Ostreid herpesvirus-1 infection in Pacific Oysters (*Crassostrea gigas*) - New Zealand

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Abstract

Ostreid herpesvirus-1 (OsHV-1) was associated with summer mortalities in New Zealand Pacific oysters in 2010-2011. During the mortality investigation, a cohort of Pacific oyster spat, negative with OsHV-1 from a South Island hatchery, were followed forward after transfer to a grow-out farm with high oyster mortalities in the North Island. One important finding in this short longitudinal study was the temporality of OsHV-1 nucleic acid detection by real time PCR assay and onset of Pacific oyster mortality. The research described in this thesis was undertaken to further support the causal link between OsHV-1 infection and oyster mortality. To achieve this aim, an *in situ* hybridisation (ISH) assay was developed to elucidate OsHV-1 infection in Pacific oysters collected from the same short prospective study. OsHV-1 presence and distribution in spat indicated by ISH signal were then correlated with the existence of any histopathological findings in oyster tissues.

Hybridisation of the labelled probe with the target region in the OsHV-1 genome on infected cells produced dark blue to purplish black cell precipitates during colorimetric detection. *In situ* hybridisation signals were seen predominantly in the stroma of the mantle and gills at day 5. Towards day 7 and 9, OsHV-1 infected cells were distributed in various tissues as indicated by the widespread distribution of ISH signals. Histopathological abnormalities were mostly non-specific, however, a progressive pattern of focal and mild to widespread haemocytosis seemed to coincide with the appearance of OsHV-1 infected cells in spat collected at different time-points. The results of this study further supported the view that OsHV-1 was causally involved in summer mortalities observed in farmed oysters in New Zealand. Further studies to

elucidate OsHV-1 pathogenesis in Pacific oysters in association with other causal variables such as elevated temperature are recommended.

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1 Introduction

The Pacific oyster (*Crassostrea gigas*, Thunberg, 1793) is the most dominant shellfish species for aquaculture farming worldwide. According to the Food and Agriculture Organisation of the United Nations (2005), the global output of Pacific oyster in 2003 was about 4.4 million tons. China produces 84% of the total production followed by Japan, Korea and France, with more than 100 thousand tons of oyster per year from each country. Pacific oysters are native to the Western Pacific, along the coast of Japan, Korea and China, and their cultivation has been part of Japanese culture for centuries (Aranishi & Okimoto, 2004). *Crassostrea gigas* was introduced to Europe, North America and Australia to replace dwindling native oyster stock (Miossec, Le Deuff *et al.*, 2009b). As an example, the Pacific oyster was transplanted from Japan to France in the 1960s as a substitute for the diminishing population of Portuguese oyster, *Crassostrea angulata*, in decline due to iridoviral infection (Renault, 1996). Other countries intentionally introduced *C. gigas* as a new species for experimental cultivation, like Brazil and Argentina, although it was later discontinued (Miossec *et al.*, 2009b).

The Pacific oyster has also been translocated accidentally. In New Zealand, Pacific oysters were inadvertently introduced by trading ships during the late 60s and was discovered for the first time at Mahurangi harbour in 1971 (Dinamani, 1971). After a few years, Pacific oysters were detected in Four Fathom Bay of the South Island (Jenkins & Meredyth-Young, 1979). The Pacific oyster is now the major species for aquaculture in New Zealand, dislodging the native "northern rock oyster" (*Saccostrea glomerata, Gould, 1850*), due to its hardiness and faster growth rate to market (Dinamani, 1987).

1.1 Mass Mortalities

Mortality events in Pacific oysters have been a frequent phenomenon for the past several decades in Japan (Mori, Imai *et al.*, 1965), Europe (Cotter, Malham *et al.*, 2010; Goulletquer, Soletchnik *et al.*, 1998) and North America (Chávez-Villalba, Villelas-Ávila *et al.*, 2007; Friedman, Beattie *et al.*, 1991). Mortalities usually occur in summer, thus commonly referred to as "summer mortalities" (Davis, Downing *et al.*, 1988). Summer mortality events can be loosely categorised into two different clinical observations. The earliest descriptions according to Davis *et al.* (1988), pertain to mortality rates of more than 30%, and are normally seen in adult Pacific oysters, two years of age and older. On the other hand, summer mortalities of about 80-100% in the last two decades, largely involve spat or young Pacific oysters (Miossec, Gwenhael *et al.*, 2009a).

One of the earliest risk factors investigated with regards to adult oyster mortality in summer is the physiological and metabolic demand of reproduction (Imai, Mori *et al.*, 1969; Mori *et al.*, 1965). Characteristics of high gonad activity with extremely low levels of glycogen reserves was histologically evident in Pacific oysters from area of high mortalities (Berthelin, Kellner *et al.*, 2000). In the same study by Berthelin *et al.* (2000), oysters gathered from locations of low mortalities had less gonad volume with a relatively rich amount of glycogen. A high gonad to glycogen reserve ratio was reported in Pacific oysters affected by mortality events in North America in the late 1960s (Lavoie, 2005). Lavoie (2005) believed that the decline in mortality rates in the mid 70's was attributed to natural selection of physiologically adapted populations of Pacific oysters. Similarly, recent genetic studies using a divergent selection format for Pacific oysters (Huvet, Normand *et al.*, 2010) showed that a resistant line of Pacific oysters had significantly smaller gonadal area in

comparison to the susceptible line of oysters. Hence, this difference in reproductive effort among oysters, although not directly related to other causal factors to summer mortalities, is being studied as a basis for selection of genetically resistant lines (Huvet *et al.*, 2010).

The reproductive cycle of oyster bivalves is believed to be driven by two factors, namely, trophic resource abundance or phytoplankton biomass in the water column (Lambert, Moal *et al.*, 2008) and temperatures of around 19°C (Moal, Lambert *et al.*, 2008). An abundant, good quality food supply in spring stimulates the oyster to invest in production of gonadal tissue, leading to high gametogenic activity in summer (Enríquez-Díaz, Pouvreau *et al.*, 2009). However, the metabolic cost of gamete formation and eventual expulsion during spawning is very demanding, and requires mobilisation of energy reserves, particularly glycogen (Pouvreau, Bourles *et al.*, 2006). As a consequence, a state of energy deficit exists in oysters, which is further exacerbated by their inability to readily assimilate nutrition during the post spawning stage (Lambert *et al.*, 2008). Carbohydrate depletion is a very precarious state for Pacific oysters, making them vulnerable to the effect of other stressors.

The detection of herpesvirus-like particles in Pacific oysters in the early 1990s changed the clinical profile of summer mortality events in this species. In comparison to the "summer mortality" syndrome in adult oysters, mortality events associated with herpesvirus-like infection, later named as Ostreid herpesvirus-1 (OsHV-1), affected larvae, spat and young oysters (Garcia, Thebault *et al.*, 2011; Hine, Wesney *et al.*, 1992; Nicolas, Comps *et al.*, 1992). Massive die-offs occurring in spat, was also referred to as "summer seed mortality" (Burge, Judah *et al.*, 2007).

1.2 Pacific oyster mortality outbreak in New Zealand

New Zealand was not spared from this phenomenon as high mortalities in juvenile Pacific oysters *(Crassostrea gigas)* have been investigated during the summer months of 2010-2011 by the Investigation and Diagnostic Centre & Response of the Ministry for Primary Industries (Bingham, Brangenberg *et al.*, 2013; Johnston, Keeling *et al.*, 2011). The disease epidemic affected 73% of oyster growing areas in the north of the North Island and was characterized by an acute onset of mortalities, particularly in cohorts of recently deployed spat. Mortalities were estimated to be between 15-100% in spat and juveniles and between 5-60% in adult oysters (Bingham *et al.*, 2013). Moribund animals showed gaping behaviour during emersion and a slow or reduced closing reaction to gentle-tap stimuli during immersion (Johnston *et al.*, 2011). Various risk factors were implicated including elevated seawater temperature, decreased water quality as well as various disease causing organisms exotic to New Zealand (Bingham *et al.*, 2013) . One of the important findings was the high prevalence of OsHV-1 nucleic acid, detected by PCR, in farms experiencing mortalities. In addition, *Vibrio* spp. *(V. splendidus*, *V. aesturianus, V. alginolyticus V. chagassi, V. alginolyticus)* bacteria were consistently isolated from the same affected farms, suggesting that a possible co-infection may have contributed to the mortality syndrome (Keeling, Brosnahan *et al.*, 2014). Information on concurrent risk factors such as temperature, salinity or phytoplankton blooms that can be associated with oyster mortality, however, was very limited (Bingham *et al.*, 2013).

1.3 Interaction between mortality risk factors

Although, the recent outbreak of Pacific oyster mortality in New Zealand was directly associated with the presence of OsHV-1 (Bingham *et al.*, 2013), the extent of

summer mortality events are often due to an inter-relationship of environment, pathogen and host specific factors (EFSA Panel on Animal Health and Welfare (AHAW), 2010). Because of such complexities involving multiple risk variables, elucidation of aetiological causation in this setting is very challenging. Making a diagnosis becomes difficult especially when virus detection occurs for the first time in previously considered naïve population, and or if the viral pathogen, when present, does not have immediate consequences for the target host. A correlation between OsHV-1 DNA prevalence and high oyster mortalities should be corroborated by other diagnostic tools to support diagnosis of infection.

The research described in this thesis was undertaken in parallel with the juvenile oyster mortality events as a follow up of the investigation into the epidemic Pacific oyster mortality in New Zealand. It aimed to further support the causal link between OsHV-1 infection and oyster mortality. More specifically, an *in situ* hybridisation (ISH) technique was developed to elucidate OsHV-1 infection in Pacific oyster from areas experiencing high mortalities. Using a DIG labelled DNA specific probe, sites of early viral infection and target tissues were described. Results of OsHv-1 presence and distribution were then correlated with the existence of any histopathological findings in oyster tissues and other available information to infer causality.

In Chapter 2 of the thesis, a brief review on the biology and diseases affecting Pacific oyster particularly those associated with OsHV-1 infection, is presented. Chapter 3 deals with methodology including description of the short longitudinal study conducted to detect OsHV-1 and infer the role of pathogens during the mortality investigation. In this chapter methods used to develop and validate ISH assay to detect OsHV-1 in Pacific oyster tissues are described, as well as design of a short prospective study to investigate the association between OsHV-1 infection and disease. Chapter 4

includes all the findings and results of this study while chapter 5 concludes the thesis with a general discussion of the results. Unless stated otherwise, the term oyster mentioned through-out the paper refers to Pacific oyster.

2 Review of Literature

2.1 Introduction

The ubiquitous Pacific oyster is very hardy and can establish in a wide range of marine conditions, creating reefs of wild base populations in new regions. However, in spite of the oyster's trait of extreme tolerance to temperature, salinity, suspended sediments and dissolved oxygen, it has not been spared from mass mortality events (Miossec *et al.*, 2009b). Massive die-offs in this bivalve mollusc, a commercially important species, often lead to severe economic repercussions, especially in major Pacific oyster producing nations (Goulletquer *et al.*, 1998).

Multiple risk factors are believed to be involved in recent Pacific oyster mortality occurrences in Europe, USA and Australia (*Final report OsHV-1µVar International OsHV-1 Workshop*, 2011). Ostreid herpesvirus-1 was considered to be a necessary cause in these mortality events, in correlation with other primary risk factors such as high water temperature (Burge, 2010), oyster genetic variability (Samain, Dégremont *et al.*, 2007), husbandry practices (Peeler, Allan Reese *et al.*, 2012) as well as the presence of other pathogens such as *Vibrio* sp. bacteria (Friedman *et al.*, 1991; Saulnier, De Decker *et al.*, 2010). Other external factors aggravating the intensity of oyster losses include water quality and food availability (Malham, Cotter *et al.*, 2009), environmental stresses (Soletchnik, Lambert *et al.*, 2005), including artificial eutrophication (Mori, 1979) and harmful algae (Kondo, Nakao *et al.*, 2012),

Although the intricate interplay of multiple factors differs in various marine settings, a model depicting these relationships (**Figure 1**) was proposed by Samain et al.

(2008). This interaction model was derived from the results of years of multidisciplinary research collaboration in France, initially to investigate the physiological basis of summer mortality and to deconstruct the relative influence of high water temperature, nutrition, environment and oyster genetics on the occurrence of mortality events. The suggested model hypothesised that high quantities of available food materials, such as phytoplankton or detritus in the water column, occur as a result of organic materials flowing from watersheds into estuarine environments (Samain & McCombie, 2008). This abundant source of nutrition provides an increased stimulus for reproductive development in Pacific oysters during spring. Certain genetic stains of *C. gigas* undergo substantial gonadal development without accumulating enough glycogen reserves (Samain, Degremont *et al.*, 2006). Gonadal maturation and spawning usually occurs in summer and especially when a threshold temperature of 19°C is reached. This reproductive process of gametogenesis and release is metabolically demanding, such that susceptible strains of Pacific oysters with low energy reserves are highly vulnerable to other stressors and opportunistic pathogens (Samain *et al.*, 2006). What has been difficult to determine is the relative contribution of these stressors to the demise of susceptible populations.

This chapter provides a review of several infectious organisms that have been linked to the occurrence of massive losses of Pacific oysters. The first section presents briefly the biology of Pacific oyster, followed by a review of viral diseases affecting bivalves, particularly *C. gigas*. In the last section a review of our current knowledge on OsHV-1 and the various methods of diagnosing this infection is presented. Recognised causal variables in the interaction model, although considered important in diagnosis, are beyond the scope of the literature review.

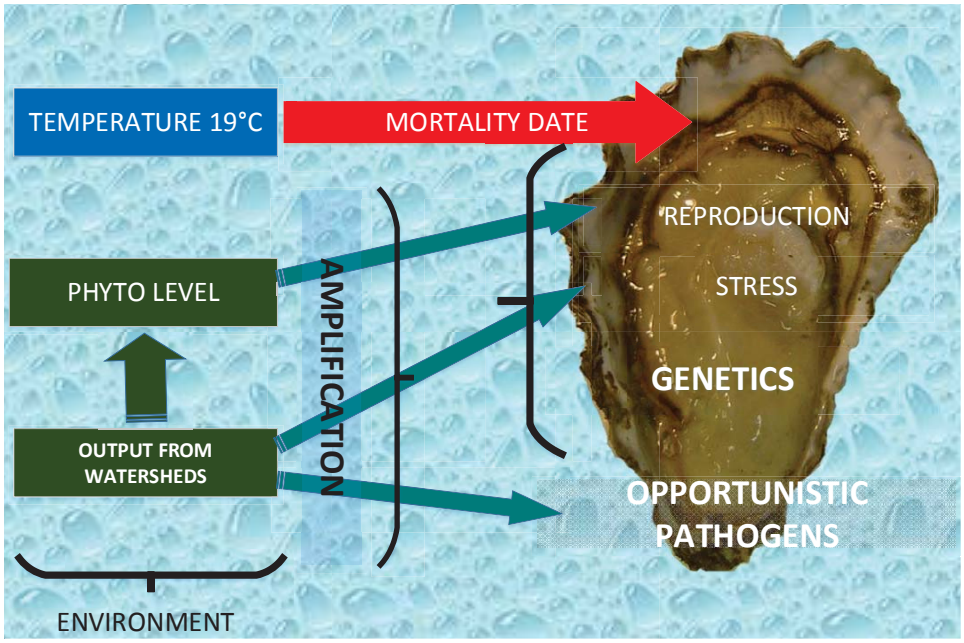


Figure 1. Interplay of various factors in precipitating mortality events in Pacific oysters. Adopted from Samain *et al*. (2008)

2.2 Pacific oyster

Crassostrea gigas (Thunberg, 1793) (*Mollusca: Ostreidae*) is commonly known as "Pacific oyster" or "Pacific giant oyster". Other familiar names of *C. gigas* are Japanese oyster, Pacific cupped oyster and Miyagi oyster.

2.2.1 Basic anatomy

Galtsoff (1964) described extensively the anatomy and physiology of eastern oyster *Crassostrea virginica*, comparatively similar to *C. gigas*. The shell is composed of two valves (Figure 2). The right valve is the smaller, slightly convex upper valve. The right valve covers the larger, cup shaped lower or left valve. Unless grown singly in a commercial growing set up, the left valve is normally cemented to a hard surface

in the substratum or onto wooden sticks found in growing farms. Morphologically, the shell surface of the Pacific oyster is rough, elongated and highly variable in conformation. According to Miossec *et al.* (2009b), oyster shape is highly dependent on how they sit together with adjacent marine bivalves or other materials in their natural environment. The adult lengths vary between 200 to 300 mm (Miossec *et al.*, 2009b).

Figure 2. The left image is a side view of a Pacific oyster showing the cup shape lower valve while on the right is a top view of the slightly convex upper valve.

Removal of the right valve reveals the soft anatomy of an oyster cradled by the left valve (Figure 3). Two fleshy mantle folds envelope and contain the visceral mass of the oyster (Eble & Scro, 1996). The mantle has several functions including shell secretion, screening particles for ingestion, storage of glycogen, gamete dispersion as well as internal defence via mucous secretions (Grizel, 2003). Crossing the body cavity is the highly visible adductor muscle, responsible for opening and closing the shell.

Between the right and left mantle skirt, the pallial cavity is formed, where a pair of gills can be found at one end and a pair of labial palps on another end, adjacent to the hinge. The gills, in concert with the mantle edges and shell opening-closure function of adductor muscle, regulate the water flow going inside the bivalve for feeding, respiration and excretion (Galtsoff, 1964). Gill filaments, generate cilliary currents to direct food particulates towards the labial palps for ingestion into the oral orifice (Grizel, 2003). Gonads are large, diffusely scattered and make up most of the oyster's bulk during reproductive development (Miossec *et al.*, 2009b).

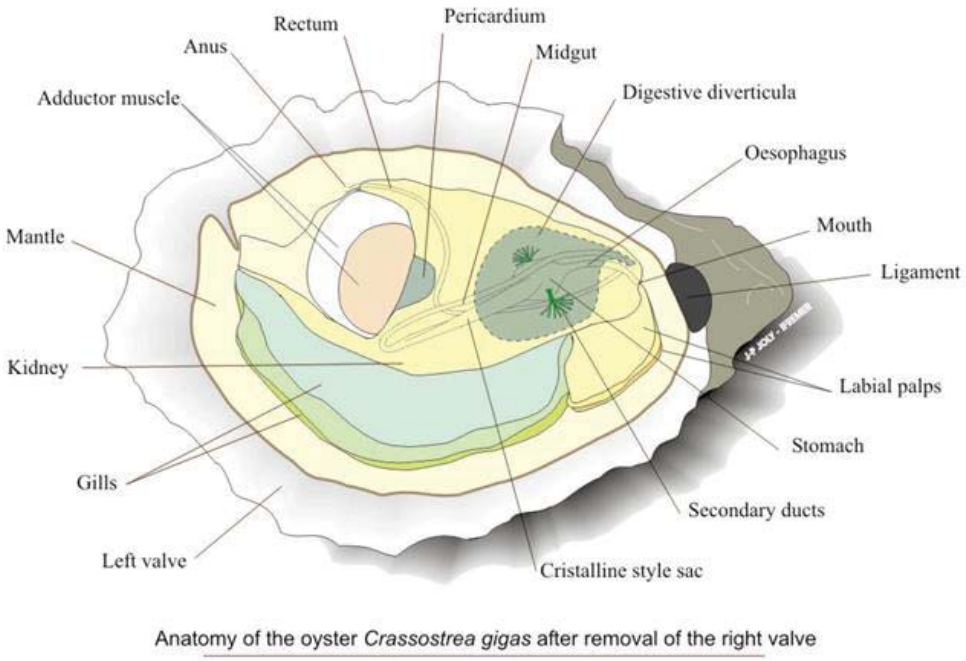


Figure 3. Anatomy of Crassostrea gigas (European Union Reference Laboratory, 2013).

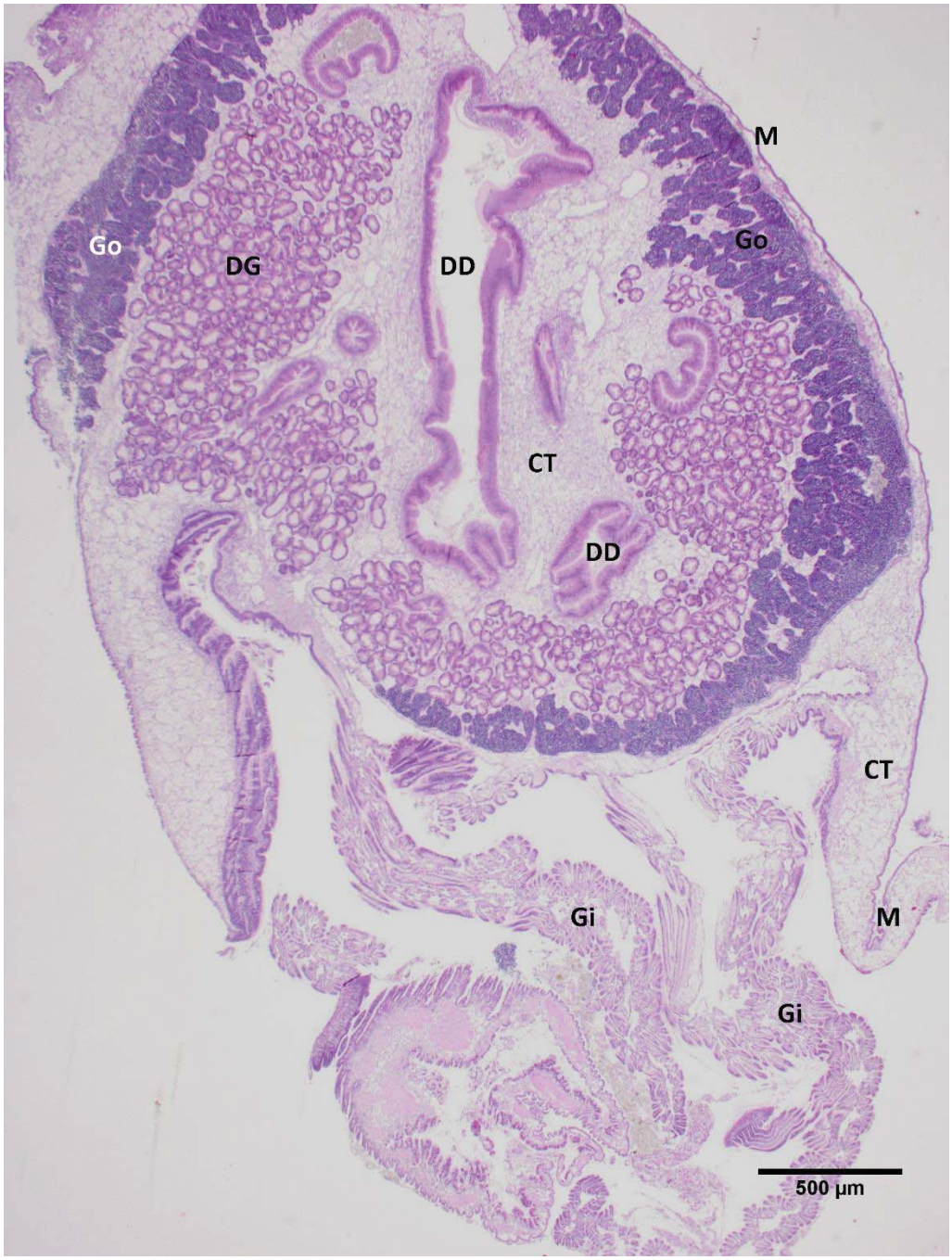


Figure 4. A typical transverse section of a Pacific oyster spat stained with H & E, Go - gonad, DG - digestive glands, DD - digestive ducts, CT - connective tissue, M - mantle, Gi - gills (scale bar - 500 µm).

A typical transverse histology section of *C. gigas* spat (Figure 4) may include various organs such as the mantle, the outer covering of the oyster, gills (Figure 4 and Figure 5), and major sections of the stomach, intestines and the digestive glands or diverticula (Figure 6). The gonads (Figure 7) or the germinal reproductive centres, usually male at initial stage, surround the digestive system and occupy a large part of the visceral mass. In between these major organs is a network of structural elements referred to as vesicular cells by Galtsoff (1964). Vesicular cells are large globular or oval body with small nucleus. Embedded within the vesicular connective tissue, are the vascular ducts (Figure 8) and a nerve tissue (Figure 9). Haemocytes (Figure 10) were commonly observed within the vascular duct, stroma of the connective tissues or around the basal membrane of the intestinal epithelium (Figure 11), the mantle and gills. Occasionally in some oyster sections, the heart, the adductor muscle and the labial palps were included.

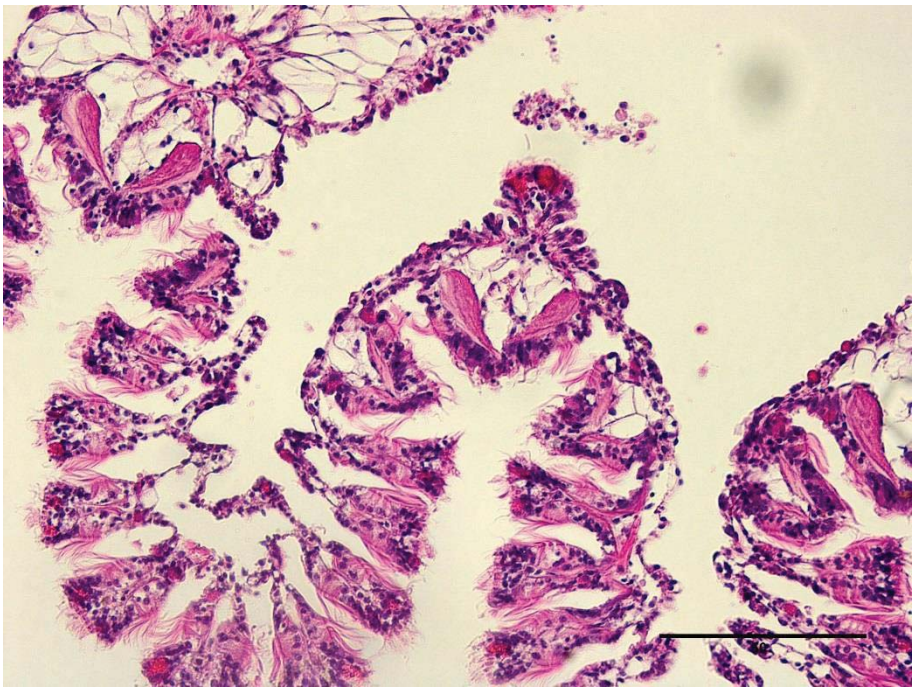


Figure 5. Normal gill section in H & E (scale bar = 50 µm).

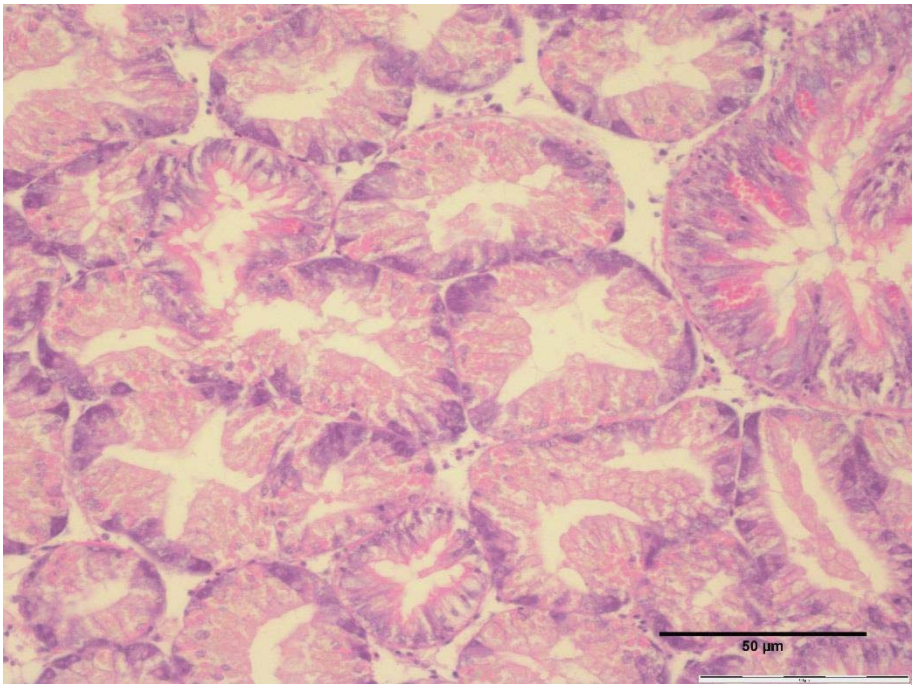


Figure 6. Normal didestive gland or diverticulae with star shape lumen, H & E (scale bar = 50 µm).

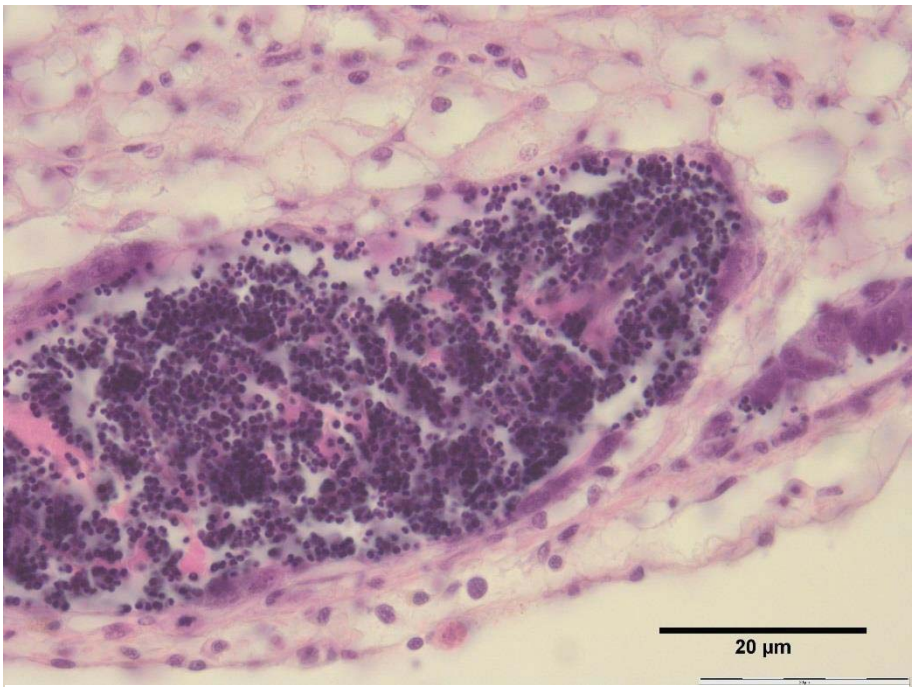


Figure 7. Male gonad containing spermatocytes and a germinal reproductive centre, H & E (scale bar = 20 µm).

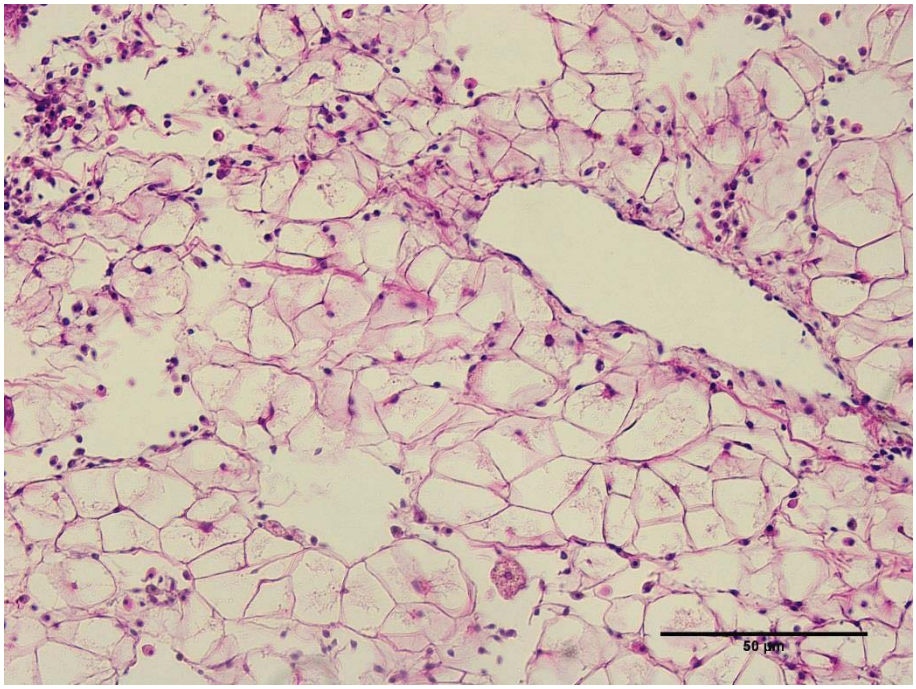


Figure 8. Connective tissue stroma containing vesicular cells which surround a haemovascular duct, H & E (scale bar = 50 µm).

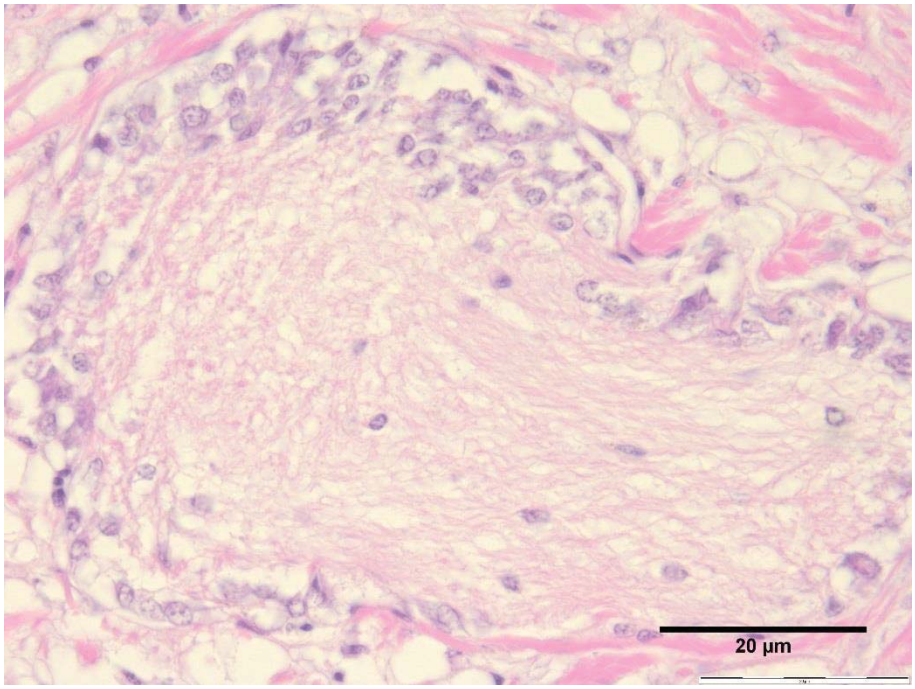


Figure 9. A nerve tissue embedded in the stroma of the mantle, H & E (scale bar = 20 µm).

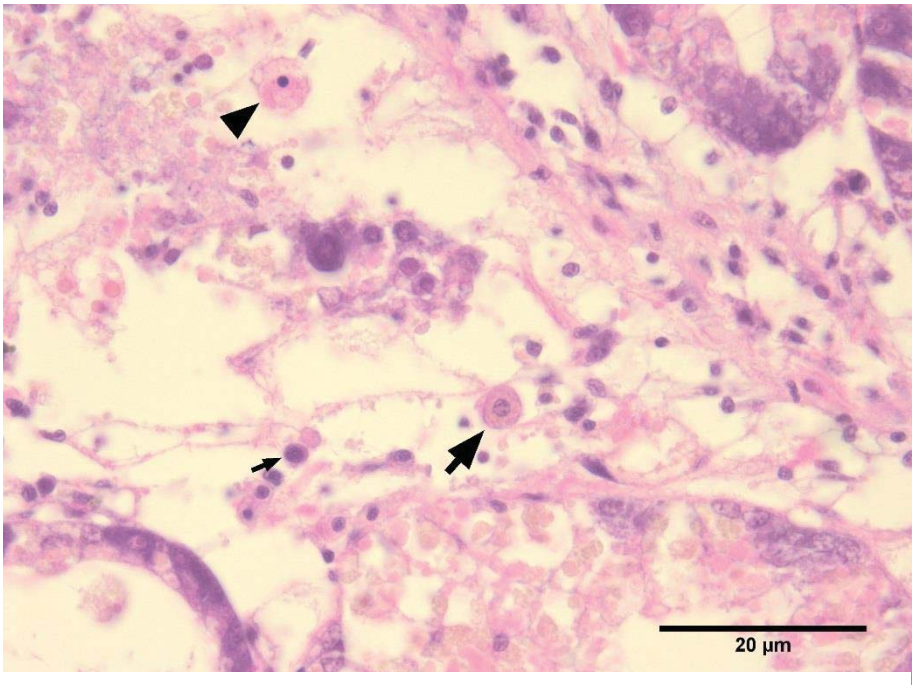


Figure 10. Different types of haemocytes located in the interstitial connective tissue (small arrow - small haemocyte with high nucleus to cytoplasm ration; big arrow - an agranulocyte; arrowhead - a granulocyte), H

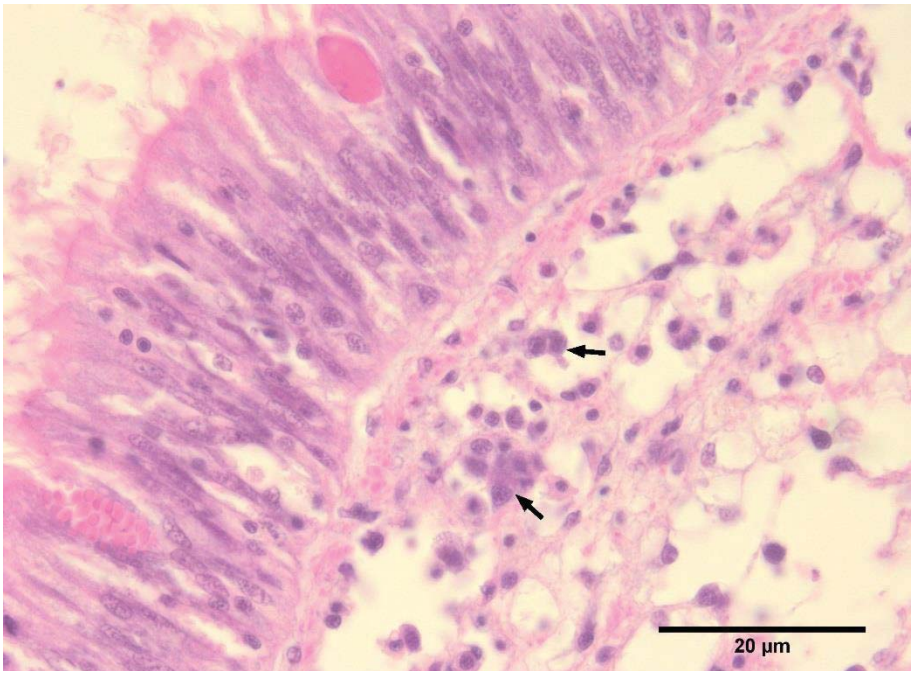


Figure 11. Epithelial lining of the digestive tract consisting of columnar cells with haemocytes (arrows) aggregating beneath the basal membrane, H & E (scale bar = 20 µm).

2.2.2 Reproduction

The Pacific oyster is a protandric hermaprodite, switching from an initial release of male gametes (male sex) on first spawning to becoming female for subsequent years and for the succeeding release of gametes. Gametogenesis is generally dependent on temperature intensity and time rather than a certain temperature threshold (Steele & Mulcahy, 1999). For example during experimental wintering conditions, eight months was needed to ripen oysters (state when oysters are ready for spawning) at a temperature range of 8-10°C (Fabioux, Huvet *et al.*, 2005). Spawning of *C. gigas* usually occurs within a temperature range of 18-22°C. (Miossec *et al.*, 2009b). The reproductive capacity of the female form is very high, producing 100 million eggs per release into the water column, where fertilisation occurs. After fertilisation, large numbers of fertilized eggs develop into free swimming larvae. At 2-3 weeks of age and when conditions are favourable, larvae settle and anchor on hard surfaces where they metamorphose into juvenile oysters, commonly known as spat (Miossec *et al.*, 2009b). The etymology of the term "spat" is uncertain and it is used to refer to both the spawn of shellfish, oysters in particular, as larvae and to juveniles up to 25 mm in length. It is almost invariably applied as a plural and implies a large and relatively uncountable number. In common usage, oyster spat can either refer to the larval form or juvenile to sub-adult bivalve shellfish. In natural environment it is more common to refer to spat as being attached to a substrate, although this can be temporary in the case of recently settled stock, versus free living in the ocean.

2.2.3 Nutrition

Similar to other bivalves, the Pacific oyster is a filter feeder. Bivalves filter various food materials such as phytoplankton, zooplankton and dissolved organic

materials in the water column. Particulates trapped in the gills are directed towards the palps for sorting and unwanted materials are rejected as pseudofaeces (Gosling, 2002). Food ingested via the mouth are digested and expelled as faeces.

An oyster filters about 0.5 litres of seawater per hour (Bougrier, Geairon *et al.*, 1995). This method of nutrient assimilation enables bivalves to ingest food and inorganic particulates currently suspended in the water column, including microorganisms, some of which may be harmful to them (Negri, Bunter *et al.*, 2004).

2.2.4 Immunity

The defence mechanism of bivalve molluscs against foreign invaders is similar to innate immune mechanism in mammals in having a cellular and humoral components, but without the capability to develop acquired immunity (Bachère, Mialhe *et al.*, 1995). Cellular defence function is carried out by haemocyte cells that are present in the haemolymph. Haemolymph is the fluid equivalent to blood, that circulates to an open vascular systems and tissues of bivalves (Auffret, 1988). According to a review made by Fisher (1986) and Hine (1999), the haemocyte cell has two basic function as a result of its phagocytic activity, digestion of nutrients and defence against invading microorganisms. Haemocytes are responsible for recognition and phagocytosis of ingested material, including potential pathogens in the digestive glands, followed by digestion and release of nutrient in the different tissues. At the same time, this cell type removes unwanted material or debris and carries it out into the lumina of digestive ducts for excretion through the process of diapedesis. A certain degree of excretion in this manner can occur in the epithelium of the mantle and gills (Fisher, 1986). As a consequence of this function, haemocyte may be able to spread infectious organisms such as viruses easily.

Haemocytes in bivalves are broadly classified into two morphological cell types, granulocytes and the agranulocytes (Hine, 1999). Granulocytes contain granules in the cytoplasm in varying amounts. These granules in *C. virginica,* are characterised as containing B-glucuronidase, acid, phosphatase, alkaline phosphatase, lipase, aminopeptidase, and lysozymes that can enzymatically digest particles, including pathogens. During the exocytosis or degranulation process these enzymes are released in the haemolymph with cidal properties against bacteria and other microbes (Fisher, 1986). On the other hand, agranulocytes, also known as hyalinocytes, are haemocytes with clear cytoplasm and with very minimal granules. Although agranulocytes are believed to have a phagocytic function as well, the exact role of this cell group is unclear.

Recently, the haemolymph of *C. gigas* was demonstrated to contain a soluble protein, cavortin, with an anti-viral activity against herpes simplex virus 1 replication (Green, Robinson *et al.*, 2014b). Pacific oysters also expressed genes homologous to virus recognition receptors, TLR (toll like receptor) and MDA5, after 24 hours following the primary injection of double stranded ribonucleic acid (dsRNA) (Green, Benkendorff *et al.*, 2014a). In the same study by Green *et al.* (2014a) putative anti-viral signalling molecules (IRF & SOC-1) and effector (PKR, viperin & Mpeg1) genes were up-regulated up to 168 hours after inoculation. A synthetic viral analogue, poly I:C, also induces the expression of TLR3 like homologue, if injected to Pacific oysters (Green & Montagnani, 2013).

The expression of TLR, signalling molecules and antiviral effector genes in *C. gigas* were hypothesised to be analogous to the type-1 interferon response of vertebrates (Green & Montagnani, 2013). In vertebrate animals, the initial recognition of invading pathogens is a function of "sentinel cells" such as macrophages, dendritic cells and mast cells. These sentinel cells possess pattern recognition receptors (PAMS)

that can detect pathogen associated molecular patterns unique to bacteria, fungi and viruses. Pattern recognition receptors, also called toll like receptors can be found on cell surfaces to identify extracellular invaders or within cells to spot viruses. Binding of TLRs and PAMS triggers a series of signalling system that induces inflammation and other mechanism to defend the body against foreign invaders.

Pauley and Sparks (1966) demonstrated the acute inflammatory reaction of Pacific oysters in response to turpentine injection. It was shown in this study that the pathological process of inflammation is similar to vertebrate animals but without the manifestation of reddening and heat. Histological changes as a reaction to the irritant within few hours (8-16 hours) include oedema, general infiltration of leucocyte or haemocyte, congestion of vascular channels and cellular migration towards the site of injection. A characteristic walling-off with thick layers of haemocytes around the wound was seen after 48 hours. Multi-nucleated giant cells and diapedesis of haemocytes across the gut epithelium was noted after 64-72 hours. Giant cells are described to be aggregates of haemocytes containing engulfed necrotic debris and it is a normal component of post mortem change (Sparks, 2012).

2.2.5 New Zealand Pacific Oyster Industry

The Pacific oyster growing areas of New Zealand are primarily located in the Northland, Auckland and Coromandel regions of the North Island and in the Marlborough Sounds in the South Island. The Pacific oyster industry had an annual output of about three million dozen oysters and the export market for oysters earned a revenue of 18 million dollars at the end of the 1st quarter of 2011 (Aquaculture New Zealand, 2011a). Although the current contribution of farmed oysters to New Zealand's seafood exports has declined significantly, Pacific oyster cultivation, together with salmon production and the green-lipped mussel industry, is expected to help achieve

New Zealand's vision for a sustainable one billion dollar aquaculture industry sector by 2025 (Burrell & Meehan, 2006).

Oyster growers source their spat either from the wild or by supply from a commercial hatchery (Aquaculture New Zealand, 2011c). The sizes of spat for on-growing are usually within the range of 10-20 mm. During the spawning months of January to March (summer months in the Southern hemisphere), spat are collected in the wild using wooden sticks. These are re-deployed on grow-out farms located in sheltered harbours or estuaries during late spring to early summer (Aquaculture New Zealand, 2011c). The wooden racks are placed inter-tidally where they are subjected to two tides in a day. In contrast, hatchery supplied spat are cultured as "singles" or unattached in baskets, mesh trays or bags. Single oysters grow sub-tidally at a faster rate but still require 1-3 months of inter-tidal "hardening" in order to better condition them for the market. Transplanted spat, later referred to as juvenile oysters, are grown to a market size of about 80-100 millimetre in 12-20 months (Hay & Lindsay, 2004).

In contrast to the commercially cultured Pacific oysters, an iconic New Zealand delicacy, *Ostrea chilensis*, commonly known as "bluff oysters" are fished by dredging in the Foveaux Strait (Yang, Frazer *et al.*, 2010). While these oyster 'farming' enterprises utilize different species and entirely different management systems (bluff oysters are harvested in line with a quota system rather than farmed *per se*), both the oyster populations and the industries founded on them are highly susceptible to infectious disease. The bluff oyster harvest has been closed or subject to fishing restrictions due to the effects of the parasite *Bonamia exitiosa*.

As a result of the 2010 mortality outbreak in juvenile Pacific oysters, anecdotal evidence of reduced volumes of harvested oysters in the succeeding two years was

available but not officially reported at that time, resulting in loss of jobs and even closure of oyster farming enterprises. Estimates for the type and extent of contraction to the Pacific oyster industry vary widely and range from 50 to 60 % drop in production (Anonymous, 2013). Due to the extent of the impact of OsHV-1 and because individual livelihoods and an industry were at stake, mitigation of the economic consequences posed by the incursion of OsHV-1 associated mortality in Pacific oyster is essential.

2.3 Pathogens of oyster bivalves

According to the European Union Reference Laboratory (2013), about 28 individual or groups of microorganisms are considered pathogenic to bivalve molluscs. More than half of the list members are protozoan (16) and the rest are bacteria, viruses and metazoans. Several important diseases affecting Pacific oysters in Australia could pose a potential threat to New Zealand oyster aquaculture. These include the protozoan *Microcytos mackini*, *Perkinsus marinus*, *Marteilioïdes chungmuensis*, *Vibrio* spp. bacteria and OsHV-1. In addition, although not reported to infect *C. gigas,* two protozoans *Marteilia sydneyi* and *Microcytos roughleyi,* have adverse effects on other oyster species such as *Saccostrea glomerata* (Sydney rock oyster). Some organisms, for example *Haplosporidium nelsoni*, do not trigger diseases in Pacific oysters, but may be carried out of the original geographic range during translocations, causing severe mortalities in other oyster bivalve hosts (Burreson & Ford, 2004).

Prior to the summer mortality event in New Zealand Pacific oysters, reports of infectious disease causing substantial mortalities to other bivalves in New Zealand were limited. Notable of these are infections associated with the presence of herpesvirus-like particles (Hine *et al.*, 1992), *Bonamia exitiosa* (Hine, 1991) and *Perkinsus olseni*. Of the three, only herpesvirus-like infection was associated with Pacific oyster mortalities. The protozoan parasite, *Bonamia exitiosa* causes significant

mortalities in flat oyster *Ostrea chilensis* (Doonan, Cranfield *et al.*, 1994) and *Perkinsus olseni* infects other bivalve molluscs including cockles (Dungan, Reece *et al.*, 2007) and more recently the green-lipped mussels and scallops but without clinical disease (PromedMail, 2014). The lack of organised disease surveillance strategies in New Zealand's wild and farmed aquatic animal populations however does not enable the presence of a potential pathogens to be discovered at a stage where risks and risk avoidance can be factored into the farming operation. An example of recent accidental finding that may have economic impact in the shellfish industry was the detection of *Bonamia ostreae* for the first time in flat oysters (*Ostrea chilensis*) from Marlborough Sounds of the South Island during an on-going research project (PromedMail, 2015). No clinical signs however was observed in the two affected marine area, but being an OIE reportable disease pathogen, it instigated a surveillance activity to describe its epidemiology and to identify possible movement control zones. The Haplosporidian protozoa, *Bonamia ostreae* caused high mortalities by infecting the haemocytes of European flat oyster (*Ostrea edulis*) in France over the period of 1980-1983 (Bucke, 1988).

In an article by Elston (1997) and Renault (2008), viruses morphologically similar to members of *Iridoviridae, Herpesviridae, Papoviridae, Reoviridae, Birnaviridae*, and *Picornaviridae* families, were detected in molluscs, few of them in association with mortalities events. However, except for ultrastructural and histopathological characterisation, molecular studies and elucidation of pathogenesis for most of these viruses are lacking (Renault, 2008) and need further research. In parallel, other viruses for example norovirus causing human enteritis and hepatitis A virus found in sewage contaminated seawater are also sequestered by oysters during filter feeding, but have not been shown to be able to establish infection in oysters' tissues (Jones,

2011). Thus, evidence of virus particles in oysters should be distinguished between bio-accumulated virions and those associated with true infection.

2.3.1 Picornavirus

Picorna-like viral particles were reported in several species of bivalves. According to Jones, Scotti *et al*. (1996), non-enveloped virion particles, 25-45 nm in diameter were recovered from farmed seed and adult green lipped mussel (*Perna canaliculus*) experiencing summer mortalities in New Zealand. The mussel pathology was manifested by massive inflammatory reactions and cell death of the digestive gland epithelia, interstitial and basal cells. Hine and Wesney (1997) also characterized virus-like particles (22-30nm) interpreted as enteroviruses (*Picornaviridae*) in the digestive glands of New Zealand scallops (*Pecten novaezelandiae*, Reeve, 1853) and toheroa (*Paphies ventricosum*, Gray, 1843). Scallops and toheroa examined in this report were obtained in areas suffering mass mortalities. A free non-enveloped virion particles (27-35 nm) in the cytoplasm and or in connection with endoplasmic reticulum within connective tissue cells in carpet-shell clam *Ruditapes decussatus,* similar to picornavirus like particle in scallops and toheroa in New Zealand, were also described Spain (Novoa & Figueras, 2000). Similar viral particles (19-21 nm in length) in paracrystalline arrays were also associated with large foci of haemocytic infiltrations in cockles (*Cerastoderma edule*) in the same location, Galicia in Spain (Carballal, Villalba *et al*., 2003). More recently, ultrastructural observation of brown muscle disease in Manila clams (*Ruditapes philippinarum)* was suggested to be due to the presence of virus like particles akin to a member of the *Picornaviridae* (Dang, Gonzalez *et al*., 2009). Brown coloration and degenerative lesions of the adductor muscle in cockles was pathologically similar to Akoya viral disease in Japanese pearl oysters *Pinctada fucata martensii* (Jones, 2007**)**. Akoya virus has been described to have a

diameter of approximately 30 nm based on the investigation of the intrasarcoplasmic inclusion bodies of necrotic muscle cells. It has an unknown taxonomic affiliation (Miyazaki, Goto *et al.*, 1999). As of this time, evidence of picornavirus-like infection has not been reported in Pacific oysters.

2.3.2 Papovavirus

Virus-like particles with morphology similar to papilloma or polyomaviruses were described to be present in basophilic intranuclear inclusions in both male and female gametes of sick Pacific oyster (Garcia, Robert *et al.*, 2006). The disease was called viral gametocytic hypertrophy (VGH) due to the hypertrophied appearance of gamete cells. The small, non-enveloped icosahedral virions reported in France by Garcia *et al.* (2006) measured between 44 to 56 nm in diameter. This virus was believed to be similar to those described in earlier reports in the US in *C. virginica* (Elston, 1997) and in recent publications of viral gametocytic hypertrophy in *C. gigas* in Ireland (Cheslett, McKiernan *et al.*, 2009), Korea (Choi, Lee *et al.*, 2004), Alaska (Meyers, Burton *et al.*, 2009) and in East Frisian coast of Germany (Watermann, Herlyn *et al.*, 2008). Viral gametocytic hypertrophy lesions were also noted in *C. gigas* in Galicia, Spain during a comprehensive histological survey from 2004-2009 to establish prevalence level of diseases and potential pathogens (Iglesias, Rodríguez *et al.*, 2012). Similar basophilic inclusion bodies in the epithelial cells of the digestive gland of razor clam *Ensis arcuatus* were also noted in a separate survey in Galicia (Ruiz, Darriba *et al.*, 2011). Such intranuclear bodies were found to contain icosahedral viral particles comparable with non-enveloped viruses in the *Papillomaviridae* or *Polyomaviridae* families. While the size and morphology of the particles may suggest a polyomavirus, papillomaviruses (PPVs) will often induce characteristic changes to the host integument and stratified squamous epithelia. As such PPVs are frequently detectable

with degenerate primer sets, as an important and widely studied pathogen group of humans. Polymaviruses appear to be distributed in nature on a more irregular basis and are not as characteristically associated with a host response. The use of molecular tools would significantly assist in the identification of a polyomavirus and whether or not the size of the virions inducing gamete hypertrophy is a co-incidence.

2.3.3 Iridovirus

Three types of iridovirus-like virions based on pathological lesions were implicated in major epidemics of mass mortalities in oysters in France (Comps, 1988). According to Comps (1988), gill necrosis virus (GNV) was observed in necrotic ulcerations of the gills' marginal lamellae and labial palps. Another iridovirus-like virus, designated haemocytic infection virus, induced an acute inflammatory response with the proliferation of atypical haemocytes in the connective tissues in *Crassostrea angulata*. Gill necrosis virus and haemocytic infection virus mainly affected the Portuguese oyster during the late 60s to 70s, although Pacific oysters also succumbed to the viral disease. Iridoviral infection was believed to be the cause of Portuguese oyster's disappearance in France.

Oyster velar virus disease (OVVD) was reported in hatchery reared Pacific oyster larvae with velar and mantle erosion in the US (Elston, 1979). Intra-cytoplasmic virus-like particles (228 nm diameter) with icosahedral symmetry, morphologically similar to iridovirus were detected in tissues of affected oysters (Elston & Wilkinson, 1985).

2.3.4 Other viruses

A few other viruses have been reported in other marine molluscs. A marine birnavirus (MABV) was associated with mortality events involving the Japanese pearl

oyster (*Pinctada fucata*) (Suzuki, Kamakura *et al.*, 1998).This virus group belong to the genus Aquabirnavirus which includes the infectious pancreatic necrosis virus (IPNV) of salmonids. Marine birnavirus was described as a non-enveloped double stranded RNA virus, consisting of two segments of the viral genome. However, MABV was considered an opportunist pathogen (Kitamura, Tomaru *et al.*, 2002) and its virulence was believed to be triggered by the presence of other stressors (Kitamura, Jung *et al.*, 2000). Low grade, persistent infection in haemocytes was reported by Kitamura *et al.* (2000) during summer in Japan with the virus invading the parenchymal cells in winter. In contrast to other reported viruses in oysters, MABV was isolated (Kitamura *et al.*, 2000) using CHSE-214 cells. This virus type was known to infect various species of fish and marine invertebrates (Kitamura *et al.*, 2000; Meyers *et al.*, 2009).

Baculovirus-like particles were also discovered in the digestive gland epithelium of an apparently healthy scallop, *Patinopecten yessoensis* in Japan *(Chang, Huh et al., 2002)*. According to Chang *et al.* (2002), this virus was ultra-structurally alike to baculovirus detected in shrimp affected with the white spot syndrome disease (Chang, Lo *et al.*, 1996). In this report, no pathology was observed and the virion particles were considered to represent a "transient" virus. Retrovirus-like element was also implicated in an epidemic of disseminated neoplasia in cockles in Spain (Romalde, Luz Vilariño *et al.*, 2007). Characterisation of the viral particles in neoplastic cells as retroviruses was done using transmission electron microscopy and reverse transcriptase activity detection. Aquareovirus-like infection was described in geoduck clam, *Panope abrupta* in Alaska (Meyers *et al.*, 2009). The virus was isolated in BF-2 cells after blind passages from survey samples submitted from 1997 to 2009. Cytophatic effect (CPE) was characterised by large syncytial cell plaques after 7 days in monolayer cells. Infected BF-2 cells contained intra-cytoplasmic arrays of viral particles with double capsid morphology and a diameter of 63-68 nm. However, no

cytopathology in geoduct clam was associated with the reported aquareovirus-like particles by Meyers *et al.* (2009).

2.3.5 Ostreid herpesvirus-1

Ostreid herpesvirus-1 (OsHV-1), commonly known as oyster herpesvirus induces massive mortalities in bivalve molluscs, particularly the ubiquitous Pacific oyster, *C. gigas*. Herpesvirus-like particles in bivalves were accidentally discovered in the Eastern oyster, *C. virginica,* during an experiment on the effect of a high temperature environment on oyster growth and survival (Farley, Banfield *et al.*, 1972). In this study, examination by light and electron microscopy of tissues from oysters that died at an elevated temperature of 28-30°C showed intranuclear inclusion bodies and virus particles with herpesvirus-like structure. *In situ* hybridisation testing of archival European flat oyster (*Ostrea edulis*) tissue from 1976 provided the earliest evidence for the presence of oyster herpesvirus genetic material (Davison, Trus *et al.*, 2005), corroborating the findings of Farley et. al. (1972). However, it was not until the early 1990's that oyster herpesvirus manifested itself as an important pathogen of bivalve molluscs. In New Zealand, for the first time in 1991, a batch of hatchery reared *C. gigas* larvae incurred a 100% mortality event that was associated with a herpesvirus-like particle observed by electron microscopy (Hine *et al.*, 1992). This was followed by a reported outbreak of mortality in Pacific oyster larvae in France a year later (Nicolas *et al.*, 1992). High mortalities in Pacific oyster spat grown along the French Atlantic coast have also been associated with detection of a herpesvirus-like pathogen (Renault, Cochennec *et al.*, 1994a; Renault, Le Deuff *et al.*, 1994b). Since then, oyster producing regions in the US, France, Spain, Ireland, Mexico, New Zealand and Australia have experienced periodic losses due to oyster herpesvirus epidemics (Friedman, Estes

et al., 2005; Keeling, Brosnahan *et al.*, 2011; Roque, Carrasco *et al.*, 2011; Vásquez-Yeomans, García-Ortega *et al.*, 2010).

Oyster herpesvirus-like infection has been reported in different bivalve mollusc hosts, including other ostreid species, clams and scallops; for example the flat oyster in New Zealand, *Tiostrea chilensis* (Hine, Wesney *et al.*, 1998); the Australian flat oyster, *Ostrea angasi* (Hine & Thorne, 1997); the European flat oyster, *Ostrea edulis* (Renault, Le Deuff *et al.*, 2000a); the Manila clam, *Ruditapes philippinarum* (Renault, Lipart *et al.*, 2001); the European clam, *Ruditapes decussatus* (Arzul, Renault *et al.*, 2001b); and French scallops, *Pecten maximus* (Arzul, Nicolas *et al.*, 2001a). Interestingly, a previously reported virus in 2001 with unknown taxonomic affiliation called acute viral necrotic virus (AVNV) was associated with mass mortalities of Zhikong scallops (*Chlamys farreri*), an important shellfish in China (Tang, Liu *et al.*, 2010). Similar to OsHV-1, AVNV infection caused widespread necrosis in the epithelium and connective tissues of the mantle, gills and hepatopancreas of the Zhikong scallops (Tang *et al.*, 2010). AVNV morphology was previously described as spherical with a diameter of 130-170 nm (Wang, Wang *et al.*, 2001). Recent complete genome sequencing of AVNV was found to be 97% similar to OsHV-1 and thus this virus was concluded to be a variant of Ostreid herpesvirus (Ren, Chen *et al.*, 2013). In Taiwan and in Australia disease outbreaks of abalone, mortality have been attributed to a herpesvirus like virus causing ganglioneuritis (now called AbHV-1). The virus was described by Tan, et al. (2008) and was found to be distantly related to ostreid herpesvirus through varying homology of the virus genome including similarity to several herpesvirus proteins (Savin, Cocks *et al.*, 2010) such as the DNA packaging ATPase subunit of (putative) terminase and DNA polymerase.

In spite of the wide distribution and apparent wide host range of OsHV-1 in shellfish of commercial importance, the natural host of this herpesviruses is still

unknown (Renault, 2008). One of the characteristic traits of mammalian herpesviruses is the high level of host specificity and adaptation, but oyster herpesvirus is unique in that it has an extremely wide host range. When extreme mortality events occur, these can often be linked to an upset of the balance between the host, the environment and the infectious agent. If the change involves the agent in such a way that it is proximate to a naive susceptible population or an ability to infect a new host species, then mortality is typically high.

2.3.5.1 Impact of OsHV-1 Infection

A sudden and abnormally high mortality in young oysters is the most obvious clinical presentation associated with OsHV-1 infection. The duration of the acute mortality phase may range between 5-14 days with an average of 10 days for both larvae and spat (Burge *et al.*, 2007; Sauvage, Pépin *et al.*, 2009; Schikorski, Faury *et al.*, 2011a; Schikorski, Renault *et al.*, 2011f). Hatchery-reared larvae and newly deployed spat and juveniles are affected most severely and mortality events are usually preceded by a rise in seawater temperature (Burge, Griffin *et al.*, 2006; Renault, Bouquet *et al.*, 2014; Samain *et al.*, 2006). Although reported cumulative mortality varies, losses appear to plateau at 40% in some cases, or go on to reach 100% especially when a new variant of the virus, for example OsHV-1 µVar, is involved (Martenot, Oden *et al.*, 2011). Mortality also occurs in adult oysters and scallops (Arzul *et al.*, 2001a; Arzul, Renault *et al.*, 2002; Hine & Thorne, 1997), but is generally lower than losses in larvae and spat populations.

2.3.5.2 Virion Classification

Ostreid herpesvirus-1 is assigned to the genus *Ostreavirus,* and the only member of the recently designated *Malacoherpesviridae* family (Davison, Eberle *et al.*, 2009).

Malacoherpesviridae is one of the three families under *Herpesvirales*, a new order created from former family *Herpesviridae* (McGeoch, Davison *et al.*, 2008). The newly revised *Herpesviridae* family includes mammal, bird and reptile viruses while the other family, *Alloherpesviridae,* contains fish and frog viruses (Davison, 2010; McGeoch *et al.*, 2008). The gastropod mollusc virus, abalone herpesvirus (AbHV-1) is a potential candidate for inclusion within the *Malacoherpesviridae* family (Savin *et al.*, 2010). Criteria for inclusion will likely rest with sequence and genome structure similarities between candidate members rather than host characteristics.

2.3.5.3 Morphology

Herpesvirus genomic DNA is densely wound in toroid and contained within an icosahedral capsid composed of 162 surface capsomeres (150 hexamers and 12 pentamers) each with diameter of 115-150 nm (McGeoch *et al.*, 2008). Surrounding the capsid is the tegument, a coat of amorphous protein material. A lipid bilayer embedded with various glycoproteins then envelopes the whole virion, increasing the final diameter up to 200 nm (McGeoch, Rixon *et al.*, 2006). In the past, this distinctive structure and morphology has been the main basis of membership in the group of herpesviruses (McGeoch *et al.*, 2006). Many illustrative examples of icosahedral capsid architecture have been published in previous studies of herpesviruses from diverse host species, for example Equine herpesvirus-1 (Baker, Newcomb *et al.*, 1990) and Channel catfish virus (Booy, Trus *et al.*, 1996). Characterization of OsHV-1 by cryo-electron microscope images shows icosahedral configuration, a capsid diameter of about 116 nm and a triangulation number of T=16 (Davison *et al.*, 2005). The capsid's morphology thus, classifies OsHV-1 as a herpesvirus.

2.3.5.4 Genomic Structure

Herpesvirus DNA is linear and double stranded, with a size range of between 125 and 295 kbp (Davison *et al.*, 2009). A preliminary approximation of the OsHV-1 genome size suggested a length of 180 kbp based on the sizes of restriction endonuclease fragments (Le Deuff & Renault, 1999). Completion of sequencing work by Davison, et al., (2005) revealed a larger genome (~207 kbp) containing 38.7% G+C nucleotides. The genome of OsHV-1 is represented by the following structural formula, TR_L-U_L-IR_L-X-IR_s-U_S-TR_s, (Davison *et al.*, 2005). Two unique regions, a long (U_L, 167.8 kbp) and a shorter (U_s, 3.4 kbp) segment are both sandwiched by inverted terminal and internal repeats TR_L/IR_L and TR_s/IR_s (7.6 and 9.8 kbp, respectively). A third unique region X (1.5 kbp), then joins internal copies of the repeats, IR_L and IR_s. This organisation of viral genome is similar to that of herpes simplex virus type 1 (HSV-1) and human cytomegalovirus (HCMV) (Davison *et al.*, 2005; McGeoch *et al.*, 2006).

Overall, the 207 kbp ostreid herpesvirus genome sequence encodes at least 124 genes (Davison *et al.*, 2005). A large group of 38 unique protein coding genes belong within 12 families of related genes. According to Davison, et al. (2005) these families are associated with helicases, inhibitors of apoptosis, deoxyuridine triphosphatase and RING-finger proteins, in addition to membrane proteins. Open reading frame (ORF) 109 encoding a putative ATPase sub-unit of terminase found in OsHV-1 is also present in other herpesviruses. In spite of commonality in herpesvirus capsid structure that is presumed to originate from sets of homologous genes for capsid proteins (McGeoch *et al.*, 2008), there is relatively little similarity between OsHV-1 and other known mammalian or fish herpesviruses based on amino acid sequence data (Davison *et al.*, 2005).

2.3.5.5 Genotypes

The completed molecular characterization of an oyster herpesvirus isolate from a Pacific oyster larva collected in 1995 (Davison *et al.*, 2005; Le Deuff & Renault, 1999; Segarra, Pépin *et al.*, 2010) became the basis for the establishment of the reference OsHV-1 strain (GenBank Accession no. AY509253). Variant viruses have been observed with base pair deletions, substitutions and/or insertions in the genome sequence. In 2001, the first variant of OsHV-1, referred to as OsHV-1 Var was detected from *C. gigas*, *R. philippinarum* and *P. maximus* larvae (Arzul *et al.*, 2001b). The variant was detected in Pacific oysters and Manila clams that had originated from a hatchery with a history of mortalities. In comparison with the reference strain, the aberrant variant, OsHV-1 Var, has a 2.8 kbp deletion close to the C2 fragment of the two inverted repeat regions (Arzul *et al.*, 2001b).

Identified genotypes of OsHV-1 are based mostly on polymorphism of the C region of the OsHV-1 genome. The C region, close to ORF 4, is present twice in the inverted repeats, TR_L and IR_L, encoding two proteins of undetermined functions. France experienced unusually high mortalities in Pacific oyster spat and juveniles and a dramatic increase in the detection rate of OsHV-1 beginning in 2008. Molecular characterization of OsHV-1 from this outbreak revealed a third emergent genotype, the OsHV-1 µVar [GenBank HQ842610] (Martenot *et al.*, 2011; Segarra *et al.*, 2010). Polymorphism was observed between the C2 and C6 segments with 12 successive nucleotide deletions on a microsatellite region, depicted by CTA repeats followed by one adenine deletion (Martenot *et al.*, 2011). Recently, New Zealand (Keeling *et al.*, 2014) and Spain (Roque *et al.*, 2011) reported OsHV-1 associated mortalities in Pacific oysters in which viruses with the sequence homologous to the OHV-1 µVar strain in the C region were detected. Around the same time, OsHV-1 µVar was also identified in Italy from *C. Gigas* spat of French origin. In contrast with the Spain and New Zealand

outbreaks, there were no accompanying mortalities reported in Italy (Dundon, Arzul *et al.*, 2011). Interestingly, a further study done by Martenot, et al. (2011) on 300 samples of *C. gigas* positive for OsHV-1 µVar revealed yet another new genotype. This new emerging variant, OsHV-1 µVar Δ9, is characterized by nine consecutive deletions in the C region and was only detected in five samples. The existence of OsHV-1 variants based on polymorphisms in the nucleotide sequence of the A region has also been described in California, and from oysters that originated in Japan, China and Korea (Friedman *et al.*, 2005; Moss, Burreson *et al.*, 2007). Region A encodes a protein with an undetermined role (Batista, Arzul *et al.*, 2007). In a recent study done by Renault, Moreau *et al.* (2012) on 72 OsHV-1 infected oyster samples, apart from the polymorphism shown in the C region, sequence variations were also seen in ORFs 35/36/37/38 and ORFs 42/43, which might provide useful basis for constructing virus genogroups. The 72 samples were collected over an 18 year period from various geographical locations. The majority of samples originated in France, and the rest were collected in Ireland, USA, China, Japan and New Zealand. Given the complexity and cost of outbreaks to the industry, a comparison of OsHV-1 genotypes based on their entire sequence is warranted. Such an endeavour will further aid strain differentiation and phylogenetic studies (Segarra *et al.*, 2010).

2.3.5.6 Pathology

Clinical signs due to physiological alterations are very general and non-specific. Non-attached moribund *C. gigas* larvae manifested reduction in feeding, circular swimming behaviour and loss of motility (Hine *et al.*, 1992; Le Deuff, Renault *et al.*, 1996; Renault *et al.*, 1994b). Other bivalve species also exhibit similar clinical symptoms in previous reports of larval losses related to herpes-like virus infection (Renault *et al.*, 2000a). Once attached to a substrate, weak and diseased spat display

gaping behaviour during emersion (Bondad-Reantaso, MccGladdery *et al.*, 2001). When a gentle tap stimulus is applied during immersion, the moribund spat respond with a protracted closing reaction of the shell; which is atypical.

Pathological changes in Pacific oyster larvae includes detachment of the velar portion (Le Deuff, Nicolas *et al.*, 1994). Most cellular abnormalities are limited to connective tissues (Renault *et al.*, 2000a). Fibroblast-like cells are characterized by enlarged nuclei with nuclear alterations such as chromatin margination. Pyknosis or chromatin condensation was also observed in ovoid cells construed to be haemocytes. Similar alterations were also described in Manila clams infected with OsHV-1 and consequent to induced mortalities (Renault *et al.*, 2001). In spat, cells in the connective tissue of the mantle, labial palps and digestive glands often contained abnormally large nuclei with marginated chromatin (Renault *et al.*, 1994a; Renault *et al.*, 2000a). Condensed nuclear material in circulating haemocytes was observed although the numbers of inflammatory cells were greatly reduced. Cowdry type A inclusion bodies were observed in herpes-like virus infection in *C. gigas* larvae (Hine et al., 1992) and spat (Renault *et al.*, 1994b); and in *O. angasi* (Hine & Thorne, 1997). Cowdry type A intranuclear, eosinophilic inclusion bodies are composed of viral nucleic acids and proteins accumulated in infected cells and are common findings in HSV, Varicella-zoster virus, and HCMV infection. However, these intranuclear inclusions were not detected in recent histological studies on *C. gigas* in Australia (Jenkins, Hick *et al.*, 2013).

Comparatively, histological lesions in larvae are more diffuse in distribution while cellular changes in spat are more focal (Renault *et al.*, 1994b). Other microscopic changes associated with OsHV-1 infection include cuboidal metaplasia and tubule dilatation of digestive glands, diffuse haemocyte infiltration and cell necrosis in connective tissues, particularly the mantle, and enlarged haemocytes with a small

nucleus to cytoplasm ratio (Burge *et al.*, 2006; Friedman *et al.*, 2005). These abnormalities are relatively non-specific and have also been attributed to other disease causing agents, stress factors and occasionally to normal physiological events (Pearce, Handlinger *et al.*, 2005; Watermann *et al.*, 2008).

2.3.5.7 Methods to detect OsHV-1 infection

The main means of detecting herpesvirus-like infection in Pacific oysters rely on traditional methods such as histology and transmission electron microscopy. Early presumptive diagnosis can be made if histological and ultrastructural examination reveal inclusion bodies or viral particles indicative of herpesvirus-like infection (Batista *et al.*, 2007). Because cytological and histological alterations are usually non-specific and can be attributed to a number of aetiologies, further confirmatory tests are necessary. Confirmation using electron microscopy requires more specialist skills and equipment (Hine *et al.*, 1992; Renault *et al.*, 1994b) . Both techniques require time and have relatively poor sensitivity (Renault, Le Deuff *et al.*, 2000d).

Lack of continuous bivalve cell lines prevents isolation of the virus (Burge & Friedman, 2011). This limitation was partially resolved with the purification of OsHV-1 particles from infected larvae using a sucrose gradient density sedimentation technique (Le Deuff & Renault, 1999). Elucidation of the OsHV-1 genome from the purified virion by Le Deuff and Renault (1999) and Davison *et al.* (2005) provided a platform for the development of nucleic acid based diagnostic tools. To date, OsHV-1 infection in Pacific oysters is commonly diagnosed by molecular techniques. Molecular assays reviewed by Batista *et al.* (2007) include both qualitative and quantitative PCR techniques to detect and quantify herpesviral DNA in oysters. Conventional and nested PCR was used to assess OsHV-1 prevalence especially during periods of high virus multiplication (Burge *et al.*, 2006; Renault *et al.*, 2000d). Although, conventional or

nested PCR do not necessarily delineate an active infection from a latent or low grade infection, real time quantitative PCR technique employed in recent studies, have the ability to determine the number of viral copies (Burge & Friedman, 2011; Sauvage *et al.*, 2009; Schikorski *et al.*, 2011a). An indication of a productive infection was illustrated in a recent epidemiological study demonstrating the relationship between high OsHV-1 viral load shown in qPCR and the consequent occurrence of elevated mortalities in Pacific oysters (Oden, Martenot *et al.*, 2011). Furthermore, an RT-qPCR (reverse transcription quantitative PCR) was able to show production of viral mRNA over the time course of infection, signifying active infection (Burge & Friedman, 2011). A rapid loop-mediated isothermal amplification (LAMP) assay was also developed for practical field diagnosis to detect OsHV-1 (Ren, Renault *et al.*, 2010)

In situ hybridisation can also reveal the presence of OsHV-1 DNA in Pacific oyster tissues. The basic principle is the complementary pairing of labelled DNA probes with target nucleic acid sequences on tissue sections (Gall & Pardue, 1969). Although the technique was discovered several decades ago, utilisation of ISH as a diagnostic assay came late in the 90s. This type of pathological investigation has the benefit of correlating the progression or distribution of infection with identifiable anatomical and morphological alterations in tissues (Chang *et al.*, 1996). Rather than just denoting the mere presence of the viral genome in nucleic acid detection assay, visualisation of specific viral DNA or RNA sequences by ISH indicates replication activities within tissue cells, suggesting real infection. Furthermore, detection of OsHV-1 by PCR or ISH can be complemented by immunohistochemistry where the antigen targets are generally viral proteins indicating active infection as opposed to latency.

The virulence and pathogenicity of OsHV-1 is not yet well understood and is hampered by the lack of bivalve cell lines for virus isolation. Detection of OsHV-1 in *C. gigas* does not necessarily correspond to an active infection. Although transmission

trials of OsHV-1 among larvae and spat demonstrate the ability of OsHV-1 to induce infections, the presence of the oyster herpesvirus alone does not invariably generate abnormally high mortality. In adult oysters, OsHV-1 can be detected without clinical signs indicating either a low grade infection or latent infection. Asymptomatically infected adult oysters are hypothesised to be a source of OsHV-1 for horizontal (Burge *et al.*, 2006) or vertical transmission (Arzul *et al.*, 2002). In addition, predisposing factors such as high seawater temperature coupled with stress, such as translocation of spat, allow for rapid viral replication. Furthermore, a threshold level of OsHV-1 in terms of DNA copy number has been suggested to indicate an increased risk of significant mortalities.

To address the deficiency of classical virological techniques due to the absence of feasible tissue culture to grow OsHV-1, molecular techniques must be relied on to a greater extent to supplement diagnosis; for example, quantitative PCR, ISH, immunochemistry and reverse transcription qPCR.

2.4 Aim of the study

The results of previous studies using ISH assay for investigation of OsHV-1 infection in oysters suggested that infection of adults may be asymptomatic. However, viral nucleic acid distribution in spat was suggestive of pathogenic insult ((Arzul *et al.*, 2002; Barbosa-Solomieu, Miossec *et al.*, 2004; Lipart & Renault, 2002). This information, in particular details of infection, susceptibility and tissue distribution by age, is essential in managing emerging aquatic animal diseases. Rapid discrimination of the true causative agent from secondary pathogens leads to effective approaches in disease mitigation. While novel pathogens constantly emerge from the diversity of microorganisms in the aquatic environment, nucleic acid detection by PCR alone cannot establish causation or differentiate virulent aggressive infections from

asymptomatic infections or carrier states (Burge *et al.*, 2006). Molecular 'identification' has to be complemented with other diagnostic tools whereby infection and pathogenic consequences are associated (Batista *et al.*, 2007). It can be similarly difficult to discriminate mild or initiating pathogens that weaken the host immune system from secondary pathogens which exploit the host in this weakened state. Demonstration that histopathological changes in tissues are co-localized with detection of the pathogens of interest is a key strength of visual techniques involving tissues in their organized, albeit diseased state.

To support the causal association of OsHV-1 and mortality event in juvenile Pacific oysters in New Zealand, the aims of this study were 1. to develop an *in situ* hybridisation assay to detect OsHV-1 in Pacific oysters, 2. to demonstrate and describe the progression of OsHV-1 infection from spat collected during a longitudinal study, and 3. to investigate an association between presence of OsHV-1 nucleic acids and histopathological changes in oysters infected with a reportedly more pathogenic variant of the virus. It was hoped that the data would provide valuable information on the pathogenesis and spread of virulent OsHV-1 infection, strengthening the suspected aetiological link between infection with OsHV-1 and oyster die offs.

3 Methodology

3.1 Background of the longitudinal study

The Investigation and Diagnostic Centre & Response (IDC&R) of the Ministry for Primary Industries (formerly Ministry of Agriculture and Forestry) investigated the occurrence of high mortalities in farmed Pacific oysters after a notification in November, 2010 (Bingham *et al.*, 2013). Juvenile cultured Pacific oysters were primarily affected, although adult oysters also succumbed to the unknown disease problem in the Northland and Coromandel regions. Juvenile oysters were defined in the outbreak as spat measuring less than 20 mm and small oysters between 20 mm to 80 mm. The mortality events were acute and characterized by large losses. Die offs occurred one week after batches of susceptible spat were introduced to the farms. Oyster farmers reported mortalities ranging from 15% to 100%. Mortality events were widespread and affected 15 out 18 oyster growing locations in the North Island. Inspection of growing sites revealed the presence of large numbers of moribund oysters, gaping animals (incomplete shell closure), shells containing decomposed tissue and empty shells. Influential environmental factors were initially considered to moderate the pattern of die off events, however, preliminary PCR tests revealed the presence of OsHV-1 nucleic acids.

As part of the investigation, a longitudinal study was conducted by IDC&R to describe the temporal development of the mortality syndrome, detect associated pathogens and their distribution within the host using a serial sampling strategy. The results of the first part described the high prevalence of OsHV-1 µVar genotype in the collected samples; and temporality of OsHV-1 nucleic acid detection by qPCR and onset

of Pacific oyster mortality (Keeling *et al.*, 2014). Keeling *et al.* (2014) also reported the possible involvement of *Vibrio* spp. in the mortality syndrome.

This study was the second part of the longitudinal study in order to detect the presence of OsHV-1 in Pacific oyster tissues, describe the progression and spread of viral infection in oyster tissues and investigate the relationship of OsHV-1 presence to pathological changes.

3.1.1 Longitudinal Study

The longitudinal study followed the transplantation of approximately 17 000 Pacific oyster spat from an OsHV-1 free commercial hatchery in the South Island to an affected oyster farm (Bay of Islands) in the North Island. The recipient farm met the case definition of being a farm experiencing high mortality and where OsHV-1 had also been identified by real time PCR assay. It was assumed that the placement of spat on the farm to be co-incident with the onset of exposure. After arrival to the farm, spat were segregated into mesh bags containing approximately 300 shellfish per bag. Mesh oyster bags were then deployed and arranged in two lines with 28 bags per row in a marine environment adjacent to older oysters that had been presumably exposed and surviving to the pathogens involved in the high mortality event. The newly transferred spat served as the study population in this investigation.

In this study, serial sampling of the newly transferred spat population were done regularly for laboratory testing during the follow up period. Serial sampling of susceptible naïve spat would permit the presence and increase of a specific pathogen to be followed over time and associated with pathological changes to affected tissues. This approach would further allow discrimination of a pathogen from opportunist or background organisms.

The approach for each serial sampling was focused on pathogen detection and was based on 10% expected prevalence. Thirty (30) spat (~20 mm) were sampled on each collection day beginning with a control group sampled at the nursery prior to transfer (-1 day). After arrival at the farm, cohorts of 30 exposed spat were sampled on days 1, 3, 5, 7, 9 and 13 following introduction. The method of sampling consisted of random selection of 10 spat from each mesh bag located on either end of one line and another 10 spat from one of the middle bags. There was no attempt to deliberately select or target moribund or gaping oysters at the time of sampling. Collection of samples was discontinued after thirteen days post transfer due to high mortality experienced by the naive hatchery-sourced spat, with resultant dead spat being generally unsuitable for testing. A total of 210 samples were collected at the end of the sampling period.

Oyster farms in New Zealand do not have systems in place to capture mortality data. In order to derive a mortality estimate, a sub-cohort was selected from the newly introduced spat to enumerate mortalities. Four selected mesh bags, each containing 253 to 302 oysters spat initially, were designated for incidence of mortality observation. The number of dead oysters, characterised by opened shells that may have contained decomposing animal tissues, were recoded and removed from the bag during inspection.

3.1.2 Processing of spat samples

Spat samples received at Animal Health Laboratory-IDC&R, Wallaceville were processed inside PC3+ laboratory facilities to avoid the risk of potential emerging pathogens escaping to the environment. Spat were opened and initially observed for gross abnormalities, followed by sterile incision and swabbing for bacterial culture (for a separate study). A 2-5 mm thick sample of the oyster tissue was obtained from a

transverse cut between the labial palps and adductor muscle of the spat and fixed in 10% formalin in sea water (see Figure 3). Remaining oyster tissues, including the gills, mantle, adductor muscle and digestive diverticula, were then dissected and allocated to different downstream testing protocols for PCR as well as for storage for future testing.

3.2 Designs of the current study

To examine the co-incidence and significance of OsHV-1 detection in the spat samples using real time PCR, an *in situ* hybridisation technique was developed and optimised. Once optimised, ISH was used to demonstrate the presence of OsHV-1 in Pacific oyster tissues and to elucidate the timing of infection in serially sampled spat from the longitudinal study in section 3.1.1. In addition, fixed tissue sections were stained using the haematoxylin and eosin (H&E) method and examined in order to determine if there was any correspondence (co-localization) between the pathological findings and the presence of OsHV-1 signal by ISH.

Eighty seven (87) fixed tissue blocks, generated from the collection of spat over a two week period and subsequently processed, were used in this study (Table 1). Fixed tissues were embedded in paraffin and sectioned at the Institute of Veterinary, Animal and Biomedical Sciences (IVABS), Massey University. One set of 5 µm thick tissue sections was used for the *in situ* hybridisation assay and another set of tissue sections (3 µm) stained with H&E.

Development of the *in situ* hybridisation assay, testing of the samples and microscopic examination of H&E stained slides was carried out at the Animal Health Laboratory–IDC&R at Wallaceville and at the Institute of Veterinary, Animal and

Biomedical Sciences (IVABS), Massey University, Palmerston North. This study was carried out from March, 2011 to November, 2012.

Table 1. Formalin fixed paraffin embedded tissues from the longitudinal study examined for *in situ* hybridisation.

Day of sampling post introduction	No. of FFPE* tissues
day -1 (pre-transfer)	5
day 0 (transfer date)	0
day 1	7
day 3	7
day 5	17
day 7	17
day 9	17
day 13	17

*FFPE - formalin fixed paraffin embedded
** day 1,3,5,7,9,13 - days of post transfer in the high mortality affected farm

3.3 Development of *in situ* hybridisation for OsHV-1 detection

Development of OsHV-1 ISH consisted of two steps. Firstly, a non-radioactive DIG labelled DNA probe was generated. Secondly, various steps in ISHC protocol were optimised.

3.3.1 Construction of DNA hybridisation probe

Previously published PCR protocols for the identification of OsHV-1 were adapted for this study. The approximately 700 bp PCR product generated using

primers C2 (5' CTCTTTACCATGAAGATACCCACC 3') and C6 (5' GTGCACGGCTTACCATTTTT 3') described by Arzul *et al.* (2001b) was selected for use as a probe.

3.3.1.1 Amplification of C2/C6 region from OsHV-1 infected Pacific oysters

Genomic DNA from six Pacific oysters were screened for the presence of the OSHV-1 C2/C6 region (ORF 4). All six Pacific oyster DNA samples were previously positive for OsHV-1 nucleic acid in a real time PCR (Keeling *et al.*, 2014) using BF and B4 primers targeting the B region of OsHV-1 which encodes a putative apoptosis inhibitor (Martenot, Oden *et al.*, 2010).

PCR conditions and parameters for amplifying C2/C6 products were adopted from an in-house assay developed and used at AHL-IDC&R (Keeling *et al.*, 2011). The PCR assay was executed using Kapa 2G Fast Hotstart Ready mix PCR kit (Kapa 2G, KappaBiosystems, USA). Each PCR reaction consisted of 12.5 µL of 2X Kapa2G Fast Hotstart mix, 0.1 µM of each primer (C2 forward and C6 reverse), and 1 µL (20-200 ng) DNA template in a total volume of 25 µL. A negative (water) and a positive control were included in the run. The positive control DNA was extracted from the New Zealand reference isolate containing the sequenced C2/C6 segment similar to the µvar OsHV-1 (Keeling *et al.*, 2014). All DNA amplifications were performed in an AB Veriti 96 well Thermal Cycler (Applied Biosystem). For this confirmatory PCR the following conditions were used: an initial denaturation at 95°C for 2 minutes followed by 25 cycles of 15 seconds denaturation at 95°C; 15 seconds annealing at 50°C; and 1 second extension at 72°C. An aliquot (10 µL) of each PCR reaction was subjected to electrophoresis through a 1.5% agarose gel in 1xTAE buffer containing 0.01% GelRed® Nucleic Acid stain (Biotium) at 100 V for 50 minutes. Amplicon size was estimated by comparison with a molecular ladder (100bp Trackit™ Invitrogen). Samples were

considered positive if approximately 700 bp amplicons were visible and positive and negative controls showed the expected results. The C2/C6 amplicon was used as template and tried for PCR probe labelling but was unsuccessful.

3.3.1.2 Cloning of C2/C6 Amplicons

Two OsHV-1 positive DNA samples were re-amplified using the PCR conditions and parameters described in 3.3.1.1, with an additional final extension time of 30 seconds at 72°C. All 25 µl of the PCR reaction product was subjected to electrophoresis through a 1.5% agar gel containing 0.01% GelRed® Nucleic Acid stain (Biotium) in TAE buffer) for 80 minutes at 100V. DNA was extracted from the gel slice with the use of Zymoclean™ Gel DNA Recovery kit (Zymo Research Corporation, USA) and eluted with 10 µL of PCR-grade water. Eluted DNA was used immediately for the cloning reaction.

Cloning reactions were performed using TOPO TA Cloning Kit® for Sequencing (Invitrogen) according to the manufacturer's protocol. Ligations were performed using 4 µL of the purified DNA eluted from the agar gel, 1 µL salt solution, and 1 µL of plasmid vector pCR®4-TOPO® for 5 minutes at room temperature.

Transformation was done by adding and mixing gently 2 µL of the ligation mix to a vial of One Shot® TOP10 chemically competent *E. coli* on ice and incubating the mixture for 5 minutes. After incubation, *E. coli* cells were heat shocked at 42°C in a water bath for 30 seconds and immediately transferred to the ice bath. Two hundred and fifty microlitres of S.O.C. medium (Super Optimal broth with Catabolite repression) were added into the each vial, and the vials were capped and incubated at 37°C with shaking horizontally at 200 rpm. Three volumes (10 µL, 30 µL, 50 µL) of the transformed *E. coli* cells were plated separately onto pre-warmed (37°C for 30 minutes) selective Luria Bertaini (LB) agar plates containing 50 µg/mL ampicillin, for

an overnight incubation at 37°C. The following morning, 10 colonies were randomly selected and individually inoculated into 10 vials containing 2 mL of LB broth with 50 µg/mL ampicillin each. After another overnight incubation at 37°C, a reading of 4 in McFarland turbidity tubes indicated good growth in the LB liquid media. One millilitre was then transferred to a microfuge tube for plasmid purification and the remaining of the bacteria were stored in ceramic beads (Cryobank™) at -80°C for future use.

3.3.1.3 Plasmid Purification

Plasmid DNA was purified from 1 mL of an overnight *E. coli* culture using PureLink™ Quick Plasmid Miniprep Kit (Life Technologies), according to the manufacturer's instructions. Briefly, the bacterial culture was centrifuged at 6,000 x g for 3 minutes and the pelleted cells were broken down with alkaline lysis buffer. DNAs were bound to a silica membrane on a spin column and recovered after two series of washing by elution using 75 µL Tris-EDTA buffer (TE buffer). The isolated plasmid DNA was analysed by PCR and sequencing. PCR screening assays were done using both the vector primers (M13 forward: 5'-GTAAAACGACGGCCAG-3' and M13 reverse: 5'-CAGGAAACAGCTATGAC-3') and OsHV-1 specific C2/C6 primers. The PCR parameters described in section 3.3.1.1 were used for both sets of primers and 1 µL of plasmid DNA template was used as a template in each reaction. The concentration of the M13 forward and reverse primers was 1.0 µM each. The PCR product amplified with M13 primers was recovered from the gel and sent for sequencing to the EcoGene Sequencing Service in Auckland, (together with M13 primers). DNA sequence chromatograms were assembled and edited using the Geneious® 6.1.7 Software (Biomatters Ltd). The identity of amplified DNA was confirmed by comparison with sequences available in GenBank using BLAST algorithms (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.3.1.4 Generation of OsHV-1 specific, DIG-labeled PCR probe

DIG labelled PCR probes were prepared using a DIG PCR probe synthesis kit (Roche) according to the manufacturer's instructions. The labelling reaction consisted of 5 µL of 10X PCR buffer with 1.5 mM MgCl; 5 µL labelling mix, 0.5 µM each primer (C2 forward and C6 reverse); 0.75 µL enzyme mix (Roche Expand High Fidelity PCR system); and 3 µL (~80 pg) template DNA (plasmid containing C2/C6 insert). The labelling mix contained 200 µM each of dATP, dCTP, and dGTP, and either 65 µM dTTP/35 µM DIG-dUTP or 130 µM dTTP/70 µM DIG-dUTP. A separate PCR reaction was prepared replacing the labelling mix with a standard 200 µM each of the dNTPs. The PCR cycling conditions consisted of an initial denaturation for 2 minutes at 95°C followed by 38 cycles of denaturation (30 seconds at 95°C), annealing (30 seconds at 60°C) and elongation (40 seconds at 72°C), and the final elongation for 1 minute at 72°C. An aliquot (2 µL) of each PCR product was subjected to gel electrophoresis as described in 3.3.1.1. The efficiency of the labelling reaction was judged based on the comparison of sizes of the labelled product to the unlabelled control. The DNA concentration of DIG labelled probe was measured by Qubit™ Fluorometer (Life Technologies) and the probe was stored at -20°C.

3.3.2 In situ hybridisation steps optimisation

In situ hybridisation procedures were adapted with modifications from published protocols (Arzul *et al.*, 2002; Barbosa-Solomieu *et al.*, 2004; Lipart & Renault, 2002; Meyer, Bower *et al.*, 2005). Paraffin embedded tissues from four Pacific oysters from the longitudinal study and juvenile oyster mortality investigation were used as a starting material for the development and optimization step. Equivalent tissues from the selected samples were positive for OsHV-1 in the real-time PCR assay.

Likewise, tissues from three Pacific oysters negative for OsHV-1 served as negative controls.

3.3.2.1 Deparaffinization

Oyster tissue sections (5 µm) on SuperfrostPlus® slides were dewaxed by immersing slides in xylene twice for 5 and 3 minutes, followed by rehydration in a series of graded ethanol solutions (100% ethanol 2 x 1 minute; 96% ethanol 1 x 1 minute; 80% ethanol 1 x 1minute; 50% ethanol 1 x 1 minute). The slides were then rinsed with distilled water for 1 minute and equilibrated with 1X PBS (phosphate buffered solution, 1 minute).

3.3.2.2 Proteinase K digestion

Proteinase K (PK) digestion was performed and optimized by adding 100 µl of PK onto the tissue section followed by a coverslip. Incubation in a humid chamber was standardized at 37°C. The optimum conditions for PK digestion were determined by testing three different PK concentrations (50, 75, 100 µg/mL) with serial sections of an OsHV-1 positive oyster. After selecting the optimum PK concentration, two different incubation times (15 & 25 min) were trialled. Protein hydrolysis was stopped by using a standard washing step with 1.0% glycine in 1X PBS for 5 minutes.

3.3.2.3 Pre-hybridisation and denaturation

Following PK digestion, tissue sections were air dried briefly before the addition of 100 µL of pre-hybridisation buffer (50% formamide, 10% dextran sulphate, 4X SSC or saline sodium citrate buffer, 250µg/ml yeast tRNA, and 10% Denhardt solution). Either Hybri-slips® or a cut-out PARAFILM® M was used to cover the section and the slides were incubated in a humid chamber at 42°C oven for 30 minutes. The initial

concentration of Denhardt' solution used was 1%, but it was increased to 10% in further experiments to reduce non-specific background staining. After the pre-hybridisation step, coverslips were removed and excess liquid from around the edges blotted dry using lint-free tissue paper. A midsize Gene Frame® (ABgene Ltd) was mounted around the tissue and the resultant trough was saturated with 60 µL of hybridisation solution consisting of the pre-hybridisation buffer with 2.3 ng/µl of DIG-labelled DNA probe. The Gene Frame was sealed with the coverslip, and the slide was incubated at 95°C for 5 minutes on a flat solid metal platform placed inside the oven and cooled immediately to 4°C for 5 minutes on ice.

3.3.2.4 Hybridisation

The slides were then moved to a humidified box and incubated overnight at the pre-determined hybridisation temperature. The optimal hybridisation temperature was estimated by subtracting 20-25°C from the melting temperature (Tm) of the PCR probe (Coulton, 1990), which was calculated to be 64°C from the formula Tm = 81.3 + 16.6 log (molarity of monovalent cation) + 0.42 (%GC)- 0.61 (% formamide) - % base mismatch (Schwarzacher & Heslop-Harrison, 2000). Based on this theoretical derivation and the ISH protocol published by other authors (Lipart & Renault, 2002), two hybridisation temperatures (40°C and 42°C) were tested.

3.3.2.5 Post-hybridisation wash

Post hybridisation stringency washes were done in 2X SSC (5 minutes), followed by 1X SSC (5 minutes) at room temperature. The final wash (10 minutes) was done at 42°C and different salt concentrations of the SSC for the final wash (1X SSC, 0.75X SSC & 0.5X SSC) were tested and the concentration that optimally removed non-specific signal was used for further experiments.

3.3.2.6 Probe detection

The DIG label on tissue bound probes was detected by immunohistochemical methods. After the final wash, tissue sections were equilibrated with 80-100 µL Solution I (100 mM maleic acid, 5M NaCl, pH 7.5) for 5 minutes at room temperature, and incubated with Blocking solution (Roche Diagnostics) for 30 minutes in a humidified chamber (room temperature). PARAFILM® M (Bemis Company) was used as an alternative cover slip material throughout the experiment to avoid electrostatic tissue disruption by ordinary coverslips. The hybridised probe was detected following incubation of sections with 80-100 µL of the anti-DIG alkaline phosphatase (AP) conjugate (diluted 1/500 in blocking solution) for an hour at room temperature. The antibody was removed by two washes (1 minute each) with Solution I and the slides then equilibrated with 100 µL Solution II (1M Tris pH 8, 5M NaCl, 1M MgCl2, pH 9) for 2 minutes at room temperature. To visualise the hybrid molecules, 80-100 µL of BCIP (5-bromo-4-chloro-3-indolyl phosphate, Roche)/NBT (nitroblue tetrazolium salt, Roche) diluted 1/50 in Solution II, was dropped on the section and incubated at room temperature for one hour in the dark. This enzyme catalysed reaction produced an insoluble dark blue indigo precipitate. Colour development was stopped by a brief rinse in distilled water and 1X PBS. The section was counterstained with Bismarck Brown Y (Sigma-Aldrich) for 1 minute and was dehydrated by a series of ethanol washes of increasing concentration (96% ethanol 2 x 1 minute; absolute ethanol 3 x 15 seconds). After clearing twice with xylene, tissue sections were mounted with a drop of Eukitt® resin (Sigma-Aldrich) and a cover slip.

Slides were examined for the presence of dark blue to black precipitates in cells labelled by the probe. To demonstrate reproducibility of the optimised protocol, the same experiment was conducted by another technician in the laboratory.

3.3.2.7 Non-specific reactors

OsHV-1 negative oyster tissue were tested to determine if there was any non-specific binding of the probe. In addition to this trial, non-specific binding of the DIG labelled probe was determined in the ISH assay by testing two serial sections of the same OsHV-1 positive tissue. The developed ISH procedure was implemented for both sections except that for one tissue section, the DIG labelled probe was omitted in the hybridisation solution. In the same experiment, non-specific staining of any endogenous alkaline phosphatase in the oyster tissue was examined using the same format of testing two consecutive OsHV-1 positive tissue sections. For both sections, the labelled probe was added but one section did not receive the anti-DIG alkaline phosphatase conjugate during testing.

3.4 Demonstration of OsHV-1 infection in New Zealand Pacific Oysters by ISH

Eighty seven 5 µm thick sections of oyster tissue from the longitudinal study (Table 1) were tested and analysed by the newly developed ISH assay described in section 3.3. Once the mountant was dry, slides were examined using an Olympus BX51 microscope and images were documented with a Colorview Soft Imaging camera in analySIS docu 5.0 software (Olympus Soft Imaging Solutions).

The distribution of OsHV-1 DNA in the oyster tissues, represented by the presence of dark blue labelled cells was assessed semi-quantitatively according to the intensity of staining and the number of cells with ISH signals per organ. A grade of +, ++, and +++ was assigned for low, moderate or high degree of ISH labelling, respectively. A specific tissue with more than 10 positive cells per slide was rated with +++, 5-10 positive cells with ++ and 1-5 cells with +.

The general distribution and specific location of ISH signal was then used to inform investigation of the corresponding histological slide sections stained with H&E. A total of 33 stained slides were examined from oysters at the same time point of the longitudinal study. These H & E slides were used to determine if a relationship between cellular and histological anomalies and the presence of OsHV-1 co-existed or were co-localized.

3.5 OsHV-1 real time PCR

Real time PCR (qPCR) was conducted earlier on fresh tissues from the same oysters prior to fixation and use for the ISH study (Keeling *et al.*, 2014). The primers and probe targeting the B region, encoding a putative apoptosis inhibitor of the OsHV-1 genome were used (Martenot *et al.*, 2010). Using a reaction volume of 10 µL, the Bio-rad SsoFast probe mix was optimised on the Bio-rad CFX96 (Bio-Rad, USA). The DNA concentration was 50-100 ng/µL and the primer and probe concentrations was 20 µM and 10 µM respectively. The PCR conditions were 95ºC for 120 sec followed by 40 cycles of 95ºC for 5 sec and 60ºC for 5 sec. Samples were assayed in duplicate. The results were recorded as positive if both duplicates had a Cq value of ≤ 39. A negative result was noted down if at least one replicate had a Cq value >39.

3.6 Assay performance and comparison

The results of the ISH assay in the longitudinal study were statistically compared with the results of a real time PCR (qPCR) test conducted earlier using equivalent fresh tissues as described by Keeling *et al.* (2011). Relative agreement beyond chance between ISH and qPCR was analysed by a calculated *Kappa* value. Initially, the frequency data from the two tests results were classified into a 4 cells of a 2X2 table (*a*, both test positive; *b*, qPCR positive and ISH negative; *c*, qPCR negative

and ISH positive and d, both test negative). The K value was calculated using the EpiTools developed by Ausvet Animal Health Services for diagnostic evaluation and comparison of two tests (http://epitools.ausvet.com.au/content.php?page=home). The *Kappa* value can range from -1 to +1. Agreement was evaluated with the following interpretation (Viera & Garrett, 2005) : *Kappa* value of <0 is less than chance agreement, 0.01-0.20 has slight agreement, 0.21-0.40 has fair agreement, 0.41-0.60 is moderate agreement, 0.61-0.80 is substantial agreement and > 0.8-1 is almost perfect agreement.

Similarly, preliminary estimates of diagnostic sensitivity and specificity of ISH for the study population were described with qCPR as the reference method. The OsHV-1 probe based Taqman qPCR developed by AHL-IDC&R has a sensitivity of 95% and specificity of 99% (S. Keeling personal communication, March, 2011). The Epitools software was also used for this calculation (http://epitools.ausvet.com.au/content.php?page=TestEvaluation).

4 Results

4.1 Generation of a DIG-labelled probe

4.1.1 C2/C6 PCR

Two out of six *oysters* were positive for OsHV-1 PCR with C2/C6 primers, with an approximately 700 bp product amplified and clearly visualized in the agarose gel (Figure 12). The C2/C6 primer product was subsequently cloned into TOP10 chemically competent *E. coli* cells. The recombinant plasmid contained the C2/C6 product insert, as was demonstrated by conventional PCR. Approximately 850 bp band was amplified from one of the recombinant plasmids using M13 primers (Figure 13) and approximately 700 bp product was amplified from the plasmid with the C2/C6 primers (Figure 14). Both results were consistent with the insertion of the OsHV-1 amplicon into the plasmid, which was further confirmed by sequencing.

Assembled and edited sequence 'reads' of the plasmid containing the cloned C2/C6 region demonstrated the presence of an 837 bp insert. The insert was 99-100% identical to a number of submitted OsHV-1 DNA sequences of the C region available in the GenBank (example GenBank accession AB734065.1, KF185070.1, JN800122.1 and JQ959598.1).

4.1.2 Non-radioactive labelling of DNA probe

The DIG-labelled probe (Table 2) was successfully constructed by integrating non-radioactive DIG-dUTP into OsHV-1 specific DNA by PCR. The DIG-labelled probe

demonstrated similar intensity but had a higher molecular weight compared to the non-labelled control amplicon as shown in Figure 15.

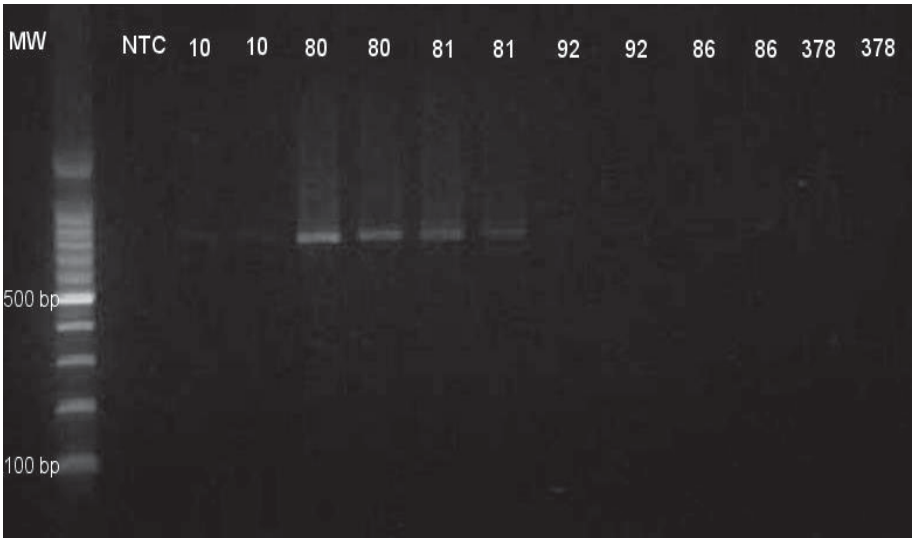


Figure 12. Two Pacific oysters genomic DNA (80 & 81) contained the amplified region between the primer C2 and C6 (approximately 700 bp). Oyster 80 is the New Zealand isolate with sequence similar to OsHV-1 µVar strain.

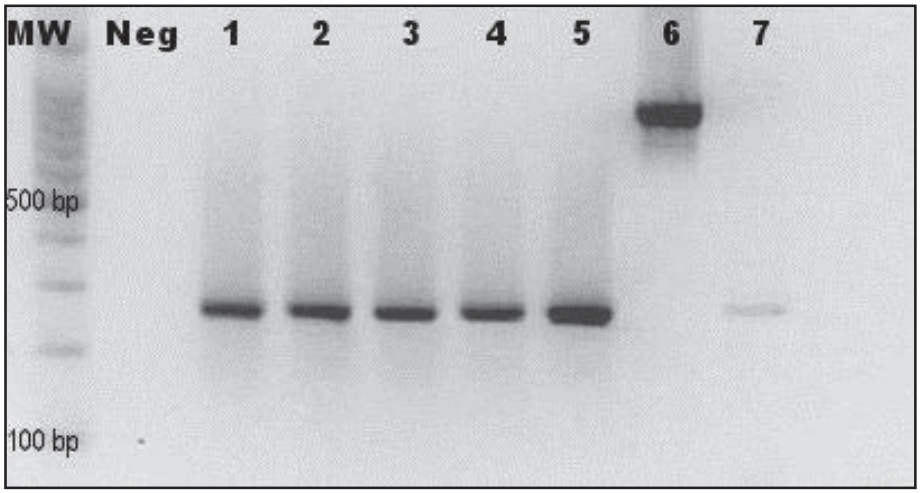


Figure 13. An approximately 850 bp band containing the C2/C6 segment insert is clearly visible in Lane 6 when amplified with M13 forward and reverse primers. Other visible bands were product inserts of the B region.

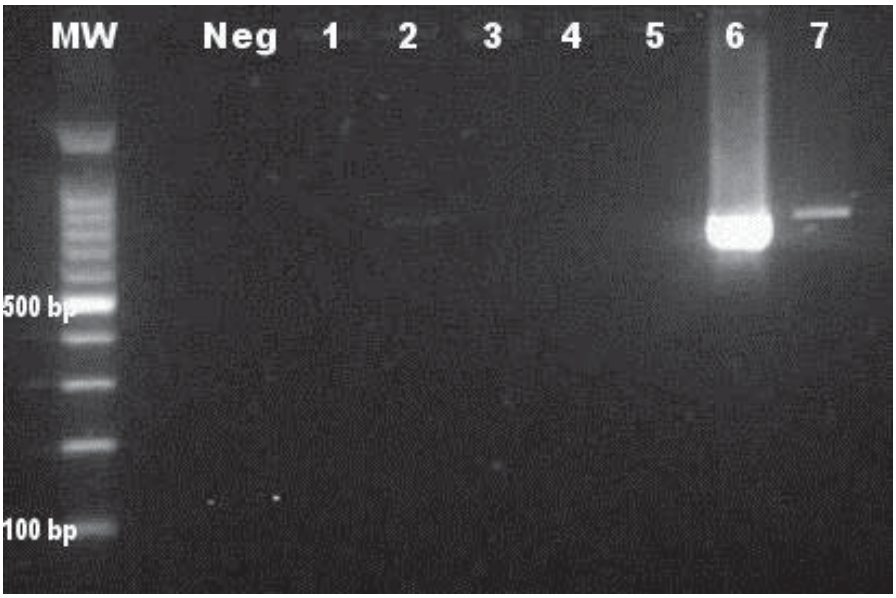


Figure 14. An intense band at lane 6 with approximately 700 bp product of the primer pair C2/C6 segment amplified from the recombinant plasmid.

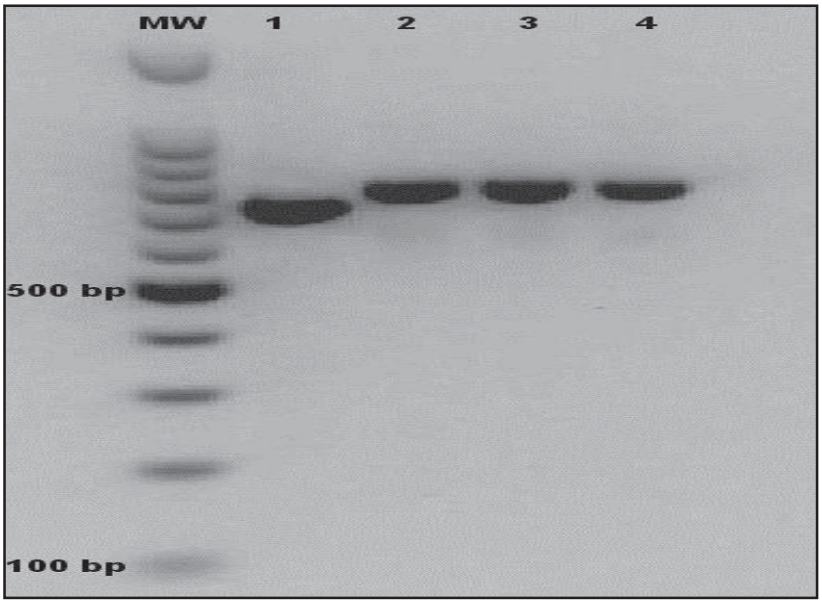


Figure 15. The labelled amplicons (lane 2, 3, 4) have slightly higher molecular weight due to incorporation of DIG molecules but with similar intensity to the control band (lane 1) without DIG-dUTP.

Table 2. Nucleotide sequence of the probe (695 bp) generated from the sequence reads of the recombinant plasmid containing the C2/C6 region insert. Nucleotides in red letters are the forward and reverse primers.

CTCTTTACCATGAAGATACCCACCAATGTGGTAAAGACGGAACAATCTTT
TTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAAA
GTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATGGC
AAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTCGA
TTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAACCCACATG
GGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGCGAT
TTGTCAGTTTAGAATCATACCCACACTCAATCTCGAGTATACCACAACTG
CTAAATTAACAGCATCTACTACTACTACTGAAAAATGCAGCCTTTCACAG
AATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTC
ATCAACCAGACGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTT
CGATTGCGAAGATAAAGTCGTGGCATCATTGGCTGCAGTCAGATCTGACA
TACCCATAGAAGTCACGGAACGCAAAGACCTGAACCTCCTCGACCTGATC
CAGTTCTTCGAAAAGAAGATAGAGTTTACCACTCTCATTGACGAATTGTT
CACTGCCCACAAAGACCATTGTCAGAAAAATGGTAAGCCGTGCAC

4.2 Development of *in situ* hybridisation

Four OsHV-1 positive formalin fixed paraffin-embedded Pacific oyster tissues and three OsHV-1 negative fixed tissues were used as starting material for the development of ISH assay. Positive hybridisation of the DIG labelled probe to target sequences was demonstrated by the presence of dark bluish coloration in cells of Pacific oyster tissues positive for OsHV-1 (Figure 16 and Figure 17). Labelling was initially observed in the nucleus of cells located in the connective tissues of the mantle, gills and digestive ducts. For OsHV-1 negative oysters, there were no hybridisation signals seen (Figure 18).

After an initial detection of hybridisation signals in the tissues of OsHV-1 positive oysters, several key steps of the *in situ* hybridisation protocol were optimised. Permeabilisation of tissue by enzymatic digestion using 100 µg/mL of PK yielded the best results and showed a stronger signal when compared to treatments with 75 µg/mL or 50 µg/mL of PK (Figure 19). Stronger signal was revealed as a greater density or opacity of chromogen and a shift in the colour spectrum from dark blue toward dark purple or black. In contrast, a difference in the intensity of ISH signals was not clearly observed when PK treatments of 15 and 25 minutes were compared. Non-specific staining was apparent when overnight hybridisation step was done at 40°C compared to 42°C. A final post-hybridisation wash using a concentration of 0.75X SSC at 42°C reduced the amount of background signal.

Repeatability of the optimized ISH protocol was demonstrated when duplicate tissue sections yielded similar labelling of cells in the same assay. The reproducibility of the technique was demonstrated when the same results from duplicate tissues were obtained by another technical staff (images not shown).

4.2.1 Specificity

Specificity of the probe was determined when no ISH reactions were observed in OsHV-1 negative tissue section. Labelling of cells in the ISH reaction was also not observed in OsHV-1 positive oyster tissue section when the probe was omitted during the hybridisation step (Figure 20). In this slide, absence of non-specific staining indicated that no DIG-like epitopes were present in the tissue subjected to the optimised pre-treatment protocol. In a parallel experiment using the same protocol, the anti-DIG alkaline phosphatase conjugate was excluded in one of the duplicate slide. The tissue section without the conjugate showed no hybridisation signal and no background staining (Figure 21). Altogether, these results indicate that the optimised ISH protocol was specific for detection of OsHV-1 DNA.

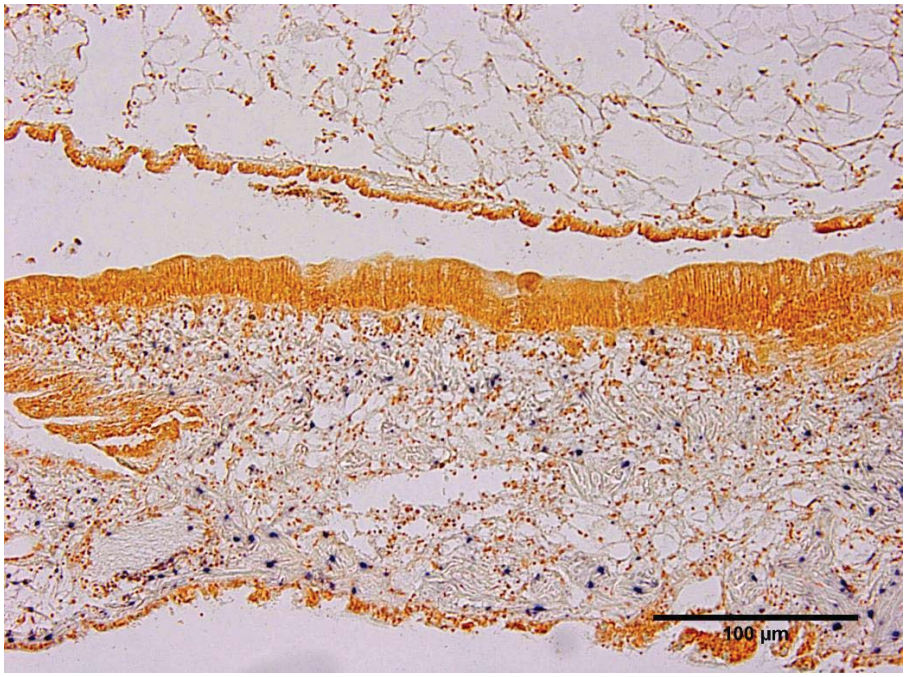


Figure 16. Positive hybridization of the C2/C6 probe and the target nucleic acid segment was demonstrated by dark blue precipitate in the nucleus of the cells (scale bar = 100 µm).

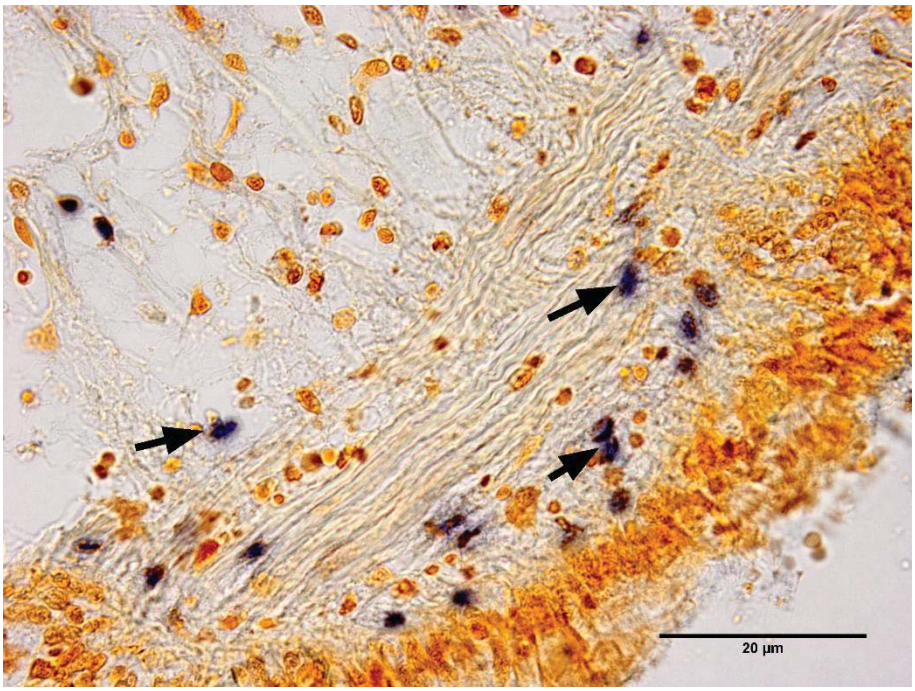


Figure 17. Strong ISH labelled cells along the muscle fibres within the connective tissues of the mantle margin of the Pacific oyster (scale bar = 20 µm).

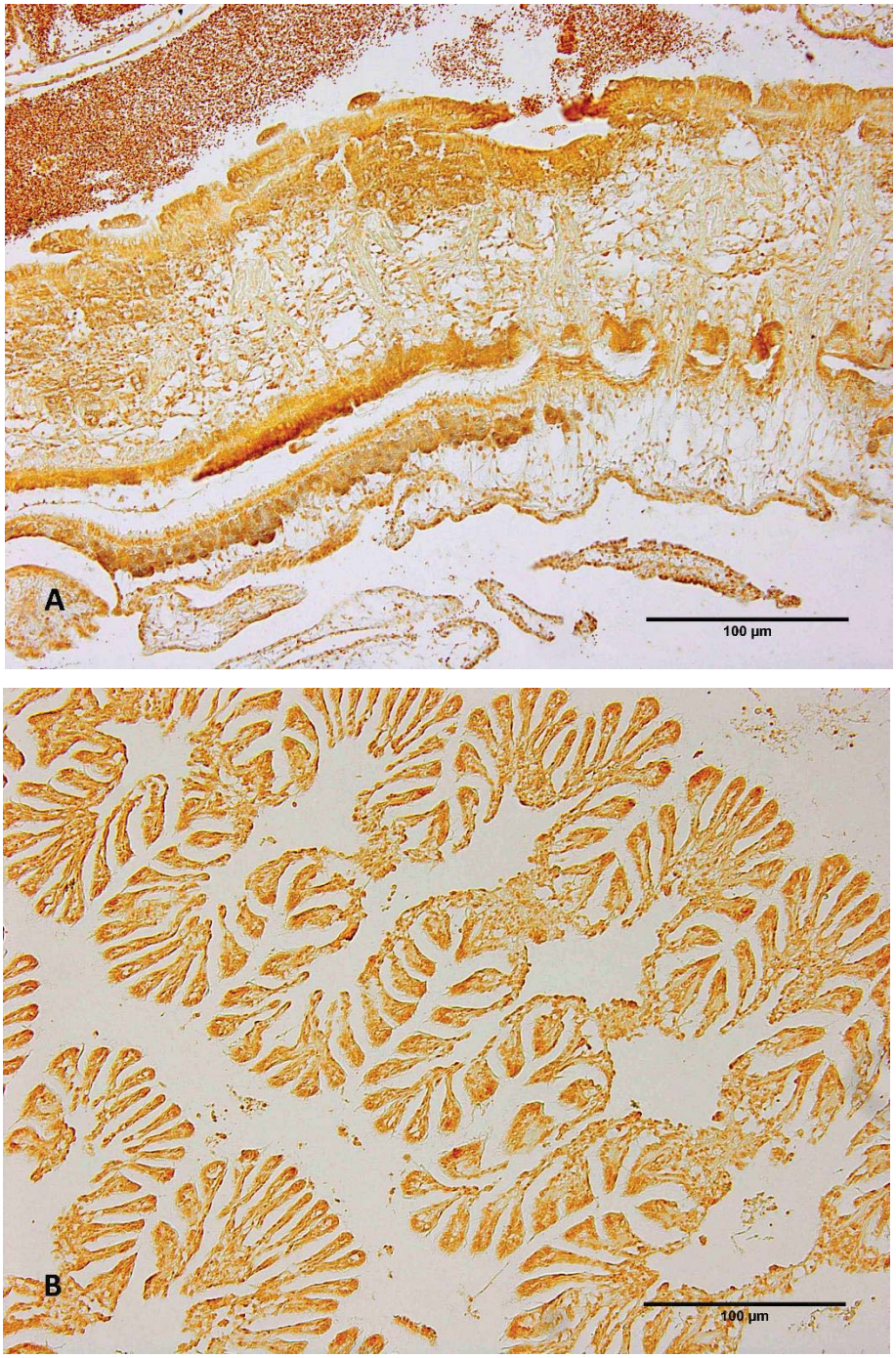


Figure 18. OsHV-1 negative oyster tissues. Mantle (A) and gills (B) showed no signals in the ISH assay using DIG labelled C2/C6 probe (scale bar = 100 µm).

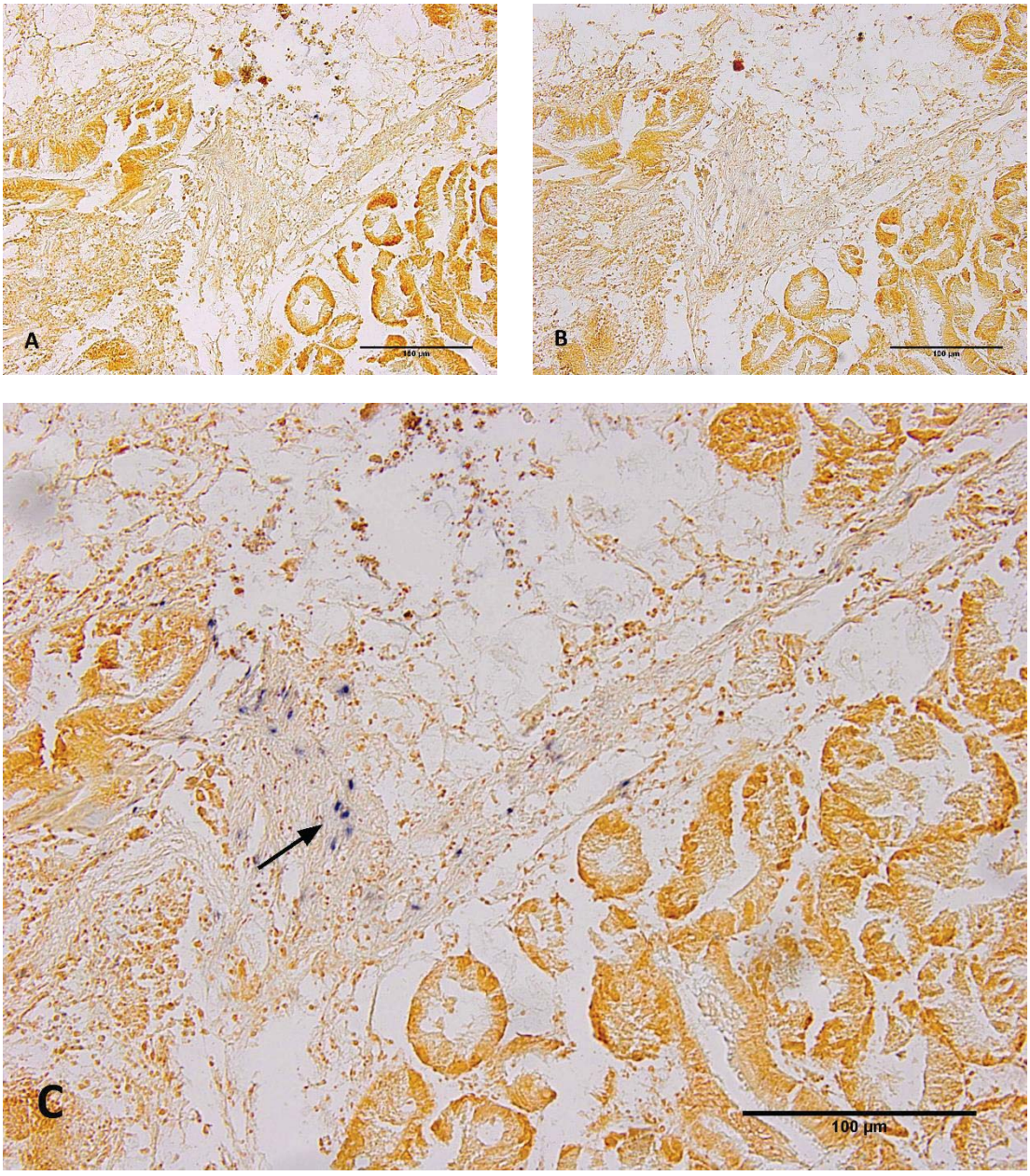


Figure 19. Permeabilisation by proteinase K (PK) digestion to promote probe penetration in tissues. A PK concentration of 100 µg/mL (C) is more efficient, as shown by a more intense dark blue staining in cells (arrow) as compared to 75 µg/mL (B) and 5µg/mL (A) (scale bar = 100 µm).

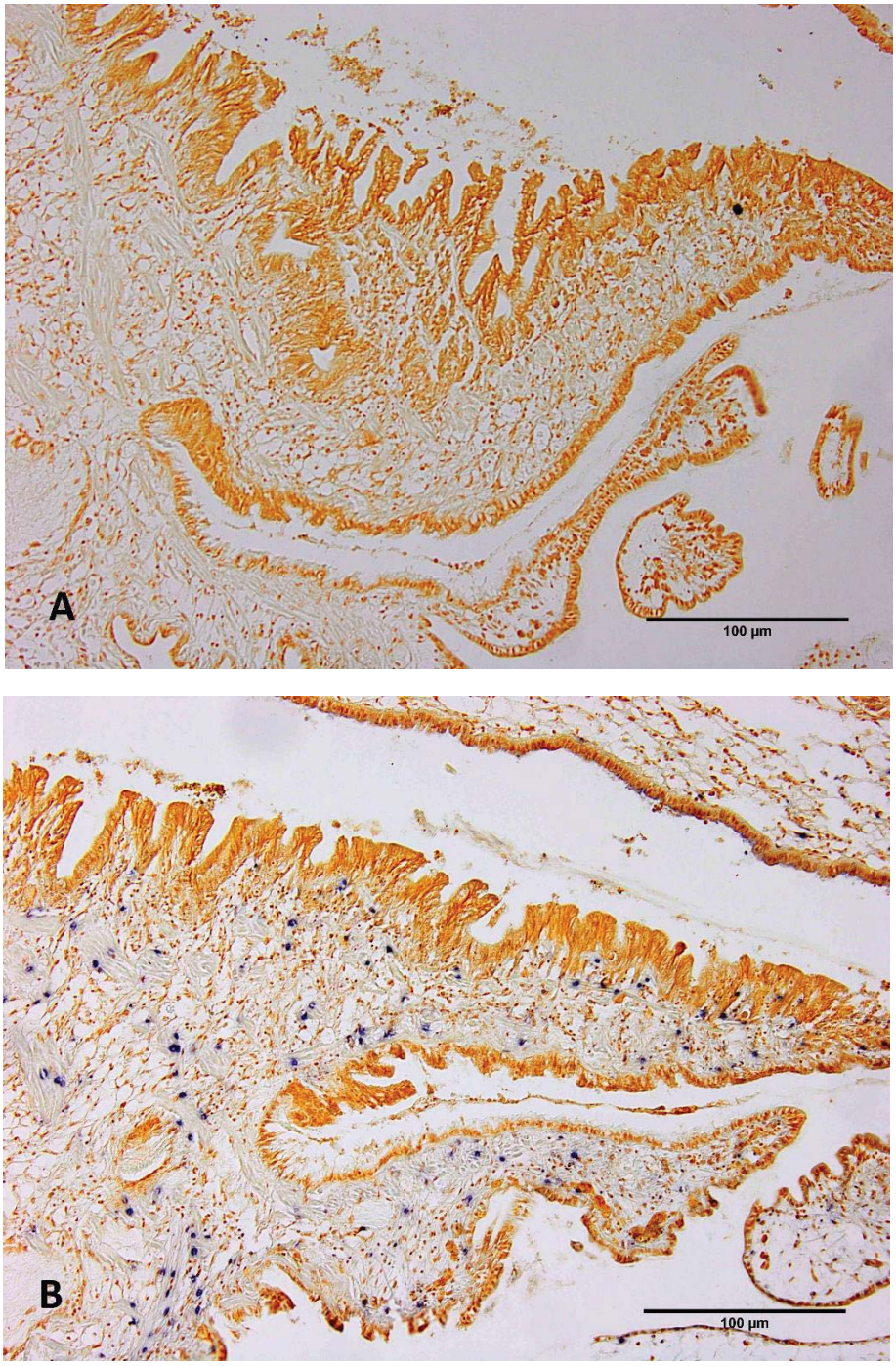


Figure 20. Non-specific reaction of the anti-DIG conjugate is not evident when the probe was omitted in one of two serial tissue sections from the same oyster. Tissue section B has numerous dark blue ISH precipitates while no signal was observed in section A when the probe was not included during hybridisation (scale bar = 100 µm).

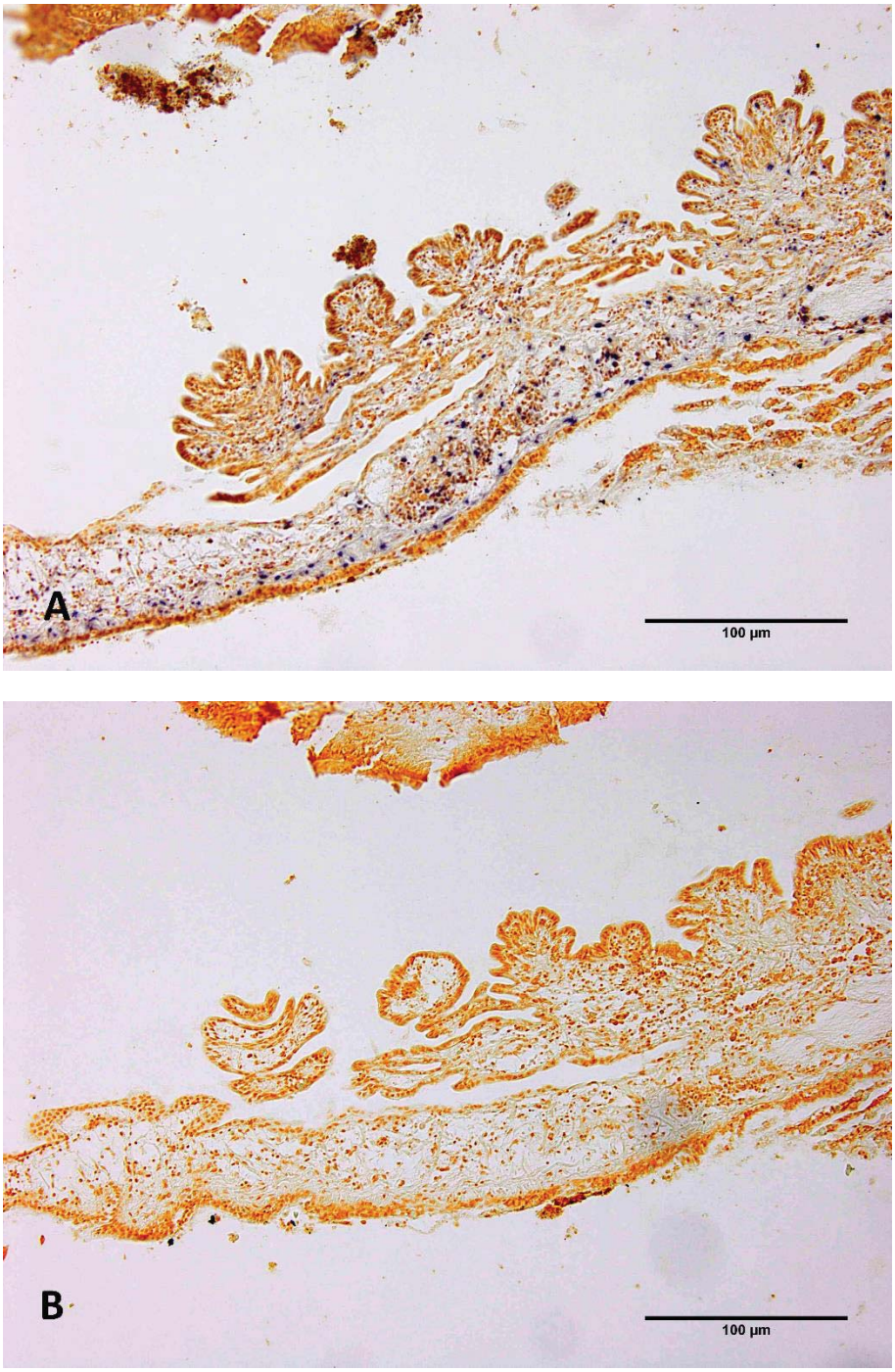


Figure 21. In two (A and B) serial section from the same OsHV-1 positive oyster, abundant dark blue ISH precipitates are noticeable in tissue section A while no signal was observed in B when the anti-DIG conjugate was omitted prior to colorimetric development (scale bar = 100 µm).

4.3 Demonstration of OsHV-1 nucleic acids in New Zealand Pacific Oysters

4.3.1 ISH and PCR assay

Individual results of testing spat samples by the ISH assay are shown in Appendix 1. An oyster spat was considered positive if dark blue to purplish black stained cells were observed as a consequence of the complementary hybridisation of the C2C6 DIG labelled probe with the OsHV-1 viral genome. No labelling was detected in controls such as the OsHV-1 negative oyster tissues, and OsHV-1 positive tissues with probe omitted during hybridisation or OsHV-1 positive tissues with the anti-DIG conjugate omitted prior to development using enzyme substrate during immuno-histochemical detection. Thirty two of 87 (36.78%) oyster tissues were positive for OsHV-1 DNA by ISH. Fifty five of 87 (63.91%) were negative for OsHV-1 DNA by ISH. One spat sample collected at day 1 post transfer and another at day 3 post transfer showed ISH labelling in the digestive lumen but not within individual cells and were considered negative with OsHV-1 infection.

The proportion of samples positive for OsHV-1 was higher when fresh tissues were tested by qPCR than when equivalent formalin fixed paraffin embedded tissues were tested by ISH. The OsHV-1 prevalence by qPCR was 71.26% (62/87), with 28.73% (25/87) negative samples (Table 3). The total number of observed agreement between the two tests was 55 (63.22%), including 31 samples positive for OsHV-1 by both qPCR and IHC, and 24 samples negative for OsHV-1 by both tests (Appendix B). Most of the disagreements included samples that were positive for OsHV-1 by qPCR, but negative by ISH. However, one spat negative for OsHV-1 by qPCR was positive with hybridisation signal by ISH. A fair agreement between the two tests, demonstrated by *Kappa* value of 0.339 (C.I. 0.195-0.483) was observed. The preliminary diagnostic sensitivity and

specificity obtained for the ISH assay relative to qPCR were 50% and 96.00% respectively (Table 4).

Table 3. Interpreted results of the ISH assay and real time PCR (qPCR).

Sample	ISH	qPCR*	Sample	ISH	qPCR	Sample	ISH	qPCR
1586-1	-	-	1620-17	+	+	1651-9	+	+
1586-2	-	-	1620-19	+	+	1651-12	+	+
1586-3	-	-	1620-21	-	+	1651-13	+	+
1586-4	-	-	1620-22	-	-	1651-14	-	+
1586-5	-	-	1620-23	-	-	1651-18	+	+
1601-11	-	-	1620-24	+	+	1651-19	+	+
1601-12	-	-	1620-27	+	-	1651-20	-	+
1601-13	+**	-	1636-1	+	+	1651-21	-	+
1601-18	-	-	1636-2	-	+	1651-24	+	+
1601-24	-	-	1636-4	-	+	1651-25	+	+
1601-27	-	-	1636-5	+	+	1651-26	+	+
1601-30	-	-	1636-6	+	+	1651-29	-	+
1618-1	-	-	1636-7	+	+	1671-1	-	+
1618-2	-	-	1636-8	-	+	1671-2	-	+
1618-3	-	-	1636-9	+	+	1671-5	+	+
1618-4	-	-	1636-10	+	+	1671-6	-	+
1618-5	+**	-	1636-11	+	+	1671-7	-	+
1618-11	-	-	1636-13	+	+	1671-12	-	+
1618-14	-	-	1636-14	+	+	1671-17	-	+
1620-1	-	+	1636-15	-	+	1671-19	-	+
1620-2	+	+	1636-17	-	+	1671-21	-	+
1620-3	-	+	1636-19	+	+	1671-22	-	+
1620-10	+	+	1636-26	-	+	1671-23	-	+
1620-11	-	+	1636-27	-	+	1671-24	+	+
1620-12	-	+	1651-2	+	+	1671-25	-	+
1620-13	+	+	1651-3	+	+	1671-26	-	+
1620-14	-	+	1651-4	+	+	1671-27	-	-
1620-15	+	+	1651-7	-	+	1671-28	-	-
1620-16	-	+	1651-8	+	+	1671-30	-	-

*qPCR is a probe based Taqman real time PCR developed and validated by AHL-IDC used to test for OsHV-1 infection during the juvenile Pacific oyster mortality investigation (Keeling *et al.*, 2014). Samples were tested in duplicate and were considered positive (+) for OsHV-1 if both replicates had a cut-off Cq value of ≤39, and negative (-) if both or one replicate has more than 39 Cq value.

** ISH labelling were detected in digestive lumen but not within tissues, and the spat was considered not infected with OsHV-1.

Table 4. Calculated sensitivity and specificity of ISH assay relative to qPCR as reference method.

	Point Estimate	Lower 95% CL	Upper 95% CL
Sensitivity (DSe)	0.50	0.3702	0.6298
Specificity (DSp)	0.96	0.7965	0.999
Likelihood ratio +ve	12		
Likelihood ratio -ve	0.5208		

4.3.2 Spread of infection and mortality

ISH detected OsHV-1 viral DNA in various tissues of samples collected. Dark blue to purplish black staining were observed in the nucleus and cytoplasm of cells located in the connective tissues of the mantle (Figure 22), gills (Figure 23A), beneath the primary digestive tract (Figure 23B) and secondary digestive ducts (Figure 23C). Labelled cells interpreted as haemocytes (Figure 23D) were also observed within the lumen of the gonad follicle. Some stained cells were also detected within the gonad (Figure 24A & Figure 24B) and nervous tissue (Figure 24C & Figure 24D). Intensity of labelling signals in tissues was variable but highly noticeable was the abundance of positive ISH signals in the connective tissue of the mantle (Figure 25). In addition, a more intense hybridisation signal was observed in the nuclei of cells dispersed within the muscle fibres in the mantle tissue (Figure 25B). These cells were interpreted as OsHV-1 infected haemocytes.

The distribution and sequential spread of OsHV-1 infection in various oyster tissues and organs is depicted in Figure 26. No labelled cells were detected in spat collected a day prior to placement (day -1). At day 1 post transfer to the farm, one spat out of 7 tested (1/7) demonstrated positive ISH signal in the lumen of the digestive

tract (Figure 27A). On the 3rd day post transfer, one spat out of 7 tested for ISH also demonstrated ISH signal in the intestinal lumen (Figure 27B). OsHV-1 infected cells were detected at day 5 post farm introduction. Out of 17 spat tested, 8 (47%) showed blue to purplish black precipitates in various tissues. The incidence of ISH positive oysters increased to 59% (10/17) at day 7 and 71% (12/17) at day 9 post transfer. However, only 2 out of 17 oysters (12%) were ISH positive at day 13 post placement.

Based on the intensity of staining and the number of cells infected (+++), severe infections were noted on spat collected from days 5, 7 and 9. During these periods, ISH labelled cells were predominantly confined to the mantle and gills, followed by occasional signals in the connective tissue surrounding the different parts of the digestive tract. Observed mortalities of 14% were recorded initially on day 6 post transfer. At day 9 and 13, cumulative mortalities were 50% and 70%, respectively. In this prospective study, temporal relationship was observed between detection of OsHV-1 infection shown by ISH labelled cells at day 5 and occurrence of mortalities at day 6 (Figure 28).

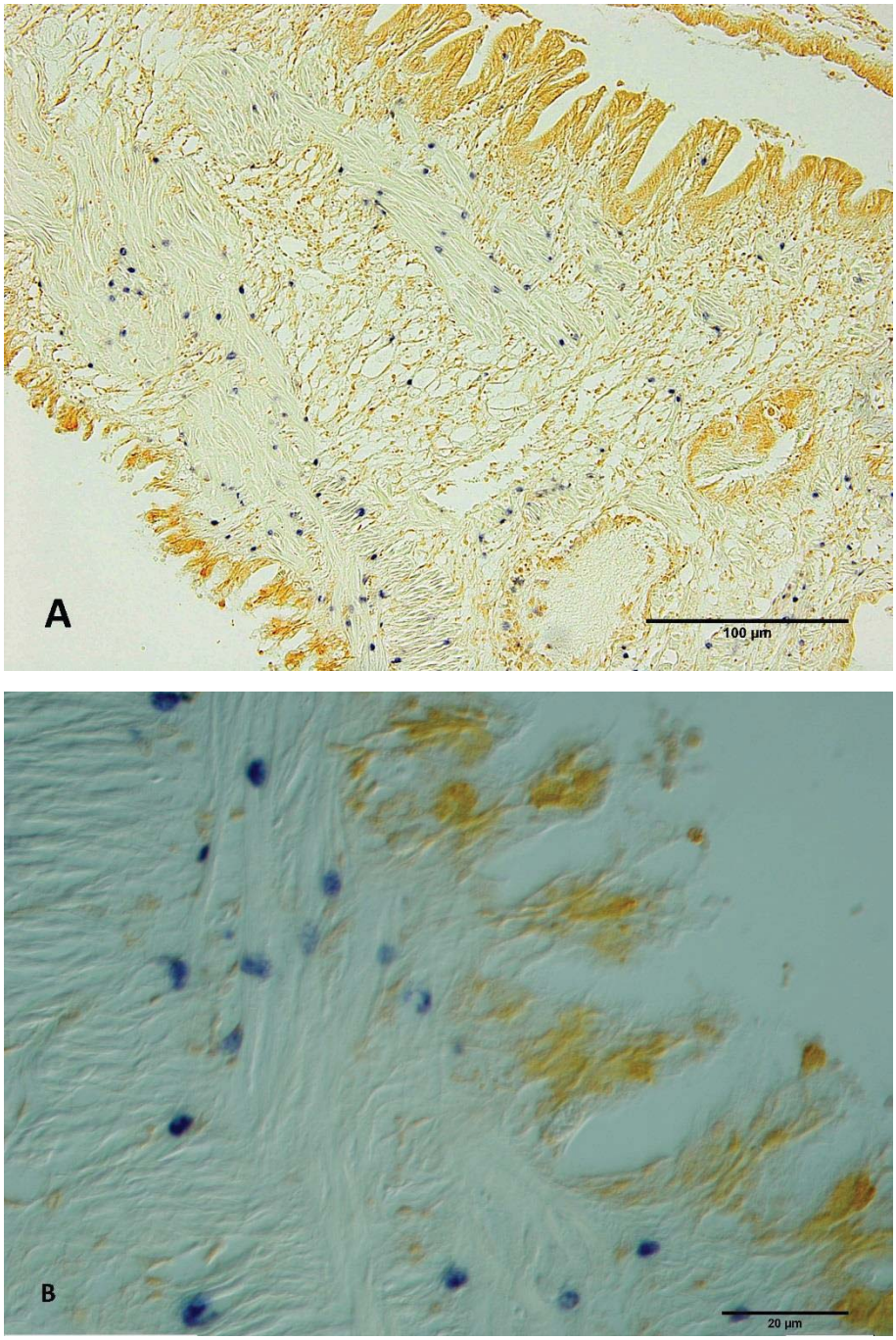


Figure 22. Dark blue signal in cells in the stroma of the same section of the mantle. Section B image was taken using differential phase contrast (scale bar = 100 µm (A) and 20 µm (B).

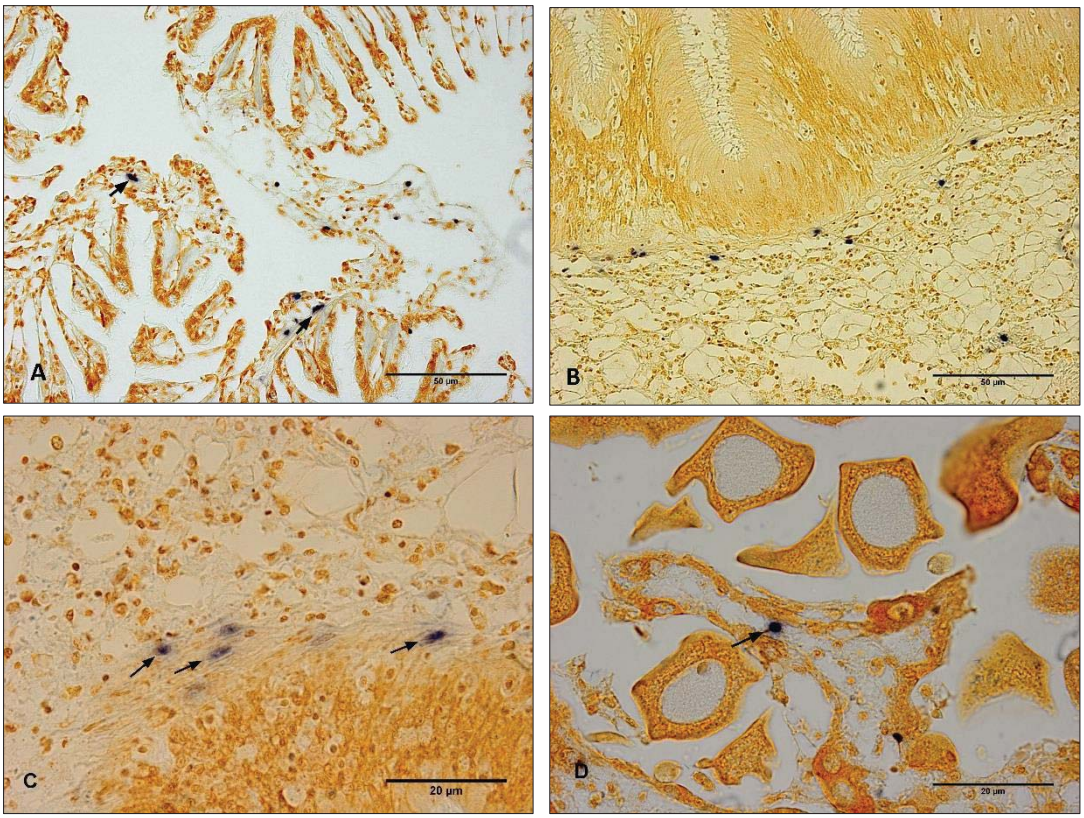


Figure 23. ISH signal (dark blue labelled cells with arrow) in the connective tissue of the gills (A), Leidig tissue and basal membrane of the digestive tract (B and C), and in the lumen of gonad follicles interpreted as haemocyte (D) (scale bar = 50 µm (A, B and D), 20 µm (C)).

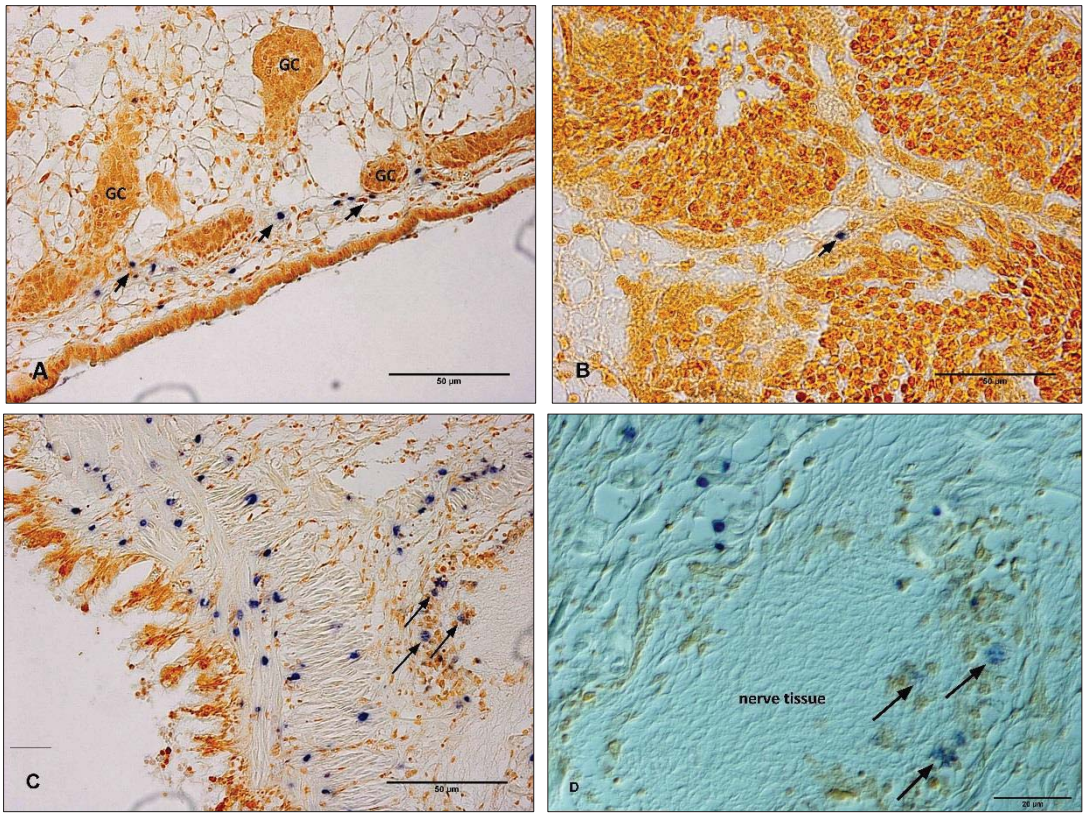


Figure 24. Labelled cells associated with germinal centres (GC) in A (with arrows) and within interstitial space of the male gonad in B. ISH signals in C and D were also observed in cells (arrows) within the periphery of the nerve tissue (scale bar = 50 µm (A, B and C); 20 µm (D - taken using differential phase contrast)).

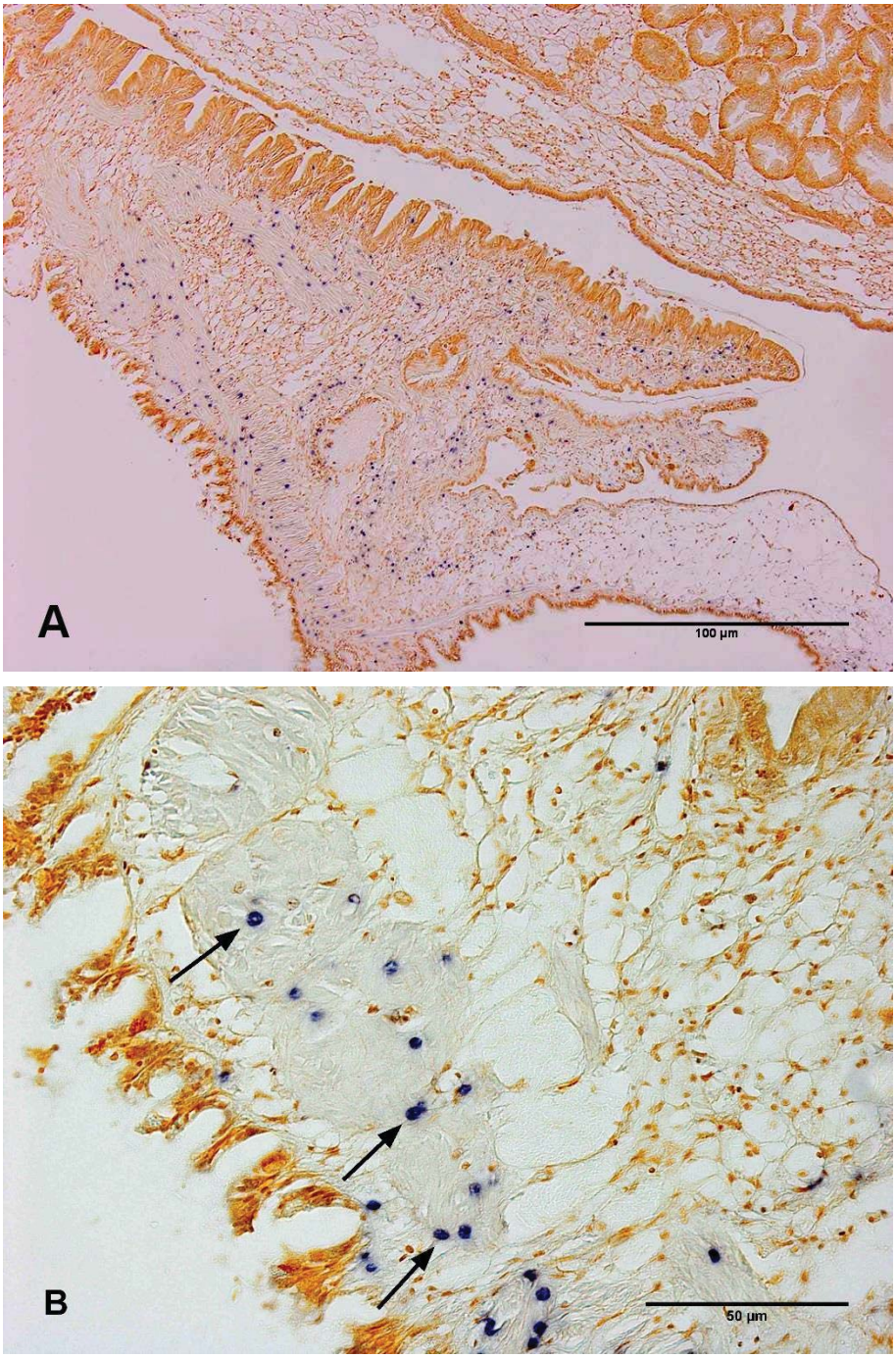


Figure 25. A. Abundant hybridisation signal (blue labelled cells) within the connective tissue of the mantle (scale bar = 100 µm). B. Hybridisation signal in the muscle fibres of the mantle are most likely haemocytes (arrows) (scale bar = 50 µm).

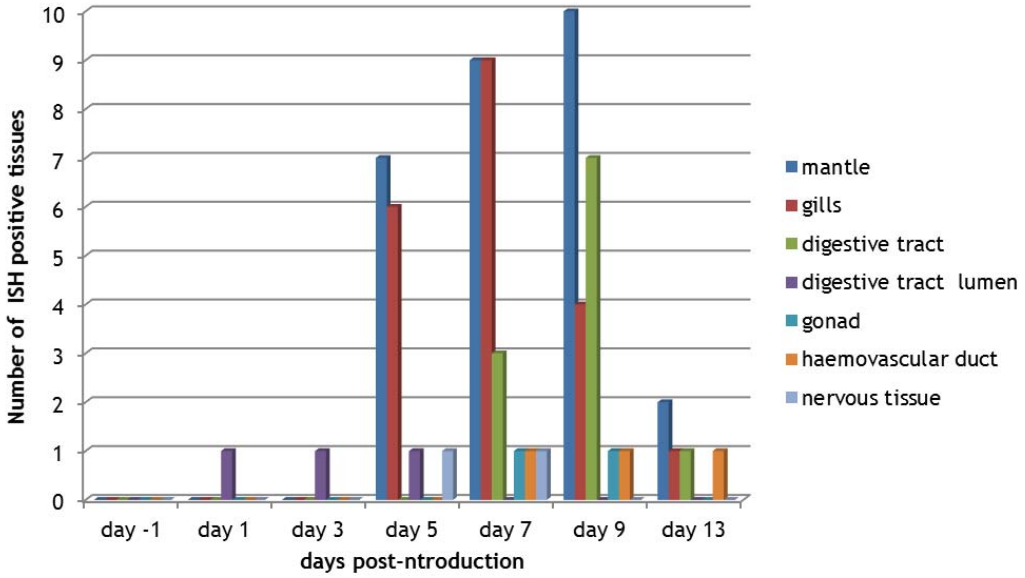


Figure 26. The graph shows the chronological appearance of hybridisation signals in different Pacific oyster tissues during the follow up period of the study. At day 1 and 3 post introduction, hybridisation signal were detected initially in the lumen of the intestine. On subsequent sampling days (5, 7, 9), ISH labelling were predominantly confined in the connective tissue of the mantle, gills and in the sub-epithelium of the digestive tract. Occasional ISH signals were also seen in nerve tissue, gonad and haemovascular ducts.

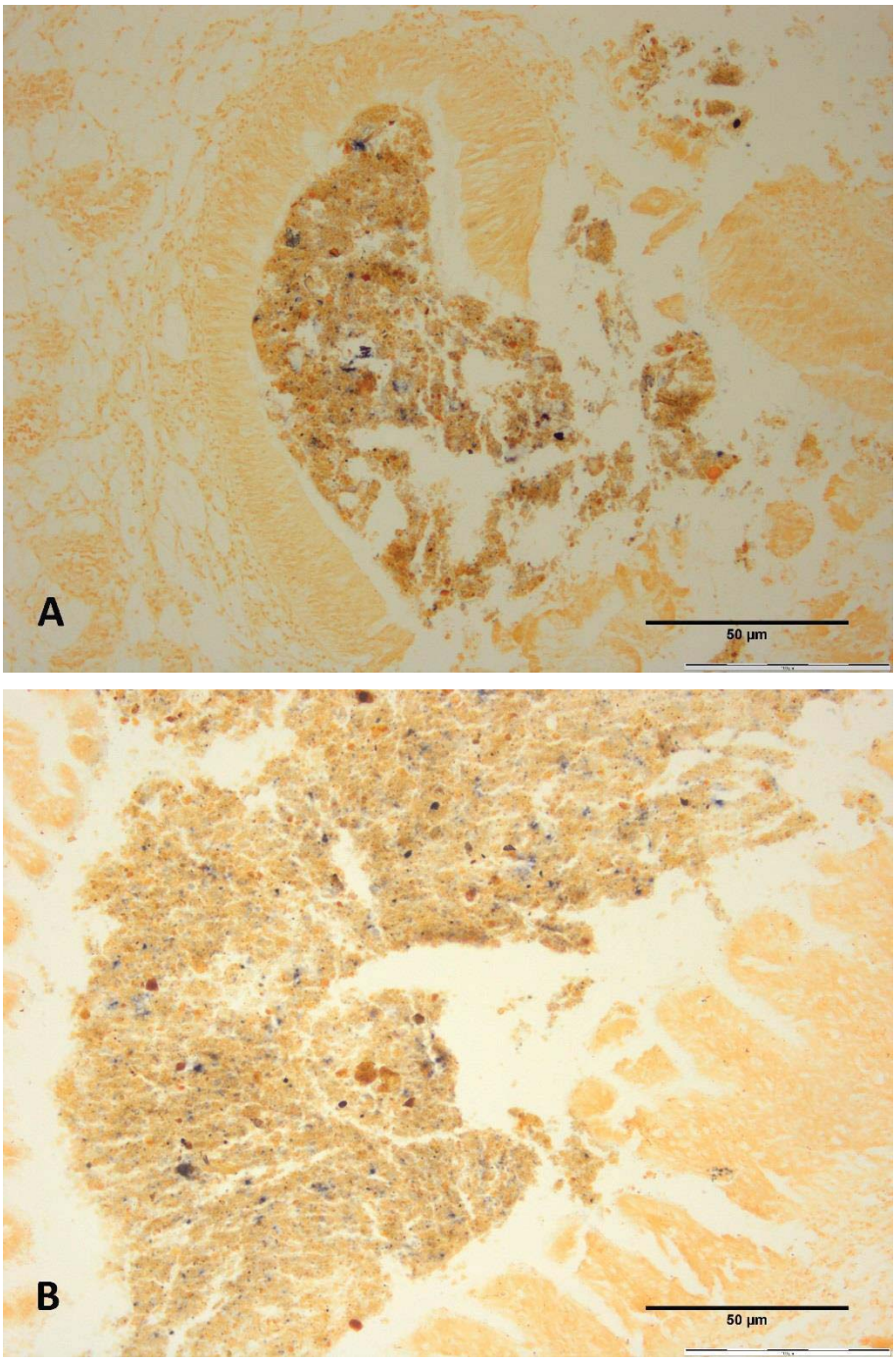


Figure 27. ISH signal in the digestive lumen of a spat collected at day 1 (A) post transfer and at day 3 (B) post transfer (scale bar = 50 µm (A and B)).

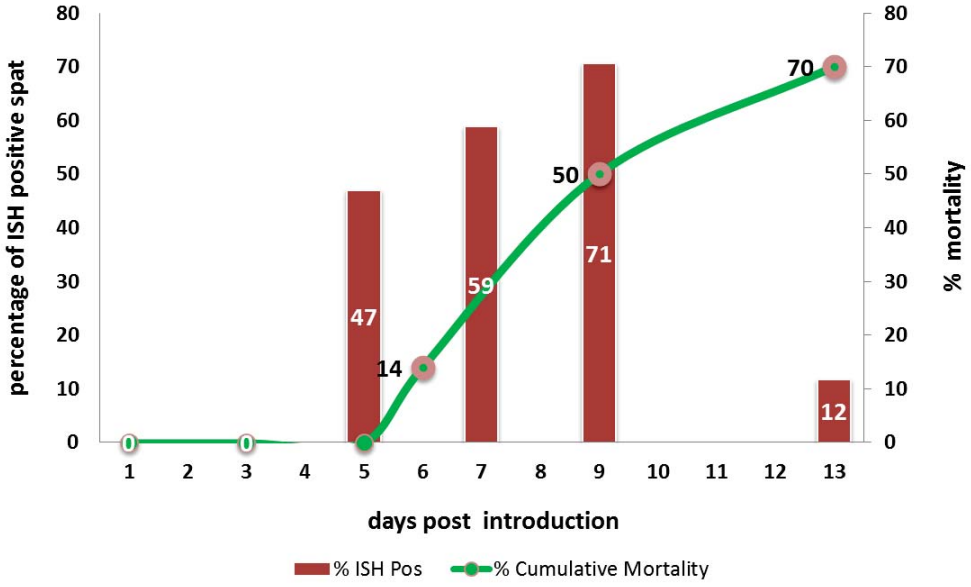


Figure 28. The proportion of OsHV-1 ISH positive spat were 47% at day 5, 59% at day 7 and 71% at day 9 after introduction to an infected farm. However, only 12% were found to be ISH positive at day 13. Low detection rate was possibly due to mostly dead spat collected at this time point, making the material unsuitable for testing. Viral DNA in autolysed tissues may have been degraded and or very low in copy numbers, such that ISH detection was not possible. The appearance of OsHV-1 ISH positive spat at day 5 post introduction and the onset of mortality at day 6 indicated the temporal relationship between the two variables.

4.3.3 Histopathology

Progressive pathology was difficult to ascertain, although several non-specific histological changes were observed in oyster tissue sections from the prospective study. One prominent example was the aggregation of haemocytes in tissues (haemocytosis), taken at day 1 and 3 post transfer. However, this was observed also from spat taken from the hatchery. Haemocytosis was seen as either focal sites of inflammation (Figure 29A and Figure 29B) or diffuse and systemic cellular infiltration in the stromal connective tissues (Figure 29C). Cell aggregations were particularly noticeable around major digestive ducts and tubules (Figure 30A) and in the stroma of the mantle sub-epithelium (Figure 30B). Overall, it appeared that the degree of severity of haemocytosis in the various samples transitioned from focal, light and moderate diffuse haemocytosis (hatchery samples at day -1, day 1, day 3) to moderate and severe diffuse haemocytosis (day 5,7,9) (Figure 31). In spite of these inflammatory changes however, discernible association with any pathologic agents, detectable by light microscopy was not detected in the H & E stained sections.

Degenerative lesions were also noted in the digestive glands in most tissues at all collection time-points with no obvious pattern being discerned. These included different stages of epithelial atrophy of the diverticula, even within individual samples. In the normal digestive diverticula, epithelial cells are tall and with triradiate or quadriradiate lumen (Figure 6 and Figure 32A). Some of the apparently abnormal tissues had sloughed gland epithelial cells in the lumen (Figure 32B) and others had dilated appearance due to reduction in height of the epithelium (Figure 32C). Atrophy was seen predominantly in the peripheral region of the digestive glands although in some slides atrophy had a more general distribution.

Other occasional non-specific changes seen were signs of foci of cell death such as pycnosis and karyorrhexis observed in spat collected at days 1 and 3 and diapedesis of haemocytes towards the digestive duct lumen across the different samples (Figure 33). Focal and diffuse necrotic cell infiltrates in tissues in the mantle (Figure 34), and loss of tissue architecture of the gills (Figure 35) and digestive ducts (Figure 36) at days 5 and 7 were also seen. Intranuclear inclusion bodies were not detected in any of the samples but some chromatin margination in cells (Figure 37) were seen in day 7 samples. Another observation was the apparent increase in the number of brown pigmented cells also called, "brown cells", in areas with necrosis (Figure 38).

4.3.3.1 Co-location of ISH signal with histological changes

Tissues taken at days 5 and 7 post transfer revealed widespread cellular degeneration and necrotic foci in the connective tissues of the mantle and gills (Figure 39). In several instances, cellular changes were co-localised with the presence of OsHV-1 infected cells as determined by ISH. *In situ* hybridisation signal in gills with an extensive cellular necrosis on equivalent serial H & E stained section from day 7 samples were observed. Necrotic cells were also noted in the connective tissues of the mantle region with extensive positive hybridised cells. In a mantle section taken at day 7 with haemocytic infiltration, the stromal tissue contained OsHV-1 infected cells distributed throughout the sub-epithelium and around vascular duct (Figure 40 and Figure 41). In some slides, large cells with strong dark blue ISH precipitates within the muscle fibre bundles of the mantle palial lobe were surrounded with cellular aggregates of haemocytes cells (Figure 42).

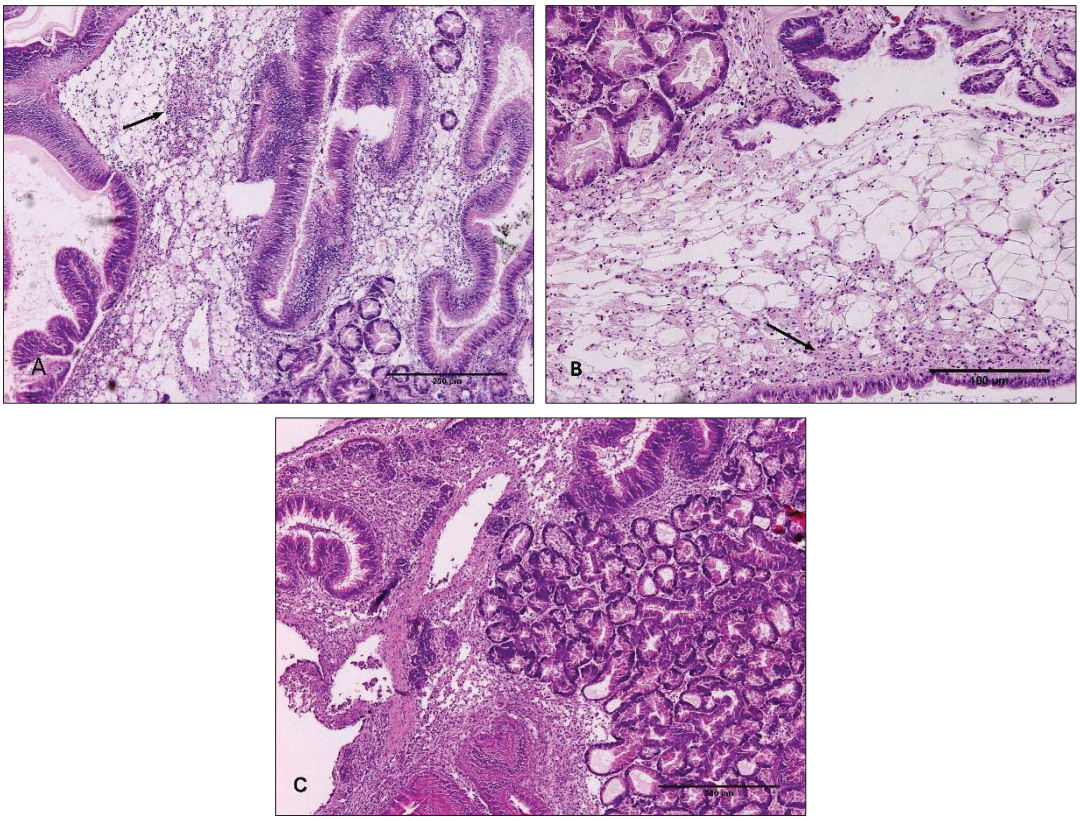
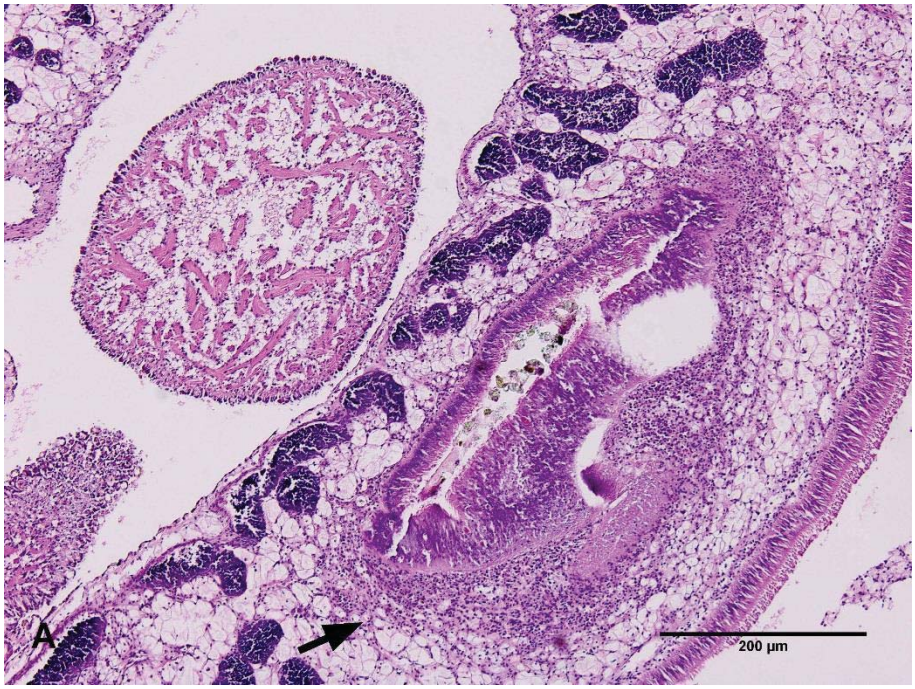


Figure 29. Foci of haemocyte aggregations are usually seen from spat collected at day 1 and 3 post farm introduction (A and B) while the spat section (C) with severe haemocytosis in the stromal connective tissue was from day 5 sampling (C), H & E (scale bar = 100 µm (A and B), 200 µm (C)).



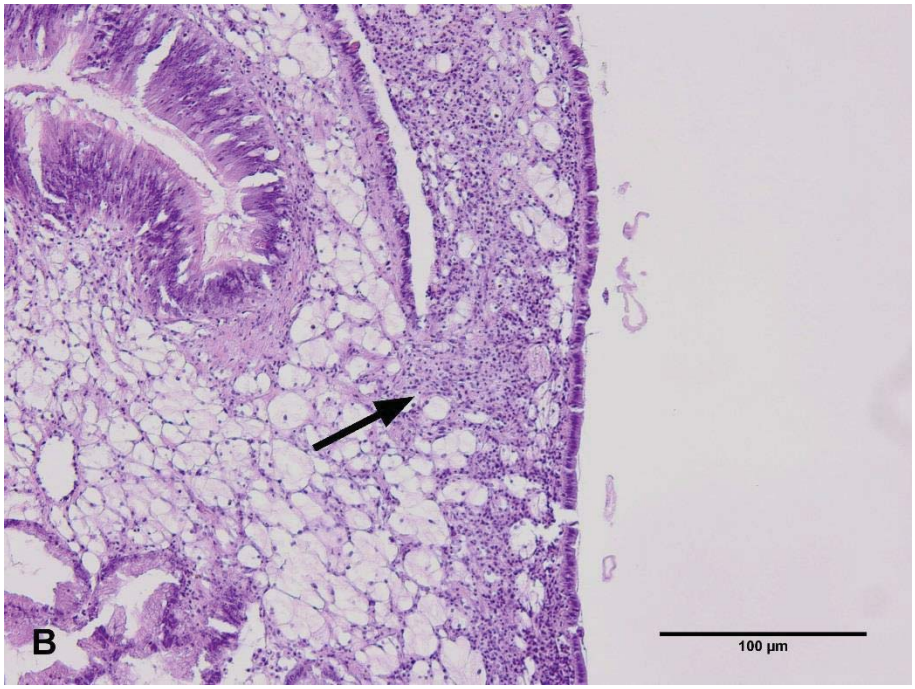


Figure 30. Cellular aggregation around digestive duct (A), and haemocytosis in the mantle region (B), H & E (scale bar = 200 (A), 100 µm (B)).

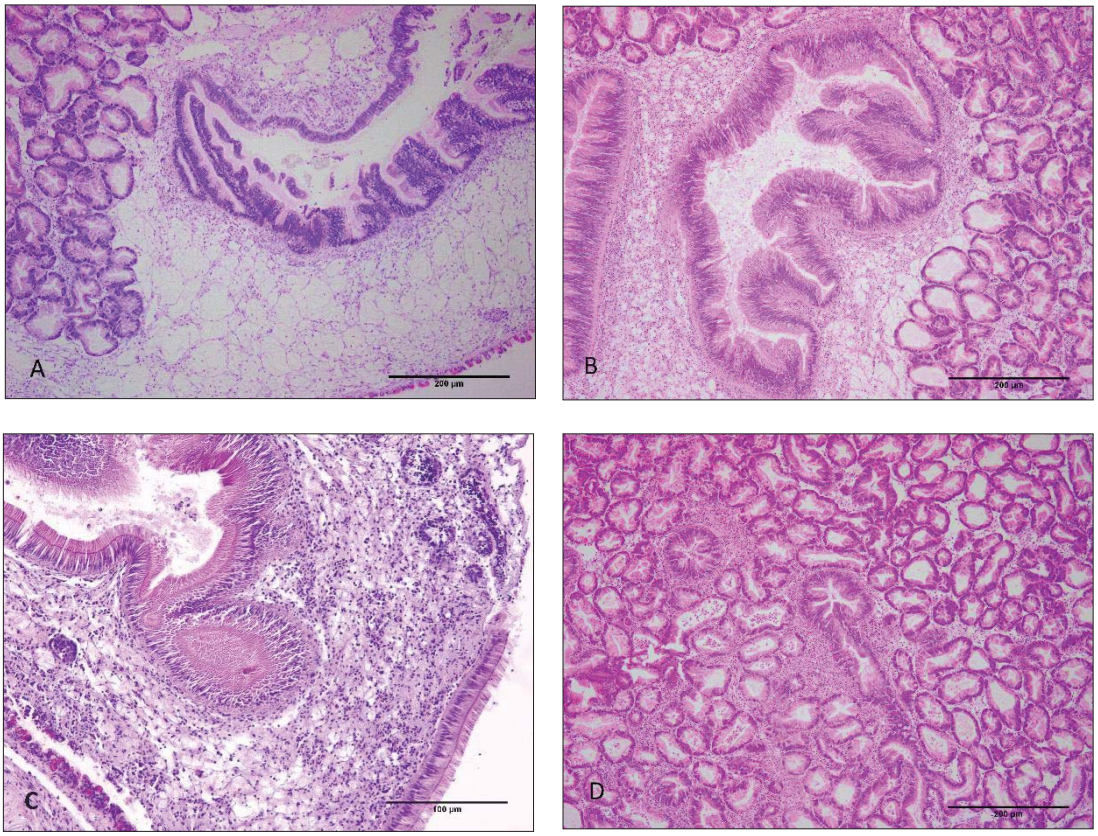


Figure 31. Degree of haemocytosis severity in *C. gigas* spat was described as mild to focal (A), moderate (B), and severe (C and D). Mild to focal haemocytosis was common from day 1 and 3 spat samples, and transitioned to moderate and severe from day 5 spat samples onwards, H & E, (scale bar = 200 µm (A,B,D) and 100 µm (C)).

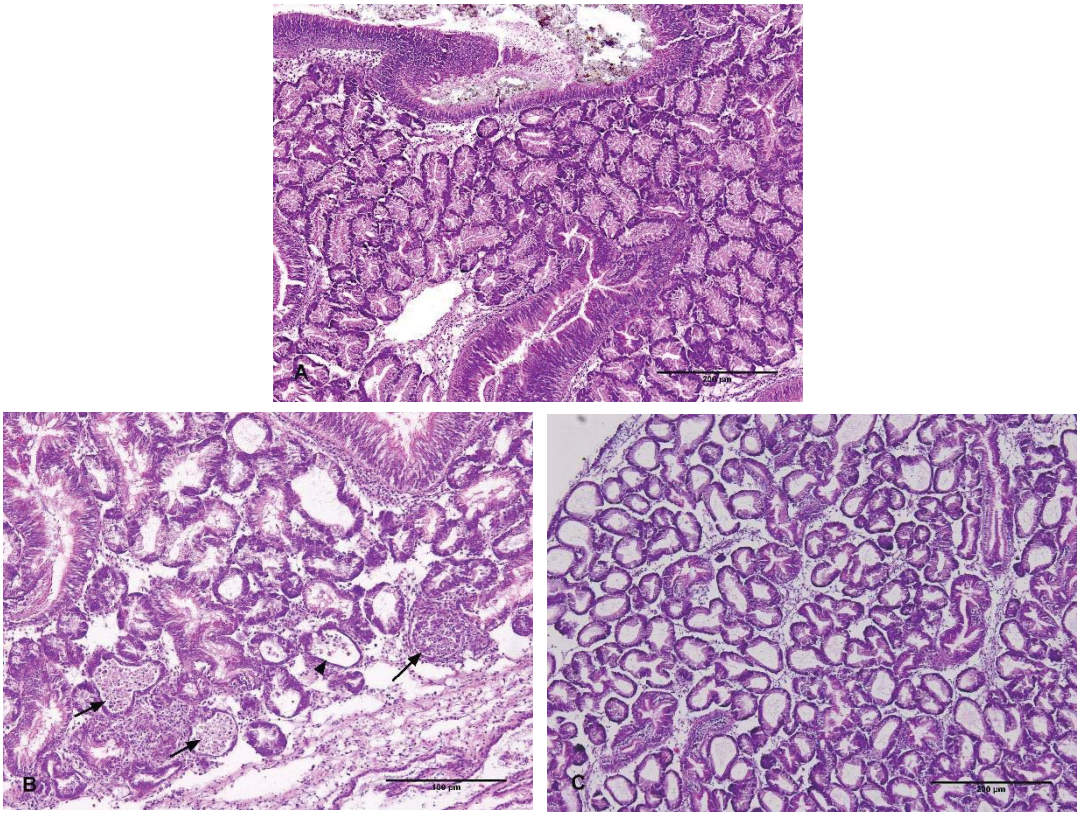


Figure 32. A. Normal digestive glands or diverticulae, H & E (scale bar = 200 µm). B. Non-normal digestive glands (arrow) with sloughing of epithelial cells leading to thin wall (arrowhead), H & E (scale bar = 100 µm). C. Dilated digestive glands, H & E (scale bar =200 µm).

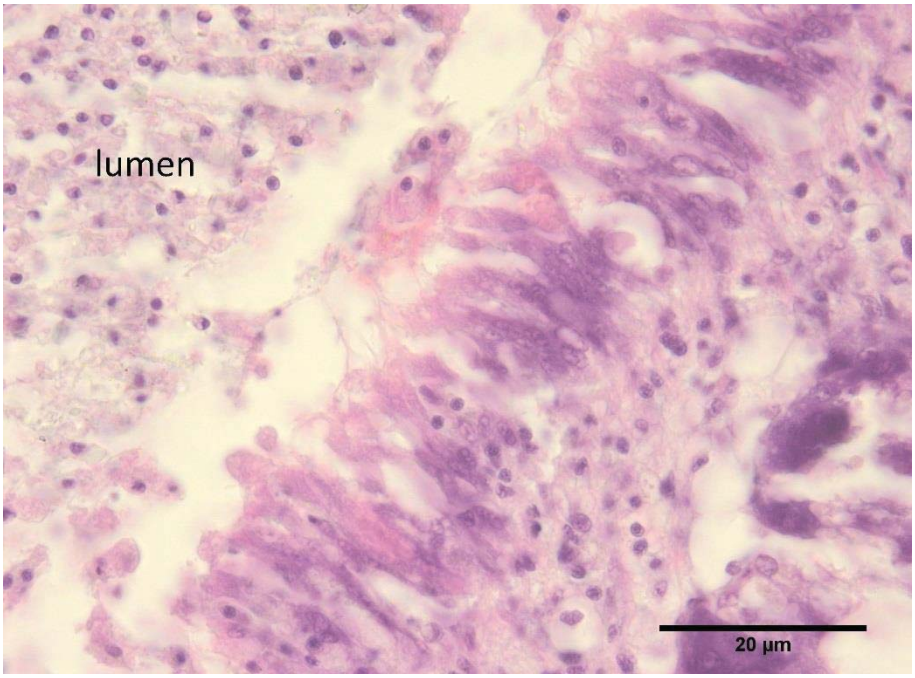


Figure 33. Haemocytes in the lumen of the digestive tract due to diapedesis, H & E (scale bar = 20 µm).

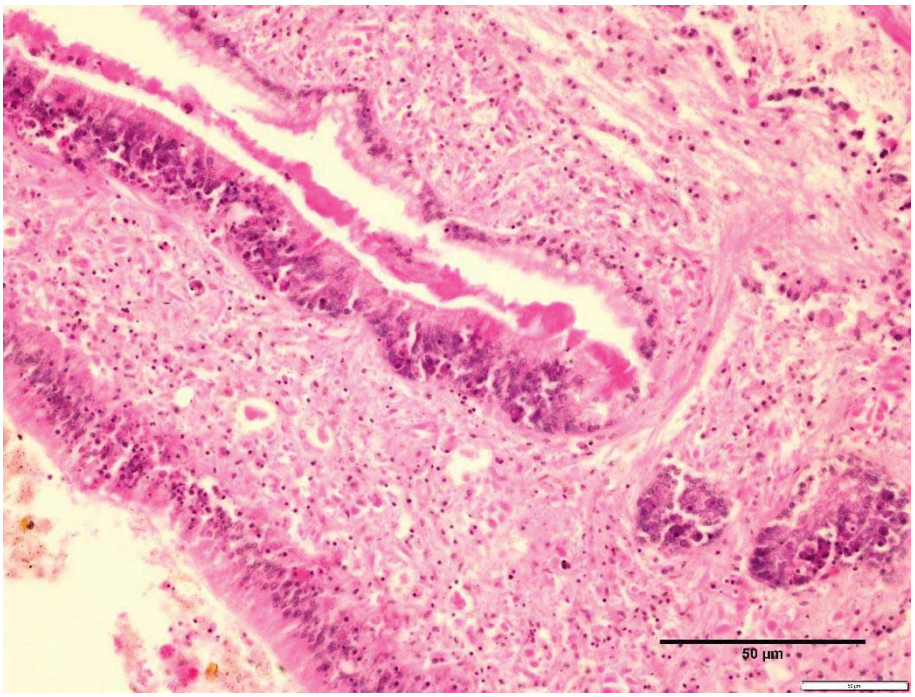


Figure 34. Necrotic cell infiltrates in the mantle connective tissue. Note the loss of cellular architecture of muscle cells or myocytes and the epithelial cells of the mantle, H & E (scale bar = 50 µm).

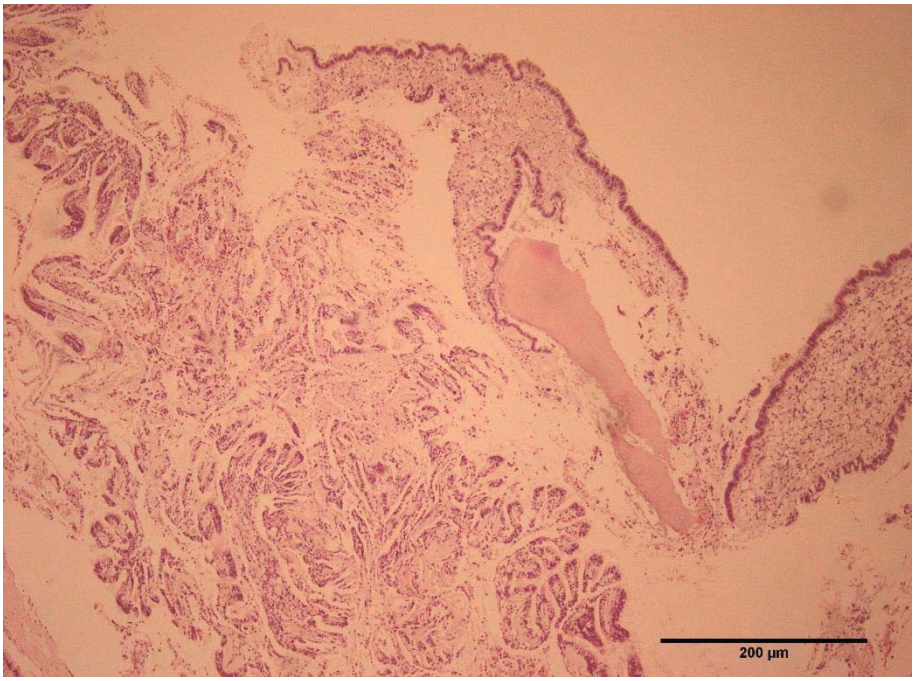


Figure 35. Loss of tissue architecture in the gills, H & E (scale bar = 200 µm).

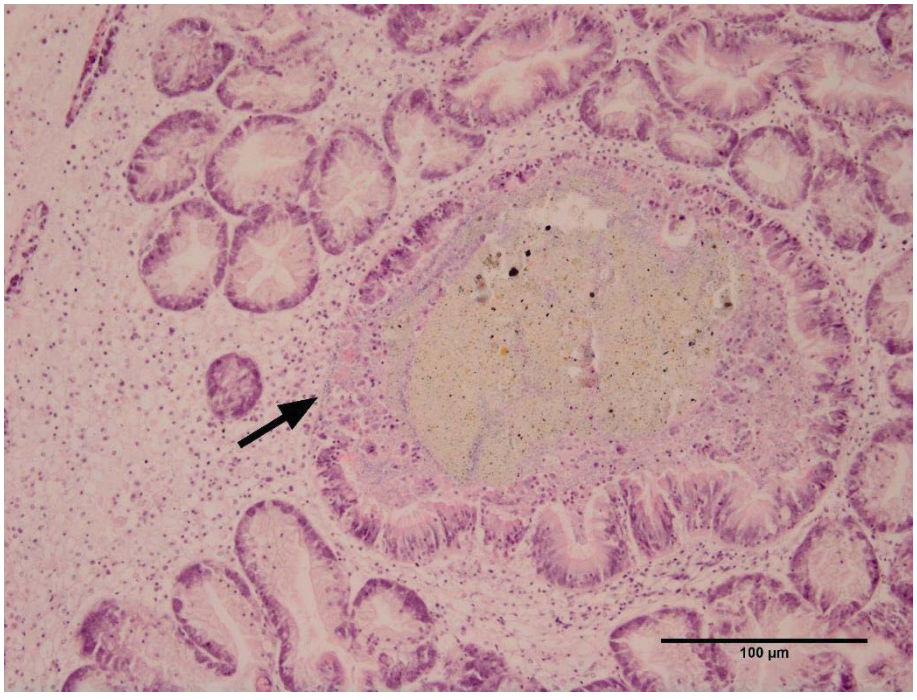


Figure 36. Necrotic epithelial wall of a digestive duct, H & E (scale bar = 100 µm).

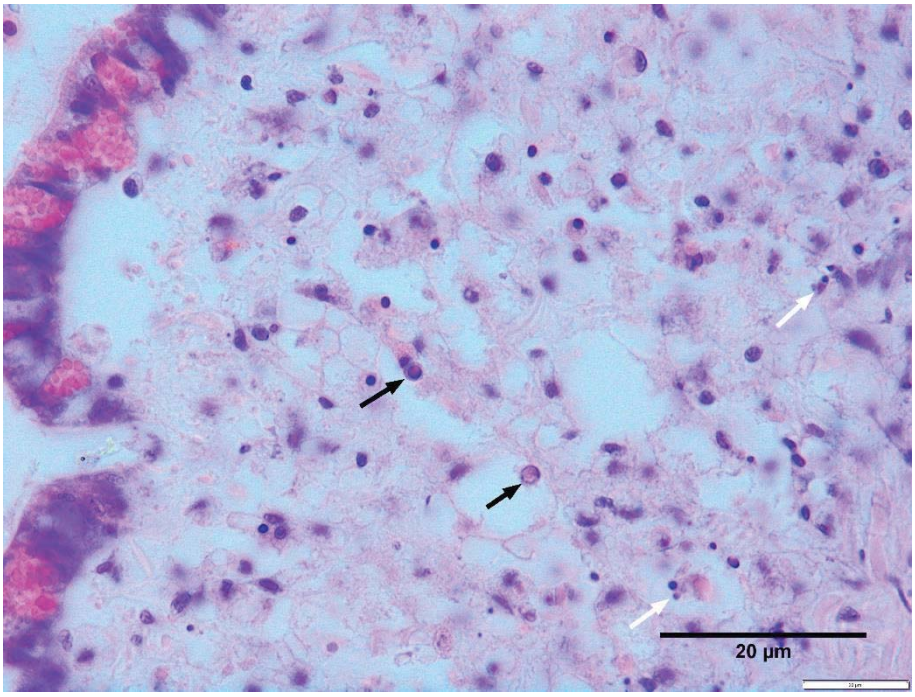


Figure 37. Chromatin margination in cell nuclei (black arrows) and isolated necrotic cells (white arrow), H & E (scale bar = 20 µm).

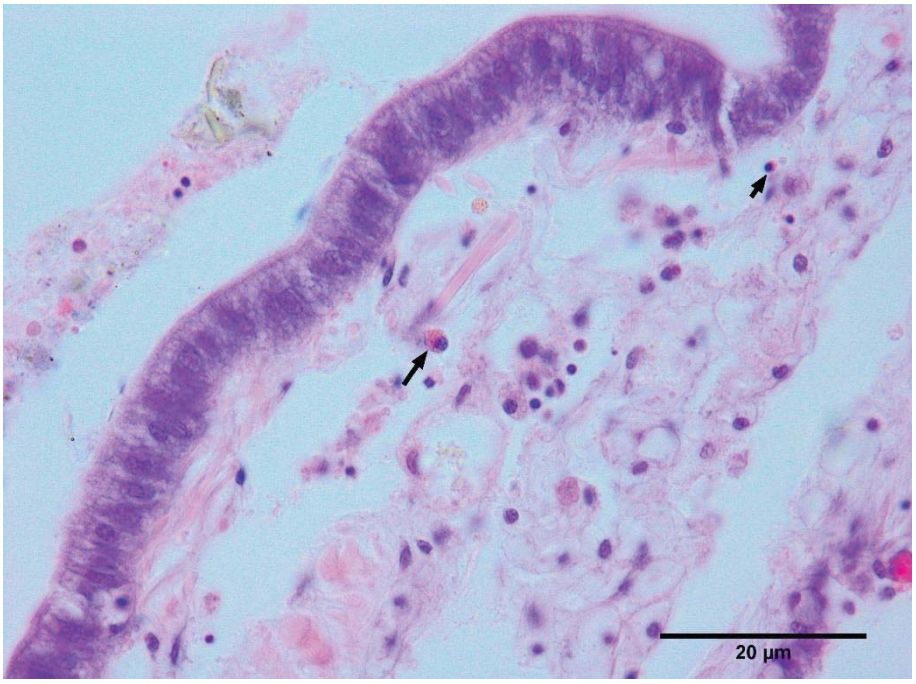


Figure 38. Brown pigmented cells or "brown cells" in the sub-epithelium of the mantle, H & E (scale bar = 20 µm).

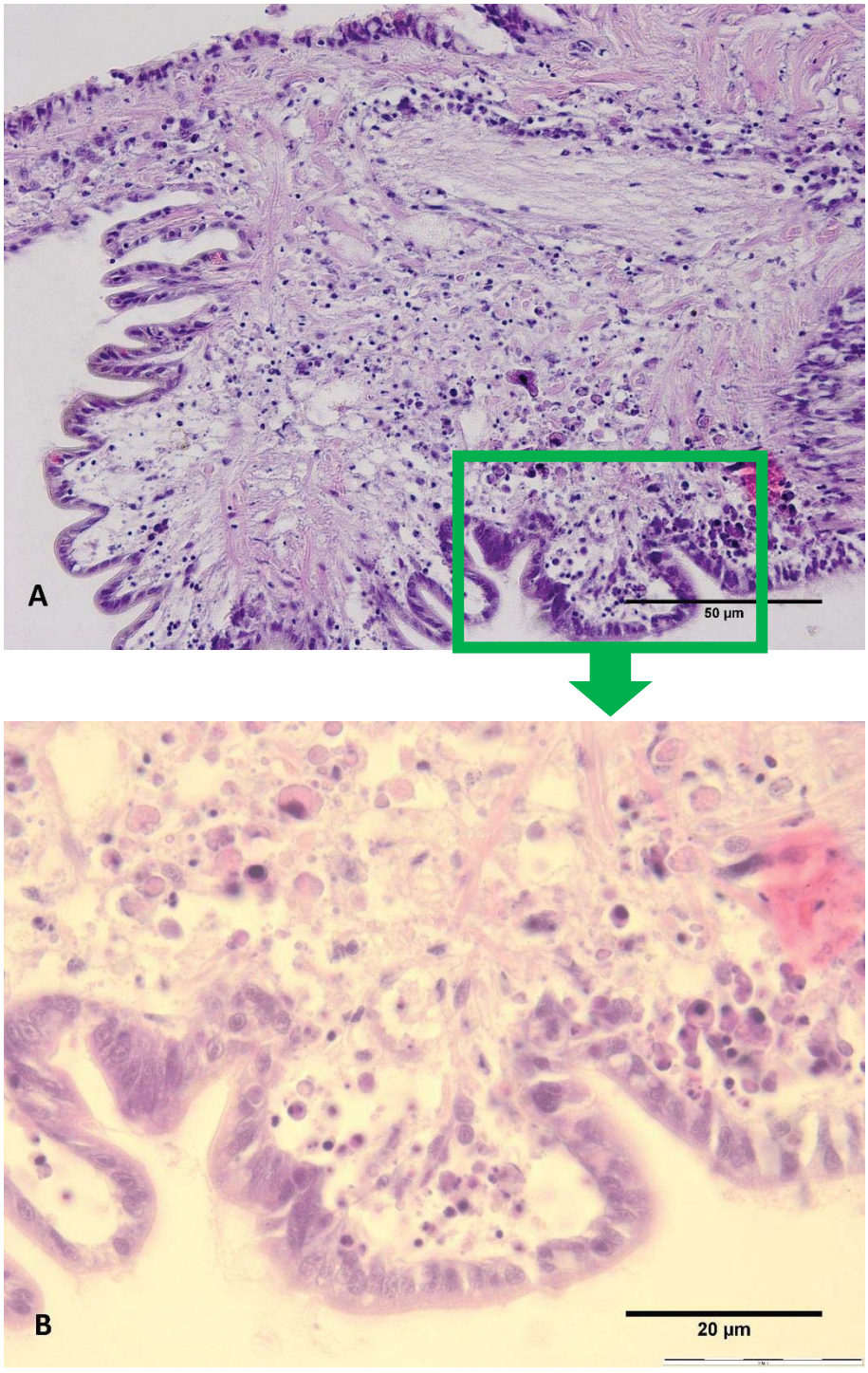


Figure 39. Cellular degeneration and tissue necrosis in the mantle of spat collected at day 5 post-transfer, H & E (scale bar = 50 µm (A) and 20 µm (B))

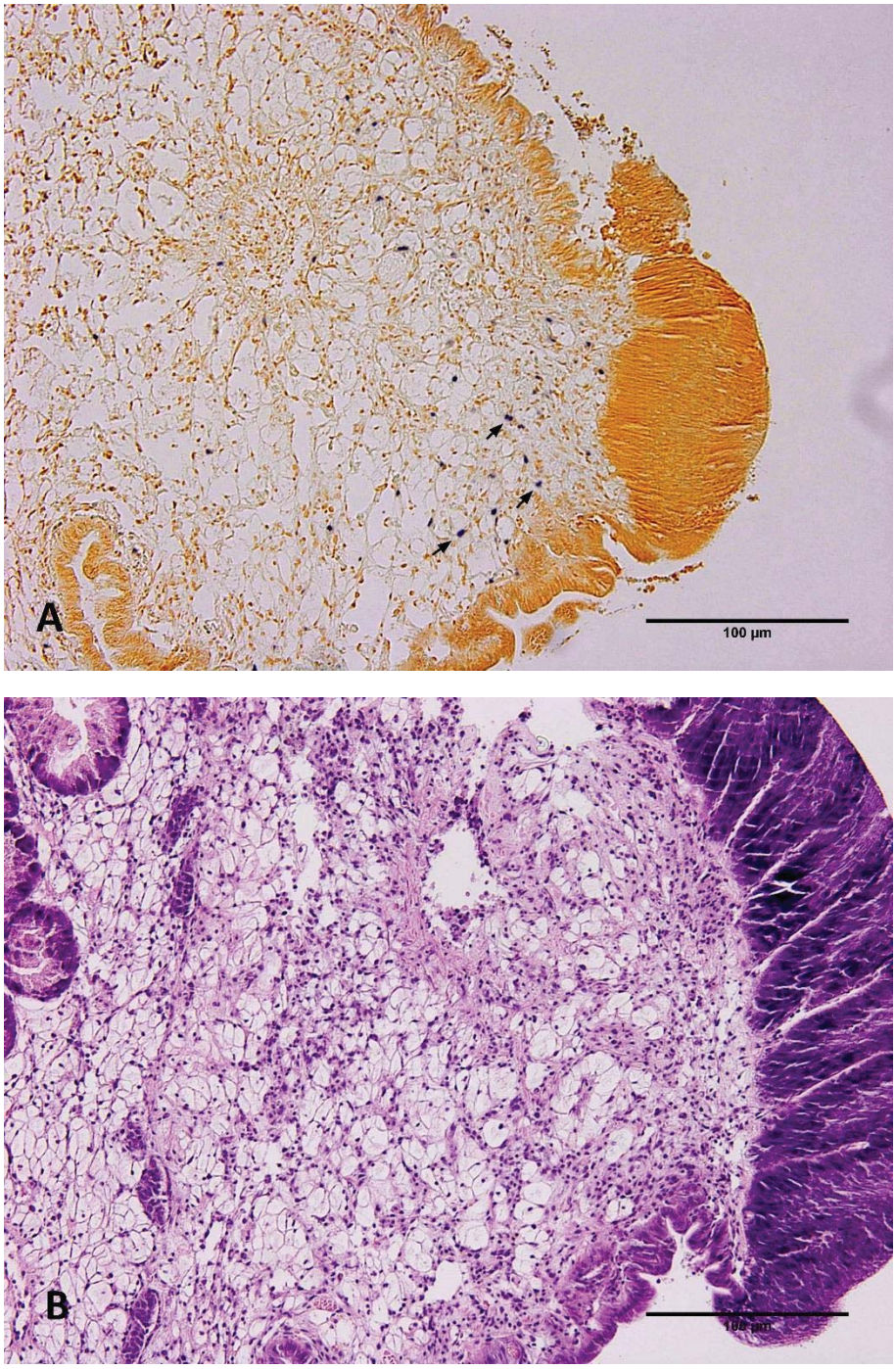


Figure 40. OsHV-1 infected cells (bluish to black cells in arrows) in the connective tissue (A) of the mantle were apparently surrounded by haemocytic infiltrates in consecutive serial section (B) stained with H&E (scale bar = 100 µm (A & B)).

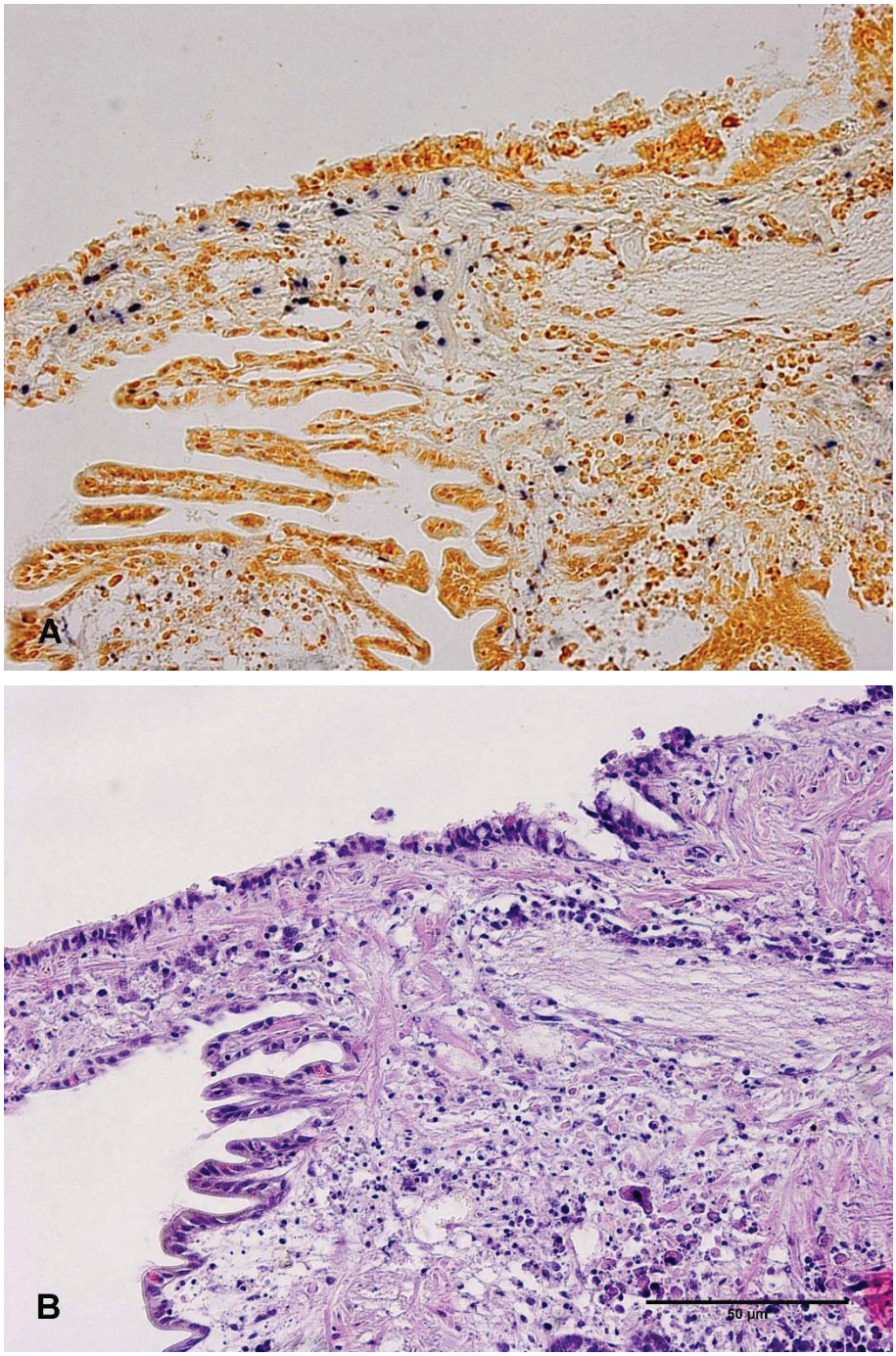


Figure 41. Blue labelled cells in the mantle (A) with tissue necrosis on the same consecutive H & E section (B) (scale bar = 50 µm (A & B)).

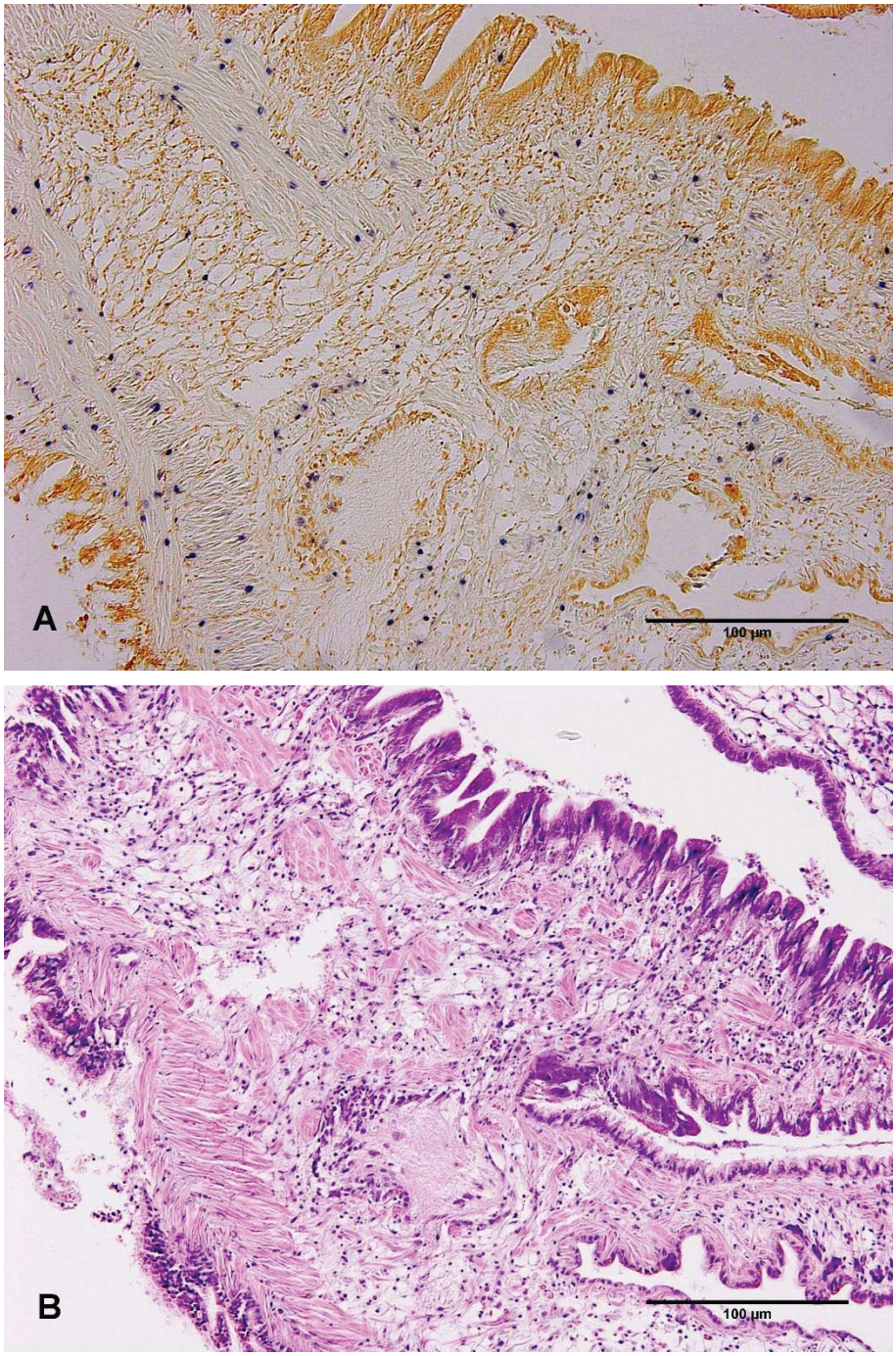


Figure 42. OsHV-1 infected muscle fibres demonstrated by the bluish ISH signal (A) with cellular aggregates in equivalent H & E section (B) (scale bar = 100 µm (A and B)).

5 Discussion

In this study, *in situ* hybridisation signals of purplish to black precipitate in cells demonstrated the complementary pairing of the DIG labelled probe and the target OsHV-1 nucleic acid sequences in various oyster tissues. The ISH signal was first detected at day five, a day prior to a noted increase in recorded mortalities, thus providing evidence of temporal relationship between OsHV-1 infection of *C. gigas* and the onset of massive mortality. The presence of OsHV-1 in New Zealand was confirmed during the 2010-2011 investigation of summer mortality events in Pacific oysters. The first report of a herpesvirus-like infection dates back to 1991 (Hine *et al.*, 1992) when high mortalities in a batch of hatchery-reared *C. gigas* larvae were described. However, a PCR-based survey failed to detect OsHV-1 infection in molluscs, including Pacific oysters in 2007 (Webb, Fidler *et al.*, 2007). Results of subsequent testing of archived paraffin embedded oysters tissues at IDC&R by C. Brosnahan (personal communication, 2011) and Renault *et al.* (2012) suggested that OsHV-1 was present in New Zealand prior to the 2010-2011 outbreak. At least three lines of evidence suggested that OsHV-1 was a necessary cause of Pacific oyster mortalities in the initial outbreak (C. Johnston, personal communications, 2011). One was the consistent and widespread detection of OsHV-1 nucleic acids in oysters from mortality affected farms (Bingham *et al.*, 2013). Another was the preliminary characterization of the New Zealand OsHV-1 µVar isolate as genetically similar to an emerging, apparently more pathogenic genotype from France (Keeling *et al.*, 2014). Lastly, results of the short prospective study demonstrated that the sudden increase in the numbers of OsHV-1 PCR-positive oysters was followed by a corresponding increase in oyster mortality (Keeling *et al.*, 2014).

The current work was undertaken to further our understanding of the pathogenesis of OsHV-1 µVar infection in oysters and its association with disease. To address this task, an ISH assay was developed to detect OsHV-1 in Pacific oyster tissues. This test was then used to demonstrate the presence of OsHV-1 DNA in various tissues of spat at several time-points before and after transfer to the affected farm. Histopathological changes in equivalent samples were also examined to determine whether or not OsHV-1 infection is associated with pathological changes in diseased oysters.

The discussion in this final chapter consists of two sections. The first part examines the utility and development of ISH in demonstrating OsHV-1 infection in Pacific oysters. The second part discusses the application of the test to the investigation of OsHV-1 pathogenesis in Pacific oysters.

5.1 *In situ* hybridisation assay development

The application of ISH for demonstrating viral infections has been described by others (Deim, Szeredi *et al.*, 2006; Heino, Hukkanen *et al.*, 1989; Mabruk, 2004). The test permits an investigator to determine which cells are susceptible to viral infection and characterise kinetics of the viral spread in tissues. This aspect of viral disease pathogenesis is very important especially when studying emerging viruses (Chang *et al.*, 1996). The development of an ISH based method to detect the presence of OsHV-1 infection in *Crassostrea gigas* consisted of development of the probe and optimisation of the hybridisation steps. During this study, a 695 bp DNA probe from the OsHV-1 C2/C6 region was DIG-labelled by PCR. Optimisation of hybridisation procedures was aimed at improving accessibility of the probe to nucleic acid targets in cells by PK digestion and increasing stringency of hybridisation to reduce background staining. The experiments conducted included determination of critical conditions such as

hybridisation temperature and the post hybridisation washes. The absence of ISH signal in OsHV-1 PCR negative control oyster, omitted probe control and omitted anti-DIG control demonstrated the absence of non-specific reactors.

In situ hybridisation is a complex technique such that a variety of conditions can influence the sensitivity and specificity of hybridisation (Schwarzacher & Heslop-Harrison, 2000). Important components of this technique include design and construction of the probe, accessibility of nucleic acids in tissue preparation, and optimisation of various conditions in *in situ* hybridisation steps. Three types of probes, each having its own unique advantage and disadvantages are used for *in situ* virus detection in formalin fixed paraffin embedded tissue. Double stranded DNA probes can be labelled conveniently by PCR amplification, with a relatively high degree of specificity and sensitivity. In comparison, RNA probes can form more stable hybrid duplexes to target RNA than DNA-DNA complexes (McGee & Polak, 1998). However RNA-RNA hybrids have the ability to formed non-specific binding resulting in higher background staining (Jin & Lloyd, 1997). At the same time, RNA probe and target sequences are prone to RNAse digestion (McNicol & Farquharson, 1997). Oligonucleotide single stranded DNA probes are synthetically made, usually between 20-50 bp. Because of their short sizes, they can easily penetrate cells to their target nucleic acids. However, oligonucleotide probe lack specificity due to non-specific binding and also low sensitivity due to low amount of reporter molecules in the short nucleotide sequence (Jin & Lloyd, 1997). These limitations of oligomers are overcome by using multiple oligo probes targeting sequential region of the gene segment of interest.

The selection of a dsDNA probe construct in this study utilised published PCR method based on the C2/C6 primers. Specific sequences of OsHV-1 C2/C6 region of the New Zealand isolate (personal communication, Keeling, S. 2011) was found to be

genetically similar to the µVar strain involved in the *C. gigas* mortality episodes in France from 2008-2010 ((Martenot *et al.*, 2011; Segarra *et al.*, 2010). The labelled C2/C6 probe consisted of 695 bp nucelotides with 42% GC (guanine and cytosine) content. Previously, it was suggested that the probe optimum length is between 200-500 bp for easier tissue penetrability (Jin & Lloyd, 1997). The labelled DIG-DNA probe was able to localise OsHV-1 nucleic acid sequences in Pacific oysters, which were observed in the connective tissues of the mantle, gills and digestive ducts. Performance of the labelled DNA probe in this study was in agreement to the findings of Lipart and Renault (2002), when a similarly prepared probe was first used to investigate OsHV-1 infection in France. One of the advantage of this DNA probe is that the target C region is present in both inverted repeats flanking each of the two unique region of the OsHV-1 genome (Davison *et al.*, 2005). This means that the C2/C6 probe has better chances of complimentary pairing to target nucleic acids in the oyster tissues.

Formalin fixation of oyster tissues immediately after dissection is required to preserve cell morphology and to prevent nucleic acid loss. However, this protein crosslinking process incapacitates tissue penetration of the probe. Crosslinking is also influenced by fixation time. In order to improve probe permeability and expose target DNA inside the cells without loss of cellular detail, the amount of proteinase K for digestion need to be optimised (Nakamura, 1990). In this study, the PK concentration of 100 µg/mL at 37ºC for 15 minutes was found to be optimal for elucidation of hybridisation signal with minimal tissue disruption. In most ISH studies, PK concentration for protein hydrolysis is in the range of 1-50 µg/mL for a treatment period of 5-30 minutes (Jin & Lloyd, 1997).

The complementary pairing of a single strand DNA probe and the target nucleic acid after denaturation is affected by the reaction conditions during hybridisation

(Nakamura, 1990). Probe-target hybrid molecule is more stable at higher stringency and can be carried out at higher temperature during renaturation, reducing salt concentration, using formamide as reaction solvent and a more alkaline pH (Schwarzacher & Heslop-Harrison, 2000). Empirical determination of these conditions ensure optimum probe specificity without, or with minimal, background staining (Alonso, Cano *et al.*, 2004). In this study, two hybridisation temperatures of 40ºC and 42ºC were compared. It may seem that a two degree difference may not produce very different results, however, annealing at 42ºC produced less background staining than 40ºC. According to Schwarzacher and Heslop-Harrison (2000), 1% increase in hybridisation temperature equates to 1% increase in stringency. Another parameter optimised in the ISH development was the concentration of monovalent cations, namely sodium, in the post hybridisation washes. Binding of the DNA hybrid molecule is better at higher ionic concentration and worse at low ion concentration (Tan & Chen, 2006). Low ion concentration of the final wash eliminates weak probe-target complexes with low homology. Three sodium concentrations of the final wash with saline sodium citrate buffer were trialled (1X SSC, 0.75X SSC & 0.5X SSC) at 42ºC. At 0.75X SSC, non-specific staining was reduced to the greatest extend.

5.2 Comparison of ISH performance with qPCR results

The results obtained showed that the ISH technique had fair agreement (kappa=0.339 (95 % C.I. = 0.195-0.483) with qPCR. Fair implies a reasonable level of disparity which may be explained in more than one way. Firstly, by the type of material used for testing. Real time qPCR used fresh tissues whereas the ISH technique utilised an equivalent paraffin embedded formalin fixed tissues. Formalin fixation degrades nucleic acids in tissues and at the same time, the cross linking of proteins around the remaining segments of nucleic acids, renders it unavailable for detection (Bancroft &

Gamble, 2001). In addition to incomplete target sequences, failure to optimally expose DNA during tissue pre-treatment processes such as proteinase k digestion reduces the sensitivity of the technique (Lu, Lawson *et al.*, 1995). Finally, PCR techniques involve exponential amplication of low copy numbers to a point where detection is possible whereas ISH allows for only modest and more linear signal amplification based on the number of DIG labels per strand and the constraints imposed by antibody / enzyme binding and chromogen build up. In Situ PCR is a technique that could increase the sensitivity of tissue section based diagnosis and the agreement between qPCR and ISH. This method was not considered technically feasible in the current study and is prone to greater numbers of non-specific reactions.

Despite the fact that developed OsHV-1 ISH assay has low diagnostic sensitivity and high specificity relative to qPCR it may be a test of choice for selected purposes, for example for ruling in an OsHV-1 infection in an outbreak situation.

5.3 Localisation and spread of OsHV-1 in Pacific oysters

The precise events on the fate of OsHV-1 after ingestion by Pacific oyster are poorly understood. Schikorski et al. (2011a) suggested that the course of viral infection begins when the OsHV-1 particles entering the mouth manage to cross the digestive tract barrier and infect haemocytes present among the epithelial cells. During the first few hours, infected haemocytes are circulated in the haemolymph which allows the virions to be distributed throughout the body. The virus is then able to infect cells in various tissues where secondary replication may occur. It is also possible for virions to be disseminated from the digestive gland freely via the open network of haemolymphatic vessels.

In addition to ingestion, assimilation due to a long period of exposure to the virus and/or compromise in the integrity of the epithelial lining of the mantle and gills may provide another point of entry for viral invasion. Environmental conditions may contribute to the weakening or compromise of various epithelial barriers, which may have the effect of increasing the number of virus particles able to gain entry from the water column. In an article on juvenile oyster disease in *Crassostrea virginica,* Ford and Borrero (2001) reported the penetration of protozoa and bacteria as a consequence of mantle epithelium degeneration and erosion.

In the current prospective study, a hybridisation signal for OsHV-1 in the gut lumen of one naïve spat (1/7), a day after exposure to an infected marine farm, indicated that the earliest noticeable event of viral entry was via the digestive tract. It is also possible, although no ISH signal observed during the early period of exposure that OsHV-1 particles crossed the thin epithelial border of the mantle and gills. Haemocytes are present within and underneath the epithelium in normal conditions and may have facilitated the sequestration and distribution of the virus from the initial entry point. In this scenario, OsHV-1 would to be both 'epithelial' and more widespread throughout the oyster's tissues, albeit at a level below the detection limit for ISH. The low detection level of OsHV-1 at day one was likely influenced by different disease transmission mechanisms at play in an aquatic environment such as hydrodynamics and density of infective virions (Paul-Pont, Dhand *et al.*, 2013). OsHV-1 particles in the intestine may have been ingested during filter feeding of the bivalve as soon as they were dispersed into the water columns. The role of water as a default medium for OsHV-1 transmission was previously elucidated in closed environment co-habitation trials (Schikorski *et al.*, 2011a) and in water sampling from open estuarine setting with high oyster mortality incidence (Evans, Paul-Pont *et al.*, 2014).

In comparison, Pacific oysters are able to bio-accumulate human pathogens such as norovirus, poliovirus and hepatitis A virus within 12-24 hrs after exposure to contaminated water. (Le Guyader, Loisy *et al.*, 2006; McLeod, Hay *et al.*, 2009; Seamer, 2006). Although, virus uptake mechanisms may require further elucidation, norovirus can localise within 12 hours in epithelial cells throughout the digestive tract and in cells in the underlying connective tissue around the basement membrane (McLeod *et al.*, 2009). According to Le Guyader *et al.* (2006), noroviruses bind to carbohydrate structures with a terminal N-acetylgalactosamine residue in an α linkage present in the digestive tracts. This ligand is similar to human histo-blood group antigen recognition site. Phagocytes present in the intestinal border also sequester the virus and possibly distribute it to various tissues (Le Guyader *et al.*, 2006). McLeod *et al.* (2009) and Seamer and Hay (2007) further suggested that this model of virus uptake may be the same for some viruses such as poliovirus and cricket paralysis virus.

5.3.1 Tissue tropism

Despite of the presence of viral DNA in the intestinal lumen of two spat at day 1 and 3 after cohabitation with Pacific oysters from the farm that has been experiencing increased rates of oyster mortalities, evidence of OsHV-1 infected cells were only shown at day 5 post transfer. The interval between sampling (36-48 hours) in the longitudinal study was determined to be too long, limiting the possibility to observe important steps in the spread and localisation of OsHV-1 in Pacific oysters. A more appropriate experimental design to demonstrate early events in the pathogenesis of this viral infection, considering the oyster's small body size, should have involved a more frequent sampling. For example, Chang *et al.* (1996) used a gap of 12 to 18 hours in between sampling from the time of exposure or inoculation to described the pathogenesis of white spot syndrome associated baculovirus in black tiger shrimp by

ISH. Whilst in conducting the longitudinal study, the original intent was not to elucidate pathogenesis using a time course study but to support the association of OsHV-1 as a causal agent in the mortality outbreak in *C. gigas*.

Detectable individual cells containing OsHV-1 nucleic acids at day five post transfer were found mostly in the connective tissue elements of the mantle and gills. Infection of epithelial cells was not observed in this study. The positive ISH labelling thus implies that these organs (mantle and gills) with a transient population of haemocytes are preferentially infected with the virus, and thus comprise a good material for future OsHV-1 diagnostic testing. Infected cell types observed in these tissues were small round cells possibly of haemocyte class, fibroblast-like connective tissue cells, and cells in the muscle fibres. The ISH signal in these tissues was particularly abundant and prominent especially in the cell nuclei. These observations are in agreement with the early reports (Arzul *et al.*, 2002; Lipart & Renault, 2002) wherein connective tissues of *C. gigas* spat and adult were the main target of OsHV-1 infection. Interestingly, the type of infected cells with large nuclei associated with muscle bundles in the mantle was difficult to establish. Initially, labelled cells were thought to be myocytes but later interpreted to be more likely of haemocyte type. In contrast to our findings, Corbeil, Faury *et al.* (2014) corroborated the earlier report made by Lipart and Renault (2002) on the detection of OsVH-1 DNA by ISH in adductor muscle, muscle cells in the mantle and cells in the heart ventricle.

Some infected cells were also located within the periphery of nervous tissue but not as abundant when compared to positive cells located in the mantle and gills. Although latency genes have not been found (Davison *et al.*, 2005; Segarra, Baillon *et al.*, 2014), establishment of latency in the nerve tissue is a possibility. Alpha herpesviruses that infect vertebrate animals are known to be neurotropic and have predilection for establishing active infection in epithelial cells and establishing latency

in neuronal cells (Krummenacher, Carfí *et al.*, 2013). Persistence of OsHV-1 in adult Pacific oysters without associated clinical signs and mortalities has been previously reported (Arzul *et al.*, 2002) and the nerve tissue was a suggested site for latency (Lipart & Renault, 2002). Recrudescence of virus from a hypothesized viral latency state due to stress factors such as elevated temperatures would likely promote horizontal spread of infection in aquatic surroundings. In the current study however, cells in the nerve tissue were more likely haemocytes that were associated with the haemolymph supply. Another mollusc virus, the abalone herpesvirus (AbHV) also favours infecting cells within the nervous system of the gastropod, abalone (Hooper, Hardy-Smith *et al.*, 2007).

Occasional labelling of cells was also observed around and within the male and female gonads, including the germinal reproductive areas, however the significance of these findings remains unclear. OsHV-1 vertical transmission from asymptomatic parent stock was considered when a herpesvirus-like infection was detected in early larval states (Barbosa-Solomieu, Dégremont *et al.*, 2005; Le Deuff *et al.*, 1996). This hypothesis was further reinforced by ISH and immunohistochemistry detection of viral DNA and proteins in the connective tissues of male and female gonads, including female egg cells of adult Pacific oysters (Arzul *et al.*, 2002). The infected gametes could contaminate developing embryos during discharge and spawning.

5.3.2 Histopathology

In general, non-specific histological changes were detected in all tissues examined. A pattern of progressive pathological anomaly as is commonly observed in disease processes of vertebrate animals was not evident in this study. However, it is largely viewed that tissue changes such as aggregation of haemocyte cells, epithelial thinning or atrophy of the digestive gland and foci of tissue lysis are a physiological,

perhaps immunological, coping mechanism of bivalve molluscs against environmental stresses, infection with primary pathogenic agents or concurrent secondary infections (Sparks, 2012). Thus, such findings in this study, although seems unclear, may have been important pieces in the larger pathogenesis story of OsHV-1 infection. Interestingly, deformation of the gill's inter-lamellar junction is a prominent feature of OsHV-1 infected adult Pacific oysters in Mexico (Vásquez-Yeomans *et al.*, 2010). However, the association of the gill lesion development and the presence of OsHV-1 in tissues was uncertain.

Haemocytosis or the massive infiltrations of haemocytes in all organs, although common across the different sampling time-points, appeared more pronounced and severe in spat collected at day 5 and onwards. In bivalve molluscs, the aggregation of haemocytes represents a host cellular response to destroy, dilute and isolate unwanted or injurious agents (Sparks & Morado, 1988). This cell type is also responsible for clearing dead cells and tissue debris. Hence, the increase in the proportion of spat with widespread haemocyte cellularity is probably due to a marked inflammatory reaction to virus infected cells and or necrotic tissue. The presence of either an injurious agent or an autolytic process, resulting from an environmental change, could elicit this cellular response, thus Sparks and Morado (1988) advised caution in interpreting haemocytosis. This is also important in differentiating between post mortem changes and a reaction to injury and wound repair (Sparks, 2012).

This study's finding of haemocytosis is in common with various described reports of OsHV-1 infections (Burge *et al.*, 2006; Jenkins *et al.*, 2013). Widespread haemocytic infiltration surrounding the ulcerated labial palps and necrotic gills were also seen in Portuguese oyster with gill necrosis virus (GNV) (Comps, 1988). In the same report by Comps (1988), marked inflammatory responses were also noted in haemocytic infection viruses (HIV) in *C. angulata*. Interpretation of this immunological

event, however, was complicated by the detection of *Vibrio* spp. bacteria in the study cohort as shown in a separate report (Keeling *et al.*, 2014). *Vibrio alginolyticus* was detected in spat from the hatchery followed by identification of several species of Vibrios (*splendidus, chagassi, aestuarianus*) on subsequent sampling days by sequencing of the *atpA* gene. Although the presence of this bacterial species may have contributed to the observed histological alterations, haemocyte infiltration around bacterial foci was not evident as described by Elston, Beattie *et al.* (1987). A cluster of gram positive, acid fast bacteria was found to induce focal areas of haemocyte cells in Pacific oysters (Elston *et al.*, 1987). Severe bacteraemia induced multiple foci of haemocytosis across different tissues or regionalised multifocal infectious zones when it involves extensive area of connective tissues, digestive glands and mantle sub-epithelium (Elston *et al.*, 1987).

Various degrees of digestive gland atrophy were found in almost all samples in the prospective study, although areas of thinner epithelium were observed predominantly in samples from day 9 post transfer. Atrophy of the digestive gland is attributed to various factors and considered to be a good indicator of unfavourable environmental conditions (Sparks, 2012). Anoxic exposure at high summer temperature (Fogelson, Rikard *et al.*, 2011), poor nutritional state and spawning stress (Kang, Chu *et al.*, 2010), salinity changes (Knowles, Handlinger *et al.*, 2014), and toxin producing dinoflagellates (Pearce *et al.*, 2005) were shown to induce sloughing of gut cells resulting in a thin, dilated digestive glands in oyster bivalves. Thinning of the digestive diverticulae was also reported to be an early feature, within eight hours, of acute inflammation in *C. gigas* (Sparks & Morado, 1988). In addition, post mortem change in this organ could occur 48 hours after somatic death (Sparks, 2012). It should also be noted that poor fixation may be reflected as degeneration of the digestive glands and may provide confusion in the interpretation of this anomaly.

In the current study, areas of tissue necrosis were observed at day 5, 7 and 9 post transfer and usually involved the mantle, gills and alimentary tract. Mantle and gills are the most affected organs in terms of the numbers of infected cells and the high intensity of positive signals. An interesting finding here was the co-location of positive hybridisation ISH signal in areas with haemocyte aggregations and cell necrosis. Although the significance of this discovery is difficult to ascertain, it indicates that haemocyte infiltrations may have occurred as a response to the presence of virus infected cells or necrotic debris from cell destruction in the wake of viral replication. Notably, spat samples from day 9 appeared to be more intact and morphologically normal as compared to spat from day 5 and 7. It is presumed that in this single time cohort, a group of resistant oysters started to emerge and the highly susceptible spat were diminished due to mortalities. Certain individuals or age classes of oysters are more likely to survive infection, continue to produce virus particles and or act as asymptomatic carriers (Degremont & Benabdelmouna, 2014).

Another microscopic finding was the prominent appearance of two types of cells, multi-nucleated cells and large cells with brown pigmented cytoplasm, at day 9 post transfer. These cell types are believed to participate in clearing cellular debris (Sparks, 2012; Sparks & Morado, 1988). Brown cell numbers were observed to increase during inflammation, which suggest that they may participate in tissue defence (Zaroogian & Yevich, 1994). According to a study by Zaroogian and Yevich (1994) in *C. virginica*, brown cells contain lysosomes which help in the detoxification and degradation of internalised soluble foreign debris from the hemolymph.

5.3.3 Incubation to mortality event

A substantial mortality (14% & 50%) was not seen in the study cohort until the 6th and 9th day after transfer to the disease affected farm. Given the right

environmental temperature threshold of at least 16°C, it transpired that OsHV-1 infection could be acquired within 24 hours of exposure to a contaminated seawater environment and that the incubation period from the estimated start of infection to the start of significant mortality was 5 days. This is compatible with other descriptions of high mortalities in oyster larvae due to a herpesvirus-like agent that occured within 6-7 days from the beginning of spawning (Hine *et al.*, 1992; Le Deuff *et al.*, 1996; Nicolas *et al.*, 1992). A cohabitation trial between OsHV-1 infected and non-infected spat in a closed water environment also resulted in a cumulative mortality of 50% after 8 days (Schikorski *et al.*, 2011a). In a recent study under field condition, substantial mortalities were recorded only after two weeks post-deployment (Degremont & Benabdelmouna, 2014; Dégremont, Ledu *et al.*, 2014). These infection patterns are similar to a reported 10 days average incubation period on oyster farms in natural environments (Sauvage *et al.*, 2009). In contrast, experimental inoculation of healthy spat by injection with tissue homogenates containing a known viral load based on the levels of viral DNA resulted in mortalities after two days (Schikorski *et al.*, 2011f). This observation by Schikorski *et al.* (2011f) is in agreement with an earlier transmission trial of a herpesvirus-like agent to axenic Pacific oyster larvae in a glass container with sterile sea water (Le Deuff *et al.*, 1994).

The two day interval from inoculation to mortality may represent the minimum incubation period of OsHV-1 (Sauvage *et al.*, 2009). This interval allows the replication of the virus in oysters to progress up to a point of causing overwhelming cell and tissue damage and subsequent somatic death (Sauvage *et al.*, 2009). However, the variation in incubation period and in the levels of OsHV-1 associated mortalities under natural, as opposed to experimental conditions can be due to the differences in the initial infective dose of the virus and the prevailing temperature. Lower level exposure may require the virus to complete replication cycles for longer periods before it can cause

substantial damage and subsequent mortality to its bivalve hosts. As such, the cumulative viral load is affected by the incremental exposure or contact time between the virus and the susceptible host as well as by temperature. In a closed water system, the exposure to an infectious agent(s) is greater when the water environment is not changed during experimental infection by cohabitation. Manipulation of the environment ensures availability of infective viruses and increases the contact time between the virus and the oysters. In contrast, oysters reared in normal farm settings are subjected to hydrodynamic factors which effectively dilute the virus concentration in water columns (Schikorski *et al.*, 2011a).

5.4 Conclusions

Establishing causation in any disease outbreak is an important endeavour to develop effective intervention strategies. However, the elucidation of cause and effect is not a straight forward process and requires a body of compelling arguments derived from observational events and experimental evidence to prove causal link. The association of Ostreid herpesvirus-1 to summer mortalities in New Zealand Pacific oysters in 2010 to 2011 is an example of this predicament. The aim of this research was to further support the causal link between OsHV-1 infection and oyster mortality.

OsHV-1 was concluded to be a necessary cause, in association with other multiple risk factors including elevated temperature, for the summer mortalities among the farmed Pacific oysters in New Zealand in 2010-11 (Bingham *et al.*, 2013). This inference was attributed to consistent detection of viral nucleic acid in oysters from all affected farms, the detection of OsHV-1 during the short longitudinal study conducted (Keeling *et al.*, 2014) and the similarity of the virus detected from the outbreak to the sequenced µVar strain that caused widespread mortality of *C. gigas* in France in 2008 (Segarra *et al.*, 2010). In the current study, we have demonstrated that

Pacific oysters were in fact infected with OsHV-1 using ISH technique. The results of this study further supported the view that OsHV-1 was causally involved in summer mortalities observed in farmed oysters in 2010-2011. Whilst the positive ISH result does not necessary detect viral replication, the visualisation of an increasing number of OsHV-1 infected cells in spat prior to marked increase in mortality indicated an on-going active infection. Such temporal relationship is an important criterion of insinuating a strong evidence to corroborate the causal link of OsHV-1 and spat mortalities. Further studies to elucidate OsHV-1 pathogenesis in association with elevated temperature is recommended in Pacific oysters.

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Appendix A

List of Pacific oysters spat collected from the different time point in the longitudinal study showing the various locations of hybridisation signals if present when tested with the newly developed ISH.

sample	days Post Infection	tissue/organ with associated ISH signal							ISH interpretation
		mantle	gills	digestive ducts	digestive lumen	gonad	nerve tissue	vascular duct	
1586-1	day -1	neg	neg	neg	neg	neg	neg	neg	neg
1586-2	day -1	neg	neg	neg	neg	neg	neg	neg	neg
1586-3	day -1	neg	neg	neg	neg	neg	neg	neg	neg
1586-4	day -1	neg	neg	neg	neg	neg	neg	neg	neg
1586-5	day -1	neg	neg	neg	neg	neg	neg	neg	neg
1601-11	day 1	neg	neg	neg	neg	neg	neg	neg	neg
1601-12	day 1	neg	neg	neg	neg	neg	neg	neg	neg
1601-13	day 1	neg	neg	neg	**+2**	neg	neg	neg	**neg**
1601-18	day 1	neg	neg	neg	neg	neg	neg	neg	neg
1601-24	day 1	neg	neg	neg	neg	neg	neg	neg	neg
1601-27	day 1	neg	neg	neg	neg	neg	neg	neg	neg
1601-30	day 1	neg	neg	neg	neg	neg	neg	neg	neg
1618-1	day 3	neg	neg	neg	neg	neg	neg	neg	neg
1618-2	day 3	neg	neg	neg	neg	neg	neg	neg	neg
1618-3	day 3	neg	neg	neg	**+2**	neg	neg	neg	**neg**
1618-4	day 3	neg	neg	neg	neg	neg	neg	neg	neg
1618-5	day 3	neg	neg	neg	neg	neg	neg	neg	neg
1618-11	day 3	neg	neg	neg	neg	neg	neg	neg	neg
1618-14	day 3	neg	neg	neg	neg	neg	neg	neg	neg
1620-1	day 5	neg	neg	neg	neg	neg	neg	neg	neg
1620-2	day 5	**1+**	**1+**	neg	neg	neg	neg	neg	**pos**
1620-3	day 5	neg	neg	neg	neg	neg	neg	neg	neg
1620-10	day 5	**3+**	neg	neg	neg	neg	neg	neg	**pos**
1620-11	day 5	neg	neg	neg	neg	neg	neg	neg	neg
1620-12	day 5	neg	neg	neg	neg	neg	neg	neg	neg
1620-13	day 5	**3+**	**2+**	neg	**+1 sus**	neg	**1+**	neg	**pos**
1620-14	day 5	neg	neg	neg	neg	neg	neg	neg	neg
1620-15	day 5	**1+**	neg	neg	neg	neg	neg	neg	**pos**
1620-16	day 5	neg	neg	neg	neg	neg	neg	neg	neg
1620-17	day 5	**3+**	**3+**	neg	neg	neg	neg	neg	**pos**
1620-19	day 5	**3+**	**3+**	neg	neg	neg	neg	neg	**pos**
1620-21	day 5	neg	neg	neg	neg	neg	neg	neg	neg
1620-22	day 5	neg	neg	neg	neg	neg	neg	neg	neg
1620-23	day 5	neg	neg	neg	neg	neg	neg	neg	neg
1620-24	day 5	**2+**	**2+**	neg	neg	neg	neg	neg	**pos**
1620-27	day 5	neg	**1+**	neg	neg	neg	neg	neg	**pos**
1636-1	day 7	**3+**	**3+**	**2+**	neg	**1+**	neg	neg	**pos**
1636-2	day 7	neg	neg	neg	neg	neg	neg	neg	neg
1636-4	day 7	neg	neg	neg	neg	neg	neg	neg	neg
1636-5	day 7	**3+**	**3+**	**1+**	neg	neg	neg	neg	**pos**
1636-6	day 7	**1+**	neg	neg	neg	neg	neg	neg	**pos**
1636-7	day 7	**3+**	**3+**	neg	neg	neg	neg	neg	**pos**
1636-8	day 7	neg	neg	neg	neg	neg	neg	neg	neg
1636-9	day 7	neg	**1+**	neg	neg	neg	neg	neg	**pos**
1636-10	day 7	**2+**	**2+**	neg	neg	neg	neg	neg	**pos**
1636-11	day 7	**2+**	**1+**	neg	neg	neg	neg	neg	**pos**
1636-13	day 7	**3+**	**3+**	neg	neg	neg	**1+**	**1+**	**pos**
1636-14	day 7	**3+**	**3+**	**2+**	neg	neg	neg	neg	**pos**
1636-15	day 7	neg	neg	neg	neg	neg	neg	neg	neg
1636-17	day 7	neg	neg	neg	neg	neg	neg	neg	neg
1636-19	day 7	**2+**	**2+**	neg	neg	neg	neg	neg	**pos**
1636-26	day 7	neg	neg	neg	neg	neg	neg	neg	neg
1636-27	day 7	neg	neg	neg	neg	neg	neg	neg	neg

Appendix A. continuation

sample	days Post Infection	tissue/organ with associated ISH signal							ISH interpretation
		mantle	gills	digestive ducts	digestive lumen	gonad	nerve tissue	vascular duct	
1651-2	day 9	**1+**	neg	**1+**	neg	neg	neg	neg	**pos**
1651-3	day 9	**3+**	**1+**	neg	neg	neg	neg	neg	**pos**
1651-4	day 9	**1+**	**1+**	neg	neg	neg	neg	neg	**pos**
1651-7	day 9	neg	neg	neg	neg	neg	neg	neg	neg
1651-8	day 9	**2+**	neg	**2+**	neg	neg	neg	neg	**pos**
1651-9	day 9	**2+**	neg	neg	neg	neg	neg	neg	**pos**
1651-12	day 9	neg	neg	**1+**	neg	neg	neg	neg	**pos**
1651-13	day 9	**1+**	**1+**	**1+**	neg	neg	neg	**1+**	**pos**
1651-14	day 9	neg	neg	neg	neg	neg	neg	neg	neg
1651-18	day 9	**1+**	**1+**	**1+**	neg	neg	neg	neg	**pos**
1651-19	day 9	**2+**	neg	neg	neg	neg	neg	neg	**pos**
1651-20	day 9	neg	neg	neg	neg	neg	neg	neg	neg
1651-21	day 9	neg	neg	neg	neg	neg	neg	neg	neg
1651-24	day 9	**1+**	neg	neg	neg	**1+**	neg	neg	**pos**
1651-25	day 9	**1+**	neg	**1+**	neg	neg	neg	neg	**pos**
1651-26	day 9	neg	neg	**1+**	neg	neg	neg	neg	**pos**
1651-29	day 9	neg	neg	neg	neg	neg	neg	neg	neg
1671-1	day 13	neg	neg	neg	neg	neg	neg	neg	neg
1671-2	day 13	neg	neg	neg	neg	neg	neg	neg	neg
1671-5	day 13	**1+**	neg	neg	neg	neg	neg	neg	**pos**
1671-6	day 13	neg	neg	neg	neg	neg	neg	neg	neg
1671-7	day 13	neg	neg	neg	neg	neg	neg	neg	neg
1671-12	day 13	neg	neg	neg	neg	neg	neg	neg	neg
1671-17	day 13	neg	neg	neg	neg	neg	neg	neg	neg
1671-19	day 13	neg	neg	neg	neg	neg	neg	neg	neg
1671-21	day 13	neg	neg	neg	neg	neg	neg	neg	neg
1671-22	day 13	neg	neg	neg	neg	neg	neg	neg	neg
1671-23	day 13	neg	neg	neg	neg	neg	neg	neg	neg
1671-24	day 13	**2+**	**1+**	**2+**	neg	neg	neg	**1+**	**pos**
1671-25	day 13	neg	neg	neg	neg	neg	neg	neg	neg
1671-26	day 13	neg	neg	neg	neg	neg	neg	neg	neg
1671-27	day 13	neg	neg	neg	neg	neg	neg	neg	neg
1671-28	day 13	neg	neg	neg	neg	neg	neg	neg	neg
1671-30	day 13	neg	neg	neg	neg	neg	neg	neg	neg

Appendix B

Cross tabulation of ISH and qPCR results to assess agreement using *Kappa* value. In this table, signal in the digestive lumen were considered test negative for the ISH assay.

		qPCR		
		T+	T-	
ISH	T+	**31**	**1**	32
	T-	**31**	**24**	55
		62	25	87

*T+, test positive; T-, test negative

Number of observed agreements: 55 (63.22%)

Number of agreements expected by chance: 38.6 (44.38%)

Kappa: 0.339 (0.195-0.483)

SE of *Kappa*: 0.073

The strength of agreement is considered to be “fair”.

Appendix C

Cross-tabulation of the results of ISH and reference test method qPCR to assess test performance. In this table, signal in the digestive lumen were considered as OsHV-1 not infected or ISH negative.

		reference test		
		qPCR		
		T+	T-	
ISH	T+	31 (a)	1 (b)	32 (a+b)
	T-	31 (c)	24 (d)	55 (c+d)
		62 (a+c)	25 (b+d)	87 (a+b+c+d)

*T+, test positive; T-, test negative

Sensitivity (a/a+c) Specificity (d/b+d)