0 for negative, 1, 2, 3 for changes level 1, 2, 3, respectively (refer to **Table 2-2** for histological changes ranking). Then the possibility statistical method countif was applied in order to determine the prevalence distribution of these observations for each organ or pathogen. The results of this analysis were then expressed as a percentage in the tables and graphs presented.

CHAPTER 3: RESULTS

3.1. Gross signs

A total of 270 barramundi fingerlings were collected and examined in this study. The fish varied in size from 5 g to 10 g and were aged between one to three months old. During the nine months sampling period, there were two occasions in March and May 2012 when darkened colour, lethargy, and whirling swimming behaviour were evident. However, there were no external signs of skin ulceration or haemorrhaging. All fish collected were examined for histological changes in the retina, brain, spinal cord, gill, liver, kidney, and spleen using H&E staining as described previously.

3.2. Routine histology (H&E staining)

Observation of histological changes in all samples are described in **Table 3-1** below. Overall, there were only 18 out of 270 samples that showed abnormal histological changes during the nine months that samples were collected. Those 18 samples were mainly from the batches in March and May 2012 where fish also showed abnormal swimming pattern and skin discoloration. When the retina and the brain of those samples were examined with H&E staining vacuoles were observed, potentially indicative of Betanodavirus infection. These 18 samples went on to be tested for Betanodavirus infection with IHC in order to confirm the VNN diagnosis (these results are described later).

During the examination of the 270 fish, the highest prevalence (49%) and level of tissue changes was found to have occurred within the gill (**Table 3-1**). The kidney and retina followed as the second and third most affected organs accounting for 32% and 20% of observed changes, respectively.

Table 3-1: Prevalence of each level of histological change in samples examined (n=270, 4 replicate sections for each fish was examined.1=mild, 2=moderate, 3=marked)

	Prevalence of histological changes			
Organ	**1**	**2**	**3**	**TOTAL**
Retina	16%	4%	0%	20%
Brain	14%	0%	0%	14%
Spinal cord	4%	0%	0%	4%
Gill	38%	8%	3%	49%
Kidney	27%	5%	0%	32%
Liver	13%	3%	0%	16%
Spleen	11%	1%	0%	13%

3.2.1. Retina

Fish retina is made up of nine layers: the pigment epithelium layer, the rods and cones layer, the outer nuclear layer, the outer plexiform, the inner nuclear layer, the inner plexiform layer, the ganglion cell layer, the ganglion axon layer and the vitreous body (Ferguson, 2006). The normal structure of fish retina would be to have all layers in perfect condition without any abnormalities such as haemorrhage, swollen cells, deconstructed layers, vacuolation. **Figure 3-1** shows normal structure of fish retinal tissue whereby most layers are easily observed, and with no vacuoles occurring in the inner nuclear layer.

Vacuolation (**Figures 3-2, 3-3 and 3-4**) and hypertrophy **(Figure 3-4**) were common observations of tissue abnormality within the retina that were observed in 18 of 270 fish. These are also signs that are indicative of a VNN infection in the barramundi. Histological changes occurred chiefly in the inner nuclear layer (**Figures 3-2, 3-3 and 3-4**). The other layers of the retina randomly showed abnormal structures such as increase in size of nucleus and erythrocyte aggregation, particularly in the flexiform layer (**Figure 3-4**). However, the occurrence of swollen blood capillaries was less frequent than vacuolar degeneration. There was no observation of changes in the photoreceptor layer.

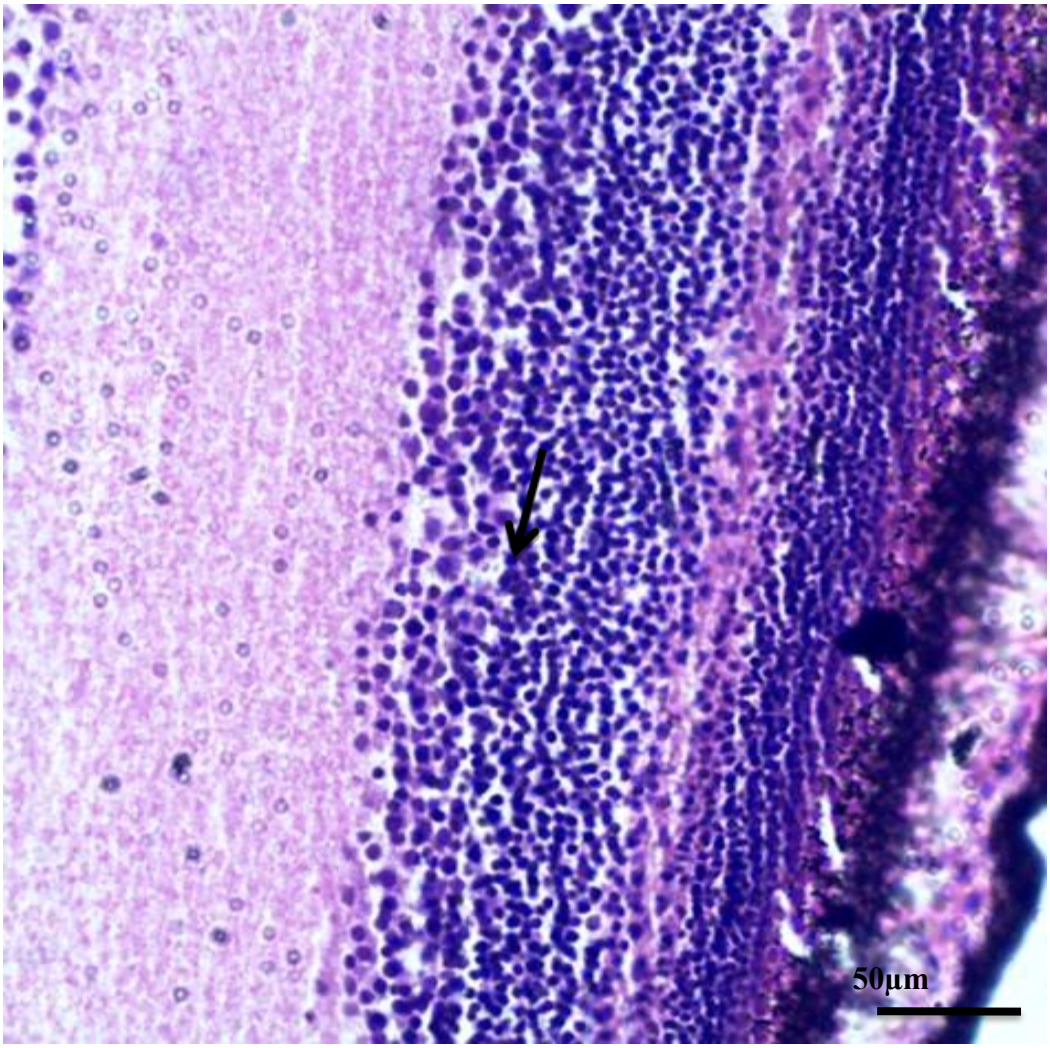

Figure 3-1: Normal structure of the *L. calcarifer* retina without vacuoles and hypertrophy. Most layers of the retina can be observed here. Note the normal inner nuclear layer (black arrow) H&E staining, 40X, as per method described in **Appendix 6-1.**

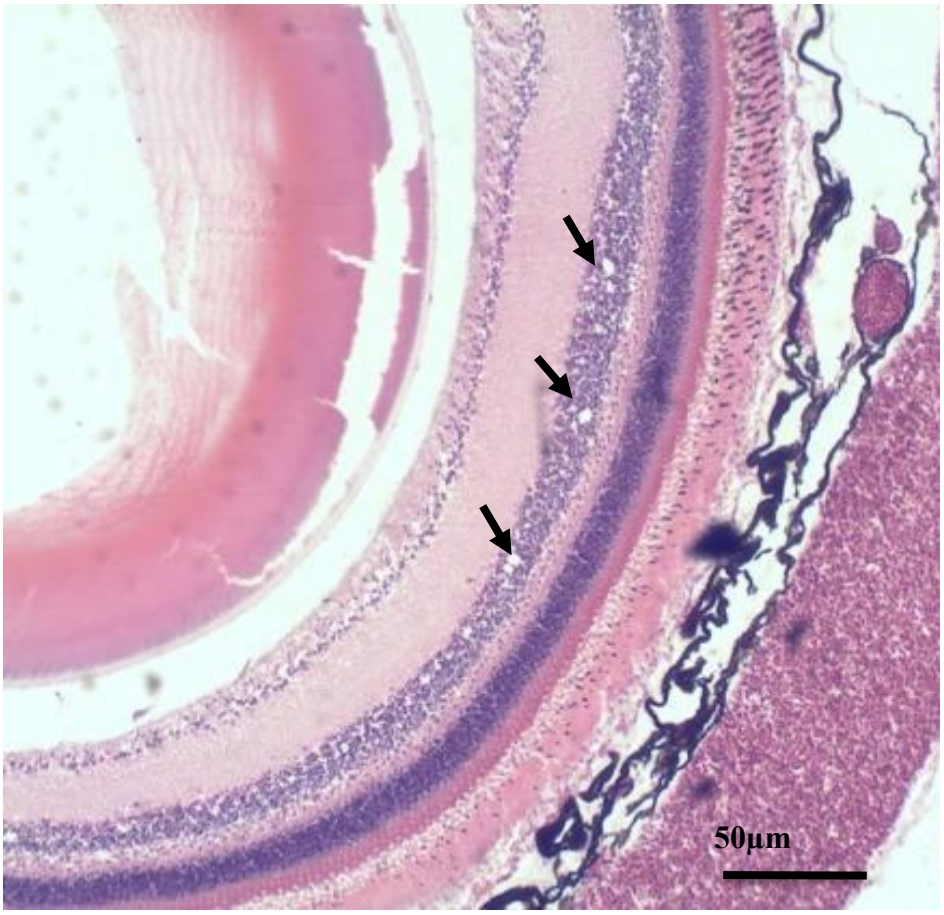

Figure 3-2: Abnormal layers observed in a *L. calcarifer* retina. Vacuolation occurs (black arrows) in the inner nuclear layer, H&E, 10X, as per method described in **Appendix 6-1.**

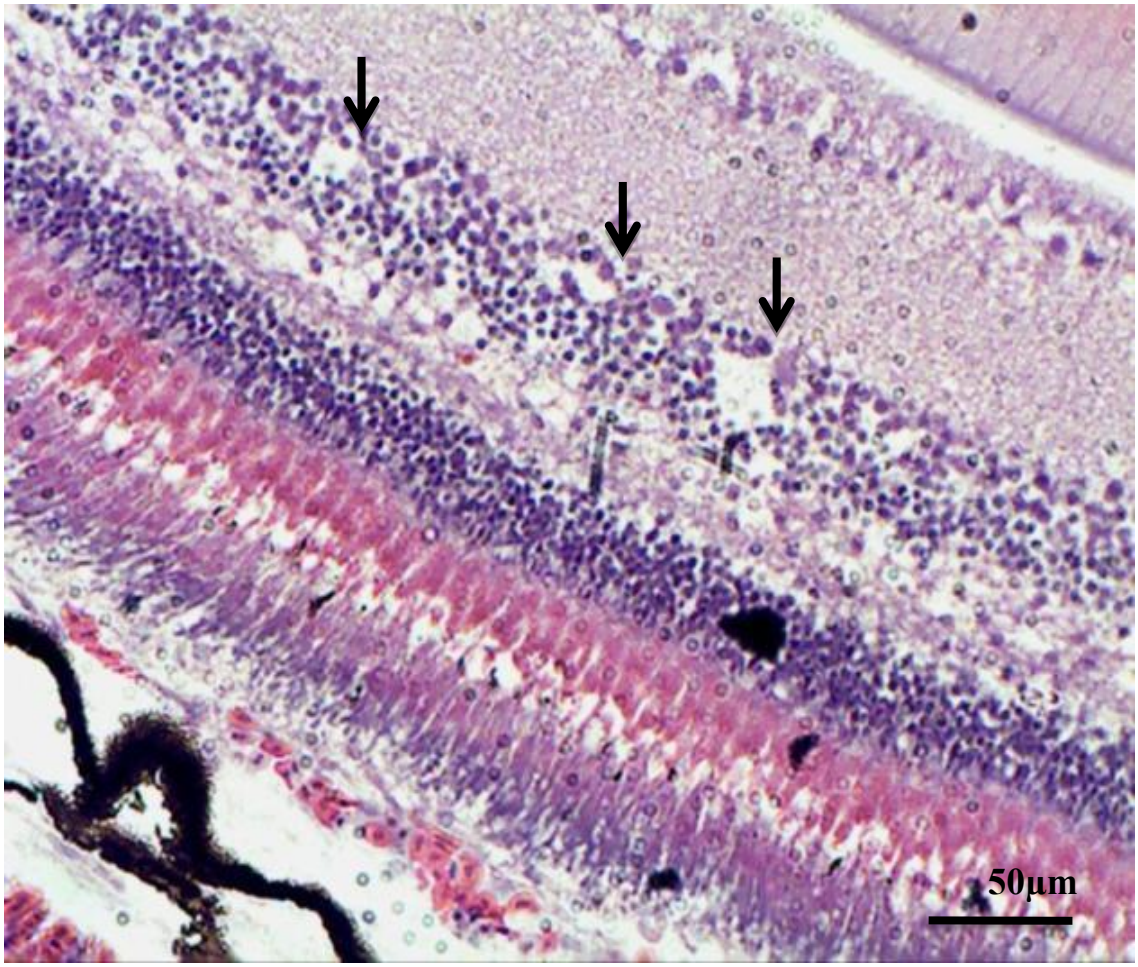

Figure 3-3: Vacuoles observed within the retina inner nuclear layer (arrows) of *L. calcarifer* presenting with VNN disease symptoms. The fish showed signs of abnormalities such as lethargy, darkened pigment, belly-up swimming and cloudy eyes, H&E, 40X, as per method described in **Appendix 6-1.**

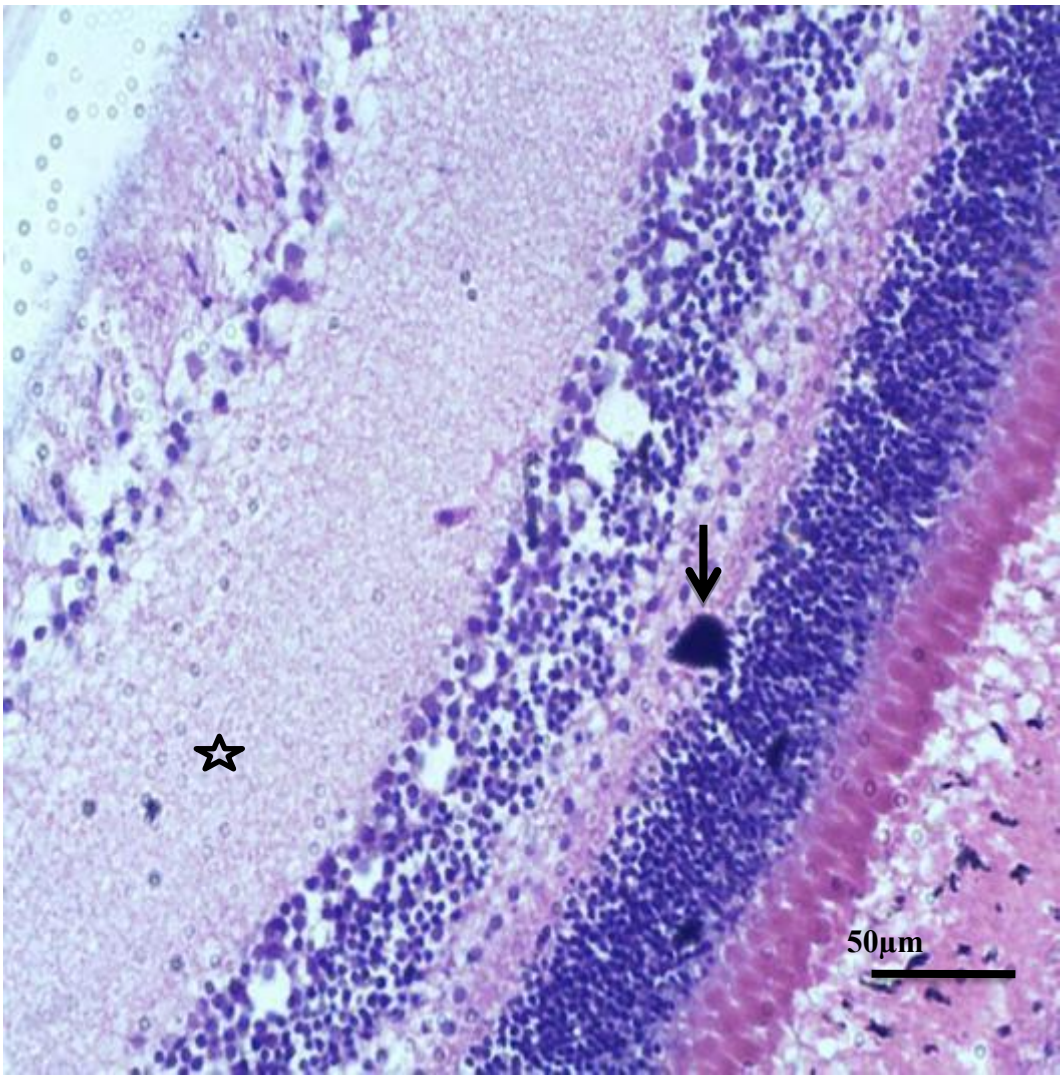

Figure 3-4: Abnormal pathology observed in *L. calcarifer* retina. Vacuolation was observed in the inner nuclear layer (black arrow) and swelling in the flexiform layer (*), H&E, 40X, as per method described in **Appendix 6-1**.

3.2.2. Brain

The normal structure of the optic lobe in *L. calcarifer* is shown in **Figure 3-5**. In this study, hypertrophic brain cells and granulomatous inflammation (**Figure 3-6**) were found within brain structures, particularly in the optic tectum. These changes accounted for the majority of pathological degeneration observed in the brain. Granulomatous inflammation characterized by a loose aggregate of granules in conjunction with fiberized epithelium, haemorrhage and vascular inflammation were found within the nervous tissue. Vacuoles also were observed in the cerebellum of the brain which is illustrated by **Figure 3-7**. Swollen capillaries or inflammation and vacuoles occurred but with lower frequency (**Figure 3-8**). The level of abnormalities that are observed in the brain were of mild histological grade and only occurred in 37 out of 270 fish examined (**Table 3-1**). Overall, the brain was the third least organ where changes were observed after the spinal cord (4%) and the spleen (13%).

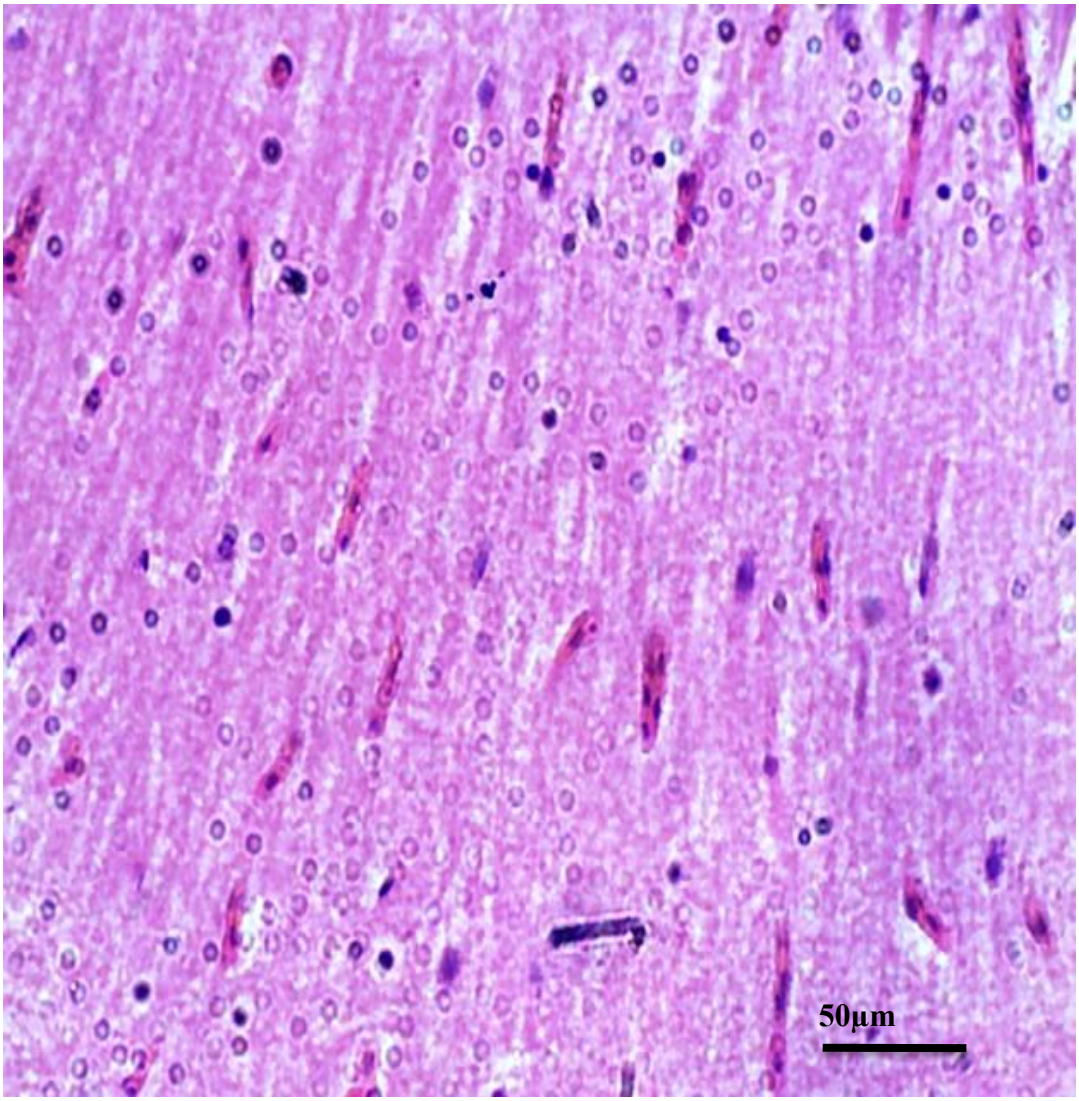

Figure 3-5: Normal tissue from the optic lobe of *L. calcarifer* brain. Sections were stained with H&E, 40X, as per method described in **Appendix 6-1**.

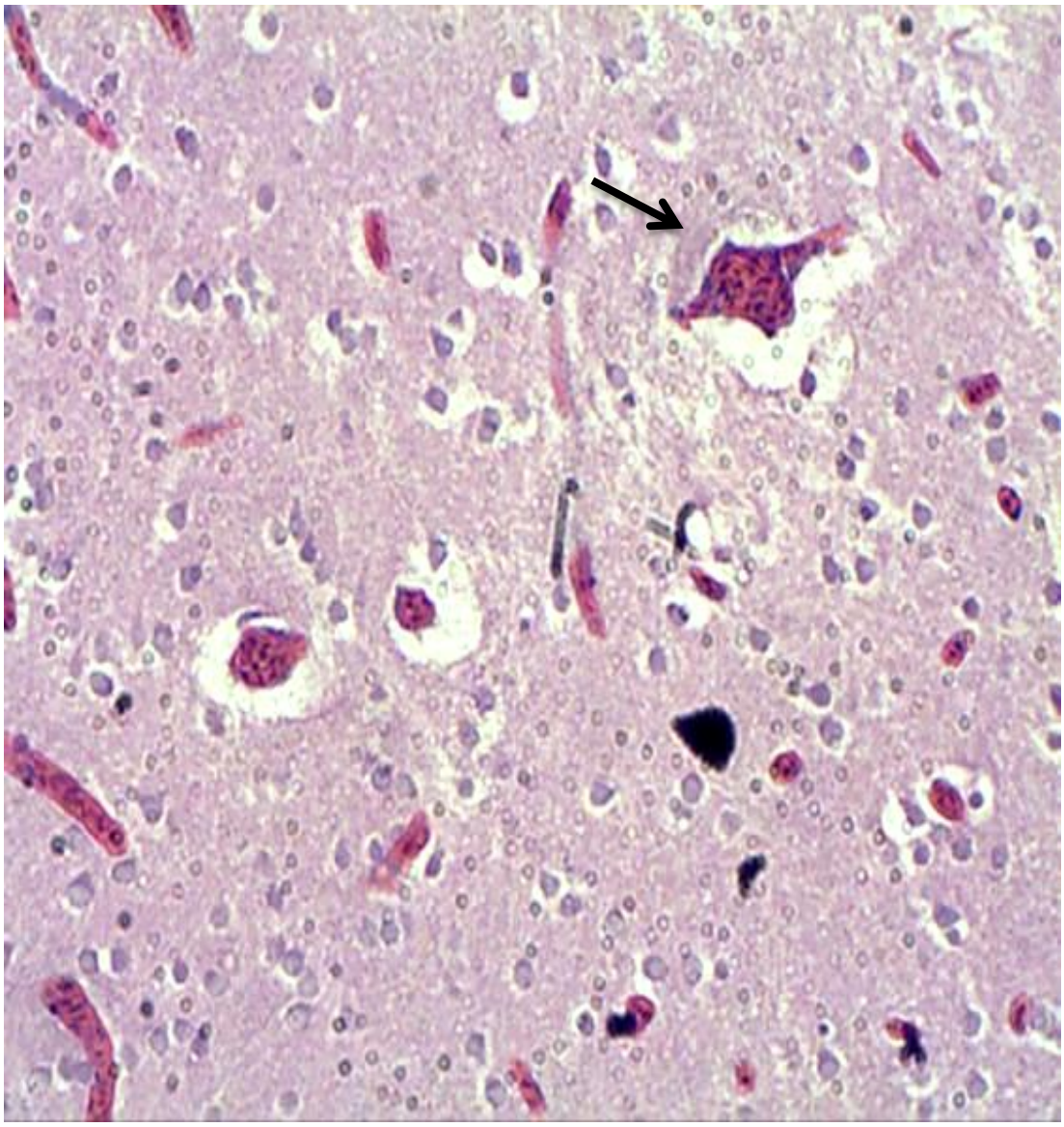

Figure 3-6: Abnormal changes in the optic lobe of *L. calcarifer* brain. Neuronal shrinkage and vascular congestion in the optic lobe (black arrow). Sections were stained with H&E, 40X, as per method described in **Appendix 6-1**.

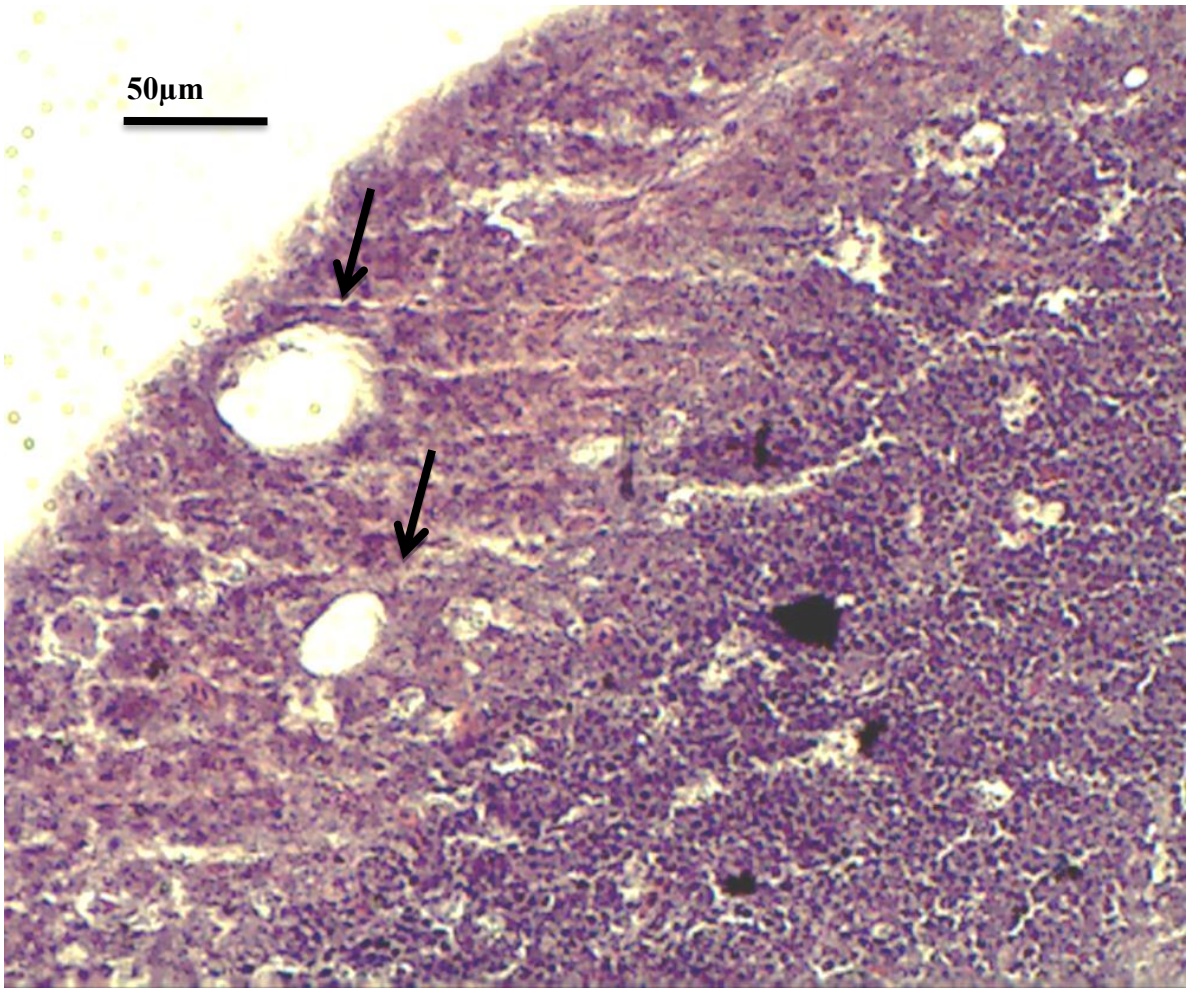

Figure 3-7: Abnormal changes observed in the cerebellum of *L. calcarifer* brain. Vacuoles that occurred are indicated by the arrows. Sections were stained with H&E, 40 X, as per method described in **Appendix 6-1**.

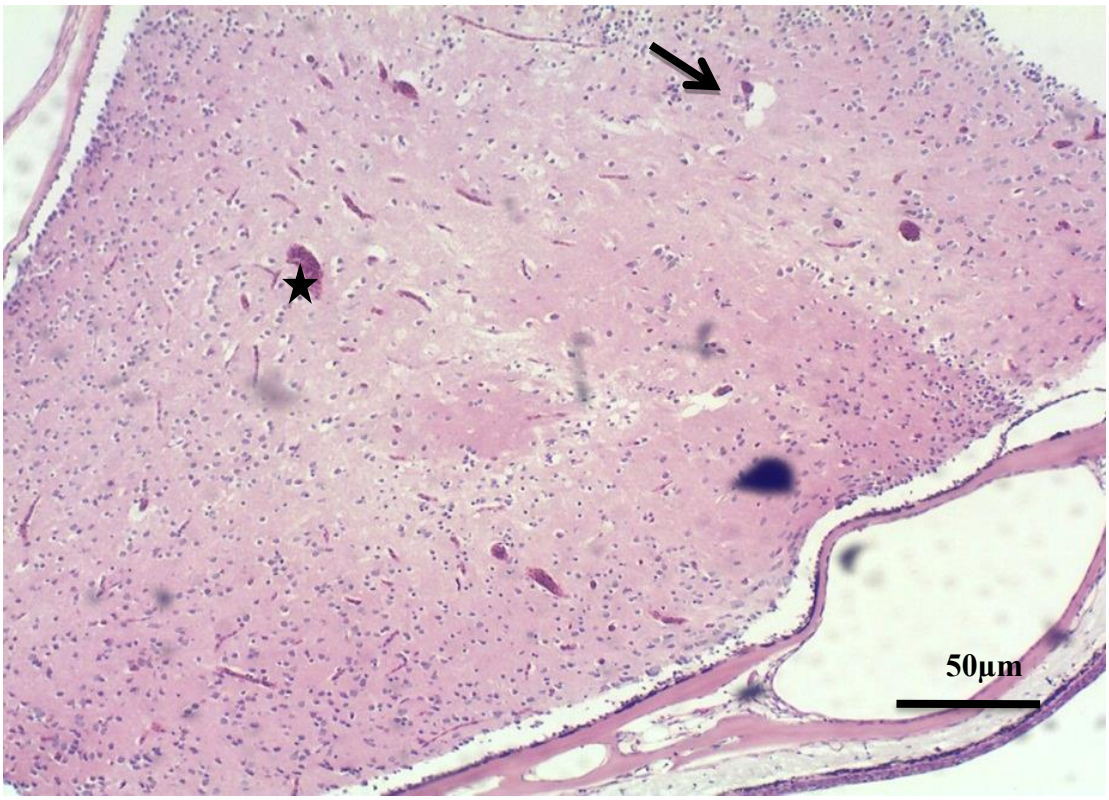

Figure 3-8: Abnormal changes observed in the diencephalon of the *L. calcarifer* brain. Swollen capillaries (*) and vacuolation (black arrow) in the diencephalon. Sections were stained with H&E, 40X, as per method described in **Appendix 6-1**.

3.2.3. Spinal cord

Eleven fish (4%) out of the 270 samples examined exhibited mild abnormalities within the spinal cord. This was the lowest prevalence of histological changes observed amongst the organs. Histologically, abnormality was chiefly observed via the occurrence of vacuoles (data not shown). There was no evidence of haemorrhage or melanin aggregates in the spinal cord of examined fish.

3.2.4. Gill

The gill was the only organ where marked or severe histological change were observed (level 3). These histological changes were observed in the gill of 132 out of 270 fish samples (49%). Typical changes observed in gill tissue were hyperplasia (**Figure 3-9**), melanin aggregation (**Figure 3-10**), vacuolation (**Figure 3-11**), necrosis and haemorrhage. These changes occurred in association with parasite infections described later in this thesis.

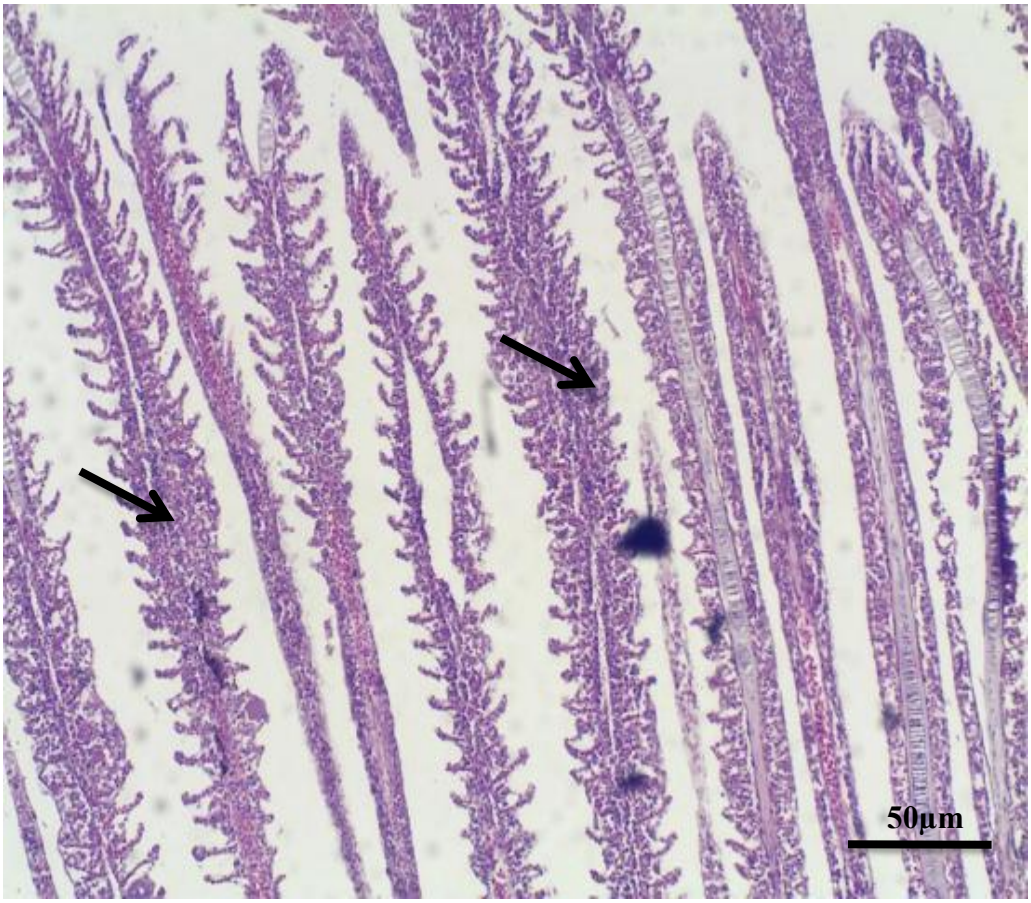

Figure 3-9: Abnormal histological changes observed in *L. calcarifer* gill. Hyperplasia (arrows) in gill. Sections were stained with H&E, 40X, as per method described in **Appendix 6-1**.

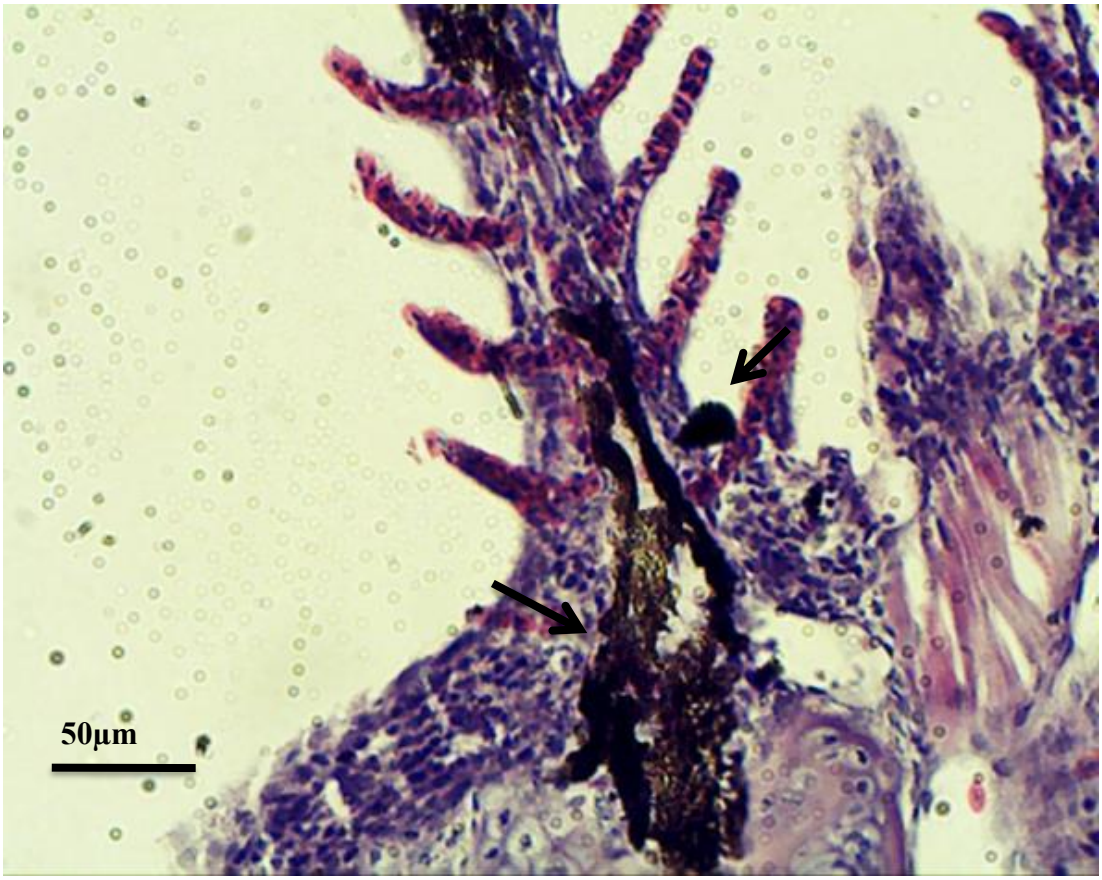

Figure 3-10: Abnormal histological changes observed in the *L. calcarifer* gill. Melanin aggregation (black arrows) in gill. Sections were stained with H&E, 40X, as per method described in **Appendix 6-1**.

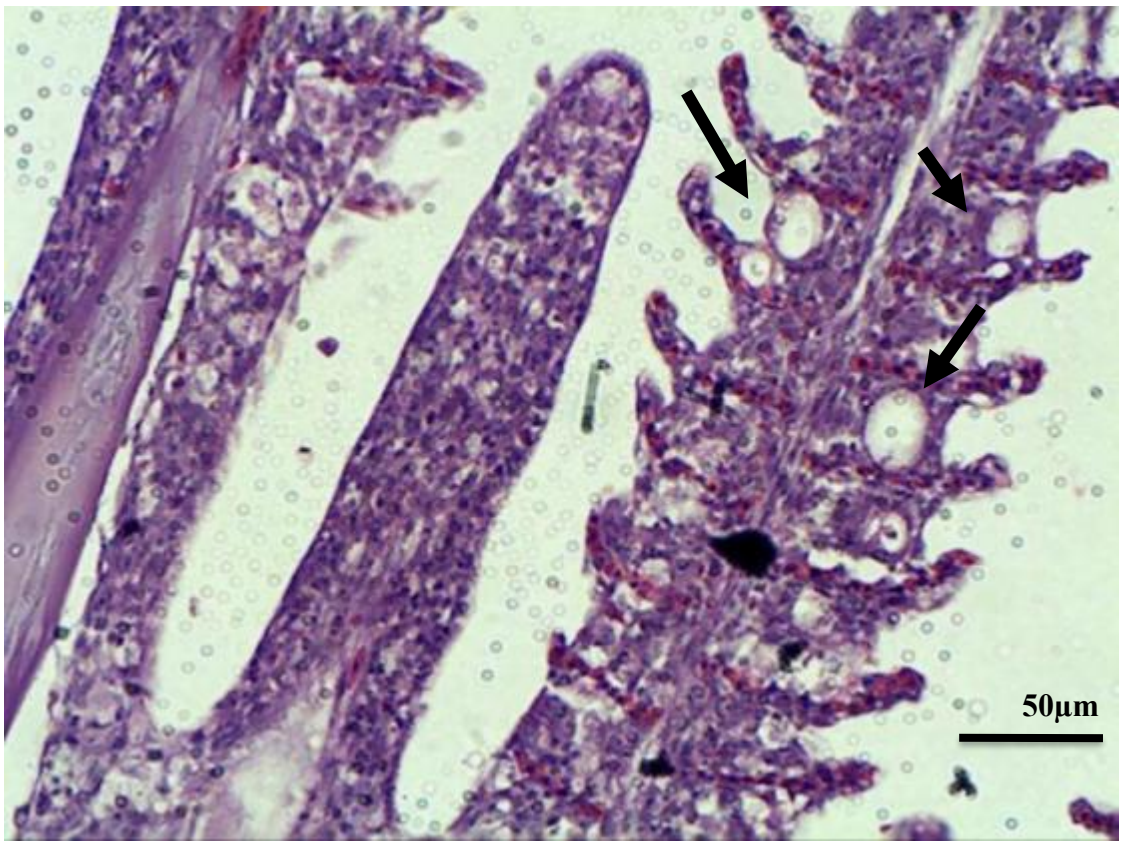

Figure 3-11: Abnormal histological changes observed in the *L. calcarifer* gill. Vacuoles (black arrows) vary in size within the gill. Sections were stained with H&E, 40X, as per method described in **Appendix 6-1**.

3.2.5. Liver

There were 43 fish observed with changes within the liver tissues, accounting for 16% of overall histological changes. The primary abnormality observed in liver tissue was necrosis of the hepatic veins and epithelium (**Figure 3-12**). Necrotic cells showed decreased nuclear size. The concentration of chromatin and eosinophilic tissue is typical for liver necrosis. Necrosis was found mainly as focal necrosis (**Figure 3-13).** Lipid droplets were also observed in some livers (**Figure 3-14)**. These types of changes occurred in conjunction with necrosis or vacuolar degeneration. Additionally, congestion occurred in the epithelium, veins, arteries and bile ducts of the liver.

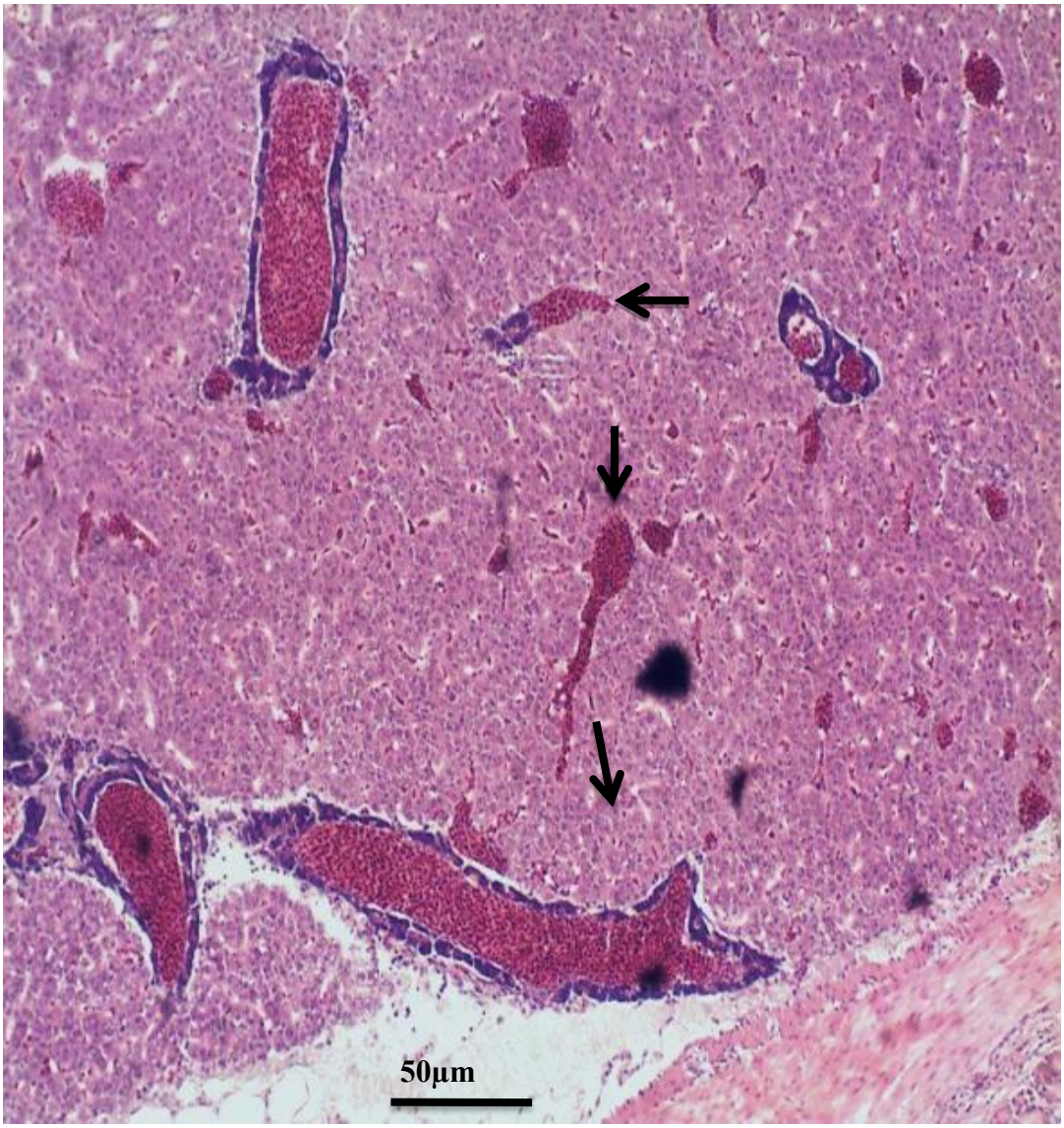

Figure 3-12: Abnormal histological changes observed in *L. calcarifer* liver. Haemorrhaging (arrows) within the liver was observed by the appearance of erythrocyte staining outside of the major vessels**.** Sections were stained with H&E, 40X, as per method described in **Appendix 6-1**.

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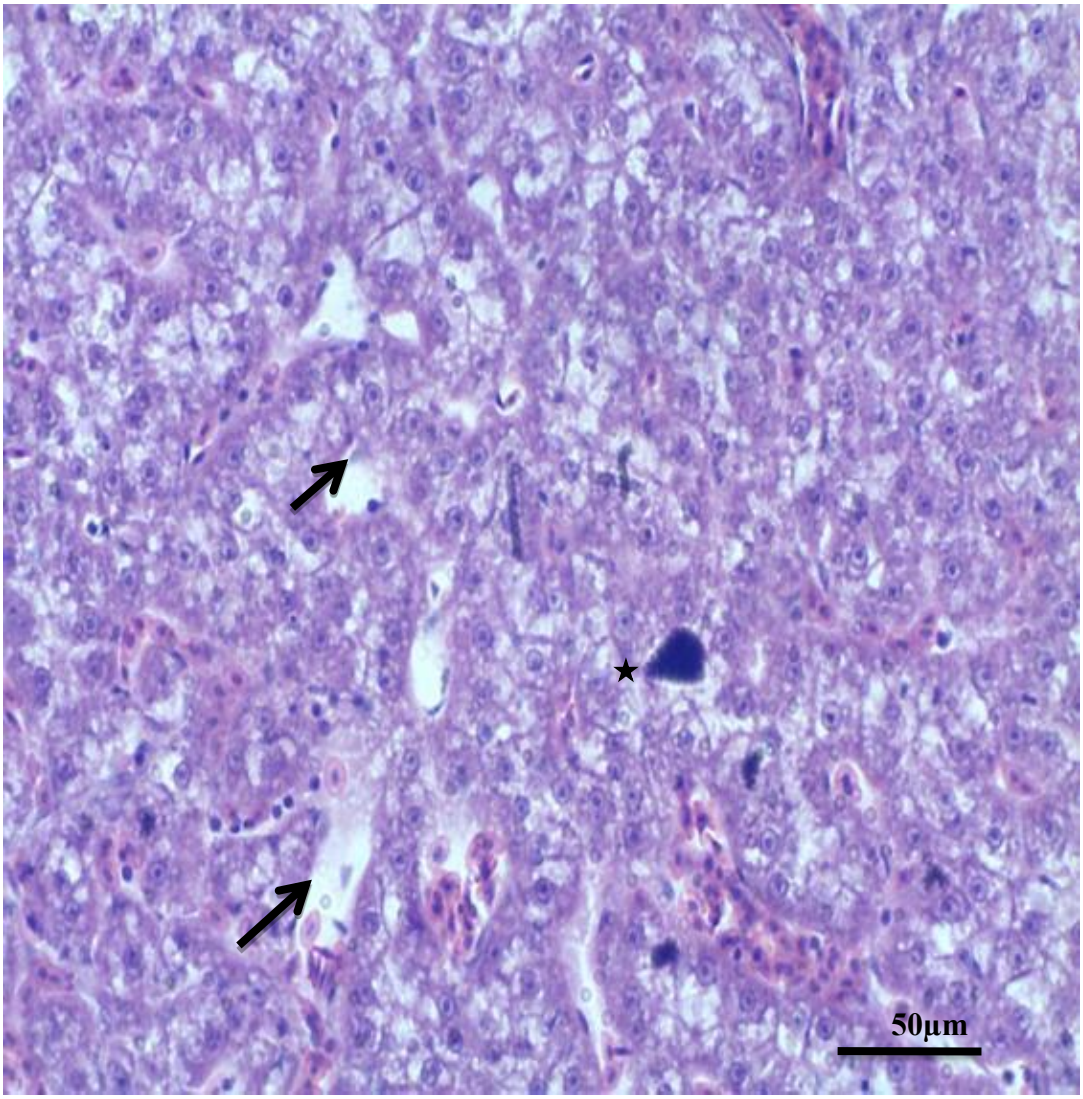

Figure 3-13: Abnormal histological changes observed in *L. calcarifer* liver. Hyaline droplet (arrows) and focal necrosis (star) in liver epithelium. Sections were stained with H&E, 40X, as per method described in **Appendix 6-1**.

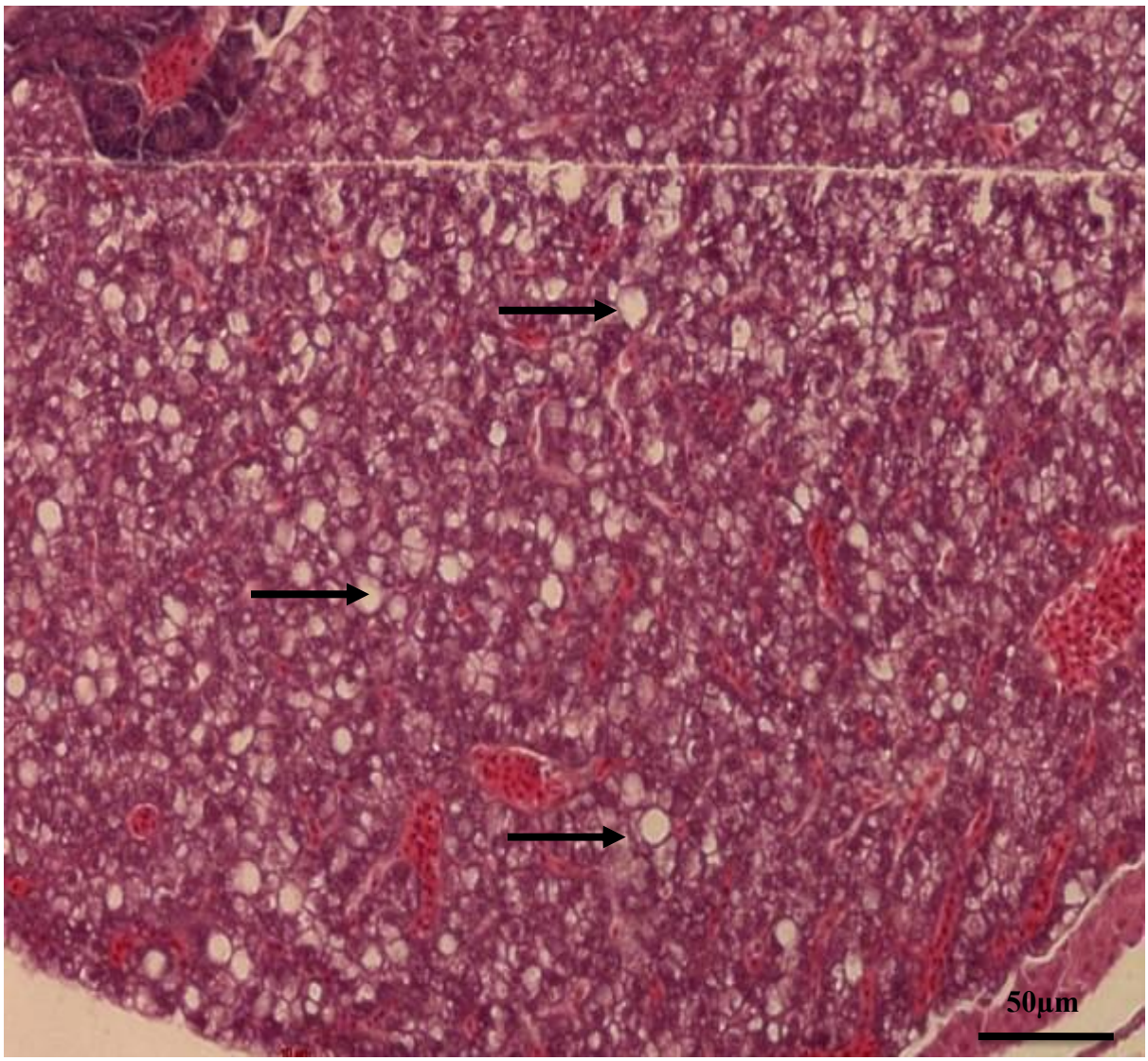

Figure 3-14: Fat deposits (arrows) observed in *L. calcarifer* liver. Sections were stained with H&E, 40X, as per method described in **Appendix 6-1**.

3.2.6. Kidney

There were 86 fish that had histological changes within their kidney tissues, accounting for 32% of all changes observed. This makes the kidney the second most changed organ histologically. Haemorrhage and melanomacrophage centres were the most common histological changes observed in the anterior kidney (**Figure 3-15**). Vacuolation (**Figure 3-15)** was also found in severely damaged renal tissue in both anterior and posterior kidney. In terms of histological changes in posterior kidney, increases of spaces in the corpuscles and in renal tubules were typical. In addition, eosinophilic fragmentation was evident in necrotic renal tubules. Bowman's space was expanded (**Figure 3-16)** and the space between Bowman's capsule and glomerulus was congested. Moreover, the capillaries of the glomerulus were swollen. Hyaline droplet degeneration of renal tubules was not observed.

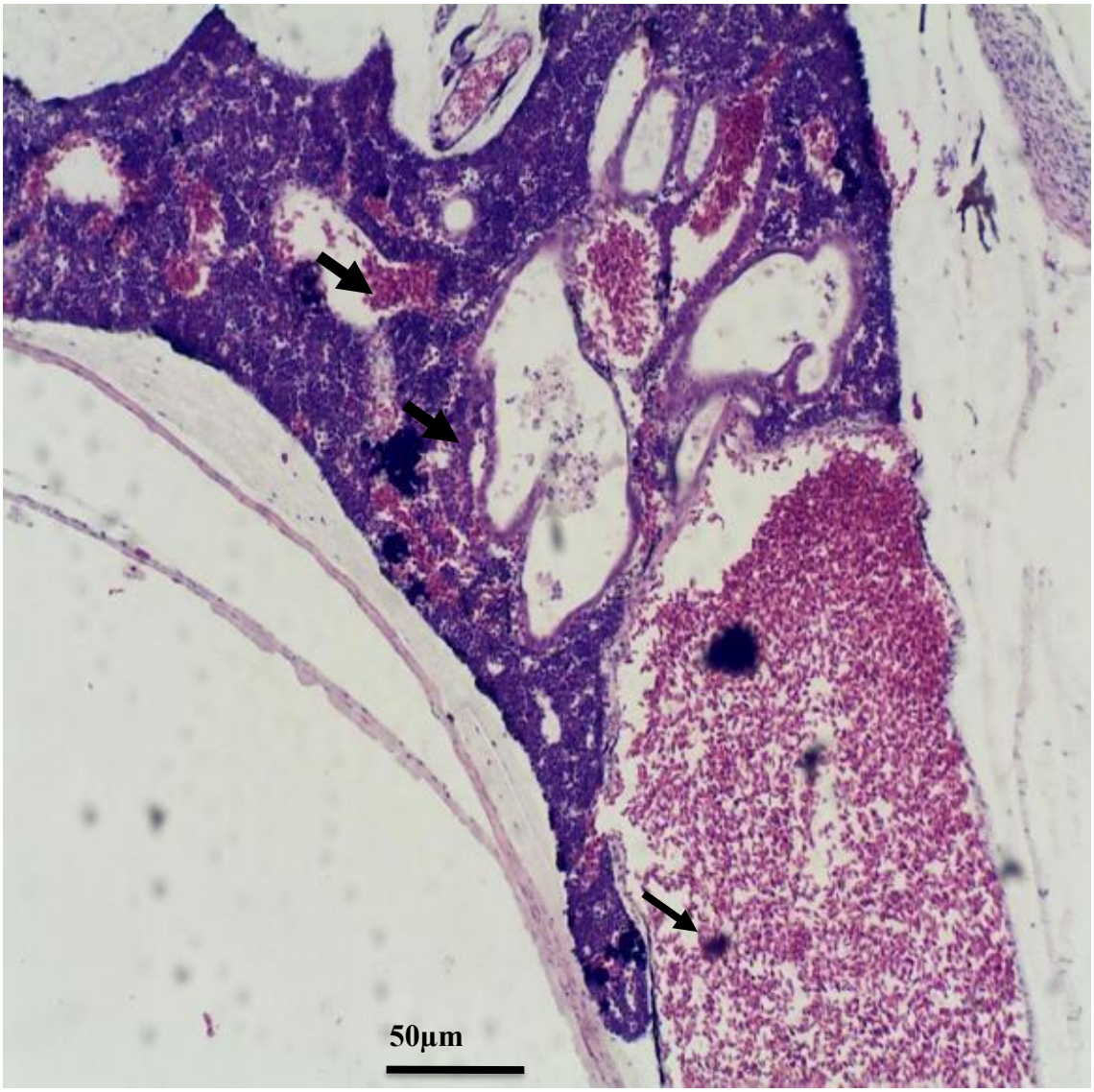

Figure 3-15: Severe and extensive necrosis and blood vessel congestion in *L. calcarifer* anterior kidney. Note the appearance of melanomacrophage cells (black arrows)**.** Sections were stained with H&E, 40X, as per method described in **Appendix 6-1**.

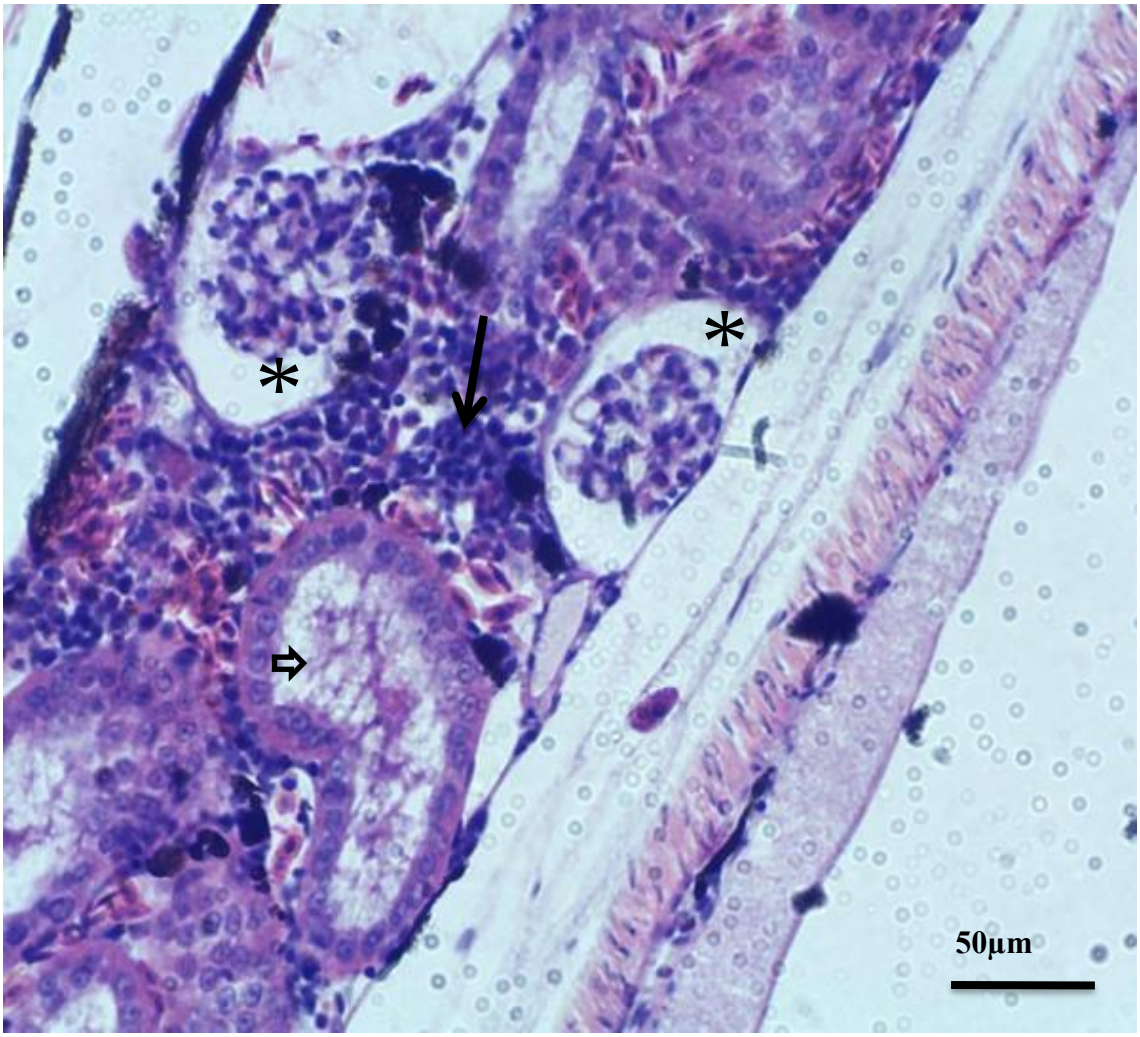

Figure 3-16: Abnormal pathology observed in *L. calcarifer* posterior kidney. Granules and melanin aggregate in the interstitium tissue (arrow) and the increase of Bowman's space (*). Note the eosinophilic fibre inside the tubule (arrowhead ⇨). Sections were stained with H&E, 40X, as per method described in **Appendix 6-1**.

3.2.7. Spleen

There were 35 out of the 270 fish sampled (13%) that had marked histological changes recorded within their spleen tissues. Inflammation and vacuolation (**Figure 3-17**) were observed in spleen tissue with low prevalence. The aggregation of melanomacrophage centres in the spleen was also observed.

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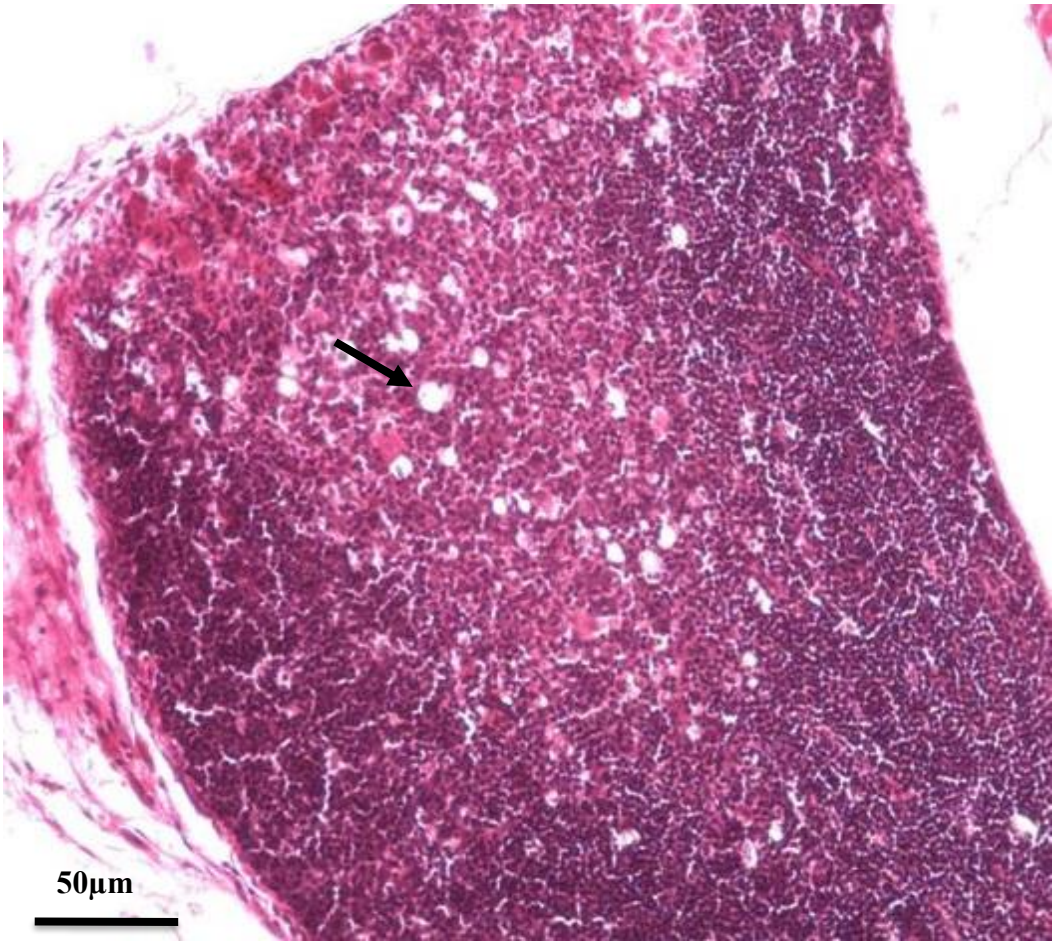

Figure 3-17: Abnormal histological changes observed in *L. calcarifer* spleen tissue. Vacuolation (arrow) occurred mainly at the white pulp area of the spleen. Sections were stained with H&E, 40X, as per method described in **Appendix 6-1**.

3.3. Using IHC to confirm VNN infection

There were 18 samples that showed abnormal changes in the histological sections stained with H&E that were consistent with what was expected during a Betanodavirus infection based on descriptions of Betanodavirus pathogenesis in the literature. To be more specific, vacuoles were observed in the brain, retina and the spinal cord of 18 of 270 fish. In addition, severe histological changes were recorded extensively in liver, kidney and gills such as haemorrhage, melanin aggregation and hyperplasia of the same fish. IHC was then performed on all 18 of these samples using an antibody that detects the Betanodavirus coat protein. Using this approach Betanodavirus immunopositivity was not observed in optic lobe or retinal samples from any of these 18 fish (**Figures 3-18 & 3-19**).

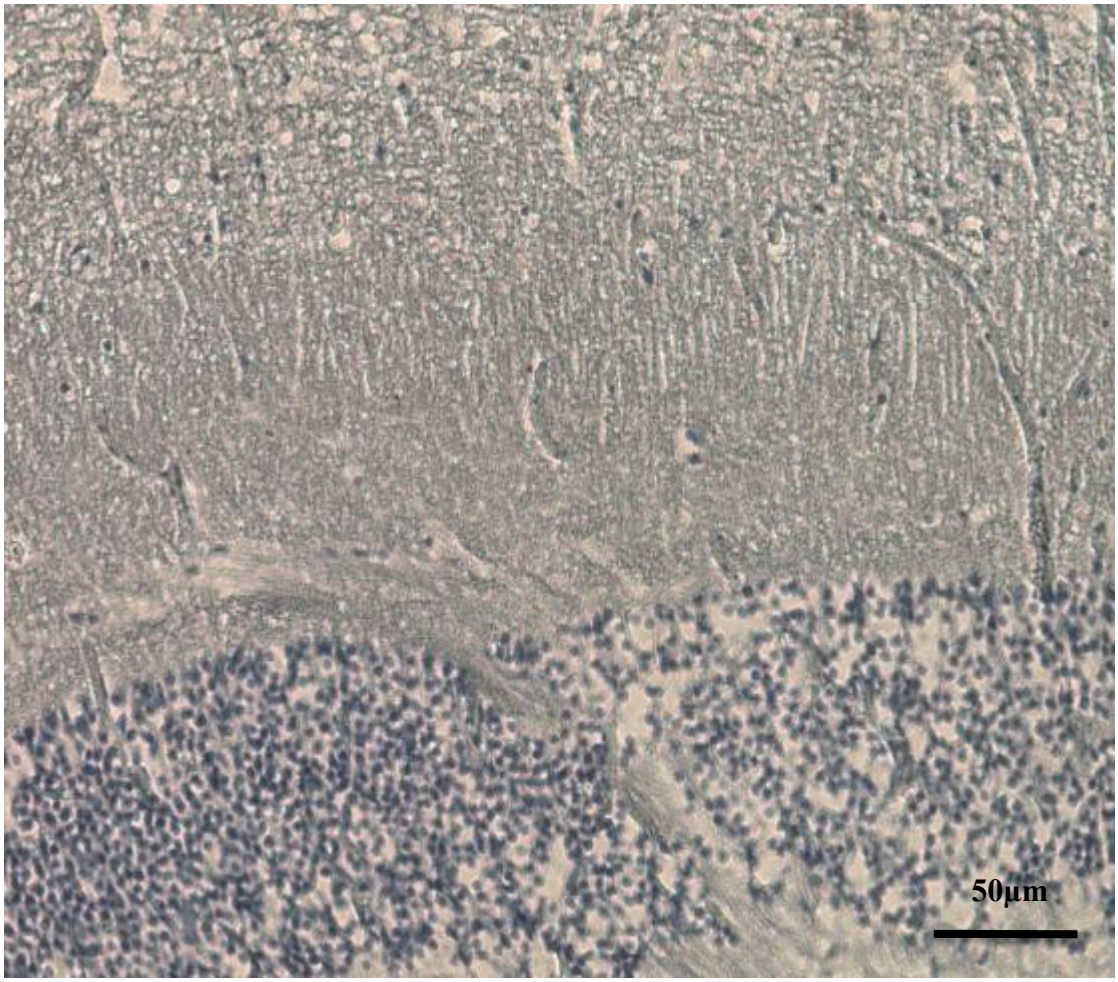

Figure 3-18: Optic lobe of *L. calcarifer* brain showing that Betanodavirus was not detected using IHC staining. 40X, sections were stained as per IHC method in **Appendix 6-2**.

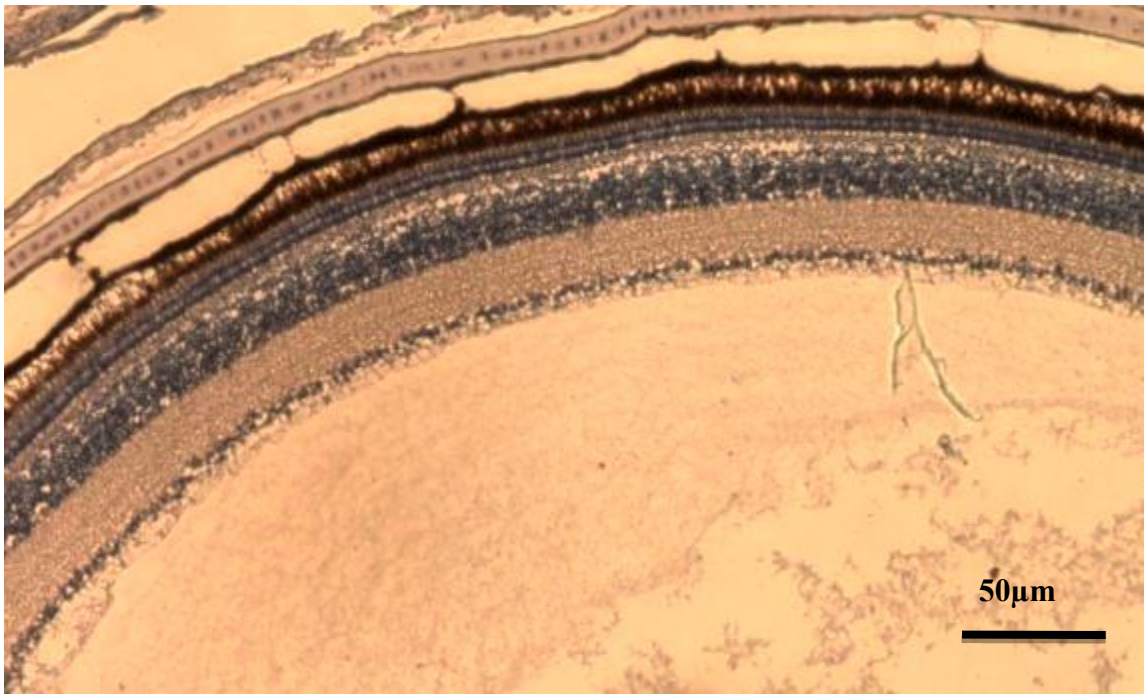

Figure 3-19: No positive immunoreactivity was observed within the *L. calcarifer* retina using IHC staining, 10X, sections were stained as per IHC method in **Appendix 6-2**.

3.4. Validation of the negative VNN-IHC results

Paraffin blocks containing specimens from four Betanodavirus positive and two negative fish were obtained from the CSIRO's Animal Health Laboratories, at Geelong, in Victoria and examined. The Betanodavirus status of these fish had been pre-determined in these laboratories using the IHC method described in the methods and used in section 3.3. These samples were from larval barramundi and were about five cm long and in the same age range as the specimens (3-5cm) collected in this thesis. The four positive blocks 1, 2, 3, 4: showed positive immunoreactivity broadly distributed within the central nervous system (all parts of the brain), different layers of the retina and along the spinal cord (**Figures 3-20, 3-21& 3-22**). The most severely infected area was the optic lobe. Positive staining representing Betanodavirus infection within cells (antibody recognises the Betanodavirus outer coat protein) appeared dark brown in colour. No positive staining

was observed in the gill, liver, kidney, spleen tissue of the control fish. The two negative blocks 1 and 2: showed no staining although some vacuole pathology was seen in the brain, retina and spinal cord.

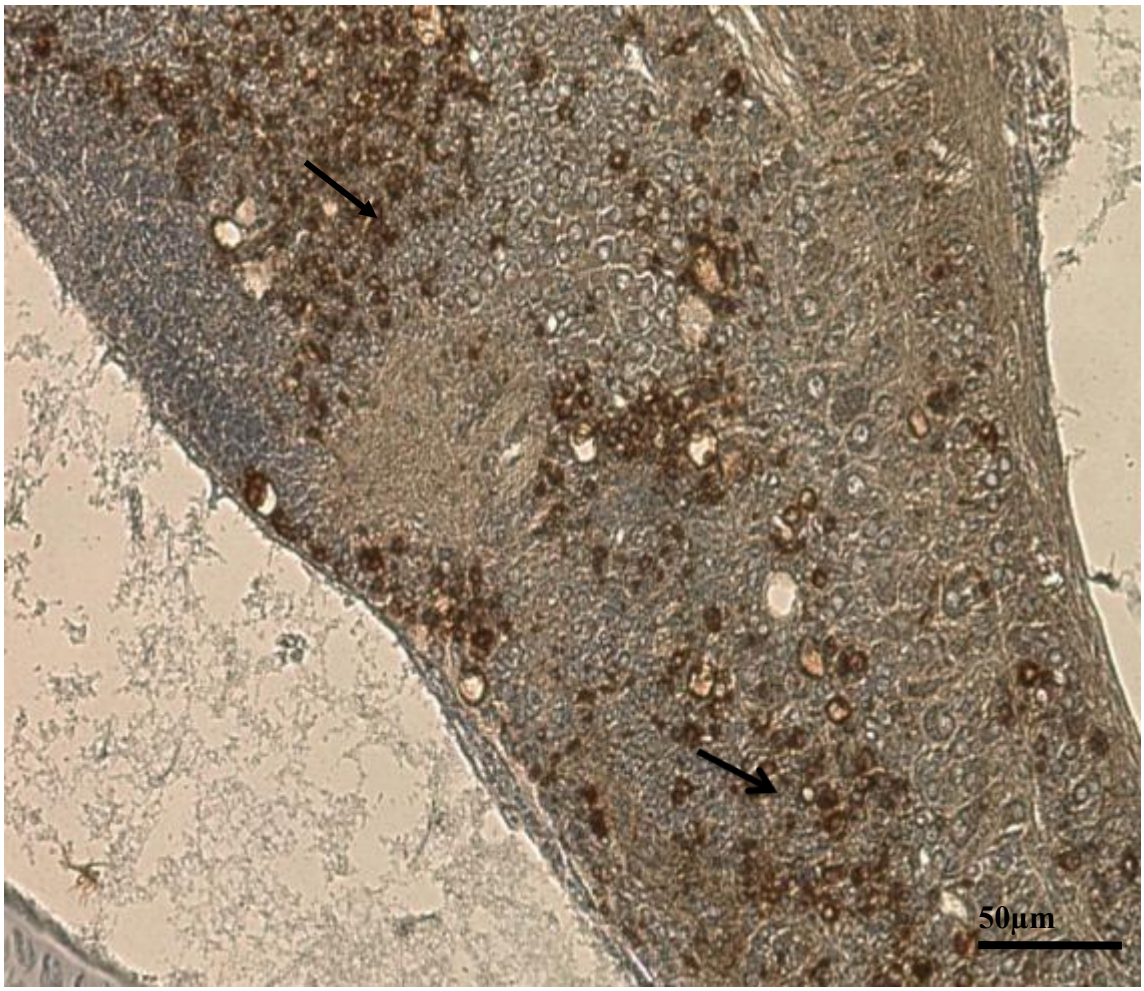

Figure 3-20: Detection of Betanodavirus positivity in a Betanodavirus positive control *L. calcarifer* brain sample stained with primary sheep anti-BNNV recombinant coat protein. Infected cells (arrows) were distributed extensively thorough the diencephalon. 40X, sections were stained as per IHC method in **Appendix 6-2**.

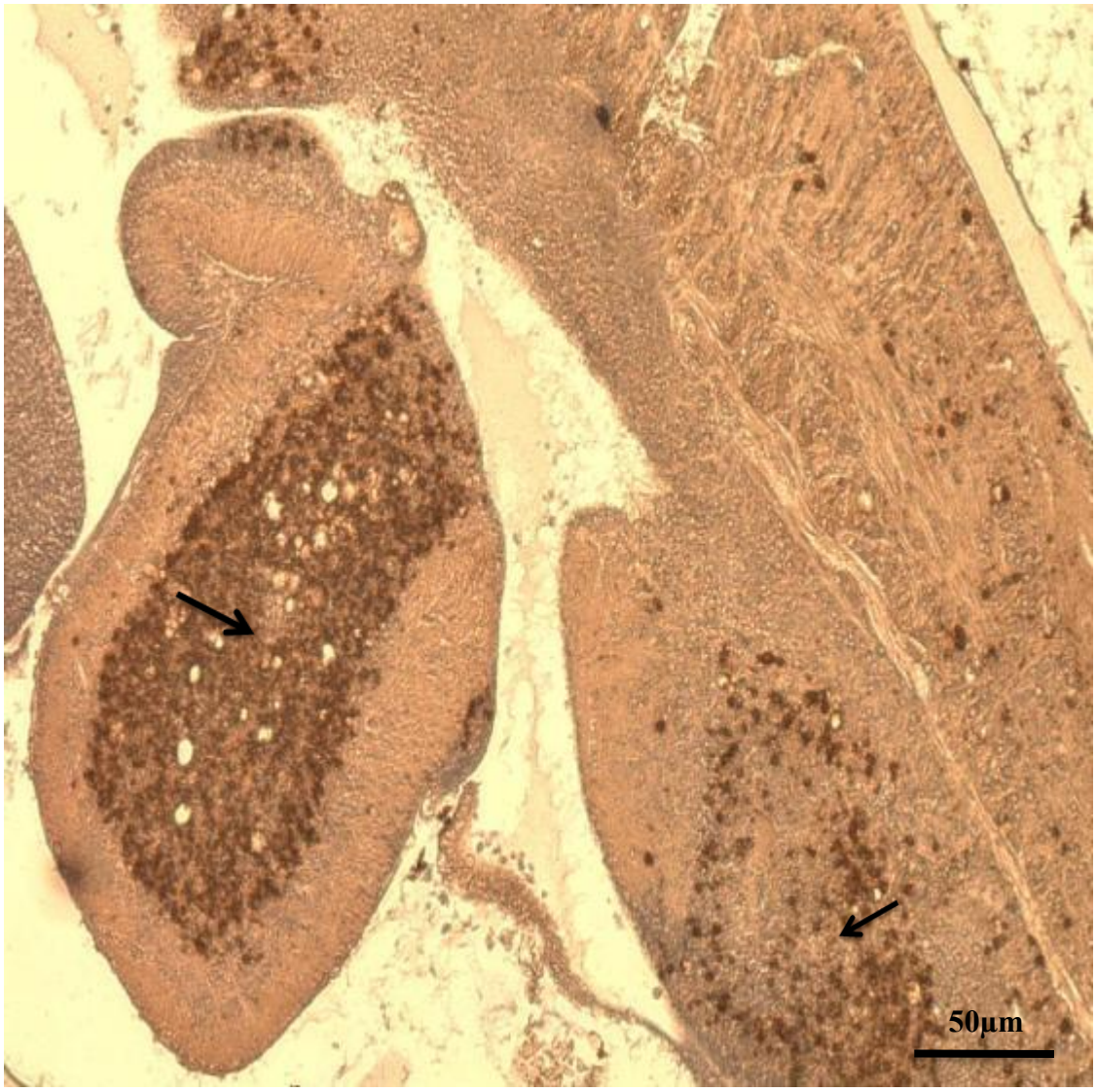

Figure 3-21: Detection of Betanodavirus immunopositivity in Betanodavirus positive control *L. calcarifer* cerebellum and optic lobe stained with primary sheep anti-BNNV recombinant coat protein. Betanodavirus viral coat protein (arrows) was detected in foci of vacuolation, 10X, sections were stained as per IHC method in **Appendix 6-2**.

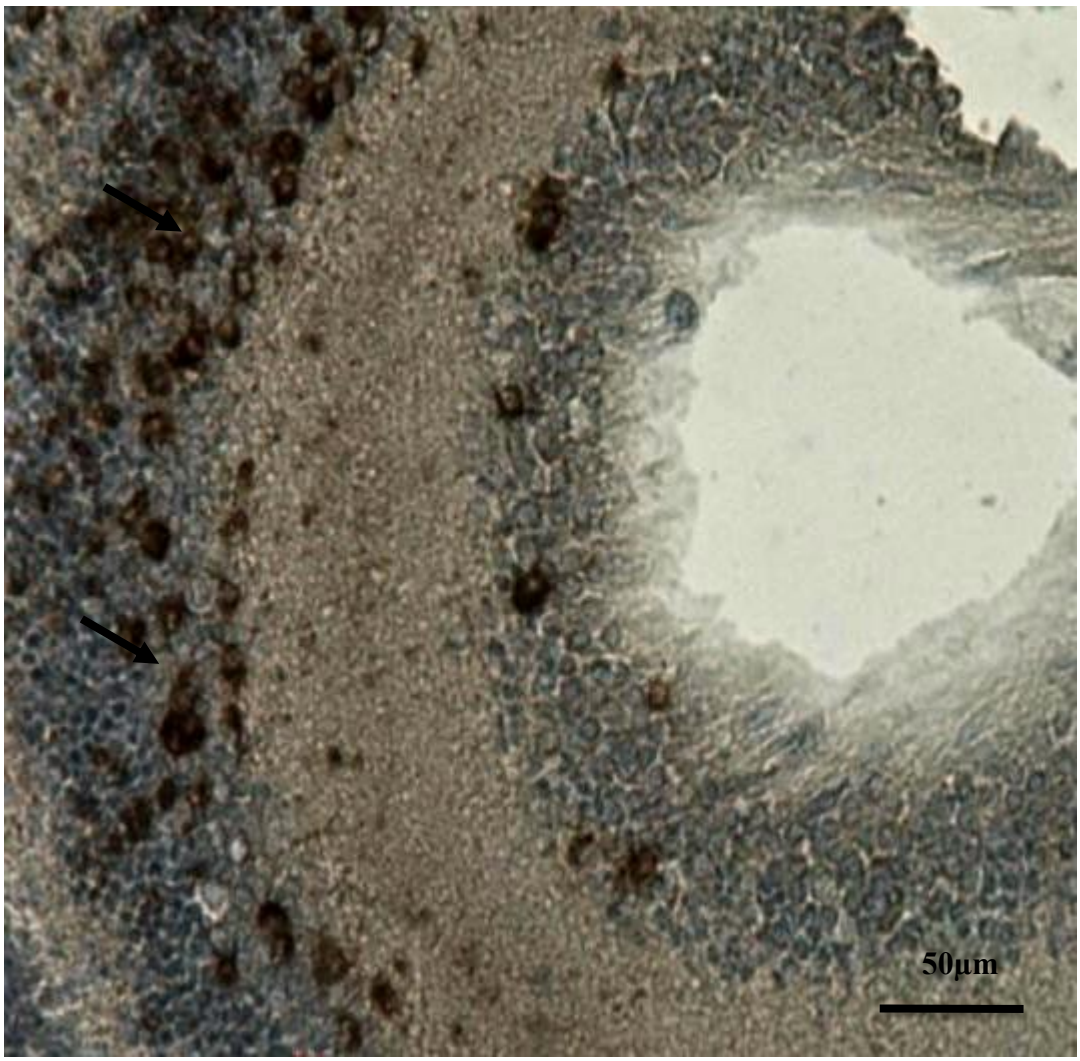

Figure 3-22: Detection of Betanodavirus immunopositivity in retina from a positive control *L. calcarifer* sample. Betanodavirus viral coat protein was detected in the inner nuclear layer of the retina (arrows), 40 X, sections were stained as per IHC method in **Appendix 6-2**.

H&E staining was also performed on the negative and positive samples received from AAHL, Victoria in order to examine the effect of virus infection on general fish histopathology. Intriguingly vacuoles were found within the brain, spinal cord and retina of both positive and negative fish samples but at varied levels of severity. Tissues from fish samples positive for Betanodavirus showed a more severe vacuolation (**Figure 3-23**),

characterised with clusters of vacuoles in bigger size as well as rounder compared to negative tissue, with occasional giant cell granulomas (**Figure 3-24**). Other organs showed melanomacrophage aggregation, necrosis and haemorrhage appeared in kidney and liver while melanin aggregation and hyperplasia were evident in the gill.

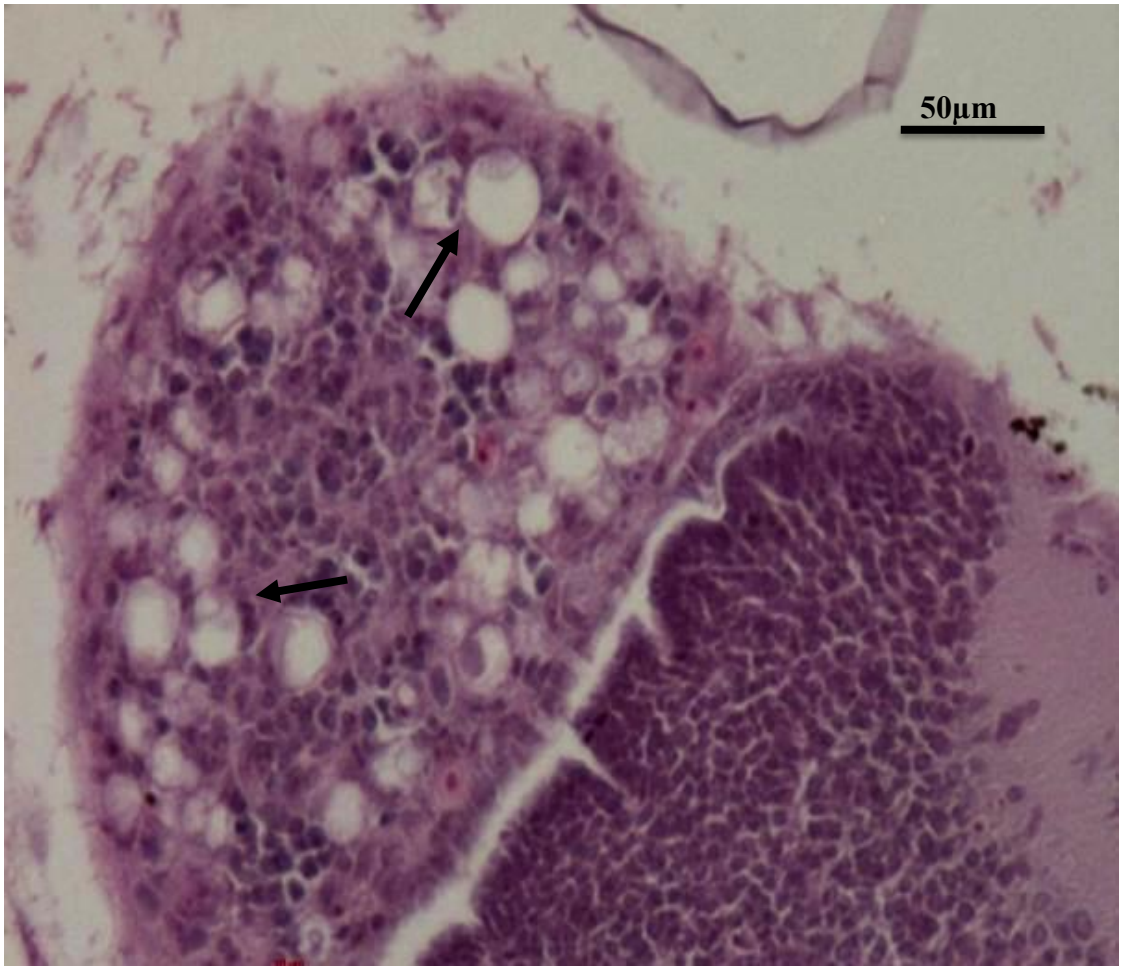

Figure 3-23: Cluster of vacuoles (arrows) within the neuropil of the cerebellum in a brain from an IHC Betanodavirus positive *L. calcarifer*. Sections were stained with H&E, 40X, as per method described in **Appendix 6-1**.

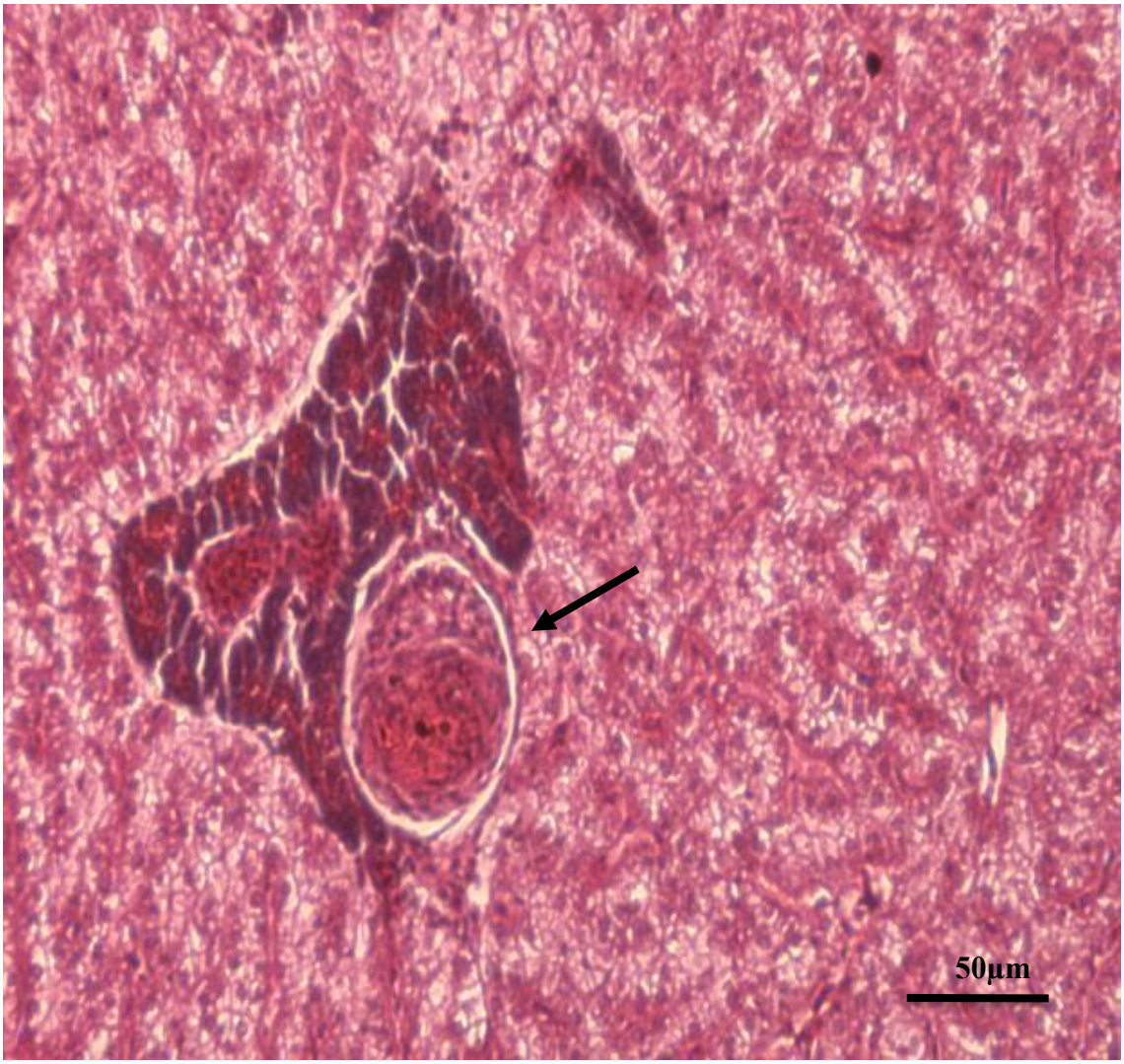

Figure 3-24: Abnormal histological changes in liver from an IHC Betanodavirus positive *L. calcarifer*. Granuloma (arrow) found within liver tissue. Sections were stained with H&E, 40X, as per method described in **Appendix 6-1**.

3.5. Other pathogenic organisms

While samples were being examined histologically for signs of Betanodavirus symptoms it became apparent that the while the fish where not infected with the virus there were numerous signs of infections via other pathogenic organisms. All together 154 samples out of 270 (57%) samples exhibited histological signs of infections which included epitheliocystis, monogeneans and some unknown parasites (**Table 3-2**). Epitheliocystis occurrence made up most infected samples, totalling 118 samples (77%) and the remaining 36 samples (23%) exhibited fluke infection. The unknown parasite-like agents are described later in this section but without quantitative data as the frequency of those infections were low and their identities remained unknown. In terms of periodic collection, it was coincident that epitheliocystis appeared every second time from a total nine fish collections spaced approximately month apart.

3.5.1. Epitheliocystis

A variety of cyst shapes and sizes were observed depending on which developmental cycle the cysts were at. Young branchial cysts were small (10 -15µm) and contained a central inclusion surrounded by a thickened host epithelium. Mature cells (60 - 400µm) were surrounded by layers of normal epithelium with remnants of the host cell. In total there were 118 epitheliocystis infected fish samples (**Figure 3-25**). Regarding severity of parasite infection, level 1 was observed in 47 % of samples, following by 36 % for level 2 and 17% for level 3 in 118 total epitheliocystis infected samples (**Table 3-2**).

The locations of infected cells within the gill was varied and included the gill arch, the tip of the gill lamella (**Figure 3-26**), within the gill filament (**Figure 3-27**), tip of the gill filament (**Figure 3-28**), within the gill lamellae and between gill lamellae (**Figure 3-29**).

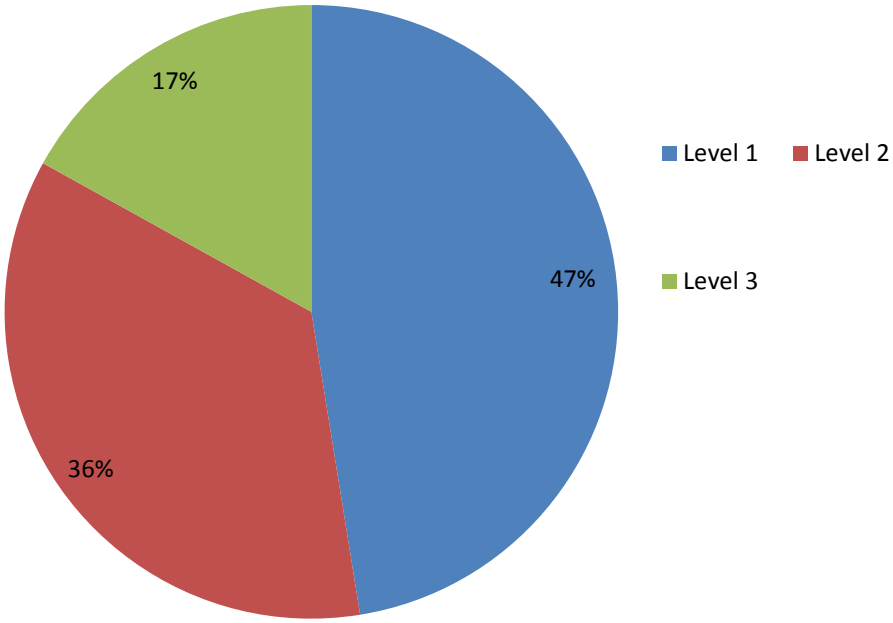

Figure 3-25: Pie chart shows the percentage of epitheliocystis infection within the gills at various levels of severity. Level 1: < 30%, level 2: from 30%-60%; level 3: >60%. N=118

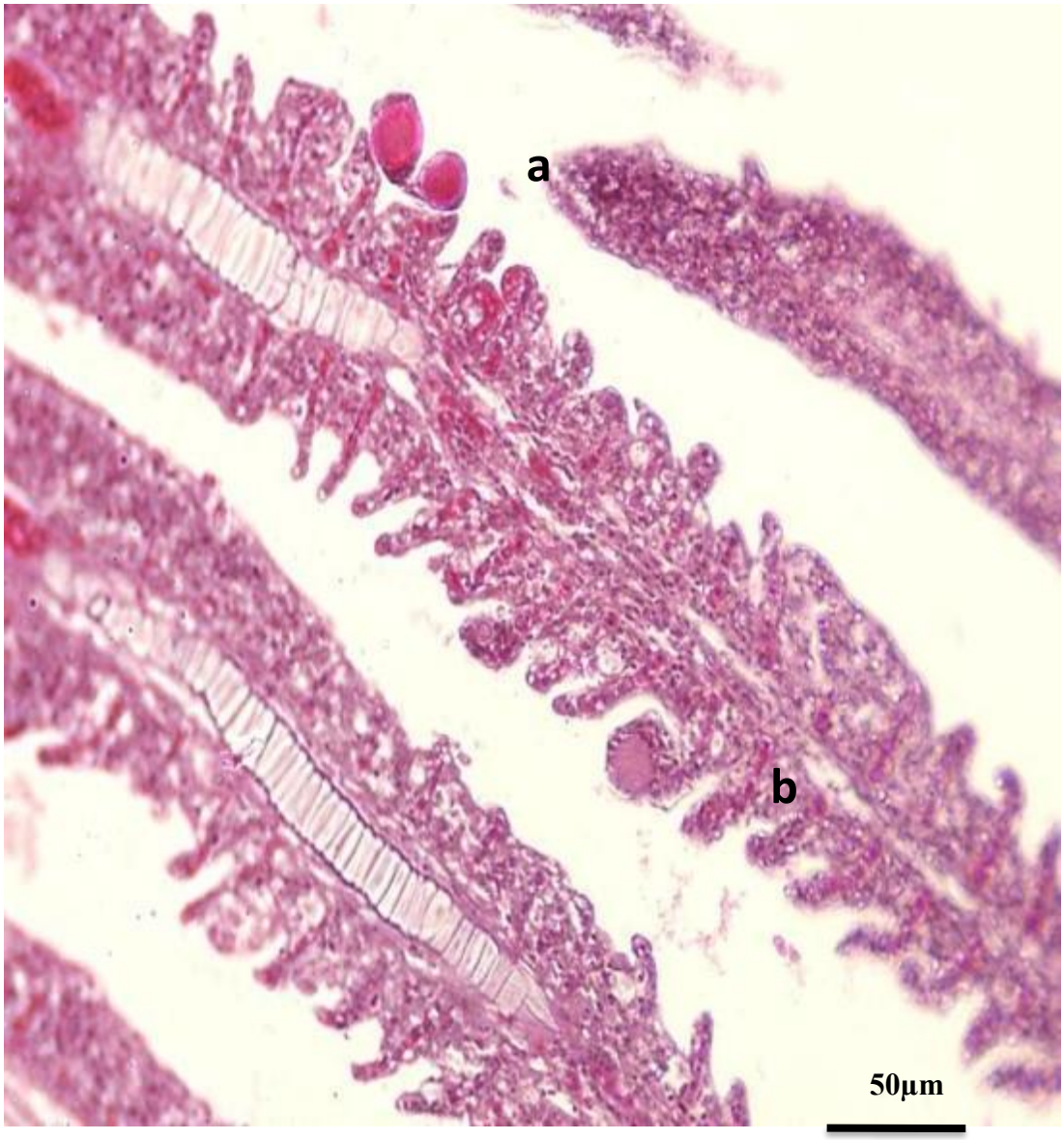

Figure 3-26: Epitheliocystis cysts found within *L. calcarifer* gill filament in association with gill hyperplasia. Note the free cyst with eosinophilic colour and bell shape (a) and a cyst appearing in the epithelial cells (b) with the outstanding basophilic-colour inclusion which is the elementary body, the first shape of epitheliocyst after infecting the host cell. Sections were stained with H&E, 10X, as per method described in **Appendix 6-1**.

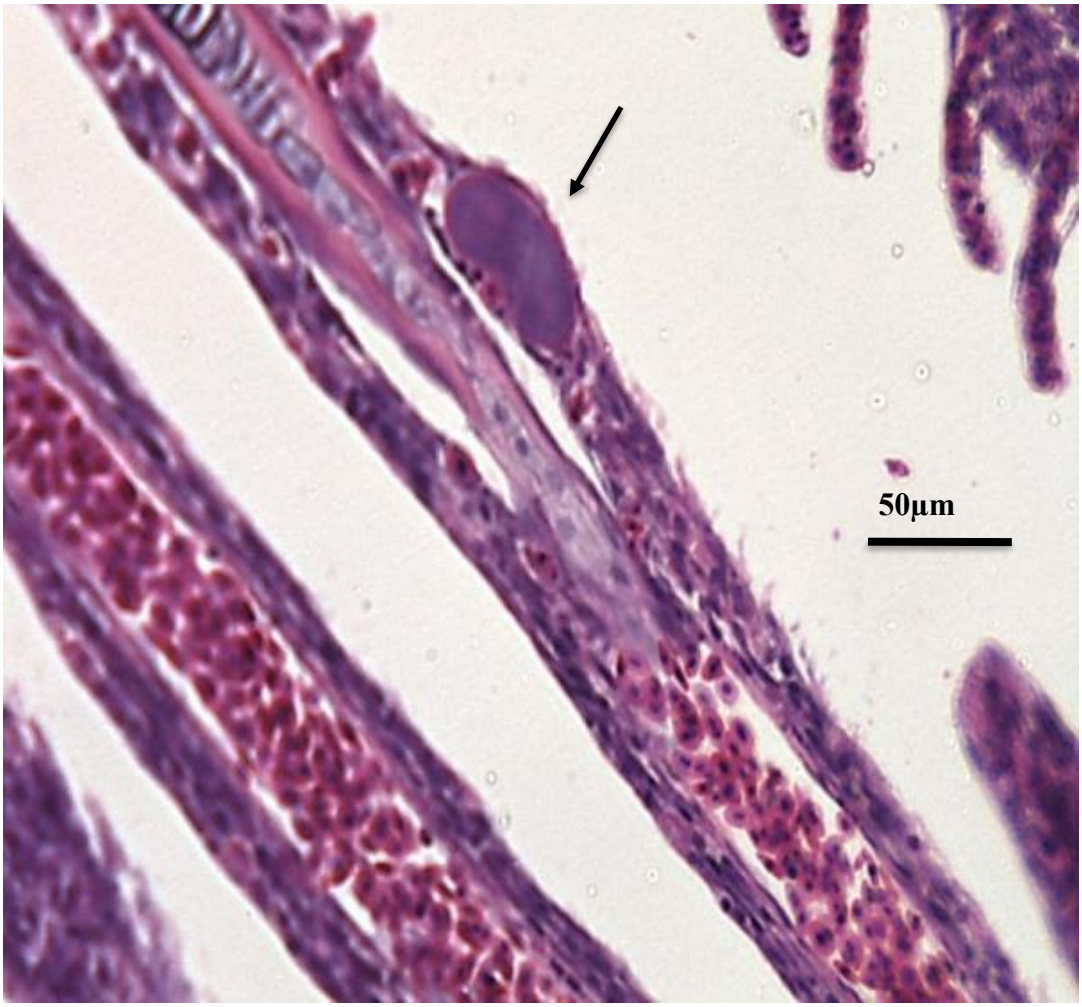

Figure 3-27: A reticulate body (arrow), the second stage of epitheliocystis development cycle 1 within a *L. calcarifer* gill filament. Sections were stained with H&E, 40X, as per method described in **Appendix 6-1.**

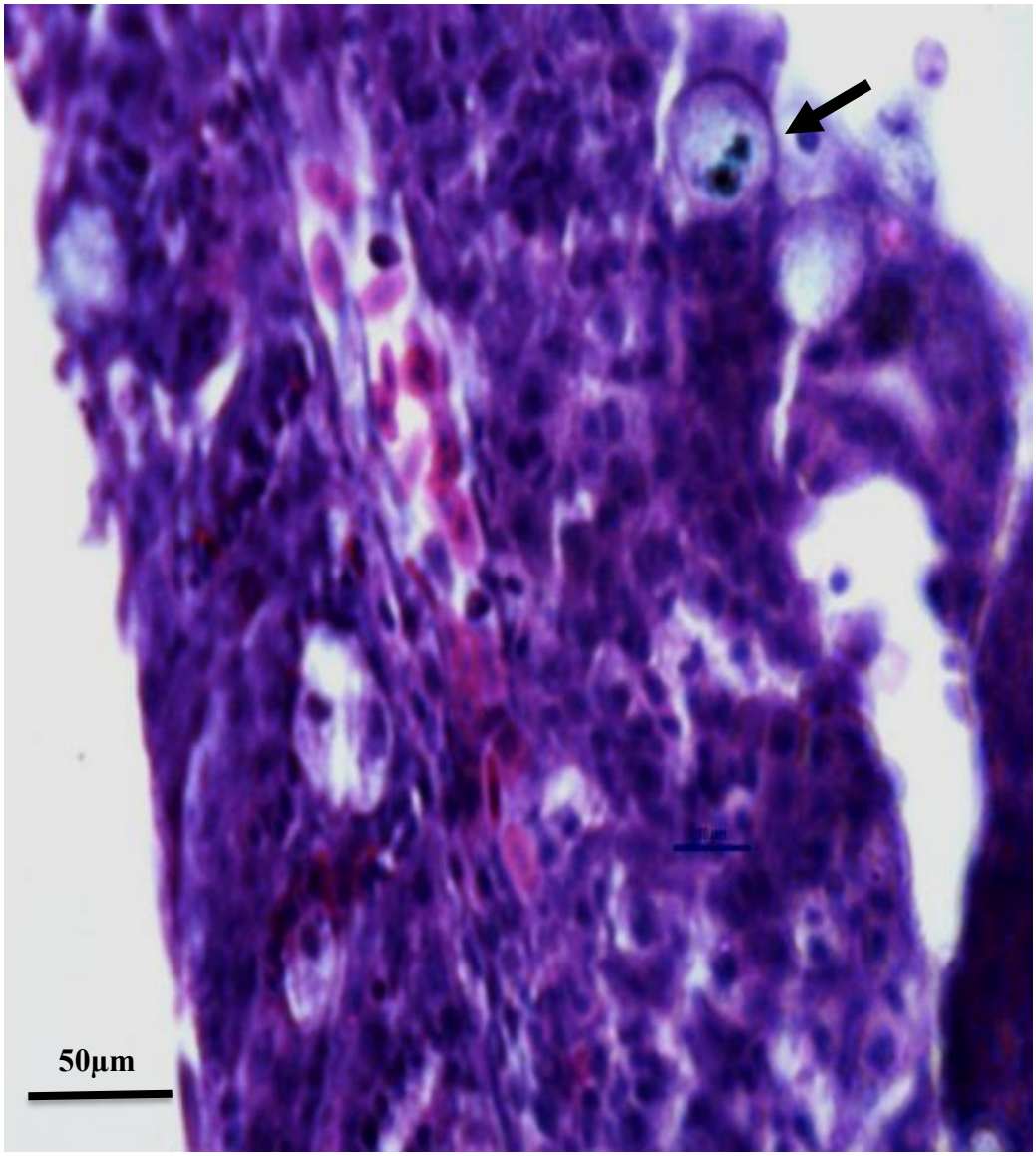

Figure 3-28: Intermediate body (arrow) in the *L. calcarifer* gill, a phase of epitheliocystis developmental cycle 2. Sections were stained with H&E, 100X, as per method described in **Appendix 6-1**.

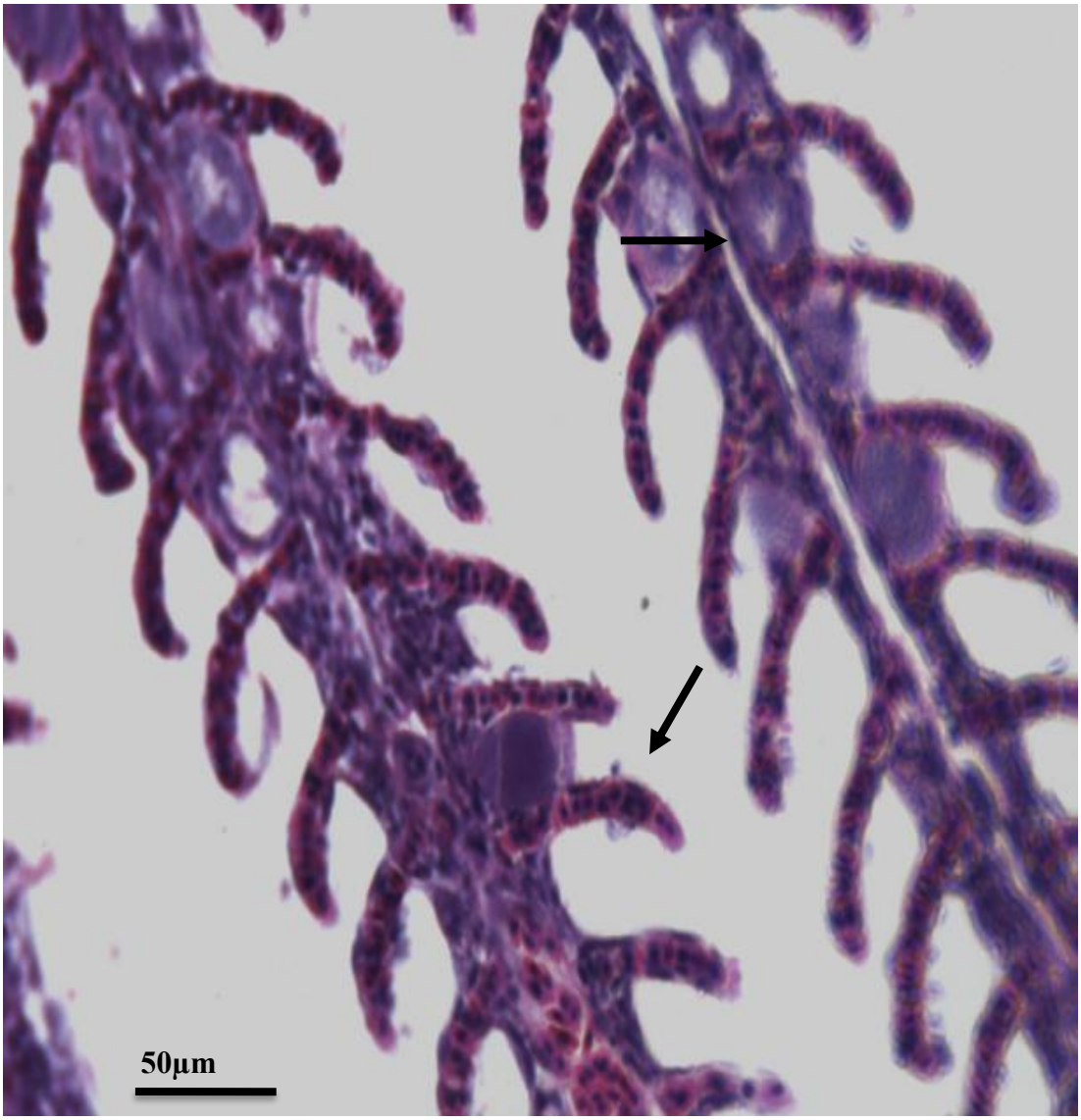

Figure 3-29: *L. calcarifer* gills heavily infected with epitheliocystis (arrows) at different stages of life cycle (developmental 1 and 2). Sections were stained with H&E, 40X, as per method described in **Appendix 6-1.**

3.5.2. Monogenea

Flukes were observed in the gill filaments and lamellae of fish samples. Results for monogenea infections are shown in the pie chart below (**Figure 3-30**) and in **Figures 3-31** and **3-32**. Infection level 1 made up the majority of observed infections, accounting for 83 % of observed infected samples. It was followed by 11 % and 6 % for the 2 and 3 levels of infection respectively in a total of 36 infected samples (**Table 3-2**).

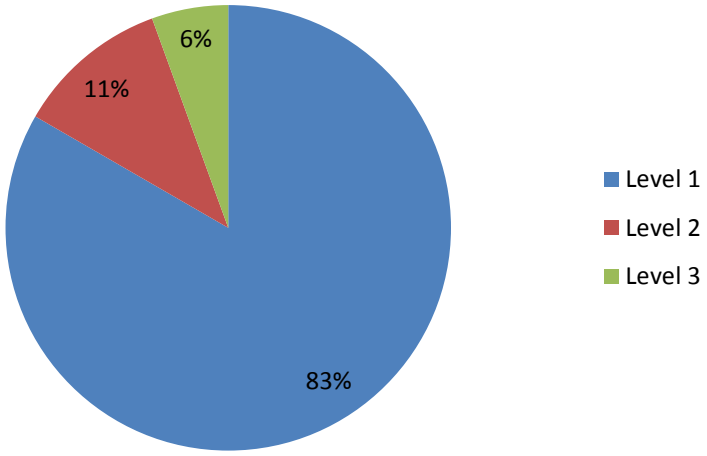

Figure 3-30: Pie chart of different levels of fluke infection in percentage. Level 1: < 30%, level 2: from 30%-60%; level 3: >60%. N=36

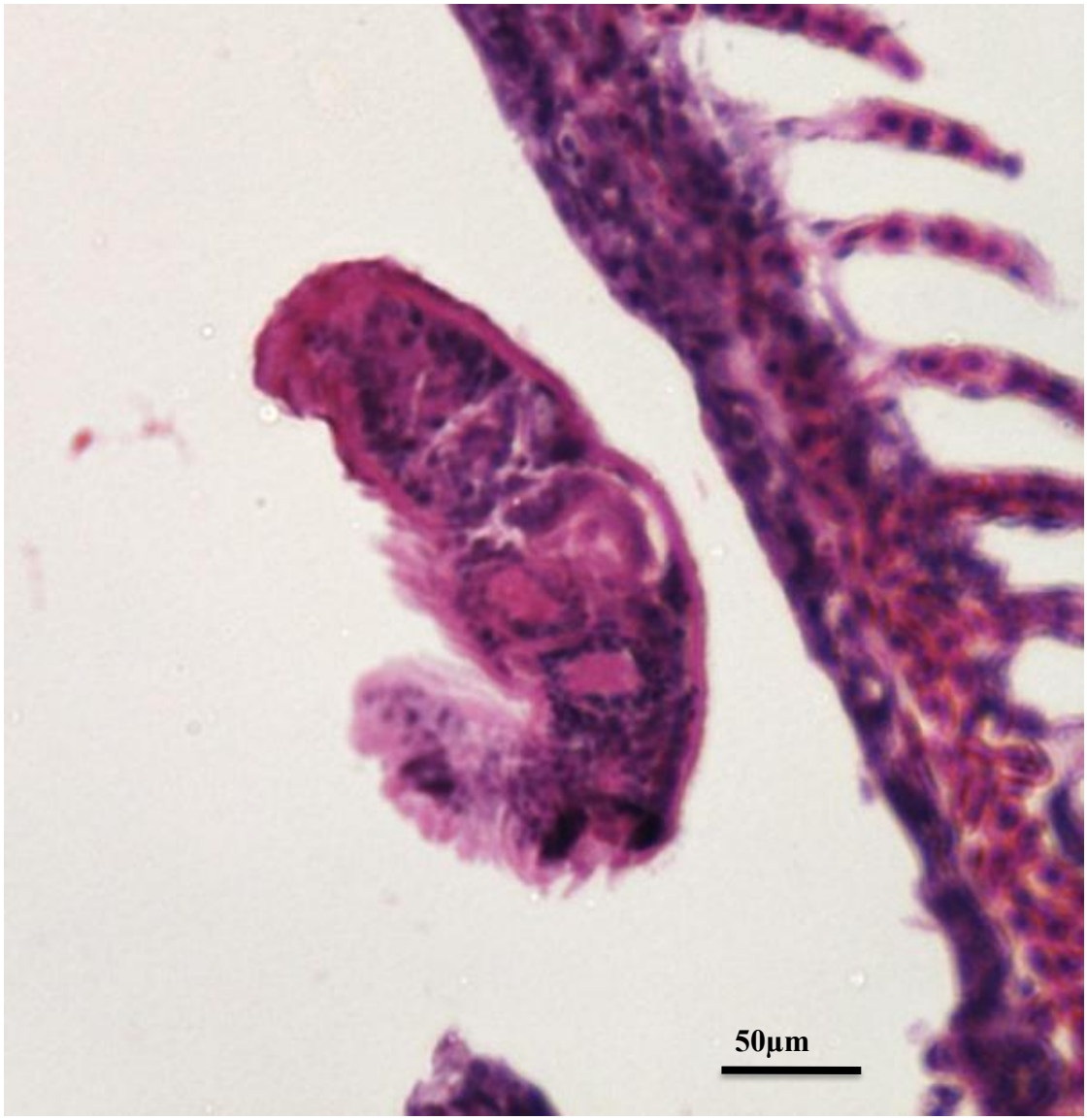

Figure 3-31: A *Diplectanidae* sp. within the gill with angled view. H&E, 40X, as per method described in **Appendix 6-1**.

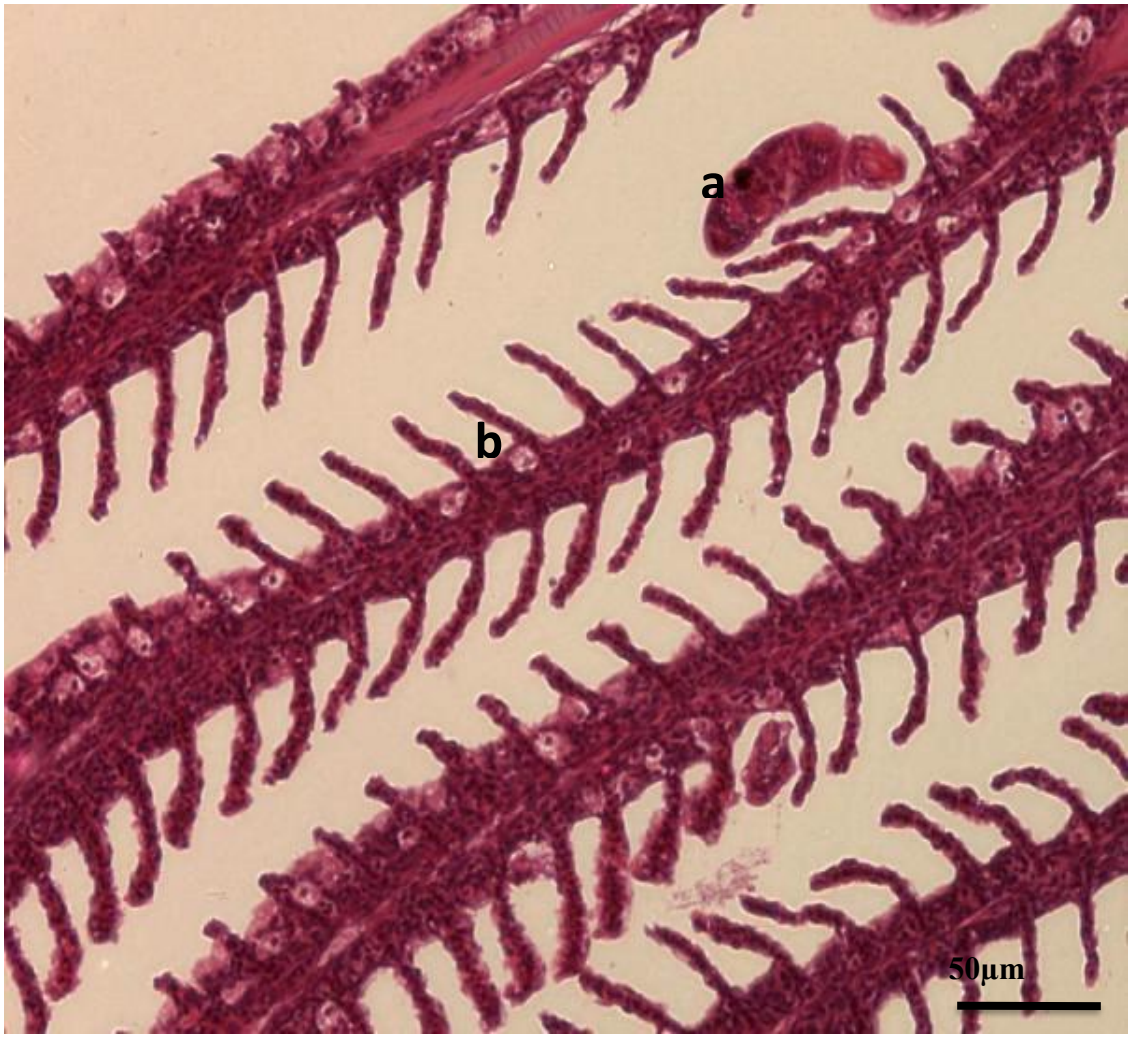

Figure 3-32: Fluke (a) and epitheliocystis (b) infection in fish gill with mild hyperplasia. H&E, 10X, as per method described in **Appendix 6-1.**

3.5.3. Unknown organisms

Unknown infections appeared less frequently than epitheliocystis or flukes. The unknown parasite was distributed extensively within the gills in conjunction with other parasitic diseases or alone. They occurred in clusters of small and elongated eosinophilic shapes. At higher magnification, the individual was myxosporidian like but lacked polar capsules (**Figure 3-34**). This unknown organism was also found within the central nervous system. They appeared in larger size clusters than that observed in the gills. Their occurrences were associated with brain inflammation in some cases (**Figure 3-35 & 3-36**).

In addition, in a total of 31 out of 270 fish a condition known as telangiectasis was observed, characterised by swelling of blood vessels within lamella or lamella dilation, such that this abnormality appeared like a glomerulus (**Figure 3-37, 3-38**). This, additionally, increased the size of a normal gill lamella. Moreover, telangiectasis was present in every second fish collection time when there were no parasitic infections as shown in **Table 3-2** below.

Table 3-2: Prevalence of pathogenic agents that occurred in each collection.

The shaded periods are when abnormal behavioural signs were observed in fish

Periods	No. of samples	Prevalence of epitheliocystis occurrence	Prevalence of monogenean occurrence	Prevalence of telangiectasis occurrence
27/09/2011	1-30	28	5	0
09/11/2011	31-60	1	0	5
8/12/2011	61-90	27	2	0
02/03/2012	91-120	0	0	11
22/03/2012	121-150	28	12	0
18/04/2012	151-180	0	0	7
16/05/2012	181-210	11	9	0
20/07/2012	211-240	0	0	8
11/08/2012	241-270	23	8	0

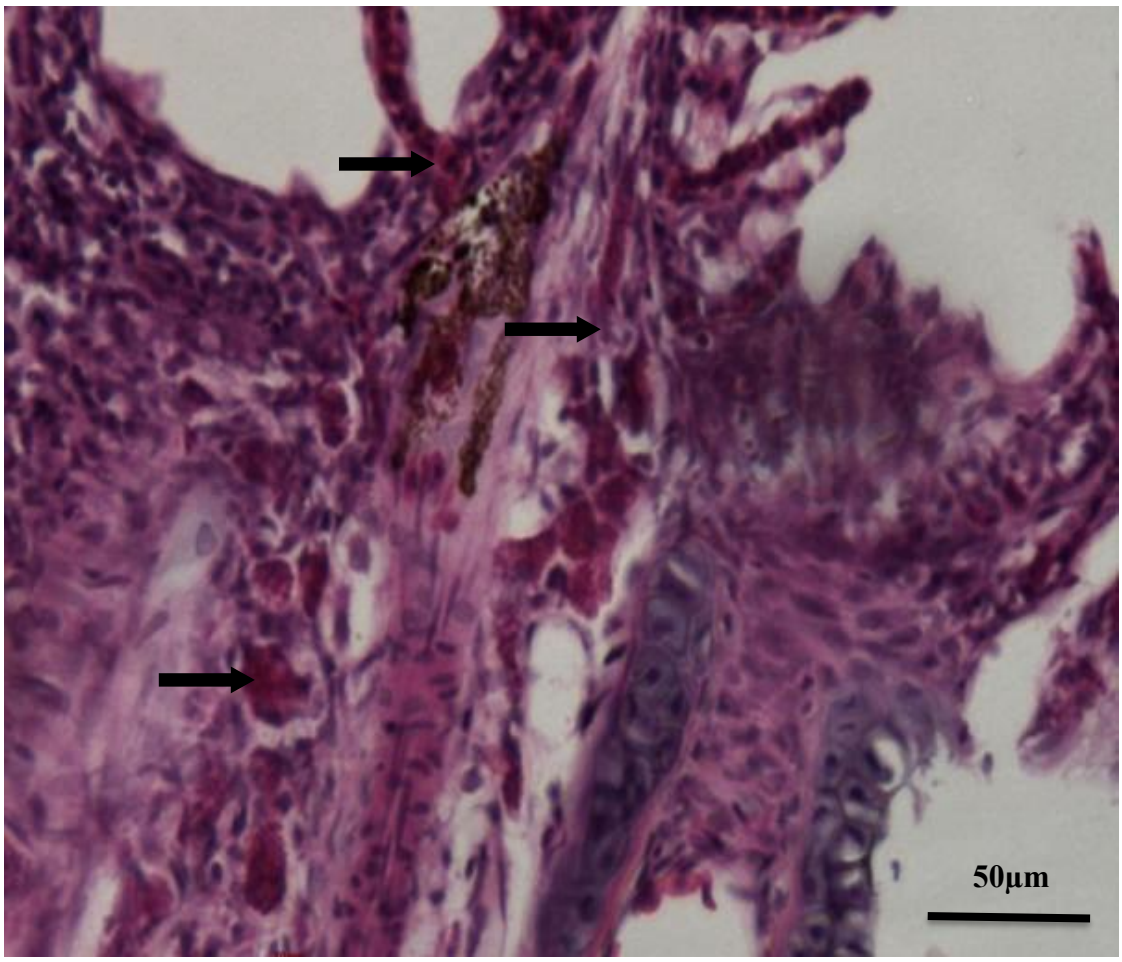

Figure 3-33: Unknown organisms appeared in clusters (arrows) surrounded by melanin aggregation in a gill filament. H&E, 100X, as per method described in **Appendix 6-1.**

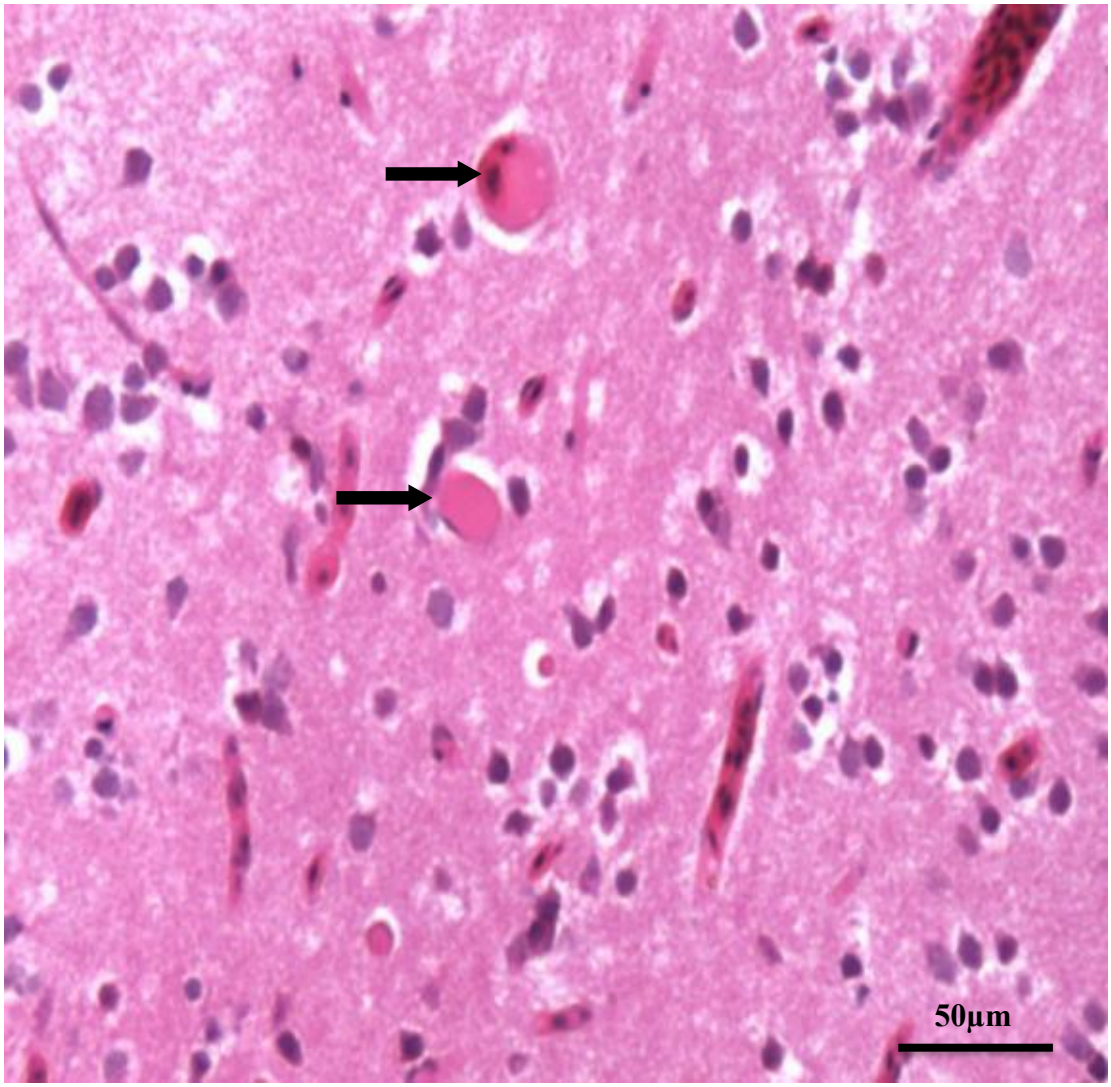

Figure 3-34: Unknown structure (arrows) in diencephalon in the brain. H&E, 100X**,** as per method described in **Appendix 6-1**.

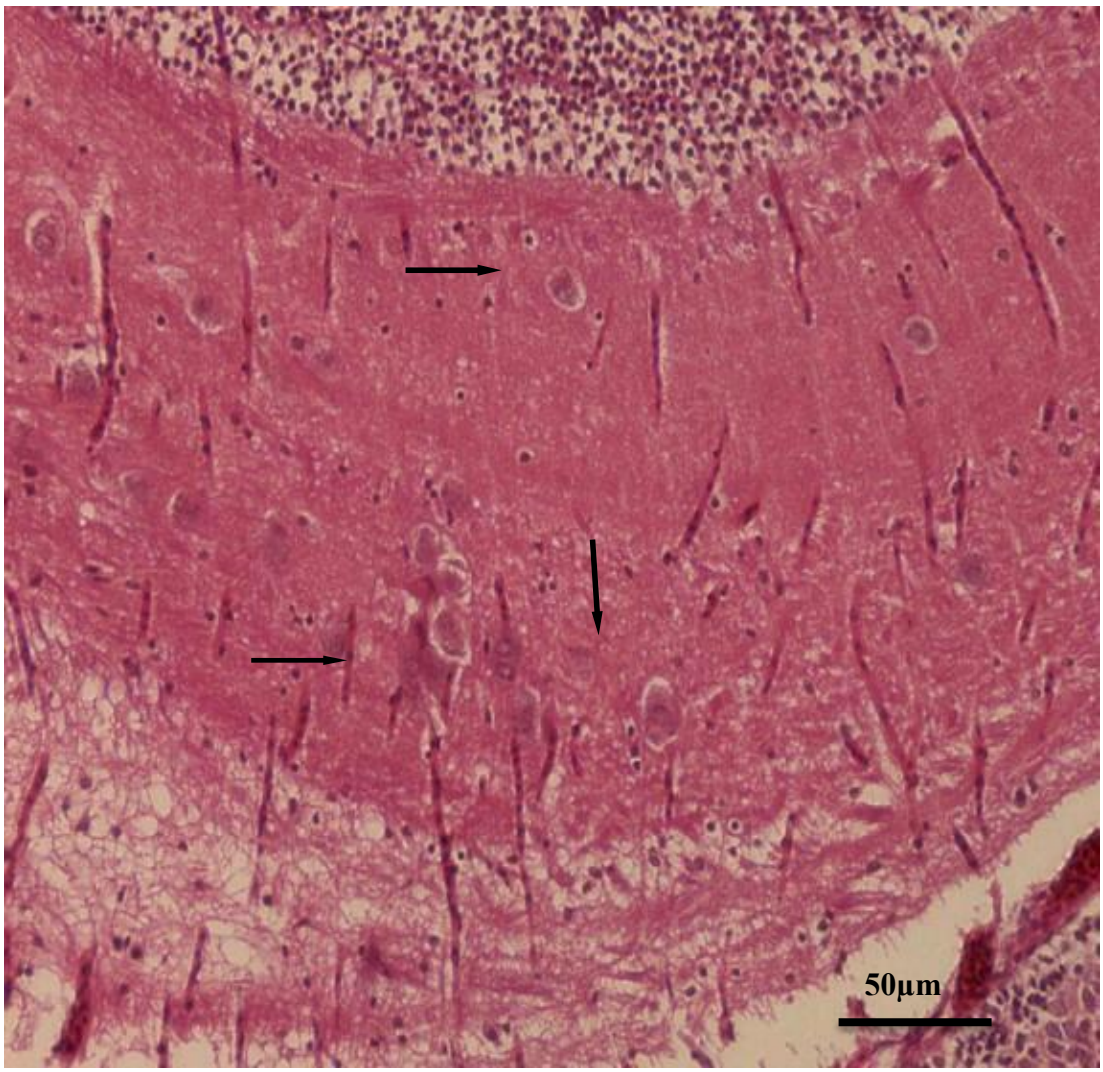

Figure 3-35: Unknown organisms in the brain optic lobe (arrows). H&E, 100X**,** as per method described in **Appendix 6-1**.

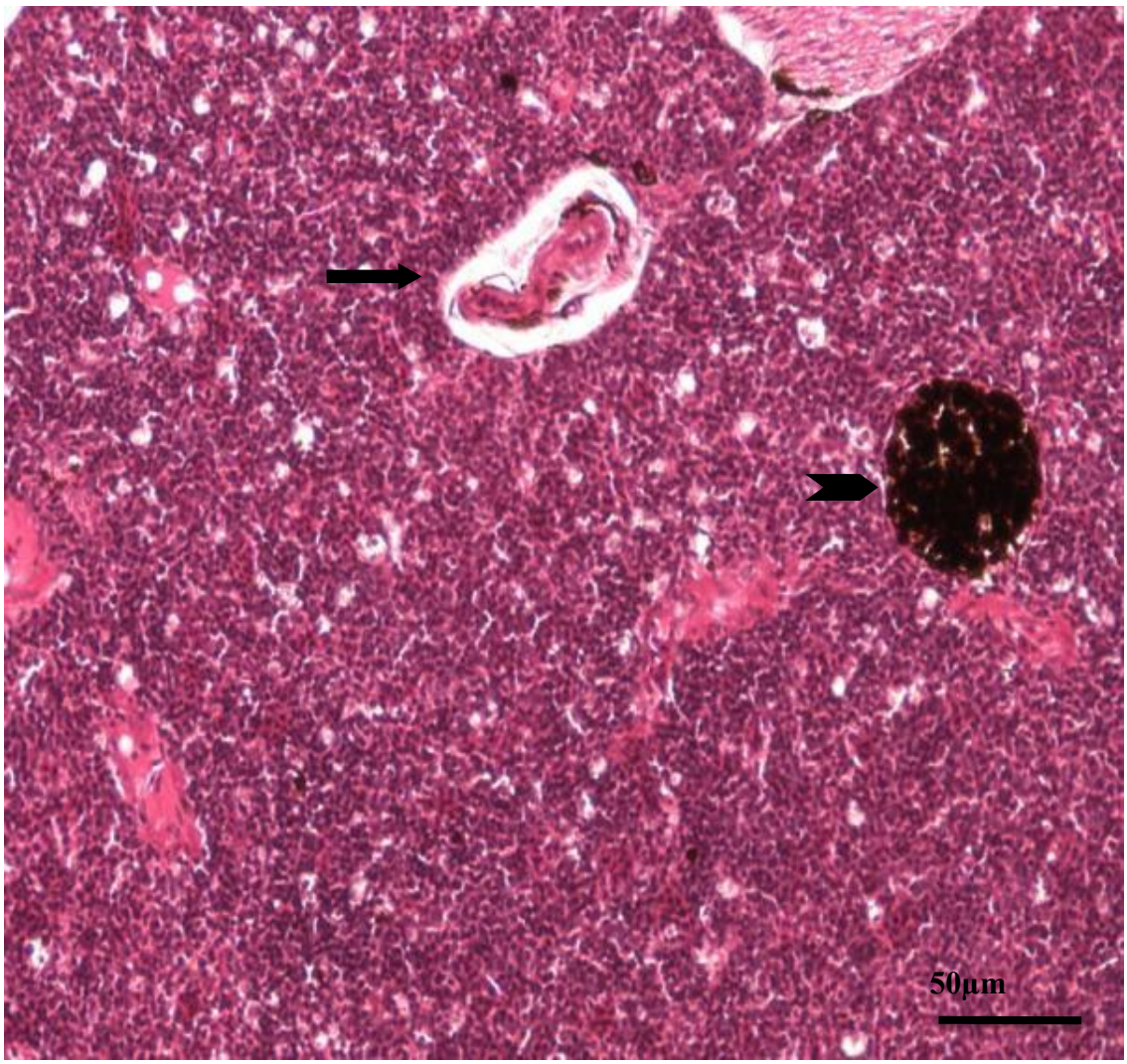

Figure 3-36: A strange agent (arrow) occurred in association with melanomacrophage centre (arrowhead) and vacuolation in the anterior kidney tissue. H&E, 10X, as per method described in **Appendix 6-1**.

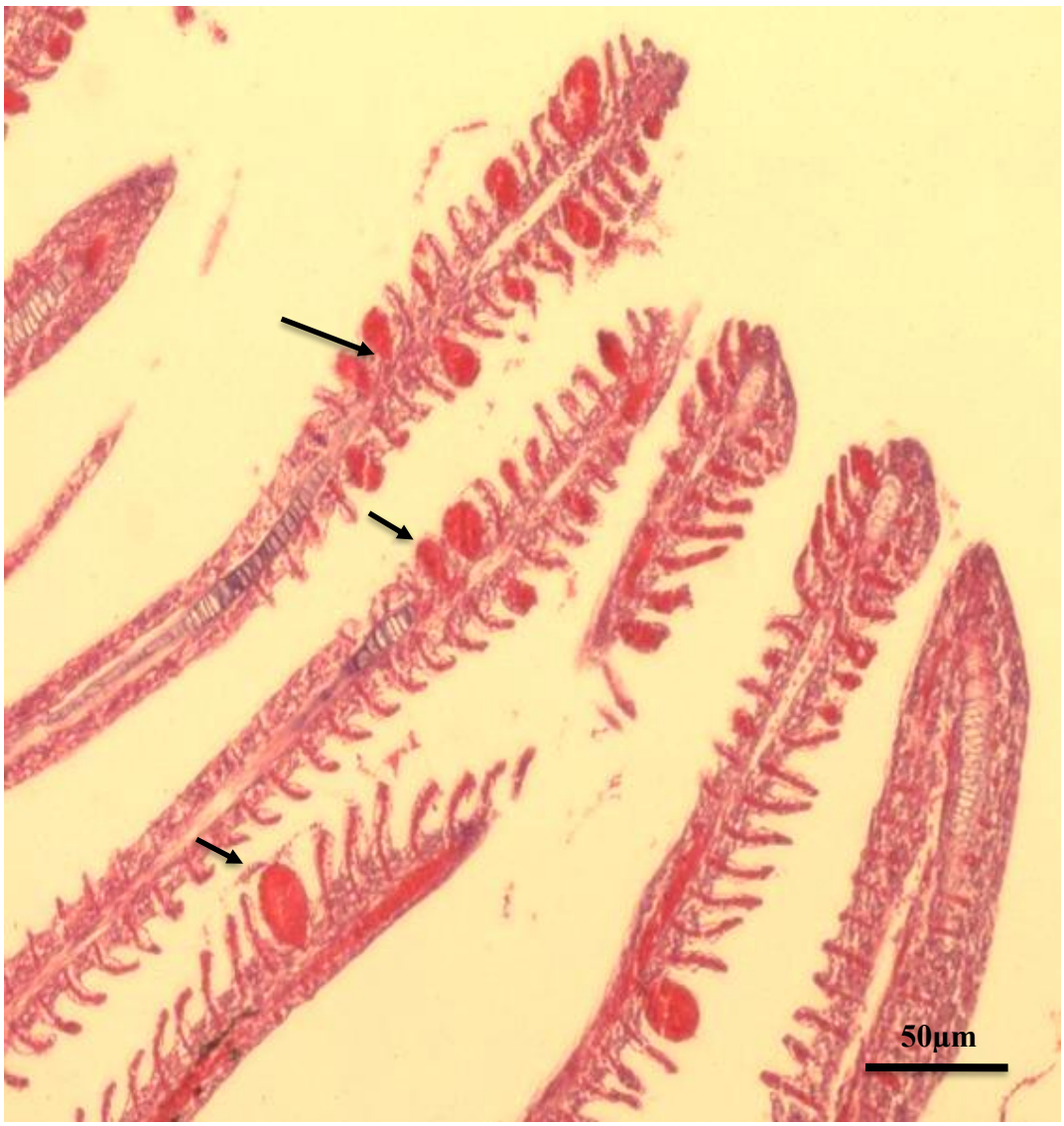

Figure 3-37: Moderate telangiectasia (arrows) in gill filaments with mild hyperplasia. H&E, 10X, as per method described in **Appendix 6-1**.

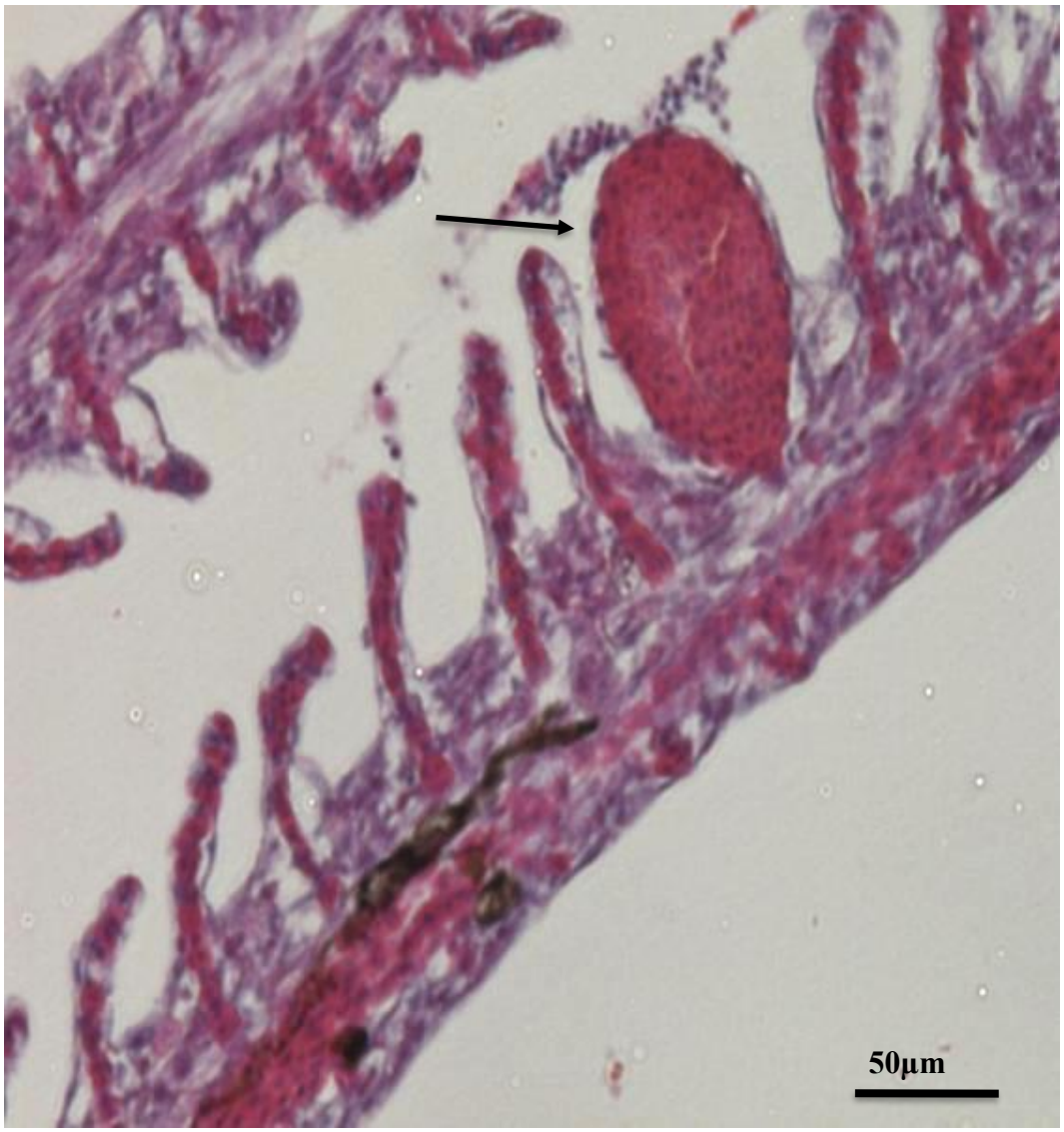

Figure 3-38: Telangiectasis (arrow) at higher magnification occurring in the gill, characterised by blood cell aggregation and swollen lamella. H&E, 40X, as per method described in **Appendix 6-1**.

CHAPTER 4: DISCUSSION

In this study extensive disease surveillance was conducted in *L. calcarifer* fingerlings from a facility at West Beach, South Australia. Over a nine-month period, 270 fish were examined using H&E staining, with the most common histopathological changes including vacuolation in the brain and retina, melanomacrophage aggregation and inflammation in different tissues. Surveillance also revealed epitheliocystis, parasites and other abnormalities. These and other pathological changes observed with different severity in fish tissues were also similar to those reported in the literature as being present in Betanodavirus infected fish. The gold standard immunohistochemistry detection method was then used to investigate Betanodavirus infection in these fish. No VNN positivity was observed in fish collected in this study. Identifying the cause of these pathological changes using other methods such as PCR, bacterial isolation or TEM was beyond the scope of this work.

4.1. Histopathological changes

4.1.1. By occurrence

Vacuolation has been reported in many previous published papers as a feature of Betanodavirus infection (Thiery et al., 2004, Gomez et al., 2007 and Gomez et al., 2008). Most researchers reported that vacuoles were a typical histopathological change in infected tissues. In most of the research reports the occurrence of vacuoles is associated with nervous tissues located in the retina, brain and spinal cord of fish (Munday et al., 1992, Thiery et al., 2004). Specifically, lesions are generally less severe in older fish than those seen in juvenile fish (Arimoto et al., 1992). Depending on fish age, the severity of vacuolation can vary from one or two affected cells to necrosis of whole areas of the retina and brain. In the nervous system, vacuolation appears more frequently in the cerebellum and optic tectum of barramundi than in the medulla oblongata and telencephalon (Moody

& Horwood, 2008). In fact, reports of VNN in sea bass and grouper state that vacuolated cells have the look of a sphere with a marginally constricted or pyknotic nucleus. Vacuolated cells form extensive areas termed spongy lesions in which microglia have restricted basophilic cytoplasm (Mladineo, 2003, Tanaka et al., 2004).

During this project, vacuolations were recorded in tissues examined from 18 (of 270) fish. Brain, retina and spinal cord of examined fish were affected with variable levels of vacuolation, whilst individual cells in these tissues demonstrate pyknosis. All the samples that contained vacuolation underwent IHC with an antibody that recognised Betanodavirus coat protein but no Betanodavirus particles were present in these samples. In positive control VNN samples that were obtained from Geelong, H&E staining revealed similar vacuolation patterns in brain and retinal in these samples to that observed in our fish. Thus, vacuolations appear to occur in both our Betanodavirus- negative and the Betanodavirus positive samples (from Nick Moody of AHHL). At this point, it is clear that the presence of vacuoles and necrosis in nervous system tissue is not strong enough evidence to indicate Betanodavirus infection. Another explanation for this result is that vacuoles can occur when there have been abnormalities or disease pathology other than VNN infecting the fish. Further examination of the tissues from the 18 samples indicated that epitheliocystis, parasite(s) and other unknown abnormalities were present in these fish. This indicates that vacuolation could be part of a normal immune response that results when the fish immune system is presented with foreign agents. This hypothesis agrees with several researchers who have examined histopathological changes in fish (Hibiya, 1995, Bernet et al., 1999 and Ferguson, 2006). These authors all confirm that vacuoles are a typical sign that is detected when fish tissues are examined and is often an indicator of poor fish health. These combined results suggest that H&E staining by itself

is not specific and sensitive enough to detect Betanodavirus infection but is still very useful in showing pathological changes that may be suggestive of diseases such as Betanodavirus and other viral or parasitic infections.

Apart from vacuolation, other changes such as granuloma formation, melanomacrophage aggregation, blood vessel congestion, haemorrhage and hyperplasia were evident in fish tissues in this study. These changes mentioned above were believed to be the normal response of the fish immune system to foreign agents. For instance, granuloma formation was a response observed in cod (*G. morhua* L.) to a typical furunculosis, while in the case of Atlantic salmon, haemorrhage and localized cellular necrosis were predictable signs of the same type of infection (Magnadottir, 2010). Blood vessel congestion in the brain is a sign of abnormalities of the diencephalon where capillaries distribute extensively and play an important role in fluid-brain exchange (Ferguson, 2006).

Histopathologically, the diverse changes observed above indicate that the fish sampled were potentially unhealthy. For instance, in this study, melanomacrophage aggregations appeared within the kidney and gill of the infected fish of the positive VNN control samples. Tanaka et al. (2004) described Betanodavirus infected cells as containing cellular debris and with macrophages infiltrating the lesions as well as haemorrhage of which are similar to those changes that being observed in this project in the absence of Betanodavirus infection.. All in all, the changes observed in the current study can be considered as indications of unhealthy fish. However, the specific cause of pathological changes could not be determined since the samples were VNN- negative. The occurrence of other bacterial and parasitic agents may have led to the pathology observed and this is discussed in detail later.

4.1.2. By examined organs

Histologically, the gill was the most affected tissue among those organs studied. The gill made up 49 % of affected tissue (organ) and was the only type of tissue that showed histological change at level 3, the highest degree. This was followed by the kidney with 32 % of changes, the retina with 20 % of changes while the spinal cord showed the lowest percentage of changes at 4 %. This result agrees with the current knowledge of the fish immune system in regard to the gill and the skin playing a crucial part as a primary barrier against pathogens in fish. Gills are also a very delicate structure comprised of many immunological factors and are reported to be the portal of entry of pathogens (Nguyen et al., 1996, Whyte, 2007). Ferguson (2006) claimed that the epithelial layer of the gill is remarkably metaplastic in response to environmental and physiological triggers as well as during diseases and is frequently targeted by infectious or non- infectious mechanisms.

4.1.3. Immune response and histological changes observed in fish adapting to pathogens

During this study, the importance of understanding the histological changes as well as immune response of fish to pathogens and the ability to link those responses and histological changes in particular tissues was highlighted when interpreting samples using histology. For instance, Tanaka et al. (2004) described Betanodavirus infected cells as containing cellular debris and with macrophages infiltrating the lesions as well as haemorrhage of which are similar to those changes that being observed in this study in the absence of Betanodavirus infection. Melanomacrophage aggregations also occurred in the positive VNN control samples (from Nick Moody of AHHL) in this project within the kidney and gill of the infected fish. Other changes such as granuloma formation, melanomacrophage aggregation are also evident in our samples. The immunological

responses of teleost fish species against viral diseases have been of many research interests. For example, Workenhe et al. (2010) confirmed that teleost fish have both adaptive and innate immunity although the adaptive immune response lacks isotype switching to produce virus specific antibodies. The susceptibility of fish to viral infection is dependent on enhancement of the interferon system. Furthermore, acute infection during the larval and juvenile stages is largely due to inactivation of the interferon response. It is concluded that an interferon related gene/s are not activated during barramundi larval stages (Lu et al., 2008). This may explain why larval and juvenile barramundi are highly sensitive to Betanodavirus infection and that is why larvae barramundi were examined in this research.

4.2. IHC results

IHC is the gold standard test for validating the presence of VNN disease in barramundi. This method was established in the lab and used successfully to confirm four positive and two negative control samples (which had similar age and length compared to our samples) from the AHL in Victoria. It has also been reported by numerous researchers that Betanodavirus can be diagnosed by immunostaining methods. This protocol (2008, updated in 2014) has been used previously to diagnose VNN outbreaks in South Australia. Regarding the results of this project, all 18 samples that indicated VNN by H&E staining were negative when tested by IHC. This might be due to the pathogenic organisms other than Betanodavirus causing similar histological changes to that previously observed with Betanodavirus infection. However, it could have been possible that the stage of the VNN present in the fingerlings was too early to be detectable by IHC. For instance, Tanaka et al. (2004) revealed that specific Betanodavirus targeted cells in the central nervous system and retina have not been found in larval or juvenile fish associated with VNN using IHC. RT-PCR in this case might have been a more efficient method to detect VNN in this cohort as several papers also report that VNN can be identified by the more sensitive PCR

test, from fish without any clinical signs (Iwamoto et al., 2001, Gomez et al., 2004 and Gomez et al., 2008). Moreover, in this study, it needs to be acknowledged that only the 18 fish that had abnormal pathology were tested by IHC for Betanodavirus. Although these fish were negative for Betanodavirus, the remaining fish did not undergo this testing, so it is still possible that they may have tested positive for Betanodavirus by IHC or have had early VNN infection that may be picked up at early stages if PCR methods were utilised.

4.3. Other pathogenic agents

4.3.1. Epitheliocystis

Epitheliocystis is caused by intracellular Gram-negative bacteria and is found in both freshwater and marine species, including fish. Fish mortalities that are associated with epitheliocystis infections have been reported in wild and farmed fish (Paperna & Alves de Matos, 1984, Nowak & LaPatra, 2006). However, there were no deaths observed in this study despite the high prevalence of epitheliocystis infection recorded. Epitheliocystis mainly targets the gill and skin of fish. Gill infestations may be linked with epithelial hyperplasia, necrosis and degeneration, together with haemorrhage (Nowak & LaPatra, 2006).

Epitheliocystis can infect the gill in various areas. This project revealed a number of infected sites within the gill such as within the filament, gill arch, tip of lamellae and between lamellae. Histological changes associated with epitheliocystis infection included hyperplasia, melanin aggregation and vacuolation. Epitheliocystis was the most prevalent pathogen observed in our tissue samples. It was observed in 118 samples out of the total 270 samples and was accounted for 77% of the pathological changes (out of 154 abnormal samples) examined and the severity of those infections were high. Specifically, 47% of the samples (N=56) illustrated infection level 1, 36% (N=42) illustrated infection level 2

and just 17% (N=20) for the lowest level of infection (**Figure 3-25**). Host cells included coccobacillary or coccoid organisms that finish a pleomorphic developmental phase with specific morphological characteristics reliant on the stage of intracellular growth phase. Most stages of epitheliocystis life cycle were present in the gill structure of studied fish indicating they had long term exposure to the causative agent.

A classic developmental cycle contains a reticulate body, infective elementary body and intermediate body (round cell) (Avakyan & Popov 1984). In this project, most stages of the developmental cycle were evident within the gill of fish except for primary long cells and intermediate long cells which are the reproductive forms of epitheliocystis. Moreover, in some cases, monogenean flukes as well as unknown organisms occurred in the infected gills where epitheliocystis was found. This indicated that either epitheliocystis or another pathogen could be the primary gill pathogen and may increase the opportunity for secondary pathogens to affecting the fish gills later. Regarding the high number of fish (118) found with epitheliocystis compared to that of 36 associated with flukes, it is possible that epitheliocystis was the primary pathogen in this case. This finding demonstrates that disease surveillance should play an important role in aquaculture, where early abnormalities can be detected efficiently and then hatcheries can apply treatments as appropriate.

The lack of primary long cells of the epitheliocystis is perhaps due to the age of the examined fish (juvenile) or the condition of their environment (low to no stress factors) as it is believed that stress or fish age or environmental conditions change (Crespo et al., 1999) and types of cell infected (Paperna & Alves de Matos, 1984) are causative factors in the switching between the two developmental cycles. Further work is still required to fully understand the epitheliocystis life cycle in fish. Epitheliocystis has been related with

mortality and morbidity in cultivated fish species. However, the direct connection between mortalities and epitheliocystis has not been proven due to the absence of an experimental challenge template. This is due to the inability to culture the organism/s associated with epitheliocystis (Stride et al., 2014). This also explains why histopathology, especially H&E staining is still the most common and powerful method to observe epitheliocystis. Several authors have published the importance of histology use during disease diagnosis and surveillance; otherwise, disease can be accidently missed (Egusa, 1983, Kusuda & Kawai, 1998). Thus, this can lead to misleading results where fish are examined superficially and assumed to be uninfected and healthy, as discussed in Nowak & LaPatra (2006).

4.3.2. Monogenea

Monogeneas are flat worms that complete their life cycle in a single host. Monogenean infection has been described in various fish species around the world and is associated with fish mortality (Brunol et al., 2006). This group of parasites are also commonly found with other bacterial or parasitic diseases. Histologically, monogenean infection in the gills shows decrease epidermal thickness and reduced amounts of mucous cells, related with parasite attachment. These parasite infections can result in destroying epithelial cell layers, reduce the number of chloride cells on gills, trigger over-production of mucus and finally lessened respiratory function of infected fish (Whittington, 2005). This can weaken the host's regulatory and metabolic processes and lead the host being susceptible to other opportunistic pathogens. In agreement, Trujillo-González et al. (2015) showed that a major drop in the amount of cutaneous mucous cells in infected fish skin could alter the fish's ability to survive other opportunistic pathogens.

Monogenean infection leads to lesions which are indicative of necrosis and also primarily haemorrhage of epithelial tissue of the gill and skin (Brunol et al., 2006). These symptoms are similar to those observed in infected samples in this study. Diplectanid spp.

monogeneans were detected and lesions typical of this organism were present in 23% of samples (36 samples out of 154 samples) in the gill. Additionally, 29 of those 36 monogenean infected samples also exhibited epitheliocystis in their gills. The prevalence of high severity level 3 monogenean infections in this study was low. In fact, there were only 2 out of 36 infected samples that had severe infection (6%). This observation can be explained as follows. First, the infection may have been at its early stage therefore there were not many severely infected samples observed. Second, co-infection with epitheliocystis may reduce the severity of its transmission as epitheliocystis were dominant with a great number of infected samples (118 samples). Thirdly, the use of anaesthetic and histological techniques to detect monogeneans could lead to the loss of monogeneans during the fixation process. Several researchers such as Brunol et al. (2006), Ferguson et al. (2011) and Sitja-Bobadilla & Alvarez-Pelliterio (2009) have noted that fixation techniques can affect the detection of monogenea. In this study, parasites were evident using H&E staining. Therefore, this technique is still useful and practical for fish health surveillance.

In this project, both epitheliocystis and flukes were evident within the gill of examined barramundi. These pathogens were observed when the fish appeared unhealthy with darkening skin and swirling swimming behaviour. Interestingly, the co-infection of epitheliocystis and fluke occurred two months apart and was only observed every second collection. The appearance of epitheliocystis infection in conjunction with monogenean infection has also been reported in another fish species gilthead seabream (Sitja-Bobadilla & Alvarez-Pelliterio, 2009), and previously in farmed and wild barramundi (Griffiths, 2011).

Regarding the finding of fluke and epitheliocystis co-infection in this research, it is important to understand more about the optimal fish living conditions and environment,

required to host fluke and epitheliocystis; especially if co-infection could benefit either or both pathogens. Thus, further research is required to understand why flukes and epitheliocystis infections appeared together in this study.

4.3.3. Telangiectasis in gill

Coincidently telangiectasis appeared every time when sampled barramundi appeared healthy. Telangiectasis is characterised by the swelling of blood vessels within lamellae or lamella dilation, making this abnormality look like a glomerulus and increasing the size of a normal gill lamellae. Telangiectasis was previously reported in barramundi from the same hatchery as the one used in the current research (Griffiths, 2011). Their results agreed with the results reported here in such that telangiectasis was not associated with any specific disease on examined barramundi. Griffiths (2011) suggested that telangiectasis was possibly caused by high stocking rates, or suspended solids or mud in the supplied water. However, given that pathogens were not detected in these sample during this project, the causes of telangiectasis might be due to chemical remnants in the water supplied to the tanks. The theory relates to the absence of observed abnormalities in the tissue of every new batch of barramundi every 2 months. Instead, was the appearance of telangiectasis in the gill. It might be because the hatchery is using a new water supply disinfected by chemicals at the time of our second periodic sampling. Crespo et al. (1999) and Ferguson (2006) have discussed previously the acute response of fish gills to chemicals in water. Also, when it comes to fish exposure to pollutants, De Almeida et al. (2014) stated that gills and livers are considered primary markers or responders to aquatic pollution. The gills have a large surface area, and they risk direct contact with chemicals diluted in water; the liver is the first organ to come into contact with chemicals after uptake. Highly contaminated water can induce pathological changes in fish gills if they are frequently exposed to it. That is why gill tissues are commonly examined when

investigating environmental impacts. However, this theory needs more research and examination. The development of a survey about hatchery facilities and conditions may be considered for interested researchers in future.

4.4. General discussions about disease surveillance at a local South Australian barramundi hatchery

In the period of 9 months, we collected 30 juveniles per month for disease surveillance with the focus on detection of VNN. Within Australia, incidences of the VNN have been reported in South Australia, the Northern Territory, Queensland, Tasmania and Western Australia in both freshwater and marine finfish species (Munday et al., 2002). VNN affects at least 70 species of aquatic animals around the world in which there are about 50 marine species. VNN has been considered as one of the most severe viral diseases affecting larval and juvenile marine fish (Grove et al., 2003, Doan et al., 2017 and Bandin & Souto, 2020). Therefore, it is best to have early diagnosis of VNN as the mortality caused by VNN can reach 100%. In South Australia, Moody et al. (2009) reported that a barramundi Betanodavirus infection occurred in 2004. Furthermore, the Betanodavirus isolates from South Australia belong to RGNNV genotype, one of five genotypes of Betanodavirus. In this project, we collected 270 samples of approximate 30 days old barramundi from a local hatchery within a period of nine months for disease surveillance in order to detect early signs of any abnormalities. Although, similar signs of Betanodavirus infections such as swirling, skin discolouring and haemorrhage were observed in fish during this study, tissue samples from these fish were VNN negative using the IHC test that detects VNN viral coat protein. Having said that, it is believed that Betanodavirus can be detected from fish with or without clinical signs as well as natural or experimental infections (Gomez et al., 2004, Tanaka et al., 2004). Clinical disease mostly occurs in juvenile and larval finfish, and this is the situation in Australia, although

clinical disease in mature fish has also been observed (Moody & Crane, 2014). High mortality rates (up to 100%) are mainly observed in larval fish and mortalities tend to reduce as the size of the infected fish grows (Shetty et al., 2012). Fish about 30 days old are believed to be highly susceptible to Betanodavirus invasion. Therefore, it was crucial that juvenile barramundi should be regularly examined by other techniques of higher sensitivity such as IHC or PCR in order to detecting VNN early and for general disease surveillance because H&E staining is not sufficient enough to detect VNN based on the outcome of this study.

Apart from pathogen surveillance, the methods of detection applied are vital to the success of early diagnosis. In summary this work indicates that H&E is not suitable for VNN confirmation but is the most appropriate tool to detect pathological changes, such as epitheliocystis. H&E can also detect parasites and abnormalities such as telangiectasis that can be underestimated when using other methods such as IHC or PCR.

Epitheliocystis and fluke infections have been recorded with high prevalence and periodically in this South Australia hatchery. This indicates poor quality of barramundi fingerlings products as these were being bred for commercial purpose. However, no deaths were observed although abnormal behaviours of fish were evident. This could lead to fingerlings being sold to a larger scale facility for farming and could infect that farm causing financial loss. However, we have reported the infections to authorities at the hatchery as soon as we discovered the issues with our sampled barramundi. In this case, mitigation measures to reduce disease transmission for hatcheries created by Department of Agriculture should be applied (Landos et al., 2019).

CHAPTER 5: SUMMARY, LIMITATIONS AND FUTURE DIRECTIONS

Barramundi and its reputation in commercial markets are recognised worldwide. In Australia and Asia Pacific region, it is considered as an important species in aquaculture (Lawley, 2010). Barramundi, however, are susceptible to variety of pathogenic organisms such as viruses, bacteria, parasites and fungi. The original findings of this study are: (a) Gills had the most histological changes but observed to a lesser extent in brain and retina (b) VNN detection was negative by IHC test. This suggests that the appearance of vacuolation in the brain, retina and central nervous system tissues is not sufficient enough to identify VNN in barramundi fingerlings at a South Australian hatchery. It could be either a new agent presenting similar pathology to VNN or the previous Betanodavirus has mutated so that is not detectable with our detection methods. In this study, vacuoles were evident in our (VNN negative tested by IHC) samples and both the positive and negative control samples. Clinical symptoms such as skin darkening, lethargy, swimming in a swirling pattern are not pathogen-specific as they were observed in the absence of VNN and could be symptomatic of the epitheliocystis and parasitic infections observed. (c) Epitheliocystis and parasitic infections with high prevalence were periodically recorded (every 2 months) without telangiectasis and vice versa in barramundi fingerlings at a South Australian hatchery.

Regarding VNN disease surveillance, the most common histological changes described for VNN disease were seen in the brain and retina of 18 out of the 270 fish examined during our nine month study. Other pathological changes similar to those from previous reports about Betanodavirus infection in fish were also recorded with different severity in the fish tissue examined. After applying the IHC method, no positive identifications for Betanodavirus were observed in these 18 fish. This led to the concerns about the limitation of H&E studies for Betanodavirus detection as we had samples which showed

vacuolation, melanomacrophage aggregation and inflammation all previous signs observed in Betanodavirus infected fish. The fact that abnormal pathology, vacuolation occurred within the central nervous system, it appears that these vacuoles may be caused by different factors (biological or non-biological agents). Furthermore, those abnormal behaviors (of the 18 samples fish) that were described previously, maybe are the symptoms of other infections as those 18 fish are in the same batches with those that were evident with epitheliocystis and parasitic infection. However, answering that question was beyond the scope of this study.

Moreover, other pathogens were also observed during tissue examination. An intracellular bacteria, of unknown origin caused a disease, known as epitheliocystis in the majority of abnormalities observed in gill tissues accounting for 44% (118 fish) of total samples (270) and showed most stages of its developmental cycle, except for primary long cells and intermediate long cells which are considered as its reproductive cells. The bacterial disease epitheliocystis might explain some changes observed in other fish tissue apart from the infected site- the gill. Yet, there have been no reports about the association between epitheliocystis in gill and histological abnormalities in other organs in fish. Previous research about epitheliocystis has focused on the morphology of infected cells and its developmental cycles. Thus, routine histopathology is a powerful and compulsory method for diagnosing epitheliocystis (Draghi et al., (2007) even when the causative agent has not been cultured successfully.

Monogenean and unknown organisms were evident in the fish examined in this project. Monogeneans occurred with a low frequency (36 fish) and were mostly associated with epitheliocystis in this study. The H&E staining produced an adequate result for monogenean detection in this project, but the staining process can cause the loss of monogeneans so could not provide a proper observation for taxonomic identification.

Some unknown organisms were observed in the brain, gill and kidney of the fish but those cases only appeared occasionally in a minor number of fish. However, the identification of this/these unknown agent/s would require the use of a variety of techniques, such as electron microscopy, cell culture and PCR test. Although, these detecting methods are efficient in identifying the two emerging diseases in barramundi (scale drop disease and "big belly" syndrome), the pathological changes and clinical signs that observed in our samples indicates that they are not related to those two diseases. That is why, this work would be interesting for future research in fish pathology.

Interestingly, the appearance of pathogens detected in this project was periodic, and although sampling of larval barramundi occurred monthly, they were collected from different larval batches and disease was only detected every second collection. This instantly raises the question why is it periodic? Is it because the samples for each collection come from different brood stocks? Or is it water treatments (for parasites) were delivered every two months? In addition, telangiectasis, which is characterised by swollen blood vessels within lamella or lamella dilation, was found during this study and it is possible that chemical remnants present in the rearing water may have contributed to this abnormality rather than pathogens, but confirmation would require further study.

In this study, after nine months of regular sampling from West Beach hatchery, there were no positive observations of VNN disease in examined fish using routine histology and IHC test. This may indicate that Betanodavirus infection is less prevalent in that local area and the South Australian's governments management of VNN is highly effective. It could also be because the stocking rate of barramundi is appropriate (not overcrowded) as it is believed that the likelihood for a Betanodavirus infection is high when barramundi is reared in overcrowded populations with poor environmental management. Thus,

conditions for VNN disease occurrence were not occurring at the West Beach hatchery facilities during the period of collecting samples for this project.

Nonetheless, the fact that no infected samples were collected increased the limitation of this study. Only 18 (out of 270) samples (ones that indicated Betanodavirus infection based on pathology observed with H&E) were tested for VNN using IHC (OIE - Manual of Diagnostic Tests for Aquatic Animals, 2019). Thus, it is possible, there are fish with VNN that have not been detected in this work. Although, there are abnormal behaviours in our sampled fish, no deaths were observed. This means the relationship between external disease signs and the 18 fish identified with pathology is unclear. It may be more effective to apply techniques such as transmission electron microscopy in future studies thanks to its high magnification in order to provide a proper examination of fish tissues. However, this was beyond the scope of this study. Methods that require usage of fresh tissues (PCR) cannot be applied for our samples now as we stored them as fixed tissues.

Understanding the pathogenesis of Betanodavirus is still a compelling aspect to complete our current understanding about VNN disease and continues to be an important area of research for industry. In addition, it would be very useful to identify the organism/s that led to the observation of epitheliocystis in our study and future work could be conducted into different methods of detecting this organism, and its association and effect on epitheliocystis on other fish organs warrants further investigation to protect the future of the barramundi fish industry from unwanted disease outbreaks. In 2014, histological analysis combined with IHC test in fixed or fresh tissue to detect viral antigen, was still the valid recommended options for Betanodavirus detection in terms of cost and efficiency by the Australian government (Moody & Crane, 2014). The work described here demonstrates that histology alone is not recommended for detection of Betanodavirus. In future I would also recommend using Real-time RT-PCR to detect

Betanodavirus from brain and eye tissue rather IHC and cell culture to isolate the virus as it requires a little less resources and can accurately detect VNN as outlined in ANZSDP 2014 (Moody & Crane, 2014).

CHAPTER 6: LIST OF REFERENCES AND APPENDIXES

LIST OF REFERENCES

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APPENDIX 6-1: H&E STAINING PROCEDURE

1. Calcification

After removing fins and excess internal fluid, fish were placed in decalcification liquid (DECAL solution) for at least 24h. Then washed under tap water for at least 4h.

DECAL solution: Dissolve 2g EDTA in 1,860 mL of distilled water. Once dissolved add 140mLs of concentrated HCl

2. Processing

Alcohol 70% (1h) -> alcohol 80% (1h)-> alcohol 90%(1h)->absolute alcohol (1h)-> absolute alcohol (1h)-> Chloroform (overnight)-> paraffin (30min)->paraffin (1h)->paraffin (1h)-> embedding.

3. Sectioning

Using microtome to make a 5μm thick slide, 4 slides for 1 sample. All slides were kept in 37^{o}C oven overnight.

4. Staining

Xylene (2min)->Xylene (2min)->absolute alcohol (2min)->90% alcohol (2min)->80% alcohol (2min)->70% alcohol (2min)- deinonized water (2min)->Haematoxylin (10min)-> tap water until clear-> Acid-alcohol (2min)-> Lithium carbonate (2min)-> deionized water (2min)-> Eosin (3min)-> tap water until clear-> absolute alcohol (1min) for 3 times->Xylene (2min) 2 times-> coverslip with mounting medium.

APPENDIX 6-2: IMMUNOHISTOCHEMISTRY TEST FOR BETANODAVIRUS

1. Principle of the test

This test uses polyclonal antibodies raised in sheep against the recombinant coat protein of a barramundi or sleepy cod Betanodavirus isolate and an anti-sheep IgG horseradish peroxidase conjugated secondary antibody, to localise viral coat protein in histological sections containing nervous tissue of finfish. The test is used to confirm or exclude Betanodavirus as the agent causing lesions observed in H&E stained sections, or to diagnose Betanodavirus infection in tissue sections in the absence of histology expertise.

This protocol is from Moody & Crane (2014). More detail can be found at Australian Government Department of Agriculture, Water and the Environment, Betanodavirus infections of finfish, accessed 17 February 2021, https://www.agriculture.gov.au/animal/health/laboratories/procedures/anzsdp/Betanodavirus-finfish.

2. Reagents

- Polyclonal antibody

  The primary sheep anti-BNNV rCP or sheep anti-SCNNV rCP polyclonal antibody is available from the address of the authors at the end of this protocol.
- Peroxidase conjugated secondary antibody.

  Commercial anti-sheep IgG [H+L] horseradish peroxidise conjugate can be used. New batches should be tested using positive and negative control slides.
- Tris buffered saline (TBS; 20mM Tris, 500mM NaCl)

Tris	9.68 g
NaCl	116.9 g
Deionised water	4L

- 0.1% trypsin in TBS

Trypsin (1:250)	0.2 g
TBS	4L

- 3% H_2O_2 in methanol

H_2O_2	6.0 mL
Methanol	200 mL

- 5% bovine serum albumin (BSA) in TBS

BSA	2.5 g
TBS	50 mL

- 2.5 % BSA in TBS

BSA	1.25 g
TBS	50 mL

- ImmunoPure ®Metal Enhanced DAB Substrate Kit (Pierce, USA)
- Deionised water
- Mayer's haematoxylin

Aluminium ammonium sulphate	10 g
Deionised water	200 mL
Haematoxylin	0.2 g
Sodium iodate	0.4 g
Citric acid	0.2 g
Chloral hydrate	10 g

Dissolve the aluminium sulphate in the distilled water using a magnetic stirrer and large stir bar. Do not heat. When completely dissolved, add the haematoxylin. Once the haematoxylin is completely dissolved, add in the following order: sodium iodate, citric acid and chloral hydrate. Ensure that all chemicals are completely dissolved.

- Lithium carbonate

Lithium carbonate	2.8 g
Deionised water	200 mL

- Mounting medium

3. Equipment

- Deparaffinised, rehydrated tissue sections mounted on positively charged glass histology slides.
- Hydrophobic marker
- Humidified 37°C chamber
- Compound microscope

4. Test procedure

a) Circle the tissue sections with a hydrophobic pen marker. Tissue sections should not be allowed to dry at any stage

b) Add 1 mL 0.1 % trypsin to each tissue section and incubate at 37ºC for 30 minutes in a humidified chamber

c) Wash three times with TBS

d) Block endogenous peroxidase by immersing the tissue sections in 3 % H_2O_2 in methanol at room temperature for 20 minutes

e) Wash three times with TBS

f) Block non-specific binding sites by incubating each tissue section with 1 mL 5% BSA in TBS in a humidified chamber at 37ºC for 20 minutes.

g) Wash three times with TBS

h) Add 1 mL Sheep®α-NNV rCP polyclonal antibody, diluted 1/1000 in 2.5 %

i) BSA in TBS, to each tissue section and incubate in a humidified chamber at 37ºC for 60 minutes

j) Wash three times with TBS

k) Add 1 mL of Rabbit®α-Sheep IgG [H+L] HRP conjugate, diluted 1/1000 in 2.5% BSA in TBS, to each tissue section and incubate in a humidified chamber at 37ºC for 60 minutes

l) Wash three times with TBS

m) Prepare the ImmunoPure® Metal Enhanced DAB Substrate Kit according to the manufacturer's instructions. Add 1 mL to each tissue section and incubate for 10 minutes at room temperature. Stop development by immersing the slides in deionised water.

n) Counterstain tissue sections with Mayer's haematoxylin for 60 seconds, rinse in tap water for 60 seconds, blue in lithium carbonate for 60 seconds and rinse in tap water for 60 seconds

o) Mount tissue sections under a coverslip using an aqueous mounting medium and examine with a compound microscope.

5. Quality control aspects

Positive and negative control slides must be included in every test. Ideally, a positive slide showing a low level of infection should also be included.

For the test to be valid, dark brown or black staining of neuronal cells must be observed in the nervous tissue of the spinal cord, brain and/or retina of the positive control slides No specific staining should be seen in the negative control slide. Some non-specific staining may be seen in the stomach.

6. Interpretation

Any positive staining indicates the presence of the Betanodavirus coat protein and the fish is considered to be undergoing an active infection. When a single fish from a larger group is positive in an IHCT, that is sufficient evidence to consider the entire group is infected.

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APPENDIX 6-3: RECODING TABLE OF PARASITES AND EPITHELIOCYSTIS

Number of samples	Epitheliocystis	Fluke
1	1	0
2	3	0
3	2	0
4	2	0
5	2	0
6	1	0
7	2	0
8	1	0
9	1	0
10	1	0
11	2	0
12	1	0
13	1	0
14	2	2
15	1	0
16	3	3
17	1	0
18	2	0
19	1	0
20	3	3
21	1	0
22	0	0
23	1	0
24	1	0
25	2	0
26	0	0
27	1	1
28	2	1
29	1	0
30	1	0
31	0	0
32	0	0
33	0	0
34	0	0
35	1	0
36	0	0
37	0	0
38	0	0
39	0	0
40	0	0
41	0	0
42	0	0
43	0	0
44	0	0
45	0	0
46	0	0
47	0	0

48	0	0
49	0	0
50	0	0
51	0	0
52	0	0
53	0	0
54	0	0
55	0	0
56	0	0
57	0	0
58	0	0
59	0	0
60	0	0
61	2	0
62	2	0
63	1	0
64	3	0
65	2	0
66	3	1
67	1	0
68	3	1
69	2	0
70	1	0
71	1	0
72	1	0
73	2	0
74	0	0
75	2	0
76	1	0
77	1	0
78	3	0
79	1	0
80	1	0
81	2	0
82	1	0
83	2	0
84	3	0
85	2	0
86	2	0
87	3	0
88	2	0
89	0	0
90	0	0
91	0	0
92	0	0
93	0	0
94	0	0
95	0	0
96	0	0
97	0	0

98	0	0
99	0	0
100	0	0
101	0	0
102	0	0
103	0	0
104	0	0
105	0	0
106	0	0
107	0	0
108	0	0
109	0	0
110	0	0
111	0	0
112	0	0
113	0	0
114	0	0
115	0	0
116	0	0
117	0	0
118	0	0
119	0	0
120	0	0
121	0	1
122	2	0
123	0	0
124	1	1
125	1	2
126	1	0
127	2	0
128	2	0
129	1	0
130	3	1
131	1	0
132	2	1
133	2	1
134	3	0
135	3	1
136	3	0
137	2	0
138	2	0
139	1	0
140	2	1
141	1	0
142	3	0
143	2	0
144	3	0
145	3	2
146	3	1
147	2	0

148	3	1
149	3	1
150	3	0
151	0	0
152	0	0
153	0	0
154	0	0
155	0	0
156	0	0
157	0	0
158	0	0
159	0	0
160	0	0
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162	0	0
163	0	0
164	0	0
165	0	0
166	0	0
167	0	0
168	0	0
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171	0	0
172	0	0
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175	0	0
176	0	0
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183	0	1
184	0	1
185	1	0
186	0	0
187	0	0
188	0	0
189	0	0
190	0	0
191	0	0
192	2	0
193	1	0
194	0	0
195	0	1
196	0	0
197	0	0

198	0	2
199	1	1
200	2	0
201	1	0
202	0	0
203	1	0
204	2	0
205	1	1
206	0	0
207	0	1
208	0	1
209	2	0
210	1	1
211	0	0
212	0	0
213	0	0
214	0	0
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233	0	0
234	0	0
235	0	0
236	0	0
237	0	0
238	0	0
239	0	0
240	0	0
241	0	0
242	0	0
243	2	1
244	2	1
245	1	1
246	1	0
247	1	1

248	1	0
249	2	0
250	2	0
251	1	1
252	1	0
253	2	0
254	1	0
255	2	0
256	0	0
257	1	0
258	0	0
259	0	0
260	1	0
261	1	0
262	0	0
263	0	0
264	1	1
265	1	1
266	1	1
267	2	0
268	1	0
269	2	0
270	1	0