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Susceptibility of European crustaceans to White Spot Syndrome Virus (WSSV), a non-exotic, EC Directive-listed pathogen

by

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Abstract

This project provides a definitive statement on the susceptibility of ecologically and economically important European crustacean species to White Spot Syndrome Virus (WSSV). Exposure trials revealed universal susceptibility to WSSV infection in seven hosts, and that relative susceptibility varies significantly between species. To determine whether WSSV infection would remain in the tissues of crabs as persistent infections (or whether these crabs would clear the viral infection from their systems over time) crabs were fed with WSSV-infected tissues and then observed in tanks for 3 months. Results suggested that the carcasses of infected (but not diseased) shore and edible crab crabs appear to pose a limited risk of transmission to susceptible hosts within the 3 month timeframe of the study.

The European shore crab (*Carcinus maenas*) was shown to display a lower susceptibility to White Spot Syndrome Virus (WSSV) when compared to other European decapod species. Despite showing signs of infection with WSSV, the shore crab appeared resistant to the development of disease and was highlighted as a possible asymptomatic carrier of the virus. *Carcinus maenas* individuals which had been injected with WSSV and then exposed to varying temperature stress conditions were compared. This suggested that crabs could be divided into two groups (high and low responders) according to differences in pathogenesis and viral replication. The response was not dependent on the presence or absence of an external stressor but was more likely an inherent capacity within individual crabs.

A small scale survey of supermarket commodity shrimp confirmed the presence of WSSV-contaminated products within shrimp imported for human consumption, demonstrating that frozen commodity shrimp is a route of entry for WSSV into Europe. Experimental trials showed that this virus is viable and that the commodity products were infective and could passage the infection to naive crustaceans that are known to be susceptible to the virus, highlighting this practice needs to be considered and controlled in risk assessments. Analysis of WSSV Variable Nucleotide Tandem Repeats (VNTR) within ORF 94 following passage through different hosts revealed subtle differences in WSSV VNTR types present in crab and crayfish tissues suggesting that the host may influence which viral type propagates during infections. These studies also identified and described two novel virus infections. The viral infections mimic the appearance of WSSV and highlight the importance of being able to fully characterise virus infections when they are first identified.

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Academic Thesis: Declaration of Authorship

I, KELLY BATEMAN

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Susceptibility of European crustaceans to White Spot Syndrome Virus (WSSV), a non-exotic, EC Directive-listed pathogen

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
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6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as:

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Signed: .....

Date: 05/06/15 .....

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Chapter 1.

Introduction

1.1 Food security

Crustaceans are an important source of aquatic food protein and play a large role in securing global food security. In 2009 crustacea contributed 11.2 million tonnes to the global fisheries and aquaculture production, 5.9 million tonnes were generated from capture fisheries and 5.3 million tonnes originated from aquaculture (Bondad-Reantaso *et al*., 2012) with a first sale value of $40bn (Stentiford *et al*., 2012). Between 2000 and 2008 crustacea experienced a massive increase in production compared to previous decades; production in this sector growing faster than molluscan and finfish during this period, largely accounted for by the culture of *Litopenaeus vannamei* in China, Thailand and Indonesia (Bondad-Reantaso *et al*., 2012). Aquaculture production currently contributes 48% of aquatic animal food destined for human consumption; this figure is expected to exceed 50% by 2015 with an increase in crustacean culture thought necessary to help fill this gap in supply (Bondad-Reansanto *et al*., 2012). Despite the importance of this product in terms of global food security and high economic value current estimates predict that up to 40% of total shrimp production is lost annually (>$3bn) and this loss is mainly due to viral infections. Viral pathogens appear to exert the most significant constraints on the growth and survival of crustacea under culture conditions (Flegel, 2012; Lightner *et al*., 2012; Stentiford *et al*., 2012).

Unless farmed, mass mortalities of marine animals are rarely detected and studied so it is difficult to assess the impact viral infections can have upon a population. Virus infections have been identified and studied mainly from commercial or larger species of decapod crustaceans; we have limited information on viral infections of other crustacean species or of other animals that may be living in the same environment. These organisms may be reservoirs or be involved in ecology of known viruses. A recent example of this is the Sea-star Wasting Disease (SSWD), SSWD has been used to describe die-off of sea stars in the Northeast Pacific since the 1970's. Following a recent mortality event (June 2013) this condition has now been linked to a densovirus (*Parvoviridae*), Sea star associated densovirus (SSaDV) (Hewson *et al*., 2014).

Recent work has also highlighted a difference in pathogens present in juvenile and adult populations of wild crustaceans (Bateman *et al*., 2011; Behringer, 2012). The ability to

assess the effects that viral disease may have on wild fisheries in terms of production losses is far more challenging than in farmed stocks, as a result there is limited information available on the pathogens and disease of wild crustaceans and this has led to a deficit in knowledge on causes of mortalities within the wild populations.

Crustaceans from either fisheries or aquaculture can be delivered into the food chain as live or raw product (Neil, 2012; Stentiford *et al*. 2012). The short-term holding of individuals for future sale to market is a unique situation that exists for the crustacean supply chain (Neil, 2012; Sinderman, 1988). Whilst not classified as 'farming' (due to the fact that animals are often not fed) animals held in these conditions are nevertheless exposed to conditions that allow for exposure to pathogens, enhanced infection pressure, and the development of disease (Neil, 2012; Stentiford *et al*. 2012). The effects and potential spread of viral infections in animals held within these environments and the subsequent impacts upon product quality is currently unknown.

1.2 White Spot Disease

Viruses appear to be the most abundant biological entities on the planet, substantially outnumbering cells in most well-studied habitats (Voonin and Dolja, 2013). The growth in penaeid shrimp aquaculture has been mirrored by the emergence of numerous diseases, the majority viral in origin (Peeler, 2012). White Spot Disease (WSD) caused by the agent White Spot Syndrome Virus (WSSV) for example, has rapidly spread across the globe through movement of infected stock and commodity products (Durand *et al*., 2000; Jones, 2012; Nunan *et al*., 1998; Peeler, 2012). In recognition of its significance, WSD has been listed as a notifiable disease by the World Organisation for Animal Health (Office International des Epizooties (OIE)) for over a decade (OIE, 2006).

Despite almost two decades since its discovery, WSD is still considered the most significant known pathogen impacting the sustainability and growth of the global penaeid shrimp farming industry. WSSV infection occurs in all tissues of mesodermal and ectodermal origin (e.g. gills, lymphoid organ, cuticular epithelium, sub-cuticular connective tissues). Infected nuclei become hypertrophied with marginalized chromatin, and contain inclusion bodies (Lightner, 1996a). Virions measure 120-150nm in diameter and 270-290nm in length, possess a tail-like projection and a striated nucleocapsid (Vlak *et al*., 2005; Wang *et al*., 1995). In contrast to the penaeids, relatively little information exists on the pathogenesis and outcome of WSD in non-penaeid decapod species (including crabs, lobsters and crayfish) and further, the utility of recommended

confirmatory diagnostic tests for diagnosis of infection and disease in these non-model hosts (OIE, 2006). Such information is now deemed vital given the global distribution of WSSV and potential for interaction with novel host species (farmed and wild) in new locations i.e. areas distant from those traditionally associated with its presence (Baumgartner *et al*., 2009). Several authors have provided some useful information on this issue by demonstrating susceptibility to WSD in certain decapod crustacean species found in European marine habitats (Corbel *et al*., 2001). Furthermore, studies demonstrate that WSSV can infect and cause disease in freshwater hosts maintained at ambient temperatures found within European regional waterways (Du *et al*., 2008; Jiravanichpaisal *et al*., 2001).

The emergence of White Spot Disease (WSD) is suggested to have been due to the feeding of imported frozen crabs to shrimp broodstock in Asia (Stentiford *et al*., 2012). Viruses which are morphologically similar to WSSV have been identified in portunid crabs in Europe and it has been suggested that these may be ancestral forms of WSSV (Bonami and Zhang, 2011). There are numerous reports of viral infections caused by DNA viruses within crustaceans, very little is known about these. With the expected increase in crustacean aquaculture and the propensity to house farmed stocks adjacent to and in direct contact with natural waterways it is possible that the next emergent disease may be an opportunistic pathogen which infects the surrounding fauna of these farms (Stentiford *et al*., 2012).

1.3 EC Council Directive 2006/88/EC

More recently, WSD has also been listed in regional legislation within the European Union (EU) via its inclusion in European Directive 2006/88EC (on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals). This legislation provides guidelines for EU Member States with regard to designation of their national status for specific listed diseases (including WSD) and the measures that should be applied to ensure that any outbreak of a listed disease is handled appropriately. Furthermore, it stipulates the requirements that must be met by importing nations (outside of the EU) wishing to trade susceptible live aquatic animals and their products to Member States within the EU, and to Member States wishing to trade susceptible species and products to other EU Member States. The overall aim is one of improved biosecurity for the listed globally significant pathogens within the European Union (Stentiford *et al*., 2010).

As highlighted by Stentiford *et al.* (2010) and Stentiford and Lightner (2011), since WSD has been listed in European Directive 2006/88EC as a 'non-exotic' disease within Europe, Member States are required to declare a national status for WSD (Table 1); categories ranging from Category I (free from disease) to Category V (infected). As highlighted within Table 1 the classification of disease free and infected could have major impacts upon the trading of crustaceans between member states, import and export restrictions being enforced depending upon the classification. The current situation, prevalence and spread, of WSD within Europe is currently unknown. Although not officially reported, outbreaks of WSD were observed in shrimp farms in Southern Europe in the late 1990s; the last known outbreak of the disease was in 2001 on a shrimp farm in Italy (Stentiford and Lightner, 2011). One of the duties listed under this Directive is that EU Member States must declare their status for WSD. In order to declare disease freedom Member States must undertake a 2 year surveillance programme. In order to do so, Member States may be required to undertake an epidemiologically- rigorous surveillance programme which utilises appropriate diagnostic tests designed to detect WSSV if present. EC Directive 2006/88/EC currently lists 'all decapod crustaceans' (>20,000 extant species) as potentially susceptible to WSD. Numerous other aquatic hosts including rotifers, bivalves, polychaete worms, non-decapod crustaceans and some aquatic insects were considered as mechanical vectors for WSSV (Stentiford *et al*., 2009). In this context, an improved understanding of host susceptibility, pathogenesis of WSD within non-model (but susceptible) hosts, manifestation of disease under ambient conditions not considered optimal for WSSV replication, and the suitability of current diagnostic methodologies for its detection, are urgently required.

Table 1. Health status of aquaculture zones or compartments and the corresponding import and export restrictions to be enforced within these areas. Recreated from EU Council Directive 2006/88/EC.

Category	Health Status	May introduce animals from	Health Certification		May dispatch animals to
			Introduction	Dispatching	
I	Disease Free	Category I Only	Yes	No when dispatching to category III or V Yes when dispatching to categories I,II or IV	All categories
II	Not declared disease free, undergoing a surveillance programme to declare disease freedom	Category I Only	Yes	No	Categories III and V
III	Undetermined, not known to be infected but not subject to a surveillance programme	Categories I, II or III	No	No	Categories III and V
IV	Known to be infected but undergoing an eradication programme	Category I Only	Yes	Yes	Category V only
V	Infected	All categories	No	Yes	Category V only

1.4 WSD, an issue for Europe?

The European crustacean industry centres on imports and wild fisheries, with limited aquaculture production of crustaceans. Wild fisheries for marine crustaceans are considered key resources in the European area; total fishery production of crustaceans from European waters in 2008 was 120,940 metric tonnes (www.fao.org/figis). Import of commodity products has been highlighted as a possible route of introduction of WSD (Durand *et al*., 2000; Nunan *et al*., 1998), despite there being a large market for tropical commodity shrimp in Europe relatively little research has been directed towards susceptibility of European crustaceans and the potential for establishment in new hosts (Stentiford *et al*. 2009). Shrimp imported for human consumption and being used as angling bait has been identified as a relevant risk for introduction of viral crustacean pathogens (BA, 2009). There is considerable anecdotal information that uncooked tropical shrimp are being used as angling bait in the UK freshwater and marine environments, however the extent of this practice is unknown.

As previously highlighted, relatively little information exists on the pathogenesis and outcome of WSD in non-penaeid decapod species (including crabs, lobsters and crayfish). The aim of this thesis is to address some of the keys questions, are European crustaceans susceptible to WSD? Is the pathological manifestation of the disease similar to that seen in penaeid decapod species? What is the true risk of importing infected commodity product? By addressing some of these key issues it is hoped that this work will be able to feed into risk assessments and provide an enhanced appraisal of WSD in Europe.

Chapter 2.

DNA Virus pathogens of Decapod Crustaceans

2.1 Introduction

Vago described the first invertebrate virus from the marine environment in 1966 from *Macropipus depurator* caught on the French Mediterranean coast (Vago, 1966). Prior to this discovery viral infection were described in terrestrial insects and mites. Diseased crabs showed a slow development of paralysis, haemolymph taken from these diseased crabs and injected into healthy crabs provoked the same symptoms. Negative staining revealed numerous paraspherical bodies 50-60µm in diameter. Bonami later rediscovered this virus infecting *M. depurator* sampled from the same area and the virus was classified as a reovirus (Bonami, 1973). Since this initial discovery there have been numerous crustacean viruses described over a relatively short space of time. Some of these viruses have had devastating consequences on the crustacean farming industry whilst their effects within wild populations remain relatively unstudied.

Few viruses from marine invertebrates have been assigned to a particular family of viruses with certainty because biochemical, biophysical and immunological data are incomplete or completely lacking. This deficit has been due largely to the lack of crustacean cell cultures, and to the difficulties in producing viruses *in vivo* using wild host animals. The problem of multiviral infections which are very frequent in crustaceans (Johnson, 1984; Johnson, 1983; Mari and Bonami, 1986) is another complicating factor and appears one of the most restricting factors for pathological and virological studies in this invertebrate group (Mari and Bonami, 1988). Viruses have so far been tentatively assigned to families based upon morphological and developmental characteristics and their location of infection within the host cell. Several reviews of the subject have been presented though rates of discovery outpace theses synopses (Bonami and Zhang, 2011; Brock *et al*., 1983; Couch, 1981; Edgerton, 1999; Johnson, 1984; Johnson, 1988; Johnson and Lightner, 1988)

Viruses appear to be the most abundant biological entities on the planet, substantially outnumbering cells in most well-studied habitats (Voonin and Dolja, 2013). The growth in penaeid shrimp aquaculture has been mirrored by the emergence of numerous diseases, the majority viral in origin (Peeler, 2012). Some of which, White Spot Syndrome Virus (WSSV) for example, have rapidly spread across the globe through

movement of infected stock and commodity products (Durand *et al*., 2000; Jones, 2012; Nunan *et al*., 1998; Peeler, 2012). There are numerous reports of viral infections caused by DNA viruses within crustaceans, as highlighted earlier, very little is known about these. With the expected increase in crustacean aquaculture and the propensity to house farmed stocks adjacent to and in direct contact with natural waterways it is possible that the next emergent disease may be an opportunistic pathogen which infects the surrounding fauna of these farms (Stentiford *et al*., 2012). Here I present a review of the current status and knowledge of DNA viruses in decapod crustaceans.

Table 1. Summary of known DNA viruses of decapod crustaceans, text highlighted red indicates an OIE notifiable disease. It can be clearly seen that the majority of crustacean viruses described have not been officially classified by the ICTV (-) and as such have been presumptively placed within viral families.

Family (Presumptive)	Virus	Type Species	Virion Size (width x length, nm)	Virion Shape	Replication	ICTV Classification (King *et al.*, 2012)	Initial Publication
Nimaviridae	White Spot Syndrome Virus (WSSV)	*Marsupenaeus japonicus*	80-120 x 270-290	Ovoid or ellipsoid with thread like extension at one end	Mesodermal and ectodermal tissues	✓	Takahashi *et al.*, 1994
	B Virus	*Carcinus maenas*	75-80 x 230-280	Rod shaped, sometimes curved	Haemocytes and connective tissues	Tentative (Vlak *et al.*, 2005)	Bazin *et al.*, 1974
	RV-CM	*Carcinus maenas*	95-110 x 235-280	Rod shaped, nucleocapsids occasionally u or V shaped	Haemocytes and cells in haematopoietic tissue	Tentative (Vlak *et al.*, 2005)	Johnson, 1988
	B2 Virus	*Carcinus mediterraneus*	90-110 x 340-380	Rod shaped	Haemocytes and connective tissues	Tentative (Vlak *et al.*, 2005)	Mari and Bonami, 1986
	Baculo-B	*Callinectes sapidus*	85-100 x 370-390	Rod shaped	Haemocytes and cells in haematopoietic tissue	Tentative (Vlak *et al.*, 2005)	Johnson, 1988
	Baculo-A	*Callinectes sapidus*	70 x 285	Rod shaped	Hepatopancreatic epithelial cells	Tentative (Vlak *et al.*, 2005)	Johnson, 1976a
	Tau virus	*Carcinus mediterraneus*	80 x 350	Rod shaped and bow shaped	Hepatopancreatic epithelial cells	Tentative (Vlak *et al.*, 2005)	Pappalardo *et al.*, 1996

Baculoviridae	*Penaeus duorarum* single nucleopolyhedrovirus (PdSNPV)	*Penaeus duorarum*	50 x 270	Rod shaped, Tetrahedral occlusion bodies	Hepatopancreatic epithelial cells	-	Couch, 1974a
	Penaeus plebejus baculovirus (PBV)	*Penaeus plebejus*	50 x 440	Rod shaped, Spherical occlusion bodies	Hepatopancreatic epithelial cells	-	Lester *et al*., 1987
Nudivirus	*Penaeus monodon* Nudivirus (PmNV)	*Penaeus monodon*	69 x 275	Rod shaped, Spherical occlusion bodies	Hepatopancreatic epithelial cells	-	Lightner & Redman, 1981
Intranuclear bacilliform viruses	Baculoviral mid-gut gland necrosis (BMN)	*Penaeus japonicus*	72 x 310	Rod shaped	Mid – gut	-	Sano *et al.,* 1981
	Baculo -PP	*Paralithodes platypus*	37-40 x 190-210	Rod shaped	Hepatopancreatic epithelial cells	-	Johnson and Lightner, 1988
	Crangon crangon bacilliform virus (CcBV)	*Crangon crangon*	72 x 215	Rod shaped with distinctive bulb of envelope at one end	Hepatopancreatic epithelial cells	-	Stentiford *et al*., 2004a
	Pandalus montagui bacilliform virus (PmBV)	*Pandalus montagui*	55 x 250	Rod shaped with distinctive kink in centre of nucleocapsid	Hepatopancreatic epithelial cells	-	Bateman *et al*., 2007

	Cancer pagurus bacilliform virus (CpBV)	*Cancer pagurus*	60 x 210	Rod shaped	Hepatopancreatic epithelial cells	-	Bateman and Stentiford, 2008
	Carcinus maenas bacilliform virus (CmBV)	*Carcinus maenas*		Rod shaped	Hepatopancreatic epithelial cells	-	Stentiford and Feist, 2005
	Scylla baculovirus (SBV)	*Scylla serrata*	44 - 67 x 253	Rod shaped, wide translucent area between inner and outer envelopes	Hepatopancreatic epithelial cells	-	Anderson and Prior, 1992
	Cherax quadricarinatus bacilliform virus (CqBV)	*Cherax quadricarinatus*	100 x 260	Rod shaped	Hepatopancreatic epithelial cells	-	Anderson and Prior, 1992
	Cherax destructor bacilliform virus (CdBV)	*Cherax destructor*	68 x 304	Rod shaped	Hepatopancreatic epithelial cells	-	Edgerton, 1996
	Astacus astacus bacilliform virus (AaBV)	*Astacus astacus*	71 x 343	Rod shaped with unilateral subapical expansion at one end	Hepatopancreatic epithelial cells	-	Edgerton *et al*., 1996
	Austropotomobius pallipes bacilliform virus (ApBV)	*Austropotombius pallipes*	67 x 360	Rod shaped with lateral expansion at one end	Hepatopancreatic epithelial cells	-	Edgerton *et al*., 2002b
	Pacifastacus leniusculus bacilliform virus (PlBV)	*Pacifastacus leniusculus*	72 x 229	Rod shaped	Hepatopancreatic epithelial cells	-	Hedrick *et al.,* 1995

Parvoviridae	*Penaeus stylirostris brevidensovirus* (PstDNV)	*Penaeus stylirostris*	Type 1, 27, within cytoplasm Type 2, 17, within membrane bound inclusions Type 3, 20, within nuclei	Icosahedral, non-enveloped	Hypodermal and Haematopoietic tissues	Listed as a member of the genus *Brevidensovirus* but has not been approved as species	Lightner *et al*., 1983
	Hepatopancreatic Parvovirus (HPV)	*Penaeus merguiensis/ Penaeus indicus*	22 - 24	Icosahedral	Hepatopancreatic epithelial cells	-	Chong and Loh, 1984
	HPV from *P. chinensis* (Pchin DNV*)*	*Penaeus chinensis*	22	Icosahedral	Hepatopancreatic epithelial cells	may be a member of the subfamily *Densovirinae* but has not been approved as a genus or a species	Bonami *et al*., 1995b
	HPV from *P. monodon* (PmDNV)	*Penaeus monodon*	22 - 24	Icosahedral	Hepatopancreatic epithelial cells	May be a member of the subfamily *Densovirinae* but has not been approved as a genus or a species	Sukhumsirichart *et al.,* 1999

	Penaeus merguiensis densovirus (PmergDNV)	*Penaeus merguiensis*			Hepatopancreatic epithelial cells	May be a member of the subfamily *Densovirinae* but has not been approved as a genus or a species	La Fauce *et al*., 2007a
	PC84 Parvo-like virus	*Carcinus mediterraneus*	23 -27	Icosahedral	Connective tissues	-	Mari and Bonami, 1988
	Lymphoidal parvo-like virus	*Penaeus merguiensis, P. monodon and P. esculentus*	18 - 20	Icosahedral	Lymphoid organ	-	Owens *et al*., 1991
	Spawner-isolated mortality virus (SMV)	*Penaeus monodon*	20	Icosahedral	Systemic	-	Fraser and Owens, 1996
	Cherax destructor systemic parvo-like virus (CdSPV)	*Cherax destructor*	20	Icosahedral	Systemic	-	Edgerton *et al*., 1997
	Parvo-like virus	*Cherax quadricarinatus*	20	Icosahedral	Gills	-	Edgerton *et al*., 2000
	Cherax quadricarinatus parvo-like virus (CqPV)	*Cherax quadricarinatus*	19.5	Icosahedral	Ectodermal, endodermal and mesodermal tissues	-	Bowater *et al*., 2002
	Virus- like particles in *Penaeus aztecus*	*Penaeus aztecus*	23	Icosahedral	Cytoplasmic vacuole of phagocyte	-	Foster *et al*., 1981
	Bay of Piran Shrimp Virus (BPSV)	*Palaemon elegans*	22 - 27	Icosahedral	Hepatopancreatic epithelial cells	-	Vogt, 1996
	Parvo-like virus	*Nephrops norvegicus*	22	Icosahedral	Haemocytes	-	Unpublished data

Herpesviridae	*Panulirus argus* virus 1 (PAV-1)	*Panulirus argus*	182	Hexagonal	Haemocytes	-	Shields and Behringer, 2004
	Bi-facies virus	*Callinectes sapidus*	184 - 214	Hexagonal Type A possess inner and outer envelope Type b inner envelope only	Haemocytes and haematopoietic tissues	-	Johnson, 1976b
	Herpes-like virus	*Rhithropanopeus harrisii*	100 - 110	Hexagonal	Mesodermal cells of testes	-	Payen and Bonami, 1979
	Herpes-like virus	*Paralithodes platypus*	140 - 165	Hexagonal	Antennal gland	-	Sparks and Morado, 1986
	Herpes-like virus	*Carcinus maenas*		Hexagonal	Haemocytes and haematopoietic tissues	-	This thesis
Iridoviridae	*Protrachypene precipua* Iridovirus	*Protrachypene precipua*	122 edge to edge 136 point to point	Hexagonal	Cuticular epithelium, gills and stomach epithelium	-	Lightner and Redman, 1993
	Sergestid Iridovirus (SIV)	*Acetes erythraeus*	140	Hexagonal	Cuticular epithelium	-	Tang *et al*., 2007c

2.2 *Nimaviridae*

2.2.1 White Spot Syndrome Virus (WSSV)

White Spot Disease (WSD) is caused by the virus White Spot Syndrome Virus (WSSV) and is probably the most extensively studied crustacean virus to date due to the devastating effect this virus has had on the shrimp farming industry, cumulative losses exceeding $10bn since 1993 (Stentiford *et al*., 2012). The name of the disease refers to the clinical signs that have been reported in some (not all) species of shrimp, white spots associated with deposition of calcium appear on the inner surface of the cuticle. The virus was originally discovered in 1991 in *Penaeus japonicus* in China and Taipei, spreading rapidly throughout Asia and then the Americas (Cai *et al*., 1995; Stentiford *et al*., 2009). The virus has been isolated from various crustacean hosts in China, Japan, Southeast Asia, the Indian continent, the Mediterranean, the Middle East and the Americas (Vlak *et al*., 2005). Due to the rapid spread of the virus and the isolation and identification by numerous laboratories the virus has been referred to by a variety of different names, Chinese Baculo-like virus (Nadala *et al*., 1997), White Spot Syndrome (Chou *et al*., 1995), White Spot Bacilliform virus, White Spot Syndrome Baculovirus (WSBV) (Chang *et al*., 1996; Lightner, 1996; Wang *et al*., 1995), Penaeid rod shaped DNA virus (PRDV) (Inouye *et al*., 1996), Rod-shaped virus of *P. japonicus* (RV-PJ) (Inouye *et al*., 1994), Penaeid haemocytic rod-shaped virus (PHRV) (Owens, 1993), *P. monodon* non-occluded baculovirus I and III (PmNOBIII) (Wang *et al*., 1995), Systemic Ectodermal and Mesodermal Baculovirus (SEMBV) (Wongteerasupaya *et al*., 1995), Hypodermic and Hematopoietic Necrosis Baculovirus (HHNBV) (Huang *et al*., 1995). It was later agreed that all of these infections were caused by the same agent and the name White Spot Syndrome Virus (WSSV) was adopted.

WSSV is a large dsDNA virus and the sole member of the virus family Nimaviridae (King *et al*., 2012). It is known to cause mass mortalities within shrimp ponds and has had a devastating effect on the industry since its emergence in the late 1990's. WSSV replicates within the nucleus and infects all tissues of mesodermal and ectodermal origin. It is a lytic virus and in the late stages of infection causes infected cells to disintegrate causing destruction of affected tissues and organ failure (Leu *et al*., 2010).

Gross signs of disease are lethargy (Durand *et al*., 1997), reduced feeding, reddish colouration (Lightner *et al*., 1998), loose cuticle (Lo *et al*., 1996a), enlargement and yellowish discolouration of the hepatopancreas (Sahul Hameed *et al*., 1998) and a thinning and delayed

clotting of the haemolymph (Wang *et al*., 2000). The formation of white spots cannot be used as a definitive diagnostic tool as these white spots can also be caused by bacterial infections and are not always present in infected shrimp. WSD results in very acute, rapid, high mortalities and can cause 100% mortality within infected shrimp ponds within 3-10 days (Lightner, 1996). Clinical signs usually appear within farmed stocks 14 - 40 days after stocking (Stentiford *et al*., 2009). Mortalities within crabs, crayfish, spiny lobsters, freshwater prawns and clawed lobsters is highly variable (Cai *et al*., 1995; Chen *et al*., 2000; Hossain *et al*., 2001; Jiravanichpaisal *et al*., 2001; Momoyama *et al*., 1994; Nakano *et al*., 1994; Rodriguez *et al*., 2003; Sahul Hameed *et al*., 2000; Takahashi *et al*., 1994; Yoganandhan *et al*., 2003; Bateman *et al*. 2012a [this study]). The virus is transmitted *per os* by predation on diseased individuals or via the water through the gills. Survivors of an infection may carry the virus for life and may pass the virus to progeny via vertical transmission (Lo *et al*., 1996b; Lo *et al*., 1997). Birds may also act as vectors for the virus as they gather around ponds where mortalities are occurring and eat dead shrimp (Vanpatten *et al*., 2004).

WSSV infects all tissues of mesodermal and ectodermal origin, such as gills, lymphoid organ, midgut but especially cuticular epithelium and sub-cuticular connective tissues. WSSV also infects the haemocytes, causing acute anaemia, as highlighted by Owens (1993) and Inouye *et al*. (1996). TEM revealed hypertrophic nuclei to be filled with virions, ovoid or ellipsoid to rod-shaped, consisting of an electron dense nucleocapsid, with a tight fitting capsid layer, surrounded by a loose-fitting trilaminar envelope. Virions measured 120-150nm in diameter and 270-290nm in length and nucleocapsids measured 65-70nm in diameter and 300-250nm in length (Vlak *et al*., 2005). In some cases a tail like projection can be seen extending from one end. WSSV virions possess a very distinctive capsid layer giving the DNA core a cross-hatched or striated appearance (Vlak *et al*., 2005). The capsid was described as being composed of rings of subunits in a stacked series. Rings were 20nm thick, perpendicular to the longitudinal axis of the capsid (Wang *et al*., 1995). This feature is similar to that described in B virus of *Carcinus maenas* (Bazin *et al*., 1974; Bonami and Zhang, 2011) and B2 virus in *Carcinus mediterraneus* (Bonami and Zhang, 2011; Hernandez-Herrera *et al*., 2009; Mari and Bonami, 1986) suggesting that these viruses may be closely related to WSSV.

All decapod and non decapod crustaceans are listed by the OIE as being susceptible to this virus (OIE, 2012). To date, over 100 species of arthropod have been reported as hosts or carriers of WSSV either from culture facilities, the wild or from experimental infection (Sánchez-Paz, 2010). Stentiford *et al.* (2009) identified 98 potential host species from the scientific literature, of these 67 could be described as susceptible using the European Food Standard Agency (EFSA) principles. Numerous aquatic organisms, rotifers (Yan *et al*., 2004),

bivalves, polychaete worms (Vijayan *et al*., 2005) and non-decapod crustaceans such as *Artemia sp*. and copepods were also reported as potential mechanical vectors (Stentiford *et al*., 2009). Although these have been shown to accumulate large concentrations of viable virus there has been no evidence of replication within these hosts hence they are currently classed as mechanical vectors (Chang *et al*., 2002; Li *et al*., 2003; Lo *et al*., 1996b; Yan *et al*., 2004). Desrina *et al*. (2013) detected the presence of WSSV infected nuclei within the foregut epithelium of a polycheate species (*Dendroneireis* spp.), confirming that the virus can replicate within this species. This is the first report of WSSV replication being shown in a non-crustacean host suggesting that WSSV is more of a generalist virus than initially thought and highlights this virus has a wider host range than crustaceans alone.

WSSV was originally classified as an unassigned member of the *Baculoviridae* family. It was later reclassified within a new genus *Whispovirus* and family *Nimaviridae,* and named White Spot Syndrome Virus 1 by the ICTV (Mayo, 2002a; Mayo, 2002b; Vlak *et al*., 2005). It is currently the only member of this genus but Vlak *et al*. (2005) state that since this is a newly recognised family, its membership is likely to be augmented in the future as new taxa are discovered. Vlak *et al*. (2005) also tentatively list B virus, B2 virus, τ (tau) virus and Baculo-A and Baculo-B viruses as putative members of the genus and family.

Numerous studies have contributed towards its characterisation. WSSV virions have a buoyant density of 1.22g/cm^3 in CsCl gradients and nucleocapsids have a buoyant density of 1.31g/cm^3. Virions are sensitive to detergents indicating the presence of lipid within the WSSV envelope. Nucleocapsids contain a circular dsDNA strand approximately 300kbp in length. Virions contain at least 5 major and at least 13 minor polypeptides ranging in size from 14 to 190 kDa. Envelopes contain two major proteins VP28 and VP19 and the nucleocapsid consists of 3 major proteins VP26, VP24 and VP15. VP28, VP26 and VP24 are related (Vlak et al., 2005). Numerous diagnostic tools have also been developed based upon detection of these viral components (Durand *et al*., 1996; Durand and Lightner, 2002; Liu *et al*., 2002; Lo *et al*., 1996b; Poulos *et al*., 2001) with the method described by Lo *et al*., (1996a) for detection of VP28 via PCR being the confirmatory diagnostic method listed by the OIE (OIE, 2012). However Claydon *et al*. (2004) showed that this test could result in false positive results when used with *Cherax quadricarinatus* samples, highlighting a weakness of the Lo *et al*. (1996b) technique. Nunan and Lightner (2011) further developed and optimised the PCR procedure by adjusting the annealing temperature and shortening the cycling times. This modified assay was compared to the Lo *et al*. (1996b) protocol and was shown to be faster and just as sensitive.

The WSSV genome has been fully sequenced and 184ORFs have been identified. Genes have been shown to encode structural proteins and those involved in DNA replication and protein modification, however most remain unassigned. The genome contains nine homologous repeat regions. There are three fully sequenced genotypic variants known to date. They can be discriminated by restriction fragment length polymorphism and on the basis of the complete genome sequence. The variants range in size from 297,967 nt (WSSV-Th) to 305,107nt (WSSV-Ch) and 307,287nt (WSSV-Tw). These differences in size can be attributed to insertions/deletions located at map position +10.6. It will be interesting to see whether other viruses tentatively assigned to this genus/family (such as B virus from *C. maenas*) have similar genomic structuring to WSSV and further, whether they may be shown to be ancestral forms of the emergent WSSV 1 forms circulating in global shrimp aquaculture.

WSSV is listed as an OIE notifiable disease and as a non-exotic disease within Europe (EC Council Directive 2006/88). Although not officially reported, outbreaks of WSD were observed in shrimp farms in Southern Europe in the late 1990s - this evidence providing the basis for the classification of WSSV as a 'non-exotic' disease within the EU. However the prevalence and spread of this virus within Europe is not clear; the last known outbreak of the disease was in 2001 on a shrimp farm in Italy (Stentiford and Lightner, 2011). One of the duties listed under this Directive is that EU Member States must declare their status for WSD; categories ranging from Category I (free from disease), to Category V (infected). In order to declare disease freedom Member States must undertake a 2 year surveillance programme. Commodity products contaminated with WSSV have been highlighted as a possible route of introduction of the pathogen to non-farming regions such as the EU (Bateman *et al*., 2012b [this thesis]; Durand *et al*., 2000; Nunan *et al*., 1998). However, despite the large market for tropical commodity shrimp entering Europe, relatively little research has been directed towards assessment of susceptibility of European crustaceans to WSSV and to potential establishment in host populations (Stentiford *et al*. 2009). Corbel *et al*. (2001) demonstrated the susceptibility of some European species and other studies have shown WSSV can infect and cause disease at temperatures found within Europe (Du *et al*., 2008; Jiravanichpaisal *et al*., 2001).

2.2.2 B Virus

Bazin *et al.* (1974) described a viral infection of *Carcinus maenas* from the coast of France. B virus was discovered during a study of limb regeneration and was first noticed infecting the nuclei of haemocytes and connective tissues formed during early stages of regeneration. Histology revealed hypertrophied nuclei with marginalized chromatin. TEM showed viral particles within these enlarged nuclei, appearing in parallel arrays. Virions were often

associated with vesicles of various sizes within the nucleus, were 300-320nm in length, rod-shaped and sometimes appeared curved. Particles consisted of a club-shaped electron dense capsid, 230-280nm in length and 75-80nm in diameter, surrounded by an envelope measuring 8nm. The size of the virus, its intranuclear location, structure and morphology suggested that it shared closest affinity with members of the *Baculoviridae* (Bazin *et al*., 1974). However because of the association between virions and vesicles within the nucleus and the absence of any inclusion body, it was alternatively suggested to be closer to those viruses infecting rhinoceros beetles, *Oryctes rhinoceros*, (Huger, 1966), whirligig beetles, *Gyrinis natator*, (Gouranton, 1972), European red mites, *Panonychus ulmi*, (Bird, 1967) and citrus red mites, *P. citri*, (Reed and Hall, 1972), rather than the nuclear polyhedrosis virus infecting pink shrimp, *Penaeus duorarum* (Couch, 1974a). After purification, virus particles inoculated to healthy crabs reproduced the typical symptoms of the disease (Bonami, 1976). Taxonomic position of this virus has never been defined but it was tentatively classified within the *Nimaviridae* by the ICTV (Vlak *et al*., 2005). Interestingly, B virus possesses the unique cross-striated capsid structure observed in WSSV (Bonami and Zhang, 2011). Due to lack of evidence, the classification of B virus within the *Nimaviridae* has been suspended pending additional study (Lo *et al*., 2012).

2.2.3 Rod shaped virus of *Carcinus maenas* (RV-CM)

Johnson (1988) described a similar infection in to B virus in invasive populations of *C. maenas* from the Atlantic coast of the USA. She named this virus rod-shaped virus of *Carcinus maenas* (RV-CM), although morphologically similar to B virus from European *C. maenas,* size differences and certain other characteristics suggest that RV-CM may be distinct. The virus was present at low apparent prevalence (1/29 crabs) in a single sample set (of 75) obtained from Woods Hole, Massachusetts, USA during the period 1982-1983. The infected crab had been held in the laboratory for 12 days and showed no external signs of disease. Upon dissection, the haemolymph was ‘milky’, had the expected complement of haemocytes but also contained numerous small granules. The haemolymph of the infected crab did not clot properly with only small aggregates of haemocytes forming. A complicating factor was that the crab appeared to be co-infected with a second virus, a rhabdo-like virus similar to EHV in blue crabs (Johnson, 1983; Johnson, 1984).

RV-CM infects haemocytes and cells in the haematopoietic tissue. Some infected nuclei are hypertrophic but many appear to be of relatively normal volume. All stain positively with Feulgen, indicating presence of DNA within the nuclear inclusion. Virions measure 95-110nm in diameter and between 190-540nm in length (majority measure between 235-280nm). Newly

completed nucleocapsids are straight with the envelope fitting loosely along its length. The envelope measures 7-9nm thick and becomes spherical when closed forcing the nucleocapsid into a curved shape. Curvature of the particles can be so pronounced that occasionally the nucleocapsids bend into U or V shapes. The taxonomic position of this virus has never been defined but it was tentatively classified within the *Nimaviridae* by the ICTV (Vlak *et al*., 2005). Similar to B virus, this classification has been suspended pending further study (Lo *et al*., 2012).

2.2.4 B2 Virus

B2 virus, also putatively assigned to the Baculoviridae at time of discovery was described infecting *Carcinus mediterraneus* from the French Mediterranean coast by Mari and Bonami (1986). Despite the different host, this virus appeared identical to that of B virus infecting *C. maenas*. Numerous similarities exist between B, B2 and WSSV at histological and ultra-structural level. B2 targets haemocytes and connective tissues leading to damage of the interstitial tissues within the hepatopancreas and the gills. Infected haemocytes display hypertrophied nuclei containing virus particles at different stages of development with many rod shaped structures. B2 virus particles measure 340-380nm in length and 90-110nm in diameter. Nucleocapsids measure 280-320nm long and 70-80nm in diameter with 10nm space between capsid and envelope. The two membranes of the nuclear envelope are disconnected, membranes only connection being the nuclear pores. Similar observations of this peri-nuclear space were reported for B virus by Bazin (1974) and Baculo-B virus by Johnson (1983). Virions are organised in parallel groups within infected nuclei, capsids enclosing an electron dense nucleoprotein surrounded by a trilaminar envelope. Negative staining showed that the particles are more or less ovoid but did not show any internal structure. If the envelope was broken, negative staining with phosphotungstic acid (PTA) revealed a pile of ring-shaped segments terminated at one end by a flat edge and the other a rounded edge, identical to that observed in WSSV and B virus, with only the number of segments variable between viruses. In all three cases, virogenesis is similar, with development of viral envelopes within the nucleus and the formation of electron-dense nucleocapsids and trilaminar envelopes with characteristic membrane extensions.

Professor JR Bonami presented a paper at the EAFP conference highlighting the similarity of B, B2, and Baculo-B viruses (see below), to WSSV (Hernandez-Herrera *et al*., 2009). Despite the clear ultrastructural similarity, a lack of molecular data for the B/B2/Baculo-B isolates has detracted from direct comparison of these viruses with WSSV infecting penaeid shrimp. Hernandez-Herrera *et al*. (2009) presented data obtained during the first stages of infection of

B2 virus highlighting the possible role played by the tail-like structure in host cell infection, particularly attachment to the plasma membrane. They hypothesised that WSSV may deploy a similar attachment process. In addition, Hernandez-Herrera *et al*. (2008) carried out a dot-blot hybridisation between WSSV and B2 using probes listed by Durand *et al.* (1997) and Shi *et al.* (2000). Samples of gill and hepatopancreas were taken from crabs which had been experimentally exposed to B2 virus by injection of inoculum arising from naturally infected *C. mediterraneus*. Samples had initially been generated in the 1980's and stored for over 20 years at -20˚C. Despite this, positive results were obtained from using crab tissues treated with WSSV probes. This data provides further evidence that B2 and WSSV at least are closely related and perhaps that B2 is an ancestral form of WSSV 1 (Bonami and Zhang, 2011).

2.2.5 Baculo-B

The rod-shaped virus that infects the haemocytes of the blue crab *Callinectes sapidus* (Johnson, 1984; Johnson, 1988; Johnson, 1983) is also very like B/B2 viruses described infecting European *Carcinus* species. Baculo-B was described infecting undifferentiated haemocytes and other cells within haematopoietic tissues of crabs from Chesapeake Bay and Chincoteague Bay, USA. It occurred in apparently normal crabs although crabs infected with Baculo-B also harboured other pathogenic viruses and protistan pathogens. Overtly infected cells can be easily distinguished; nuclei are hypertrophied, up to 1.6 times normal size and stain strongly positive with Feulgen stain (with multiple small, intensely stained inclusions in some cases). Multiple viral infections did not alter the ultra-structural appearance of Baculo-B or the appearance of Baculo-B infected nuclei. First signs of infection are similar to RV-CM, with an additional marginal band of variable width next to the nuclear membrane which occupied all the nuclei not filled by the stroma. Completed capsids possess squared or bluntly rounded ends and normally measure 220-280nm in length and 53-60nm in diameter. Enveloped virions measure 370-390nm in length and 85-100nm in diameter, with envelope measuring 5-6nm thick. Before or after envelopment thin sectioned nucleocapsids of Baculo-B and RV-CM are polarised because of the specialised apex. In some infected nuclei either the inner or outer nuclear membrane is missing in areas leaving a single membrane to separate the nucleus from the cytoplasm. Nuclear pores in these nuclei are larger than normal.

B, B2, RV-CM and Baculo-B viruses are similar in terms of size, shape and morphogenesis. Negative staining of isolated virus particles provided greater detail of viral structure with the tail-like structure varying in length and appearing to be formed by prolongation of the envelope. The nucleocapsid is characterised by one rounded extremity with the other blunt-ended by a

trilaminar structure 15-18nm think. The surface of the nucleocapsid appears crosshatched in a perpendicular fashion according to its longitudinal axis. This structure is composed of stacked rings arranged in 14 striations of approximately 22nm thick. As reported for B/B2 viruses, this is a common trait with WSSV; in fact these viruses appear morphologically indistinguishable (Bonami and Zhang, 2011).

B, B2 and Baculo-B have previously been considered as non-occluded baculoviruses (classified in sub-group C of the Baculoviridae). However, as noted, their taxonomic position may be more aligned with the *Nimaviridae* (Vlak *et al*., 2005; Lo *et al*., 2012).

2.2.6 Baculovirus A (Baculo-A)

Baculo-A is a nonoccluded virus infecting the hepatopancreatic epithelial cells of the blue crab *Callinectes sapidus* (Johnson, 1984; Johnson and Lightner, 1988; Johnson, 1976a; Johnson, 1983). The prevalence of Baculo-A ranged from 4 to 20% in crabs sampled from Chesapeake and Chincoteague Bays (overall apparent prevalence of 6% in 1500 crabs sampled). Infected host nuclei appeared over 2 times larger than normal and stain Feulgen positive. Although Baculo-A has not been associated with mass mortalities, infected cells appear to not function normally As for numerous gut infecting viruses of crustacean hosts, transmission through contaminated faecal matter appears most likely. Although it is currently unknown how viral particles pass from one epithelial cell to another, the focal nature of infection suggests a likely route may be cell-cell gap junctions or other inter-membrane transport. As for B/B2 and Baculo-B viruses, the taxonomic position of this virus has not been defined and although tentatively assigned to the *Nimaviridae* (Vlak *et al*., 2005), this designation has been revoked due to lack of evidence (Lo *et al*., 2012).

2.2.7 τ (tau) virus

The τ (tau) virus was isolated from diseased *Carcinus mediterraneus* from the Mediterranean coast by Pappalardo *et al*. (1986). The virus was named after the lagoon of Thau where it was first observed. Diseased crabs were less aggressive, were lethargic with loss of appetite and died within several days of exposure. Healthy crabs sampled from a separate area were used in challenge trials, crabs being fed and injected with infected material. Histology shows lesions within the hepatopancreas and midgut, nuclei appeared hypertrophied, with marginated chromatin and loss of nucleoli. Infected nuclei appeared twice as large as uninfected nuclei and during advanced infections host nuclei appear uniform and stain intensely Feulgen positive. The cytoplasm of infected host cells appeared highly vacuolated and disorganised,

particularly in the basal region adjacent to the basement membrane. Changes in nuclear structure were associated with enlargement of the perinuclear cisternae and migration of the nucleus to the apical part of the cell. Host cell rupture was followed by the release of cellular contents into the tubule lumen. Inclusion bodies were not observed in either infected nuclei or the cytoplasm of infected cells. All major cell types of the hepatopancreas (E, R, F and B) were found to be equally infected in distal, median and proximal parts of the tubule. Nuclear alterations were present in the B and F cells and these cells frequently became detached from the basal lamina and ruptured. Identical pathological changes were present in the midgut but at a lower intensity.

TEM revealed hypertrophic nuclei containing rod-shaped and bow-shaped viral particles, some of which are enveloped. Particles measure 350nm in length and 80nm in diameter. Viroplasm contained membranous structures 6-7nm thick and spherules 20-25nm in diameter. Tubular structures 50-60nm in diameter but of variable length were observed in parallel arrays within the viroplasm. Particles accumulate at the nuclear periphery, fibrillar stroma occupying the centre. The two membranes of the nuclear envelope appear to be held together by the pore structures, in heavily infected nuclei the outer envelope appears dilated and expands into a membranous labyrinth.

Pappalardo *et al*. (1986) highlighted that the lesions observed during exposure studies were characteristic of baculoviruses which did not produce occlusion bodies and pointed out the similarities to insect baculoviral infections. They also highlighted similarities between τ (tau) virus and Baculo-A but noted that although repair and regeneration appeared to occur in infected blue crabs, the viral disease described here was rapid and apparently fatal. Pappalardo *et al*. (1986) also pointed out similarities between τ (tau) virus and B virus (although they develop in different tissues). Again, the tentative positioning of this virus within the *Nimaviridae* (Vlak *et al*., 2005) was revoked due to lack of evidence (Lo *et al*., 2012).

2.3 Nudiviruses

Nudiviruses are a diverse group of rod-shaped viruses with large circular closed dsDNA genomes. These viruses have previously been named as non-occluded baculoviruses (Huger and Krieg, 1991). The term nudiviruses emerged during the 1990s, the prefix *nudi* meaning ‘bare’, ‘naked’ or ‘uncovered’ (Burand, 1998) to depict their apparent lack of occlusion body (Huger and Krieg, 1991). Alternative terms such as intranuclear bacilliform viruses (IBV) have also been used (Evans and Edgerton, 2002). The similarity of these viruses, occurring in a wide range of (mainly insect) host species was based upon structural characteristics and

similarities in replication alone since no genetic data was available at the time. Due to this lack of genetic data the *Nudibaculoviridae* subfamily of *Baculoviridae* was dissolved in 1995 and viruses that had been tentatively placed in this taxon remain unclassified (Murphy *et al*., 1995; King *et al*., 2012). Jehle (2010) highlights the fact that the so called 'non occluded baculoviruses' are in fact an assemblage of different viral taxa, most likely comprising several divergent lineages. The relationship between these viruses and to 'true' baculoviruses was not confirmed until the genome sequences of certain isolates were obtained. Comparative phylogenetic analysis of gene and genome sequences showed that at least five of the known types formed a monophyletic clade considered as the 'nudiviruses': *Oryctes rhinoceros* nudivirus (OrNV), *Heliothis zea* nudivirus 1 (HzNV-1), *H. zea* nudivirus 2 (HzNV-2), *Gryllus bimaculatus* nudivirus (GbNV) and the *Penaeus sp*. baculoviruses (PvSNPV & PemoNPV). The latter two are crustacean-infecting viruses and actually possessed occlusion bodies (Jehle, 2010). Sequence comparisons and phylogenetic analyses of PemoNPV and HzNV-1 and HzNV-2 showed that that they are closely related and it is suggested that PemoNPV should be considered as an 'occluded nudivirus' (Wang and Jehle, 2009; Yang *et al*., 2014).

In further work, Jehle (2010) states that nudiviruses are likely a highly diverse group of arthropod viruses with a very wide host range covering host taxa within the Crustacea, Coleoptera, Orthoptera, Lepidoptera and possibly other arthropod orders. To date four insect nudivirus genomes have been fully sequenced. In these cases, although gene order is poorly conserved, all contain a characteristic gene cluster of *helicase*, 16*KDa* and/or *lef-5*. This is similar to a gene cluster found in the genome of the baculoviruses. Although this feature alone was not considered sufficient to indicate a common origin of baculoviruses and nudiviruses (as these clusters could have been horizontally transferred among virus ancestors), the presence of 20 baculovirus core genes within the nudivirus genomes could not be explained by this phenomenon since genes within this core are involved in fundamental virus functions (e.g. DNA replication, nucleotide metabolism, transcription, virus morphogenesis, structure and *per os* infectivity (Jehle, 2010; Wang and Jehle, 2009). Based on gene sequences and ontogeny Jehle (2010) therefore proposed that nudiviruses and baculoviruses are sister lineages of dsDNA viruses and share a common ancestor. Some gene homologies with shrimp whispoviruses (WSSV) suggest that all these viruses may have derived from a circular dsDNA marine arthropod virus (Jehle, 2010).

2.3.1 *Penaeus monodon* Nudivirus (PmNV)

An occluded bacilliform virus found to infect *P. monodon* was described by Lightner and Redman (1981) following mortalities in laboratory-reared populations of shrimp. Originally

thought to be specific to this species it has since been discovered in several penaeid shrimp from different geographic regions. So called Monodon baculovirus (MBV) is known to infect *P. merguiensis, P. semisulcatus, P. kerathurus, P.indicus* (Johnson and Lightner, 1988; Lightner *et al*., 1983b; Lightner and Redman, 1981; Sindermann, 1988; Vijayan *et al*., 1995), *Metapenaeus ensis* (Chen *et al*., 1989), *M. bennettae* (Spann and Lester, 1996), *M. monoceros* and *M. elegans* (Manivannan *et al*., 2004). MBV was associated with significant losses in Indo-Pacific shrimp culture facilities (Lightner *et al*., 1990) where prevalence has previously reached 100% in hatcheries and farms in Asia (Baticados *et al*., 1991). Infection can result in substantial economic losses due to poor growth and reduced survival of post larvae. It was suggested that MBV is well tolerated by *P. monodon* in low to moderate infections if other conditions are optimal (Lightner *et al*., 1987). Severely affected *P. monodon* at any life stage are lethargic, do not feed and seldom preen resulting in increased surface and gill fouling (Lightner *et al*., 1983b). Environmental stressors such as crowding and handling, and biological stressors such as gill fouling, have been shown to enhance the severity of infection in *P. monodon* (Natividad and Lightner, 1992). The simplest method to detect infection is examination of freshly minced tissue squashes prepared from the central portion of the hepatopancreas. A 0.05% aqueous solution of malachite green is used to prepare the tissue squash to aid the observation, spherical occlusion bodies stain rapidly and uniformly bright green, distinguishing them from lipid droplets, nucleoli etc. (Lightner *et al*., 1983b).

Histology revealed lesions within the hepatopancreas in older shrimp and in both hepatopancreas and anterior midgut in young post larvae. Infection is characterised by the presence of intranuclear, spherical inclusion bodies within hypertrophied nuclei of the hepatopancreatic epithelial cells. Cells stained with H&E in the first cytopathic stage (stage 1) of development show slight hypertrophy of the nucleus, peripherally displaced or non-discernable nucleolus and margination of chromatin. Second stage cells are similar but nuclei are further hypertrophied and contain one or more weakly eosinophillic stained inclusion bodies. Cells in the third stage of development show greatly hypertrophied nuclei with several large eosinophillic inclusion bodies. Cytoplasm of these cells is reduced and appears more basophilic than normal cells. Infection is not observed in any other tissues or organs in infected shrimp (Lightner *et al*., 1983b).

Lightner and Redman (1981) re-processed tissues from Davidsons fixed material for electron microscopy and this revealed the inclusion bodies to be composed of an electron dense amorphous matrix some of which contained virus particles. Virions both free in the viroplasm and occluded within the inclusion bodies were rod-shaped, consisting of a relatively thin

trilaminar envelope around an electron dense nucleocapsid. Nucleocapsids measure 24 nm in diameter; enveloped virions measure 69 nm in diameter and 275nm in length. Epithelial cell nuclei with inclusions are occasionally seen within the tubule lumen, released by necrosis and lysis of the epithelial cell. Lightner *et al.* (1983b) reported complete virions measure 324 ± 33nm in length and 75 ± 4nm in diameter, nucleocapsids measure 246 ± 15nm in length and 42 ± 3nm in diameter; larger than that originally described (Lightner & Redman, 1981). This can be accounted for by the different fixation techniques used as the original description was based upon material re-processed from Davidson's fixative.

Vickers *et al*. (2000) described naked nucleocapsids free in the cytoplasm, attached to the nuclear membrane. This was the first description of such an observation in MBV and the authors suggested this as a means by which the viral genome is transported from the plasma membrane of the hepatopancreas to the nucleus. They suggested that virions lost their envelope at the plasma membrane, nucleocapsids travelling through the cytoplasm to attach to the nuclear membrane, the viral genome then entering the nucleus. This supported evidence provided by previous studies of baculoviral infections in crustacea (Johnson and Lightner, 1988). Insect viruses have been shown to lose the envelope at the cellular membrane, nucleocapsids travelling through the cytoplasm and attaching via a filament at a nuclear pore and releasing the nucleic acid into the nucleus. Observations presented by Vickers *et al*. (2000) suggested a similar mechanism in MBV.

Detection of occlusion bodies via histology required a high level infection so molecular diagnostic techniques were developed as more sensitive methods to aid the diagnosis of this disease, PCR techniques (Belcher and Young, 1998; Chang *et al*., 1993; Lu *et al*., 1993), ELISA (Hsu *et al*., 2000) and *in situ* hybridisation *(Poulos et al., 1994)*. However not all known MBV isolates were detected by these techniques, Surachetpong *et al*. (2005) developed an improved PCR diagnostic method which was shown to amplify MBV isolates from all known geographic regions. Nimiphak *et al*. (2010) developed a loop-mediated isothermal amplification combined with a lateral-flow dipstick (LAMP-LFD) method that proved to be ten times more sensitive than one-step and nested PCR reactions, enabled faster results and did not require expensive equipment. Nimiphak *et al*. (2010) suggested this may be a suitable method for shrimp farms as an alternative to traditional PCR techniques.

The virus was originally named and is commonly known as Monodon Baculovirus (MBV) (Lightner & Redman, 1981). Spherical baculovirosis (MBV) was listed as an OIE notifiable disease until 2009. Mari *et al*. (1993) purified and characterised the virus and suggested renaming the virus *P. monodon* single nucleopolyhedrovirus (PmSNPV). The virus is now

named *P. monodon* nucleopolyhedrovirus (PemoNPV) and was tentatively listed as a member of the *Nucleopolyhedrovirus* genus of the *Baculoviridae* family by the ICTV (Theilmann *et al*., 2005). Mari *et al*. (1993) described PemoNPV particles as possessing characteristic appendages at each extremity resembling those described in some non-occluded baculoviruses. PemoNPV nucleocapsids were shown to be similar to the τ (tau) virus from *C. mediterraneus* due to the superficial helical structure and the 2 layered caps that enclosed the extremities. Some PemoNPV nucleocapsids were found to release a braided-like structure, interpreted as the nucleoprotein, similar to the τ (tau) virus and the *Oryctes rhinoceros* baculovirus. These properties led Mari *et al*. (1993) to suggest that PemoNPV may be more closely related to the non-occluded crustacean baculoviruses than to the occluded type A baculoviruses. Mari *et al.* (1993) suggested using this isolate as a reference strain to which other MBV-type isolates could be compared. Yang *et al*. (2014) have recently examined the viral genome features and completed a phylogenetic analysis, they concluded that PemoNPV is not a baculovirus, and that it should be renamed *Penaeus monodon* Nudivirus (PmNV) and be assigned to a new genus (*Gammanudivirus*) within the proposed *Nudiviridae* family.

For the purpose of this review the remaining viruses that form occlusion bodies have been grouped within the genus *Gammabaculovirus* of the *Baculoviridae* according to current ICTV classifications. In contrast, rod-shaped viruses lacking an occlusion body are considered as intranuclear bacilliform viruses since this group (mainly infecting decapod crustacean hosts) require closer taxonomic treatment before they can be classified within the *Nudiviridae*. Although commonly observed, our understanding of the taxonomy of the intranuclear bacilliform viruses is limited, their separation from one another being based solely on structural characteristics. They do however form excellent models for further research in to the relationship between large DNA virus pathogens of crustaceans and insects.

2.4 *Baculoviridae - Gammabaculovirus*

2.4.1 *Penaeus duorarum* single nucleopolyhedrovirus (PdSNPV)

Baculovirus penaei (commonly known as BP) was first discovered by Couch (1974a) in pink shrimp *Penaeus duorarum* experimentally exposed to the polychlorinated biphenyl (PCB), Aroclor 1254. BP was renamed *Penaeus duorarum* single nucleopolyhedrovirus (PdSNPV) and tentatively assigned in the *Nucleopolyhedrovirus* genus by the ICTV (Francki *et al*., 1991). The *Baculoviridae* have since been reclassified (Jehle *et al*., 2006) and is now composed of four genera *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus* and *Deltabaculovirus*.

PdSNPV is now likely to sit within the genus *Gammabaculovirus*, however this classification is not present in the most recent report of the ICTV (Herniou *et al*., 2012). Infected shrimp showed no gross signs of infection but histology revealed large triangular occlusion bodies within hypertrophied nuclei of hepatopancreatic epithelial cells. Nuclei contained one or more occlusion bodies, in moderate to light infections the inclusions occurred in foci whereas in heavy infections inclusions were distributed throughout the hepatopancreas (Couch, 1974b). Reserve cells (R-cells) appeared the principle site of infection but in heavy infections the virus was also present within epithelial cells of the midgut. Occlusion bodies appear tetrahedral (pyramidal) in fresh tissue squashes and range in size from 0.5µm to 20µm. Growth of the inclusion body within infected cells results in rupture of the host cell and liberation of occlusion bodies to the tubule lumen (and faeces).

Electron microscopy revealed rod-shaped non-occluded and occluded virions within these hypertrophied nuclei. Non-occluded virions were rod-shaped and consisted of an electron dense nucleocapsid surrounded by a trilaminar envelope. Some displayed a protruding structure at one end of the virion, the functional significance of which is unknown (Couch, 1974b). Virions measure 269.6 ± 20.7nm by 50.3 ± 4.8nm. Occluded virions are of similar dimensions and structure to non-occluded virions within the same infected nuclei. Infected nuclei are 1.5-2 times larger than uninfected nuclei. Heavily infected nuclei show a distinct loss of heterochromatin, nucleolar degeneration and a proliferation of the nuclear membrane forming a multi-membranous labyrinth (Couch, 1974a). These changes were similar to those observed in insect cells infected with *Baculovirus* so the name *Baculovirus penaei* was proposed by Couch (1974b). Prior to this there had been no reports of viruses that resembled *Baculoviruses* in any animals other than insects or mites. Couch stated that the discovery of this virus indicated that marine crustaceans are potential hosts for viruses similar to certain viruses infecting insects and mites.

Within shrimp farming industry BP has caused serious epizootics in the larval, postlarval and juvenile stages of *Penaeus duorarum, P. aztecus, P. vannamei, P marginatus, and P. stylirostris* and has also been observed infecting *P.setiferus and P.californiensis* (Brock *et al*., 1986; Lightner, 1988; Lightner *et al*., 1989). Transmission of BP is horizontal via cannibalism, ingestion of faeces, occlusion bodies or contaminated detritus or water. Prevalence may be highly variable (<1% in wild populations and up to 100% in cultured populations). Besides a high mortality rate, gross signs of infection in shrimp farms include reduced feeding and growth rates and increased surface and gill fouling. Severe crowding stress has been shown to enhance establishment of viral infections. Inactivation of BP is possible using disinfectants, low pH, heat and UV irradiation (LeBlanc and Overstreet, 1991). The disease can be controlled

by quarantine of imported stocks or potential carriers, destruction of known contaminated stocks and disinfections of contaminated facilities. The pathogen (Tetrahedral baculovirosis, BP) was listed by the OIE until delisting in 2009.

BP was accepted as a member of the *Baculoviridae* family on the basis of morphological features (Couch, 1974b) and the demonstration that its genome was a large circular dsDNA molecule of 75 x 10^6 Da (Summers, 1977). Later, Bonami *et al.* (1995a) highlighted that the specific type of occlusion body was not an accurate discriminator for relatedness of specific viral taxa. Rather, that analysis and characterisation of genomic elements were mandatory for their identification. The discovery of multiple susceptible shrimp species coupled with the wide geographic spread of the BP virus led Bonami *et al* (1995) to suggest that these viruses would eventually be found to be different from one another. There have been three geographically distinct 'strains' of BP detected: 1) South-east Atlantic and Gulf of Mexico coasts of USA and Caribbean (PdSNPV) 2) Pacific coast of South, Central and North America (*Penaeus vannamei* single nucleopolyhedrovirus (PvSNPV)) (Bonami *et al*, 1995) and 3) Hawaii (*Penaeus marginatus* single nucleopolyhedrovirus (PmSNPV)) (Brock *et al*., 1986). PCR techniques and application of gene probes for use as diagnostic tools such as *in situ* hybridisation have been developed to enable researchers to distinguish between the strains (Bruce *et al*., 1993; Durand *et al*., 1998; Wang *et al*., 1996) thus confirming the prediction of Bonami *et al*. (1995).

2.4.2 *Penaeus plebejus* baculovirus (PBV)

PBV was the first baculovirus to be described in Australian penaeid shrimps by Lester *et al*. (1987). The virus was discovered in post-larvae and juveniles from a hatchery in New South Wales, Australia, hepatopancreas appearing cloudy, similar to the signs of BMN infections. Histology reveals subspherical intranuclear inclusion bodies in the epithelial cells of the hepatopancreas. Infected nuclei were hypertrophied and contained single or multiple inclusion bodies which were strongly eosinophillic; few cells contained more than three inclusions. Multiple inclusion bodies were more commonly seen in post-larvae samples.

Ultrastructurally the cytoplasm of infected cells contained membranous labyrinths, little intact endoplasmic reticulum and mitochondria. Within the nuclei the inclusion bodies showed a crystalline lattice of 20nm periodicity, in some cells this lattice was orientated in different planes within the same inclusion body. Virions were rod-shaped, measure up to 440nm in length and were present both free and occluded in the inclusion body. Virions line the inner surface of the nuclear membrane, nucleocapsids measure 30 - 45nm in diameter and consist

of an electron dense core surrounded by an electron lucent margin. Virions possess a trilaminar envelope; envelope of some non-occluded virions was detached from the nucleocapsid for part of its length. 80% of infected post-larvae died but it is not clear whether this is entirely due to the presence of the virus as the hatchery had water quality, nutritional and density problems when the virus was discovered. It is considered to be distinct from PmNV due to the majority of cells containing single inclusion bodies, staining strongly and uniformly with eosin, and staining gram negative. PmNV infections usually contain multiple inclusion bodies within the nuclei, stain weakly with eosin at the periphery and appear unstained in the centre (Lightner and Redman, 1981). Ultrastructurally PBV virions are slightly larger and the periodicity of the inclusion body crystalline lattice is smaller than PmNV. These differences led Lester *et al*. (1987) to suggest that PBV may be different to PmNV.

2.5 Intranuclear bacilliform viruses (IBV's)

Due to a lack of biochemical, immunological and molecular data non-occluded baculoviruses are unassigned according to the ICTV (King *et al*., 2012). Viruses previously designated as a non-occluded baculovirus have been referred to as IBV's (Evans and Edgerton, 2002). For the purpose of this review rod-shaped viruses lacking an occlusion body are considered as intranuclear bacilliform viruses

2.5.1 Baculoviral mid-gut gland necrosis virus (BMN)

Baculoviral mid-gut gland necrosis virus was discovered in the hepatopancreatic cells of cultured *P. japonicus*, causing mortalities in larvae and post larvae (Sano *et al*., 1981). Mid-gut gland of infected shrimp appeared white and opaque (Sano *et al*., 1984) and infected post larvae were smaller with slower growth rates than uninfected post larvae. Microscopy results showed there were no occlusion bodies' present, cellular necrosis and collapse of the mid-gut gland was evident. Nuclei in this tissue were hypertrophied, up to 2-3 times the size of normal nuclei. Electron microscopy revealed rod-shaped particles within these nuclei, virions contained a rod-shaped electron dense nucleocapsid and were enveloped with an inner and outer membrane, and envelopes occasionally appeared convex or concave. Virions measure 310nm in length and 72nm in width, nucleocapsids measure on average 250nm in length and 36nm in width. Developing particles were found alongside the complete virions, a fibrillar structure possibly representing virogenic stroma was also seen in these infected nuclei. Host range of this virus has not been studied (Johnson, 1984), the virus has only been reported from shrimp-rearing facilities in Japan. Epizootics have been reported to occur almost yearly between May and September resulting in severe mortalities; this has caused difficulties for the

rearing facilities which occasionally struggled to supply farms with post larval shrimp (Sano *et al*., 1981),

2.5.2 Baculo-PP

Baculo-PP is a non-occluded virus infecting the hepatopancreatic epithelial cells of the blue king crab *Paralithodes platypus* and was described by Johnson and Lightner (1988). It is described as similar to Baculo-A due to the focal nature of the infection and the fact that it may not be pathogenic. Baculo-PP was found in at least two populations of *P. platypus,* 40% at Olga Bay, Kodiak Island in April 1982 and 20% at the Probilof Islands in June 1982. Infected nuclei were often hypertrophied, uniformly Feulgen positive with an intensely Feulgen positive margin of variable width. Marginal material often extended inwards as a scanty reticulum or as a larger inclusion.

TEM observations were based on examination of infected hepatopancreas sections from a single crab. The samples had been fixed in Helly's solution and stored in 70% ethyl alcohol before being processed for EM. Internal cell membranes were missing and definition of other structures was impaired. Virions were in groups or scattered at random and various nuclear inclusions were observed. Viral particles which were fully enveloped were associated with many of the smaller inclusions. Only enveloped particles were seen, nucleocapsids were cylindrical with squared ends and measured 190 - 210nm in length and 37 – 40nm in diameter. Virions measured 230 – 265nm in length and 70nm in diameter at their narrowest point. Envelopes were trilaminar and measured approximately 10nm thick. A dense substance was present in the space between the envelope and the nucleocapsid. Some envelopes expanded unilaterally or subapically around the apex. This expansion was flangelike along part of its length. Many virions narrow into a cone at one end, this cone sometimes narrowed to form a threadlike tail more than 200nm long and 14nm in diameter with a very narrow internal filament extending from the core down the centre of the tail. This is sometimes viewed as a dot in cross-section.

2.5.3 *Crangon crangon* bacilliform virus (CcBV)

A non-occluded intranuclear bacilliform virus was described infecting the hepatopancreatic tubule epithelia and mid-gut epithelia in the brown shrimp *Crangon crangon* by Stentiford *et al*. (2004a). It is the first report of an intranuclear bacilliform virus (IBV) in this species and was named *Crangon crangon* Bacilliform Virus (CcBV). *C. crangon* were sampled from the Clyde estuary, western Scotland for histology and electron microscopy. The virus was also

found at high prevalence in *C. crangon* stocks sampled from various estuaries around the UK (Stentiford and Feist, 2005) and in the Wash area of the North Sea (Bateman, pers. Obs.). Histology revealed a characteristic pathology in 100% of sampled shrimp. This high prevalence was seen in both spring and autumn sampling suggesting a lack of seasonality in infection prevalence. Pathology consisted of a degeneration of the hepatopancreatic tubules causing a loss of structure. Higher magnification revealed significant changes in the differentiated epithelial cells lining the tubules and the midgut. Nuclei within these cells were hypertrophied with marginalized chromatin and contained either enlarged nucleoli or an amorphous eosinophillic matrix. Strands of marginated chromatin sometimes led to the appearance of intranuclear compartmentalisation. Infected cells appeared singly or in clusters and appeared to be confined to the reserve (R) cells and fibrillar (F) cells; infection was not present in the blister (B) cells or the regenerating epithelial (E) cells. Outer membranes of infected epithelial cells were often separated from the neighbouring cells; these cells appeared to contain apoptotic nuclei and appeared to be in the process of being expelled into the lumen of the tubule. Sloughed epithelial cells could be seen within the lumen of degenerated tubules and the midgut.

TEM revealed the presence of rod-shaped and cylindrical envelope bound particles within these aberrant nuclei. Virions did not form arrays and appeared to be embedded in an amorphous matrix which corresponds to a granular viroplasm. Trilaminar envelopes surrounded the particles and were expanded at one end to form a lateral protuberance. In some cases this protuberance appeared to contain a fine tail like structure that emerged from the proximal end of the nucleocapsid. The opposite end of the virion appeared cylindrical with a closely opposed envelope. Mean length of virions was 280.2nm in length and 71.8nm in width, nucleocapsids measured 215.2nm in length and 39.9nm in width. Development appeared to take place within the granular viroplasm; detached trilaminar membranes were observed and often associated with discrete regions of dense viroplasm that appear to be generating the nucleocapsid. Putative unbound nucleocapsids were observed in association with dense regions. In heavily infected nuclei the outer envelope appeared dilated and in numerous cases was expanded into a membranous labyrinth similar to that seen in BP and MBV infections. Cells containing this feature were often rich in mitochondria and containe numerous lysosomal aggregations.

Pathogenesis trials were not carried out but it was suggested that loss of structure to the hepatopancreas and midgut would lead to dysfunction of the hepatopancreas and ultimately death of the host. Transmission may be through cannibalism, but the pathological

manifestation of CcBV would also lead to liberation of infected stages in the faeces, coprophagy and benthic scavenging by the shrimp may also lead to transmission.

2.5.4 *Pandalus montagui* bacilliform virus (*Pm*BV)

A virus similar to CcBV has been identified within the pink shrimp *Pandalus montagui* sampled from the same site as Crangon, the Wash fishery, North Sea, UK (Bateman *et al*., 2007). Ultrastructural and pathological observations suggest this virus possesses affinities to other intranuclear bacilliform viruses of crustacea. The prevalence and severity of infection of PmBV in pink shrimp appears to be less than that seen for CcBV infections of brown shrimp from the same site. Epithelial cells lining the hepatopancreatic tubules contained hypertrophic nuclei containing enlarged nucleoli and marginalised chromatin, in some cases leading to apparent compartmentalisation of the karyoplasm. Cells containing these nuclei were spread throughout the tubules, the outer membrane of the affected cells often separated from the neighbouring cells. Severely altered cells appeared to be expelled into the lumen of the hepatopancreatic tubule and sloughed cells containing enlarged nuclei could be seen within the tubule lumens. Ultrastructural observations of the infected nuclei revealed granular viroplasm containing rod-shaped virions with a distinctive kink in the middle of the nucleocapsid. These kinked nucleocapsids were bound by a simple trilaminar envelope. PmBV virions did not form arrays and were not encapsulated within occlusion bodies. This kinked appearance seems to be specific to *P. montagui*, in comparison CcBV virions are straight, rod-shaped with a distinct protrusion at one end. As with CcBV transmission may be through cannibalism, coprophagy and benthic scavenging.

Anecdotal evidence suggests cyclical population shifts between *C. crangon* dominance and *P. montagui* dominance in the Wash fishery. Both shrimp species support important fisheries in Europe and further work is needed to investigate whether these viral infections are the same or similar and have the potential to regulate population structures within these fisheries.

2.5.5 *Cancer pagurus* bacilliform virus (*Cp*BV)

Johnson (1984) highlighted a lack of reports of viruses from lobsters and crabs from the *Cancer* genus and pointed out that viruses were unknown in the blue crab *Callinectes sapidus* until juvenile crabs were studied. Bateman and Stentiford (2008) proved this point when investigating the diseases of a population of juvenile edible crabs. Edible crabs (*Cancer pagurus*) were sampled as part of a research contract studying population level effects of disease. A non occluded bacilliform virus was identified infecting the hepatopancreatic

epithelial cells of juvenile *C. pagurus*. The virus was shown to be morphologically similar to other intranuclear bacilliform viruses described from crabs, freshwater crayfish and penaeid shrimp and was named *Cancer pagurus* bacilliform virus (CpBV). Virus infection was found in 5% of crabs sampled; infected crabs displayed carapace width of between 20 and 70mm. Infected crabs showed no obvious external signs of disease. Histology revealed hypertrophied nuclei containing an eosinophillic granular matrix within the epithelial cells of the hepatopancreas. Infected nuclei possess marginated chromatin, occasionally strands of chromatin leading to the appearance of intranuclear compartmentalisation. Affected cells appeared singly or in clusters of fibrillar (F) and reserve (R) cells, the infection was not found to be present in blister (B) or regenerating epithelial cells.

TEM revealed rod-shaped virions within the nuclei, virions possess an elongated electron dense nucleocapsid with a relatively electron lucent end. Nucleocapsids are surrounded by a trilaminar envelope which expands at one end to form a lateral protuberance, inside which appears a fine tail like structure emerging from the end of the nucleocapsid. Development of virions appears to take place within the granular matrix. Complete virions measure 210.9nm in length and 60.3nm in width, nucleocapsids measure 181.6nm in length by 43.4 nm in width and the envelope measures 6nm in diameter. CpBV infection was not observed during a screening programme of edible crabs sampled from the fishery in the same area (Bateman *et al*., 2011). Bateman and Stentiford (2008) is the first report of a virus infection from wild crabs of the *Cancer* genus and highlights a need for consideration of age and size when carrying out disease surveys in wild populations. The authors state that further work was needed to assess the pathogenicity of the virus and prevalence in juveniles across the geographic range of *C. pagurus*.

2.5.6 *Carcinus maenas* bacilliform virus (CmBV)

A pathology similar to that associated with bacilliform virus infections was observed in the hepatopancreatic epithelium of shore crabs *Carcinus maenas* sampled from the Clyde and Tyne estuaries in the UK (Stentiford and Feist, 2005). The infection did not appear pathogenic in these crabs and samples for further analysis were not collected so it was not possible to confirm viral presence. However the pathology presented was very similar to that described with *Cc*BV infections. Further material was collected to try to study this infection but infection rates were low prevalence and infected epithelial cells were focal in their distribution making further study with electron microscopy and molecular techniques difficult.

2.5.7 *Scylla* Baculovirus (SBV)

A bacilliform virus was first described infecting the hepatopancreas cells of the mud crab *Scylla serrata* by Anderson and Prior (1992) and was named *Scylla* Baculovirus (SBV). Infections were observed in juvenile, sub adult and adult specimens but did not cause clinical disease at any stage and was an incidental finding in crabs caught in Darwin, Australia. Three of eleven crabs examined showed bacilliform nuclear pathology within the hepatopancreas tubules with a focal distribution of infected nuclei. Up to 36 infected cells were shown to have nuclear pathology in a transverse section of one tubule whilst surrounding tubules appeared clear from infection (Anderson and Prior, 1992). Infection was usually seen in the R cells with nuclei appearing irregular to round in shape, hypertrophied and up to five times normal size. Basophillic inclusions entirely occupied the nucleus and tended to be amorphous though a finely granular structure could be seen occasionally. Virus-related nuclear pathology is only seen within the hepatopancreas.

Electron microscopy revealed a loose granular virogenic stroma filling most of the nuclei. Chromatin that remained was marginal and nucleolar remnants if present were scattered. Virions appeared aligned in regular arrays particularly at the inner surface of the nuclear membrane. Nucleocapsids were cylindrical with slightly rounded ends, trilaminar envelopes were closely attached to the nucleocapsids, and occasionally a unilateral or apical envelope expansion was present. A central opaque dot could be resolved within several of these envelope extensions. A thin filament like structure arising from the apex of the nucleocapsid was apparent in some of these envelope expansions. Virions were present either budding or having budded through the nuclear membrane. A relatively wide translucent layer separated the inner envelope from the outer trilaminar envelope derived from the nuclear membrane. Nucleocapsids measured 24nm in diameter and 205nm in length, enveloped virions measured 44nm in diameter by 253nm in length with an envelope thickness of 10nm. After budding from the nucleus the double-enveloped virions measured 75nm in diameter.

During attempts to increase hatchery production Owens *et al*. (2010) witnessed basophilic to magenta hepatopancreatic intranuclear inclusions in both locally caught broodstock and then in progeny from these broodstock. 8.9% prevalence was reported in wild populations of mud crab within Ross River, northern Queensland, Australia. Fifteen batches of larvae were examined, two of these batches were observed to be infected, 40.7% of larvae showing hypertrophied nuclei (Owens *et al*., 2010). Fourteen adult mud crabs and larvae that were positive for bacilliform virus were also positive for PmergDNV by real-time PCR (Owens *et al*., 2010).

2.5.8 *Cherax quadricarinatus* bacilliform virus (CqBV)

Cherax quadricarinatus bacilliform virus (CqBV) was the first virus described in a freshwater crayfish (Anderson and Prior, 1992). It was originally named *Cherax* baculovirus (CBV) by Anderson and Prior but due to the fact that there were no occlusion bodies formed it is unlikely to be a baculovirus and so was renamed a bacilliform virus, CqBV (Edgerton, 1996). The infection was found in crayfish submitted from farms in Australia for diagnostic examination, sampled direct from rivers as an incidental find from a group of crayfish from experimental bacterial infections. 52% of juvenile and adult crayfish examined displayed bacilliform virus related pathology. Prevalence and intensity of the observed infections did not appear to be related to the origin of the crayfish or their disease status. Infection occured only in the hepatopancreas, hepatopancreatic epithelial cells were irregularly shaped, hypertrophied up to 2.5 times normal size. Nuclei contained amorphous, eosinophillic inclusion material, a thin basophilic band of chromatin rimmed the inner surface of the nuclear membrane, and some inclusions appeared more purple-red in colour. Only differentiating epithelial cells were infected, mainly R-cells.

Electron microscopy revealed virions to be scattered throughout the granular virogenic stroma; empty capsids and fully formed nucleocapsids were always enveloped. Nucleocapsids were cylindrical with square ends and measured 34 nm in diameter and 154nm in length, envelopes were loosely attached and measured 5.7nm. Fully formed virions measured 199nm in length and 67 nm in diameter on average. Poor fixation hindered the full ultrastructural classification in samples analysed by Anderson and Prior (1992). Edgerton (1996) identified that CqBV virus in glutaraldehyde fixed material was significantly larger than that described by Anderson and Prior (1992) fixed with formalin. Groff *et al*. (1993) and Edgerton (1996) agreed that virions measured approximately 260nm in length and 100nm in diameter. Edgerton (1996) suggested there may be several strains of CqBV corresponding to the multiple strains of *C. quadricarinatus* but could not rule out fixation artefact as the cause of these differences in measurement.

Originally described infecting wild and farmed *C. quadricarinatus* in Australia the virus has been introduced into the USA, Ecuador and Chile (Bateman *et al*., 2005; Edgerton, 1996; Edgerton and Owens, 1999; Groff *et al*., 1993; Hauck *et al*., 2001; Romero and Jiménez, 2002). Groff *et al.* (1993) postulated that the infection was spread to the USA via broodstock imported from Australia; Hauck *et al.* (2001) could not determine how the virus spread to Utah, USA. Although associated with mortalities CqBV is considered to have low virulence, however the virus has been associated with stunted growth (Groff *et al*., 1993; Edgerton *et al*., 2002).

Edgerton & Owens (1997) conducted a study to determine age at first infection of CqBV, the first juvenile infected was diagnosed 2 weeks after the juvenile moulted into stage 3 and the prevalence rose steadily to 17.1% at completion of the experiment (week 7). CqBV was detected shortly after the juveniles began to feed suggesting that the virus is transmitted *per os*. Non-diagnosis of CqBV in juveniles at week 0 suggested that transovarial infection did not occur, however the authors acknowledged that more sensitive diagnostic techniques were needed to confirm this hypothesis (Edgerton & Owens, 1997).

2.5.9 *Cherax destructor* bacilliform virus (*Cd*BV)

Cherax destructor bacilliform virus (CdBV) was described by Edgerton (1996) from *C. destructor* samples obtained from two aquaculture farms in South Australia. Infected epithelial cells were observed within the hepatopancreas; nuclei were markedly hypertrophic, with marginalised chromatin and contained an amorphous eosinophilic inclusion body. Occasionally nuclei appeared compartmentalised by chromatic strands. Basophilic nucleolar remnants usually occupied the centre of the hypertrophied nuclei and cytoplasm often consisted of a basophilic granular material, intensities of infection are low.

TEM showed rod-shaped virions within the hypertrophied nuclei, nuclei displaying rarefied chromatin and contained a granular viroplasm. Virions were scattered throughout the viroplasm, accumulating near the nuclear membrane in a random manner. Virions consisted of an electron dense core and slightly more lucent capsids surrounded by a trilaminar envelope. The envelope appeared to be closely applied over one half of the virion and loosely applied over the other. Longitudinally sectioned virions showed the nucleocapsid is bent in this region and the envelope and occasionally the nucleocapsid appeared kinked. The envelope expansion was more pronounced on one side and a tail like structure was often visible within this expansion. Virions at various stages of development were observed within the viroplasm. Enveloped particles measured 304.4nm in length and 67.7nm in diameter. Nucleocapsids measured 262.5nm in length and 49.3nm in diameter, nucleocapsid tail measured between 8.1 and 9.1nm in length. Several long enveloped nucleocapsids were also observed (Edgerton, 1996; Edgerton *et al*., 2002a).

2.5.10 *Astacus astacus* bacilliform virus (*Aa*BV)

A bacilliform virus was described during a survey of the noble crayfish *Astacus astacus* in Finland (Edgerton *et al*., 1996) forming the first description of a virus in European freshwater crayfish. Infected nuclei showed marginalized chromatin and contained an amorphous

eosinophillic inclusion. Some of the inclusions were darker toward the centre, haloed or compartmentalised by strands of chromatin. Infected nuclei were rarely hypertrophied but were irregularly shaped. Infections occurred in all cell types within the hepatopancreas including the E cells, midgut, midgut caecum and tegmental glands of both hindgut and midgut. Infections in the midgut and midgut caecum ware often intense. Infected cells were routinely observed sloughing from the epithelia, hepatopancreatic tubules were necrotic and encapsulated by haemocytes.

TEM revealed infected nuclei contained marginated chromatin, clumped chromatin and an extensive amorphous granular viroplasm contained rod-shaped virions. Virions were arranged in rows and were often associated with nucleolar remnants. Virions consisted of a rod-shaped nucleocapsid enclosed within a closely associated trilaminar envelope and a unilateral subapical expansion at one end. A reflexed tail could be seen within this expansion. The nucleocapsid was blunt at both ends; electron lucent capsid could be distinguished from the core. Apices of some capsids were thickened and presumed to be caps, no nucleocapsids appeared bent. Completed virions measured 343nm in length by 70.9nm in width, nucleocapsids measured 261nm in length and 51.4nm in width, the tail possessed a diameter of 10.3nm and the envelope measured 7.9nm wide.

A. astacus bacilliform virus is described as sharing many characteristics with gut infecting bacilliform viruses described in other crustaceans including the baculoviruses PdSNPV and PmSNPV. Tail like extensions of the nucleocapsid have been noted from penaeid baculoviruses but this is the first description of this feature within freshwater crayfish bacilliform virus. The author highlighted the need for further study to determine the prevalence of this virus within Europe and its possible role in crayfish mortalities.

2.5.11 *Austropotomobius pallipes* bacilliform virus (ApBV)

An intranuclear bacilliform virus was discovered in the white claw crayfish *Austropotomobius pallipes* by Edgerton *et al*. (2002b) associated with a mortality event in France. *A. pallipes* is listed as vulnerable by the International Union for Conservation of Nature and Natural Resources (IUCN), reduction of *A. pallipes* numbers in Europe has been attributed to habitat destruction, pollution, competitive exclusion by introduced species and epizootic disease (Edgerton *et al*., 2002b). The original description reports the association of this virus with the near extirpation of *A. pallipes* from the Nant watershed, France in 2000 and 2001 (Edgerton *et al*., 2002b). However samples taken from one moribund crayfish did not show signs of infection, suggesting that this virus was not the major cause of mortality. After conducting a

histopathological survey of *A. pallipes* from various populations Edgerton (2003) showed that *Ap*BV is in fact a common infection (100% in two populations) in *A. pallipes* populations in south-eastern France. Longshaw *et al*. (2012) have also recently identified this virus in *A. pallipes* sampled in the UK.

No clinical signs were observed in the mortalities other than a whitening of the tail muscle in one crayfish, which was later shown to be caused by a microsporidian infection. Histology revealed foci of hypertrophic nuclei in heptopancreatocytes, nuclei showed marginated chromatin and contained granular, eosinophillic inclusions. A high proportion of infected cells were seen within the midgut (Edgerton *et al*., 2002b), this was confirmed after the histopathological screening of *A. pallipes* populations, which showed that in most cases the intensity of infection in the midgut and midgut caecum was considerable higher than the hepatopancreas (Edgerton, 2003). Electron microscopy showed bacilliform virions amongst the granular viroplasm. Virions consisted of cylindrical nucleocapsids with electron dense nucleoprotein cores and less electron dense capsids. Each nucleocapsid was enveloped singly by a trilaminar envelope; this envelope was closely associated with the nucleocapsid at one end and showed a lateral expansion at the other end. Cross sections revealed the expansion was unilateral, occasionally an electron dense dot was observed in this expansion. Virions measured 62.9nm in width and 257.8nm in length, nucleocapsids measured 52nm in width and 224.8nm in length. There has been extensive relocations of crayfish throughout Europe, native and non-native species, however the *A. pallipes* sampled originally were from a region free from both *A. astacus* and *P. leniusculus* so it is thought unlikely they could have acquired the infection from another species. The spread of the mortality upstream over two successive summers suggests an infectious cause for the mortalities, studies on water quality and pollution levels suggest the water suitable for habitation.

2.5.12 *Pacifastacus leniusculus* Bacilliform Virus (PlBV)

An intranuclear bacilliform virus was reported in the signal crayfish (Pacifastacus leniusculus in the USA by Hedrick *et al*. (1995). The virus was originally reported as a personal communication in Hauck et al. (2001) without details of the virus or of the host. The virus infects the epithelial cells of the hepatopancreatic tubules; nuclei in infected cells were hypertrophied with marginalised chromatin and may be sloughed into the tubule lumens. Virions were rod-shaped, enveloped, contained an electron dense nucleocapsid which measured 189 nm in length and 44nm in diameter. Enveloped virions measured 240nm in length and 66nm in diameter. Little is known about the transmission or potential host range.

This virus has recently been discovered in *P. leniusculus* samples from the UK during an investigation into a mortality event. It is not known whether the virus is the cause or a contributory factor to the mortality event. TEM reveals rod-shaped particles that form arrays at the edge of the nuclei. Unlike other intranuclear bacilliform viruses no protuberance is present at one end and the particles appear slightly smaller than those previously described, however this could be due to differences in fixation. Virions appear to be associated with membrane and vesicle-like structures within the nuclei, similar to those described in Baculo-B infections (Bateman pers.obs.).

2.6 *Parvoviridae*

2.6.1 *Penaeus stylirostris* brevidensovirus (PstDNV)

Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV) was first described by Lightner *et al*. (1983a) from a disease outbreak in cultured *Penaeus stylirostris* in the Americas. It has since been renamed as *Penaeus stylirostris brevidensovirus* (PstDNV), *Brevidensovirus* genus in the family *Parvoviridae,* although this listing has not been completely accepted by the ICTV (Shike *et al*., 2000; Tattersall *et al*., 2005; Tijssen *et al*., 2012). It is the first example of a densovirus with a host outside the class insecta (Shike *et al*., 2000). The disease, originally named IHHNV to describe the principal lesions observed, is still referred to as IHHNV by many researchers as this is how it is commonly known. This viral infection is an OIE notifiable disease. Lightner *et al.* (1983a) described that *P. stylirostris* populations from an experimental facility in Hawaii were severely affected by a highly acute and lethal disease of viral aetiology; this was confirmed by transmission of the disease to previously unexposed shrimp by intramuscular injection (Bell and Lightner, 1987; Lightner *et al*., 1983a). Typical signs of PstDNV in *P. monodon* and *P. vannamei* were shown to develop after indirect exposure via contaminated water or direct feeding of infected *P. stylirostris* carcasses. Although multiple penaeid species appear to be susceptible to the virus and able to carry the virus, the severity of infection and mortality rate differs between species with *P. stylirosris* appearing most susceptible (Bell and Lightner, 1984; Lightner *et al*., 1983a). The *P. stylirostris* samples originally examined from Hawaii had all been imported demonstrating vertical transmission of the virus from broodstock to offspring (Lightner *et al*., 1983a; Bell & Lightner, 1987). The original study was based upon samples of larvae, post larvae and adult broodstock from various commercial and government run penaeid shrimp culture facilities across the Americas. The virus was also found to cause stunted growth and deformities called runt deformity syndrome (RDS) in *P. vannamei* and *P. monodon* (Bell and Lightner, 1984;

Kalagayan *et al*., 1991; Primavera and Quinitio, 2000). This condition has caused substantial economic losses in shrimp farming (Wyban *et al*., 1992).

PstDNV like WSSV principally attacks tissues of mesodermal and ectodermal origin, but it also infects endodermally derived tissues. Shrimp in the acute phase show necrosis and inflammation of cuticular epidermis of stomach, hindgut and gills, connective tissues, striated muscle, nerve cord, haemocytes and haematopoietic tissues. Necrotic tissues contained hypertrophied nuclei with marginated chromatin and contained an eosinophillic Cowdry Type A inclusion (Cowdry, 1934). Shrimp in the recovery phase showed melanised areas in the gill and cuticular epidermis, Cowdry Type A inclusions were present but were not common. Fixed phagocytes and nephrocytes of the gill and other areas contain large cytoplasmic inclusions in recovering animals (Lightner *et al*., 1983a).

Electron microscopy of gills, stomach mucosa, cuticular epidermis and antennal gland revealed three types of virus particles in the nucleus and cytoplasm of infected cells. Type 1 averaged 27nm in diameter, occurred in small aggregates in the cytoplasm and were the most commonly seen. Type 2 was rarely seen and was present within membrane-bound inclusions forming prominent crystalline arrays of virus like particles measuring 17nm in diameter. Type 3 particles measured 20nm in diameter and were observed in the hypertrophied nuclei of cells that contained masses of apparent virogenic stroma. These nuclei corresponded to the nuclei which contained Cowdry Type A inclusions via histology (Lightner *et al*., 1983a). Bonami *et al*. (1990) purified the virus from shrimp samples to enable full characterisation of the viral properties. Negatively stained virus suspensions showed full and empty capsids, particles measuring on average 22nm, 20nm from side to side and 22nm from point to point. The inner component of empty particles shows a diameter of 13nm. Five and six sided particles were observed indicating an icosahedral shape of the virions. PstDNV was described as an icosahedral, non-enveloped virus containing single-stranded DNA of 4.1 kb (Bonami *et al*., 1990).

Shike *et al*. (2000) purified and sequenced PstDNV; they found the virus had a buoyant density of 1.45 determined via a caesium chloride gradient. Analysis of 3873 nucletides of the viral genome revealed that the genomic organisation was similar to that of mosquito brevidensoviruses and they concluded that PstDNV is closely related to densoviruses of the genus *Brevidensovirus* in the family *Parvoviridae*. This was confirmed by Roekring *et al*. (2002) whose results showed that PstDNV grouped closely with the mosquito

brevidensoviruses using phylogenetic tree based on genomic DNA of shrimp and insect parvoviruses.

Three distinct genotypes of PstDNV have been identified (Tang *et al*., 2003; Tang and Lightner, 2002), Type 1 from the Americas and East Asia, Type 2 from South-East Asia and Type 3 from East Africa, India, Australia and western Indo-Pacific region. Type 1 and 2 are infectious to penaeids, Type 3 are not infectious to *P. vannamei* and *P. monodon* (Tang *et al*., 2003; Tang *et al*., 2007b; Tang and Lightner, 2002; Tang and Lightner, 2006). Non-infectious inserts of the partial PstDNV genome into the shrimp genome were discovered in *P.monodon* sampled in East Africa and Australia (Krabsetsve *et al*., 2004; Tang and Lightner, 2006) since these sequences could cause false positive results a modified PCR method was developed to detect the infectious type (Tang *et al*., 2007a).

2.6.2 Hepatopancreatic Parvovirus (HPV)

Hepatopancreatic Parvovirus (HPV) was first reported infecting *Penaeus merguiensis* and *P. indicus* from Singapore (Chong and Loh, 1984) and in postlarvae of *P. chinensis* in China (Lightner and Redman, 1985). Similar infections were noted in juveniles of *P. semisulcatus* in Kuwait and *P. monodon* from the Phillipines (Lightner and Redman, 1985). Although originally described infecting these species the natural host range of this virus has spread and appears to include wild and cultured shrimp from across the globe, geographically it can be found in the Indo-Pacific region, Africa, Middle East, Americas (Lightner, 1993; Lightner and Redman, 1992) and Madagascar (Tang *et al*., 2008). HPV has also been isolated from the freshwater prawn *Macrobrachium rosenbergii* (Anderson *et al*., 1990; Gangnonngiw *et al*., 2009). Lightner and Redman (1985) named the virus Hepatopancreatic Parvo-like Virus (HPV) due to the target organ and because the cytopathology and morphology of virus particles were consistent with that of parvovirus infections from other arthropods and vertebrates. Although similar in structure to PstDNV, HPV infected different target tissues.

Gross signs of disease include poor growth rates, anorexia, reduced preening activity, occasional opacity of the abdominal muscles and occasional presumed secondary bacterial and fungal infections. Mortalities are usually seen during the juvenile stages after apparent normal development through the larval and post larval stages, accumulative mortality reaching 50-100% within 4 to 8 weeks of disease onset. As seen by Chong and Loh (1984) HPV lesions display prominent intranuclear inclusion bodies in the hepatopancreatic tubule epithelial cells. HPV particles appear spherical but occasionally angular profiles were present and particle size ranged from 22.2nm to 23.9nm depending upon the fixation method used. Some HPV

particles appear to be in close association with microtubules or microfibrils within the inclusion bodies (Lightner & Redman, 1985).

HPV isolates from various shrimp species and geographic regions seem to be genetically different; seven geographic isolates are known and these can be split into three genotypes (Tang *et al*., 2008) 1) Korea (HPVchin (Bonami *et al*., 1995b)), 2) Thailand (PmDNV (Sukhumsirichart *et al*., 2006)) and 3) Australia (PmergDNV (La Fauce *et al*., 2007b)). Sensitive methods of detection such as PCR and gene probes are strain specific so may lead to negative test results, consequently the known strains of HPV may only represent a small proportion of existing strains (La Fauce *et al*., 2007a). Roekring *et al*. (2002) showed that HPVchin and PmDNV grouped together when using a phylogenetic tree based on genomic DNA from shrimp and insect parvoviruses. They suggest that this clustering, HPVchin and PmDNV group together but separately from PstDNV and SMV shows that the shrimp parvoviruses studied are of diverse origin and that they are not closely related.

Tang *et al*. (2008) compared isolates from Australia, Thailand, Korea and Tanzania to a Madagascan isolate and found a mean distance between them of 17% showing that hepatopancreatic parvoviruses appeared to have greater genetic diversity than other shrimp viruses. PstDNV displayed 4% variation and Taura Syndrome Virus (TSV) displays less than 6% difference in nucleotide sequence amongst isolates (Tang et al., 2008). Tang *et al*. (2008) suggested the wide geographic spread of HPV may account for this variation.

HPV has been attributed to mortalities reaching up to 100% and has been statistically related to retarded growth in *P. monodon* (Flegel *et al*., 1999). Diagnosis of this disease can be difficult as external signs of disease are not specific for HPV and shrimp are frequently infected by other pathogens that may mask the effect of an HPV infection (Lightner *et al*., 1993), the most common way to identify infections is via histology. Over the years multiple diagnostic techniques have been developed: *in situ* hybridisation (Pantoja and Lightner, 2001), monoclonal antibodies (Rukpratanporn *et al*., 2005), gene probes (Lightner *et al*., 1994; Mari *et al*., 1995) PCR (Pantoja and Lightner, 2000; Sukhumsirichart *et al*., 1999) nested PCR (Manjanaik *et al*., 2005) PCR-ELISA (Sukhumsirichart *et al*., 2002) and TaqMan real-time PCR (La Fauce *et al*., 2007b; Yan *et al*., 2010).

2.6.3 HPV from *P. chinensis* (*Pchin* DNV*)*

Using HPV positive samples of *P. chinensis* from Korea Bonami *et al*. (1995b) were the first to characterise the virus. They showed the virions to be rounded, probably icosahedral as some were obviously six-sided, with a mean diameter of 22nm, empty particles showed an

electron dense core approximately 18nm in diameter. Buoyant density of the virus was estimated to be 1.412 - 1.425 g/ml. SDS-PAGE revealed a single polypeptide, 54 kDa in size, which was considered to be unusual in the *Parvoviridae* as members have previously been reported as containing three (*Parvovirus* and *Dependovirus*) to four (*Densovirus*) polypeptides ranging from 60 – 90 kDa (Francki *et al*., 1991). DNA obtained by gel electrophoresis was characterised by Southern transfer. The band appeared to contain single stranded DNA therefore by convention in *Parvoviridae* it was hypothesised that this ssDNA represented the 'minus' strand corresponding to the major nucleic acid strand encapsulated by the virus. Genomic DNA size and strandedness suggested that HPV appeared to be more closely related to the genus *Parvovirus* than to the genus *Densovirus*.

HPV appears to be related to PstDNV but differs in its protein content, location in the tissues and histological symptoms produced. Although considered as a possible member of the Densovirus genus the early descriptions show that, with the exception of the protein constitution, HPV possesses more characteristics of an autonomous vertebrate parvovirus (Berns and Labow, 1987; Francki *et al*., 1991). The gene probe developed by Bonami *et al*. (1995) proved sensitive for the detection of HPV in *P. chinensis* but did not react with HPV from *M. rosenbergii* from Malaysia suggesting that there are different strains of HPV in different shrimp species (Lightner *et al*., 1994). HPVchin has been used throughout the literature to describe this virus. However as suggested by Sukhumsirichart *et al*. (2006) and using the ICTV taxonomic rules for nomenclature I suggest the name *P. chinensis* densovirus (PchinDNV) be used to describe this strain of the HPV virus. The classification of all hepatopancreatic parvoviruses still needs to be approved and accepted by the ICTV.

2.6.4 HPV from *P. monodon* (PmDNV)

Sukhumsirichart *et al*. (1999) characterised and used the name HPV-mon to describe the HPV infection in *P. monodon* from Thailand. Histology revealed typical basophilic HPV intranuclear inclusions, many infected cells containing two or more inclusions. TEM showed the inclusions comprised of large arrays of unenveloped viral particles typical of those described in other shrimp species. Negative staining showed virions to be circular to hexagonal in shape with a diameter of 22-24nm.

Sukhumsirichart *et al*. (2006) analysed the nucleotide sequence, genome organisation, structural proteins and phylogenetic tree generated from HPV-mon material. Phylogenetic tree analysis revealed that HPV-mon is more closely related to PstDNV, PchinDNV and the two mosquito viruses from the same family rather than other insect and vertebrate

parvoviruses. The total genome was 6.3kb, larger than that reported in *P. chinensis* (4kb) or other parvoviruses. This was suggested to be explained by differences in the genome organisation, non structural proteins were shown to be non-overlapping in HPV-mon. Despite the differences in genome size and organisation and based upon the phylogenetic tree analysis Sukhumsirichart *et al*. (2006) suggesting that HPV-mon is also a member of the *Densovirinae* subfamily of the *Parvoviridae*. Using the ICTV taxonomic rules for nomenclature they proposed replacing the name HPV-mon with *P. monodon* densovirus (PmDNV). This classification still needs to be approved and accepted by the ICTV.

2.6.5 *Penaeus merguiensis* densovirus (PmergDNV)

La Fauce *et al*. (2007a) proposed the name *P. merguiensis* densovirus (PmergDNV) after analysing sequence data from a HPV infection in *P. merguiensis* from Australia. The strain shows the highest nucleotide similarity with the Korean and Thai isolates (87%) and a lower similarity with the Indian isolate (83%). The authors pointed out that this similarity is indicative, only partial sequences of PchinDNV, PmDNV and PmergDNV were analysed. Phylogenetic analysis suggested that PmergDNV is more closely related to PchinDNV, again the authors highlighted this is probably because *P. merguiensis* is more closely related to *P. chinensis* than *P. monodon* and *P. semisulcatus*. They suggested that HPV strains may be following the phylogenetic relationships of the hosts rather than being geographically, nearest neighbour linked. The total genome was 6kb, larger than that reported from PchinDNV and similar in size to PmDNV.

La Fauce and Owens (2007) conducted challenge trials to determine the susceptibility of juvenile *C. quadricarinatus* to PmergDNV and the effect of stocking density on the expression of the disease. Overcrowding stress resulted in a higher diagnosis of disease, 90% of the fed crayfish in the overcrowded tanks were positive for PmergDNV compared to 47% which were kept at a lower stocking density. Histology did not show any intranuclear inclusion bodies, characteristic of PmergDNV infections, in the challenged crayfish but crayfish were shown to be suffering from a number of additional infections. In addition viral titres measured from the tissues after the challenge were lower than the original inoculum suggesting the virus was not replicating within the tissues. These results showed that juvenile *C. quadricarinatus* were not susceptible to PmergDNV but could be potential short-term carriers of the virus.

Owens *et al*. (2010) have recently discovered PmergDNV in the mud crab *S. serrata* in Australia via PCR and sequencing. Nucleotide sequence comparisons showed 99% homology with the genome of PmergDNV. However histology did not show any intranuclear

inclusion bodies, characteristic of PmergDNV infections. It is not clear whether the virus is replicating or just being sequestered.

2.6.6 PC84 Parvo-like virus of *Carcinus mediterraneus*

Mari and Bonami (1988) describe a small icosahedral unenveloped virus infection in the connective tissues of diseased Mediterranean shore crabs, *Carcinus mediterraneus*. Diseased animals came from a wild population in Prevost Lagoon, near Montpellier in France. Experimental transmissions carried out by injecting viral suspensions into apparently healthy crabs at 18-20˚C. Infected crabs displayed atypical symptoms, weakness, lack of appetite and passiveness. Crabs died between 10 and 25 days after inoculation, moribund crabs were sampled for histology and EM. Virus was found within all moribund crabs sampled 10 days after inoculation. Necrotic areas of the connective tissue of the hepatopancreas, midgut and gills were seen via histology. Defence reactions characterised by an accumulation of degenerative cells forming nodules were present in these areas, which stained Feulgen positive. No noticeable nuclear hypertrophy was present and the epithelial cells of these tissues appeared normal.

TEM showed that the chromatin within the nuclei of infected cells appeared to be replaced by an amorphous electron dense material. This material contained virus particles which were nearly spherical, nonenveloped and measured 23-27nm in diameter. The perinuclear space was enlarged and the two membranes of the nuclear envelope were held together by the pore structures, without noticeable breaking of the envelope. Particles identical in shape and size were also present in the cytoplasm of the infected cells, although no signs of development were present here. In infected cells virions were arranged in regular and even paracrystalline arrays in the intercellular spaces. Empty particles were present in both the nuclei and the cytoplasm and were as numerous as full particles. Moderately infected cells showed a diffuse and filamentous nucleoplasm containing few empty particles, lacked nucleoli and showed dispersed chromatin. At this stage few virions were present in the cytoplasm around the nucleus. Heavily infected cells possessed granular and moderately electron dense nuclei containing viral particles. Cytoplasmic virions were more abundant in these cells, suggesting a release of particles from the nucleus to the cytoplasm through nuclear pores but this process was not observed. Microtubules measuring 30nm in diameter were present within infected nuclei only, although never found associated with the virions, their presence in infected nuclei suggested a close relationship. It was suggested that they could be connected to an abnormal association of viral proteins during abortive morphogenesis (Mari and Bonami, 1988). At the end of the cellular infection viral particles were present throughout a vacuolated cytoplasm,

lacking cytoplasmic organelles. Lysed cells consisting of an infected nucleus surrounded by cytoplasmic fragments were frequently seen in the connective tissues.

Purification via a CsCl gradient produced three bands, one corresponding to empty capsids, the other two containing virions containing nucleic acid; these were designated light (L) and heavy (H) particles. Mari and Bonami (1988) suggested that PC84 be related to the *Parvoviridae* family due to its location within the nuclei, size of particles and similarity to other insect parvovirus infections.

2.6.7 Lymphoidal parvo-like virus

A parvo-like virus infecting the lymphoid organ of *P. merguiensis*, *P. monodon* and *P. esculentus* in Australia was described by Owens et al. (1991). Lymphoid organs were hypertrophied and possessed multinucleate giant cells. These cells displayed hypertrophy of the nucleus, margination of the chromatin, basophilic cytoplasm and an increase in the cytoplasm to nucleus ratio. Cells had undergone a noticeable transformation, almost neoplasia but mitotic figures were rare. Cell membranes were also rare suggesting giant cell formation. Later in the infection round pyknotic nuclei became apparent, some producing eosinophillic buds. Lymphoidal lobes that were full of cellular debris were encapsulated. Basophilic intranuclear inclusions similar to PmergDNV were seen in small pockets. Inclusions were Feulgen positive and fluoresced green with acridine orange at pH 3.8 indicating DNA. These changes were seen in healthy wild and farmed *P. merguiensis* and those concurrently infected with PmergDNV. They were also seen in apparently healthy *P. esculentus* and *P. monodon* co-infected with a baculovirus and a microsporidian. EM revealed large electron dense intranuclear inclusions with paracrystalline arrays of virus particles, measuring 18 to 20nm in diameter, on the edge of the inclusion. The small size of the virions, positive staining for DNA and the basophilic inclusions in the lymphoid organ suggested this was a parvovirus infection and was named Lymphoidal parvo-like virus (LPV) to distinguish this infection from other parvoviruses. LPV particles were similar in size to PmergDNV and shared similarities in the size of the inclusion bodies with PC84. Considerable similarities were also highlighted between LPV and PstDNV.

2.6.8 Spawner-isolated mortality virus (SMV)

Fraser and Owens (1996) reported a virus infection of *P. monodon* and named the virus spawner-isolated mortality virus (SMV) following a mortality event in broodstock. Challenge trials were conducted; shrimp were both fed and injected with infected material. Shrimp were

noted to be dark in colour and produced red faeces, they were lethargic, showed reduced feeding, lacked coordination and were noted to be falling onto their sides at the bottom of the tank. Histology revealed lesions present in multiple organs, haemocytic infiltration was observed in the gut and cellular debris was observed as being sloughed into the lumen. Hepatopancreas showed marked changes, haemocytic infiltration around the tubules, pyknotic nuclei and large areas of necrosis, some shrimp showed a reduced number of hepatopancreatic tubules with an increased haemal space. Haematopoietic tissues were depleted in some shrimp and subcuticular epidermis showed areas of extensive haemocytic infiltration and melanisation and necrosis of the underlying muscle. TEM revealed aggregations of non-enveloped virus-like particles within the cytoplasm of gut cells. Virions measured approximately 20nm in diameter and were hexagonally shaped suggesting icosahedral symmetry. Virions appeared to be passing from the nucleus, through pores in the nuclear membrane, into the cytoplasm. Filamentous material was associated with the virions; this was suggested to be uncut nucleic acid awaiting virion packaging.

Histological changes were considered non-specific and could easily be mistaken for bacteraemic shrimp. This lack of pathognomic lesions or inclusion bodies distinguishes SMV from other viral infections which have gross signs, inclusion bodies or lesions (Fraser & Owens, 1996). Fraser and Owens (1996) suggested that SMS may be a parvovirus similar to PstDNV. The virus was associated with mid-crop mortality syndrome (MCMS) by Owens *et al*. (1998), they suggested that there was considerable evidence that MCMS could be associated with a parvo-like virus and that this virus was likely to be very closely related or identical to SMV. Using *in situ* hybridisation Owens *et al*. (1998) showed that the first tissue to become infected was the gastrointestinal tract and indicated the most likely route of egress would be through the faeces. Positive signals were also obtained from the male reproductive tract at the terminal ampoule and the medial vas deferens and the ovary, it was suggested that SMV could be vertically transmitted through hatcheries. Owens *et al*. (2003) developed a PCR test for testing spawner faeces which enabled the shrimp stocks to be screened without sacrifice. Owens *et al*. (2003) showed that postlarvae from SMV-positive spawners had a poorer survival rate than those which were SMV-negative. They suggested that SMV caused 15 to 35% losses over the production period and that this could have huge financial consequences for shrimp farming industries.

Roekring *et al*. (2002) compared the sequences of parvoviruses from insects and penaeids (SMV, PmDNV and PstDNV). Phylogenetic tree based on genomic DNA gave two main clades, Clade 1 containing SMV and insect parvoviruses and Clade 2 with PmDNV and PstDNV and mosquito brevidensoviruses. The clustering of SMV with a different group of

insect densoviruses and the separation of PmDNV and PstDNV into another group suggested that these shrimp parvoviruses are of diverse origin and are not closely related.

SMV was also discovered in *C. quadricarinatus* in Australia during an investigation into a mortality event (Owens and McElnea, 2000). Crayfish showed a reduced tolerance to stress, practices which had previously caused no mortalities, were leading to 60-100% mortality over a two week period. Farmers were reporting a reduction in yield with the weight of the crayfish at final harvest equalling the weight of the crayfish brought in for stocking. Crayfish showed no external signs of infection, histology revealed haemocytic enteritis in the foregut and proximal portion of the midgut. Some crayfish had a mild atrophy of the hepatopancreas cells, leading to a larger interstitial space between the hepatopancreas tubules suggesting mild starvation. Positive signals were found using a DIG-labelled SMV probe in multiple organs of the moribund crayfish. Strong positive reaction were seen in the hepatopancreas, midgut, glands associated with the midgut, epithelium of seminal ducts and follicle cells surrounding the oocytes. In some crayfish positive signals were also present in the heart, haemocytes, connective tissue and subcuticular epithelium. The lack of external signs and the failure to identify any other pathogens in the crayfish was quoted as being reminiscent of the broodstock shrimp which had died in the hatchery where SMV was discovered (Owens and McElnea, 2000).

2.6.9 *Cherax destructor* systemic parvo-like virus (CdSPV)

Edgerton *et al*. (1997) described a systemic parvo-like virus from one moribund *C. destructor* sampled from an aquaculture farm in Australia and named the virus *C. destructor* systemic parvo-like virus (CdSPV). Intranuclear Cowdry Type A inclusions were observed systematically throughout the crayfish but were most common in the gills. Nuclei were hypertrophied, showed marginated chromatin and contained a single eosinophillic Cowdry Type A inclusion in the centre which was Feulgen negative. The nucleolus when present was closely associated with the inclusion. Smaller presumably earlier inclusions developed centrally and stained lightly eosinophillic. Gills contained extensive necrotic foci, characterised by pyknotic and karyorrhectic nuclei, haemocytic infiltration, melanisation and nodulation. Pyknotic nuclei and haemocytic nodules were also diffuse throughout non-necrotic tissues. Cowdry Type A inclusions were seen within the gill epithelium and in cells close to necrotic areas and becoming necrotic. Epithelial cells lacking inclusion bodies with hypertrophied nuclei, marginated chromatin and enlarged nucleoli were common throughout the gills. Hepatopancreas and midgut were extensively necrotic, haemocytic infiltration and melanised foci were common in the interstitial tissues of the hepatopancreas and midgut

submucosa. Inclusion bodies, pyknotic and karyorrhectic nuclei, haemocytic infiltration and nodules were also present in the epicardium, abdominal muscles and spongy connective tissues. TEM revealed a rounded granular viroplasm located centrally and closely associated with the nucleoli within gill epithelial cells. Viroplasm contained filamentous structures and rounded viral particles. Electron lucent particles presumed to be empty capsids measured 17.5nm and electron dense particles measured 20.8nm but were difficult to distinguish within the viroplasm. These particles could be visualised better in aggregates present between the viroplasm and the nuclear membrane. Some particles appeared hexagonal indicating an icosahedral symmetry. Edgerton *et al*. (1997) suggested the virus to be parvovirus-like due to the pathology, site of replication (nuclei), particle size and the close association between the nucleoli and the inclusion body. This is the only systemic virus described in freshwater crayfish.

2.6.10 Parvo-like virus of Cherax quadricarinatus

Edgerton *et al*. (2000) first reported a parvo-like virus infecting the gills of *C. quadricarinatus* following reports from farmers detailing a decreased resistance to stress in farmed crayfish stocks. Foci of hypertrophic nuclei were clearly observed within cross-sectioned gill lamellae; occasionally gill epithelial nuclei were also affected. Epithelium of heavily infected gill lamellae stained densely with H&E and was shrunken away from the cuticle. Hypertrophic nuclei contained marginated chromatin, peripheral nucleoli and the interior appeared to be vacant or contained a faintly basophillic granular substance. Staining with toluidine blue showed the nuclei to contain homogenously granular substance. Electron microscopy showed this granular substance to be full of consistently sized, electron dense, rounded virus-like particles. Particles measured approximately 20nm and some appeared to be slightly angular. Cytoplasm of affected cells was also shown to be more electron dense than uninfected cells.

2.6.11 *Cherax quadricarinatus* parvo-like virus (CqPV)

Bowater *et al*. (2002) also described a parvo-like virus infecting *C. quadricarinatus* and showed that this virus was responsible for causing mass mortalities within a farm; they named the virus *C. quadricarinatus* parvo-like virus (CqPV). Investigations were conducted to identify the cause of a cumulative mortality event in juvenile *C. quadricarinatus* (96% mortality over 2 months) and a few months' later cumulative mortalities in adult stocks from the same farm, the farm losing 50% of its stock. Diseased crayfish were anorexic, weak and did not respond with tail flicking when handled, many diseased crayfish were found at the edge of the pond or in bait traps. Histology revealed intranuclear inclusion bodies in ectodermal, endodermal and

mesodermal tissue types. Two types of inclusion bodies were described, early, nuclei which appeared normal but contained small eosinophilic inclusion bodies and stained Feulgen negative, and late, hypertrophied nuclei containing prominent basophilic inclusion bodies which filled most of the nuclei, and stained Feulgen positive. Gills, cuticular epithelium, foregut, midgut and hindgut epithelium appeared to be most heavily affected. Antennal gland, haematopoietic tissue, epithelial cells of seminiferous tubules and interstitial cells of the ovary were less commonly affected. Inclusions were not seen in the heart, hepatopancreas or neurones. *In situ* hybridisation of sections for PstDNV, HPV using DiagXotic test and SMV were all negative. Electron microscopy showed the late stage inclusions contained electron dense masses, chromatin was marginalised and the host nucleolus displaced by the inclusion. Electron dense mass contained numerous hexagonal parvo-like viral particles measuring 19.5nm in diameter.

Bowater *et al*. (2002) highlighted the similarities of this infection to previously described viral infections, HPV and CqSPV, but suggested that this was a new virus due to the differences in tissue type affected and the early and late stage development seen. They suggested that this virus infection was different to that described by Edgerton *et al*. (2000) as the gill lesions described by Edgerton *et al.* were not seen with CqPV infections. It is possible that they both describe the same virus but at different stages of the infection, Edgerton *et al*. (2000) sampled a low number of 'survivor' crayfish from a stock in poor health, where mortalities had been occurring for a considerable period, and did not conduct transmission trials with this virus. It is possible the crayfish sampled here had survived an initial infection and were in the recovery phase. However until now CqPV has only been reported from this single crayfish farm. CqPV was the first parvovirus infection in crayfish to be linked to high mortalities and shown to be highly pathogenic to red claw crayfish. Purification and nucleic acid characterisation is required to fully classify both these viruses; this would enable any similarities to each other and other parvo viruses to be determined.

2.6.12 Virus-like particles in *Penaeus aztecus*

Foster *et al*. (1981) described a viral infection of the heart cells from a single *P. aztecus* sampled from the coastal waters of Mississippi, USA. Viral like inclusions were observed in the cytoplasmic vacuole of at least three fixed phagocytes. A tightly packed aggregate of small non-enveloped electron dense particles measuring approximately 23 nm was present within these cells. Some of the particles appeared angular in profile and were arranged in paracrystalline arrays. Apart from these inclusions the affected cells appeared normal. Foster *et al*. (1981) suggested that this may be either a parvovirus-like or picornavirus-like infection.

No further classification was carried out. The lack of description of nuclear pathology suggests this may be a picorna-like virus as opposed to a parvo-like virus however due to the limited information available it is not possible to distinguish between the two.

2.6.13 Bay of Piran Shrimp Virus (BPSV)

A viral infection of the hepatopancreas from *Palaemon elegans* sampled at the Croatian coast of the Mediterranean Sea was described by Vogt (1996). Five out of seven female shrimp sampled showed pathogens (reovirus, rickettsia and hepatopancreatic brush border lysis-bacteria) within the hepatopancreas, one shrimp showed a parvo-like virus infection. This virus was described as Bay of Piran shrimp virus (BPSV). BPSV was found in the epithelial cells of the hepatopancreas but not in the surrounding musculature, connective tissues or haemolymph. BPSV virions were non-enveloped, spherical to angular, electron dense and measured between 22 and 27nm. Particles were most abundant in R-cells and less prevalent in F-cells of the hepatopancreas, on average 30% of the R-cells were visibly infected, B-cells were rarely and slightly infected. Within the R-cells the virus was mostly accumulated in the cytoplasm but was also abundant within membrane whorls. In the F-cells BPSV was mostly observed in the cisternae of golgi bodies. Paracrystalline arrays were rarely seen, occurring in degenerating R- and F-cells. Extracellular virus particles were often seen within the lumens of the tubules and attached to the microvilli. Extrusion of the infected R- and F-cells into the lumen was occasionally seen and this was thought to account for the free virus present, virions being released from lytic cells. The nuclei of the virus-infected cells appeared normal but showed chromatin condensation and a prominent lateral displacement of the nucleolus, inclusion bodies were absent. However within the nucleoplasm electron lucent particles of roughly the same size as the cytoplasmic particles were present. They were in close association with filaments and were described as developing BPSV particles. Vogt (1996) suggested that this virus was likely to be a parvovirus due to the nuclear development stage. No further classification studies have been conducted.

2.7 *Herpesviridae*

2.7.1 Bi-facies virus of *Callinectes sapidus*

Herpeslike virus (HLV) was described infecting the haemocytes of the blue crab, *Callinectes sapidus* by Johnson (1976b). This was later re-named Bi-facies virus (BFV) after further examination of the morphology and development identified 2 types of development and 2 final forms (Johnson, 1988). The virus was first found in moribund juvenile and mature wild crabs

from Chincoteague Bay, Virginia, USA and later in small juvenile crabs, wild and captive, from Assawoman Bay, Delaware, USA (Johnson, 1976b; Johnson, 1978). 13% prevalence was found in a natural population of juvenile blue crabs (Johnson, 1983). Infected crabs appeared normal until shortly before death when they became inactive and ceased feeding. Haemolymph was an opaque chalky white in appearance and did not clot. Crabs injected with this haemolymph were shown to die 30-40 days post injection (Johnson, 1978). BFV infections were shown to be present in the crabs before capture as juveniles that had been kept in the laboratory in separate containers for 50 days showed moderate to heavy infections. BFV was shown to cause an epizootic with a high mortality rate in juveniles held in separate containers but with flow through water system indicating that this virus is highly infectious by water route (Johnson, 1984).

BFV infects haemocytes and haematopoietic tissue, it can probably also be found in connective tissues and epithelial cells of the gill. Infected nuclei appeared hypertrophied with marginalized chromatin, stained homogenously with Feulgen or contained Feulgen positive granules. The cytoplasm of the infected cell was reduced to a thin rim around the nucleus; virus was released through lysis of the nucleus. Haemolymph of a terminally infected crab contained free virus and granular material probably derived from cell lysis. Fixed phagocytes in the hepatopancreas appeared swollen and were filled with a homogenous, slightly Feulgen positive material and were often necrotic.

EM revealed this material to be a mixture of viral particles and granular material. HLV particles were observed within these hypertrophied nuclei; particles consisted of a central cylindrical nucleoid which was surrounded by a toroid and an envelope consisting of two membranes. Virions were hexagonal and measured 185 - 214nm in diameter (Johnson, 1983). Development was described as taking place in these hypertrophied nuclei; viral particles at different stages of development were usually present in groups. Mitochondria, free ribosomes and various vesicles were present within the haemocytes but rough endoplasmic reticulum and Golgi apparatus were absent and the relative amount of cytoplasm was reduced. Multiple crystalline inclusions were present in infected nuclei of 2 crabs (Johnson, 1988). Large moderately electron dense and finely granular inclusions were present in the cytoplasm of almost all infected cells. Material from these cytoplasm inclusions adjacent to the nucleus appeared to enter through nuclear pores to form similar intranuclear inclusions. There appeared to be 2 kinds of completed virus particles that were morphologically identical whether they were found within the nuclei or extracellularly.

The original descriptions by Johnson (1976b; 1983; 1984) had been based on tissue that had been embedded in paraffin and then re-embedded in resin for electron microscopy; this resulted in shrinkage of the electron dense sphere (EDS). The ends of the core were described as extending well beyond the surface of the EDS leading to the description of the EDS as a torus rather than a sphere. After examining freshly prepared tissues Johnson (1988) highlighted that the morphology and development of BFV differed considerably from those of the Herpesviridae. Unlike herpesvirus BFV does not have an icosahedral capsid external to its core and internal to its envelope and BFV does not gain the envelope by budding through a host cell membrane, envelope is formed *de novo* within the nucleus. BFV was described as being a representative of an undescribed family of viruses, relatives may be among the double stranded DNA viruses of the families Iridoviridae, Poxviridae, Baculoviridae and Herpesviridae but BFV was shown to differ from known species of all these groups. Johnson described the virus found infecting the antennal gland and bladder of *Paralithodes platypus* as undoubtedly related to BFV (Johnson, 1988).

BFV has been shown to cause disease and mortality in both captive and wild caught crabs from the Chincoteague Bay system of Delaware, Maryland and Virginia, USA. It was unknown if an infected crab could recover from this infection, but was suggested that death probably occurs due to loss of haemocytes. Johnson (1988) stated that final diagnosis depended upon finding infected cells via histology but "reasonably firm diagnosis of terminal infections can be made on the basis of the haemolymph being chalky white and non-gelling." We know from previous studies that the appearance of white haemolymph cannot be used as a diagnostic tool. Multiple pathogens cause a similar appearance of white haemolymph in crustacea; *Hematodinium* sp. (Stentiford *et al*., 2002), *Rickettsia*-like organism (Eddy *et al*., 2007) and a haplosporidian infection (Stentiford *et al*., 2004b) have all been shown to cause white haemolymph in crustacea so confirmation of disease should always be made via histology and electron microscopy.

2.7.2 Herpes-like virus of shore crab *Carcinus maenas*

We have recently discovered a similar virus infecting the haemocytes of the shore crab *Carcinus maenas* sampled in the English Channel, UK and it is described here for the first time. Bang (1971) isolated a transmissible disease from a single shore crab during surveillance for a ciliate parasite. Bang (1971) described viral particles measuring 55nm to 125nm that could be isolated from a shore crab whose blood failed to clot and that the infection could be passaged. No further work was published on this virus; it is possible we have re-

isolated it here. The virus appears very similar ultrastructurally to Bi-facies virus, further work is currently underway to try to fully characterise this virus, see chapter 7.

2.7.3 Herpes-like virus in testes of mud crab *Rhithropanopeus harrisii*

A herpes-like virus was found infecting the mesodermal cells of the male gonad of mud crab *Rhithropanopeus harrisii* sampled in North Carolina (Payen and Bonami, 1979). The virus is non-enveloped when in the nucleus but has an envelope when seen in the cytoplasm of the cell. Particles were paraspherical, slightly angulate with capsids measuring 75-80nm and enveloped particles measuring 100-110nm. The envelope appears to form when the capsid buds out from the nucleus, a typical feature of vertebrate herpesviruses. Pathogenic effects appear to be limited to the degeneration of infected cells however the general tissue distribution of this virus is unknown.

2.7.4 Herpes-like virus of blue king crab *Paralithodes platypus*

Sparks and Morado (1986) discovered a herpes-like virus infection of the antennal gland and bladder in the blue king crab *Paralithodes platypus* from Alaska. Crab stocks were studied after population declines, tissues being sampled for both light and electron microscopy. Histology also revealed evidence of a viral infection in the bladder and antennal gland of red king crabs *P. camtschatica* and golden king crabs *Lithodes aequispina*. Nuclei within the bladder and antennal gland were hypertrophied with marginated chromatin and showed a variably stained eosinophillic substance with or without eosinophillic inclusion bodies. This eosinophillic substance was generally spherical in shape and seemed to be responsible for the nuclear hypertrophy. Heavily infected crabs showed loss of normal architecture of the bladder, hypertrophied nuclei eventually rupture the cell membranes and free the infected nuclei into the lumen.

TEM revealed the presence of numerous hexagonal virus particles within the nuclei of infected cells. Virus particles appeared similar in size and morphology to members of the Herpesviridae. Particles consisted of a hexagonal nucleocapsid composed of a nucleoid containing an electron dense cylinder measuring 55 to 60 by 90 to 105nm. This was surrounded by toroidal structure measuring 90 to 105nm and enclosed within a capsid composed of two distinct electron dense layers. Complete unenveloped nucleocapsids measure 140 to 165nm. The eosinophillic substance was composed of finely granular material where viral assembly takes place, virus particles at various stages of development were

observed within this virogenic stroma. Eosinophillic inclusion bodies appear similar to those described as Cowdry type B inclusions (Cowdry, 1934).

This virus appears morphologically very similar to the herpes-like virus of *Callinectes sapidus* except the nucleoid and its components are slightly smaller. It was considered that the 2 electron dense layers that encompassed the nucleoid were part of the capsid despite the space between them meaning that the viral particles were unenveloped consistent with features of the *Herpesviridae*. The relatively wide space between the 2 layers may be characteristic of the blue crab virus or possibly an artefact of fixation. It is not known whether this disease causes mortalities but it seems unlikely that the crabs could survive and repair the tissue damage seen.

2.7.5 *Panulirus argus* virus 1 (PAV-1)

Panulirus argus virus 1 (PaV-1) is the first description of a viral infection in lobsters (Shields and Behringer, 2004). The infection was initially observed in juvenile lobsters which were lethargic, displayed milky haemolymph, which did not clot and sometimes had a discoloured, heavily fouled carapace. Juveniles with light infections and adult lobsters did not show any obvious external signs of infection. Histology revealed nuclear hypertrophy with Cowdry-type A viral inclusions in infected haemocytes. In heavily infected individuals virtually all the hosts hyalinocytes and semigranulocytes were destroyed, granulocytes however were not affected. Fixed phagocytes, blood vessels and surrounding connective tissues were necrotic and destroyed in these heavily infected individuals. Li *et al*. (2008) showed that the infection initially infects the fixed phagocytes and the connective tissues within the hepatopancreas. In heavily infected lobsters virally infected cells could also be found in the spongy connective tissues surrounding most organs (Li *et al*., 2008).

EM demonstrated that the virus appeared to show a predilection for hyalinocytes and semigranulocytes (Shields and Behringer, 2004). Virions were assembled within the hypertrophied nuclei, virions and virogenic stroma being diffusely distributed around the inner periphery of the nuclear membrane. Virions are icosahedral in shape and measure approximately 182 ± 9nm, nucleocapsids measure 118 ± 4nm. Envelopes possess an electron lucent inner layer and an electron dense outer layer on which there are possible projections when located extracellularly. Nucleocapsid possesses an internal cylinder surrounded by a subcentric toroid structure similar to the classical toroid structure seen in the *Herpesviridae*. Unlike the herpes viruses there is no tegument or envelope formation around the nucleocapsid during migration through the nuclear envelope or the cytoplasmic

membrane. Aggregates of these virions are also observed in the haemolymph and virions occur freely in the haemal sinuses of the hepatopancreas in heavy infections. Fluorescent *in situ* hybridisation (FISH) techniques were developed (Li *et al*., 2006) and this confirmed that the primary site of infection was the connective tissues of the hepatopancreas. PCR techniques were also developed by Montgomery-Fullerton *et al*. (2007). Heavily infected lobsters show a lack of reserve inclusions indicating a lack of glycogen reserves, the lethargy present in these lobsters is likely to be end stage of the disease, mortality resulting from metabolic exhaustion (Shields and Behringer, 2004).

The assembly of the virion within the host nucleus, electron lucent cylindrical core surrounded by an electron dense toroid and icosahedral capsid are morphological features shared with the *Herpesviridae*. PaV1 is also similar to the *Iridoviridae* unenveloped, large icosahedral virions and accumulation of virogenic stroma in the cytoplasm. However the fact that assembly takes place within the host nucleus and the presence of the electron dense toroid are not consistent with the features of the *Iridoviridae*. The classification of this virus remains to be resolved.

PaV1 has been shown to be present throughout the Florida Keys, and infections have also been found in Belize, Mexico and the US Virgin Islands so it is thought to be widespread in the Caribbean Sea (Butler *et al*., 2008). Prevalence of PaV1 is usually highest among the smallest juveniles and has been shown to decline with increasing lobster size (Behringer *et al*., 2011; Behringer, 2012). Early benthic juveniles appear to be highly susceptible; field experiments indicated that prevalence in wild populations exceeded 50% in focal outbreaks (Butler *et al*., 2008). Histological evidence suggested that the prevalence was highest (~16%) among crevice-dwelling juveniles measuring 15-20mm carapace length. This declined to 5% once they reached 35-45mm CL and was virtually undetectable in adults (<1%). PaV1 has been shown to be transmitted to juvenile lobsters via inoculation, ingestion of diseased tissue and through close contact with infected individuals. The virus has also been shown to be transmitted via the water over small distances although contact and waterborne transmission were shown to be the least efficient methods (Butler et al., 2008).

2.8 *Iridoviridae*

2.8.1 *Protrachypene precipua* Iridovirus

Lightner and Redman (1993) described the presence of a putative iridovirus infection in the penaeid shrimp *Protrachypene precipua* sampled from the seawater supply canal of a

commercial shrimp farm in Ecuador. This is the first report of an iridovirus infecting penaeid shrimp. The shrimp farm grew primarily *P. vannamei*, *P. precipua* is commonly found in estuarine and marine environments from Ecuador to Nicaragua but due to its small size (4-6g for adults) it is not routinely cultured. *P. precipua* shrimp in the supply canal were showing signs of distress and some displayed a white colouration, moribund shrimp were sampled for histology. Moribund *P. vannamei* samples were also taken from the same supply canal and the grow-out ponds the canal supplied. Three of the eleven *P. precipua* sampled displayed prominent iridovirus lesions in the cuticular epidermis of the body, gills and stomach lining, infected cells were also present at a lower intensity in the connective tissues, fixed phagocytes in the gills, hepatopancreas, striated muscle and heart, haematopoietic tissue and antennal gland. Histologically infected cells showed large dense basophilic, intensely Feulgen positive cytoplasmic inclusions. Some cells contained discrete lesions whilst in others the inclusions filled the cytoplasm of markedly hypertrophied cells. No lesions were seen within *P. vannamei* samples.

Specific electron microscopy samples had not been taken so tissues were processed from paraffin-embedded blocks. Virus particles are organised into paracrystalline arrays in the cytoplasm of the infected cells and are hexagonal in profile. They were reported to be similar to the iridovirus previously described infecting the daphnid *Simocephalus expinosus* (Federici and Hazard, 1975). Virions were shown to contain an electron dense spherical core measuring 85nm, surrounded by an amorphous layer approximately 20nm thick and enclosed within a trilaminar membrane. Fully formed virions measured 122 edge to edge and 136nm point to point. The virus was not fully characterised due to the limited samples. The fact that this infection was not seen within *P. vannamei* samples suggested either the iridovirus did not infect other penaeid species, or these infections were not common, and were difficult to diagnose if they did occur.

2.8.2 Sergestid Iridovirus (SIV)

An iridovirus named Sergestid Iridovirus (SIV) was found to cause high mortalities within the sergestid shrimp species *Acetes erythraeus* by Tang *et al*. (2007c). Small sergestid shrimp were inadvertently introduced into commercial shrimp ponds growing *P. monodon* in Madagascar and were noticed to be suffering mortalities. Moribund shrimp were found swimming near the surface and edges of the ponds, the exoskeleton of these shrimp appeared iridescent, similar to that identified in *P. precipua* in Ecuador. *P. monodon* from the same ponds were not affected. Using PCR primers from a conserved region of the iridovirus genome Tang *et al*. (2007c) were able to confirm this was an iridovirus infection and developed

both a PCR and an *in situ* hybridisation method to detect the infection. Histology revealed basophilic inclusions within the cuticular epithelium of the gills, appendages, mouth, stomach and epidermis but were not seen in the skeletal muscle. Inclusions were extensive and occupied the entire cytoplasmic area.

Electron microscopy of the epithelial cells showed viral inclusion bodies containing packs of paracrystalline arrays of non-enveloped virions. Virions were icosahedral, possessed an electron dense core and measured approximately 140nm. Virions in various stages of development were also present within the inclusion bodies identifying this as the site for viral assembly. Several large mitochondria, containing disintegrated or empty cristae were associated with the inclusion bodies. *in* situ hybridisation showed that SIV predominantly infects cuticular epithelial cells of the gills and appendages, epicardial connective tissue but not the muscle itself and germinal cells of the ovary and testes, identified by a strong positive reaction. Connective cells of the skeletal muscle and hepatopancreas were also positive by *in situ*. No reaction was found when this probe was used on tissue sections from *P. precipua* suggesting the two viruses had a significant divergence in their genomic sequence. Tang *et al*. (2007c) sequenced the major capsid protein coding region from the shrimp and compared this to other described invertebrate iridoviruses using a phylogenetic tree. SIV clustered together with other invertebrate iridoviruses and was closest to members of the genus *Iridovirus*. Further sequence data is needed to unequivocally determine the taxonomic status.

Tang *et al*. (2007c) noted the virus was associated with high mortalities in shrimp from a high density environment, in the natural environment, open sea; such severe infections were likely to be rare. It is not known how prevalent this virus is in wild stocks, Tang *et al*. (2007c) highlighted that these pelagic shrimp are widely distributed in the world's oceans and play a vital role in marine food chains, and suggested that severe infections could potentially have a substantial impact on marine ecosystems.

2.9 Conclusions

The need to complete full characterisations and harmonise the naming of new viruses using International Committee on Taxonomy of Viruses (ICTV) guidelines is evident throughout with many different names or abbreviations being used to describe the same virus. Development of techniques will assist with these full classifications and improve diagnostic capabilities, this review also highlighted that comparisons to other invertebrate viruses such as those described in insects may assist with these characterisations. Disease emergence and spread has often been due to poor industry practices within aquaculture, and whilst in comparison relatively little

is known about viruses in the wild, it is critical that as much data as possible is collected on viral diseases in both farmed and wild populations to enable the rapid diagnosis of emerging disease issues. Rapid diagnosis and enforcement of biosecurity measures are crucial to prevent spread of viral pathogens and prevent further disease epidemics.

Despite almost two decades since its discovery, WSD is still considered the most significant known pathogen impacting the sustainability and growth of the global penaeid shrimp farming industry (Stentiford *et al*., 2009; Stentiford *et al*., 2010; Lightner, 2011). In recognition of its significance, WSD has been listed as a notifiable disease by the OIE for over a decade and more recently, WSD has also been listed in regional legislation within the European Union (EU) via its inclusion in European Directive 2006/88EC. In contrast to the penaeids, relatively little information exists on the pathogenesis and outcome of WSD in non-penaeid decapod species (including crabs, lobsters and crayfish). The remainder of this thesis will concentrate on this viral disease and will investigate the susceptibility of European decapods species to this disease and assess the likelihood of whether a WSD infection could establish within wild stocks.

Chapter 3.

Susceptibility to viral infection and pathogenicity of White Spot Disease (WSD) in non-model crustacean host taxa from temperate regions

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3.1 Abstract

White Spot Disease (WSD) is considered to be the most significant known pathogen impacting the sustainability and growth of the global penaeid shrimp farming industry. Although most commonly associated with penaeid shrimp farmed in warm waters, the virus is also able to infect, cause disease and kill a wide range of other decapod crustaceans from temperate regions, including lobsters, crabs, crayfish and shrimp. Using principles laid down by the European Food Safety Authority (EFSA), an array of diagnostic approaches were applied to provide a definitive statement on the susceptibility to White Spot Syndrome Virus (WSSV) infection in seven ecologically or economically important European crustacean species (four marine species: *Cancer pagurus*, *Homarus gammarus, Nephrops norvegicus and Carcinus maenas*; one estuarine species, *Eriocheir sinensis* and two freshwater species, *Austropotamobius pallipes* and *Pacifastacus leniusculus)*. Exposure trials based upon natural (feeding) and artificial (intra-muscular injection) routes of exposure to WSSV revealed universal susceptibility to WSSV infection in these hosts, but also that relative susceptibility varied significantly between species. This study describes the pathogenesis of WSD in these hosts and compares this to the well documented disease progression profile of model penaeid shrimp hosts.

3.2 Introduction

Since initial outbreaks of White Spot Syndrome Virus (WSSV) infections in Asian shrimp farms during the early 1990's, the disease associated with infection in farmed penaeids (White Spot Disease, WSD) has been responsible for massive economic losses in both the western and eastern hemispheres (Lightner, 2011). The name of the disease refers to the clinical signs that have been reported in relatively few of the species known to be susceptible to infection and relates to the white spots that may appear on the inner surface of the infected host cuticle. These spots are not always present and can also be caused by bacteria, stress and high alkalinity, so presence/ absence of these spots cannot be used as a reliable diagnostic test. Other signs of WSD include lethargy (Durand *et al*., 1997), reduced feeding, reddish colouration (Lightner *et al*., 1998), loose cuticle (Lo *et al*., 1996a), enlargement and yellowish discolouration of the hepatopancreas (Sahul Hameed *et al*., 1998) and a thinning and delayed clotting of the haemolymph (Wang *et al*., 2000). Patent WSD results in rapid, high mortalities, implicating up to 100% of the exposed stock within 3-10 days of initial signs (Lightner, 1996a). Numerous authors have described the epidemiology of the disease in individual farms, farm clusters and geographic regions (Corsin *et al*., 2002; Lotz, 1997) while others have detailed the prevailing practice of so-called 'emergency harvest' as a mechanism to prevent total crop loss following initial discovery of diseased animals (Turnbull *et al*., 2005). Overall, despite almost two decades since its discovery, WSD is still considered the most significant known pathogen impacting the sustainability and growth of the global penaeid shrimp farming industry (Lightner, 2011; Stentiford *et al*., 2009; Stentiford *et al*., 2010).

Since the issue of susceptibility of hosts to a particular disease is a key component of any limitation to the trading in that host or its products, recent work by the European Food Safety Authority (EFSA) has attempted to define 'susceptibility' (with regard to the listing of hosts as such in EC Directive 2006/88/EC). In terms of the listed crustacean diseases (including WSD), this work has recently been reviewed by Stentiford *et al*. (2009). Here, the available published and grey literature (for WSD) was assessed against four objective susceptibility criteria: (A) Evidence of replication or growth of the organism; (B)

Presence of a viable organism; (C) Presence of specific clinico-pathological changes; and (D) Specific location of the pathogen within the host. Using this approach, and despite the fact that EC Directive 2006/88/EC currently lists 'all decapod crustaceans' (>20,000 extant species) as potentially susceptible to WSD, scientific data were available to support designation as 'susceptible' in just 67 species. Although evidence for susceptibility was available for significantly fewer host species than suggested by the statement of 'all decapod crustaceans' in EC Directive 2006/88/EC and by the OIE (OIE, 2006), the review did however indicate a particularly wide spectrum of WSD-susceptibility across host taxa within the Decapoda. Essentially, this spanned the two major sub-orders (Dendrobranchiata and Pleocyemata), and numerous families within each of these (Stentiford *et al*., 2009). This builds upon River's postulates (Rivers, 1937) where Rivers states that

"a) a specific virus must be found associated with a disease with a degree of regularity. b) the virus must be shown to occur in the sick individual not as an incidental or accidental finding but as the cause of the disease under investigation"

Current guidelines for the methodological approach to diagnosis of WSD are based upon the detection of viral nucleic acids and the manifestation of disease in penaeid shrimp hosts (OIE, 2006). WSSV infection occurs in all tissues of mesodermal and ectodermal origin (e.g. gills, lymphoid organ, cuticular epithelium, sub-cuticular connective tissues). Infected nuclei become hypertrophied with marginalized chromatin, and contain inclusion bodies that stain intensely eosinophillic in early stage infection and basophilic in more advanced infection (Lightner, 1996a). Virions measure 120-150nm in diameter and 270-290nm in length, possess a tail-like projection and a striated nucleocapsid (Vlak *et al*., 2005; Wang *et al*., 1995), features similar to the currently unclassified B and B2 viruses from portunid crabs (Bonami and Zhang, 2011).

As highlighted by Stentiford *et al.* (2010) and Stentiford and Lightner (2011), since WSD has been listed in European Directive 2006/88EC as a 'non-exotic' disease within Europe, Member States are required to declare a national status

for WSD; categories ranging from Category I (free from disease) to Category V (infected). In order to do so, Member States may be required to undertake an epidemiologically- rigorous surveillance programme which utilises appropriate diagnostic tests designed to detect WSSV if present.

The current study has utilised an array of diagnostic approaches to provide a definitive statement on the susceptibility to WSSV infection in seven ecologically or economically important European crustacean species from freshwater, estuarine and marine habitats, based on the principles laid down by the European Food Safety Authority (EFSA) and outlined by Stentiford *et al*., (2009). Furthermore, it has described the histopathological manifestation of WSD in these hosts and compared this to disease progression within (model) penaeid hosts (Pantoja and Lightner, 2003). I chose four marine species: edible crab (*Cancer pagurus*), European lobster (*Homarus gammarus*), Norway lobster (*Nephrops norvegicus*) and shore crab (*Carcinus maenas*); one estuarine species, the Chinese mitten crab (*Eriocheir sinensis*) and two freshwater species, the white clawed crayfish (*Austropotamobius pallipes*) and the signal crayfish (*Pacifastacus leniusculus*). The Chinese mitten crab and signal crayfish are invasive species to Europe, the Chinese mitten crab is thought to have been introduced via ballast water (Clark *et al*, 1998; Rainbow *et al*., 2003) and signal crayfish were initially imported from North America for aquaculture and restocking purposes (Svärdson, 1995). However both species can now be commonly found within European waters and as such were included in this study. Exposure trials based upon natural (feeding) and artificial (intra-muscular injection) routes of exposure to WSSV revealed universal susceptibility to WSSV infection in these hosts but also that relative susceptibility (measured in terms of temporal mortality and pathogenic manifestation of disease) varied significantly between species. These results are discussed in relation to inherent problems with defining absolute susceptibility or non-susceptibility to particular pathogenic agents in crustacean hosts. Results suggest that classification schemes may need refining to include an element of relative susceptibility (high to low), particularly when considering the role of specific life history traits in host organisms with non-continuous growth (e.g. the moult cycle), may provide a more realistic interpretation of host-pathogen interaction in invertebrate disease models. The

data presented herein provide further indication that this pathogen has the potential to impact upon non-penaeid host species residing in a wide range of aquatic habitats and at temperatures considered sub-optimal for the replication of the virus *per se* (Guan *et al*., 2003; Jiravanichpaisal *et al*., 2004; Rahman *et al*., 2007a; Rahman *et al*., 2007b; Sánchez-Paz, 2010; Vidal *et al*., 2001).

3.3 Materials and Methods

3.3.1 Criteria for assessing susceptibility

Susceptibility to WSSV infection was tested in seven non-model decapod species commonly found in European marine, estuarine and freshwater habitats. Susceptibility was assessed using criteria developed by the European Food Safety Authority (EFSA) and summarised by Stentiford *et al*. (2009). For WSD, the specific characteristics utilised to test the four criteria (replication, viability, pathology and location) are given in Table 1. These characteristics were assessed in each of the non-model decapod species following their exposure to WSSV.

Table 1. Specific techniques and characteristics applicable to support criteria A – D for White Spot Disease. Recreated and adapted from Stentiford *et al*., 2009.

	A. Replication	B. Viability	C. Pathology	D. Location
WSD	Presence of characteristic intranuclear inclusion bodies. Presence of virions in inclusions bodies by Transmission Electron Microscopy (TEM). Positive labelling of inclusion bodies by In situ hybridisation (ISH) or indirect fluorescent antibody test (IFAT). Serial passage from individual to Specific Pathogen Free (SPF) individual.	Passage bioassay to a Specific Pathogen Free (SPF) susceptible host.	Eosinophilic inclusions within nuclei of target organs and tissues.	Cells of tissues of ectodermic and endodermic origin.

3.3.2 lection of samples and husbandry

Samples were collected from natural sources as follows: a total of 18 edible crabs (*Cancer pagurus*) and 18 European lobsters (*Homarus gammarus*) were captured using baited pots in the Weymouth and Portland area of the English Channel, United Kingdom (50°32’50’’N 002º11’00’’W). Sixty Norway lobsters (*Nephrops norvegicus*) were collected from the North Sea fishery (54°08’566’’N 002º35’019’’E) using a Granton trawl and transported back to laboratory in tanks with a running sea water supply. Fifteen white claw crayfish (*Austropotamobius pallipes*), were sourced from a breeding facility in Stainforth (Near Settle) Yorkshire, United Kingdom prior to transportation to the laboratory in a small amount of freshwater with an air supply. Sixty Signal crayfish (*Pacifastacus leniusculus*) were sourced from a crayfish farm in Kent, United Kingdom and transported back to the laboratory overnight. Sixty Chinese mitten crab (*Eriocheir*

sinensis) were captured using baited pots from the River Thames, London, United Kingdom (51˚27'12''N, 00˚00'44''E) and transported back to the laboratory. Sixty shore crabs (*Carcinus maenas*) were collected from the shoreline at Newton's Cove, Weymouth, UK (50º34' N, 02º22' W) or using drop-nets in Weymouth Harbour (50°36' N, 02°27' W).

All experimental trials were performed within the biosecure exotic disease facility at the Cefas Weymouth laboratory and utilised local, filtered and UV treated seawater/ freshwater. Day length was set at 14 h/day, night was at 10h with a 30 min fade to simulate dusk and dawn. Flow rate was set a 3-4 l/min and salinity during the experimental period remained constant at 35ppt. Temperature was regulated according to the experimental conditions required. All animals utilised in experimental challenge trials appeared externally healthy. Species-specific challenges using WSSV were carried out independently (no multi-species challenges). Animals were transferred into custom-made compartments within large trough tanks, with individuals separated by tank divisions to prevent conflict but sharing the same water supply. Water temperature in all tanks was held constant at 20°C for *C. maenas, E. sinensis, A. pallipes* and *P. leniusculus*, and 16°C for *N. norvegicus*. The different challenge temperature utilised for these species was chosen to reflect the maximal summer temperatures likely experienced by these species in Europe. For *Cancer pagurus* and *H. gammarus*, chelipeds were banded to prevent conflict prior to their transfer to two large tanks (n=6 per tank) and three medium tanks which had been divided in half (n=2 per tank). Water temperature in all tanks was held constant at 20°C for *C. pagurus* and at 15°C for *H. gammarus*. Once again, ambient temperatures were chosen to reflect maximal summer temperatures likely experienced by these species in Europe. All animals were acclimatised to these exposure trial condition temperatures for a minimum of one week before trials commenced.

3.3.3 eparation of viral innoculum and challenge trials

Viral inoculates of WSSV were obtained from the OIE reference laboratory for White Spot Syndrome Virus (WSSV) at the University of Arizona, USA. The OIE isolate of WSSV (UAZ 00-173B) was generated in *Litopenaeus vannamei* (Holthuis, 1980) from an original outbreak of WSD in *Fenneropeneaus chinensis*

(Holthuis, 1980) in China in 1995. Subsequent passage of this isolate into Specific Pathogen Free (SPF) *L. vannamei* held at the Cefas Weymouth laboratory have demonstrated continued virulence of this isolate (data not reported here). All challenges reported utilised WSSV-infected *L. vannamei* carcasses generated within the Cefas Weymouth laboratory. As such, WSSV-infected shrimp carcasses were prepared by injection of the UAZ 00-173B isolate into SPF *L. vannamei* obtained from the Centre for Sustainable Aquaculture Research (CSAR) at the University of Swansea, United Kingdom. Individual *L. vannamei* were inoculated via intra-muscular injection of the diluted viral homogenate at a rate of 10 μl g^{-1} shrimp weight. Following incubation, dead and moribund shrimp were removed from the experimental tanks and infection with WSSV was confirmed using histopathology, transmission electron microscopy (TEM) and PCR as appropriate (see below for techniques). Remaining tissues were stored at –80^{o}C until required. Confirmed infected and uninfected (sham-injected) carcasses were used to prepare inoculums and feed rations for challenge studies using the non-model host species. Infected and uninfected shrimp carcasses were macerated in isolated conditions using a sterile razor blade prior to homogenisation in 2% sterile saline, see appendix, (4ml of saline per gram of minced tissue) using a blender until tissues were liquefied. The homogenate was centrifuged at 5,000 x g for 20 minutes at 4°C to pellet solid debris prior to the supernatant being diluted 1:20 with sterile saline and filtered (0.2µm Minisart syringe filter, Sartorius Stedim Biotech GmbH, Germany) to form the inoculum for the injection studies. For feeding trials, confirmed infected and uninfected carcasses were macerated into approximately 2-3 mm^3 blocks using a sterile razor blade immediately prior to feeding.

For all exposure trials, a similar protocol was followed. Group 1 (negative control feed) animals were fed with a single ration of confirmed SPF shrimp tissue at approximately 5% bodyweight on Day 0 of each trial. Group 2 (WSSV positive feed) animals were fed with a single ration of confirmed WSSV-infected (but otherwise SPF) shrimp tissue at a ratio of approximately 5% bodyweight on Day 0 of each trial. Group 3 (WSSV positive injection) animals were injected with a single dose of the diluted WSSV homogenate (see above) at a rate of 10µl g^{-1}

wet body weight, on Day 0 of each trial. Since the key objective of this study was to investigate the potential for WSSV infection via a confirmed natural route (i.e. feeding), and the fact that I do not consider direct injection as a natural route of infection for WSSV (see Stentiford *et al*., 2009), Group 3 served purely to introduce WSSV into hosts for the investigation of histopathological progression of disease in non-model decapods. As such, a negative control (sham-injected) group was not included in the trials. Thereafter, samples in all tanks were fed on squid tissues at a ratio of approximately 3-4% wet body weight.day^{-1} for the remainder of the trial period. Tanks were observed regularly throughout daylight hours. Dead and terminally morbid samples were removed from each tank and dissected. At the end of each challenge trial (Day 10), and for moribund animals sampled within the trial, surviving animals were chilled on ice for 30 minutes prior to dissection. As standard, gill, epidermis, hepatopancreas, heart, gonad, nerve and muscle were placed into histological cassettes and fixed immediately in the appropriate fixative. Davidson's fixative made with seawater (Davidson's seawater, see appendix) was used for tissues from marine species and Davidson's fixative made with tap water (Freshwater Davidson's, see appendix) was used for all freshwater crayfish tissues. For molecular analyses, gill, epidermis and hepatopancreas samples were removed and placed into tubes containing 100% ethanol. For electron microscopy, gill, epidermis, hepatopancreas and heart tissues were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for electron microscopy (EM). The remaining tissues and carcasses were stored at -80°C.

3.3.4 gy and transmission electron microscopy

For histology, tissue fixation was allowed to proceed for 24 h before samples were transferred to 70 % industrial methylated spirit. Fixed samples were processed to wax in a vacuum infiltration processor (Leica Peloris) using standard protocols (see appendix). Sections were cut at a thickness of 3-5 µm on a rotary microtome and were mounted onto glass slides before staining with haematoxylin and eosin (H&E) and Feulgen stains. Stained sections were analysed by light microscopy (Nikon Eclipse E800) and digital images and measurements were taken using the Lucia™ Screen Measurement System (Nikon, UK). For electron microscopy, tissues were fixed in 2.5 % glutaraldehyde in 0.1 M sodium

cacodylate buffer (pH 7.4) for 2 h at room temperature and rinsed in 0.1 M sodium cacodylate buffer (pH 7.4). Tissues were post-fixed for 1 h in 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer. Samples were washed in three changes of 0.1 M sodium cacodylate buffer before dehydration through a graded acetone series. Samples were embedded in Agar 100 epoxy (Agar Scientific, Agar 100 pre-mix kit medium) and polymerised overnight at 60ºC in an oven. Semi-thin (1-2 µm) sections were stained with Toluidine Blue for viewing with a light microscope to identify suitable target areas. Ultrathin sections (70-90 nm) of these areas were mounted on uncoated copper grids and stained with 2 % aqueous uranyl acetate and Reynolds' lead citrate (Reynolds, 1963). Grids were examined using a JEOL JEM 1210 transmission electron microscope and digital images captured using a Gatan Erlangshen ES500W camera and Gatan Digital Micrograph™ software.

3.3.5 xtraction, PCR and sequencing

Genomic DNA was extracted from tissues using a High Pure PCR Template Preparation Kit (Roche Diagnostics) following the manufacturer's protocols. Ethanol-preserved tissues were soaked in molecular grade H_2O prior to DNA extraction, to remove trace ethanol. DNA was eluted in 100 µl elution buffer and quantified using a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific). WSSV-infected and specific pathogen free (SPF) shrimp tissue samples were obtained from the OIE reference laboratory in Arizona (Prof. Don Lightner, Aquaculture Pathology Laboratory, University of Arizona, Tucson, USA) and DNA was extracted as above. These were used as positive and negative control material for WSSV in subsequent molecular diagnostic assays. To ensure that amplifiable DNA was present in all extracted samples, genomic DNAs were assessed using the universal small subunit ribosomal RNA (SSU-rRNA) gene primers 16S-A (5'-AACCTGGTTGATCCTGCCAGT-3') and 16S-B (5'-GATCCTTCTGCAGGTTCACCTAC-3') (Medlin *et al*., 1998) with an expected amplification product of approximately 1800 bp. Each 20 µl reaction contained 1x Green GoTaq Flexi Buffer (Promega), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 1 µM of each primer, 1 unit *Taq* polymerase (Promega), and 1 µl genomic DNA (20-50 ng total). Amplifications were performed with an initial denaturation

temperature of 94 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, 45 °C for 30 s, 65 °C for 2 min, with a final elongation step at 65 °C for 5 min. Following amplification, 5 μl of each PCR product were analysed by agarose gel electrophoresis (1.5 % w/v), stained with ethidium bromide (0.625mg/ml), and viewed under a UV light source. Images were captured with a Gel Doc 2000 (Bio Rad) imaging system.

WSSV-infection status of tissues was confirmed using the nested PCR assay of (Lo *et al*., 1996b) with minor modifications (Ms. Bonnie Poulos, University of Arizona, personal communication). First, a product of 1447 bp was amplified using the primer pair 146F1 (5'-ACTACTAACTTCAGCCTATCTAG-3') and 146R1 (5'-TAATGCGGGTGTAATGTTCTTACGA-3'), followed by an approximate 941 bp product in the nested reaction using primer pair 146F2 (5'-GTAACTCCCCCTTCCATCTCCA 3') and 146R2 (5'TACGGCAGCTGCTGCACCT-TGT-3'). For the first round of amplification (primer pair 146F1/146R1) each 25 μl PCR reaction contained the following: 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 200 μM of each dNTP, 0.31 μM of each primer, 2.5 units *Taq* polymerase (Promega), and 1 μl genomic DNA (20-50 ng total). Amplifications were performed with an initial denaturation temperature of 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, 62°C for 30 s, 72°C for 30 s, with a final elongation step at 72°C for 2 min. Reaction conditions and reagent concentrations were the same for the second round of amplification using the 146F2/146R2 primer pair, however 0.5 μl of the first round of amplification was used as a template in place of genomic DNA. Following amplification, 10 μl samples of the second round PCR product were analysed by agarose gel electrophoresis as described above. WSSV-challenged tissue samples that were negative by PCR were re-analysed for a second time by the nested WSSV PCR assay to confirm the result.

3.3.6 quencing

PCR amplification products generated in non-target decapod host species exposed to WSSV via feeding of WSSV-infected shrimp tissues were sequenced for confirmation of their identity. Reactions were analysed on an ABI 3130 Avant

Genetic Analyser. The final product was compared to known sequences deposited in GenBank and EMBL, using Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990) to determine phylogenetic homology. This is in line with OIE confirmatory technique for WSSV (Claydon *et al*., 2004).

All PCR and sequencing was conducted by Mr Paul Martin.

3.3.7 oassay of non-target decapod tissues to *L. vannamei*

Following PCR confirmation of the presence of amplicons of WSSV in tissues of WSSV-exposed non-target decapods, the infectivity of these tissues (from each test species) was tested via bioassay exposure to a known WSSV susceptible species: *L. vannamei*. Tissues from each test species were homogenised using the aforementioned approach for shrimp. Control innoculum was also prepared for each test species using confirmed WSSV PCR negative samples. Innoculum was diluted using sterile saline and filtered (0.2µm Minisart syringe filter, Sartorius Stedim Biotech GmbH, Germany) prior to intramuscular injection of SPF shrimp (10µl g^{-1} body weight). Shrimp exposed to confirmed WSSV PCR positive and negative innocula were observed for a period of 5 days. A total of 14 tanks, containing 5 shrimp per tank were maintained at a water temperature of 24˚C and observed regularly throughout daylight hours. Dead and terminally moribund animals were removed from each tank and sampled as above. At the end of the challenge period (Day 5), all surviving shrimp were sampled. Pleopods were removed from each shrimp and fixed in 100% ethanol for WSSV detection by PCR (as above). The remaining shrimp carcass was injected with Davidson's seawater fixative and shrimp placed whole into the same fixative for histology (as above).

3.3.8 tatistical analysis – Kaplan Meier Survivor Estimate

The Kaplan Meier survivor estimate test was used to analyse mortality data generated throughout this study (Kaplan and Meier, 1958). Data was entered into a table listing species, number of animals entered at beginning of study, treatment, and numbers of survivors and mortalities were listed for each day of the study. For each time interval (number of days after exposure to treatment) the percentage surviving at the start of the interval is equal to the probability of

surviving each of the preceding intervals all multiplied together. All statistical tests were conducted with the assistance of Dr Allan Reese using the software package Stata Version 9.0.

3.4 Results

3.4.1 athogenicity and Replication

A full histopathology screen was conducted on tissues sampled from each of the exposed species, Table 2 summarises the findings.

Table 2. White Spot Disease pathology shown in differing tissue types for each species of non-model crustacean hosts after exposure via feeding and injection.

	Cancer pagurus		***Homarus gammarus***		***Nephrops norvegicus***		***Austropotomobius pallipes***		***Pacifastacus leniusculus***		***Eriocheir sinensis***		***Carcinus maenas***	
	Group 2	**Group 3**	**Group 2**	**Group 3**	**Group 2**	**Group 3**	**Group 2**	**Group 3**	**Group 2**	**Group 3**	**Group 2**	**Group 3**	**Group 2**	**Group 3**
Gill	*	***	*	***	*	***	***	m	***	***	***	***	-	*
Heart	-	*	*	**	-	**	*	m	*	*	***	***	-	*
Ovary	-	*	-	**	-	**	*	m	*	*	**	**	-	-
Testis	-	*	-	*	-	*	*	m	*	*	*	*	-	-
Nerve	N/A	N/A	-	**	-	*	**	m	*	*	**	**	N/A	N/A
Connective Tissue	*	**	*	**	*	**	**	m	***	***	***	***	-	-
Cuticular epithelium	*	**	*	***	*	***	***	m	***	***	***	***	-	-
Fixed Phagocytes	*	**	*	**	*	**	***	m	***	***	***	***	-	-
Haemolymph	*	**	*	*	*	*	**	m	**	**	***	***	-	-

* Few infected nuclei present
** Infected nuclei prevalent throughout tissue
*** Infected nuclei highly prevalent throughout tissue
\- Pathology not evident
m Pathology not evident due to mortalities

In all species, WSSV associated pathology was most pronounced within the cuticular epithelium of the gill (Fig. 1A). WSSV-infected nuclei were enlarged with marginalized chromatin, and often contained a distinct eosinophillic inclusion body (Fig. 1B). Infected nuclei were also identified within the cuticular epithelium (Figure 1C & D) and underlying connective tissues of the body and appendages. Haemocytes (Figure 2A) throughout the tissues and the connective tissues surrounding the blood vessels and nerves (Figure 2B) also showed signs of infection. Within the heart the spongy connective tissues within the epicardium showed classic WSD pathology (Figure 2C) as did the sarcolemma surrounding the muscle fibres of the myocardium (Figure 2D). The germinal epithelium surrounding the oocytes (Figure 3A, B) and the connective tissues in the testes in some species also showed signs of infection (Figure 3C, D).

The Lymphoid organ (LO) is found exclusively in penaeid shrimp; other crustacea such as crabs, lobsters and crayfish do not possess this organ (Rusaini and Owens, 2010) but instead possess fixed phagocytes within the haemal spaces of the hepatopancreas. These structures are thought to perform a similar role to the LO in the immune response to invading pathogens (Johnson, 1987). Fixed phagocytes within the hepatopancreas displayed enlarged nuclei containing eosinophillic inclusions (Figure 3E, F). However, despite the fixed phagocytes in this organ displaying signs of infection the hepatopancreatic tubules themselves were not infected.

Histological assessment of tissues from WSSV-injected *A. pallipes* did not show classic WSD pathology, instead there was widespread apoptosis of the tissues, especially of the haemocytes, and a heavy bacterial infection in most samples. In these cases 100% mortality occurred within 48 hours, all samples were PCR positive; however, this result is likely to be detection of residual inoculum. In *C. maenas* samples pathology was evident within the tissues of the WSSV positive injected group but at a much lower severity to that of the other species. It was extremely difficult to identify areas of pathology within this species with fewer infected nuclei being evident within the cuticular epithelium of the gill and heart tissues (Table 3).

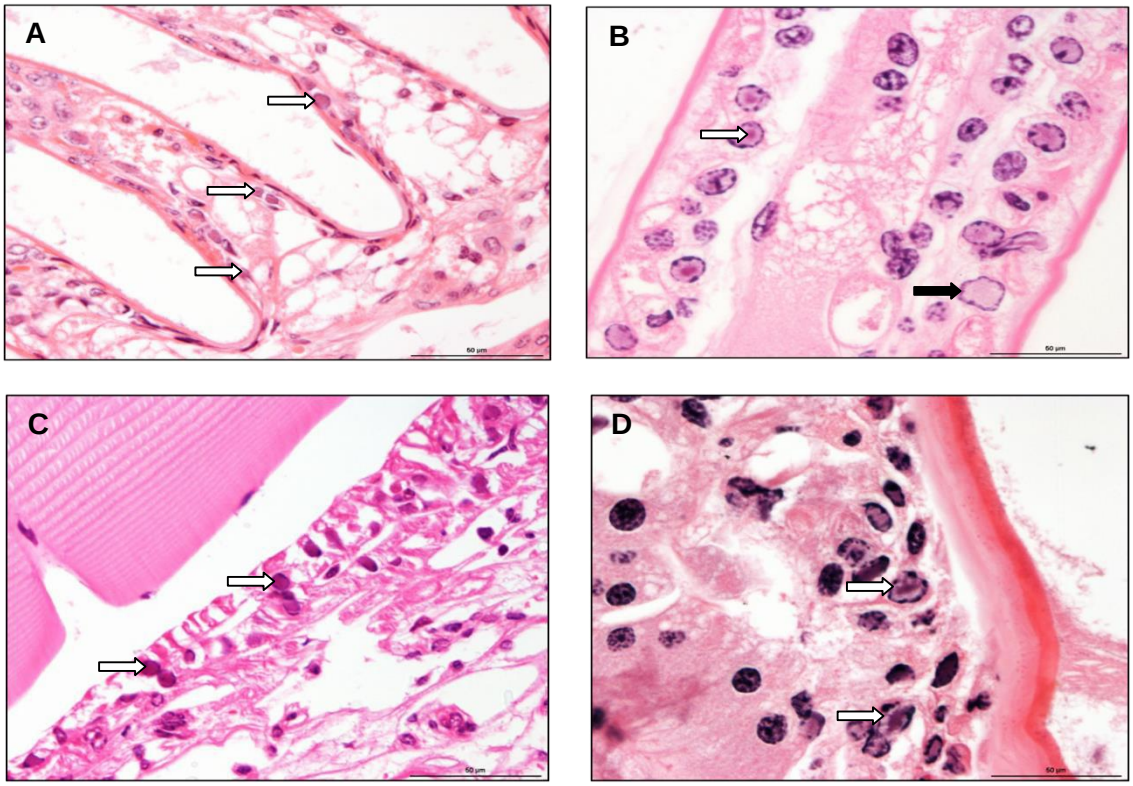

Figure 1. WSSV infected tissues, images taken from gill and cuticular epithelium. (A) Infected nuclei (arrows) can be observed scattered throughout the gill filaments of *Homarus gammarus*. Scale bar = 50µm. (B) Hypertrophied nuclei can be found within the gill filaments displaying marginalized chromatin (black arrow) and eosinophillic inclusion bodies (white arrow) in *Pacifastacus leniusculus*. Scale bar = 50µm. (C) Cuticular epithelium showed signs of infection with hypertrophied nuclei (arrows) evident dispersed throughout the tissue of *Nephrops norvegicus*. Scale bar = 50µm. (D) Cuticular epithelium showed signs of infection with hypertrophied nuclei (arrows) evident dispersed throughout the tissue of *Pacifastacus leniusculus*. Scale bar = 50µm. All images H&E stain.

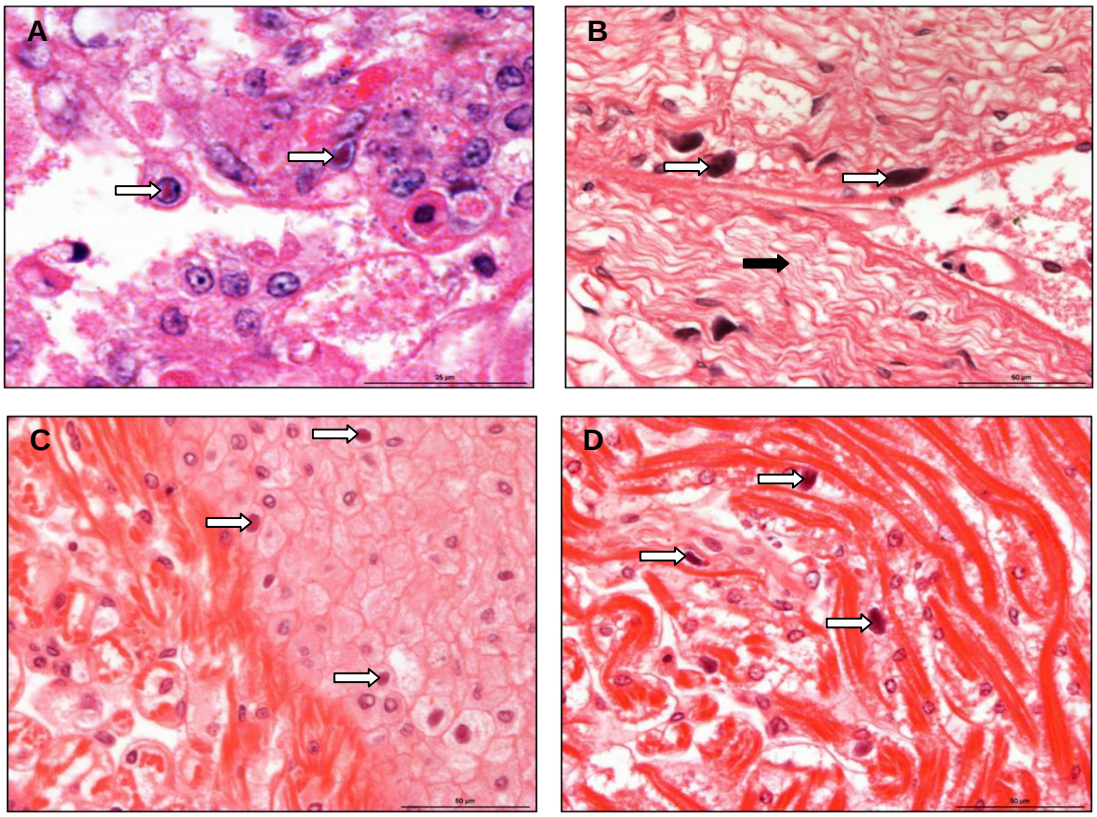

Figure 2. Representative images of WSSV infected tissues taken from a range of species. (A) Infected nuclei present within the haemocytes (arrows), haemocytes present in the haemal spaces between the hepatopancreatic tubules of *Cancer pagurus*. Scale bar = 25µm. (B) Infected nuclei (white arrows) were evident within the connective tissue surrounding the nerve fibres (black arrow) in *Eriocheir sinensis*. Scale bar = 50µm. (C) Infected nuclei (arrows) were present within the spongy connective tissue of the epicardium of *Homarus gammarus*. Scale bar = 50µm. (D) Hypertrophied nuclei (arrows) can also be observed infecting the sarcolemma of the heart muscle in *Eriocheir sinensis*. Scale bar = 50µm. All images H&E stain.

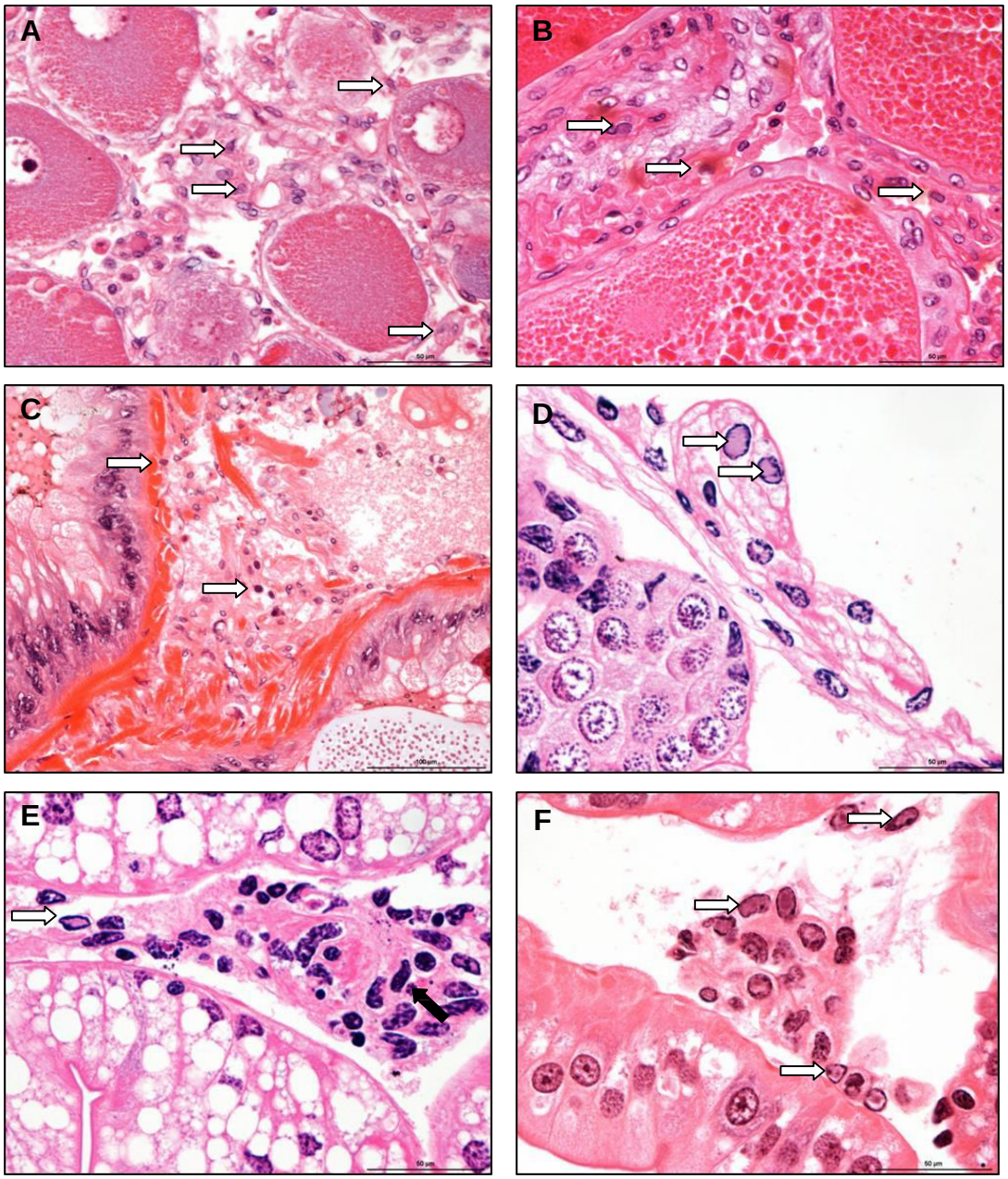

Figure 3. WSSV infected gonad and connective tissues. (A) Infected nuclei (arrows) were present within the connective tissues within the ovary and surrounding the developing oocytes of *Cancer pagurus*. Scale bar = 50µm. (B) Infected nuclei (arrows) were present within the connective tissues within the ovary and surrounding the developing oocytes of *Homarus gammarus*. Scale bar = 50µm. (C) Infected nuclei (arrows) were present within the connective tissues surrounding the testes of *Eriocheir sinensis*. Scale bar = 100µm. (D) Infected nuclei (arrows) were present within the connective tissues surrounding the testes of *Pascifastacus leniusculus*. Scale bar = 50µm. (E) Fixed phagocyte cells from *Pascifastacus leniusculus* displaying signs of infection, nuclei containing marginalized chromatin and an eosinophillic inclusion (white arrow). Areas of apoptotic cells (black arrows) were also evident within these cells. Scale bar = 50µm. (F) Fixed phagocyte cells displayed signs of infection in *Austropotomobius pallipes*, nuclei containing marginalized chromatin and an eosinophillic inclusion (arrow). Scale bar = 50µm. All images H&E stain.

All non-model host species, except *A. pallipes,* were sourced from wild fisheries in the United Kingdom; it was not possible to ensure these hosts were disease free at the start of the trial. In addition to classic WSD pathology being present

within the tissues histology also revealed other co-infections. These pathogens were pre-existing conditions and present in all exposure groups, and as such unlikely to be the cause of mortalities in WSSV exposed animals. Pink Crab Disease (Stentiford *et al*., 2002) caused by *Hematodinium* sp. was seen infecting 27% of *C. pagurus* sampled. *Nicathoë astaci* was evident in gill samples of 50% *H. gammarus* sampled. Low level infections of *Austropotomobius pallipes* Bacilliform Virus (*ApBV*) (Edgerton *et al*., 2002b) were evident within the hepatopancreas of 60% of *A. pallipes* sampled. *ApBV* infected nuclei were present in the epithelial lining of the hepatopancreatic tubules and as such could be distinguished from WSSV infection. A microsporidian infection, *Hepatospora eriocheir,* previously described by (Stentiford *et al*., 2011) was seen in the hepatopancreatic tubules in 51% of *E. sinensis* sampled. *C. maenas* samples showed a range of pathogens within the tissues including: *Microphallus primas* (Saville and Irwin, 2005), *Sacculina carcini (Boschma, 1955)*, Milky disease (Eddy *et al*., 2007), microsporidian infection of the muscle and *Hematodinium perezi* (Chatton and Poisson, 1931).

Electron microscopy of the gill tissues from each species revealed replicating virus particles within hypertrophied nuclei. Nuclei were enlarged with marginalised chromatin and contained virions at varying stages of development (Figure 4A). Fully formed virions were ovoid to elliptical in shape and contained an electron dense nucleocapsid surrounded by a trilaminar envelope (Figure 4B & 4C). Virion measurement varied according to stage of development but measurements of fully formed virions were consistent with those previously described for WSSV (Durand *et al*., 1997; Inouye *et al*., 1994; van Hulten *et al*., 2001; Wang *et al*., 1995). Patent WSSV infections in *E. sinensis* samples showed paracrystalline arrays of virus particles at the periphery of the infected nuclei (Figure 4D). Unenveloped nucelocapsid material was present within the nuclei of some hosts; this material appeared cross-hatched or striated and appeared to be composed of rings of subunits in a stacked series (Figure 4E). This is a characteristic feature of WSSV ultrastructure (Huang *et al*., 2001). Interestingly viral particles present within *C. maenas* samples appeared slightly different to classic WSSV ultrastructure shown in other species, although the unenveloped nucleocapsid material appeared identical to that in other species the viral particles themselves differed. The nucleocapsid within viral particles in various

stages of development in *C. maenas* appeared curved or ‘u’ shaped within the envelope (Fig. 4F).

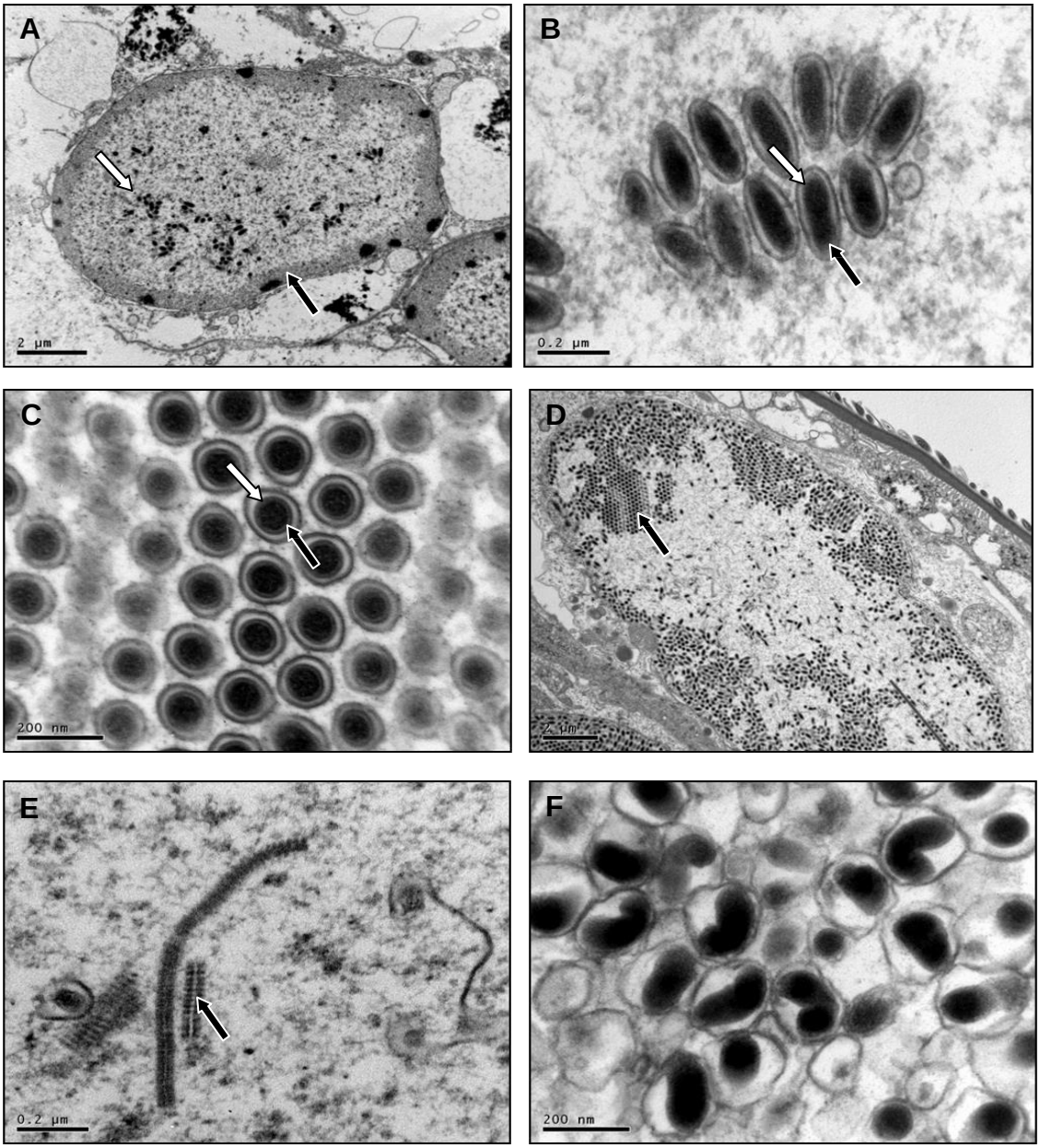

Figure 4. Transmission Electron Microscope (TEM) images of WSSV infections. (A) Infected nuclei (arrows) displaying marginalised host chromatin (black arrow) containing developing viral particles (white arrow) within gill tissue of Homarus gammarus. Scale bar = 2µm. (B) Longitudinal cross section of WSSV virions, ovoid electron dense nucleocapsid (white arrow) can be seen within a trilaminar envelope (black arrow). Scale bar = 0.2µm. (C) Transverse cross section of WSSV virions, electron dense nucleocapsid (white arrow) can be seen within a trilaminar envelope (black arrow). Scale bar = 200nm. (D) Heavily infected nucleus from Eriocheir sinensis, virions can be seen in paracrystalline arrays at the periphery of the nucleus (arrow). Scale bar = 2µm. (E) Presumptive nucleocapsid material within the nucleus prior to envelopment, material appears cross-hatched or striated and appears to be composed of rings of subunits in a stacked series (arrow). Scale bar = 0.2µm. (F) Virion particles from Carcinus maenas appeared slightly different from the classic WSSV particles, the nucleocapsids in developing particles appeared slightly curved or 'u' shaped within the envelope. Scale bar = 200nm.

3.4.2 and Sequencing

PCR analysis of gill tissues from the WSSV-injected sample group of all species revealed universal positivity for WSSV. PCR analysis of gill tissues from WSSV-fed groups of the different non-model host species revealed varying proportions of WSSV positive samples as follows: 33% C. pagurus, 20% C. maenas 17% H. gammarus, 65% N. norvegicus, 10% E. sinensis, 40% A. pallipes and 5% P. leniusculus. For confirmation of identity, a single WSSV PCR-positive sample from each of test species was sequenced. All sequenced samples showed between 99.6% and 99.8% similarity to the WSSV isolate utilised for challenge (OIE isolate of WSSV UAZ 00-173B). All crabs, lobsters and crayfish fed SPF shrimp tissues were PCR negative for WSSV.

These results confirm that the non-model species tested in this study were considered susceptible to infection with WSSV and that the virus is able to replicate and remain virulent within these species.

3.4.3 oassay

Viability of the agent (within the non-model host) was confirmed via passage bioassay of infected host tissue to SPF *L. vannamei*. In this case, the majority of SPF shrimp exposed to WSSV positive inoculum arising from non-model hosts died within 3 days post-injection. All mortalities and all remaining shrimp culled at the end of the challenge were PCR positive for WSSV. All shrimp showed classic signs of WSD infection (Lightner, 1996a) via histology (data not shown). These results confirm that these test species are susceptible to WSSV and that the virus is viable within these species and present at a high enough viral loading to cause disease.

Shore crab inoculum generated for the shrimp bioassay was originally diluted 1:20 as per other inoculates; however, all SPF shrimp died within 4 hours post-injection. This highlighted a problem with the original inoculates, shrimp most likely died from ‘protein shock’ post-injection (Lightner, 1996a). If the inoculum contained excessive amounts of proteinaceous material it could have caused systemic clotting of the haemolymph within the shrimp that led to the mortalities. The inoculum was diluted again (1:40 total dilution) and the trial repeated. 100% mortality was observed after 5 days with the second exposure and all shrimp were found to be PCR positive for WSSV.

3.4.4 *Kaplan Meier* Analysis

Figure 5 shows the Kaplan Meier survival curves generated for each species and highlight the differences seen between the treatments. No mortalities were observed within Group 1, control, in all exposed species except *P. leniusculus* where 12% mortality occurred. Histology revealed these mortalities were most likely caused by a heavy bacterial infection (Figure 6A & 6B). No mortalities were seen in Group 2, WSSV positive feed except *C. maenas* where 7% mortality was observed. Histology samples taken from these mortalities did not show classic WSD pathology, instead a heavy microsporidian infection was observed within the muscle of these crabs (Figure 6C & 6D). Group 3, WSSV positive injection caused the highest level of mortalities as highlighted. If the survival curves of each species exposed via injection are compared on a single plot (Figure 7) they form three groupings. No mortalities were seen in *E. sinensis* (Figure 7, Group A) exposures despite high levels of pathology being present within these samples. The marine species (*C. pagurus, H. gammarus, N. norvegicus* and *C. maenas*) appear to group together (Figure 7, Group B) and the freshwater species (*P. leniusculus* and *A. pallipes*) showed higher levels of mortality after WSSV injection earlier in the challenge than marine species and form the third grouping (Figure 7, Group C).

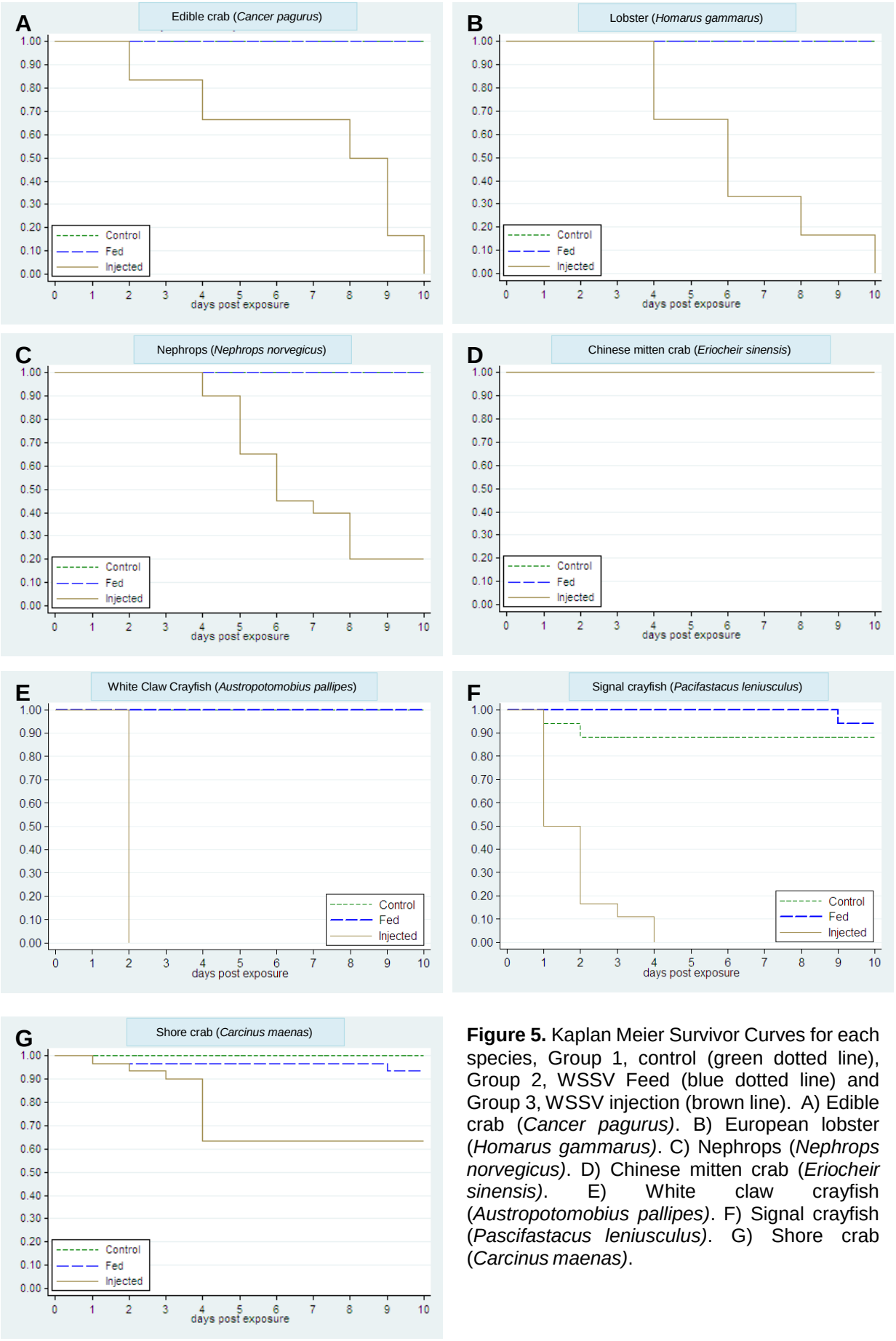

Figure 5. Kaplan Meier Survivor Curves for each species, Group 1, control (green dotted line), Group 2, WSSV Feed (blue dotted line) and Group 3, WSSV injection (brown line). A) Edible crab (*Cancer pagurus)*. B) European lobster (*Homarus gammarus)*. C) Nephrops (*Nephrops norvegicus)*. D) Chinese mitten crab (*Eriocheir sinensis)*. E) White claw crayfish (*Austropotomobius pallipes)*. F) Signal crayfish (*Pascifastacus leniusculus)*. G) Shore crab (*Carcinus maenas)*.

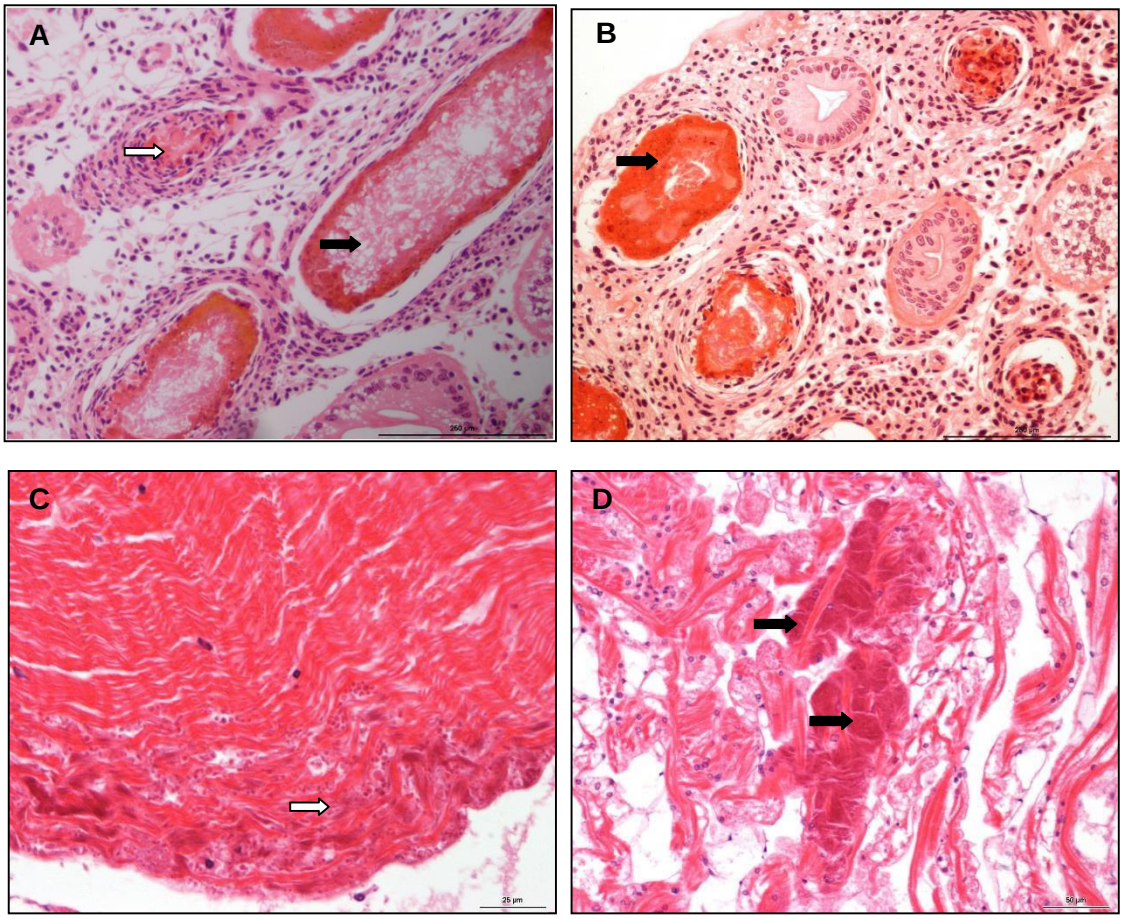

Figure 6. Histology images of likely causes of control mortalities in *Pascifastacus leniusculus* and WSSV fed *Carcinus maenas*. (A) Haemocytic host response in *Pascifastacus leniusculus*. Haemocytes can been seen encapsulating bacteria (white arrow) in the haemal sinus of the hepatopancreas. Encapsulated cells melanise to destroy the invading bacteria cells (black arrow). Scale bar = 200µm. (B) Necrotic melanised nodules (black arrow) within the haemal sinuses of the hepatopancreas of *Pascifastacus leniusculus*. Scale bar = 200µm (C) Microsporidian infection of body muscle from *Carcinus maenas*. Spherical inclusions can be seen invading the muscle fibres (white arrow). Scale bar = 25µm. (D) Microsporidian infection of heart muscle from *Carcinus maenas*. Early infection, confined to the sarcolemma of heart myofibres as shown by dense staining (black arrow). Scale bar = 25µm. All images H&E Stain.

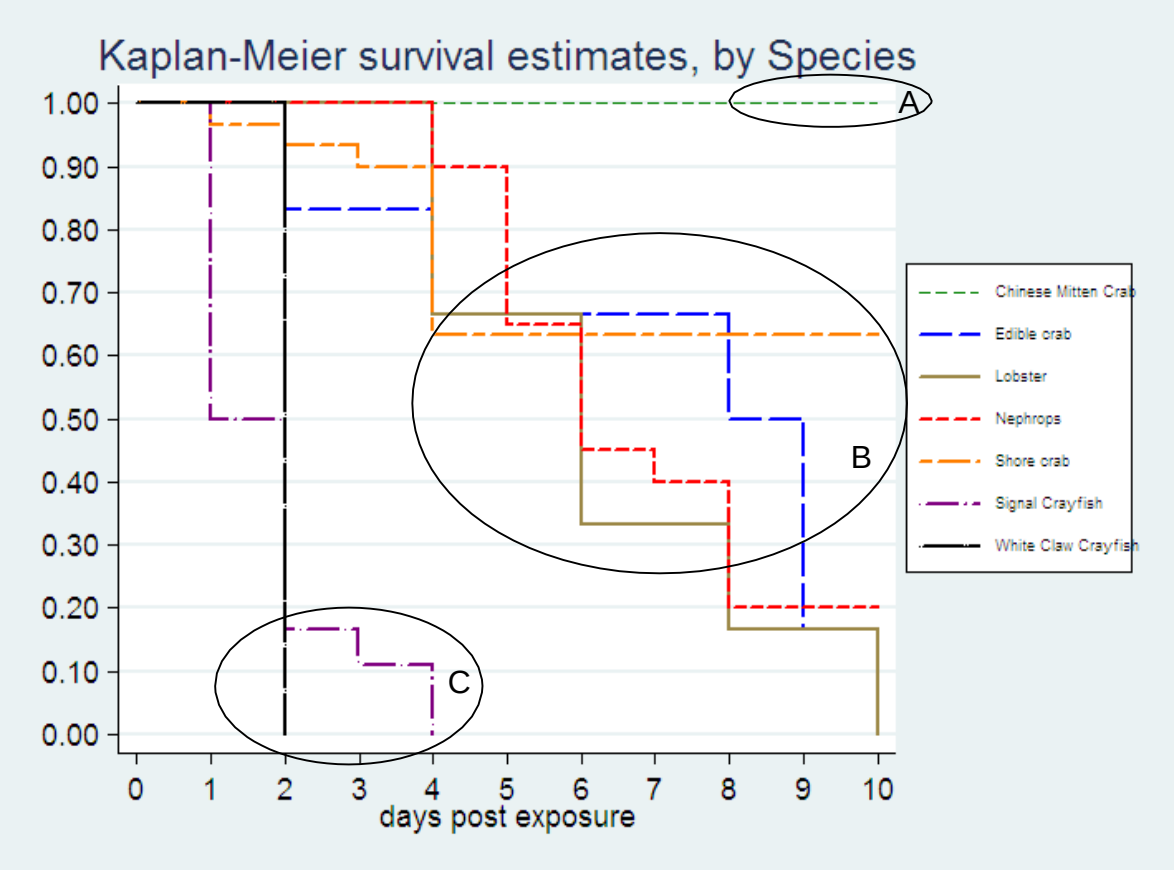

Figure 7. Kaplan Meier Survivor Curves for each species after exposure to WSSV via injection. Group A highlights that no mortalities were observed in *Eriocheir sinensis*. Marine species highlighted by Group B (*Cancer pagurus, Homarus gammarus, Nephrops norvegicus* and *Carcinus maenas*) appear to show similar pattern of survival as do freshwater species *Austropotomobius pallipes* and *Pascifastacus leniusculus*) as highlighted by Group C.

3.4.5 ceptibility to infection

Using the EFSA categories (Table 1) replication, pathology and location were shown in all species via the presence of intranuclear inclusion bodies throughout the tissues and via TEM. Although this study shows fulfilment of the EFSA WSD susceptibility criteria there were considerable differences between pathological outcomes in the respective species, particularly when the mode of virus dose delivery is considered (Table 3).

Table 3. Implementation of EFSA criteria A-D for exposure of non-model crustacean hosts to WSD. Table shows criteria that were fulfilled for each species and exposure route, asterisks (*) highlight where a positive result was shown.

	A. Replication	B. Viability	C. Pathology	D. Location
Cancer pagurus				
WSSV positive feed	*	*	*	*
WSSV positive injection	*	*	*	*
Carcinus maenas				
WSSV positive feed	-	*	-	-
WSSV positive injection	*	*	*	*
Homarus gammarus				
WSSV positive feed	*	*	*	*
WSSV positive injection	*	*	*	*
Nephrops norvegicus				
WSSV positive feed	*	*	*	*
WSSV positive injection	*	*	*	*
Eriocheir sinensis				
WSSV positive feed	*	*	*	*
WSSV positive injection	*	*	*	*
Austropotamobius pallipes				
WSSV positive feed	*	*	*	*
WSSV positive injection	-	*	-	-
Pacifastacus leniusculus				
WSSV positive feed	*	*	*	*
WSSV positive injection	*	*	*	*

All non-model species, apart from *C. maenas* and *A. pallipes* fulfilled all four susceptibility criteria via feeding and injection. In the case of *C. maenas*, replication was not observed in target tissues from animals fed with WSSV, however when tissues from *C. maenas* formed the basis of an innoculum for bioassay to SPF *L. vannamei,* characteristic signs of WSD, and mortalities occurred in shrimp. This finding is suggestive of infection in *C. maenas* with viral loading below that required to cause pathology in target tissues of this species within the time frame of the study. Replication in injected *A. pallipes* could not be

confirmed due to a rapid, non-specific mortality following injection of innoculum. Replication was demonstrated however following feeding.

3.5 Discussion

This study demonstrates universal susceptibility to WSSV infection in a range of European decapod crustaceans, exposed to WSSV via a natural (feeding) and artificial (injection) route. Furthermore, it shows that WSSV can replicate and cause disease within European crustacean species at ambient water temperatures encountered in European waterways. The manifestation of the disease differs considerably between species; freshwater crayfish undergoing more rapid mortality and development of pathognomonic signs than most of the marine species tested. Despite becoming infected with WSSV following exposure, the European shore crab (*Carcinus maenas*) appeared most resistant to the development of disease and could be considered as an asymptomatic carrier under the conditions utilised in these trials. Stentiford *et al*. (2009) have previously listed all the species that have been shown by experimental and natural exposures to be susceptible to WSD using the EFSA guidelines. Not all listed species listed in Stentiford *et al*. (2009) fulfilled the categories A-D and this was recorded as 'no data available'. Following work by Corbel *et al*. (2001) replication and partial demonstration of location were shown in *C. pagurus* and *C. maenas*. Exposures described here have repeated this work and have shown that WSD can replicate, cause pathology and the virus is viable (by bioassay), fulfilling all four categories within the EFSA criteria. There have been several reports of WSD susceptibility in freshwater crayfish species (Baumgartner *et al*., 2009; Edgerton, 2004; Huang *et al*., 2001; Jiravanichpaisal *et al*., 2001; Soowannayan and Phanthura, 2011). *P. leniusculus* is listed as fulfilling three of the four categories, replication, pathology and location but not viability. Exposures with *P. leniusculus* were repeated in this study and showed that the virus is still viable after passage within this species. This study shows that *H. gammarus, C. pagurus, C. maenas, N. norvegicus, E. sinensis, P. leniusculus* and *A. pallipes* are susceptible to WSD according to the criteria highlighted by EFSA. This is the first description of WSD susceptibility for *H. gammarus, N. norvegicus, E. sinensis* and *A. pallipes.*

Studies with penaeid shrimp have shown that WSSV targets crustacean tissues of ectodermal and endodermal origin. It is most commonly detected in the cuticular epithelium, particularly associated with the gill and other appendages, or in the epithelial layer surrounding the stomach (Lightner, 1996a). Despite the wide susceptible host range to WSSV, few studies have focussed on the pathognomonic signs of infection (and disease) in these species. In the current study, no definitive external signs (such as white spots) were associated with infection or disease in non-model hosts. WSSV infection and WSD could however be detected using histology in a manner similar to that previously described for penaeid shrimp (Lightner, 1996a; Lo *et al*., 1997; Wongteerasupaya *et al*., 1995). Characteristic eosinophillic inclusion bodies within enlarged nuclei were evident in epithelial cells of the gill and carapace, connective tissues, fixed phagocytes, haemolymph, heart, nerve, ovary and testes. In penaeids, such eosinophillic bodies are usually observed in early stage WSSV infection, these staining more basophilic as disease progresses (Lightner, 1996a). Although this indicates that the pathology within individual cells of WSSV-infected non-model hosts were at a relatively early stage, longer term exposure trials may have led to a higher frequency of basophilic types as observed in penaeid hosts. Infection levels within these tissues were variable; some tissues such as the cuticular epithelium and the underlying connective tissues displayed more infected nuclei than nerve or heart tissues.

Variation was also seen between the fed and injected animals with fewer infected nuclei evident in fed animals; however, this did not apply to all (*P. leniusculus, A. pallipes* and *E. sinensis*) suggesting that some species develop infections quicker than others and may require a lower viral loading for an infection to develop. Injected animals are being given inoculums straight into the haemolymph; virions can be transported directly to target tissues. Feeding represents a more realistic route of entry for pathogens (such as WSSV), than that provided by direct injection. In order to cause an infection, the pathogen must cross the intestinal wall and reach target tissues still in a viable form, this method would likely take longer for an infection to establish. Not all of the individuals fed on WSSV positive shrimp became infected with the virus. This could be due to a number of reasons, although they were all given the same amount of food (5% body weight) some did not eat all of the food provided. Shrimp were not homogenised prior to feeding

so it is possible that there was variation in viral loading between feed portions. However, Oidtmann & Stentiford (2011) have shown that viral load did not appear to vary greatly between different shrimp tissue types. It is likely that at the individual level the viral loading required to establish an infection, latent or patent will vary greatly according to that individual's health status as highlighted below.

WSD nuclei were evident within the epithelium and connective tissues surrounding the oocytes and spermatozoa as highlighted by Lo *et al*. (1997). It is not known whether WSD can be vertically transmitted (Lo *et al*., 1997; Stentiford *et al*., 2009); further work would be needed to establish whether this is a possibility in these species. The absence of a lymphoid organ in hosts other than members of the Superfamily Penaeoidea (including the genus *Penaeus*) (Rusaini and Owens, 2010) prevent this organ being used as a sentinel for viral infection in non-model hosts. However, characteristic pathognomonic signs comparative to those observed within penaeids during WSD (Lightner, 1996a), were seen in the fixed phagocytes of the hepatopancreas of non-model hosts, suggesting that this system is progressively degraded during pathogenesis of WSSV infection in non-model hosts. It is likely therefore that the fixed phagocyte system provides a comparative target (to the lymphoid organ) for WSSV in non-model crustaceans.

This study has highlighted differential levels of pathology seen between the different species and routes of exposure. By examining these data I have been able to define three broad categories of relative susceptibility to WSSV infection and disease in crustacean hosts (Table 3). Determining susceptibility of a species to a certain disease is a far more involved process than a simple 'yes or no' classification as there are multiple variables which need to be taken into account. The concept of 'susceptibility' in hosts should not only be considered at the species level but also at the level of the individual within that species. For example route of exposure, viral loading of inoculates, mortalities, viability, pathology and pre-existing disease status all need to be considered to evaluate whether an individual is susceptible or not and the level of susceptibility. Susceptibility appeared to differ between individuals of the same species, possibly affected by the individuals pre-existing disease profile at point of infection. This study has clearly shown that some species such as the freshwater

crayfish species *P. leniusculus* and *A. pallipes* display characteristic signs of infection and undergo rapid mortality following WSSV exposure so in this context are classified alongside penaeid shrimp as highly susceptible. In contrast *C. maenas* does not display signs of infection nor does it undergo significant mortalities when exposed to WSSV, this species is therefore considered to display low susceptibility to WSD. Low susceptible hosts can presumably harbour low viral titres, but sufficient to cause disease and mortality in highly susceptible hosts, such as *L. vannamei* during passage and as such may be considered as asymptomatic carriers for WSSV. It is important to note that WSSV has previously been shown to be carried in shrimp populations at low intensities in low-stress culture conditions without mortality events occurring (Tsai *et al*., 1999). It is possible therefore that even highly susceptible species may be able to tolerate infection while conditions are favourable, but may succumb to the disease when sub-optimal conditions occur. Disease outbreaks on shrimp farms are known to be induced by stressors such as rapid change in salinity and drop in temperature (Granja *et al*., 2003; Guan *et al*., 2003; Vidal *et al*., 2001). Corteel *et al*. (2009) have shown that shrimp were more susceptible to WSD infection via immersion after moulting than in the period before moulting and that wounding aided infection. How this latent disease or 'carrier' status will alter during stressful events such as moulting, reproduction and disease with non-penaeid species is unclear and requires further studies. Due to limited tank availability the studies presented here ran for a maximum of 10 days, it is unknown whether latent infections shown here would persist or whether the disease would develop over time. Longer term studies than those presented here are necessary to address the issue of whether low-level infections in wild (non-model) crustaceans would persist as such for extended periods or whether stressors cause progression of infection to disease at the individual and population levels. For example, mortalities from oral infection did not occur within this time period within some species such as *E. sinensis* however the level of pathology seen within these samples suggest that the crabs would have succumbed to the disease with mortalities occurring should the study have continued.

Variable results were seen with the PCR assays. In some cases PCR negative results were obtained despite pathology being seen within the tissues. This may be due to a variety of reasons but it is important to highlight that there is a

possibility that the PCR primers are not 100% reliable when used in analysis of non-penaeid samples resulting in false negative results. The method described by Lo *et al*. (1996b) is that recommended in the OIE diagnostic manual (2006) and was developed and optimised in penaeid shrimp. Claydon *et al*. (2004) showed that this test could result in false positive results when used with *Cherax quadricarinatus* samples, highlighting a lack of specificity due to the prescribed low annealing temperature in the Lo *et al*. (1996b) technique. An optimised method has recently been published by Nunan and Lightner (2011); however, this method was also optimised in penaeid shrimp. All positive results seen during this study were confirmed via sequencing; however false negatives may have occurred. Further work is required to confirm specificity and sensitivity of these methods in non-penaeid samples to ensure that the PCR methods are working correctly. This is essential for Member States wishing to conduct surveillance programmes to declare freedom from WSSV to eliminate any chance of false positive results. It is important to remember that detection by PCR alone is not adequate to confirm susceptibility. This technique identifies presence or absence of elements of the viral genome but does not indicate whether this material is present intracellularly (i.e. causing an infection) or as an incidental finding e.g. on the surface of the gut. For this reason, PCR data is not utilised to assess host susceptibility according to EFSA (see Stentiford *et al*., 2009). Presence needs to be confirmed via histology and electron microscopy as highlighted by the EFSA categories.

One species, *Carcinus maenas*, clearly showed a lower susceptibility to WSSV when compared to other European decapods species, this supports the findings of Corbel *et al*. (2001). WSSV has been classified within a new genus *Whispovirus* in the family *Nimaviridae* and was named White Spot Syndrome Virus 1 by the ICTV (Mayo, 2002a; Mayo, 2002b; Vlak *et al*., 2005). It is currently the only member of this genus but Vlak *et al*. (2005) state that as this is a newly recognised family the species organisation may change once existing and new isolates are discovered. Vlak *et al*. (2005) also tentatively list B virus from *Carcinus maenas*, B2 virus and τ (tau) virus from *Carcinus mediterraneus* and Baculo-A and Baculo-B viruses from *Callinectes sapidus* within this genus. B, B2, and Baculo-B viruses are extremely similar in terms of size, shape and morphogenesis to that reported from WSSV; in fact these viruses appear

morphologically indistinguishable and it has been suggested that these viruses may be ancestral forms of WSSV (Bonami & Zhang, 2011). Anecdotal evidence suggests that during initial outbreaks of WSSV in Asian penaeid farms, broodstock were fed with carcasses of portunid crabs (Prof. Grace Lo, pers. comm.). It may be possible that the virus now plaguing the global penaeid shrimp farming industry may have its origins in crabs, as such these ancestral hosts may well possess genome-based mechanisms to deal with viruses such as WSSV; prior exposure to B virus which is similar if not identical to WSSV may have given this species a competitive edge. Further work is required to rediscover B/B2 virus and to investigate why this species in particular appears to be less susceptible to this virus.

Although most commonly associated with disease in tropical shrimp, one of the first reports of WSSV described mortalities of *Marsupenaeus japonicus* in water temperatures of 19˚C (Takahashi *et al*., 1994). This falls within the water temperature range commonly seen within Europe and is a species which is known to be farmed within European waters (Stentiford and Lightner, 2011). Natural variations in temperature are seen throughout Europe; seasonal and climate variations at the time of exposure to WSD would likely have an effect upon the progression of the disease. Temperature has been shown to affect expression and development of WSD, penaeid shrimp and crayfish have been shown to suffer reduced mortality rates and lower viral loading in both hypothermic and hyperthermic conditions, when these individuals were returned to standardised conditions the disease developed quickly (Du *et al*., 2006a; Du *et al*., 2008; Granja *et al*., 2003; Granja *et al*., 2006; Jiravanichpaisal *et al*., 2004; Rahman *et al*., 2006; Rahman *et al*., 2007a; Rahman *et al*., 2007b; Vidal *et al*., 2001). These findings suggest that species which are exposed to WSD at low or elevated temperatures may not show signs of disease, mortalities may not occur. However, they may serve as a reservoir to spread the virus and cause disease if the temperature returns to ambient conditions Seasonal and regional climate variations may be important factors in assessing the risk of this disease to Europe.

This study has shown that European species are susceptible to this virus after a single feed and that the virus is viable, even when the viral loading was low and

animals show no characteristic signs of infection the virus could be transmitted to SPF shrimp. However, it is not known whether infection would lead to serious disease with high mortalities. There are no published studies to determine whether an infection within a population of non-penaeid species would spread and continue or die out. Chapman *et al*. (2004) comment that severe infections are rarely seen in the wild and highlight this may be due to limited sampling of wild populations, vulnerability of diseased individuals to predation, swift progression of the disease or the rarity of the event occurring. Chang *et al*. (2001) have shown that WSSV is present within wild populations of blue crabs (*Callinectes sapidus*) along the coast of the USA but these populations do not appear to be symptomatic; it was suggested they act as reservoir hosts. Progression of WSD outbreak would likely depend upon the level of mortalities and whether or not predation on these carcasses occurred. Wu *et al*. 2001 showed that high cumulative mortalities were associated with cannibalism and that the duration of mortality was extended by additional viral loading via cannibalism. This study has shown that the virus is viable after a single feed so the likelihood is that the carcasses will be consumed and infection passed on, it is likely that multiple feeds of WSSV infected tissues will result in an increased viral loading and increased mortality. It is important to note that WSSV has previously been shown to be carried in shrimp populations at low intensities in low-stress culture conditions without mortality events occurring (Tsai *et al*., 1999). Disease outbreaks on shrimp farms are known to be induced by stressors such as rapid change in salinity and drop in temperature (Granja *et al*., 2003; Guan *et al*., 2003; Vidal *et al*., 2001). Longer term studies than those presented here are necessary to answer whether fed animals would succumb to an infection or whether the infection would persist at low-levels within a population and to identify the effect stressors would have on latent infections.

3.6 Conclusion

Using the four EFSA categories Replication, Viability, Pathology and Location this study shows that *H. gammarus, C. pagurus, C. maenas, N. norvegicus, E. sinensis, P. leniusculus* and *A. pallipes* are susceptible to WSD. The manifestation of the disease differs considerably between species; with one species *Carcinus maenas* clearly showing a lower susceptibility to WSSV. Further work such as longer term exposure trials and the possible effects of

temperature and stress are needed to identify whether this disease could affect population levels of European crustacean species and to investigate why *C. maenas* in particular appears to be less susceptible to this virus.

Chapter 4.

Per os exposure and long term pathogenesis study of WSSV in *Carcinus maenas* and *Cancer pagurus*

4.1 Abstract

White Spot Syndrome Virus (WSSV) is thought to cause persistent infections in crustaceans. Results from previous studies (Bateman *et al*., 2012a) showed that although there were no mortalities after exposure to WSSV via feeding, edible and shore crabs (*Cancer pagurus* and *Carcinus maenas*) were found to be positive for the infection via histology and PCR after 10 days. The aim of this study was to determine whether WSSV infection would remain in the tissues of edible crabs (*Cancer pagurus*) and shore crabs (*Carcinus maenas*) as a persistent infection or whether these crabs would clear the viral infection from their systems over time. Thirty edible crabs and fifty shore crabs were fed with WSSV-infected tissues and crabs were then observed in tanks for 3 months. At the end of the study, crabs were sampled for histology and PCR and tissues used in a bioassay with specific pathogen free (SPF) *Litopenaeus vannamei* to identify whether the crabs could act as vectors for dispersal of the disease in the event of an outbreak in Europe. At the end of the study only two shore crabs and three edible crabs were PCR positive for WSSV. There were no signs of infection in tissue sampled from shore crabs despite the tissues being shown to be PCR positive for the virus. This suggests that the virus may be present as a persistent infection within the shore crab tissues; the virus replicating at low levels without causing any detrimental effects on the host. Edible crabs did show signs of replication via histology but only in a few cases; these crabs also testing positive for WSSV via PCR. However, when these tissues were homogenised and inoculated in to SPF shrimp, no mortalities or pathology was observed and shrimp tissues were PCR negative for the virus. These results suggest that whilst crabs may remain infected for extended time periods, viral loading may be too low to cause infection and disease in a known susceptible host species (shrimp). Alternatively, viral attenuation within infected crabs may prevent onwards passage to susceptible hosts. In conclusion, the carcasses of infected (but not

diseased) shore and edible crab crabs appear to pose a limited risk of transmission to susceptible hosts within the 3 month timeframe of the study.

4.2 Introduction

Viral infections of individuals and the eventual outcome of infection depends upon a variety of factors such as initial exposure dose, host immune defences, population density, host-host interactions and the prevailing environmental conditions. Within aquaculture settings, many viral pathogens (e.g. WSSV) appear to cause self-limiting, acute infections which result in either clearance of the pathogen from the host system or host death. However some viruses are able to establish persistent infections (Fenner *et al.*,1993). (Figure 1).

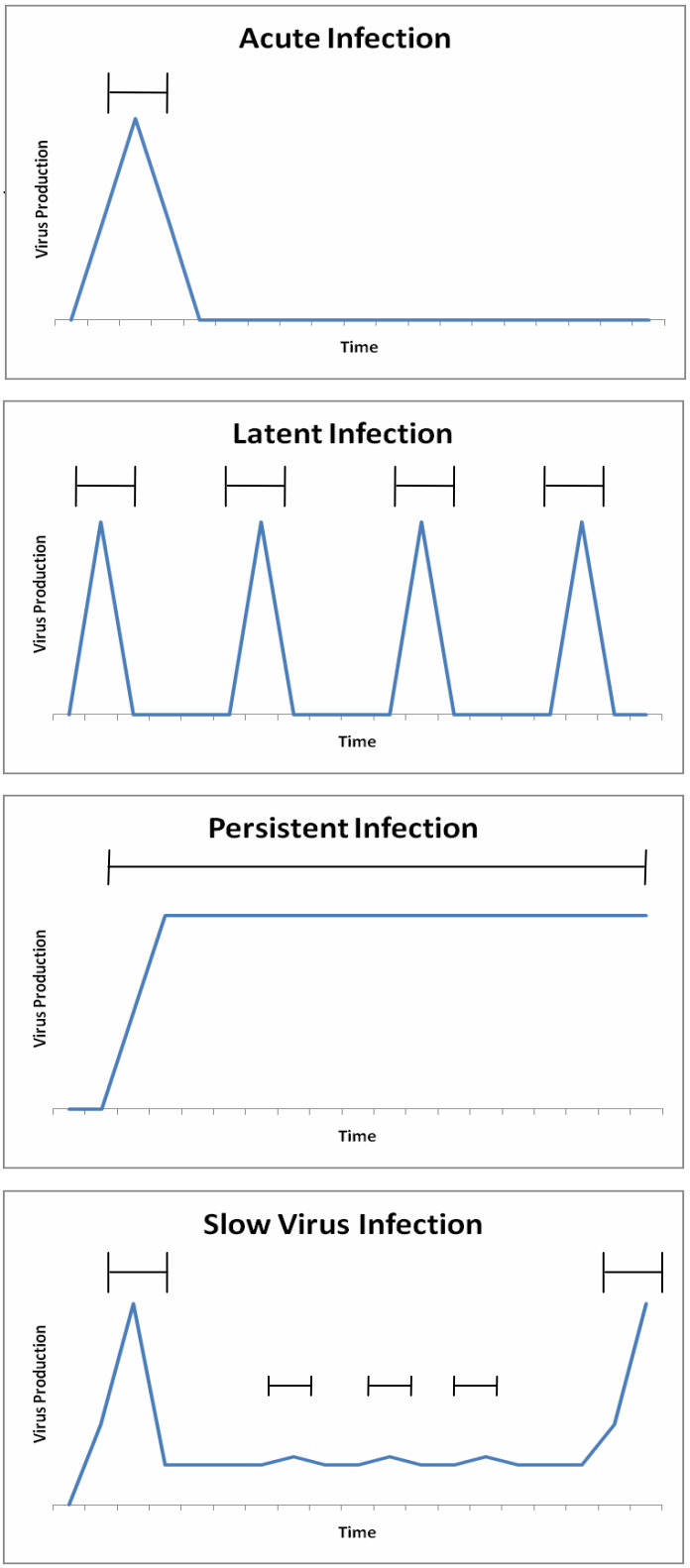

Figure 1. General patterns of viral infection. Relative virus production is plotted as a function of time after infection, period when infectious virions are released indicated by the brackets. Acute infections are represented by rapid production of virus, symptoms appear and then the virus is either cleared from the system or the host dies. Persistent infections result in continuous production of infectious virions. Latent infections are depicted by an initial acute infection followed by a quiescent phase and repeated bouts of reactivation. Reactivation may or may not be associated with symptoms but does result in production of infectious virions. A slow virus infection indicates production of virions over extended periods (up to years) and usually intervene a typical acute infection and the usually fatal appearance of symptoms. Production of infectious virions may be continuous or absent, brackets placed arbitrarily to indicate this. Adapted from Fenner *et al*. (1993).

Persistent infections are usually initiated via an acute infection using an array of different mechanisms including non-productive infection (e.g. herpesvirus), proviral integration into the host genome (e.g. retrovirus) or continuous viral replication (e.g. flaviviruses, arenaviruses and polyomaviruses) (Kane and Golovkina, 2010). Some viral infections replicate without causing host cell death whilst others induce cytopathic effects in target cells (Kane and Golovkina, 2010). Although each virus will have evolved distinct mechanisms to enable persistence there appear to be common themes which link persistent infections. These include modulation of viral gene expression, viral subversion of cellular apoptotic pathways and avoidance of clearance by the host immune system (Kane and Golovkina, 2010).

Persistent infections are not simply the result of viral evasion of host-triggered apoptosis but the outcome of a combined host-viral process that has evolved to 'dampen' the rate of apoptosis and lead to rapid development of mutual existence (Flegel, 2007). Table 1 highlights the general characteristics of arthropod (insect and crustacean) responses to viral infections when compared to vertebrates. The insects and Crustacea lack an inflammatory response to viral pathogens, can display single to multiple persistent viral infections and appear to be able to 'tolerate' the viral pathogens (with survivors remaining infectious to other naïve individuals). It should be noted here that Crustacea elicit host responses to bacterial and parasitic infections so this feature of 'tolerance' appears to be unique to viral pathogens (Flegel, 2007). In contrast vertebrates elicit immune responses, clear the viral pathogen from their tissues leading to loss of host infectivity.

Table 1. General characteristics of insect and crustacean responses to viral pathogens compared with vertebrates. Recreated from Flegel (2007).

	Insects and Crustacea	**Vertebrates**
Inflammatory response	Uncommon or missing	Common
Survivors	Commonly infected	Not infected
Infectivity of survivors	Infectious	Non-infectious
Tolerance to virus	Common	Uncommon
Virion production	High	Low
Multiple infections	Common and normal	Uncommon and rare

Herpesviruses provide a good example of infectious agents able to establish latent infections. In the aquatic environment Koi Herpes Virus (KHV) can cause latent infections with KHV DNA remaining at low copy number in latently infected host tissues (Eide *et al*., 2011). Channel Catfish Virus (CCV) has also the potential for latency following initial primary infection (Gray *et al*., 1999). If latency is to have any value to the virus a method of reactivation must exist. In many cases, this follows trauma, stress or other conditions which render the host unable to control the latent infection. White Spot Syndrome Virus (WSSV) is thought to cause persistent infections; it is probable that the WSSV genome resides in hosts either in a quiescent state or by remaining as a persistent infection (Khadijah *et al*., 2003). Khadijah *et al*. (2003) showed that WSSV genes could be detected in otherwise Specific Pathogen Free (SPF) shrimp. They proposed that three viral genes (WSV151, WSV366 and WSV 427) may play a role in subsequent activation of the virus within latently infected hosts. This report was in agreement with the results of others describing the prevalence of WSSV in the ecosystem and its predominance in shrimp aquaculture (Thakur *et al*., 2002). Latency (and toleration) may also play a role in adaptation of farmed populations to initially pathogenic virus strains. As shown with *Penaeus stylirostris* Densovirus (PstDNV), Yellowhead Virus (YHV) and Taura Syndrome Virus (TSV) WSSV-infected penaeid shrimp experienced initial periods of massive mortalities following the initial emergence of the disease but was followed after 1-2 years by an apparent reduced susceptibility of farmed shrimp populations. In these cases, subsequent harvests were successful despite the presence of infected shrimp within many pond systems (Flegel, 2007).

Crustaceans have been shown to mount a strong innate immune response against viral pathogens (Hauton, 2012; Liu *et al*., 2009), of which programmed cell death (apoptosis) is a frequent antiviral defence. Apoptosis is a natural mechanism used to protect the organism as a whole by removing unnecessary or potentially harmful cells (Liu *et al*., 2009). An acutely infecting virus must prevent apoptosis of infected cells long enough to produce sufficient numbers of daughter virions. Many viruses have evolved to survive this mechanism of host apoptosis with numerous examples of apoptosis inhibitors (e.g. mimics of the antiapoptotic protein) present within viral genomes (Goldmacher *et al*., 1999). In addition, host and viral miRNAs have also been shown to be utilized by persistent viruses to inhibit translation of cellular pro-apoptotic genes (Kane and Golovkina, 2010). WSSV infection has been shown to suppress apoptosis in infected shrimp tissues. Characteristic signs of apoptosis can be identified within non-infected 'by-stander cells' whilst WSSV-infected cells themselves remain non-apoptotic. This incidence of apoptosis in crustacean tissues has been shown to be species-specific and tissue-specific and is also related to the severity of WSSV infection, how WSSV induces apoptosis in by-stander cells remains unknown (Leu and Lo, 2011).

Flegel (2007) highlighted how persistent viral infections in insects and crustaceans have been overlooked in many previous studies (concentrating on the resistance determining survival rather than whether survivors were infected and infective). Previous studies have shown differential host responses seen after WSSV exposure of different species (Bateman *et al*., 2012; Flegel, 2007; Hameed *et al*., 2003; Kanchanaphum *et al*., 1998; Rajendran *et al*., 1999; Sahul Hameed *et al*., 2000; Sarathi *et al*., 2008; Supamattaya *et al*., 1998; Wang *et al*., 1998) with some crustacean species apparently demonstrating higher tolerance to infection (and disease) than others. Suppamattaya *et al*. (1998) showed that sand crabs (*Portuna pelagicus*) and mud crabs (*Scylla serrata*) could be active carriers of WSSV infection, without showing mortality or patent disease, though these studies only ran for 9 days. Suppamattaya *et al*. (1998) suggested that these crab species could be considered as viral 'reservoirs' and were capable of carrying persistent infections for significant time periods in the shrimp farm environment.

Kanchanaphum *et al*. (1998) also showed that three crab species (terrestrial crab *Sesarma* sp., mud crab *Scylla serrata* and fiddler crab *Uca pugilator*) could be carriers of WSSV after injection studies; these studies running for a longer period of 45 days. Here, haemolymph samples taken throughout the study period showed how crabs became infected with the virus but there were no mortalities, despite histology depicting large numbers of infected cells. Shrimps kept as cohabitants with the infected crabs began to die within 3 days. This indicated that although the crabs were not resistant to infection by, or replication of, the virus, they did appear to somehow inactivate the severe morbidity factors affecting other host taxa. In these cases however, the virus did not appear to be altered via passage through the crabs and remained virulent to shrimp (even though these co-exposure studies were run 96 h p.i. rather than at the end of the 45 day exposure period). Both Suppamattaya *et al.* (1998) and Kanchanaphum *et al.* (1998) suggest that wild crabs pose a significant threat to shrimp farming and farmers should prevent wild crabs from entering the shrimp ponds during cultivation cycles to limit spread of WSSV.

Macrobrachium rosenbergii has been shown to be less susceptible to WSSV infection than penaeid shrimp hosts (Sahul Hameed *et al*., 2000). Sarathi *et al.* (2008) injected *M. rosenbergii* with WSSV and showed that the shrimp became lethargic and stopped feeding after 3 days p.i. However no mortalities occurred and no further signs of disease were observed over the 100 day experimental period. PCR samples from various tissues were tested throughout the experimental period, depicting that all tissues were positive for WSSV between 3 and 25 days p.i. However, by 50 days p.i. only soft tissues from the head region tested positive for WSSV and by 75 and 100 days p.i. all tissues sampled tested negative, suggesting that host had cleared the virus. Haemolymph sampled inoculated *M. rosenbergii* was used in a bioassay; haemolymph collected 5 days p.i. caused 100% mortality whereas that collected 10 days p.i. failed to cause any mortality in a susceptible species (*L. monodon*). Sarathi *et al*. (2008) suggested that the viral loading in the haemolymph at day 10 p.i. was too low to cause infection.

In Chapter 2 results showed that although there were no mortalities after exposure to WSSV via feeding, crabs were found to be positive for the infection via histology and PCR after 10 days. The aim of the current study was to determine whether WSSV infection would remain within tissues of edible crab (*Cancer pagurus*) and shore crab (*Carcinus maenas*) as persistent infection or whether these crabs would clear the viral infection from their systems over an extended study period (3 months). At the end of the exposure period, crabs were sampled for histology and PCR and tissues used in a bioassay with SPF *L. vannamei* to identify whether the crabs could act as a vector for dispersal of the pathogen in the event of a WSSV outbreak in Europe.

4.3 Materials and Methods

All experimental trials were performed within the biosecure exotic disease facility as described in Chapter 3. Temperature was maintained at 20°C throughout the study.

4.3.1 eparation of viral inoculums and challenge trials

Viral inoculates of WSSV were prepared as described by Bateman *et al*. (2012a), see Chapter 2 for full details.

4.3.2 Long Term Feed Study

One hundred shore crabs (*Carcinus maenas*) and sixty edible crabs (*Cancer pagurus*) were collected from the shoreline at Newton's Cove, Weymouth, UK (50^{o}34' N, 02^{o}22' W). Crabs were transferred into custom-made compartments within large trough tanks, with individuals separated by tank divisions to prevent conflict but sharing the same water supply, with a maximum of 15 crabs per trough system.

Negative control crabs were fed with a single ration of confirmed SPF shrimp tissue at approximately 5% bodyweight on Day 0. WSSV exposed crabs were fed with a single ration of confirmed WSSV-infected (but otherwise SPF) shrimp tissue at a ratio of approximately 5% bodyweight on Day 0 of each trial. Thereafter, samples in all tanks were fed on squid tissues at a ratio of approximately 3-4% wet body weight.day^{-1} for the remainder of the trial period. Tanks were observed regularly throughout daylight hours. Dead and terminally

morbid samples were removed from each tank and dissected. At the end of each challenge trial (3 months), and for moribund animals sampled within the trial, surviving animals were chilled on ice for 30 min prior to dissection. As standard, gill, epidermis, hepatopancreas, heart, gonad, nerve and muscle were placed into histological cassettes and fixed immediately in Davidson's seawater (see appendix). For molecular analyses, gill samples were removed and placed into tubes containing 100% ethanol. For electron microscopy, gill, muscle and hepatopancreas tissues were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for electron microscopy (EM). The remaining tissues and carcasses were stored at - 80°C.

4.3.3 oassay

Following PCR confirmation (see section 3.3.6) of the presence of WSSV in tissues of edible and shore crabs at the end of the 3 month study period, the infectivity of crab tissues was tested via bioassay exposure to a known WSSV susceptible species (*Litopenaeus vannamei)*. Individual SPF *L. vannamei* (approximately 5g in weight) were obtained from the Centre for Sustainable Aquaculture Research (CSAR) at the University of Swansea, United Kingdom. Tissues from each crab species were homogenised using the aforementioned approach for shrimp. A control innoculum was also prepared for each crab species using confirmed WSSV PCR negative samples. Innoculum was diluted using sterile saline and filtered (0.2µm Minisart syringe filter, Sartorius Stedim Biotech GmbH, Germany) prior to intramuscular injection (3rd abdominal segment) of SPF shrimp (10µl g^{-1} body weight). Shrimp exposed to confirmed WSSV PCR-positive and -negative innocula were observed for a period of five days. A total of eight tanks (three SPF-fed crab tissues and five WSSV-fed crab tissues); containing five shrimp per tank were maintained at a water temperature of 26˚C and observed regularly throughout daylight hours. Dead and terminally moribund animals were removed from each tank and sampled as above. At the end of the challenge period (Day 5), all surviving shrimp were sampled. Pleopods were removed from each shrimp and fixed in 100% ethanol for WSSV detection by PCR. The remaining shrimp carcass was injected with Davidson's seawater fixative and shrimp placed whole into the same fixative for histology.

4.3.4 ology

For histology, fixation was allowed to proceed for 24 h before samples were transferred to 70 % industrial methylated spirit. Fixed samples were processed to wax in a vacuum infiltration processor using standard protocols. Sections were cut at a thickness of 3-5 µm on a rotary microtome and mounted onto glass slides before staining with haematoxylin and eosin (H&E) and Feulgen stains. Stained sections were analysed by light microscopy (Nikon Eclipse E800) and digital images and measurements were taken using the Lucia™ Screen Measurement System (Nikon, UK).

4.3.5 xtraction

Initially tissue was weighed and diluted 1:10 in G2 buffer (Qiagen, West Sussex, UK) and 10 µl Proteinase K. The sample was homogenised using a Fast prep FP120 machine (MP biomedicals) at the highest setting for 2 minutes. This homogenate was incubated at 56^{o}C for at least 3 hours to allow lysis to occur. The sample was then centrifuged at 9000rpm for 2 minutes and 50 µl of the supernatant was added to 150 µl of G2 buffer. Total DNA from this 200 µl sample was then extracted using the EZ1 DNA tissue kit (cat no 953034) and the EZ1 advanced Biorobot (Qiagen) following the manufacturer's instructions. DNA was eluted in 50 µl elution buffer and quantified using a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, UK).

4.3.6 and Nested PCR

Separate first and second round (nested) PCR reactions were performed on each DNA extract using the OIE recommended WSSV primer sets (Lo *et al*., 1996a), (see Table 1 for primer sequences). Reactions were performed in 50µl reaction mix consisting of 1 X Green Go Taq flexibuffer (Promega), 2.5mM $MgCl_2$, 0.25mM dNTPs, 100 pmol each of the forward and reverse primer, 0.25 units Go Taq Flexi (Promega), and 2.5µl extracted nucleic acid. Amplifications were performed using the following WSSV thermal cycler program on a Peltier PTC-225 thermal cycler: 94^{o}C x 2 minutes followed by 29 cycles of 94^{o}C x 30 seconds, 62^{o}C x 30 seconds and 72^{o}C x 30 seconds, followed by 72^{o}C x 2 minutes and held at 4^{o}C. Reaction conditions and reagent concentrations were the same for the second round of amplification using the 146F2/146R2 primer pair; however 0.5 µl of the first round

of amplification was used as a template in place of genomic DNA. Following amplification, 10 μl of each PCR product were analysed by agarose gel electrophoresis (1.5 % w/v), stained with ethidium bromide (0.625mg/ml), and viewed under a UV light source. Images were captured with a Gel Doc 2000 (Bio Rad) imaging system.

Table 2. WSSV PCR primers (Lo *et al*., 1996a)

Primer name	Sequence
WSSV 146 F1	ACTACTAACTTCAGCCTATCTAG
WSSV 146 R1	TAATGCGGGTGTAATGTTCTTACGA
WSSV 146 F2	GTAACTGCCCCTTCCATCTCCA
WSSV 146 R2	TACGGCAGCTGCTGCACCTTGT

All PCR was conducted with advice and assistance from Dr Michelle Pond (Cefas).

4.4 Results

4.4.1 vival data

SPF-fed edible crabs exhibited low level mortality throughout the course of the 3 month study with 96% survival being observed. SPF-fed shore crabs displayed a slightly lower survival (85%) until the end of the 3 month study period. WSSV-fed crabs displayed lower levels of survival, 76% of edible crabs, and 73% of shore crabs surviving the study period (Figure 2).

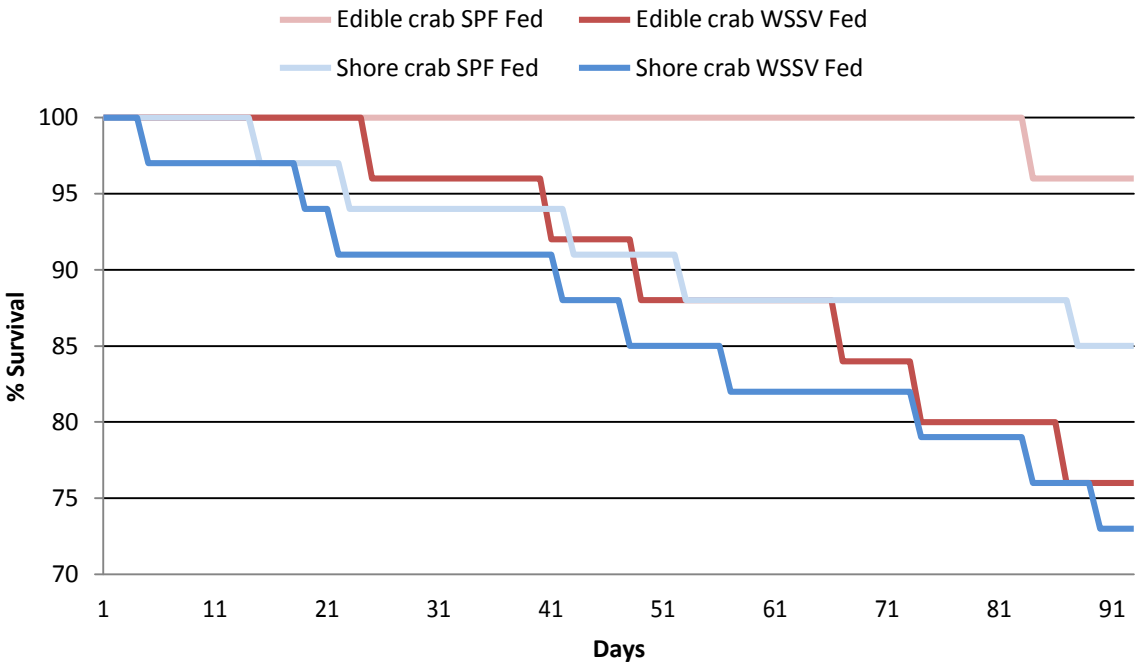

Figure 2. Survival curves from shore crab (*C. maenas*) and edible crabs (*C. pagurus*) exposed to WSSV infected shrimp tissues via feeding at 20°C.

4.4.2 gy

At the end of the exposure study hypertrophied nuclei with eosinophillic inclusion bodies could be seen within the tissues in four of the thirty edible crabs which had been exposed to WSSV (Table 3). Hypertrophied nuclei were observed within the gill epithelium, in the connective cells of the heart and within the epithelial cells of the antennal gland (Figure 3). No histological evidence of WSSV replication was observed in shore crab organs and tissues.

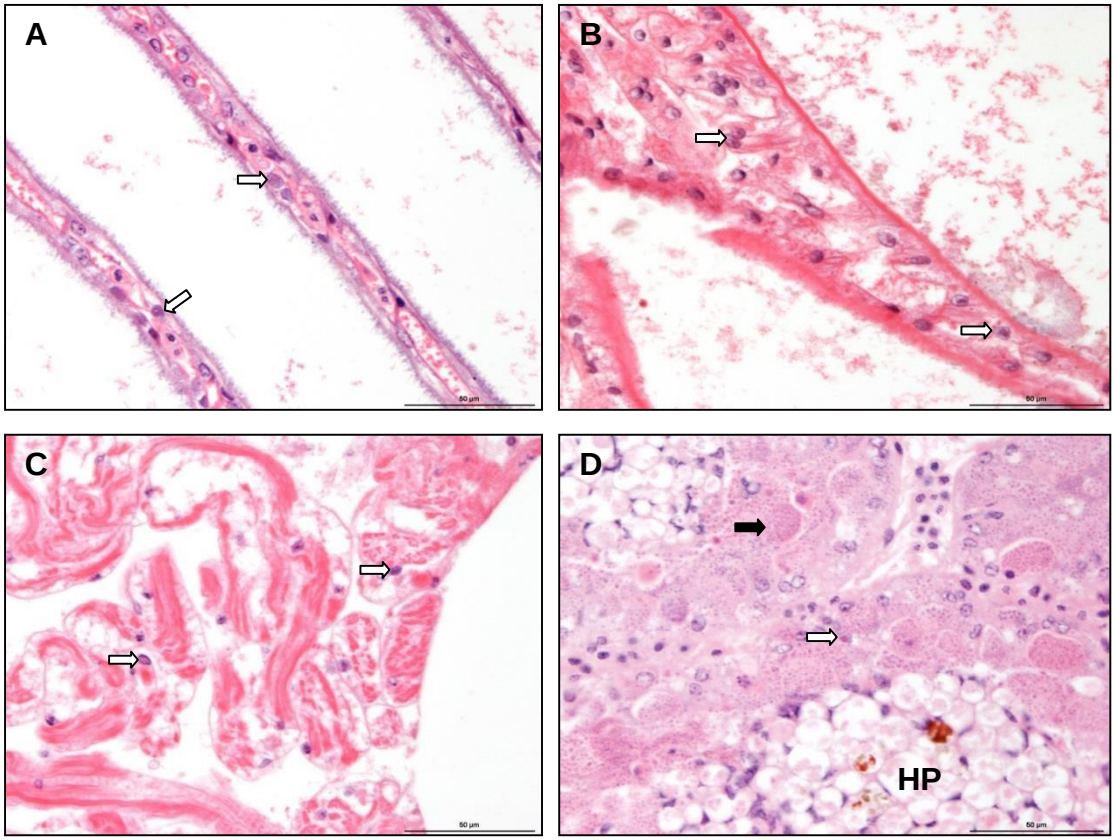

Figure 3. WSSV replication in tissues from edible crab *Cancer pagurus*. (A) Infected nuclei (arrows) can be observed scattered throughout epithelial cells in the gill lamellae of *C. pagurus*. Scale bar = 50µm. (B) Hypertrophied nuclei displaying marginalized chromatin and eosinophillic inclusion bodies (arrows) can be observed within epithelial cells of the gill lamellae Scale bar = 50µm. (C) Heart tissue showed signs of infection with hypertrophied nuclei (arrows) within haemocytes dispersed throughout the haemal spaces. Scale bar = 50µm. (D) Hypertrophied nuclei can be seen within the connective tissues of the antennal gland (white arrow). Antennal gland tissues showed massive proliferation due to co-infection with the pathogen *Paramikrocytos canceri* (black arrow). Hepatopancreatic tissues (HP) were unaffected by WSSV or *Paramikrocytos canceri*. Scale bar = 50µm. All images H&E Stain.

In addition to evidence of viral replication, histology also identified the presence of several other co-infecting pathogens in edible and shore crab tissues (Table 3).

Table 3. Pathogens present during histological screen of tissues from edible and shore crabs. Grey boxes indicate where pathogens are not found in that species.

	Edible crab (*Cancer pagurus*)		Shore crab (*Carcinus maenas*)	
	SPF Fed (30 crabs total)	WSSV Fed (30 crabs total)	SPF Fed (50 crabs total)	WSSV Fed (50 crabs total)
WSSV in Gill	0	7%	0	0
WSSV in Heart	0	7%	0	0
WSSV in Antennal gland	0	4%	0	0
Microphallus spp.	54%	56%	93%	100%
Hematodinium sp.	8%	0	0	0
Paramikrocytos canceri	84%	40%	-	-
Hepatospora sp.	4%	0	-	-
Sacculina carcini	0	0	3%	3%
Ameson pulvis	-	-	5%	14%
Milky Disease	-	-	10%	0

Microphallus primus is a digenean parasite that has a complex life cycle involving multiple hosts, metacercarial stages of the parasite were present distributed throughout both edible and shore crab tissues sampled (Saville and Irwin, 2005). *Paramikrocytos canceri* (Hartikainen *et al*., 2014) was seen at high prevalence in edible crab, this pathogen causing a massive proliferation of the antennal gland epithelium (Figure 4). The parasitic dinoflagellate *Hematodinium* sp. (Stentiford *et al*., 2002) was present within edible crabs sampled but was not identified in shore crab tissues. Two microsporidian infections were diagnosed during this study; one (*Ameson pulvis*) infecting the musculature of shore crabs (Stentiford *et al*., 2013) and another (*Hepatospora* sp.) infecting the hepatopancreas of edible crabs (Stentiford *et al*., 2011). The parasitic barnacle *Sacculina carcini* (Boschma, 1955) and the bacterial infection Milky disease (Eddy *et al*., 2007) were also observed infecting shore crab tissues (Figure 5).

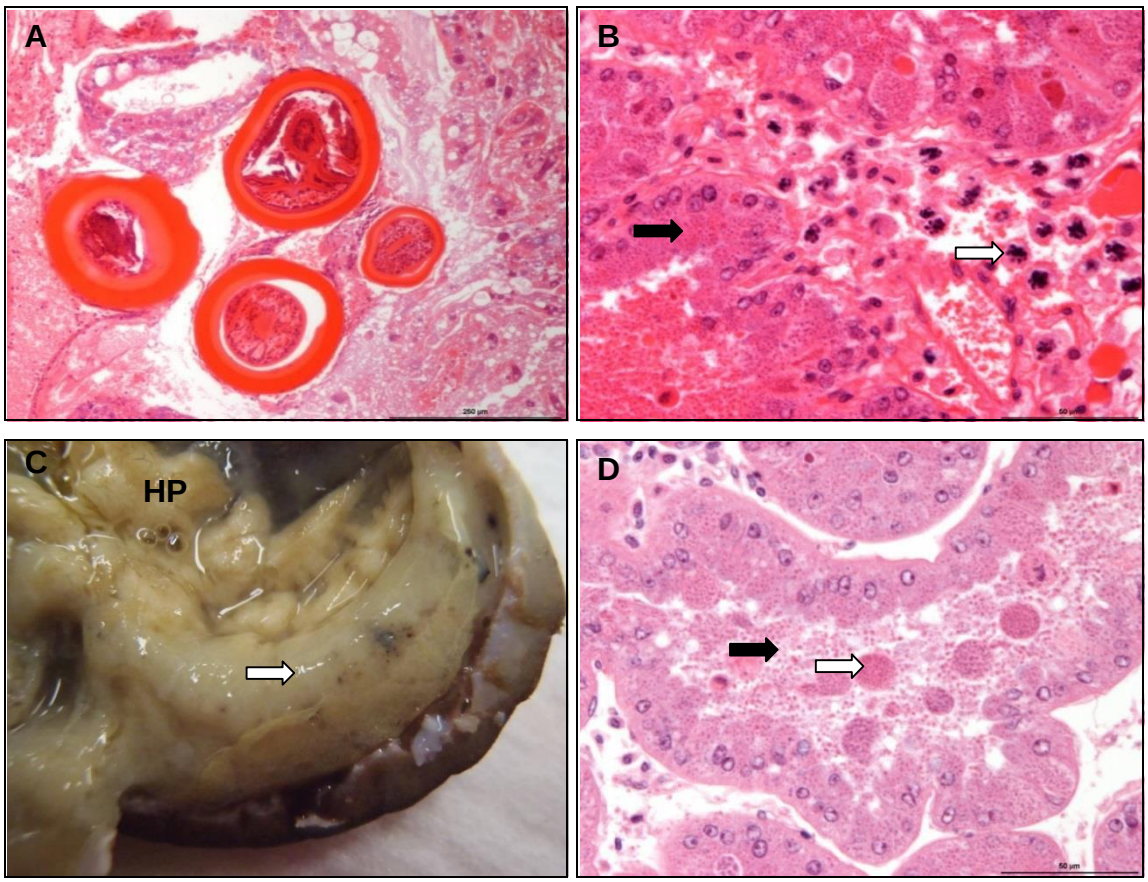

Figure 4. (A) Metacercarial stages of *Microphallus primas* in shore crab (*Carcinus maenas*) tissues. Scale bar = 250µm. (B) *Hematodinium* sp. (white arrow) can be seen in the haemal sinuses, this edible crab (*Cancer pagurus*) had a co-infection with *Paramikrocytos canceri* (black arrow). Scale bar = 50µm. (C) Antennal gland of the edible crab (*C. pagurus*) showed a massive proliferation when infected with *Paramikrocytos canceri* and was observed upon dissection as a yellow gelatinous tissue (arrow) which could be distinguished from the hepatopancreas (HP). (D) Lumen of antennal gland contains multiple plasmodia stages (white arrow) and unicellular stages (black arrow) of *Paramikrocytos canceri*. Scale bar = 50µm. All images H&E stain.

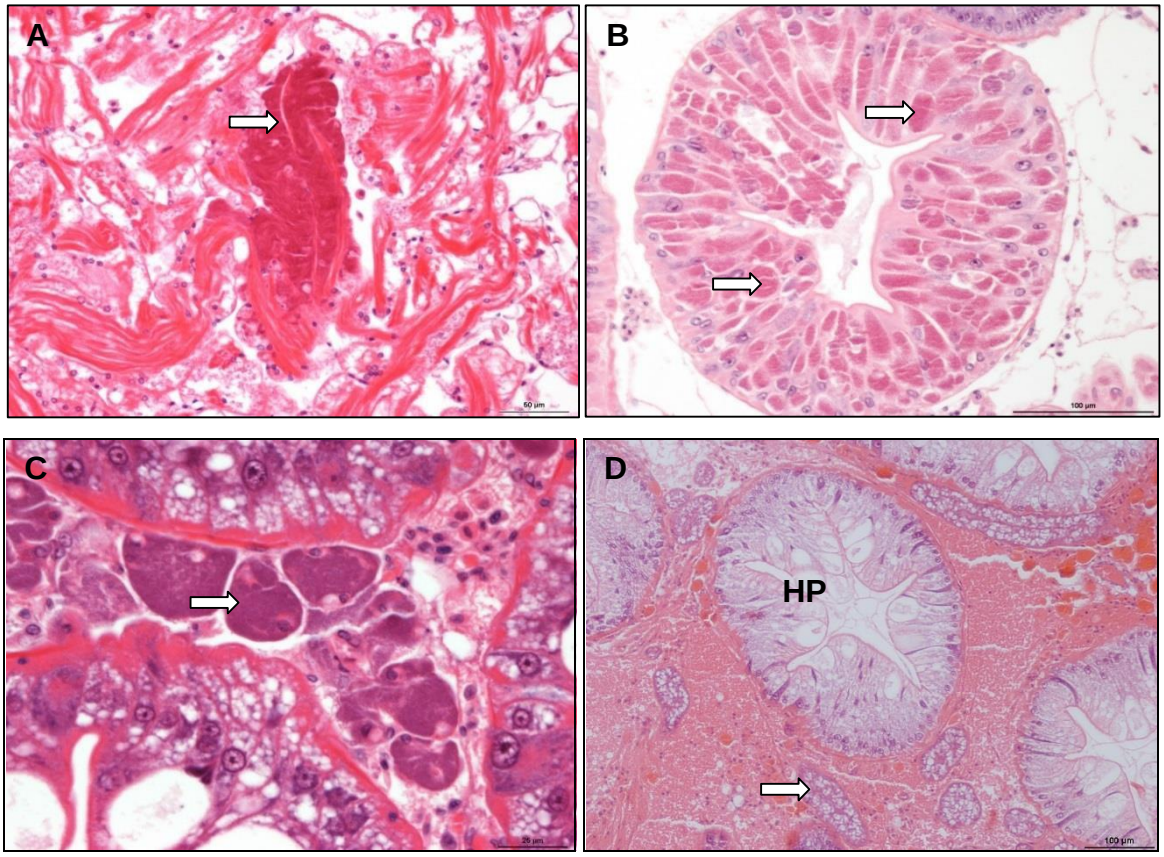

Figure 5. (A) *Ameson pulvis* infection of heart muscle (arrow) of shore crab (*C. maenas*). Early infection of sarcolemma of muscle fibres. Scale bar = 50µm. (B) Hepatopancreatic tubule of edible crab (*C. pagurus*) heavily infected with microsporidian *Hepatospora* sp. Epithelial cells of hepatopancreatic tubule contain large granular inclusions (arrows). Scale bar = 100µm. (C) Milky disease caused by a Rickettsia-like organism in shore crab (*C. maenas*). Intracellular bacterial infection of fixed phagocytes (arrow) within the haemal sinuses of the hepatopancreas. Scale bar = 25µm. (D). *Sacculina carcini* infection of the hepatopancreas of shore crab (*C. maenas*); rootlets (arrow) can be seen within the haemal sinuses between the hepatopancreatic tubules (HP). Scale bar = 100µm. All images H&E stain.

4.4.3 ted PCR

All SPF-fed edible and shore crabs were negative for presence of WSSV via first round of nested PCR. Two shore crabs and one edible crab were positive for WSSV in the second round of the nested PCR assay, with a WSSV-characteristic 941bp product observed (Figure 6).

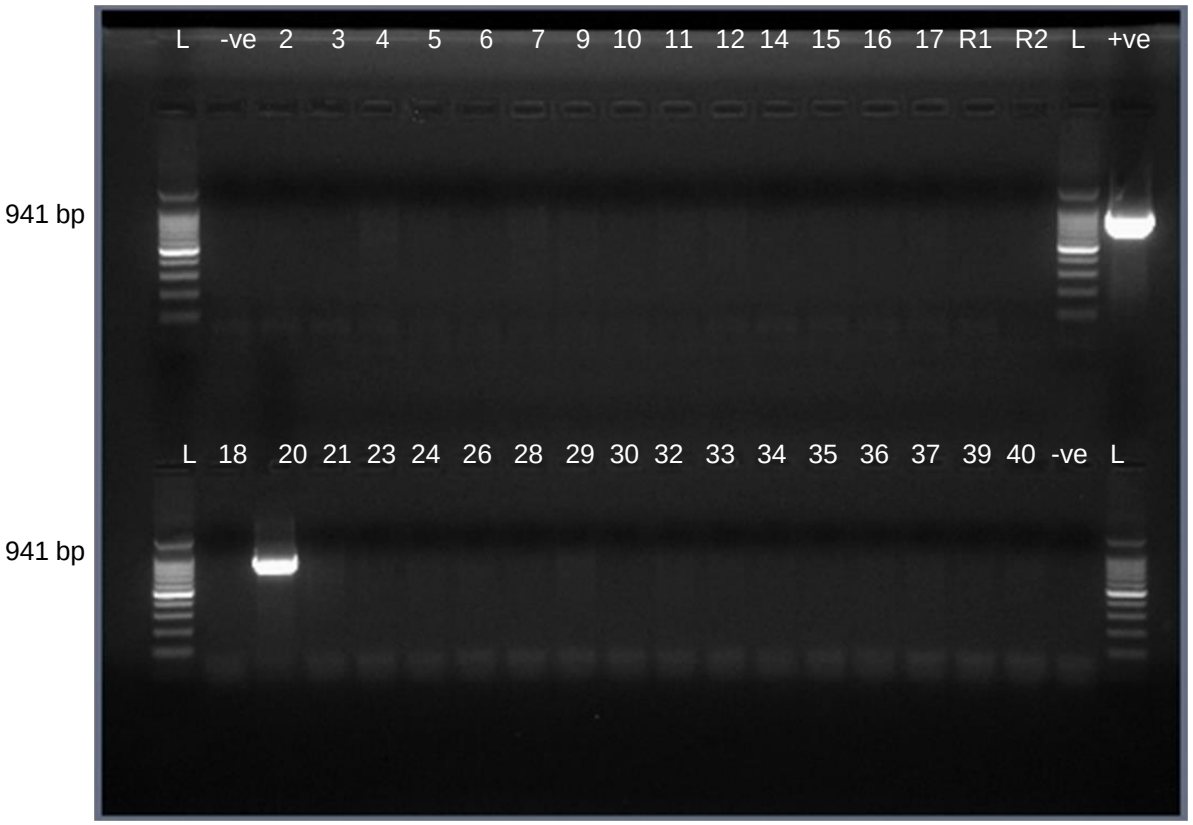

Figure 6. A representative ethidium bromide stained agarose gel showing DNA fragments produced by second round WSSV PCR assay of gill tissues from crabs exposed to WSSV. Crab 20 shows a clear band at approximately 941bp corresponding to the positive PCR sample. L) 100bp DNA Ladder. R1) First round PCR reagent negative. R2) Second round PCR reagent negative. -ve) Extraction reagents negative. +ve) WSSV PCR positive.

4.4.4 sage studies

Two shore crabs (crab #20 and #41) and three edible crabs (#43, #47 and #48) which had been exposed to WSSV were homogenised and injected into SPF *L. vannamei* (5 shrimp per sample). After 5 days, no mortalities were observed and all survivors were sampled. No evidence of WSSV infection was present within the shrimp tissues via histology, PCR analysis (Figure 7) highlighted a potential positive response in lane 3, (depicted by a faint band of approximately the correct

size for the WSSV amplicon). The PCR was repeated on this sample, giving a negative PCR result. Following agreed protocols for confirmation, the result was presumed to be negative. No mortalities or pathology were evident in shrimp exposed to SPF fed crab tissues.

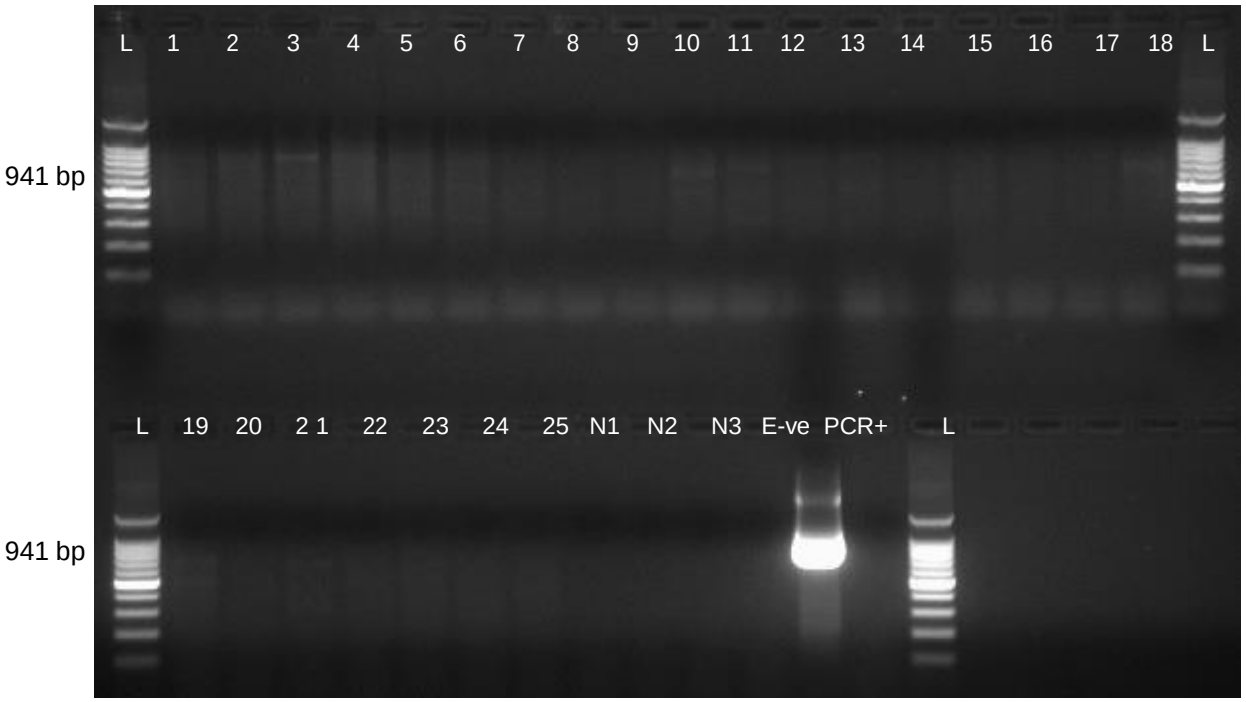

Figure 7. An ethidium bromide stained agarose gel showing DNA fragments produced by second round WSSV PCR assay from bioassay shrimp. Lane 1-5 shrimp exposed to shore crab 20. Lane 6-10 shrimp exposed to shore crab 41. Lane 11-15 shrimp exposed to edible crab 43. Lane 16-20 shrimp exposed to edible crab 47. Lane 21-25 shrimp exposed to edible crab 48. L) 100bp DNA Ladder. N1, N2, N3) Reagent Negatives. E -ve) Extraction reagents negative. PCR +) WSSV PCR positive.

4.5 Discussion

At the end of the study (3 months) only two shore crabs and three edible crabs were PCR positive for WSSV. There were no signs of infection in tissue sampled from shore crabs despite the tissues being shown to be PCR-positive for the virus. This suggested that the virus may exist as a persistent infection within the shore crab tissues; the virus is being replicated at low levels without causing any detrimental effects on the host. It is important to note here that this low level infection was seen in two crabs only; a larger sample size would be needed to confirm this. It is also possible that the shore crab is capable of eradicating the virus from its tissues, and could explain why we did not see any signs of infection in the majority of the samples, however the mechanism to enable this is unknown

and further work would be needed to investigate this. Edible crabs did show signs of replication via histology but only within a few crabs, these crabs also tested positive for WSSV via PCR. WSSV infection has been shown to induce apoptosis in shrimp (Leu and Lo, 2011) there were no obvious signs of apoptosis in either shore or edible crab tissues suggesting this mechanism was at a very low level. When the WSSV positive crab tissues were homogenised and inoculated into SPF shrimp no mortalities or pathology was observed and shrimp tissues were PCR negative for the virus at the end of the 5 day study. These results suggest that the virus was not passaged and so indicates that the infected shore and edible crab carcasses pose a limited risk. However, it is not clear whether the virus was present but below level of detection in the shrimp tissues, further studies such as larger sample numbers and longer term exposures would be needed to confirm that there was no passage.

Over the course of the study no mortalities were observed and crabs did not show any signs of ill health such as lethargy or lack of feeding. It has been suggested that persistent infections may become acute infections following a stress event such as moulting. This did not appear to be the case during this study as some of the crabs moulted during the study with no apparent ill effects. The presence of other pathogens did not appear to enhance the effects of a viral infection either as multiple pathogens were detected in the crabs used during this study. Moser *et al*. (2012) collected and held WSSV-free wild organisms at 18°C followed by a two day incubation period at 29°C; they found that when the organisms were re-tested for WSSV following the incubation at 29°C positive PCR results were present. This study was conducted at 20°C and crabs did not show signs of infection, it would have been interesting to see if raising the temperature at the end of the 3 month study (or any other stressor) would cause any potential infections to become expressed i.e. switch from persistent to acute.

There are three main requirements to ensure a successful infection in an individual host. There must be a sufficient viral dose to initiate an infection, cells at the site of infection must be susceptible to infection, and the host antiviral defence systems must be absent or defective. In principle a single virus particle should be able to initiate an infection but the physical and immune defences of

the host coupled with the complexity of the infection process itself usually demands the presence of higher numbers of viral particles (Flint *et al*., 2000). From our previous studies, we have demonstrated that edible crabs and shore crabs are differentially susceptible to WSSV infection (Bateman *et al*., 2012a). However, due to the nature of the feed production there may have been some variation between doses. It is important to note that the feed was generated from infected shrimp which were macerated into small pieces to form the feed; it has been suggested that WSSV loading can vary between shrimp and between the different tissues present (Kou *et al*., 1998), although Oidtmann & Stentiford (2011) have shown that viral load did not appear to vary greatly between different shrimp tissue types. As multiple shrimp were used to form the feed, each of which could possess varying viral load, shrimp were macerated and the macerated tissue mixed prior to feeding, the aim of this was to ensure that each crab received a similar initial feed and hence a similar initial viral loading. It was assumed that all crabs ate the feed that was presented as no feed remained in the pots after 12 hours; however, cannot be sure that all food was consumed as some may have been lost when the crabs macerated the feed as part of the feeding process. Pots were designed to limit this as drainage holes were at the side of the pots as opposed to the bottom where the feed sat until consumption. A single dose of feed was administered on day 0 and the crabs had been starved for 24 hours prior to this feed.

In the natural environment crabs are opportunistic feeders and feed on carcasses in the local environments. It is likely that in the wild the crabs would have multiple feeds from infected carcasses until carcass is consumed so would likely receive a higher viral dosing than that administered here. They may well choose which tissues to consume, viral loading is known to vary between tissue type meaning they may receive higher or lower doses depending upon the tissue they consume (Chang *et al*., 1998; Kou *et al*., 1998; Lo *et al*., 1997). Of course they may actually avoid infected carcasses completely as shown with *Panulirus argus* Virus 1 (PAV1) infection in lobsters (Behringer *et al*., 2006; Shields and Behringer, 2004) where healthy lobsters were shown to avoid cohabiting with infected individuals. This suggests that lobsters possess a way of determining infected or sick individuals and then avoid these individuals; this would also imply that these

lobsters would not consume an infected individual. The study presented here did not determine whether the crabs would choose to eat infected feed and a possible continuation of this work would be to give crabs and crayfish an option of infected and uninfected feed. This study would determine whether the crabs avoid contaminated feed and how much is consumed by an individual to determine whether the infection would pose a true disease risk in the wild.

4.6 Conclusion

At the end of the three month study only two shore crabs and three edible crabs were PCR positive for WSSV. There were no signs of infection in tissue sampled from shore crabs suggesting that the virus may be present as a persistent infection within the shore crab tissues; the virus is being replicated at low levels without causing any detrimental effects on the host. It is important to note here that this low level infection was seen in two crabs only; a larger sample size would be needed to confirm this. Edible crabs did show signs of replication via histology but only within a few crabs; these crabs also testing positive for WSSV via nested PCR. However, when the WSSV-positive crab tissues were homogenised and inoculated into SPF shrimp, no mortalities or pathology were observed and shrimp tissues were PCR negative for the virus at the end of the five day study. These results suggest that the virus was not passaged and so indicates that the infected shore and edible crab carcasses pose a limited risk after 3 months.

Chapter 5.

Effect of temperature stress on WSSV replication in a low susceptible host, *Carcinus maenas*

5.1 Abstract

The main aim of this chapter was to determine whether environment temperature had any effect on White Spot Syndrome Virus (WSSV) replication in a low susceptible European crustacean species, the shore crab (*Carcinus maenas*), and to determine whether the shore crab is truly resistant to WSSV infection. The aim of this study was to show whether the disease could be expressed after previously infected crabs were exposed to a stressor (elevated temperature). Exposure trials were designed in which shore crabs were exposed to WSSV and either maintained at 20°C for the duration of the study or exposed to an increasing temperature (20°C-26°C), at a rate of 2°C increase per week. Following administration of a standardised WSSV dose (2.75 x 10^4 copies per µl, 10µl g^{-1} wet body weight); subsequent assessment of viral load per mg of tissue, and histopathology of gill, connective tissues and heart suggested that individual crabs exhibited different disease development. Further, this individual variability was not significantly altered by exposure to temperature stress as outlined. Crabs could be divided into two groups, one group termed 'high responders' developed high viral loadings and WSSV pathology within target tissues. Conversely, 'low responders' showed very low (to absent) viral loadings and infrequent pathognomonic signs of WSSV infection. To compare viral replication in shore crabs (low susceptibility species) with a highly susceptible species such as shrimp (*Litopenaeus vannamei*) a subsequent study was designed in which both species were exposed to WSSV and samples were taken at set sampling points throughout a 48 hour period for comparison. This study revealed that viral loading in shrimp tissues increased dramatically over the period whereas the viral loading decreased in shore crabs over the same period. Results from these studies indicate that low-susceptible individuals of shore crab are able to control viral replication in the very early phases of infection. This ability contrasts that of highly susceptible penaeid shrimp. For the first time, the study also demonstrates

significant inter-individual variability in the response of hosts to infection by WSSV.

5.2 Introduction

Disease results from a complex interaction between the host, the pathogen and the environment in which both reside (Figure 1). There is considerable evidence to support links between the exposure of hosts to environmental stressors and the propensity for infection to progress to disease (Snieszko, 1974). This was expanded by Shields (2013) to include a fourth factor, anthropogenic, stating that factors such as overfishing, contaminants and global warming can affect host populations as well as pathogens.

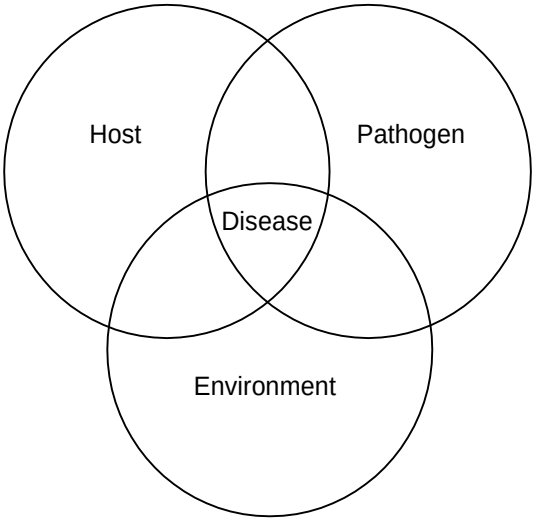

Figure 1. Stress diagram, recreated from Snieszko (1974). An overt infectious disease occurs when a susceptible host is exposed to a virulent pathogen under permissive environmental conditions.

Events such as temperature fluctuation can act as a stressor to the extent that they tax or exceed the adaptive resources of the individual. In this respect, stress determines the outcome of a host-pathogen interaction with highly infectious diseases showing the strongest association with stress (Hinkle, 1987; Korte *et al*., 2005; Maes *et al*., 1987). The role of stress in the host response to infectious disease has been long realised, as expressed by Dubos (1955):

> "There are many situations in which the microbe is a constant and ubiquitous component of the environment but causes diseases only when some weakening of the patient by another factor allows infection to proceed unrestrained, at least for a while. Theories of disease must account for the surprising fact that, in any community, a large percentage of healthy and normal individuals continually harbours potentially pathogenic microbes without suffering any symptoms or lesions."

Individuals experiencing sub-optimal environmental conditions should be more susceptible to disease. In addition, stress is more likely to increase the severity of that disease if the stressor acts to increase host susceptibility (Lafferty and Holt, 2003). Stressors such as increased temperature from climatic variation and global warming act to increase host susceptibility by weakening the host immune defences or by increasing the availability, transmission and severity of pathogens (Shields, 2013). Unpublished information mentioned in the paper by Flegel and Pasharawipas (1998) suggested that innocuous WSSV infections could be induced to disease and mortality by application of an appropriate stressor. It is important to note that WSSV has previously been shown to be carried in shrimp populations at low intensities in low-stress culture conditions without mortality events occurring (Tsai *et al*., 1999). Disease outbreaks on shrimp farms are known to be induced by stressors such as rapid change in salinity or reductions in temperature (Vidal *et al*., 2001; Granja *et al*., 2003; Guan *et al*., 2003).

The effect of water temperature on viral replication and pathogenicity is well known and clear correlations have been shown for aquatic viruses such as Koi Herpes Virus (Gilad *et al*., 2003), Cyprinid Herpes Virus (Sano *et al*., 1993), Infectious Hematopoietic Necrosis Virus (Amend, 1970), Viral Hemorrhagic Septicemia Virus (Castric and de Kinkelin, 1984) and Infectious Pancreatic Necrosis Virus (Dorson and Touchy, 1981). Temperatures above 16°C and below 32°C have been shown to be suitable for WSSV replication to occur in susceptible hosts (Bateman *et al*., 2012a; Bateman *et al*., 2012b; Corbel *et al*., 2001; Guan

et al., 2003; Jiravanichpaisal *et al*., 2004; Jiravanichpaisal *et al*., 2006; Rahman *et al*., 2006). The effect of temperature on WSSV infections is well documented with the prevalence of WSSV reduced in higher water temperatures (i.e. warm seasons in grow out ponds and hatcheries) (Du *et al*., 2008; Rahman *et al*., 2007b; Rodriguez *et al*., 2003; Withyachumnarnkul *et al*., 2003). Research in to the effects of temperature on WSSV infection has led to recommendations for use of high water temperature culture (≥32°C) as a method of controlling the disease and mortality associated with infection in tropical shrimp farming nations (Rahman *et al*., 2006; Vidal *et al*., 2001). Mortality of WSSV-infected shrimp (*L. vannamei*) or crayfish (*Pacifastacus leniusculus, Astacus astacus, Procambarus clarkii*) was reduced or even totally absent at higher (32°C - 33°C) or lower temperatures (<15°C) in comparison to the optimum temperature range (26°C - 27°C). Low temperatures (10°C - 15°C) have also been shown to reduce or delay mortality in susceptible hosts (Du *et al*., 2008; Guan *et al*., 2003; Jiravanichpaisal *et al*., 2004; Vidal *et al*., 2001). However, infection has been shown to remain latent at these lower temperatures. When WSSV-infected crayfish were held at 10°C for 24 days no mortalities were seen, but once transferred to 24°C, 100% mortality was observed within 6 days (highlighting the persistent nature of the infection in crayfish). Du *et al*. (2008) showed that low temperature reduced rather than stopped viral replication; results indicated that freshwater crayfish (*P. clarkii*) infected with WSSV but held at low temperature may serve as a reservoir to spread the virus and to cause disease outbreaks if temperature was to rise at a later date. In addition, even at optimum temperature range (26°C - 27°C) for WSSV replication, differences in virulence between specific strains have been reported (Rahman *et al*., 2006; Wang *et al*., 1999). The suggested mechanisms to explain the effects that temperature may play in affecting WSSV have been suggested to be replication (Du *et al*., 2006), apoptosis (Granja *et al*., 2003; Granja *et al*., 2006) and altered gene expression of WSSV (Reyes *et al*., 2007). Severity of WSSV infection may be due to environmental factors, for example temperature could increase susceptibility of the host as well as the viral replication process (Moser *et al*., 2012).

Moser *et al*. (2012) tested a range of larval, juvenile and adult wild crustacean species (*Callinectes sapidus*, *Cancer* species, *Clibanarius vittatus*, *Litopenaeus stylirostris, Litopenaeus vannamei, Farfantepenaeus californiensis, Macrobrachium tenellum*); holding them at 18°C followed by a 2 day incubation period at 29°C. They found that when the crab and shrimp were initially tested for WSSV (via PCR) on day 0 they were negative however when re-tested for WSSV following the temperature increase, numerous hosts tested positive for WSSV via the same diagnostic. These findings suggest that replication was mitigated by retaining animals at lower temperatures. It has been suggested that WSSV may enter target tissues and replicate at an increased rate at higher temperatures while lower temperatures may decrease infection because the virus attaches to the cell surface without entering and replicating (Moser *et al.*, 2012). Moser *et al*. (2012) suggested raising temperature to 29°C for 48 hours before testing batches of hosts for WSSV using PCR; this serving to reduce false negatives due to low viral load or viral latency within these hosts.

Whilst WSSV has a wide host range among decapod crustaceans (Bateman *et al*., 2012a; Corbel *et al*., 2001; Du *et al*., 2008; Jiravanichpaisal *et al*., 2001; Stentiford *et al*., 2009), some species appear to be less susceptible than others. *Macrobrachium rosenbergii* is considered to be tolerant to WSSV infection, however the mechanism of resistance is unknown (Sahul Hameed *et al*., 2000). The European shore crab *Carcinus maenas* has also been shown to be less susceptible to disease associated with WSSV infection when compared to other marine crab species (Bateman *et al*., 2012a) although again, the mechanism of resistance is unknown. WSSV is currently the only member of the Nimaviridae genus, a situation that is acknowledged to be unusual by the International Committee on Taxonomy of Viruses (ICTV) (Vlak *et al*., 2005). The ICTV has predicted that that this may change as new strains and isolates are discovered. Vlak *et al*. (2005) have also tentatively listed B virus and RV-CM from *Carcinus maenas* (Bazin *et al*., 1974; Johnson, 1988), B2 virus and τ (tau) virus from *Carcinus mediterraneus* (Mari and Bonami, 1986; Pappalardo *et al*., 1986) and Baculo-A and Baculo-B viruses from *Callinectes sapidus* (Johnson, 1976a; Johnson, 1983) within this genus. B, RV-CM, B2, and Baculo-B viruses are

extremely similar in terms of size, shape and morphogenesis to that reported for WSSV; in fact these viruses appear morphologically indistinguishable and it has been suggested that these viruses may be ancestral forms of WSSV (Bonami and Zhang, 2011). If this is the case then it is conceivable that previous exposure to an ancestral form of the virus may provide the crab with mechanisms to deal with exposure to similar viruses such as the WSSV. Prior exposure to B virus which is similar if not identical to WSSV (Bonami and Zhang, 2011) may have provided *Carcinus maenas* with a genomic basis for relative resistance to WSSV.

The main aim of this study was to determine whether *Carcinus maenas* is functionally resistant to WSSV infection or whether disease can be induced in infected animals following exposure of the host to a stressor. Temperature was used as the stressor and trials were designed in which shore crabs were exposed to WSSV and either maintained at 20°C for the duration of the study or exposed to an increasing temperature regime of 20°C - 26°C (2°C increment per week). The temperature range of *Carcinus maenas* in its native range falls within average summer surface- temperatures of around 22°C (Cohen *et al*., 1995). The higher temperature was chosen to reflect possible summer maxima in shallow coastal lagoons and rockpools of Europe (Cohen *et al*., 1995). Since shore crabs would be likely to experience such temperatures for relatively short periods within a given day, maintenance for extending periods at this temperature was deemed likely to impart significant stress effects on the host. In order to compare viral replication in shore crabs (low susceptible species) with a highly susceptible species such as shrimp (*Litopenaeus vannamei*) a study was designed where both species were exposed to WSSV and samples were taken at set sampling points throughout a 48 hour period for comparison.

5.3 Materials and Methods

All experimental trials were performed within the biosecure exotic disease facility as described in chapter 3. Temperature was regulated according to the experimental conditions required (details below in section 5.3.5).

5.3.1 eparation of viral innoculum

WSSV inoculum was prepared as in Bateman *et al*. (2012a). See Chapter 3 for full details.

5.3.2 hore crab collection

Shore crabs *(Carcinus maenas)* approximately 30mm in carapace width, were collected from the shoreline at Newton's Cove, Weymouth, UK (50º34' N, 02º22' W). All animals utilised in experimental challenge trials appeared externally healthy. To prevent conflict, shore crabs were housed individually in custom-made compartments within large trough tanks, with individuals separated but sharing the same water supply. A maximum of 15 crabs were housed per trough. All animals were acclimatised to the trial start condition (20˚C) for a minimum of one week before trials commenced.

5.3.3 Viral loading in shrimp tissues over 48 h period

Sixty SPF *L. vannamei* (sourced from the Centre for Sustainable Aquaculture Research (CSAR) at the University of Swansea, United Kingdom) were split into two treatment groups (n=30 per treatment) and water temperature was held constant at 27˚C for the duration of the trial. Shrimp were inoculated via intramuscular injection within the 3rd abdominal segment with either saline or a single dose of the diluted WSSV homogenate at a rate of 10µl g^{-1} wet body weight on Day 0. Shrimp were sampled at 0, 6, 12, 24, and 48 hours post injection. For molecular analyses, pleopod samples were removed and placed into tubes containing 100% ethanol; gill tissues were snap frozen in liquid nitrogen and were stored in -80°C freezer until analysis. For histology shrimp were fixed whole by injecting Davidson's seawater fixative at multiple sites throughout the body and immediately placing carcass into a pot of Davidson's seawater fixative.

5.3.4 ral loading in shore crab tissues over 48 h period

Sixty shore crabs (*Carcinus maenas*), were split into two treatment groups (n=30 per treatment) and water temperature was held constant at 20˚C for the duration of the trial. Crabs were injected at the base of the second walking leg with saline

or a single dose of the diluted WSSV homogenate at a rate of 10µl g^{-1} wet body weight on Day 0. Crabs were sampled at 0, 6, 12, 24, and 48 hours post injection. As standard, gill, hepatopancreas, heart, gonad, connective tissues and muscle were dissected and placed into histological cassettes and fixed immediately in Davidson's seawater fixative. For molecular analyses, gill samples were removed and placed into tubes containing 100% ethanol. Gill tissues were also snap frozen in liquid nitrogen and stored at -80°C. The remaining tissues and carcasses were stored at -80°C.

5.3.5 hore crab - temperature stressor study

Two hundred and forty shore crabs (*Carcinus maenas*) were split into four treatment groups (n=60 per treatment). Crabs were injected with saline (see appendix) or a single dose of the diluted WSSV homogenate (see section 5.3.1) at a rate of 10µl g^{-1} wet body weight on Day 0. For the remainder of the trial (30 days) samples in all tanks were fed on squid tissues at a ratio of approximately 3-4% wet body weight.day^{-1}.

Group 1 animals were injected with saline and temperature was held constant at 20˚C for the duration of the trial. Group 2 animals were injected with saline; water temperature was held constant at 20°C for 1 week, and increased by 2˚C at day 7 (to 22˚C), day 14 (to 24˚C) and day 21 (to 26˚C). Group 3 animals were injected with a single dose of the diluted WSSV homogenate; water temperature was held constant at 20˚C for the duration of the trial. Group 4 animals were injected with a single dose of the diluted WSSV homogenate; water temperature was held constant at 20°C for 1 week and increased 2˚C at day 7 (to 22˚C), day 14 (to 24˚C) and day 21 (to 26˚C).

All exposure groups were observed regularly throughout daylight hours. Dead and moribund animals were removed from each tank and dissected immediately. At the end of each challenge trial, and for moribund animals sampled within the trial, animals were chilled on ice for 10 minutes prior to dissection. As standard, gill, hepatopancreas, heart, gonad, connective tissues and muscle were dissected and placed into histological cassettes and fixed immediately in Davidson's seawater fixative. For molecular analyses, gill and hepatopancreas

samples were removed and placed into tubes containing 100% ethanol. The remaining tissues and carcasses were stored at -80°C.

5.3.6 gy

See Chapter 4 for full details.

5.3.7 xtraction

See Chapter 4 for full details.

5.3.8 uantitative PCR (qPCR)

Samples were analysed via qPCR using the OIE recommended qPCR protocol for the detection of WSSV (Durand and Lightner, 2002). Each 20µl qPCR reaction contained 0.8µl of WSSV probe (100nM), 0.8µl of WSSV qPCR forward primer (300nM), 0.8µl of WSSV qPCR reverse primer (300nM) (Table 1), 10µl of Taqman master mix (Applied Biosystems, Cheshire, UK), 5.1µl of molecular grade water and 2.5µl of each sample. Each sample was run in triplicate.

Table 1. WSSV qPCR primers and probes (Durand and Lightner, 2002)

Primer and probe name	Sequence
WSSV probe	6FAM-AGCCATGAAGAATGCCGTCTATCACACA-Tamra
WSSV forward primer 1011F	TGGTCCCGTCCTCATCTCAG
WSSV reverse primer 1079R	GCTGCCTTGCCGGAAATTA

qPCR plates were run using the Applied Biosystems Stepone Plus Real Time qPCR machine using the following program parameters: 50^{o}C for 2 min, 95^{o}C for 10 min, 95^{o}C for 15 sec, 60^{o}C for 1 min. This was repeated for 40 cycles.

5.3.9 WSSV Standard Curve

In order to quantify viral load within crab and shrimp tissues, a standard curve of known amounts of plasmid DNA was generated using qPCR. Quantitation of the amount of target in unknown samples was accomplished by measuring CT and use of a standard curve to determine initial copy number. Primers (WSS1011F/WSS1079R) were used to generate a 69-bp amplicon (representing

the upstream primer plus the probe and the downstream primer) which was cloned. To make a standard curve 4µl of WSSV plasmid (1.6 x 10^9 copies/µl) was added to 156µl of molecular grade water and vortexed to give 4 x 10^7 copies/µl. Five microlitres of this mixture was added to a microfuge tube containing 45µl shore crab extract (shown to be free from WSSV via nested PCR) vortexed to mix and subsequent serial tenfold dilutions were made to give concentrations from 4 x 10^6 to 4 x 10^1 copies/µl. Each of the tenfold serial dilutions (4 x 10^7 to 4 x 10^1copies/µl) were vortexed and 2.5µl of each dilution was added in triplicate to each qPCR plate. This gave final concentrations in the standard curve of: 1 x 10^8 to 1 x 10^1 copies.

5.3.10 Statistical Analysis

Survival was measured in days, Kaplan-Meier curves were plotted for the four groups defined by treatment (Control, WSSV) and temperature (constant, increasing), and the log-rank test used to test for a statistically significant difference between these groups. To obtain further details on the relationships between treatment, temperature and survival, a Cox proportional hazards regression model was used to test the effects of treatment, temperature, and the interaction between these two terms. The viral load data was first log (10)-transformed to normalise the data. Only 58 crabs were included in this analysis per treatment as viral load was shown to be absent or below level of detection in 2 crabs from each treatment group, these were removed from the analysis. In order to ensure that the inclusion or exclusion of these animals did not change the results, we repeated the described analyses after assigning a value of 0.1 to them before the log transformation. In order to avoid biasing the results, only crabs who survived the study were included in the final analyses of viral load. Linear regression models were used to test for and measure an association between viral load and the number of tissues infected with WSSV (out of 3 possible tissues: heart, gill and connective tissues), and also to investigate whether presence or absence of WSSV in specific tissues had differing effects on viral load. Analysis of variance was used to investigate the effect of temperature, sex and initial dose on viral load at 30 days.

All statistical analyses were performed in Stata 12 with the assistance of Dr Rachel Jinks, Animal and Plant Health Agency.

5.4 Results

5.4.1 Mortality in shrimp and crabs

C. maenas which were injected with saline and held at 20˚C displayed 85% survival by day 30 of the trial, whereas crabs held at the increasing temperature regime experienced 80% survival (Figure 2, blue lines). *C. maenas* injected with WSSV inoculum and held at 20˚C experienced 67% survival whereas those held at the increasing temperature regime displayed 62% survival by day 30 (Figure 2, red lines).

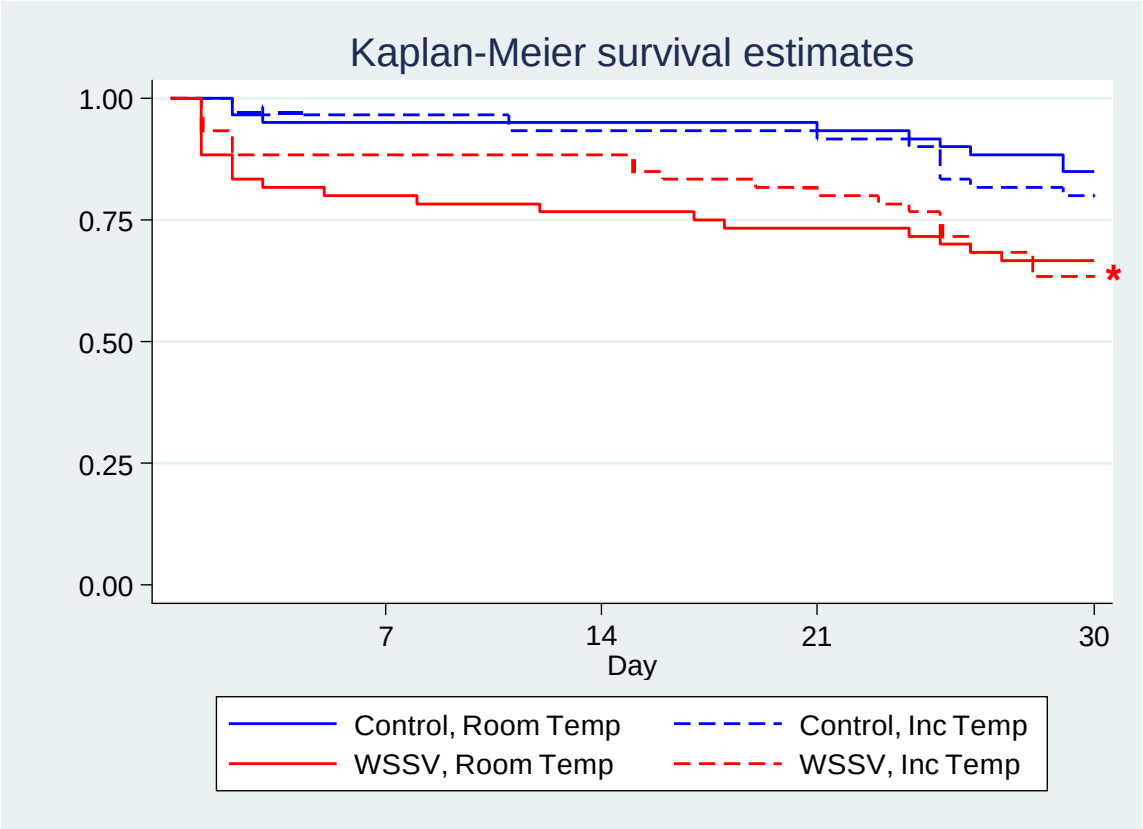

Figure 2. Kaplan-Meier plot of *Carcinus maenas* exposed to WSSV at different temperatures (n= 60). Solid lines show crabs which were kept at 20°C, dashed lines show crabs which were exposed to increasing temperatures at day 7 (22°C), day 14 (24°C) and day 21 (26°C). * indicates that survival in the WSSV exposed crabs is significantly different from the controls, WSSV exposed crabs >2.5 times more likely to die (p=0.015).

A Kaplan-Meier log-rank test revealed differences in survival between treatment groups, p=0.012. In addition, a Cox proportional hazards regression model (to detect effects of the treatment (saline injected or WSSV injected) and temperature (constant temperature or increasing temperature) on the risk of death) indicated a significant effect of treatment group (with crabs exposed to WSSV >2.5 times more likely to die compared to untreated control crabs (Hazard Ratio 2.65 [95% CI 1.12-5.84; p=0.015). In contrast, there was no significant effect of the increasing temperature regime on risk of death, in either control or WSSV-exposed groups.

5.4.2 ral Loading in shrimp and crabs

48 hour study

Initial viral load of the WSSV inoculum was determined via qPCR, allowing for calculation of the total viral load administered to each individual crab and shrimp. Individual crab and shrimp were inoculated at a dose rate of 270 copies per mg of tissue. Initial viral loading was compared to the final viral loading in individual crabs and shrimp at the end of trial (48 h). Mean viral loading increased in shrimp over the trial period, in contrast, mean viral loading decreased in shore crabs over the same period (Figure 3). Interestingly, although mean WSSV viral loading at 48 h exceeded 4500 copies per mg of tissue, viral copy number decreased at 6 and 12 h and was lower than that expected from detection of the received dose. Between 12 and 24 h, the viral loading marginally exceeds the expected loading related to the received dose whilst viral copy number increased exponentially between the 24 and 48 h time points. In comparison the viral copy number in shore crab tissues gradually reduces over the 48 hour period (Figure 3).

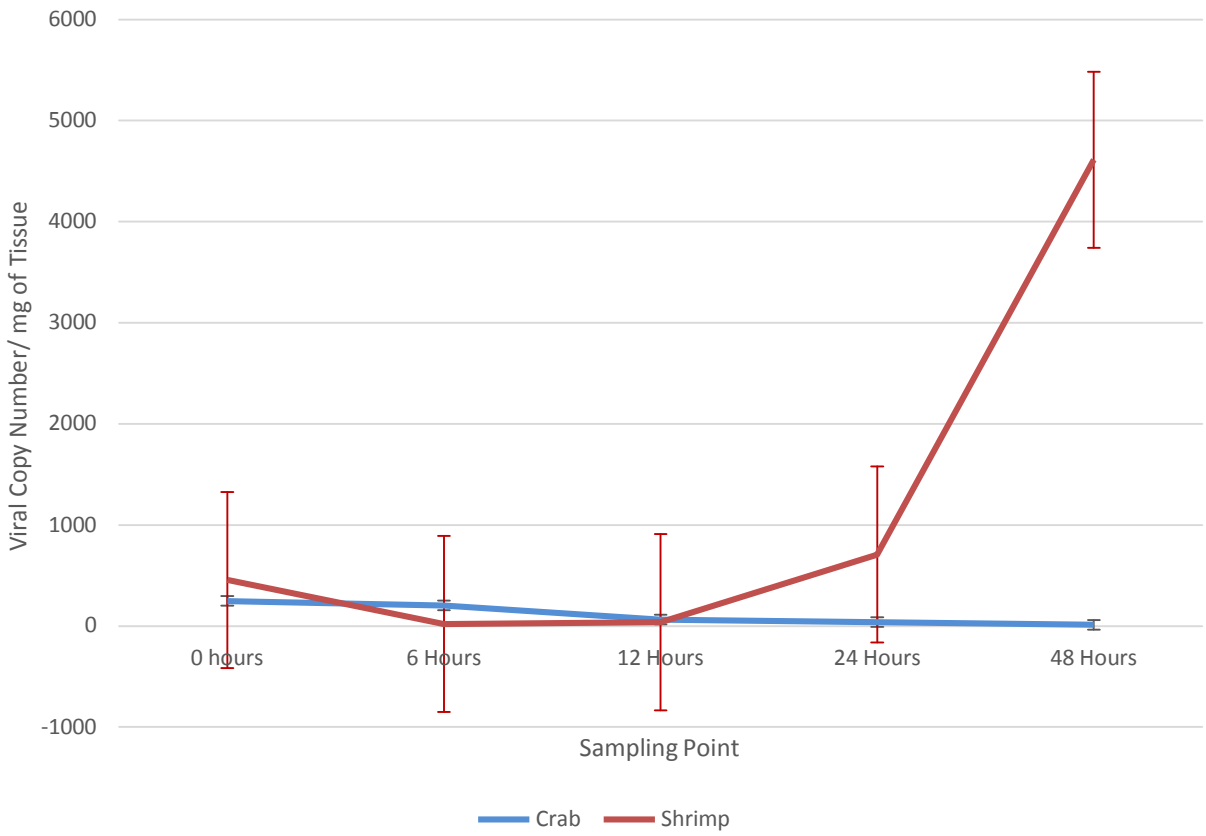

Figure 3. Mean viral loading between *C. maenas* (n=6) and *L. vannamei* (n=6) taken at set sampling points over a 48 hour period. Viral loading increasingly exponentially in shrimp tissues over the period between 24 and 48 h (red line). Viral loading in crab tissues declined steadily from the received dose at time 0 (blue line).

30 day study

Shore crabs exposed to WSSV via injection and then housed at 20°C or at the increasing temperatures regime (20°C to 26°C) displayed varying levels of WSSV viral copy number and could be split into two main groups: those which survived the study and were sampled at end of trial (day 30) and those sampled before the end of trial (moribund or recently dead). Table 2 displays the numbers of crabs sampled at end of trial (Survived) and those sampled before the end of trial (Died in trial), by exposure group.

Table 2. Comparison of crabs sampled at end of exposure (Survived) and those sampled during the exposure (Died in trial).

Group	Died in trial	Survived	Total
Control Constant Temp	9	51	60
	15.00%	85.00%	100.00%
Control Inc Temp	12	48	60
	20.00%	80.00%	100.00%
WSSV Constant Temp	20	40	60
	33.33%	66.67%	100.00%
WSSV Inc Temp	22	38	60
	36.67%	63.33%	100.00%
Total	63	177	240
	26.25%	73.75%	100.00%

If this data is plotted to show the viral loading split by temperature exposure and survival (Figure 4) there appears to be a difference between the constant temperature crabs (20°C) which were sampled before 30 days and the increasing temperature crabs (20°C - 26°C) which were sampled before day 30. Crabs exposed to higher temperature displaying a higher viral loading when compared to constant temperature crabs.

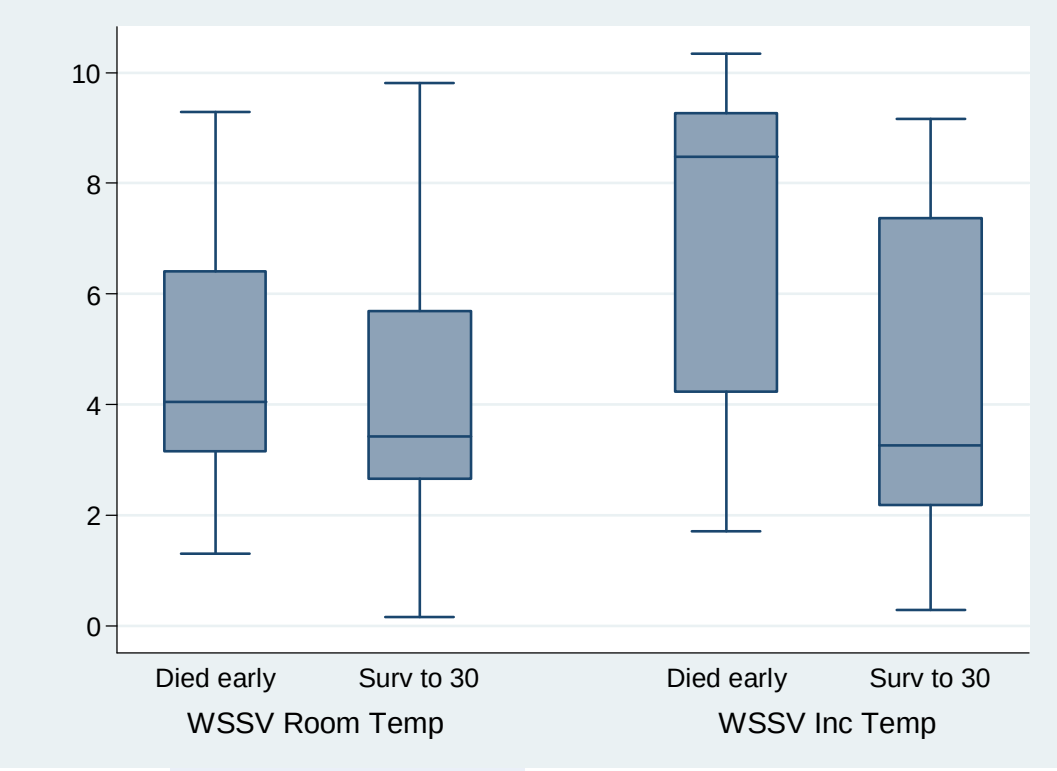

Figure 4. Final viral loading of crabs (log (final VL per mg)) which were sampled before day 30 (Died in trial) and at day 30 (Survived trial) between the exposure groups. Crabs which were sampled before day 30 showed a higher viral loading in the WSSV increasing temperature exposure group.

A t-test to detect the difference in final viral load between crabs maintained at constant temperature and those exposed to increasing temperature did not show a significant difference between the two (p=0.6571). However if the crabs which died in trial in each of these exposure groups were compared there was a significant difference between the two, crabs exposed to increasing temperature showing a significantly higher viral loading (p=0.0041).

Figure 5 displays the viral loading of all crabs between the two exposures. Only 58 crabs were included in this analysis per treatment as viral load was shown to be absent or below level of detection in 2 crabs from each treatment group, these were removed from the analysis.

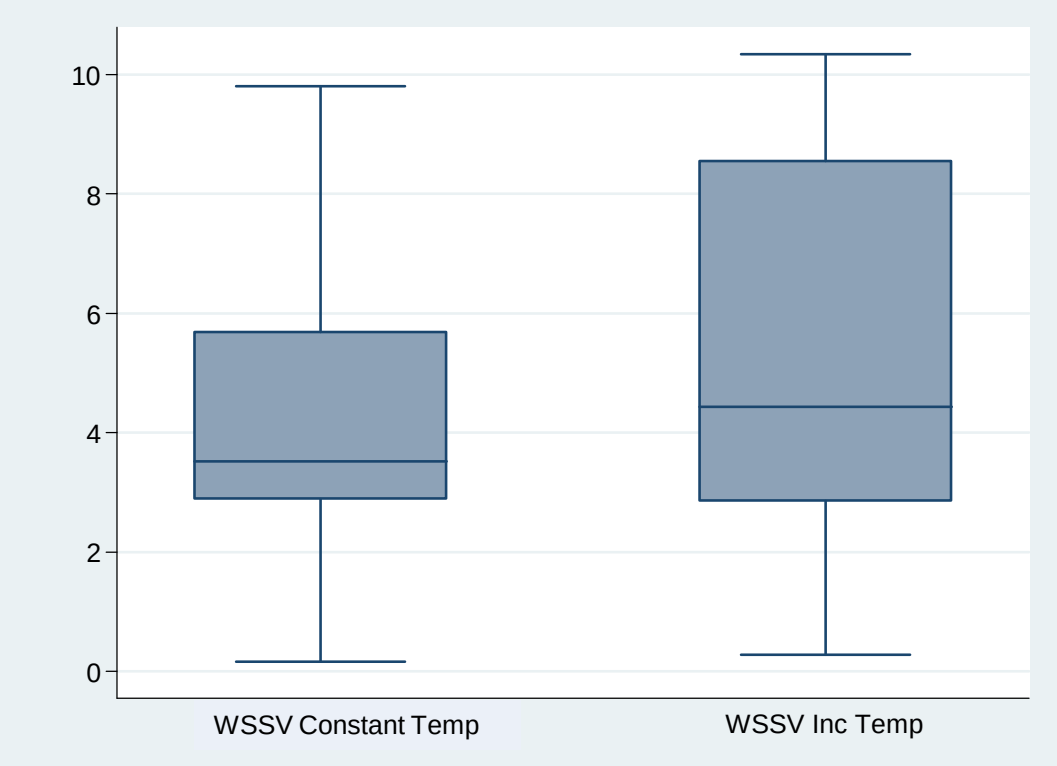

Figure 5. Final viral loading of crabs (log(final VL per mg)) which were exposed to WSSV at constant temperature (20°C) and those exposed to WSSV at increasing temperatures (20°C - 26°C). Crabs which were exposed to increasing temperature showed a significantly higher viral loading than those which were maintained at constant temperature.

If the data from all crabs (those which died in trial and those which survived trial) are included in an analysis of variance (ANOVA) there is a significant difference between the groups (p=0.0273). Crabs exposed to increasing temperatures

(20°C - 26°C) showed a significantly higher viral loading than crabs which were maintained at constant temperature (20°C).

Crabs were injected with a single dose of the diluted WSSV homogenate (2.75 x 10^4 copies per µl) at a rate of 10µl g^{-1} wet body weight on Day 0, dashed line in Figure 6 indicates mean initial viral loading administered at the start of the study. Some crabs appeared to reduce the viral loading of virus present in tissues i.e. final copy number was below that initially inoculated at day 0, represented by the red circles and blue squares below the dashed line in Figure 6. Other crabs appeared to show replication of WSSV within the tissues with the final viral loading being above that initially inoculated at day 0, represented by the red circles and blue squares above the dashed line in Figure 6. Interestingly this distribution of viral reduction or viral replication occurred in both crabs which were maintained at 20°C (blue squares, Figure 6) and those which were exposed to an increasing temperature (red circles, Figure 6). The individual crab either enables replication, showing an increased viral load over the course of the study or the individual suppresses replication, showing a reduced viral load over the course of the study. However in crabs where replication was enabled there was a significantly higher viral loading in crabs exposed to increasing temperatures (20°C - 26°C) when compared to crabs which were maintained at constant temperature (20°C).

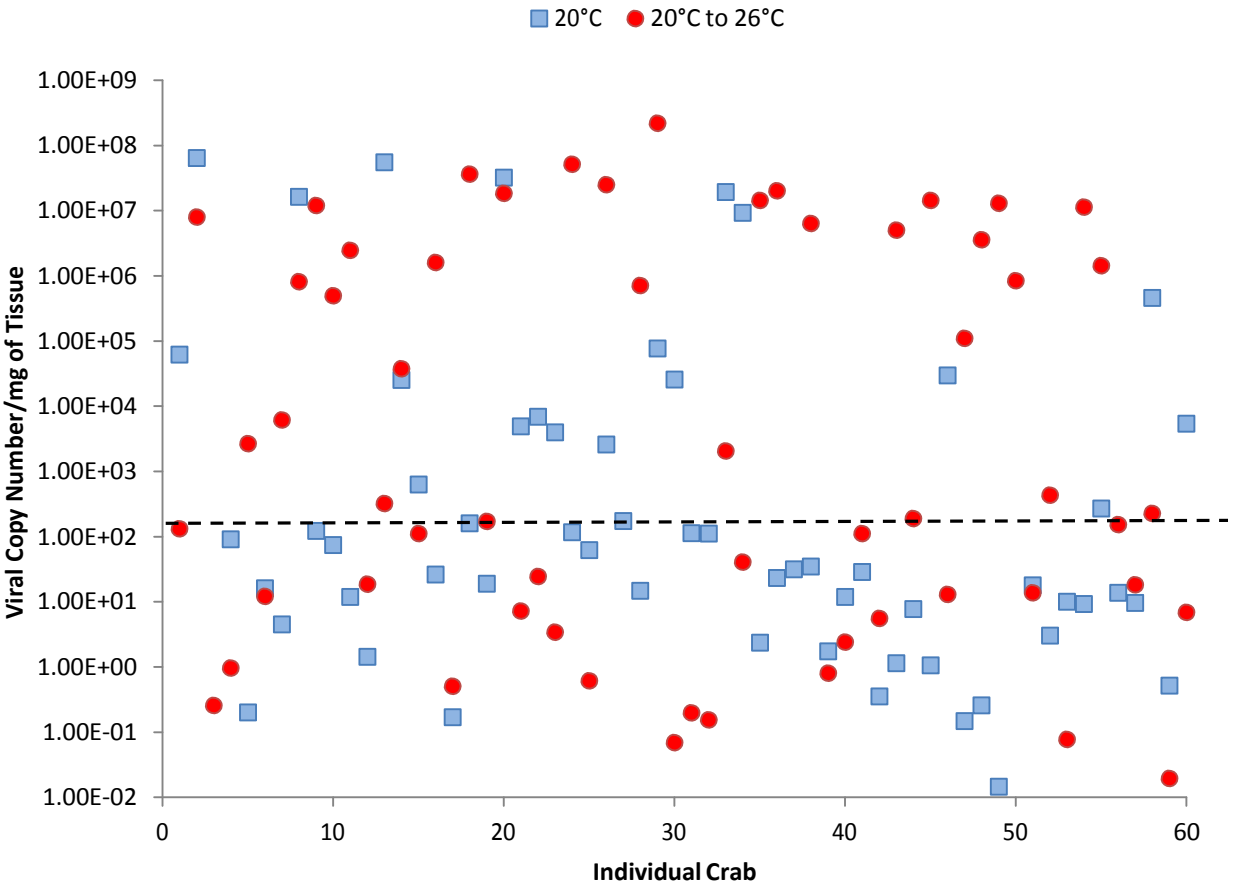

Figure 6. Total viral loading of individual shore crabs after 30 day temperature stressor study, viral copy number is expressed on logarithmic scale. Red circles indicate total viral loading in individual crabs after being exposed to WSSV at increasing temperatures (20°C - 26°C). Blue squares represent total viral loading of individual crabs after exposure to WSSV and being held at constant temperature (20°C). Dashed line indicates initial viral loading administered at the start of the study.

Total viral loading data in each crab at the end of the studies was compared with initial dose given to each crab at the start of the study, gender of the crab and exposure treatments. Using ANOVA to investigate the effect of the gender of the crab, initial dose administered and the treatments showed that gender ($p=0.3429$) and initial dose administered ($p=0.1295$) had no effect on the viral loading; however as expected differences between the exposure groups ($p=0.0111$) were significant (Table 4).

Table 3. Analysis of variance to investigate the effect of the gender of the crab, initial dose administered and the temperature treatments on total viral loading. Red line highlights that the different exposure groups had a significant effect on viral loading but gender and initial dose administered had no effect on viral loading.

```
                  Number of obs =     116     R-squared     =  0.0672
                  Root MSE      = 2.70999     Adj R-squared =  0.0422

      Source |  Partial SS    df       MS           F     Prob > F
-------------+----------------------------------------------------
       Model |  59.2256206     3  19.7418735       2.69     0.0498
             |
       group |  49.0027809     1  49.0027809       6.67     0.0111
         sex |  6.66323835     1  6.66323835       0.91     0.3429
  loginitmg  |  17.1325728     1  17.1325728       2.33     0.1295
             |
    Residual |  822.532986   112  7.34404451
-------------+----------------------------------------------------
       Total |  881.758606   115  7.66746614
```

5.4.3 gy

Carcinus maenas has been shown to be less susceptible than other decapod species, with difficulty in identifying infections via histology (Bateman *et al*., 2012a). This was also the case in crabs sampled during the 48 hour exposure where pathognomonic signs of WSSV infection were not observed. However, shrimp tissues displayed varying signs of pathology after 24 hours exposure. Pathology was observed in crabs sampled from the thirty day temperature stressor; however, it is important to note that not all crabs exposed in the stressor study displayed signs of infection via histological analysis. It was easier to identify infections in crabs which had been exposed to increasing temperatures when compared to those held at 20°C. Tissue type and the presence of viral replication were recorded. These data were then compared with the viral loading data; results indicated that as viral load increased pathology became more evident in the tissues (Figure 7).

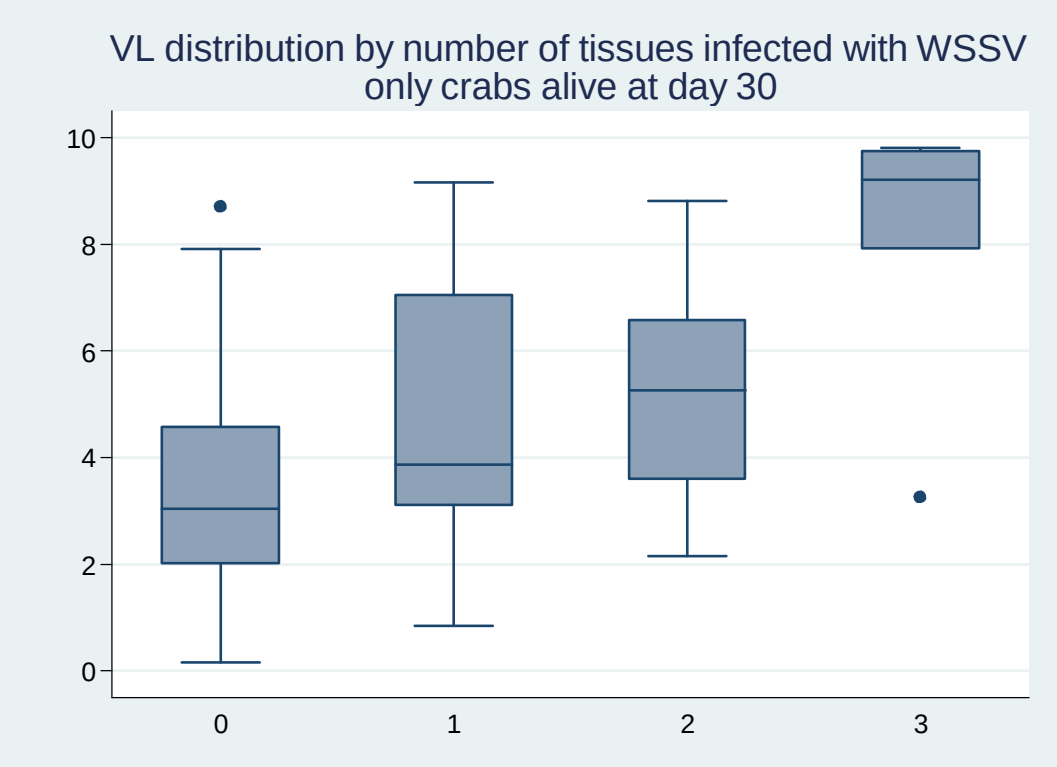

Figure 7. Comparison between viral loading (log(final VL per mg) and presence of pathology in various tissues. 0 – No Pathology seen, 1 - Hypertrophied nuclei present in gill tissues. 2 – Hypertrophied nuclei present in gill and heart tissues. 3 – Hypertrophied nuclei present in gill, heart and connective tissues. These data include crabs which were sampled at day 30 (n=75).

A linear regression model showed that mean final viral loading per mg of crab tissue was 1.20 times higher in crabs displaying pathognomonic signs of WSSV in one tissue type, 1.80 times higher in those with WSSV signs in 2 tissues and 4.71 higher in those displaying signs in 3 tissues, when compared to those showing no pathology. Further analysis suggested that tissue type where signs were noted was also an important factor in observed viral loading: Pathognomonic signs in the heart were associated with a significant increase in final viral loading (1.75, p=0.012) while presence of signs in the gills significantly increases loading by 2.52 (p=0.001). Presence of pathognomonic signs of WSSV in connective tissues was not associated with a significant increase in final viral loading (p=0.893).

As mentioned in previous studies, gill tissues were also deemed most suitable for detection of WSSV infection via histology (Bateman *et al*., 2012a; Lo *et al*., 1996a). Heart and connective tissues were also suitable for detection of

hypertrophied nuclei with eosinophilic inclusion bodies, characteristic of WSSV infection (Figure 8).

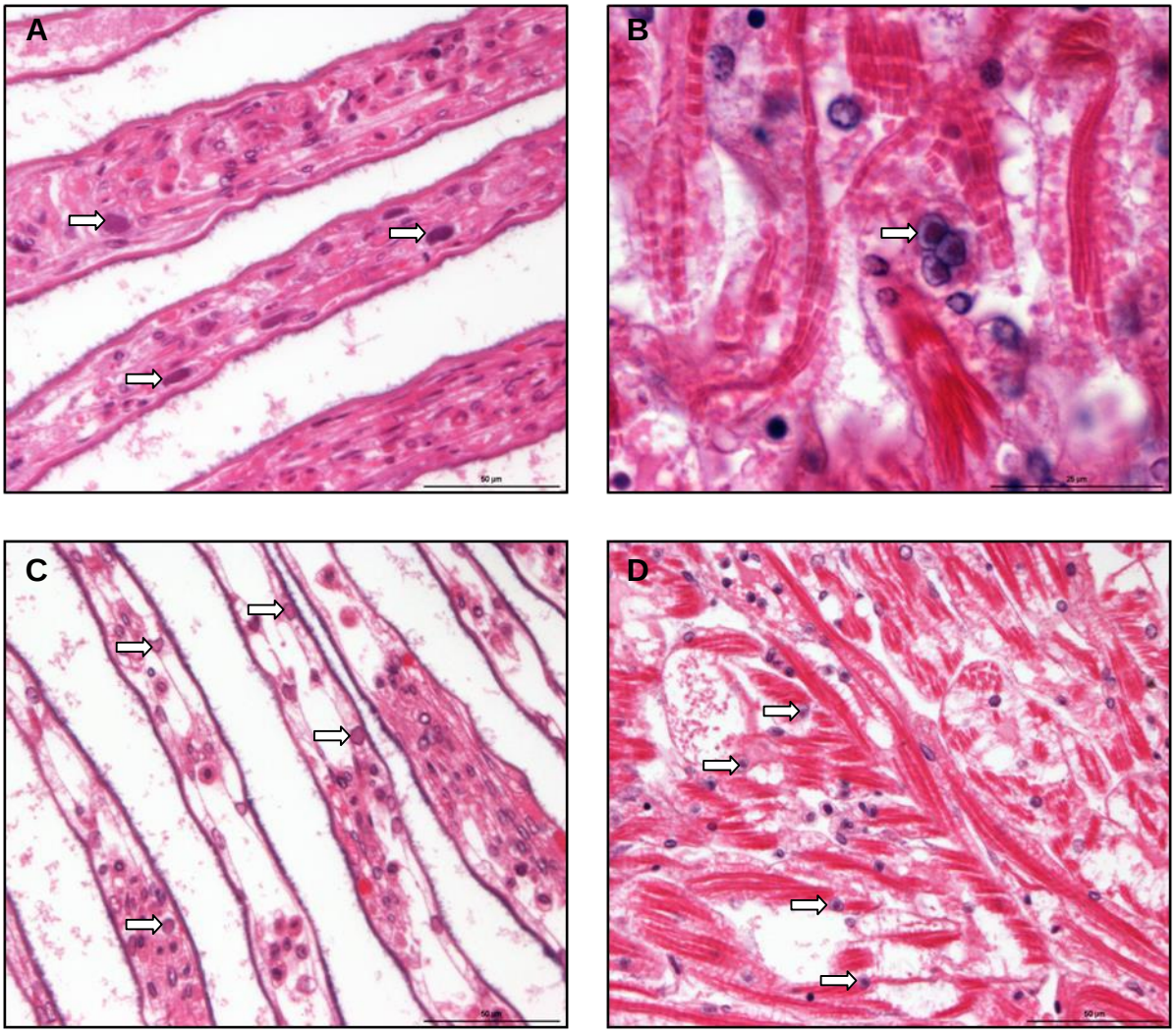

Figure 8. WSSV infected gill and heart tissues of *Carcinus maenas*. (A) Infected nuclei (arrows) can be observed scattered throughout the gill filaments of *C. maenas* exposed to WSSV and maintained at 20°C. Scale bar = 50µm. (B) Hypertrophied nuclei were present in the heart tissues of *C. maenas* exposed to WSSV and maintained at 20°C. (C) Hypertrophied nuclei can be found within the gill filaments displaying marginalized chromatin and eosinophillic inclusion bodies (white arrow) in *C. maenas* exposed to WSSV at increasing temperatures. Scale bar = 50µm. (D) Heart tissue showed signs of infection with hypertrophied nuclei (arrows) evident dispersed throughout the tissue in *C. maenas* exposed to WSSV at increasing temperatures. Scale bar = 50µm. All images H&E stain.

Histology also revealed the presence of other pathogens within shore crab tissues and organs. These data will not be presented here but, briefly, *Microphallus primus* (Saville and Irwin, 2005), *Hematodinium* sp. (Stentiford *et al*., 2002), *Ameson pulvis* (Stentiford *et al*., 2013) and Milky disease (Eddy *et al*.,

2007) were detected in shore crabs used in the current study. In addition a novel virus Herpes-like Virus (HLV) infection, somewhat mimicking the pathognomonic signs of WSSV infection was detected within haemocyte and connective tissues (in crabs retained in stressor conditions). This virus will be detailed more fully in Chapter 7.

5.5 Discussion

This study aimed to determine whether the shore crab is truly resistant to WSSV infection and whether the disease could be expressed after the crab was exposed to a temperature stressor. *Carcinus maenas* individuals were injected with WSSV and then exposed to varying temperature stress conditions. Assessment of total viral load per mg of crab tissue and histopathology from gill, connective tissues and heart suggested that crabs exhibited different disease development between individuals and this did not appear to be affected by temperature stress conditions. It was noted when pathology was present and in which tissues viral replication could be identified via histology. These data were then compared with viral loading data and results indicated that as viral load increased viral pathology became more evident in the tissues. Viral loading was higher in crabs showing pathology in heart and gills as opposed to no pathology. It was also shown that the tissue type infected, e.g. heart, is more important than the number of tissues infected.

Some crabs appeared to reduce the level of virus present in tissues i.e. final copy number was below that initially inoculated at day 0. Other crabs appeared to show replication of WSSV within the tissues with the final viral loading being above that initially inoculated at day 0 (Figure 6). However, this distribution of viral reduction or viral replication occurred in both crabs which were maintained at 20°C and those which were exposed to an increasing temperature. Total viral load data in each crab at the end of the studies were compared with initial dose given to each crab at the start of the study, gender of the crab, pathogens present and exposure treatments. There was a statistically significant difference between total viral loading between the two exposure groups (crabs exposed to WSSV at 20°C and those exposed to WSSV at increasing temperature (20°C - 26°C));

however, initial dose administered and gender of the crab was shown to have no effect on the viral loading. Crabs could be divided into two groups according to the differences in development of the disease (histopathology) and infection (viral load). One group termed high responders (HR) developed increased levels of WSSV pathology within the target tissues and higher viral loading than the second group termed low responders (LR) which showed reduced WSSV pathology and lower viral loading. The so called high responders developed severe pathology and elevated viral loading while low responders showed a reduced viral load and lower levels of pathology. High and low responders were present in stressed and non-stressed exposure groups suggesting that these individuals would either replicate the virus or not irrespective of the different stress conditions they were exposed to.

In order to compare viral replication in shore crabs (low susceptible species) with a highly susceptible species (*L. vannamei*) we exposed both species to WSSV and sampled at set points throughout a 48 hour period. This study revealed that the viral loading in shrimp tissues increased over the 48 hour period whereas the viral loading in shore crabs decreased over the same time period. Viral copy number in shrimps initially decreased between 0 and 12 hours, however at 12 hours the viral loading increased as the virus replicated within the tissues; the replication cycle of WSSV is known to be approximately 20 hours at 25°C (Chang *et al*., 1996; Chen *et al*., 2011; Wang *et al*., 2000b). Shrimp in this study were held at 27°C which could account for the slightly quicker replication rate. In comparison the viral copy number in shore crab tissues was seen to reduce over the 48 hour period. This study clearly showed that the shore crab, a low susceptibility species responds to WSSV infection in a different manner to that of a highly susceptible species. The shore crab appears to be able to limit the viral replication process in the first 48 hours of infection when compared to shrimp. The mechanism by which the shore crab achieves this is unknown and further work is needed to investigate this response.

It is important to note that this stress study would not have been possible with any other temperate marine species previously tested, edible crab (*Cancer*

pagurus), nephrops (*Nephrops norvegicus*) and lobster (*Homarus gammarus*) which have all been shown to be moderately susceptible to this virus and would have died within 10 days after being held at 20°C as shown in previous work (Bateman *et al*., 2012a).

Viral accommodation theory was originally suggested by Flegel and Pasharawipas (1998) to explain the lack of inflammatory response to viral pathogens in crustacea. Accomodation being characterised by the presence of an active mechanism to tolerate single and multiple viral infections without gross or histological signs of disease, a feature commonly seen in crustacea and insects, and by the absence of an active defence against it. This theory was updated by Flegel in 2007 to become:

> "crustaceans and other arthropods actively accommodate viral pathogens as persistent infections that act as a kind of memory that functions to specifically reduce the severity of disease and to dampen viral triggered apoptosis"

It had been shown that shrimp could carry infections of *Litopenaeus stylirostris* densovirus (*Pst*DNV) without actually becoming infected themselves, persistently infected survivors reaching adulthood and passing it onto their offspring. However when these shrimp were co-cultivated with naïve shrimp massive mortalities occurred in the naïve shrimp showing that the virus carried by persistently infected individuals was still virulent and not attenuated as thought (Flegel, 2007). A similar phenomenon was seen with Yellowhead Virus (YHV) when this virus first hit the shrimp culture industry it caused massive mortalities. However, this problem declined over a two-year period and it appeared that shrimp had developed a tolerance to infection with YHV similar to that seen with PstDNV (Chantanachookin *et al*., 1993). Longyant *et al*. (2005) revealed a difference in expression of viral proteins between individuals which did and did not show signs of Yellowhead disease after infection with Yellowhead Virus (YHV). Insects have been shown to carry a wide variety of animal and plant viruses without displaying gross signs of disease, persistently infected insects

appear unaffected themselves but continuously produce infectious virus particles that can cause disease in plants (Hogenhout *et al*., 2003) and animals similar to that shown in shrimp (Flegel, 2007). This process does not appear to be related to the number of virus particles present or the number of cells infected but on the genetics of both the virus and the host.

It was also shown with YHV that mortalities occurred in the presence of other non-lethal viral infections which appeared to be tolerated by the general shrimp population (Flegel *et al*., 2004). It has been noted previously that infection with one virus may protect against infection with a second virus. Tang *et al*. (2003b) showed that *P. stylirostris* infected with Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV) were partially protected against WSSV infection in challenge studies however *P. vannamei* without IHHNV were not protected. The underlying mechanism was unknown but it is noted that WSSV and IHHNV infect the same target tissues so presence of one virus in a cell may interfere with the entry or replication of another.

In 2006 Tang and Lightner reported the occurrence of non-infectious sequences of *Pst*DNV inserted into the genome of *Penaeus monodon* from East Africa and Australia. It was not known how this occurred but the PstDNV fragments were associated with transposable elements. PstDNV was not discovered until *P. monodon* stock was moved to the Americas and cultivated near *P. stylirostris* and caused a massive mortality. The virus has been shown to be endemic in *P. monodon* and causes no disease (Lightner, 1996b). This raised the question as to whether crustaceans and other arthropods were able to integrate viral sequences into their genome in order to possess some kind of protection from future infections, the viral sequences playing a role in subsequent occurrence of persistent infections without signs of disease (Flegel, 2009b). Chayaburakul *et al*. (2005) and Saksmerprome *et al*. (2011) screened for PstDNV in *P. monodon* using overlapping primers which had been designed to cover most of the viral genome. Individuals which tested positive with all primer sets were considered to be positive for infection whilst those that gave any negative results were analysed in detail to determine whether they showed true infection or whether the primers were actually detecting viral inserts. They showed that inserts of random

position and length were common and these could lead to false positive results with standard PCR tests and kits. It is currently unknown whether this phenomenon is present in Crustacea other than shrimp or with other shrimp and crustacean viruses. Hendrix *et al*. (2000) noted that this mechanism of incorporating genes suggests a fundamental role for viruses in the early evolution of host cells, a similar phenomenon is known from insects (Crochu *et al*., 2004; Lin *et al*., 1999) and in other systems, such as phages, that genes inserted into ancestors are retained because they increase the fitness and immunity and confer an advantageous phenotype on the host (Hendrix *et al*., 2000). It has been suggested that random integration of viral genome fragments into the host genome could lead to antisense RNA transcripts which are capable of suppressing propagation of the same virus within host tissues (Flegel, 2009b). These sequences provide protection via RNA interference (RNAi) pathways which have been demonstrated in plants, shrimp and insects (Kumria *et al*., 1998; Lu *et al*., 2004; Robalino *et al*., 2005; Saleh *et al*., 2009; Su *et al*., 2008). The host RNAi mechanism is thought to produce immunospecific RNA that binds with viral mRNA to suppress viral propagation, this leads to low-level active infections whereby the host exhibits no sign of disease but may remain infected and infectious for naïve hosts (Flegel, 2009b).

Whether the shore crab has developed some kind of mechanism to deal with WSSV infection through previous exposure to a virus which is genetically similar to WSSV needs to be investigated. It would be necessary to re-isolate the viruses which have been described as being similar to WSSV in order to determine whether they are similar to or in fact the same as WSSV as has been suggested. However, it is possible that the shore crab may have viral gene inserts within its genome from such a previous exposure and may be producing miRNA which could limit the WSSV replication within shore crab tissues. It is important to resolve this issue and determine whether WSSV viral inserts are possible and if so present in shore crabs in order to prevent false positive results in WSSV surveys of wild stocks.

We have previously shown (Chapter 2) that there is a difference in appearance of WSSV viral particles seen within *C. maenas* tissues. It is not known whether

these particles are indeed WSSV or whether they could be B virus or RV-CM; ultra structurally they appear very similar to the particles described by Johnson (1988a). The crab that displayed these particles was sampled from a WSSV study and tissues had been shown to be PCR positive for WSSV so it was assumed that they were WSSV particles. However, it is possible that these could be B virus or RV-CM particles and that the crab was initially infected with this virus when we sampled from the shoreline. It may be that this virus is extremely similar to WSSV which would account for the positive PCR results; further work would be needed to confirm this theory. Assuming this is a WSSV infection it is possible that the host may be affecting the replication cycle of the WSSV virions and these 'U' shaped particles are the result of this host response. Initially called von Magnus phenomenon, Defective Interfering (DI) virus particles are known to occur in most animal virus systems (Huang, 1973). DI particles are defective virus particles which consist of viral structural proteins and a part of the viral genome, they are capable of interfering with the growth of standard virus and may play a major part in the evolution of viral diseases (Huang and Baltimore, 1970). Species, tissue and genetic differences in host cells have been shown to affect the production of DI particles and their ability to interfere with the production of standard virus (Huang, 1973). DI particle presence is thought to play a key role in viral accommodation (Flegel, 2007) and may provide a possible explanation as to why some shore crabs respond to WSSV infection and others do not, further work would be needed to confirm whether these 'U' shaped particles are in fact DI WSSV particles and the extent to which these DI particles would inhibit replication. One of the benefits of viral accommodation is that there would be positive selection of viral variants with the least negative effect on the host and positive selection for host variants least affected by presence of the virus (Flegel, 2007). The shore crab could have developed strategies for dealing with WSSV infections via previous exposure to a virus that was genetically similar to WSSV and this strategy may well have been passed to offspring as shown in shrimp. This may explain the difference between high and low responders to the virus, high responders lacking previous exposure to a genetically similar virus infection. Further work would be needed to investigate these theories.

As highlighted by Stentiford *et al.* (2010) and Stentiford and Lightner (2011), since WSD has been listed in European Directive 2006/88EC as a 'non-exotic' disease within Europe, Member States are required to declare a national status for WSD; categories ranging from Category I (free from disease) to Category V (infected). In order to do so, Member States may be required to undertake an epidemiologically-rigorous surveillance programme which utilises appropriate diagnostic tests designed to detect WSSV if present. Should the shore crab be chosen as species for surveillance and should it be shown that viral inclusions are present in the shore crab genome these surveys could result in false positive declarations.

5.6 Conclusions

This study revealed that the viral loading in shrimp tissues increased over the 48 hour period whereas the viral loading in shore crabs decreased over the same time period, the shore crab appearing to limit replication of the virus in the early stages of infection. Assessment of total viral load per mg of crab tissue and histopathology from gill, connective tissues and heart suggested that crabs exhibited different disease development between individuals and this did not appear to be affected by temperature stress conditions. Crabs could be divided into two groups according to the differences in development of the disease (histopathology) and infection (viral load). One group termed high responders (HR) developed increased levels of WSSV pathology within the target tissues and higher viral loading than the second group termed low responders (LR) which showed reduced WSSV pathology and lower viral loading. Viral replication appeared to be limited by host response, the individual crab appearing to determine whether the virus replicated within the tissues.

Chapter 6.

Susceptibility of juvenile European lobster (*Homarus gammarus*) fed high- and low-dose White Spot Syndrome Virus (WSSV) infected shrimp products

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Bateman, K.S., Munro, J., Uglow, B., Stentiford, G.D. (2012) Susceptibility of juvenile European lobster (*Homarus gammarus*) fed high- and low-dose White Spot Syndrome Virus (WSSV) infected shrimp products. Diseases of Aquatic Organisms, 100, 169-184

6.1 Abstract

White Spot Disease (WSD) is still considered the most significant known pathogen impacting the sustainability and growth of the global penaeid shrimp farming industry. Although most commonly associated with penaeid shrimp farmed in warm waters, the virus is also able to infect, cause disease and kill a wide range of other decapod crustaceans from temperate regions, including lobsters. In 2005, the European Union imported $500m worth of raw frozen or cooked frozen commodity products, much of which originated in WSD positive regions. A small scale survey of supermarket commodity shrimp established the presence of White Spot Syndrome Virus (WSSV) within the UK food market via nested PCR. Passage trials utilising supermarket derived commodity shrimp inoculum delivered by injection to specific pathogen free (SPF) *Litopenaeus vannamei* led to rapid mortality, and pathognomonic signs of WSD in shrimp, demonstrating that WSSV present within commodity was viable. A representative commercially important European decapod crustacean (*Homarus gammarus*) was exposed to a single feeding of WSSV-positive supermarket derived commodity shrimp, and to positive control material (high dose WSSV infected *L. vannamei*). These trials demonstrated that lobsters fed with positive control (high dose) frozen raw products succumbed to WSD and displayed pathognomonic signs associated with the disease using histology and electron microscopy. Lobsters fed WSSV positive

supermarket-derived commodity shrimp (low dose) did not succumb to WSD (no mortality or pathognomonic signs of WSD) but demonstrated a low level or latent infection via PCR. This study confirms susceptibility of *H. gammarus* to WSSV via single feeds of previously frozen raw shrimp products obtained directly from supermarkets.

6.2 Introduction

White Spot Disease (WSD) caused by White Spot Syndrome Virus (WSSV) has caused devastating losses in the global shrimp farming industry since its discovery in the 1990's. WSD is estimated to have caused at least $10bn in losses to the penaeid shrimp production systems since 1993 (Stentiford *et al*., 2012). Cultivation of marine shrimp accounts for a large proportion of global aquatic food production. In 2007, global shrimp production was over 3 million metric tonnes, with a value of $13bn (FAO, 2007). Annually, the European Union (EU) imports around $500m worth of farmed shrimp commodity products, the majority of which are raw frozen or cooked frozen penaeid shrimp (Stentiford *et al*., 2010). Due to the wide global distribution of WSSV, large proportions of this product originate from regions in which WSSV is endemic (Lightner, 2003).

Aquaculture production of crustaceans is limited within the EU, only accounting for around 200 metric tonnes (Mt) per annum, (http://www.fao.org/figis). Conversely, the total fishery production of crustaceans from European waters totalled almost 400,000 Mt in 2004, with a large majority of this comprised of marine prawns (c. 200,000 Mt), lobsters (c. 60,000 Mt) and crabs (c. 85,000 Mt). In fresh waters, capture fisheries are solely comprised of crayfish (c. 6,000 Mt). As a result, wild fisheries for marine crustaceans are considered key resources in the European maritime area and in many countries (such as the UK); they rank above several important finfish species in terms of production quantity and value (Anon, 2004). Despite the high economic value of this fishery the exposure and consequence risks to wild populations of imported commodity products that may inadvertently enter the aquatic environment, are largely unknown.

The European Commission (EC) Directive 2006/88 (on animal health requirements for aquaculture animals and products thereof, and on prevention of and control of certain animal diseases) lists three notifiable crustacean diseases, all of which are caused by viral pathogens and generally associated with farmed penaeid shrimp from tropical and sub-tropical regions. These are: White Spot Disease (WSD), caused by White Spot Syndrome Virus (WSSV), Yellowhead Disease, caused by Yellowhead Virus (YHV) and Taura Syndrome, caused by Taura Syndrome Virus (TSV). All have caused severe economic losses in the global shrimp farming industry due to their transmissible nature, their potential for socio-economic impact and their likelihood for spread via the international trade of animals and animal products. The listing of these diseases implies that their detection in crustaceans from European waters would be subject to compulsory control measures. It formally recognises the potential for diseases traditionally associated with tropical penaeid shrimp to impact upon the sustainability of commercially and ecologically significant crustacean populations in the European aquatic network. YHV and TSV are listed as exotic to the EU, whereas WSSV is listed as non-exotic (Stentiford *et al*., 2010; Stentiford and Lightner, 2011). In the European context, WSSV is considered to be the most significant threat due to its wide host range, pathogenesis in temperate conditions and its potential for rapid spread (Stentiford *et al*., 2009; Stentiford *et al*., 2010).

EC Directive 2006/88 requires health certification for the import of commodity products unless these products are destined for further processing, packaged in 'retail sale' packages, and labelled in accordance with EC Regulation 853/2004. Therefore, products (live or frozen) imported directly for human consumption are not covered by the Directive and do not need to originate from areas designated free from listed pathogens, even when imported to confirmed 'disease free' Member States. Nunan *et al.,* (1998) and Hasson *et al.,* (2006) have previously demonstrated that frozen commodity shrimp imported to the USA for human consumption tested positive for WSSV by PCR. Previous transmission trials with commodity shrimp have shown that sufficient viable virus remains in frozen commodity products to induce mortality of *Litopenaeus vannamei* (Durand *et al*., 2000; Hasson *et al*., 2006). Oidtmann & Stentiford (2011) highlight the risk of these commodity products to naive crustacean populations by stating "...if such

shrimp were introduced into a country free from the pathogen, and crustaceans in the receiving country were exposed to infected tissues *per os*, there is a considerable risk that such exposed crustaceans may become infected and the infection established in domestic populations of crustaceans".

Stentiford and Lightner (2011) report the presence of WSSV within European shrimp farms in 2000 after shrimp on these farms were exposed to WSSV infected shimp carcasses; however, the virus has not been discovered in wild European crustacean populations to date. WSSV has been shown to be successfully transmitted to a range of hosts from European marine and fresh waters. These include the marine crab species *Liocarcinus depurator and Necora puber*, the commercially significant marine crab *Cancer pagurus* and freshwater crayfish of the genera *Astacus* and *Pacifastacus* (Corbel *et al*., 2001; Jiravanichpaisal *et al*., 2001; Jiravanichpaisal *et al*., 2004). These authors and others note the unusually large host range for this virus, its potential for infection of freshwater, brackish and marine species and the potential sensitivity of 'naïve' European crustaceans to WSSV. These studies also demonstrate the high potential for transmission to hosts from European waters, particularly from imported shrimp products and from imported brood stock or larvae for new cultivation ventures in the region and further. Furthermore, they demonstrate that temperature may affect host susceptibility and WSSV pathogenicity (Jiravanichpaisal *et al*., 2004).

In the current study, imported fresh and frozen shrimp products were screened for the presence of WSSV DNA. Imported commodity was also tested for potential to act as a source for transmission of WSSV to a known WSSV-susceptible host (*Litopenaeus vannamei*) and an important species in the European crustacean fishery, the European lobster (*Homarus gammarus*). Juvenile *H. gammarus* obtained from a commercial hatchery in the United Kingdom were exposed to known high-dose (high WSSV viral load) feed at two temperatures to determine initial susceptibility of this species. In addition, juvenile *H. gammarus* were fed high–dose WSSV infected *L. vannamei* carcasses and low-dose (low WSSV viral load) supermarket-derived commodity product. Viral loading in feed was assessed prior to feeding using quantitative PCR. The susceptibility of juvenile lobsters to WSSV was assessed using histopathology, electron microscopy and nested PCR

assays specific for WSSV. The susceptibility of *H. gammarus* to WSSV via feeding of low- and high-dose WSSV-infected commodity products and the potential for WSSV to impact upon native European crustacean stocks via “normal-use pathways” is discussed.

6.3 Materials and Methods

6.3.1 sampling and passage conditions

Frozen uncooked shrimp from various global production regions (Table 1, location as defined on the packaging) were purchased from supermarkets and from a large fish market in the United Kingdom. Products were tested for the presence of WSSV using a nested PCR assay recommended by the OIE (OIE, 2006) with minor modifications (see section 5.3.7). Products displaying positivity via PCR were utilised for production of inoculates and feeds for subsequent passage trials. All passage trials were conducted within the biosecure exotic diseases facility at the Cefas Weymouth laboratory and utilised local, filtered and UV treated seawater. Day length was set at 14 h/day, night was at 10 h with a 30 min fade to simulate dusk and dawn. Temperature was regulated according to the experimental conditions required for *Litopenaeus vannamei* (Holthuis, 1980) (26°C) and for *Homarus gammarus* (15°C and 22°C).

Table 1. Summary of results from a WSSV survey of commodity products. Frozen uncooked shrimp were purchased from supermarkets and a large fish market in the UK, country of origin being identified from packaging. Products were tested for WSSV by nested PCR

SPECIES	ORIGIN	DESCRIPTION	NESTED PCR (% POSITIVE)
L. vannamei	Ecuador	Supermarket- headless, shell off	65
L. vannamei	Honduras	Supermarket- headless, shell off	80
P. monodon	Indonesia	Supermarket- headless, shell off	0
L. vannamei	Thailand	Supermarket- headless, shell off	5
P. monodon	Thailand	Supermarket- headless, shell off	0
P. monodon	Vietnam	Supermarket- headless, shell off	100
P. monodon	Bangladesh	Market- whole animal	0
P. monodon	Bangladesh	Market- whole animal	0
P. monodon	Bangladesh	Market- headless, shell on	0
L. vannamei	Brazil	Market- whole animal	0
L. vannamei	China	Market- headless, shell on	0
P. monodon	India	Market- whole animal	0
P. monodon	Indonesia	Market- whole animal	0
L. vannamei	Indonesia	Market- whole animal	0
F. notialis	Senegal	Market- headless, shell on	0
P. monodon	Vietnam	Market- headless, shell on	20

6.3.2 Viability of WSSV in Commodity Products

Commodity shrimp from Ecuador and Vietnam which had been confirmed positive for WSSV via nested PCR were macerated using a sterile razor blade prior to homogenisation in sterile saline (4 ml of saline per gramme of minced tissue) using a blender until tissues were liquefied. The homogenate was centrifuged at 5,000 x g for 20 minutes at 4°C to pellet solid debris prior to the supernatant being diluted 1:20 with sterile saline and filtered (0.2µm Minisart syringe filter, Sartorius

Stedim Biotech GmbH, Germany) to form the innoculum. Individual specific pathogen free (SPF) *L. vannamei*, (approximately 5g in weight) obtained from the Centre for Sustainable Aquaculture Research (CSAR) at the University of Swansea, United Kingdom, were inoculated via intramuscular injection of the diluted viral homogenates at a dose of 10 μl g^1 shrimp weight. Water temperature was held constant at 24˚C. Shrimp were monitored throughout the day for five days, dead and moribund shrimp were removed from the experimental tanks; pleopods were fixed in 100% ethanol for molecular analysis, gills were taken for transmission electron microscopy and the carcass was fixed whole in Davidson's seawater fixative for histopathological confirmation of WSD.

6.3.3 eparation of WSSV positive low- and high-dose feeds

For preparation of high-dose feeds, viral inoculates of WSSV were obtained from the OIE reference laboratory at the University of Arizona, USA. The OIE isolate of WSSV (UAZ 00-173B) was generated in *L. vannamei* from an original outbreak in *Fenneropeneaus chinensis* (Holthuis, 1980) in China in 1995. Subsequent passages of this isolate into naïve *L. vannamei* held at the Cefas Weymouth laboratory have demonstrated continued pronounced virulence of this isolate (data not reported here). High-dose WSSV infected shrimp carcasses were prepared by direct injection of the (UAZ 00-173B) isolate into SPF *L. vannamei* as detailed above. The viral loading in high-dose feeds was assessed using quantitative PCR (qPCR). Abdominal tissues from *L. vannamei* that were confirmed positive for WSSV via histology and nested PCR were macerated into approximately 2-3 mm blocks using sterile razor blade. For preparation of low-dose feeds, individual supermarket-derived shrimp (abdominal section) originating from Ecuador, Vietnam and Honduras, and confirmed positive for WSSV via nested PCR were macerated. The viral loading in low-dose feeds was assessed using qPCR.

6.3.4 Lobster feeding trials with low- and high-dose products

Juvenile European lobsters (*Homarus gammarus*) were obtained from the National Lobster Hatchery in Padstow, Cornwall (UK). Lobsters were at moult stage 4 and approximately 2 months of age. To prevent conflict, juvenile lobsters were housed individually in custom-made 'Orkney pots' that were suspended in

the upper water column of each 30 litre experimental tank. The pots contained a perforated base to allow for water circulation.

In Trial 1, water temperature was held constant at 15^{o}C. Lobsters in Tank 1 (n = 20) received a single ration (~ 0.05 g) of high dose WSSV-infected shrimp tissue on Day 0 and a further ration on Day 7. Lobsters in Tank 2 (n = 20) received a single ration (~ 0.05 g) of uninfected shrimp tissue on Day 0 and another on Day 7. Between these times lobsters were fed on 3 mm Royal Oyster pellets (Bernaqua) at a ration of approximately 3-4% bodyweight/day for 10 days.

In Trial 2, water temperature was held constant at 22^{o}C. Lobsters in Tank 1 (n = 20) received a single ration (0.05 g) of high dose WSSV-infected shrimp tissue on Day 0 and a further ration on Day 7. Lobsters in Tank 2 (n = 20) received a single ration (0.05 g) of uninfected shrimp tissue on Day 0 and another on Day 7. Thereafter, lobsters in both tanks were fed on 3 mm Royal Oyster pellets at a ration of approximately 3-4% bodyweight/day for 10 days.

In Trial 3, water temperature was held constant at 20˚C. On day 0 lobsters in all tanks (n=20 for each tank) received a single ration of feed (0.05 g). Tank 1 received high-dose WSSV-infected shrimp tissue, Tank 2 received uninfected shrimp tissue, Tank 3 received low-dose commodity shrimp originating from Ecuador, Tank 4 received low-dose commodity shrimp originating from Vietnam and Tank 5 received low-dose commodity shrimp originating from Honduras. Thereafter, lobsters in all tanks were fed on 3 mm Royal Oyster pellets at a ration of approximately 3-4% bodyweight/day for 12 days.

In all trials, tanks were observed regularly throughout daylight hours. Dead and terminally morbid animals were removed from each tank. Cheliped samples from all dead, terminally morbid and live (at the end of the trial period) animals were fixed in 100% ethanol for PCR. Remaining carcasses were prepared for histology and selectively, for transmission electron microscopy (TEM). Juvenile lobsters were prepared for histology by making an incision along each side of the carapace, placing whole animals into histological cassettes and fixing immediately in Davidson's seawater fixative. From selected juvenile lobsters, the abdomen

was removed from the cephalothorax and the carapace opened as described for histology. The abdomen was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature, cuticular epithelium and gills were dissected out of these fixed animals and processed for electron microscopy.

6.3.5 gy

See Chapter 4 for full details.

6.3.6 ron microscopy

Tissues were fixed in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature and rinsed in 0.1 M sodium cacodylate buffer (pH 7.4). Tissues were post-fixed for 1 h in 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer. Samples were washed in three changes of 0.1 M sodium cacodylate buffer before dehydration through a graded acetone series. Samples were embedded in Agar 100 epoxy (Agar Scientific, Agar 100 pre-mix kit medium) and polymerised overnight at 60ºC in an oven. Semi-thin (1-2 µm) sections were stained with Toluidine Blue for viewing with a light microscope to identify suitable target areas. Ultrathin sections (70-90 nm) of these areas were mounted on uncoated copper grids and stained with 2 % aqueous uranyl acetate and Reynolds' lead citrate (Reynolds, 1963). Grids were examined using a JEOL JEM 1210 transmission electron microscope and digital images captured using a Gatan Erlangshen ES500W camera and Gatan Digital Micrograph™ software.

6.3.7 tection of WSSV in commodity shrimp via PCR and qPCR

Total shrimp DNA was extracted from commodity product (tail muscle) using the High Pure PCR Template Preparation Kit (Roche Diagnostics) following the manufacturer's protocol for the isolation of nucleic acids from mammalian tissue. The quality and quantity of the DNA was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific*).* For use in the qPCR, prior to analysis, total DNA was standardised at 20 ng/µl to remove variations in viral load due to tissue weight variation or extraction efficiency between samples.

6.3.8 Nested PCR for high dose fed lobsters

WSSV DNA presence was assessed using the nested PCR assay of (Lo *et al*., 1996b) Lo *et al*. (1996b) with minor modifications (Ms Bonnie Poulos, University of Arizona, personal communication). First, a product of 1447 bp was amplified using the primer pair 146F1 (5'-ACTACTAACTTCAGCCTATCTAG-3') and 146R1 (5'-TAATGCGGGTGTAATGTTCTTACGA-3'), followed by an approximate 941 bp product in the nested reaction using primer pair 146F2 (5'-GTAACTCCCCCTTCCATCTCCA-3') and 146R2 (5'TACGGCAGCTGCTGCACCT-TGT-3'). For the first round of amplification (primer pair 146F1/146R1) each 25 μl PCR reaction contained the following: 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 200 μM of each dNTP, 0.31 μM of each primer, 2.5 units *Taq* polymerase, and 1 μl genomic DNA (20-50 ng total). Amplifications were performed with an initial denaturation temperature of 94 °C for 2 min, followed by 30 cycles at 94 °C for 30s, 62 °C for 30s, 72 °C for 30s, with a final elongation step at 72 °C for 2 min. Reaction conditions and reagent concentrations were the same for the second round of amplification using the 146F2/146R2 primer pair, however 0.5 μl of the first round of amplification was used as a template in place of genomic DNA. Amplified products were resolved on a 2% (w/v) agarose/TAE (40 mm Tris-acetate, pH 7.2, 1 mm EDTA) gel containing 1.0 mg mL^{-1} ethidium bromide and visualized under UV irradiation.

6.3.9 ted PCR for commodity product and low-dose feed lobsters

The initial PCR method described above was modified to include an internal control to ensure false negatives did not occur without displaying any noticeable reduction in sensitivity (data not shown). To ensure validity of each sample, an internal control was included in each PCR sample. The internal control, consisting of a single primer set F-5'-GTGGACATCCGTAACCACCTGTACG-3' and R - 5'-CTCCTGCTTGCTGATCCACATCTGC-3' amplified a 201bp product specific towards beta-actin, which is found in all eukaryotic cells. First (primers 146F1 and 146R1) and second (primers 146F2 and 146R2) round PCR were performed in a 50μl reaction volume containing 0.15 μM of primers, 0.25 μM dNTPs, 1.25 U GoTaq (Promega), 2.5 mM $MgCl_2$, 5 x buffer and 2.5 μl of template. Amplification details as above.

6.3.10 Quantitative PCR

In individual shrimp demonstrating positivity for WSSV via nested PCR, and to ensure suitability for further utilisation for the feeding trial to naive *H. gammarus*, a DNA fragment of 69-bp representing the upstream primer plus the probe and the downstream primer was cloned into the pGEM-T easy vector (Promega Corporation) and then transformed into JM109 competent cells (Promega Corporation) following the manufacturer's instructions. The plasmid was extracted using QIAprep spin Miniprep Kit (Qiagen) and the concentration was determined using a NanoDrop spectrophotometer. The copy number of the plasmid DNA containing the 69-bp insert was estimated and a series of dilutions were made to use as standards. The primers and probe used in this study for the quantification of WSSV were developed by (Durand and Lightner, 2002)Durand and Lightner (2002). Briefly, the sequence of the primers were: WSS1011F: 5'-TGGTCCCGTCCTCATCTCAG-3' and WSS1079R: 5'-GCTGCCTTGCCGGAAATTA-3'. The TaqMan probe sequence (5'-AGCCATGAAGAATGCCGTCTATCACACA –3') was synthesized and labeled with fluorescent dyes 5-carboxyfluroscein (FAM) on the 5' end and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end. The Taqman assay used was adapted from Durand and Lightner (2002). Briefly, 100 ng of total DNA was added to Taqman Universal mastermix (Applied Biosystems) containing 0.3 μM of each primer and 0.15 μM of Taqman probe in a final volume of 25 μl. Amplification and detection were performed using an ABI Biosystems Taqman machine. The reaction mix was subjected to an initial temperature of 50°C for 2 mins, then 1 cycle at 95°C for 10 mins, 50 cycles at 95°C for 15s and 60°C for 1 min. Quantification of the number of WSSV copies in samples to be utilised for feeding trials were determined by measuring Ct values and using the standard curve to determine the initial copy number per ng total DNA. Each unknown sample was analysed in triplicate and the mean calculated.

6.3.11 Sequencing

Selected reactions were analysed on an ABI 3130 Avant Genetic Analyser to confirm the specificity of the PCR. The final product was compared to known sequences using Basic Local Alignment Search Tool (BLAST) (Altschul *et al*.,

1990) to determine phylogenetic homology. This is in line with OIE confirmatory technique for WSSV (Claydon *et al*., 2004).

All PCR and sequencing was conducted with advice and assistance from Dr Hamish Small and Dr James Munro.

6.4 Results

6.4.1 ommodity product screening for WSSV

The prevalence of WSSV in commodity shrimp imported into the UK for human consumption ranged from 0% to 100%. Sixty six percent (4/6) of the batches of shrimp purchased from supermarkets tested positive for WSSV, within bag prevalence ranging from 0% to 100%. Ten percent (1/10) of the shrimp samples purchased from fish markets were also positive for WSSV (Table 1).

6.4.2 Viability of WSSV in Commodity Products

L. vannamei injected with homogenised WSSV positive commodity shrimp from either Ecuador or Vietnam experienced 100% and 40% mortality respectively, within 3 days post injection (Fig. 1).

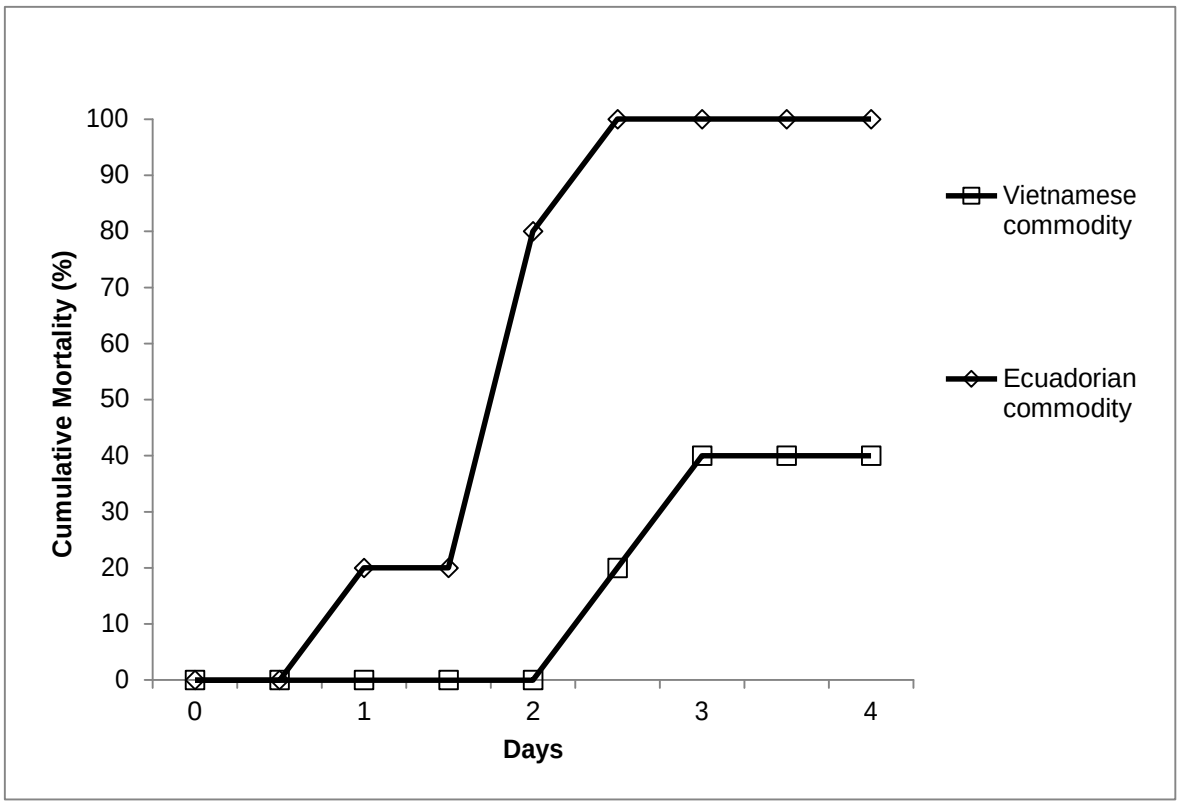


Figure 1. Mortality data of *Litopenaeus vannamei* exposed to WSSV infected commodity products from Ecuador and Vietnam.

Nested PCR analysis (Fig. 2A and B) indicated that all animals injected with commodity inoculate from Ecuador were positive for WSSV, with four of these animals displaying infection detectable in the first round of PCR, indicating a pronounced disease status (Fig. 2A). Shrimp injected with commodity inoculate from Vietnam were similarly all positive for WSSV (Fig. 2B) but only weak bands were present in the second round of PCR, suggestive of a lower level infection.

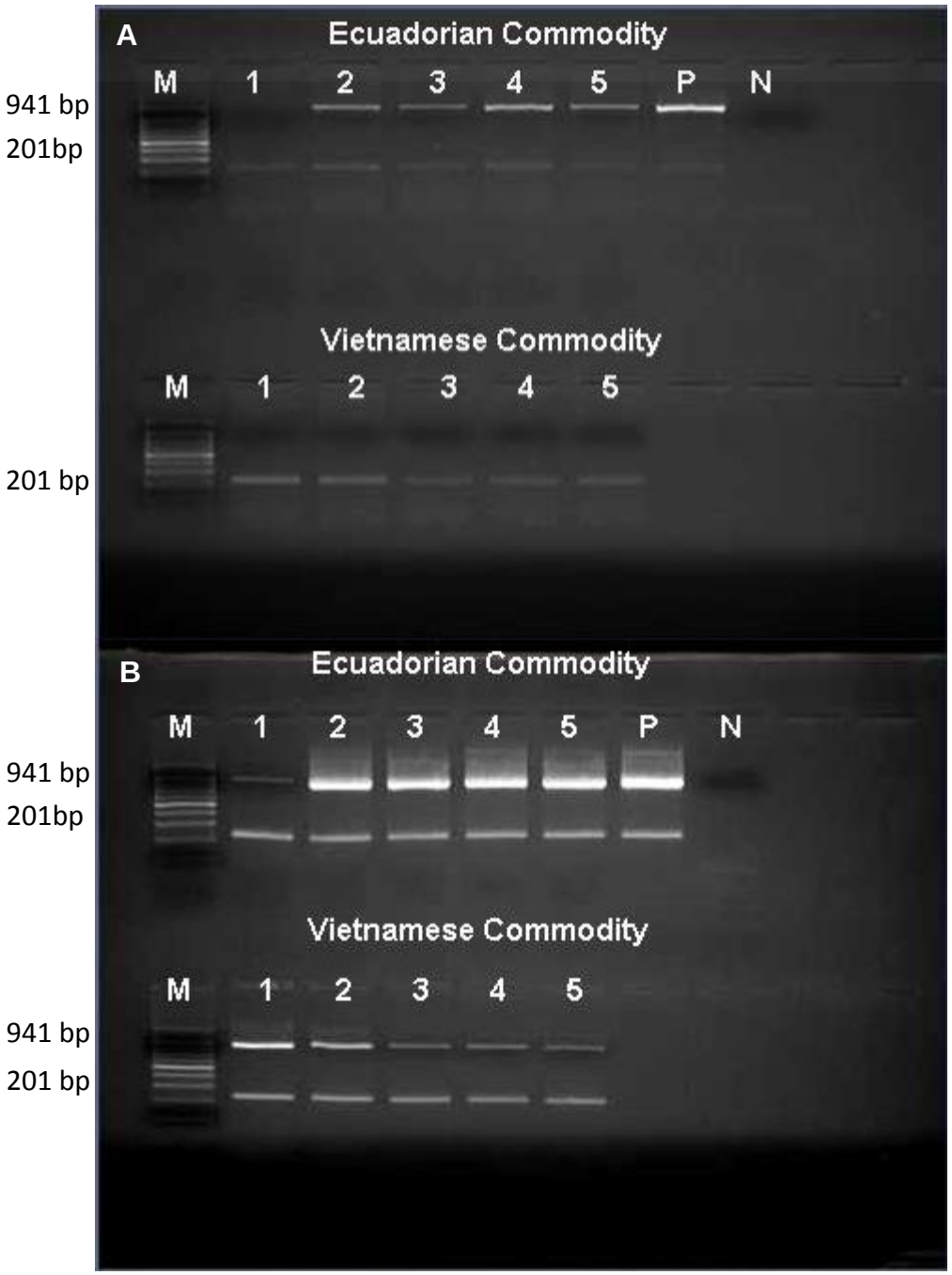


Figure 2. An ethidium bromide stained agarose gel showing DNA fragments produced by PCR amplification of WSSV infected commodity products (*Litopenaeus vannamei)* from various geographical regions. (**A**) Results from first round of nested PCR (*Lo et al*., 1996). Ecuadorian commodity: 4/5 positive for WSSV. Vietnamese commodity: 0/5 positive for WSSV. (**B**) Results from second round of nested PCR (Lo *et al*., 1996). Ecuadorian commodity: 4/5 positive for WSSV. Vietnamese commodity: 5/5 positive for WSSV. M: 100bp DNA ladder (Promega). P: positive control for WSSV. N: negative control for WSSV. Expected product sizes: 201 bp= decapod-specific DNA, 941 bp= WSSV.

Histological examination of these shrimp demonstrated the characteristic pathology associated with WSSV infection (Fig. 3); shrimp injected with commodity inoculate from Ecuador displayed signs of advanced WSD, whilst shrimp injected with commodity inoculate from Vietnam displayed lower grade, albeit characteristic lesions associated with WSSV infection. Animals displaying advanced WSD identified from histopathology were further selected for TEM analysis. Electron microscopy revealed large numbers of rod-shaped virions synonymous with WSSV in epithelial tissues (Fig. 3E and 3F). Manifestation of WSD in SPF shrimp injected with supermarket and fish market derived commodity products confirmed the viability of WSSV in such products.

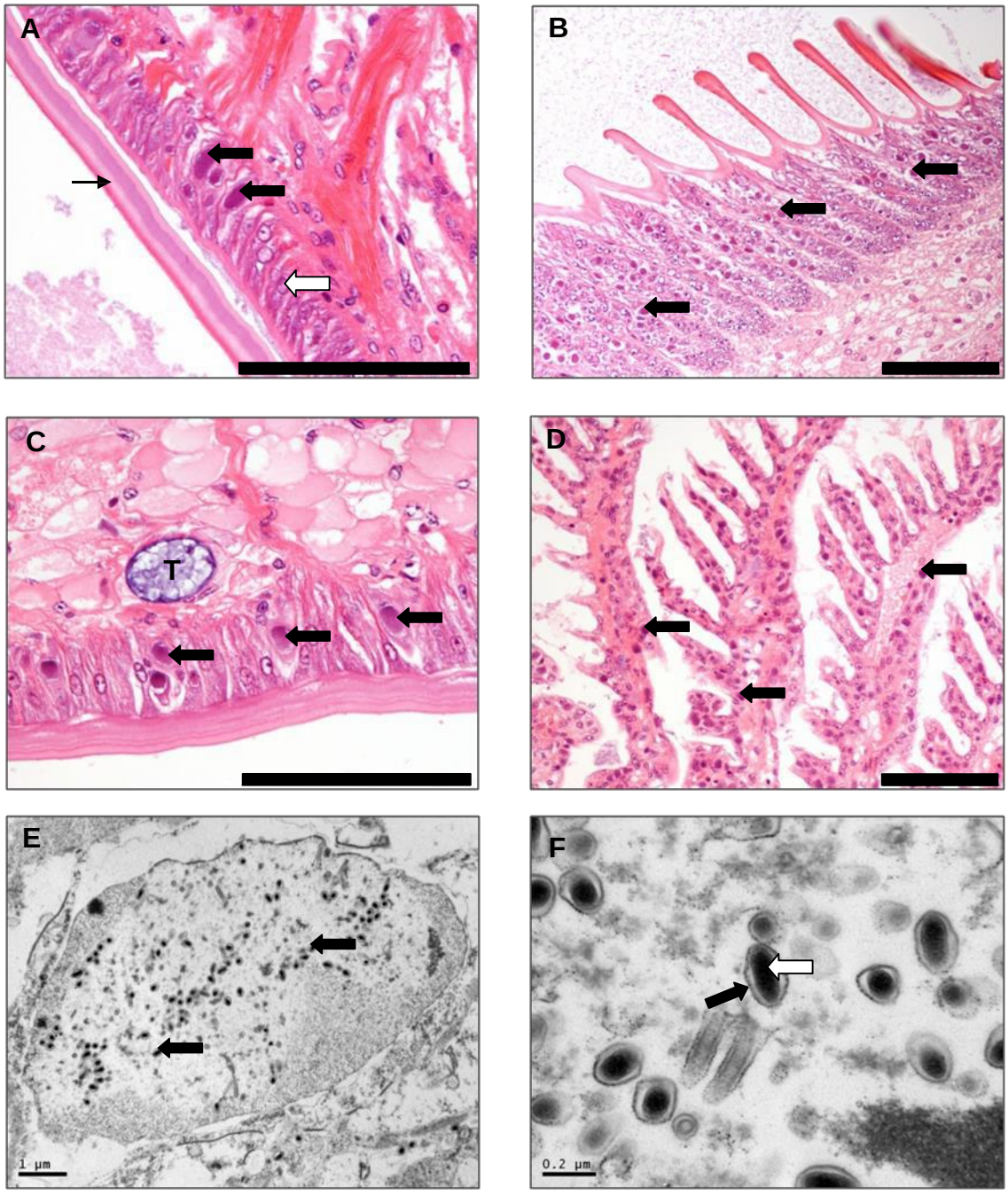


Figure 3. WSSV-infected *Litopenaeus vannamei*. (**A**) Carapace (black line arrow) and columnar epithelial cells (white arrow) showing enlarged nuclei with dense staining (black arrows). H&E stain. Scale bar = 100 µm. (**B**) Stomach epithelium showing hypertrophied nuclei with eosinophilic staining (arrows). H&E stain. Scale bar = 100 µm. (**C**) Enlarged infected nuclei (arrows) within epithelial cells, also showing tegmental gland (T). H&E stain. Scale bar = 100 µm. (**D**) Gills with enlarged, infected nuclei (arrows) distributed throughout the cuticular epithelium. H&E stain. Scale bar = 100 µm. (**E**) Infected cell within the gills of L. vannamei. Numerous viral particles can be seen within the nucleus of the cell (arrows). TEM. Scale bar = 1 µm. (**F**) Longitudinal and transverse sections of viral particles within the nucleus. Electron dense nucleocapsid (white arrow) is surrounded by a trilaminar envelope (black arrow). TEM. Scale bar = 0.2 µm.

6.4.3 Viral loading in low- and high-dose feeds

The WSSV-infected shrimp used in the high dose challenge had an equivalent viral loading of 3.65 x 10^5 copies/ng total DNA. While the supermarket-derived commodity shrimp used in the low dose feed challenge had an equivalent viral loading of 5.16 x 10^2, 4.68 x 10^1 and 1.04 x 10^2 copies/ng total DNA in the Honduran, Ecuadorian and Vietnamese shrimp respectively.

6.4.4 Lobster feeding trials: high-dose products

Cumulative mortality of WSSV-fed lobsters in Trial 1 (15ºC) reached 5% on day 1 and 10% by day 10 of the trial. In Trial 2 (22ºC), cumulative mortality of WSSV-fed lobsters reached 40% by Day 3 and 55% by day 6. No further mortalities occurred between day 6 and day 10. Cumulative mortalities in control tanks in Trials 1 and 2 reached 15% by the end of the 10 day trial (Fig. 4).

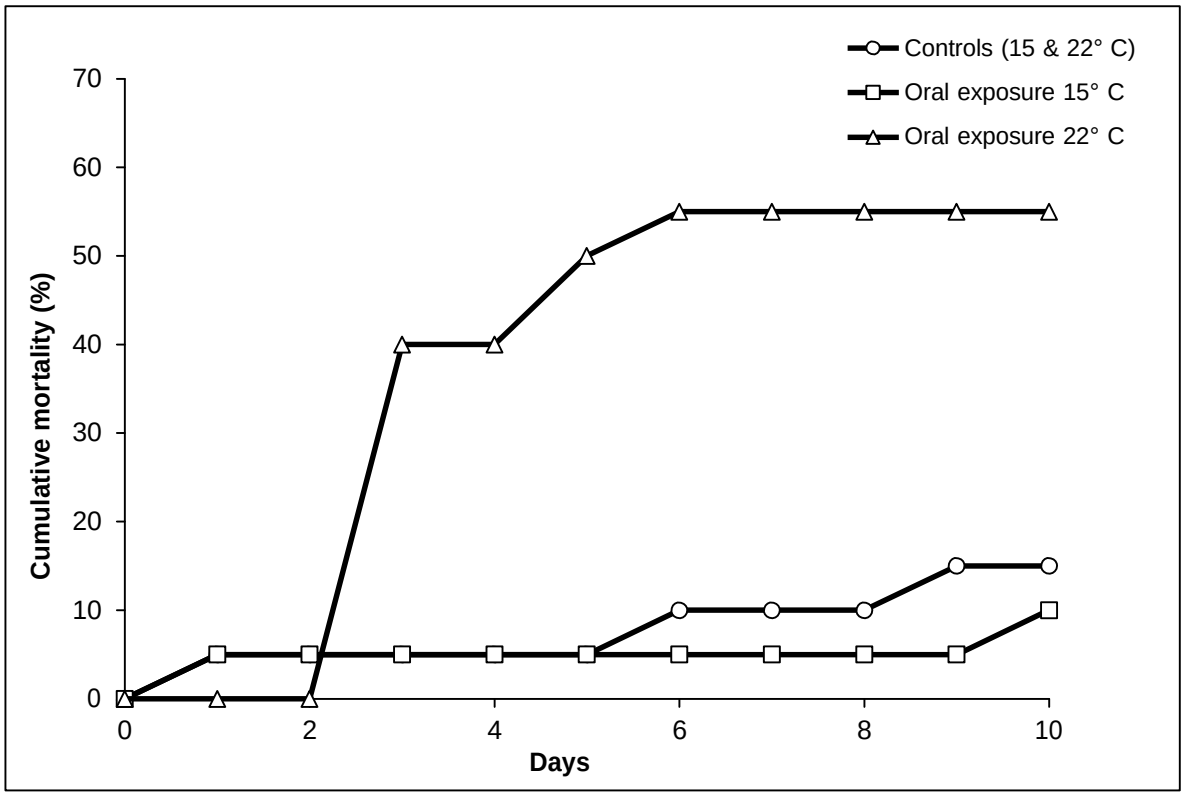


Figure 4. Mortality data of *Homarus gammarus* held at 15°C and 22°C after oral exposure to high dose WSSV infected *L. vannamei* tissues. Control animals were fed SPF *L. vannamei* tissues and held at the same temperatures; graph shows cumulative mortality in control exposure.

Two moribund or recently dead lobsters in Trial 1 (15ºC) and 8 in Trial 2 (22ºC) displayed histopathological lesions typical of WSD in other crustacean species,

including penaeids. WSD-associated lesions were most apparent in the gill, haemopoietic centres, haemocytes, connective tissues, stomach epithelium and particularly the cuticular epithelium. Cellular changes in each of these target sites included nuclear hypertrophy, margination of chromatin at the nuclear periphery and the presence of a single basophilic to strongly eosinophilic inclusion that occupied a large proportion of the host nucleoplasm. In some cases, this inclusion was separated from the nuclear periphery by an unstained zone (Fig. 5A to E). Feulgen-positive staining of affected nuclei demonstrated the DNA composition of the inclusion and of the darkly staining nuclear periphery (Fig. 5F). The connective tissues and cuticular epithelium of the limbs appeared to be particularly heavily affected with an apparent loss of tissue mass in these regions and the presence of necrotic cellular debris and remnant WSSV inclusions. The hepatopancreas, midgut and skeletal muscle of infected lobsters appeared to be unaffected. Of those lobsters that were fed high dose WSSV-infected material and survived the experimental period, none exhibited the pathologies described above.

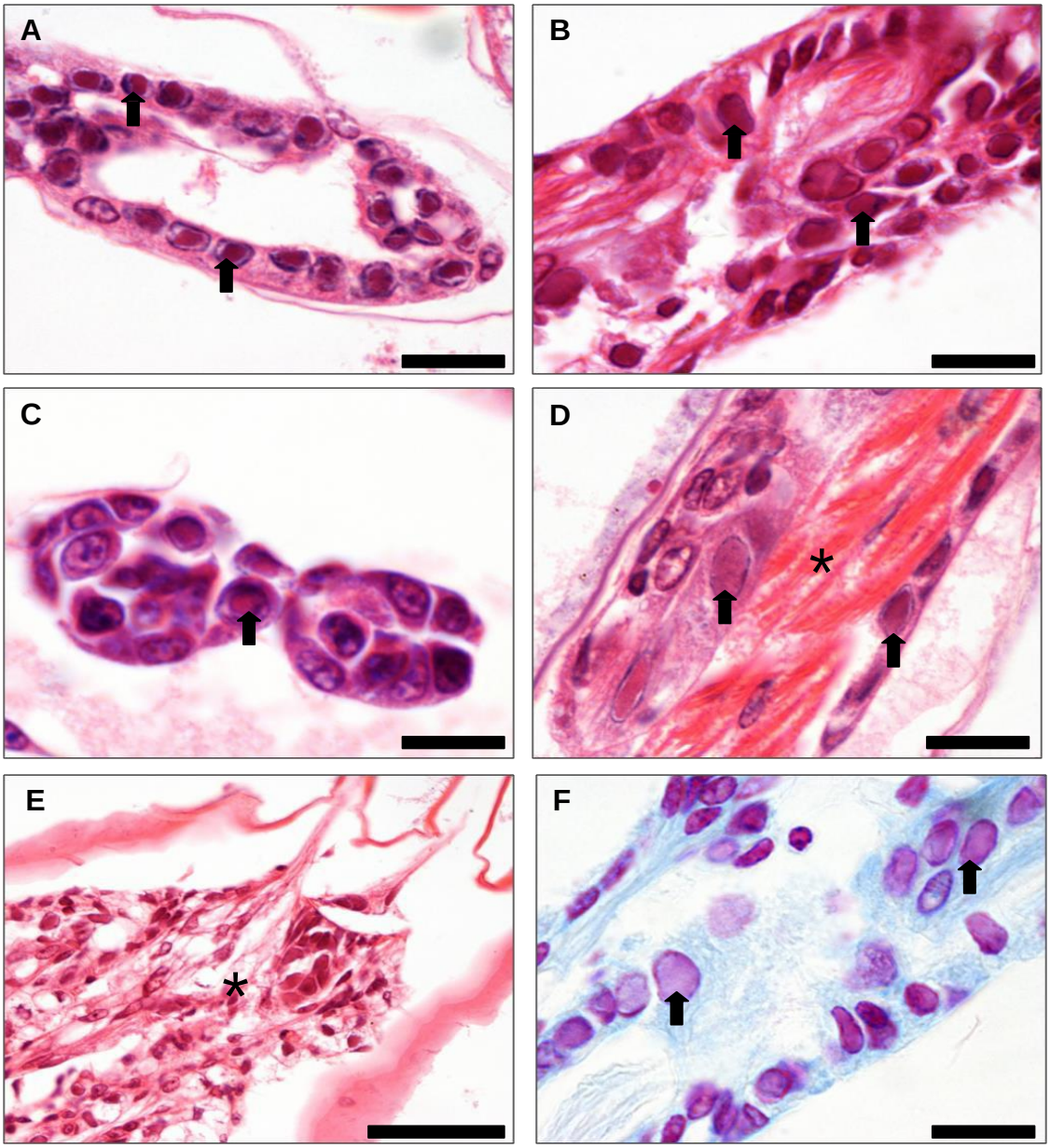


Figure 5. Histopathology of WSSV infection in *Homarus gammarus*. **(A)** Transverse section of gill secondary lamellae. Infected epithelial cell nuclei containing eosinophillic inclusions (arrows). Scale bar = 10 μm. **(B)** Epidermis and connective tissue at extremity of walking limb. Hypertrophic nuclei with marginalized chromatin (arrows). Scale bar = 10 μm. **(C)** Haemopoeitic cluster with infected germinal cells (arrow). Scale bar = 10 μm. **(D)** Mid section of walking limb with infected connective tissue and epidermal cells (arrow) but absence of infected nuclei within skeletal muscle (asterisk). Scale bar = 10 μm. **(E)** Extremity of telson containing infected connective tissue and epidermal cells (asterisk) Scale bar = 25 μm. **(F)** Feulgen-staining of hypertrophic nuclei borders and nucleoplasm of epidermal and connective tissue cells within walking limb (arrows). Scale bar = 10 μm. All H&E apart from F.

Semi-thin sections (Fig. 6A) and TEM sections (Fig. 6B-F) of lobsters displaying the aforementioned pathologies confirmed the presence of intranuclear inclusions and oval-shaped viral particles, typical of WSSV, within epithelial cell nuclei. Viral particles were elliptical to short-rod shaped in longitudinal section and round in transverse section (Fig. 6C and D). Virions consisted of an electron dense capsid, bound by a clear envelope, surrounded by a double membrane. Virions at various stages of development and apparent assembly could be observed embedded within a granular matrix that corresponded to a viral stroma within host nuclei. The presence of free capsid material (Fig. 6E) and a long rod-shaped structure (LRS) (Fig. 6F), corresponding to the viral nucleosome observed in other studies of WSSV, was also noted. Completed virions measured 280 ±5.5 nm in length and 116 ±1.4 nm in width (n=30) consistent with measurements of 270 to 300 nm x 90 to 120 nm previously described for WSSV.

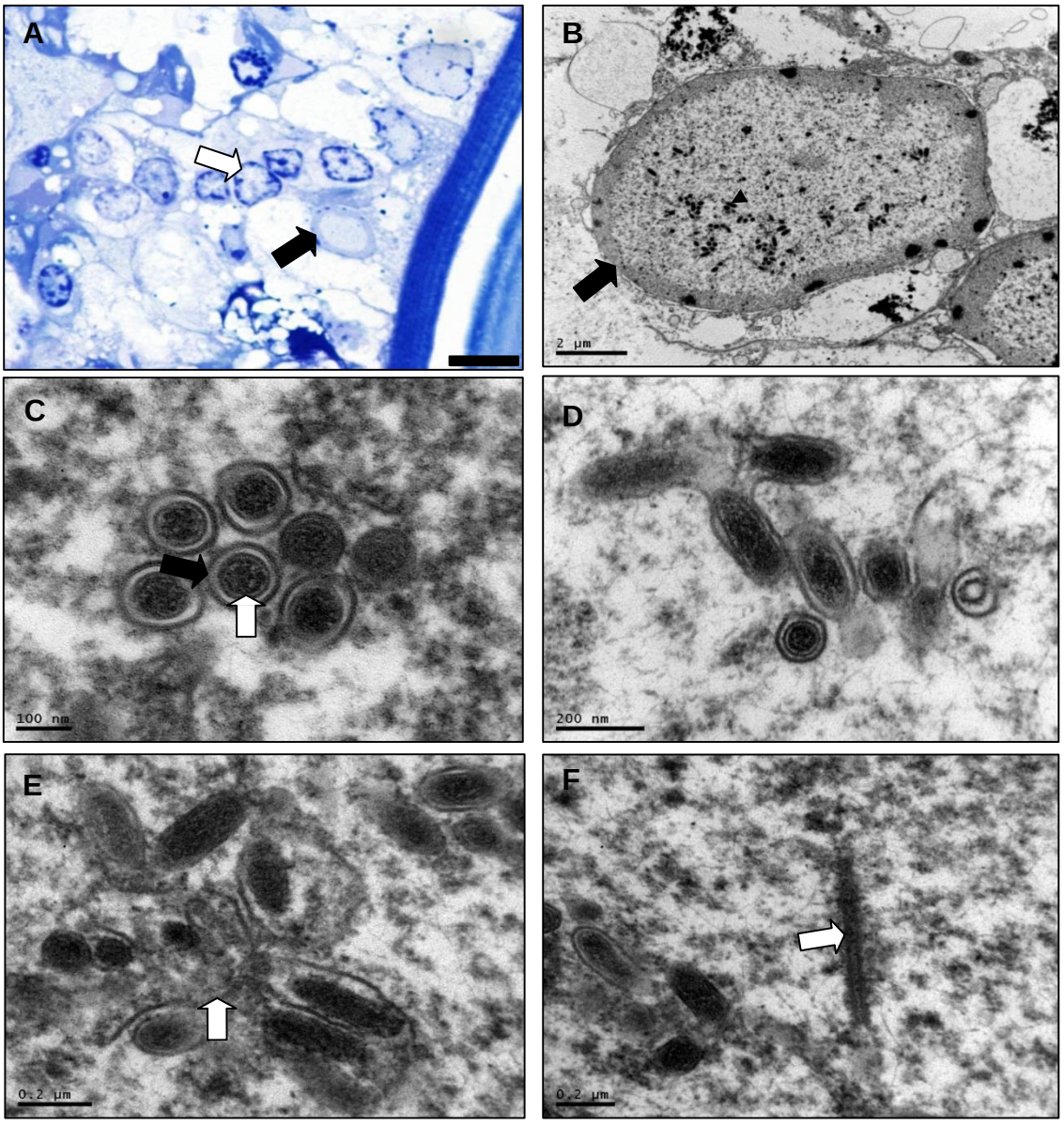


Figure 6. WSSV infection in epidermis of *Homarus gammarus*. **(A)** Semi thin section of epidermis. Viroplasm (*) can be seen within the hypertrophied WSSV infected nuclei (black arrow), uninfected nuclei (white arrow) are also present. Toluidine blue stain. Scale bar = 10 µm **(B)** Transmission electron micrograph of nucleus within the epidermis infected with WSSV. Nuclei were enlarged with marginalised chromatin (arrow) and contained viral particles (arrowhead) within the viroplasm (*). TEM. Scale bar = 2 µm **(C)** Cross section of WSSV virions within the nucleus. Virions consist of an electron dense capsid within an envelope (white arrow) that is surrounded by a double membrane (black arrow) TEM. Scale bar = 100 nm. **(D)** Longitudinal and cross section of WSSV virions in various stages of development within the nucleus. TEM. Scale bar = 200 nm **(E)** Capsid material can be seen free within the viroplasm (white arrow) surrounding viral particles in various stages of development. TEM. Scale bar = 0.2 µm. **(F)** Long rod-shaped structure (arrow) within the viroplasm of WSSV infected nucleus. TEM. Scale bar = 0.2 µm.

Lobster cheliped tissues used for DNA extraction and nested PCR yielded high quality host genomic DNA as indicated by a characteristic 1800 bp amplification product using the universal SSU rRNA gene primers. All WSSV-challenged lobsters (n=18) held at 15°C were positive for WSSV by nested PCR. Eighty % (16/20) of the WSSV-challenged lobsters held at 22°C were positive for WSSV by nested PCR. No WSSV-positive results were obtained from the negative control group samples.

6.4.5 Lobster feeding trials: low-dose products

Mortality rates in low-dose supermarket-derived commodity fed lobsters were 0%, 20% and 22% for the feed prepared from Honduran, Ecuadorian and Vietnamese shrimp respectively. Histological examination of low-dose fed lobsters did not reveal any of the characteristic signs of WSD as observed in penaeid shrimp or in high-dose fed lobsters. However, characteristic signs of WSD were observed once more in the positive control high-dose fed lobsters, particularly in the antennal gland and gill epithelium (Fig. 7). TEM of tissues from positive control animals once again revealed the rod-shaped viral particles typical of WSSV within the nucleus of infected cells (Fig. 7G and 7H).

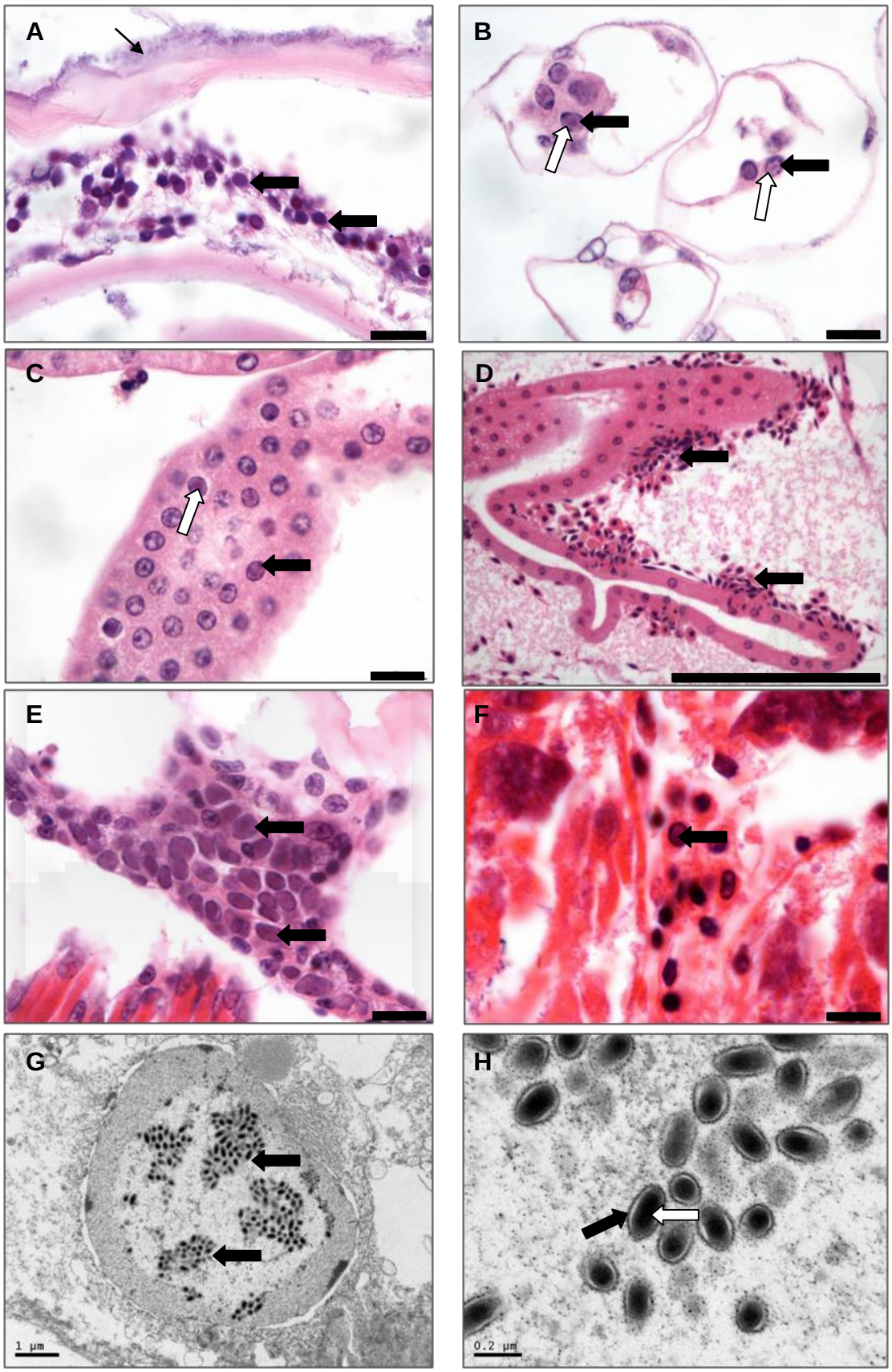


Figure 7. WSSV-infected *Homarus gammarus* tissues. (**A**) Carapace (black line arrow) and epithelial cells with marginated chromatin and eosinophilic staining (arrow). H&E stain. Scale bar = 10 µm. (**B**) Transverse sections of secondary gill filaments, showing marginated chromatin (black arrows) and eosinophillic inclusions (white arrows) within nuclei. H&E stain. Scale bar = 10

µm. (**C**) Antennal gland showing infected nuclei with marginated chromatin (black arrow) and eosinophillic inclusions (white arrow). H&E stain. Scale bar = 10 µm. (**D**) Antennal gland with haemocyte aggregations (arrows). H&E stain. Scale bar = 100 µm. (**E**) Haematopoietic tissue with numerous enlarged nuclei, with marginated chromatin and dense, uniform staining (arrows). H&E stain. Scale bar = 10 µm. (**F**) Connective tissues, with infected nuclei displayed dense uniform staining and marginated chromatin (arrows). H&E stain. Scale bar = 10 µm. (**G**) Infected cell within the gills of *Homarus gammarus*. Numerous viral particles can be seen within the nucleus of the cell (arrows). TEM. Scale bar = 1 µm. (**H**) Viral particles within the nucleus. Electron dense nucleocapsid (white arrow) is surrounded by a trilaminar envelope (black arrow). TEM. Scale bar = 0.2 µm.

Despite the lack of cellular pathology associated with WSD, nested PCR carried out on samples from the low-dose study revealed that almost 100% (16/17) of lobsters fed positive control shrimp were positive for WSSV. In addition, 70% of the lobsters fed supermarket-derived commodity from Honduras displayed positivity in the second round of PCR (sub-patent infection) whilst lobsters fed with supermarket-derived commodity from Ecuador and Vietnam displayed 30% and 45% positivity for WSSV in the second round of PCR, respectively (Fig. 8A, 8B, 8C, 8D, respectively). Sequencing of the nested PCR amplicon from one lobster in each of the treatment tanks followed by analysis using Basic Local Aligned Search Tool (BLAST) confirmed with at least 99% identity that the amplicons were from WSSV (GenBank accession AF332093.1) in all cases tested.

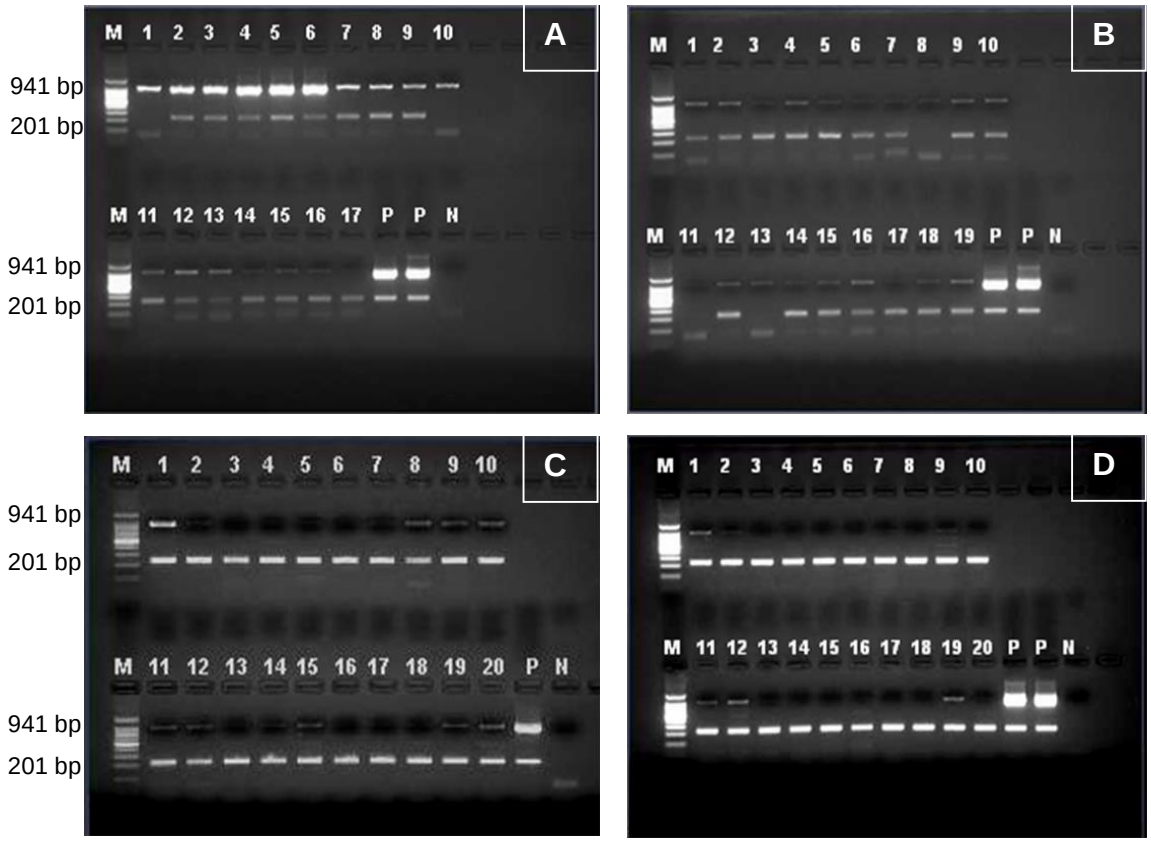


Figure 8. Ethidium bromide stained agarose gels showing DNA fragments produced by second round PCR amplification of WSSV. Results from bioassay of WSSV infected commodity to *Homarus gammarus.* (**A**) Positive control tank. 16/17 positive for WSSV. (**B**) Honduran commodity-fed tank. 13/20 positive for WSSV. (**C**) Ecuadorian commodity-fed tank. 5/20 positive for WSSV. (**D**) Vietnamese commodity-fed tank. 10/20 positive for WSSV. M: 100 bp DNA ladder (Promega). P: positive control for WSSV. N: negative control for WSSV. Expected product size: 201 bp = decapod- specific DNA, 941 bp= WSSV.

6.5 Discussion

As expected from similar surveys in the USA and Australia, a small scale survey of imported penaeid shrimp commodity products from UK supermarkets and fish markets has revealed an apparent widespread prevalence of WSSV-contaminated products. The study demonstrates that frozen commodity shrimp is a route of entry for WSSV into European Member States. The study has also demonstrated that the WSSV contaminated shrimp tissues contain viable virions that can be passaged to naive susceptible hosts via injection (*Litopenaeus vannamei*) and feeding (*Homarus gammarus*). Despite the limitations of the survey design, the underlying within-batch prevalence of the WSSV was relatively high, though in individual components of these batches (e.g. single abdominal sections), the virus was present at a relatively low viral titre, and certainly lower than positive control material generated by passage bioassays to shrimp within our laboratory. This work has also revealed relatively high batch prevalence for WSSV in supermarket-derived commodity, tested using PCR. The prevalence was apparently lower in animals sampled from a large fish market, possibly due to the larger size of specimens obtained from this location. Albeit limited, these preliminary results demonstrate a potentially large variation in the presence of WSSV in batches of commodity shrimp between type of importer, and country of origin as demonstrated in Table 1. For example, the batch of shrimp imported from Vietnam for sale in supermarkets had a within-batch prevalence of 100% while shrimp imported from the same country but sold at the fish market demonstrated a within-batch prevalence of 20%. The variation in WSSV batch and within-batch prevalence may reflect a focus on sale of relatively small shrimp, (bags containing shrimp of approximately 10 g original size) by supermarkets and a contrasting focus on larger, whole animals (15-20 g), with head and shell on, at fish markets. Whether the presence of smaller animals in batches from supermarkets represents a higher propensity for supermarket shrimp to be derived from so-called ’emergency harvest’ (rapid harvesting of shrimp from culture facilities when it is suspected that a mortality event may be impending), remains to be ascertained. The rapid harvesting of animals essentially averts financial losses but has been suggested to lead to higher viral loading in harvested animals (Flegel, 2009a).

Central to any national import risk assessment for commodity products contaminated with pathogens such as WSSV, is an assessment of the likelihood for a pathogen to establish in naïve susceptible hosts if the pathogen is released into waters in the importing nation. Considering normal shrimp farming scenarios (i.e. non-emergency harvest), it has been reported that cultivated shrimp can carry viruses (such as WSSV) at a low level (latent) infection without showing gross signs of disease for relatively long periods of time, provided that rearing conditions are favourable (Flegel *et al*., 2004). However, a change in physiological conditions, often due to environmental changes, can allow for rapid replication of the virus and the onset of disease. Whilst the loading of WSSV in commodity shrimp products analysed in the current study was apparently low in most cases, suggesting latent or sub-patent infection in those hosts, the virus remained viable and was able infect and kill SPF *L. vannamei* within a few days of exposure (albeit by a non-natural route of transmission). However, feeding trials in which lobsters were fed with the same low-dose commodity products also demonstrated that passage of WSSV could occur via this natural route and that infection could establish, albeit in latent form, in naive hosts (lobsters) held at temperatures experienced within Europe. Since infection progressed rapidly to disease in lobsters fed with high-dose products (positive control), particularly at higher temperatures, the limiting factor in the rapid appearance of WSD in lobsters is therefore the initial dose; low-level infectious dose establishing latent infection and high-level dose progressing more rapidly to disease. Fundamental studies are now required to assess the potential for latent infections to progress to a disease state and to cause mortality in lobsters retained at European temperatures. Such data are vital for accurate consequence assessment following release and establishment of WSSV infections in wild populations.

Results from the high-dose WSSV feed trial confirm the susceptibility of juvenile European lobster (*Homarus gammarus*) to WSSV. Lobsters were fed with WSSV-infected carcasses of *L. vannamei* and were shown to succumb to the disease within the time course of the experiment (10 days). However, not all of the lobsters fed with WSSV-positive commodity shrimp products became infected with the virus. This could be due to a number of reasons. Firstly, although the lobsters were provided with the same sized ration originating from the same individual

shrimp (per treatment tank), some animals did not consume all of the food provided. In addition, since the shrimp were not homogenised prior to feeding but rather, fed as blocks of tissue, the concentration of virus may also have varied in specific meals (Soto *et al*., 2001). However, the result may also indicate an inherent variation in susceptibility to viral infection between individuals of the same species. The concept of 'susceptibility' in hosts should therefore not only be considered at the species level but also at the level of the individual within that species – this is particularly important in animals displaying non-continuous growth and which must moult at relatively frequent periods. Differential pathogen prevalence related to the crustacean moult cycle has previously been demonstrated in field studies (Stentiford *et al*., 2001). In such cases, differential susceptibility likely relates directly to immune suppression around the time of ecdysis and in relation to other stressful events (Le Moullac and Haffner, 2000). Further, it is clear that European species such as *H. gammarus* can become infected with WSSV following ingestion of a single contaminated meal, though factors such as viral loading and host age or condition appear to be key variables in assessing the likelihood of progression from an infection to a disease state. This study has also shown that in *H. gammarus*, the development of WSD and associated cumulative mortality is enhanced when animals are held at 22^{o}C rather than 15^{o}C. Temperature has previously been shown to affect the development of WSD in penaeid shrimp and in crayfish, causing reduced mortality rates and lower viral loads when hosts are retained in both hypothermic and hyperthermic conditions. Interestingly, in both scenarios, disease developed rapidly when infected individuals were returned to optimal conditions for viral replication (Du *et al*., 2006a; Du *et al*., 2008; Granja *et al*., 2003; Granja *et al*., 2006; Jiravanichpaisal *et al*., 2004; Rahman *et al*., 2006; Rahman *et al*., 2007a; Rahman *et al*., 2007b; Vidal *et al*., 2001). Whether infection would maintain in a latent state in lobsters retained at 15^{o}C for longer periods of time, or would progress to disease via exposure of infected hosts to external stressors will govern the potential for WSSV to sustain a presence in natural aquatic environments of temperate regions.

Only one naturally occurring virus infection has been documented in lobsters. *Panulirus argus* virus 1 (PaV1) has been found to infect the spiny lobster species

P. argus from the Floriday Keys and the Caribbean (Shields and Behringer, 2004). To date, no viral infections have been described from homarid lobsters. Whilst this may be due to a general lack of comprehensive disease surveys or a sampling bias away from juvenile life stages (as suggested in studies on crabs by Bateman and Stentiford (2008) and Bateman *et al*. (2011)), it may also suggest some inherent non-susceptibility to viruses in this group. The latter is now apparently unlikely due to the rapid pathogenesis of WSSV in juvenile *H. gammarus* demonstrated during this study. Pathogenicity of WSSV has also been investigated in the spiny lobster species *Panulirus versicolor*, *Pa. penicillatus*, *Pa. ornatus* and *Pa. longipes, Pa. homarus* and *Pa. polyphagous* (Chang *et al*., 1998; Musthaq *et al*., 2006; Rajendran *et al*., 1999). Chang *et al*. (1998) reported that lobsters fed with WSSV-contaminated feed did not succumb (although WSSV was detected within their tissues), while Rajendran *et al*. (1999) provided some evidence that WSSV infection via feeding and ingestion could result in mortalities of *Pa. polyphagous* and *Pa. ornatus*. However, in a follow-up study by Musthaq *et al*. (2006), *Pa. homarus* and *Pa. ornatus* fed WSSV-contaminated feed did not die while those that were injected with WSSV did. These studies demonstrate the likelihood that spiny and homarid lobsters are susceptible to viral infections, and that investigations into the presence of natural viral infections in field-caught lobsters, particularly juvenile life stages, may prove fruitful.

In terms of import risk assessment for the trading of shrimp commodity products, the risks associated with viral release through shrimp packaging and re-processing plants in importing countries has previously been highlighted (Durand *et al*., 2000; Nunan *et al*., 1998). Shrimp imported for human consumption, can also be diverted into alternative uses. The use of raw, frozen shrimp products as angling bait has been identified as a relevant risk for introduction of viral crustacean pathogens in Australia (Biosecurity Australia, 2006). Furthermore, there is anecdotal evidence (e.g. via online forums) that this practice is also relatively commonplace in the United Kingdom (and likely other parts of Europe). A recent questionnaire sent to a sub-set of the United Kingdom angling fraternity (thought to exceed 4 million individuals) suggested that up to 7% of these may utilise frozen shrimp products as bait (pers. comm. Dr Birgit Oidtmann). This increased use appears to be directly associated with the current price

competitiveness between frozen, imported shrimp commodity and other types of specialist angling bait, and the ease of purchase via supermarkets.

Since *per os* feeding represents a realistic route of entry for pathogens (such as WSSV), further work is now required to investigate the likelihood for pathogen transmission between latently-infected lobster conspecifics and also between infected lobsters and other non-penaeid decapod hosts. Only when such studies are carried out will it be possible to determine the potential for the establishment of WSSV in wild populations of decapods residing in aquatic habitats of temperate regions. Further work is also required to assess the potential for differential risk associated with commodity imported from particular regions, particularly where approaches to within-country biosecurity, or emergency harvesting of infected shrimp ponds, are likely to generate products with high disease status. In addition further work is needed to investigate the potential import of other viral diseases such as Taura Syndrome and Yellowhead disease which are also listed within the European directive (EC Directive 2006/88) as exotic diseases to Europe but are also likely to be present within these products.

6.6 Conclusions

This study confirms the presence of WSSV within supermarket commodity shrimp imported for human consumption and present within the UK food market. Experimental trials showed this virus to be viable and that the commodity products were infective and could passage the infection to a naive crustacean species (*Homarus gammarus*) that is known to be susceptible to the virus. There is anecdotal evidence that the use of raw, frozen shrimp as angling bait has become common practice due to the availability of the product (easily available in supermarkets) and the low competitive price of these products when compared with other angling baits. This study has highlighted that this is an extremely high risk practice; chapter 2 has shown that crayfish are particularly susceptible to WSSV and are most likely to be the crustacean species which are exposed to these commodity products that are used as angling bait in the rivers and lakes. The use of these products as bait should be discouraged to limit the risk posed by this practice.

Chapter 7.

Genomic Variation in White Spot Syndrome Virus Following Passage through Different Crustacean Hosts - Variable Nucleotide Tandem Repeat (VNTR) Study

7.1 Abstract

The White Spot Syndrome Virus (WSSV) genome is extremely well conserved; however, differences between isolates were identified when genome sequences from different geographical areas were compared. Three variable nucleotide tandem repeat (VNTR) loci were identified on the genome open reading frame (ORF) which contained repeat units (RUs), the number of RUs corresponding to the size of the PCR fragment present. WSSV strains with smaller genomes have been found to be the fittest and most virulent forms of the virus; smaller genomes (RUs of 2-8 at ORF 94) have been shown to be dominant in outbreak ponds. It has been suggested that genomic variations seen in the number of tandem repeats present in ORF 94 may result from host selection pressures with the host playing a role in the selection of which WSSV genotype replicates within that individual. The aim of this chapter was to determine whether there were any differences in the WSSV VNTR types present after passage through different hosts: one highly susceptible to disease (signal crayfish, *Pacifastacus leniusculus*) and another low susceptible host (shore crab, *Carcinus maenas).* Following challenge with an identical dose of the same WSSV inoculum any potential variability in ORF 94 was assessed. DNA was extracted from gill tissue of ten crayfish and ten crabs, samples were cloned and sequenced before analysis. In addition, post-passage WSSV VNTR types were compared with WSSV VNTR types present in the initial inoculum. This analysis revealed some subtle differences in WSSV VNTR types present in crab and crayfish tissues following passage through these hosts. Initial inoculum showed RU's of between 0 and 3, RU's above 3 were not identified in the initial inoculum. Signal crayfish tissues showed RU's between 0 and 7, the majority of RU's present in the crayfish tissues appearing similar to that of the initial inoculum. However shore crab tissues showed RU's between 0 and 6, RUs 4, 5 and 6 appearing more frequent

relative to the initial inoculum following passage through shore crab tissues. Although limited in scope, this study reveals potential for viral population adjustment in specific hosts and further, may indicate drivers to avirulence or accommodation in certain hosts such as *Carcinus maenas*. Until now VNTR analysis has been used to determine geographic spread of the virus and to determine where outbreaks were most likely to have originated. This study highlights that the VNTR analysis can also be used to obtain information regarding host responses and their affect upon the type of virus that replicates within that individual or species.

7.2 Introduction

The White Spot Syndrome Virus (WSSV) genome is extremely well conserved amongst known isolates from different geographic locations and hosts (Lo *et al*., 1999; Wang *et al*., 2000c). The WSSV genome consists of double stranded DNA with a large circular genome approximately 300kb in size (van Hulten *et al*., 2001; Yang *et al*., 2001). However host dependant differences were found between isolates (Lan *et al*., 2002) and significant nucleotide sequence variation has been identified at five loci within the genome (Dieu *et al*., 2004; Marks *et al*., 2004). Comparison of genome sequences of isolates from Thailand, Taiwan and China have revealed three variable nucleotide tandem repeat (VNTR) loci on the genome open reading frame (ORF) 75, ORF94 and ORF125 as well as two variable regions in which major deletions occur ORF23/24 and ORF 14/15 (Figure 1.).

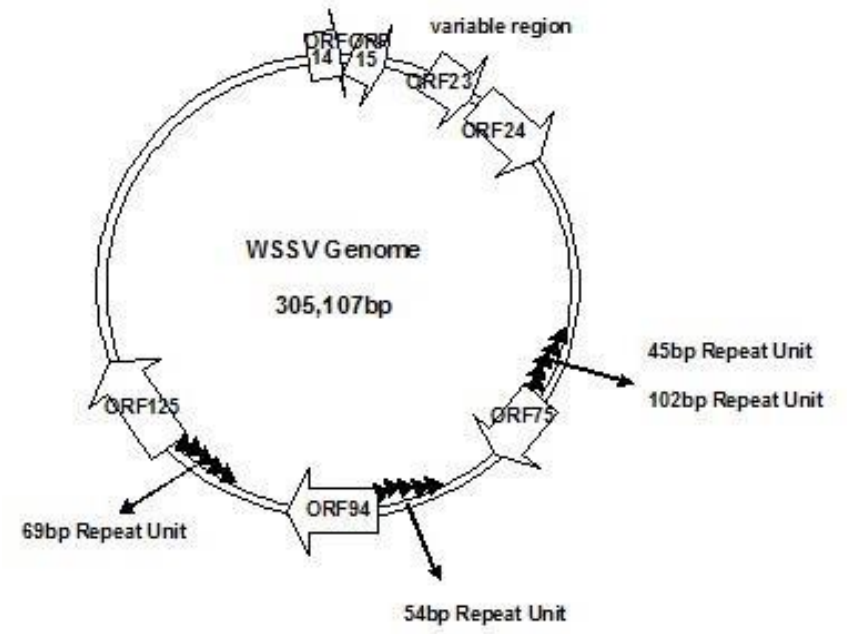


Figure 1. WSSV genome highlighting Open Reading Frame (ORF) sites, recreated from Hoa *et al*. (2011).

These regions have been used as genetic markers to characterise WSSV variants (Dieu *et al*., 2004; Marks *et al*., 2004) with VNTRs appearing more variable than deletions (Dieu *et al*., 2004). This information has been used to explain the geographical spread of the virus around the globe (Dieu *et al*., 2004; Dieu *et al*., 2010; Marks *et al*., 2004). The high uniformity in DNA sequence of WSSV suggests that a single virus species has likely been responsible for outbreaks in global shrimp farming (Wongteerasupaya *et al*., 2003).

Repeat units (RUs) are positioned within the ORFs which have non-repeated 5' and 3' ends and the number of RUs corresponds to the size of the PCR fragment generated (larger fragments corresponding to presence of multiple RUs). ORF 75 has two RUs with lengths of 45bp and 102bp, the first 45bp of the 102bp repeat being identical to the single 45bp RU (Dieu *et al*., 2004). Dieu *et al*. (2004) compared the RUs within a single isolate and found they contained single nucleotide polymorphisms (SNP) at 3, 15, 30, 40, 42 and 44 bp and the 102bp RU had an extra SNP at position 83. Each repeat unit could be recognised by its specific SNPs. The protein encoded by ORF75 has been shown to be present in WSSV virions (Huang *et al*., 2002). ORF94 has single repeat sequences of 54bp

with a SNP at position 48 which can be either Guanine or Thymine (Dieu *et al*., 2004; Dieu *et al*., 2010; Pradeep *et al*., 2008a; Syed Musthaq *et al*., 2006). The repeat region ORF94 is located between ribonucleotide reductase 1 and 2 (an enzyme that catalyses the formation of deoxyribonucleotide precursors involved in the replication process). This may indicate why the repeat structure of ORF94 is correlated to WSSV virulence (Hoa *et al.*, 2012a). However the large number of RUs within this ORF raises the question of whether ORF94 could produce a functional protein (Wongteerasupaya *et al*., 2003). ORF125 has single repeat sequences of 69bp of which the first two and last can be recognised by specific SNPs (Dieu *et al*., 2004; Dieu *et al*., 2010). The other RUs contain SNPs at positions 8, 18, 25, 66 and 69 (Marks *et al*., 2004). VNTR regions in ORF 125 and in particular ORF 94 have been found to correlate with disease outbreaks (Hoa *et al*., 2012b; Hoa *et al*., 2011) with WSSV genotypes with less than 9 repeat units (RUs) in ORF94 being shown to be dominant in ponds with outbreaks of White Spot Disease (WSD).

Pradeep *et al*. (2008b) concluded that ORF94 was the most useful molecular marker in epidemiological studies due to the wide variation typically seen. Samples collected from an outbreak pond in 2005 had 7 RUs within ORF94 as the dominant type of virion however in 2006 this had reduced to 2 RUs as the dominant form in samples from another outbreak. RUs of 2-8 were dominant in outbreak ponds and RUs of 9-16 dominant within non-outbreak ponds (Pradeep *et al*., 2008a). Table 1 provides a summary of the RUs present at the various ORFs in outbreak and non-outbreak ponds (data collated from a number of studies).

Table 1. Repeat Unit (RU) patterns in WSSV samples from outbreak and non-outbreak shrimp ponds, data collated from a number of studies (Dieu *et al*., 2004; Dieu *et al*., 2010; Pradeep *et al*., 2008a; Syed Musthaq *et al*., 2006; Wongteerasupaya *et al*., 2003). NR - not recorded.

ORF Site	*RU Size*	*Dominant RU*	*RUs not observed*	*RUs in non-outbreak ponds*	*RUs in outbreak ponds*
ORF75	*45bp* *102bp*	*NR* *NR*	*NR* *NR*	*3-16* *1-5*	*NR* *NR*
ORF94	*54bp*	*7*	*11, 15*	*2-20*	*<8*
ORF125	*69bp*	*4*	*6, 13*	*2-14*	*NR*

Hoa *et al*. (2011) showed that mixed genotype infections can occur in shrimp from ponds, by cloning and sequencing ORF75, ORF94 and ORF125 PCR products from a single shrimp they identified distinct genotypes and confirmed that these variants were unlikely to be artefacts from experimental procedures, showing that VNTR analysis was suitable for detecting mixed genotype infections. Hoa *et al.* (2011) analysed shrimp from outbreak ponds (shrimp were moribund or dead) and non-outbreak ponds (healthy shrimp with low level mortality (< 50%) They showed that individual shrimp from non-outbreak ponds were more likely to contain mixed genotype WSSV infections (see Figure 2.).

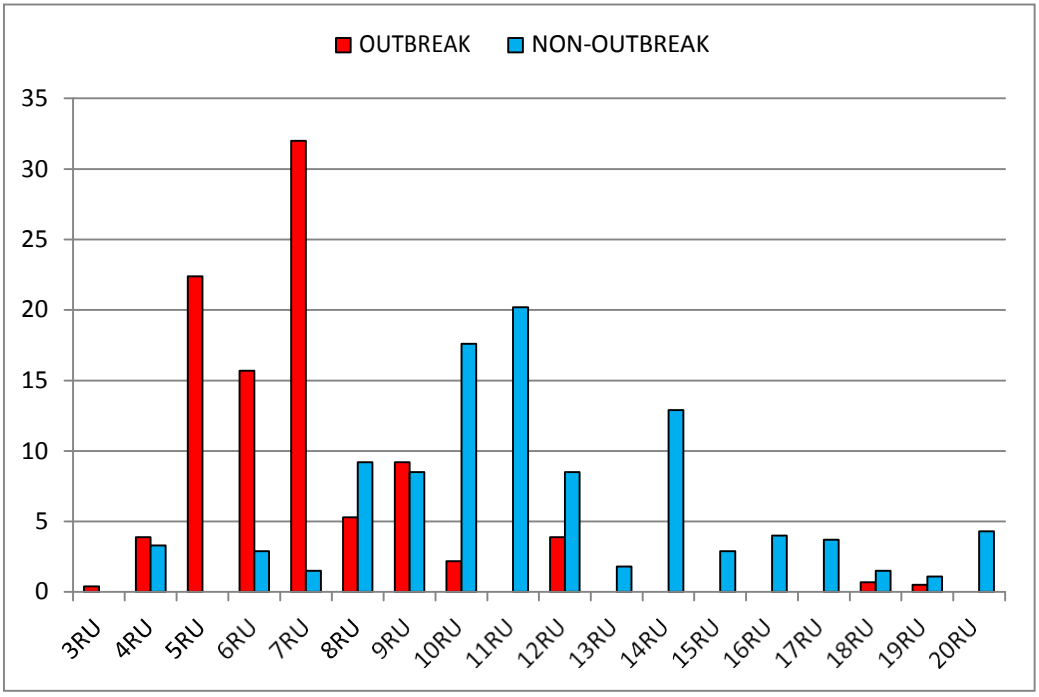


Figure 2. Distribution of WSSV genotypes among populations isolated from outbreak (red bars) and non-outbreak (blue bars) shrimp ponds analysed using ORF 94. Recreated from Hoa *et al*. (2012b).

Pradeep *et al*. (2009) have shown that WSSV strains with smaller genomes are the fittest. They conducted transmission studies using mixed strains and identified that only the strains with the smallest genome remained after two passages through shrimps and crabs. Similarly, they also caused disease after passage. This confirmed the previous findings by Marks *et al*. (2005), when comparing two WSSV strains with different size genomes, one large (~ 312 kbp) and one small (~ 293kbp). They showed a difference in virulence with the smaller genome being retained after two passages through shrimp and the larger genome being lost. Topley (1919) suggested that for high density populations the strains that replicate most rapidly are more likely to be transferred; these rapidly replicating strains also being more virulent. As such, virulent strains have a competitive edge over less virulent strains in multiple infections, leading to higher transmission. However virulence, recovery and transmission traits are a result of complex interactions with the host and are not solely determined by the pathogen (Alizon *et al*., 2009). The genetic compositions of individual hosts can also shape and 'fine-tune' pathogen virulence (Brown *et al*., 2006).

Epidemiological studies have identified different risk factors that have contributed to WSSV outbreaks. However, the mechanism for viral transmission and alteration through passage to different hosts is unknown. Wang *et al*. (2000c) compared six geographic isolates of WSSV from shrimp and crayfish. WSSV inoculums were prepared from these isolates and passed into SPF shrimp via injection. Differences between the crayfish and shrimp WSSV isolates were detected through several combinations of restriction analysis and Southern blot hybridisation. They suggested that the host species may play a role in selection of a mutant within a viral population. Differences highlighted in the crayfish isolate confirmed previous virulence and protein composition studies (Wang *et al*., 2000a; Wang *et al*., 1999). Variations in several endonucleases were identified; highlighting this was unlikely to be single point mutation randomly arising in the viral population.

Waikhom *et al*. (2006) showed that differences in numbers of tandem repeats (TR) appeared to result from host selection rather than geographical isolation. Waikhom *et al.* (2006) took four different samples of WSSV from four different regions in India and investigated the effect of passaging these through different hosts (*Portunus sanguinolentus, Portunus pelagicus, Machrobrachium rosenbergii* and *Artemia* sp.) via feeding trials. They showed that pathogenicity varied between the isolates themselves and could also be altered after passage through different species. TR present in ORF 94 were shown to vary between host species and increased when passaged isolates were compared to the original inoculums. Waikhom *et al*. (2011) suggested that genomic variations seen in the number of tandem repeats present in ORF 94 may have resulted from differential host passaging in nature and the consequent host selection pressures, especially as WSSV has such a wide host range, rather than geographical isolation.

The aim of this chapter was to determine whether there were any differences in the WSSV VNTR types present after passage through different hosts, a highly susceptible host (signal crayfish, *Pacifastacus leniusculus*) and a low susceptible host (shore crab, *Carcinus maenas*), using materials generated from previous

studies (Chapter 2). Initial inoculum samples were analysed with primers for all three ORF sites and variation was shown indicating a mixed genotype infection of the initial inoculums. However as previous studies had shown that ORF 94 was the most variable site seen within the WSSV genome (Hoa *et al*., 2012b; Hoa *et al*., 2011; Pradeep *et al*., 2008a; Pradeep *et al*., 2008b) this study concentrated on any potential effect of host on sequence variation in ORF 94.

7.3 Materials and Methods

7.3.1 Preparation of viral inoculums and challenge trials

Viral inoculates of WSSV were prepared as described in Bateman *et al*. (2012a). WSSV infected gill tissues from a highly susceptible species (*Pacifastacus leniusculus*) and a low susceptible species (*Carcinus maenas*) were generated via injection of this inoculums as described by Bateman *et al*. (2012a).

7.3.2 xtraction

Total nucleic acid was extracted from WSSV positive shrimp inoculum, shore crab tissues and crayfish tissues using an EZ1 Virus Mini Kit (Qiagen) following the manufacturer's protocols. Gill tissue from shore crabs and signal crayfish was weighed and diluted 1:10 in G2 buffer (Qiagen) and 10 μl Proteinase K. Samples were homogenised using a Fast prep FP120 machine (MP biomedicals) at the highest setting for 2 min. This homogenate was incubated at 56^{o}C for at least 3 hours to allow for lysis to occur. Samples were centrifuged at 9000rpm for 2 min and 50 μl of the supernatant was added to 150 μl of G2 buffer. Total DNA from this 200 μl sample was extracted using the EZ1 DNA tissue kit (cat no 953034) and the EZ1 advanced Biorobot (Qiagen) following manufacturer's instructions. DNA was eluted in 50 μl elution buffer.

7.3.3 ymerase Chain Reaction (PCR)

Each 50 μl reaction contained 1x Green GoTaq Flexi Buffer (Promega), 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 100 pmol of each F and R primer (see Table 2), 0.25 units *Taq* polymerase (Promega), and 2.5 μl total nucleic acid (20-50 ng total). Annealing temperatures for each primer set were as detailed in Table 2 but briefly, amplifications were performed with an initial denaturation temperature of

94°C for 5 min, followed by 34 cycles at 94°C for 1 min, annealing temperature for 1 min, 72°C for 1 min, with a final elongation step at 72°C for 10 min. Following amplification, 15 μl of each PCR product was analysed by agarose gel electrophoresis (1.5 % w/v), stained with ethidium bromide (0.625mg/ml), and viewed under a UV light source. Images were captured with a Gel Doc 2000 (Bio Rad) imaging system.

Table 2. Details of primers used in this study, Open Reading Frame (ORF) site, primer sequences and annealing temperatures used in this study.

ORF Site	Primer Name	Sequence (5'-3')	Annealing temperature	Reference
ORF75	ORF75-F ORF75-R	*GAAGCAGTATCTCTAACAC* *CAACAGGTGCGTAAAAGAAG*	50˚C	Dieu *et al.,* 2004
ORF94	ORF94-F ORF94-R	*TCTACTCGAGGAGGTGACGAC* *AGCAGGTGTGTACACATTTCATG*	61˚C	Wongteerasupaya *et al.,* 2003
ORF125	ORF125-F ORF125-R	*ACAGTGACCACACGATAATACCA* *TCGTTCACCATATCCATTGCCCT*	61˚C	Hoa *et al.,* 2011
N/A	M13-F (24mer) M13-R (22mer)	CGCCAGGGTTTTCCCAGTCACGAC TCACACAGGAAACAGCTAGAC	60°C	

7.3.4 Cloning

PCR products were purified from gels using Wizard® SV Gel and PCR Clean-Up System (Promega). DNA was ligated into the pGEM-T easy vector (Promega) and transformed into competent *E. coli* J109 High Efficiency Competent Cells using pGEM®-T Easy Vector System I kit (Promega). These were plated onto agar/ampicillin plates and incubated at 37°C overnight. White colonies were selected for plasmid isolation and M13 PCR to confirm inserts were present. Products were purified from the gel using methods as above and used in sequencing PCR using M13 primers (Promega).

7.3.5 Sequencing

PCR amplification products generated from clones were quantified using a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific). If samples were

too concentrated they were diluted to the required amount (50-100ng DNA) with molecular grade water and sequenced using M13 primers. Each 20 µl reaction contained 4µl Big Dye Terminator, 4µl Big Dye Buffer, 2µl of M13 Forward primer, 2µl of M13 Reverse primer and 10µl of sample. Reactions were analysed on an ABI 3130 Avant Genetic Analyser. Sequences were analysed using Sequencher software.

All PCR, cloning and sequencing was conducted with advice and assistance from Miss Rose Kerr (Molecular Biologist, Cefas).

7.4 Results

7.4.1 NTR of Shrimp inoculums

All primer sets utilised in this study yielded amplicons of the appropriate sizes. PCR products were cloned and subjected to a further PCR step using M13 primers (to confirm that the cloning procedure had worked and that inserts were present) (Figure 3). Product size appeared to vary between the clones present. Table 3 displays the results of the RU present at each ORF in the original shrimp inoculums used in this study. A total of 13 clones were analysed from ORF 75 and 12 clones were analysed from ORF94 and ORF 125 samples.

Table 3. VNTR results of shrimp inoculums at the three different ORF sites

ORF RU Size	ORF 75 45bp	 45bp & 102bp	ORF 94 54bp	ORF125 69bp
0 RUs	4		3	4
1 RU	3	5	3	3
2 RU	1		5	1
3 RU	0		1	1
4 RU	0		0	3
Total Clones analysed	13		12	12

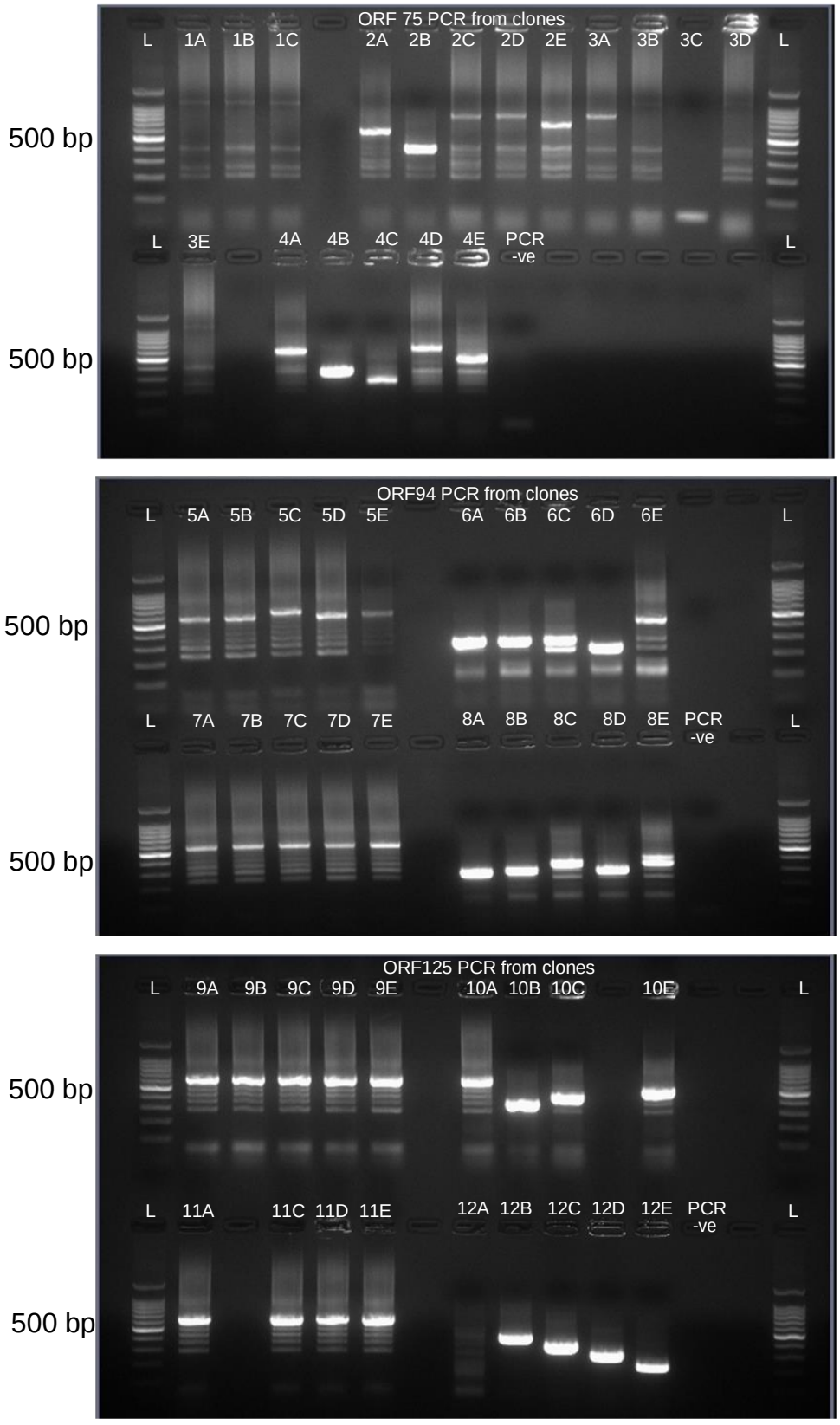


Figure 3. An ethidium bromide stained agarose gel showing DNA fragments produced by PCR amplification from ORF 94, ORF 75 and ORF 125 in shrimp inoculums. L: 100bp DNA ladder. PCR –ve: Negative control for PCR.

7.4.2 VNTRs from ORF 94 in signal crayfish (*Pacifastacus leniusculus*) tissues

Amplicons of the correct size were detected in all 10 samples of signal crayfish gill tissue (Figure 4). In some cases a smear was present below the band; both the band and the smear were selected for subsequent cloning and sequencing. Out of the 83 clones sequenced, only 15 provided data of adequate quality to allow for analysis of the RU profile. A representation of how sequences were analysed and compared is shown in Figure 6, signal crayfish tissues appearing similar to that of the initial inoculum, thymine present at bp 48 in both initial inoculum and within signal crayfish tissues. The profile of RUs present within the tissues of WSSV-infected signal crayfish is given in Figure 7.

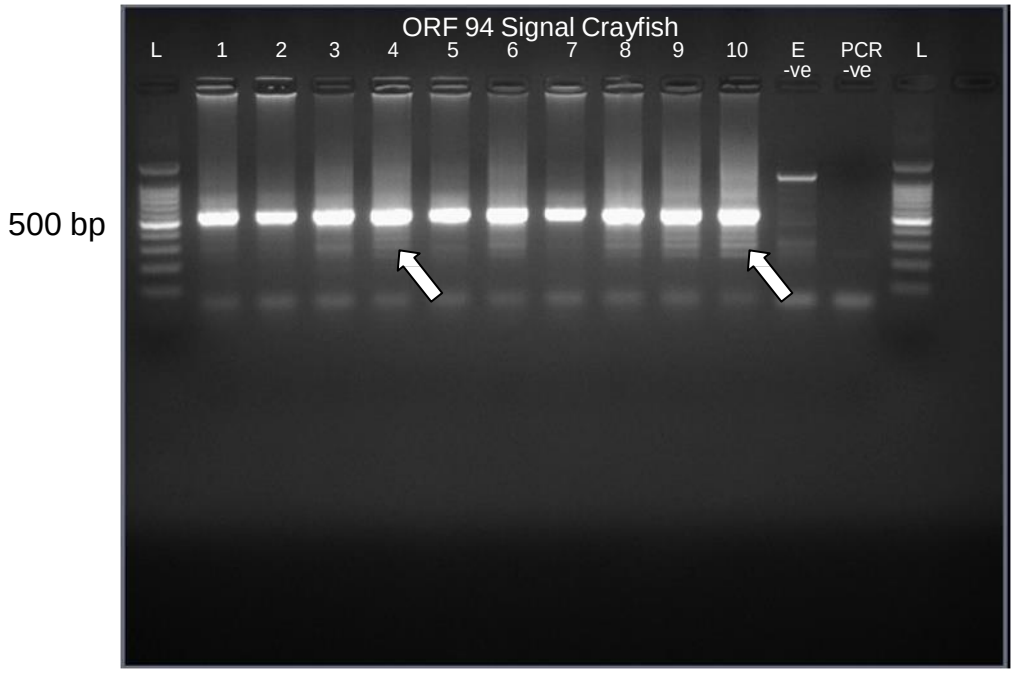


Figure 4. An ethidium bromide stained agarose gel showing DNA fragments produced by PCR amplification from ORF 94 in Signal crayfish. Products approximately 500 bp in size could be clearly seen for each sample. In some cases a smear of material was present below these bands (arrows). L: 100bp DNA ladder. E –ve: Negative control for extraction. PCR -ve: Negative control for PCR.

7.4.3 VNTRs from ORF 94 in shore crab (*Carcinus maenas*) tissues

Amplicons of the correct size were detected in all 10 samples of shore crab gill tissues (Figure 5). In some cases a smear was present above and below the band; both the band and the smears (above and below the band) were selected for subsequent cloning and sequencing. Out of the 54 clones sequenced, only 20 provided data of adequate quality to allow for analysis of the RU profile. A representation of how sequences were analysed and compared is shown in Figure 6, shore crab tissues appeared to possess more RUs than the initial inoculum and displayed variation at bp 48 within these RUs as highlighted (Figure 6). The profile of RUs present within the tissues of WSSV-infected shore crabs is given in Figure 7.

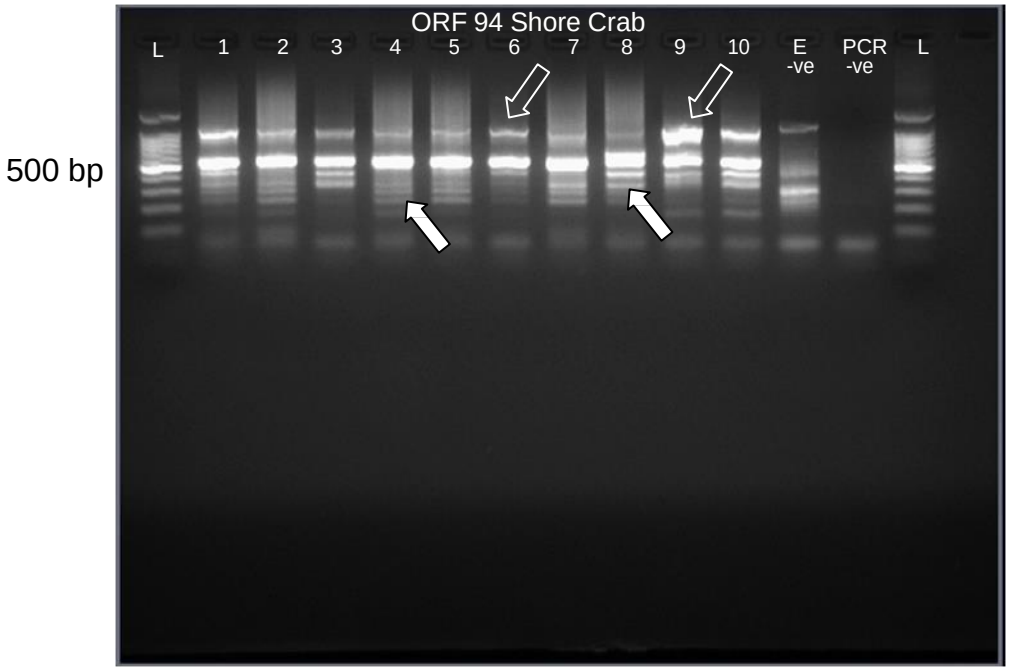


Figure 5. An ethidium bromide stained agarose gel showing DNA fragments produced by PCR amplification from ORF 94 in Shore crab. Products approximately 500 bp in size could be clearly seen for each sample. In some cases a smear of material was present above (black arrow) and below these bands (white arrows). L: 100bp DNA ladder. E –ve: Negative control for extraction. PCR -ve: Negative control for PCR.

A. ORF 94 RU

CGCAAAAAGCGTGCCGCACCTCCACCTGAGGATGAAGAAGAGGATGA**T**TTCTAC

B. Initial inoculum - 2 RUs

GACGACGACGATGACGATGGAGGAACTTTCGATACAGTAGGGTCTGGTATACTTGGA
CGCAAAAAGCGTGCCGCACCTCCACCTGAGGATGAAGAAGAGGATGATTTCTAC
CGCAAAAAGCGTGCCGCACCTCCACCTGAGGATGAAGAAGAGGATGATTTCTAC
CGCAAAAAGCGTTAAACTACGCACGAAAGTGACGGTGGTTGAAGAATAGACTAATATTG
TTGATATGTTAACCCCTTTTTTTCATGAAATGTGTACACACCTGCT

C. Signal crayfish - 2 RUs

GACGACGACGATGACGATGGAGGAACTTTCGATACAGTAGGGTCTGGTATACTTGGA
CGCAAAAAGCGTGCCGCACCTCCACCTGAGGATGAAGAAGAGGATGAGTTCTAC
CGCAAAAAGCGTGCCGCACCTCCACCTGAGGATGAAGAAGAGGATGATTTCTAC
CGCAAAAAGCGTTAAACTACGCACGAAAGTGACGGTGGTTGAAGAATAGACTAATATTG
TTGATATGTTAACCCCTTTTTTTCATGAAATGTGTACACACCTGCTA

D. Shore crab - 7 RUs

TTCTACTCGAGGAGGTGACGACGACGACGACGATGACGATGGAGGAACTTTCGATACAG
TAGGGTCTGGTATACTTGGA
CGCAAAAAGCGTGCCGCACCTCCACCTGAGGATGAAGAAGAGGATGA**G**TTCTAC
CGCAAAAAGCGTGCCGCACCTCCACCTGAGGATGAAGAAGAGGATGATTTCTAC
CGCAAAAAGCGTGCCGCACCTCCACCTGAGGATGAAGAAGAGGATGA**G**TTCTAC
CGCAAAAAGCGTGCCGCACCTCCACCTGAGGATGAAGAAGAGGATGA**G**TTCTAC
CGCAAAAAGCGTGCCGCACCTCCACCTGAGGATGAAGAAGAGGATGATTTCTAC
CGCAAAAAGCGTGCCGCACCTCCACCTGAGGATGAAGAAGAGGATGATTTCTAC
CGCAAAAAGCGTGCCGCACCTCCACCTGAGGATGAAGAAGAGGATGATTTCTAC
CGCAAAAAGCGTTAAACTACGCACGAAAGTGACGGTGGTGAAGAATAGACTAATATTGT
TGATATGTTAACCCCTTTTTTT

Figure 6. Representative sequences of WSSV VNTR analysis from ORF 94 following passage through different hosts. A. Expected sequence of 54 bp repeat unit present at ORF 94 (Hoa *et al*., 2011), bp 48 is highlighted in red as this can be either thymine (T) or guanine (G). B. WSSV sequence from the initial inoculum displaying 2 RUs at ORF 94, thymine present at bp 48. C. WSSV sequence from signal crayfish gill tissue displaying 2 RUs at ORF 94, thymine present at bp 48. D. WSSV sequence from shore crab gill tissue displaying 7 RUs, variations at position 48 are highlighted in red. RUs are highlighted in yellow to identify them within the sequences and each RU begins on a new line so sequences can be directly compared to the expected sequence (A).

7.4.4 omparison of RU profiles

Initial inoculum (blue bars, Figure 7) showed RU's of between 0 and 3. RU's above 3 were not identified in the initial inoculum; however we have to remember that the initial inoculum was sourced from shrimp in an outbreak pond so this may be expected as smaller RU's dominate in outbreak ponds. Signal crayfish tissues (red bars, Figure 7) showed RU's between 0 and 7, the majority of RU's present in the crayfish tissues were 0 and 2 similar to that of the initial inoculum. However shore crab tissues (green bars, Figure 7) showed RU's between 0 and 6, with a more even distribution of RU's between 0 and 6 when compared to the initial inoculum and the crayfish tissues. RUs 4, 5 and 6 become more frequent relative to the initial inoculum following passage through shore crab tissues.

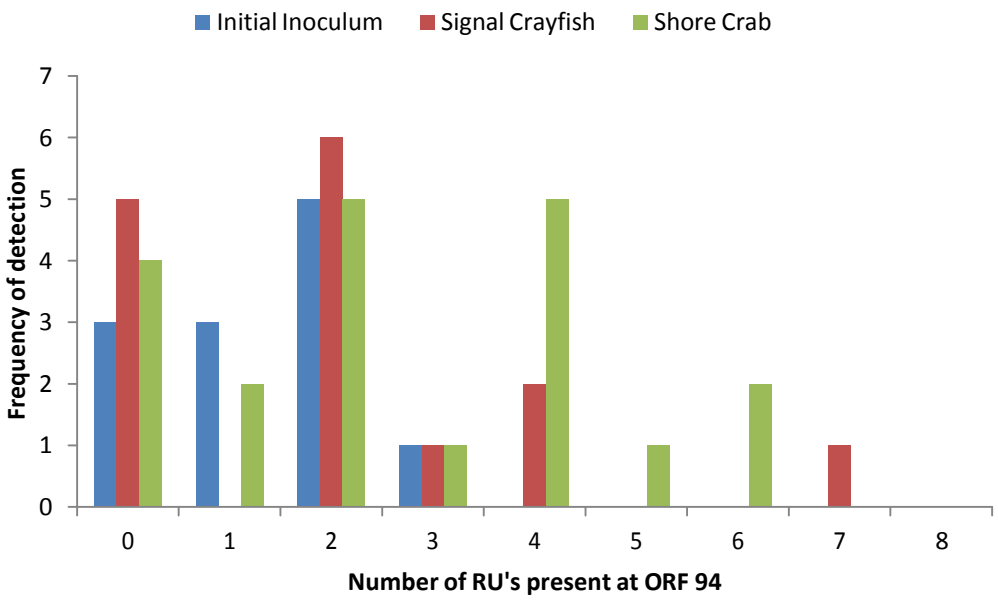


Figure 7. ORF 94 RU results comparing original shrimp inoculum (blue bars) with results obtained following passage through signal crayfish (red bars) and shore crab tissues (green bars).

7.5 Discussion

This study investigated the frequency of repeat unit (RU) profiles in 3 open reading frames (ORF 75, 94 and 125) of the WSSV genome in a stock inoculum, and of ORF 94 following viral passage through two non-target hosts (*Carcinus maenas* and *Pacifastacus leniusculus*) found in European waters. The study aimed to provide insight in to potential for alteration of the viral population during the infection process in these hosts. The initial inoculum used in this study originated from an outbreak of WSSV in China and has been repeatedly passaged through *L. vannamei* to maintain stocks, both within the donor laboratory (University of Arizona), and within our laboratory. Each animal exposed in this study was inoculated with the same batch of inoculum, originating from a single shrimp, infected with WSSV (Bateman *et al*., 2012a). In order to determine the genetic composition of the initial inoculum, extracts were analysed using primers designed to amplify ORF 75, ORF 94 and ORF125 of the WSSV genome (Dieu *et al.,* 2004; Hoa *et al.,* 2011; Wongteerasupaya *et al.,* 2003). Analysis of the initial inoculums revealed the presence of multiple ORF profiles within the stock solution. Results (Table 3) indicated that there were less than 8 RUs present at ORF 94 site, providing support that the inoculum was derived from shrimp tissues collected during a disease outbreak (see Pradeep *et al*., 2008b).

It was vital that the composition of the initial inoculums was determined to establish which genotypes were present before passage. Initial inoculum samples were analysed with primers for all three ORF sites and variation was shown indicating a mixed genotype infection of the initial inoculums. However as previous studies had shown that ORF 94 was the most variable site seen within the WSSV genome (Hoa *et al*., 2012b; Hoa *et al*., 2011; Pradeep *et al*., 2008a; Pradeep *et al*., 2008b) this study concentrated on any potential effect of host on sequence variation in ORF 94. From the data it can be seen that we only see genotypes with RU's below 8 at position 94 (Figure 7). The initial inoculum was shown to contain RU's of between 0 and 3; RU's larger than 3 were not identified in the initial inoculum. It is important to note that the initial inoculum was sourced from shrimp in an outbreak pond so this may be expected as smaller RU's dominate in outbreak ponds (Pradeep *et al*., 2008a).

Although results are limited there does appear to be a difference between the numbers of RU's present in the initial inoculum and those that are present following passage through different hosts. Both signal crayfish and shore crab individuals had been inoculated with the same initial inoculums, results were analysed to determine whether there were any differences in the viral types between a highly susceptible and a low susceptible species after passage. Despite cloning 83 samples from signal crayfish and 54 samples from shore crab tissues only 15 and 20 samples respectively provided enough material to enable sequencing. Initial inoculum shows RU's of between 0 and 3 as opposed to between 0 and 7 in signal crayfish and 0 and 6 in shore crabs, shore crabs showing more copies of the larger RU's than the other samples. Whether this is because we did not detect the larger RU's in the initial inoculum or whether the shore crab has manipulated the viral replication in some manner to slow replication is unclear and further work is needed. It is not possible to say whether there is a difference between the viral replication of WSSV in a low susceptible and highly susceptible species from the results of this study, however results may suggest subtle differences and indicate that this is a subject area worth pursuing. Until now VNTR analysis has been used to determine geographic spread of the virus and used to determine where outbreaks were likely to have originated. This study shows that the VNTR analysis can be used to obtain information regarding influences of the host themselves upon the type of virus that replicates within that individual or species.

In order to thoroughly test the theory that the host influences viral replication we should have used inoculums from a non-outbreak situation where we should have seen a broader range of genotypes present. By using an inoculum from an outbreak situation we were already biasing the genotypes present towards the disease causing, smaller and faster replicating genomes. However this study was conducted with material generated from a previous susceptibility study and the initial inoculum used needs to be considered when interpreting the results.

The initial aim of this work was to analyse material from 10 signal crayfish and 10 shore crabs, cloning and examining 16 samples from each individual, in order to

gather enough data to enable reliable statistical analysis (numbers decided following a power analysis conducted by Dr Allan Reece (Statistician, Cefas). The numbers of suitable sequences for analysis were much lower than expected and meant that interpretation of data is limited. There are a number of reasons as to why the sequencing failed to provide adequate data. Clear bands and smears of the band were taken from the different gels; these were treated separately for the cloning procedure (Figures 4 and 5). It would have probably been better to take a large section of gel containing both the smear and distinct products and purified it all together before cloning to ensure that products which were too small to see by the naked eye were captured. A different concentration of agar may also have assisted with separating out these products prior to cloning. It is possible that the material present in the smears was nonsense (host, dntps and primers etc.) and not the material that we were interested in, hence would not give clear sequences after cloning. The clones may not have replicated the area of interest within the sample and on hindsight it would have been better to work with small batches of samples as opposed to trying to clone all shore crab and all signal crayfish samples together. There was a difference in the quality of the sequences that were returned from signal crayfish when compared to the shore crab. Clear results were obtained for the M13 PCRs for the signal crayfish, the shore crab less so and in some cases the shore crab results showed binding of two forward primers rather than a forward and reverse primer. It is not clear what caused this but this work would need to be repeated with new primers to eliminate any chances this could have been due to operator error, if these results are repeated it may show some kind of preferential binding of the forward primer and optimisation issues.

Initially it was suggested to be more suitable to analyse and interpret these samples with an alternative method such as Illumina Miseq. However this would mean that a large amount of data would have been generated in the form of short reads (450 bp) and these short reads would then need to be pieced together like a jigsaw puzzle in order to get the results, this process is possible but would be quite complicated and time consuming. There are potentially 20 RUs that could be present at ORF 94 which would mean a product size of over 1000 bp and this could be too big to use this technique, maximum read size with Illumina Miseq is

900 bp (450F and 450R). An alternative method would be the Qiagen QIAxcel® system which is an automated gel electrophoresis machine that enables rapid, high throughput VNTR analysis and was used by Maeda *et al*. (2007) to analyse the VNTR for *Mycobacterium tuberculosis* isolates in Japan. This technique could provide a vast amount of information which could then be interpreted to determine the RU's present in each sample and potentially at each ORF rather than just concentrating on ORF 94. This would provide a more thorough and extensive investigation into whether passage through different hosts alters the final genotypes present.

WSSV has been shown to be taxonomically similar to members of the Baculoviridae. In fact the rapid spread of the virus when it first emerged resulted in multiple isolations by different laboratories and the use of a variety of different names suggesting it to be a baculovirus, Chinese Baculo-like virus (Nadala *et al*., 1997), White Spot Bacilliform virus, White Spot Syndrome Baculovirus (WSBV) (Chou *et al*., 1995; Lo *et al*., 1996b; Wang *et al*., 1995) , *P. monodon* non-occluded baculovirus I and III (PmNOBIII) (Wang *et al*., 1995), Systemic Ectodermal and Mesodermal Baculovirus (SEMBV) (Wongteerasupaya *et al*., 1995) and Hypodermal and Hematopoietic Necrosis Baculovirus (HHNBV) (Huang *et al*., 1995). Similar to observations in WSSV, there are also a wide diversity of baculovirus phenotypes in field populations of insects. Cory *et al*. (2005) has questioned why this diversity is maintained. There are numerous mechanisms that could explain and maintain this diversity; baculovirus diversity may be maintained by differential selection where individual variants have an advantage under different environmental conditions. The results that Cory *et al*. (2005) highlighted pointed to one obvious mechanism, the influence of virus host range. Baculoviruses that can infect more than one host show a higher fitness, different variants showing higher fitness in different host species, for example differences in virulence of baculovirus variants are shown in *Panolis flammea* (Pine Beauty Moth) and *Mamestra brassicae* (Cabbage Moth). Fitness refers to the ability of an organism to adapt and reproduce in a defined environment i.e. how well a virus can spread through a host population.

Viral ‘quasispecies’ are a large group of viruses, related by a similar mutation or mutations. High mutation rates create a “cloud” of potentially beneficial mutations at the population level affording the viral quasispecies a greater probability to evolve and adapt to new challenges during infection (Vignuzzi *et al*., 2006). The viral quasispecies theory suggests that evolution occurs through selection of interdependent viral subpopulations rather than the classic genetic concept that evolution occurs through the selection of individual viruses. Survival of a given viral population depends upon replication fidelity, however, subtle changes within the replication of this genetic material enables a reservoir of individual variants within the population. These variants are then more capable of adapting to changing environmental conditions. However having too many mutations in a genome can drive a population to extinction, and too few mutations can cause extinction, the virus being unable to survive changes in the environment or “bottlenecks” such as replication in different tissues or transmission from individual to individual. The major challenges to virus survival occur during its interactions with the host; viruses struggle with host defence mechanisms, host to host transmission and diverse cellular environments of the different tissues. It is the ability to evade these pressures which determines the pathogenic outcome of an infection (Vignuzzi *et al*., 2006). Evolving quasispecies have been shown to possess molecular memory, meaning they may be capable of swiftly reacting to a selection pressure that has already been experienced by the same viral population, even if this selection pressure had not been in operation for many viral generations (Domingo, 2000; Ruiz-Jarabo *et al*., 2000). WSSV has a remarkably broad host and temperature range, and shows wide variation in pathogenicity and virulence between host species which is a unique feature of this virus. It is quite likely that “quasispecies” features which appear to be displayed by WSSV explain how the virus is able to adapt and infect so many different species of crustaceans and inhabit so many different environments. It could also explain why some species are more affected by the virus than others, shrimp for example are highly susceptible, the virus is well suited to shrimp tissue types and overcomes the shrimp host responses. However, although the virus can infect other species such as shore crab the host responses are different and the virus is not as well suited to this environment, although the virus can persist

within shore crab tissues it does not appear to replicate as rapidly and so does not cause the same level of disease.

Mixed genotype infections are common for many viruses (Cory *et al*., 2005; Davis *et al*., 1999; Smith and Crook, 1988) and experimental work has demonstrated that mixed-genotype infections are in many instances more virulent (Hodgson *et al*., 2004; Simon *et al*., 2006; Vignuzzi *et al*., 2006). Disease outbreaks are the result of interactions between the pathogen, the host and the environment. The causes of transition from non-outbreak shrimp ponds to outbreak shrimp ponds are not known. Hoa *et al.* (2011) highlighted three possible causal relationships between mixed genotype infections and pond disease status. Firstly, WSSV genotypic composition could determine disease outbreak status since interference between different genotypes within the host may explain why the virus exhibits less virulence resulting in fewer outbreaks in mixed genotype infections. Here, host responses may be the mechanism mediating this interference. Secondly, outbreak status could determine genetic composition; there may be a strong selection for certain genotypes during an outbreak with this selection leading to displacement of other genotypes from the virus population. Thirdly, environmental conditions such as poor water quality could reduce "fitness" of the host and therefore increase the occurrence of mixed genotype infections. Hoa *et al*. (2011) stated that further work was needed to determine when the mixed genotype infections are lost at the individual level and how this relates to disease outbreaks. This study reveals potential for viral population adjustment in specific hosts and further, may indicate drivers to avirulence or accommodation in certain hosts such as *Carcinus maenas*.

7.6 Conclusion

This study has utilised ORF specific amplification to reveal evidence for genomic diversity in WSSV used as inoculum in challenge trials. This work highlights that in addition to determining the geographic spread and origins of disease outbreaks the VNTR analysis can be used to obtain information regarding host responses and their affect upon the replication of the virus. Further, by investigating ORF variants in the tissues of shrimp, crabs and crayfish exposed to the same initial

inoculum, we have provided some evidence that European hosts are able to alter the genotypes present after passage. Although limited in scope, this study reveals potential for viral population adjustment in specific hosts and further, may indicate drivers to avirulence or accommodation in certain hosts such as *Carcinus maenas*. Future studies should repeat the analysis of these samples but use the Qiagen QIAxcel® system, which is an automated gel electrophoresis machine that enables rapid, high throughput VNTR analysis. This method should provide a vast amount of information which could then be interpreted to determine the RU's present in each sample and potentially at each ORF rather than just concentrating on ORF 94. This would provide a more thorough and extensive investigation into whether passage through different hosts alters the final genotypes present.

Chapter 8.

Survey of pathogens of *Carcinus maenas* reveals a novel viral pathogen which mimics infection by WSSV

8.1 Abstract

A survey of *Carcinus maenas* collected from numerous sampling points around the UK coastline conducted between July and October 2010 revealed a range of known and novel viral, bacterial, protistan and metazoan pathogens via histology, TEM and molecular diagnostics. The prevalence and profile varied considerably between sites. As part of this survey, a novel viral infection was detected in *C. maenas*. Initially assumed to be B virus infection, TEM revealed the presence of hexagonal viral particles within the enlarged nuclei of infected haemocytes. Virions possessed a distinct brick shaped core surrounded by an electron dense sphere and both an inner and outer envelope. Due to these features the virus was deemed to most closely resemble particles described in the members of the *Herpesviridae*. The virus was thus referred to as Herpes-like virus (HLV) pending more formal characterisation. The virus causes a dramatic haemocytopoenia during patent disease states and potentially leaving infected hosts open to infection by secondary, opportunistic pathogens. Specifically, it is proposed that colonisation of the haemolymph by bacteria create a pathology previously described in this species as Milky Disease (Eddy *et al*. 2007). It is noteworthy that the pathognomonic signs of HLV in *C. maenas* mimics that of the listed pathogen WSSV. This is important since this host has the potential to be used in national screening programmes for demonstration of disease freedom for WSSV in the EU.

8.2 Introduction

White Spot Disease (WSD) is listed as an OIE notifiable disease and as a non-exotic disease within Europe, EC Council Directive 2006/88/EC. The disease was listed as a non-exotic disease in Europe after outbreaks of WSD were observed in shrimp farms in Southern Europe. However, the prevalence and spread of this virus within Europe is not clear; the last known outbreak of the disease was in 2001 on a shrimp farm in Italy (Stentiford and Lightner, 2011). One of the duties listed under the European

Directive is for Member States to declare their status for WSD. Status can range from Category I (free from disease), to Category V (infected) (see Chapter 2 for full details). In order to declare disease freedom Member States are in many cases required to undertake a two year surveillance programme, including samples collected from wild crustaceans. The European shore crab *Carcinus maenas* is ubiquitous around the coastline of the UK and Europe and is known to be susceptible to a wide range of parasites and pathogens. Due to ease of availability and low cost for obtaining samples, this species is a candidate for inclusion in such surveillance programmes.

C. maenas is known to host a range of viral diseases caused by both DNA and RNA viruses (Brock and Lightner, 1990; Johnson, 1984). B virus was discovered during a study of limb regeneration of *C. maenas*, it was first noticed infecting the nuclei of haemocytes and connective tissues formed at the beginning of regeneration (Bazin *et al*., 1974). Histology of B virus revealed hypertrophied nuclei with marginalized chromatin whilst TEM revealed parallel arrays of viral particles within these enlarged nuclei. B virus virions were often associated with vesicles of various sizes within the nucleus, measured 300-320nm in length, were rod-shaped and sometimes appeared curved (Bazin *et al*., 1974). B virus mimics WSSV both in terms of ultrastructure and pathognomonic signs of infection (see Chapter 2).

Johnson (1988a) described a similar infection in *C. maenas* from the Atlantic coast of the USA and named this virus rod-shaped virus of *Carcinus maenas* (RV-CM). Although morphologically similar to the virus from European *C. maenas,* size differences and some other development characteristics indicated that it was different. RV-CM also infects haemocytes and cells of the haematopoietic tissue. Early infections showed nuclei containing groups of vesicles that usually contained some particulate material. Viral particles and pieces of membrane were associated with the vesicles and scattered throughout the nucleus. Heavy infections showed virus and vesicles spread more evenly through the nuclei. Virions measured 95-110nm in diameter and between 190-540nm in length, the majority measuring between 235-280nm. Newly completed nucleocapsids were straight with the envelope fitting loosely along its length; however; as the envelope closed particles became spherical forcing the nucleocapsid into a curved shape. Curvature of the particles could be so pronounced that occasionally the nucleocapsids were bent into characteristic U or V

shapes. As noted in Chapters 2 and 4, U or V shaped particles were also observed within *C. maenas* exposed to WSSV.

WSSV replicates within the nucleus and infects all tissues of mesodermal and ectodermal origin, such as gills, lymphoid organ, midgut but especially cuticular epithelium and sub-cuticular connective tissues (Lightner, 1996). WSSV also infects the haemocytes as highlighted by Owens (1993). It is a lytic virus and in the late stages of infection it causes the infected cells to disintegrate causing destruction of affected tissues (Leu *et al*., 2010). Infected nuclei become hypertrophied with marginalized chromatin, and contain inclusion bodies that stain intensely eosinophillic in early stage infection and basophilic in more advanced infection (Lightner, 1996). TEM reveals hypertrophic nuclei to be filled with virions, ovoid or ellipsoid to rod-shaped, consisting of an electron dense nucleocapsid, with a tight fitting capsid layer, surrounded by a loose-fitting trilaminar envelope (Vlak *et al.,* 2005). Virions measure 120-150nm in diameter and 270-290nm in length and nucleocapsids measure 65-70nm in diameter and 250-350nm in length (Vlak *et al*., 2005). In some cases a tail like projection can be seen extending from one end and virions possess a distinctive capsid layer giving the DNA core a cross-hatched or striated appearance (Vlak *et al*., 2005). The capsid is described as being composed of rings of subunits in a stacked series; these rings are 20nm thick and perpendicular to the longitudinal axis of the capsid (Wang *et al*., 1995). This feature is characteristic to WSSV and is similar to that described in B virus of *Carcinus maenas* (Bazin *et al*., 1974; Bonami and Zhang, 2011) and the similar B2 virus in *Carcinus mediterraneus* (Bonami and Zhang, 2011; Hernandez-Herrera *et al*., 2009; Mari and Bonami, 1986).

WSSV is currently the only member of the *Nimaviridae* genus, a situation that is acknowledged to be unusual by the International Committee on Taxonomy of Viruses (ICTV) (Vlak *et al*., 2005). The ICTV has predicted that that this may change as new strains and isolates are discovered. Vlak *et al*. (2005) tentatively listed B virus and RV-CM from *Carcinus maenas* (Bazin *et al*., 1974; Johnson, 1988a), B2 virus and τ (tau) virus from *Carcinus mediterraneus* (Mari and Bonami, 1986; Pappalardo *et al*., 1986) and Baculo-A and Baculo-B viruses from *Callinectes sapidus* (Johnson, 1976a; Johnson, 1983) within this genus. This is largely due to the fact that B, RV-CM, B2, and Baculo-B viruses are extremely similar in terms of size, shape and morphogenesis

to that reported for WSSV. It has been suggested that these viruses may be ancestral forms of WSSV (Bonami and Zhang, 2011). The taxonomic position of these viruses has never been defined and the tentative classification by Vlak et al. (2005) has now been removed due to lack of evidence (Lo *et al*., 2012). Due to the potential similarities between B virus and WSSV and the potential of finding this virus during European surveillance programmes for WSSV, the main aim of this chapter was to try and re-isolate B virus in order to complete the classification of this virus and to provide material to enable a full comparison to WSSV to be completed.

Juvenile shore crabs sampled from the shoreline at Newtons Cove in Weymouth, UK displayed enlarged nuclei containing distinct eosinophilic inclusions within the haemocytes, connective tissues and haematopoietic tissues, some haemocytes displaying a distinctive binucleate appearance. The same pathology was also present in *C. maenas* sampled as part of the temperature stress study (Chapter 4), crabs were shown to possess a viral infection which upon initial histological screening appeared to be extremely similar to B virus and WSSV. Transmission Electron Microscopy (TEM) analysis however revealed herpes-like virus (HLV) particles within these enlarged nuclei, particles appearing very similar to Bi-Facies virus described infecting *Callinectes sapidus* by Johnson (1978; 1988b). Due to the histological similarities to WSSV and the potential of finding this virus during surveillance programmes the study attempted to characterise the virus with the aim of developing a diagnostic test to distinguish between this virus infection and WSSV. Passage studies were conducted to identify whether this virus could be transmitted and to increase stocks of infected material to enable for full characterisation. Samples of homogenised infected crab tissues were also prepared and submitted for 454 sequencing.

As highlighted the pathognomonic signs of HLV in *C. maenas* mimics that of the listed pathogen WSSV. This is important since this host has the potential to be used in national screening programmes for demonstration of disease freedom for WSSV in the EU and highlights the need for full characterisation of this viral infection.

8.2 Materials and Methods

8.2.1 Pathogen profile of *Carcinus maenas* from UK sites

Thirty European shore crabs (*Carcinus maenas*) were collected from the shoreline, or from offshore coastal locations collection or via the use of drop nets from nine sampling sites around the coastline of England and Wales (Table 1).

Table 1. Sampling site locations and collection method used.

Sampling Area	Location	Capture Method
1	Berwick Upon Tweed	Offshore collection via potting
2	North Shields	Offshore collection via potting
6	Blakeney Harbour, Norfolk	Drop netting
8b	West Mersea	Drop netting
9	Southend on sea	Shoreline collection
11	Rye Bay	Drop netting
12b	Poole Harbour	Drop netting
14c	Helford	Shoreline collection
20	Menai Straits	Offshore collection via potting

In addition sixty juvenile shore crabs (carapace width below 20mm) were sampled direct from the shoreline in Newton's Cove, Weymouth, UK (50º34' N, 02º22' W). Crabs were transported to the laboratory overnight and sampled the following morning. Crabs were chilled on ice for 30 minutes prior to dissection where gill, epidermis, hepatopancreas, heart, gonad, nerve and muscle were placed into histological cassettes and fixed immediately in Davidson's seawater fixative. Fixation was allowed to proceed for 24 h before samples were transferred to 70 % industrial methylated spirit. Fixed samples were processed to wax in a vacuum infiltration processor using standard protocols. Sections were cut at a thickness of 3-5 µm on a rotary microtome and mounted onto glass slides before staining with haematoxylin and eosin (H&E) and Feulgen stains. Stained sections were analysed by light microscopy (Nikon Eclipse E800) and digital images and measurements were taken using the Lucia™ Screen Measurement System (Nikon, UK).

For electron microscopy, gill, epidermis, hepatopancreas and heart tissues were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature and rinsed in 0.1 M sodium cacodylate buffer (pH 7.4). Tissues were post-fixed for 1 h in 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer. Samples were washed in three changes of 0.1 M sodium cacodylate buffer before dehydration through a graded acetone series. Samples were embedded in Agar 100 epoxy (Agar Scientific, Agar 100 pre-mix kit medium) and polymerised overnight at 60ºC in an oven. Semi-thin (1-2 μm) sections were stained with Toluidine Blue for viewing with a light microscope to identify suitable target areas. Ultrathin sections (70-90 nm) of these areas were mounted on uncoated copper grids and stained with 2 % aqueous uranyl acetate and Reynolds' lead citrate (Reynolds, 1963). Grids were examined using a JEOL JEM 1400 transmission electron microscope and digital images captured using an AMT XR80 camera and AMTv602 software.

For molecular analyses, gill, epidermis and hepatopancreas samples were removed and placed into tubes containing 100% ethanol. For PCR, tissue was weighed and diluted 1:10 in G2 buffer (Qiagen, West Sussex, UK) and 10 μl Proteinase K (Qiagen). The sample was homogenised using a Fast prep FP120 machine (MP Biomedicals) at the highest setting for 2 minutes. This homogenate was incubated at 56^{o}C for at least 3 hours to allow lysis to occur. The sample was then centrifuged at 9000rpm for 2 minutes and 50 μl of the supernatant was added to 150 μl of G2 buffer. Total DNA from this 200 μl sample was then extracted using the EZ1 DNA tissue kit (Qiagen, cat no 953034) and the EZ1 advanced Biorobot (Qiagen) following manufacturer's instructions.

For WSSV surveillance, separate first and second round (Nested) PCR reactions were performed on each DNA extract using the OIE recommended WSSV primer sets (Lo *et al.* 1996a; 1996b) (see Table 2). For the first round of amplification (primer pair 146F1/146R1) each 50 μl PCR reaction contained 1 X Green Go Taq Flexi buffer (Promega), 2.5mM $MgCl_2$, 0.25mM of each dNTP, 100 pmol each of the forward and reverse primer, 0.25 units Go Taq Flexi (Promega), and 2.5μl extracted nucleic acid (20-50 ng total). Amplifications were performed using the following WSSV thermal cycler program on a Peltier PTC-225 thermal cycler: 94ºC x 2 minutes followed by 29 cycles of 94ºC x 30 seconds, 62ºC x 30 seconds and 72ºC x 30 seconds, followed by

72ºC x 2 minutes and held at 4ºC. Reaction conditions and reagent concentrations were the same for the second round of amplification using the 146F2/146R2 primer pair; however 0.5 μl of the first round of amplification was used as a template in place of extracted nucleic acid. Amplification products were resolved on 2% agarose gels stained with ethidium bromide (0.625mg/ml) and visualised using a Gel Doc 2000 (Bio Rad) imaging system.

Table 2. WSSV PCR primers (Lo *et al*., 1996)

Primer name	Sequence
WSSV 146 F1	ACTACTAACTTCAGCCTATCTAG
WSSV 146 R1	TAATGCGGGTGTAATGTTCTTACGA
WSSV 146 F2	GTAACTGCCCCTTCCATCTCCA
WSSV 146 R2	TACGGCAGCTGCTGCACCTTGT

WSSV surveillance was conducted by the Fish Health Inspectorate (FHI) at Cefas.

8.2.2 Pathology and ultrastructure of a novel Herpes-like virus (HLV) pathogen Crabs sampled from the field and from group 2 in the stress exposure study (chapter 4) were used in this study. As standard, gill, hepatopancreas, heart, gonad, connective tissues and muscle were dissected and placed into histological cassettes and fixed immediately in Davidson's seawater fixative. For electron microscopy, gill and hepatopancreas tissues were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The remaining tissues and carcasses were stored at -80°C.

Herpes-like virus (HLV) infection was confirmed via histology and the infected carcasses were used to prepare inoculums for passage study. Infected carcasses were macerated in isolated conditions using a sterile razor blade prior to homogenisation in 2% sterile saline, see appendix, (4ml of saline per gram of minced tissue) using a blender until tissues were liquefied. The homogenate was centrifuged at 5,000 x g for 20 minutes at 4°C to pellet solid debris prior to the supernatant being diluted 1:20 with sterile saline and filtered (0.2μm Minisart syringe filter, Sartorius Stedim Biotech GmbH, Germany) to form the inoculum for the passage study.

Seventy shore crabs, carapace widths between 17 and 36 mm, were collected from the shoreline at Newtons Cove, Weymouth, UK. Crabs were transferred into custom-made compartments within large trough tanks, with individuals separated by tank divisions to prevent conflict but sharing the same water supply. Water temperature in all tanks was held constant at 20°C. Crabs were injected at the base of the first walking leg with a single dose of the diluted HLV homogenate (see above) at a rate of 10µl g^{-1} wet body weight, on Day 0. Tanks were observed regularly throughout daylight hours. Twenty crabs were sampled at day 7, day 14 and all survivors were sacrificed at the end of the challenge, day 21. Dead and terminally morbid samples were removed from each tank and dissected. Crabs were chilled on ice and dissected, with tissues being taken for histology and electron microscopy, as detailed above. The remaining tissues and carcasses were stored at -80°C.

Once infection had been confirmed via histology and TEM a heavily infected individual was selected for 454 sequencing. The carcass was macerated prior to homogenisation until tissues were liquefied. This solution was then extracted as detailed above and the extract was submitted to the University of Liverpool for sequencing.

8.3 Results

8.3.1 Pathogen profile in *Carcinus maenas*

All crabs sampled were shown to be WSSV free via PCR analysis. A wide variety of pathogens were present during this survey and these appeared to vary according to the sampling method. Figure 1 compares the different collection methods used and the prevalence of pathogens seen between each collection method. Offshore collection clearly showed fewer pathogens when compared to shoreline collection. Gill fouling organisms such as stalked ciliates (Figure 2A) and *Trichodina* (Figure 2B) were present at multiple sampling sites and frequently present within the samples. *Microphallus primus* (Saville and Irwin, 2005) is a digenean parasite that has a complex life cycle involving multiple hosts, metacercarial stages of the parasite were present distributed throughout shore crab tissues (Figure 2C) sampled from multiple sites. A few of these sites also displayed what appeared to be a hyperparasite, a microsporidian infection of the digenean parasites (Figure 2D). The parasitic

dinoflagellate *Hematodinium perezi* (Figure 2E), which is the type species of this parasite within this host (Chatton and Poisson, 1931), was identified within crabs from different sites. The bacterial infection Milky disease (Figure 2F) (Eddy *et al*., 2007), *Haplosporidium littoralis* (Figure 3A) (Stentiford *et al*., 2004a; Stentiford *et al*., 2013b) parasitic barnacle *Sacculina carcini* (Figure 3B) (Boschma, 1955), *Carcinus maenas* bacilliform virus (CmBV) (Figure 3C) (Stentiford and Feist, 2005) and a gregarine infection *Nematopsis* sp. (Figure 3D) were present within shore crab tissues. A microsporidian infection *Ameson pulvis* was diagnosed infecting the heart and body muscle of shore crabs (Figure 4A and 4B) (Stentiford *et al*., 2013a). Juvenile nematodes (Figure 4C) were witnessed in a few crabs at a few different sites and an Acanthocephalan parasite, *Profilicollis botulus* (Figure 4D) (Liat and Pike, 1980; Van Cleave, 1916) was present infecting a single crab in area 1. Apparent prevalence and pathogen profile also differed considerably between sites (Figure 5). The numbers of "healthy" crabs (i.e. crabs with no obvious signs of pathology or pathogens within histological sections) varied considerably between sites, 50% of crabs appearing healthy (pink segments, Figure 5) in Rye Bay (Area 11) compared with no healthy crabs being sampled in Blakeney Harbour, Norfolk (Area 6). Ciliates (mauve segments, Figure 5) and *Microphallus primas* (green segments, Figure 5) were present at all sites sampled. *Hematodinium perezi* (purple segments, Figure 5) was present at 7 sites along the south and east coasts, CmBV (red segments,Figure 5) was present at 2 sites along the east coast. The muscle microsporidian *Ameson pulvis* (dark orange segments, Figure 5) was present at 3 sites along the south and east coasts and Milky disease (orange segments, Figure 5) was also present at 2 sites in the south east. *Nematopsis* sp. (pale mauve, Figure 5) was only found at 1 site. It should be noted co-infections with multiple pathogens present within tissues of some crabs were evident at all sites sampled.

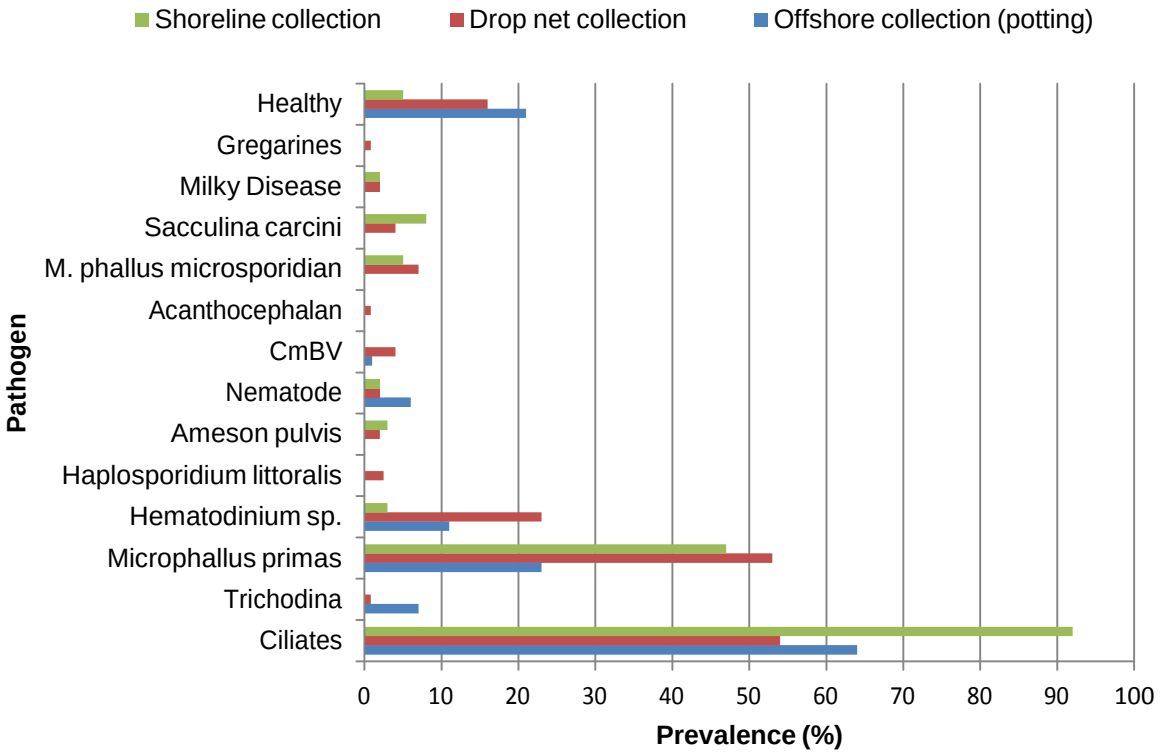


Figure 1. Comparison of the different collection methods used and the prevalence of pathogens seen between each collection method. Data for each collection method were pooled across all sampling areas.

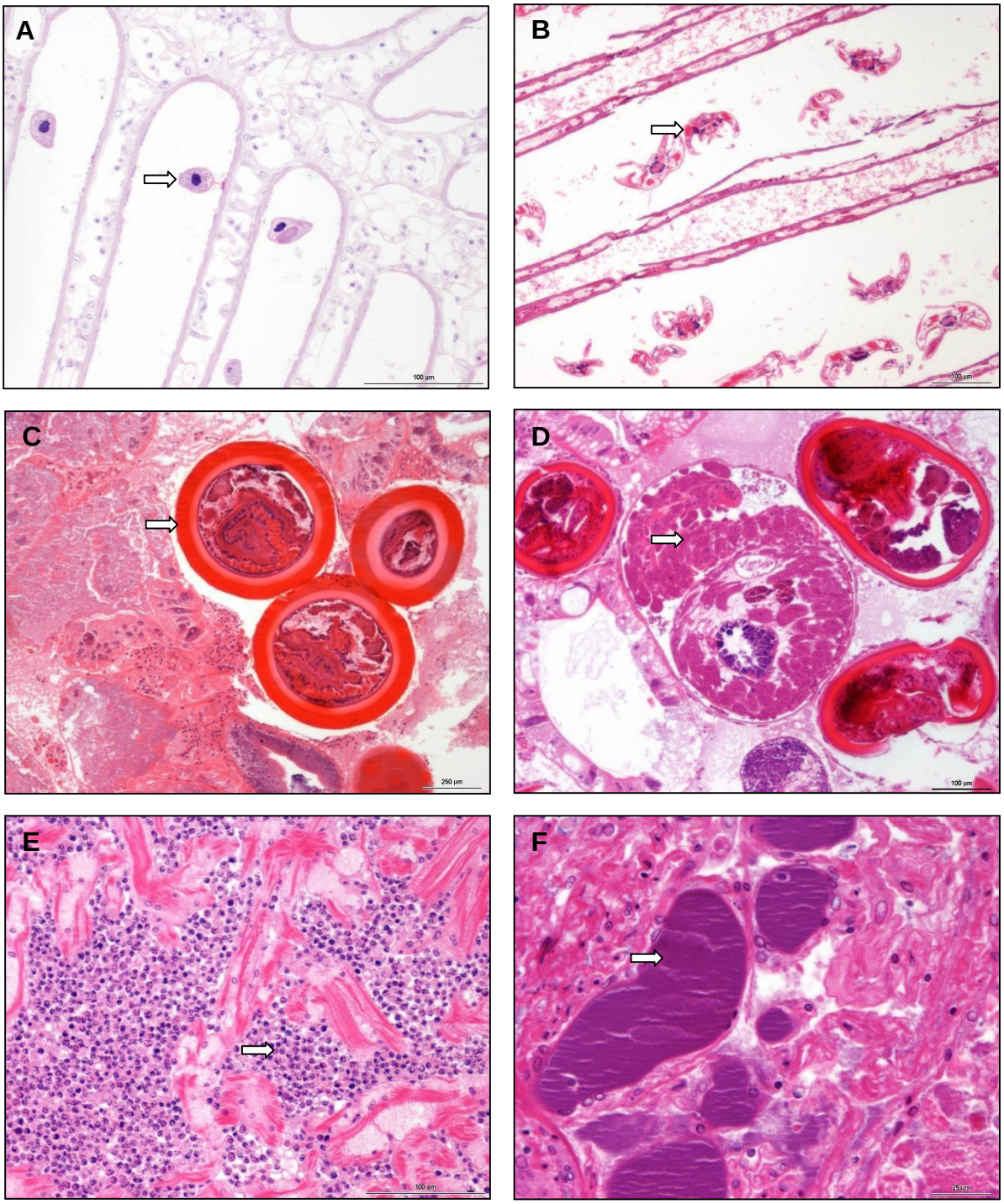


Figure 2. Pathogen descriptions. A. Most shore crabs sampled had a low level ciliate infection, images shows stalked ciliates attached to the gill (arrow). Scale bar = 100µm. B. Disc shaped trichodinids (arrow) present at gill surface. Scale bar = 100µm. C. Parasitic trematode *Microphallus primas* (arrow) within shore crab tissues, common sites of infection are the hepatopancreas and the gill. Scale bar = 250µm. D. *Microphallus primas* observed in the hepatopancreas of some shore crabs appeared to be infected with a microsporidian parasite. Infected digenea showed a loss of cuticle and internal structure appeared to be replaced with packets of small spore like structures (arrow), suspected to be microsporidian spores. Scale bar = 100µm. E. Systemic *Hematodinium perezi* infection, parasite (arrow) can be seen here within the heart tissue. Scale bar = 100µm. F. Milky disease thought to be cause by a gram negative Rickettsia-like organism. Hepatopancreas tubules show no obvious morphological changes, large numbers of swollen cells were seen in the haemal sinuses containing fibrous-like material (arrow). Scale bar = 25µm. All images H&E Stain.

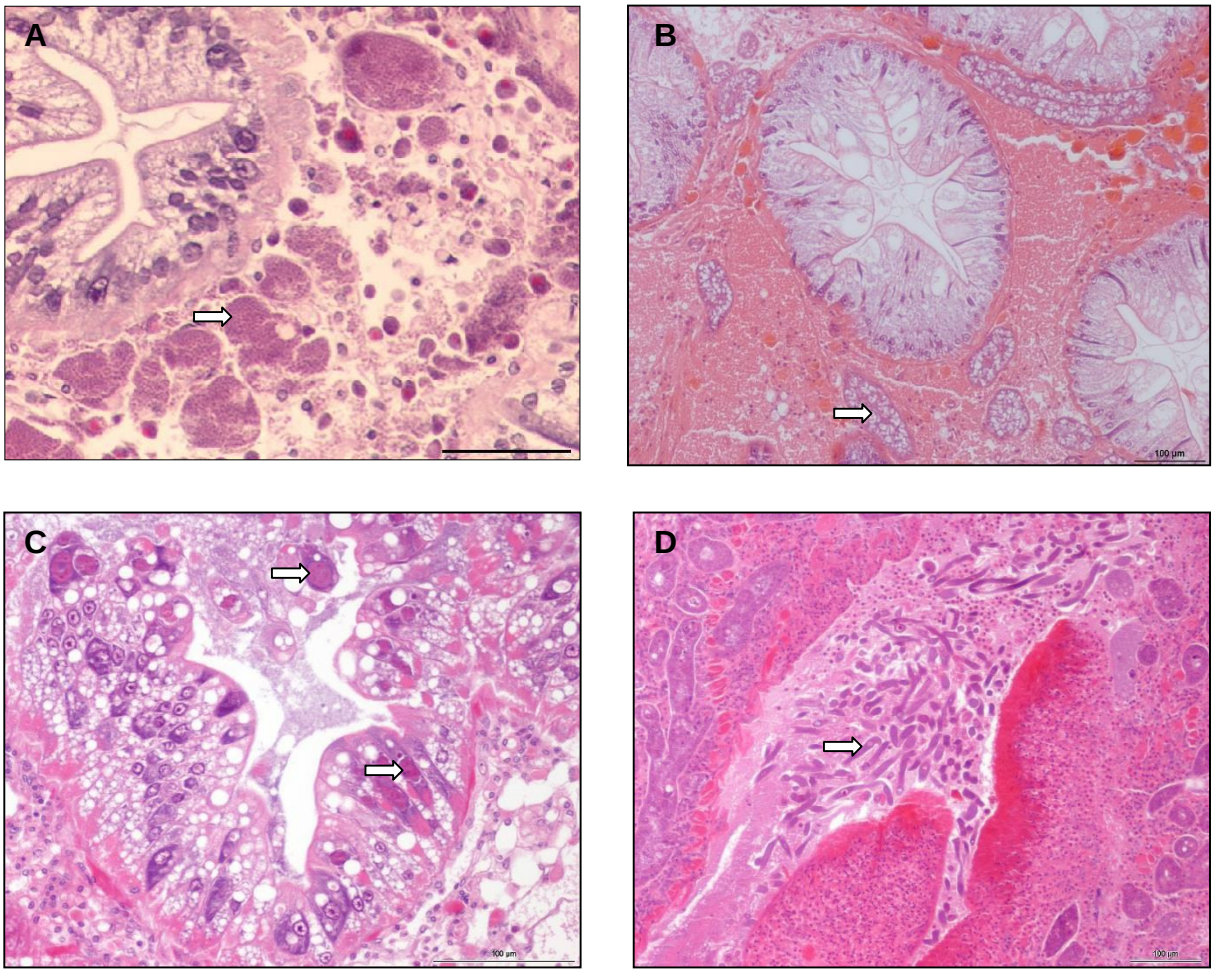


Figure 3. Pathogen descriptions. A. *Haplosporidium littoralis* infection, intracellular stages of the parasite are found within reserve inclusion cells (arrow), connective tissue and muscle cells, while free forms can be found within haemal spaces. Scale bar = 100µm. B. *Sacculina carcini* infection, rootlets of interna (arrow) develop a systematic network within crab often displacing internal organs. Scale bar = 100µm. C. Intranuclear bacilliform virus infection of the hepatopancreas tubules. Infected tubular epithelium cells become hypertrophied with marginalised chromatin and contain an eosinophillic inclusion body (arrow). Scale bar = 100µm. D. Gregarine infection, *Nematopsis* sp., within the midgut of the shore crab (arrow). Scale bar = 100µm. All images H&E Stain.

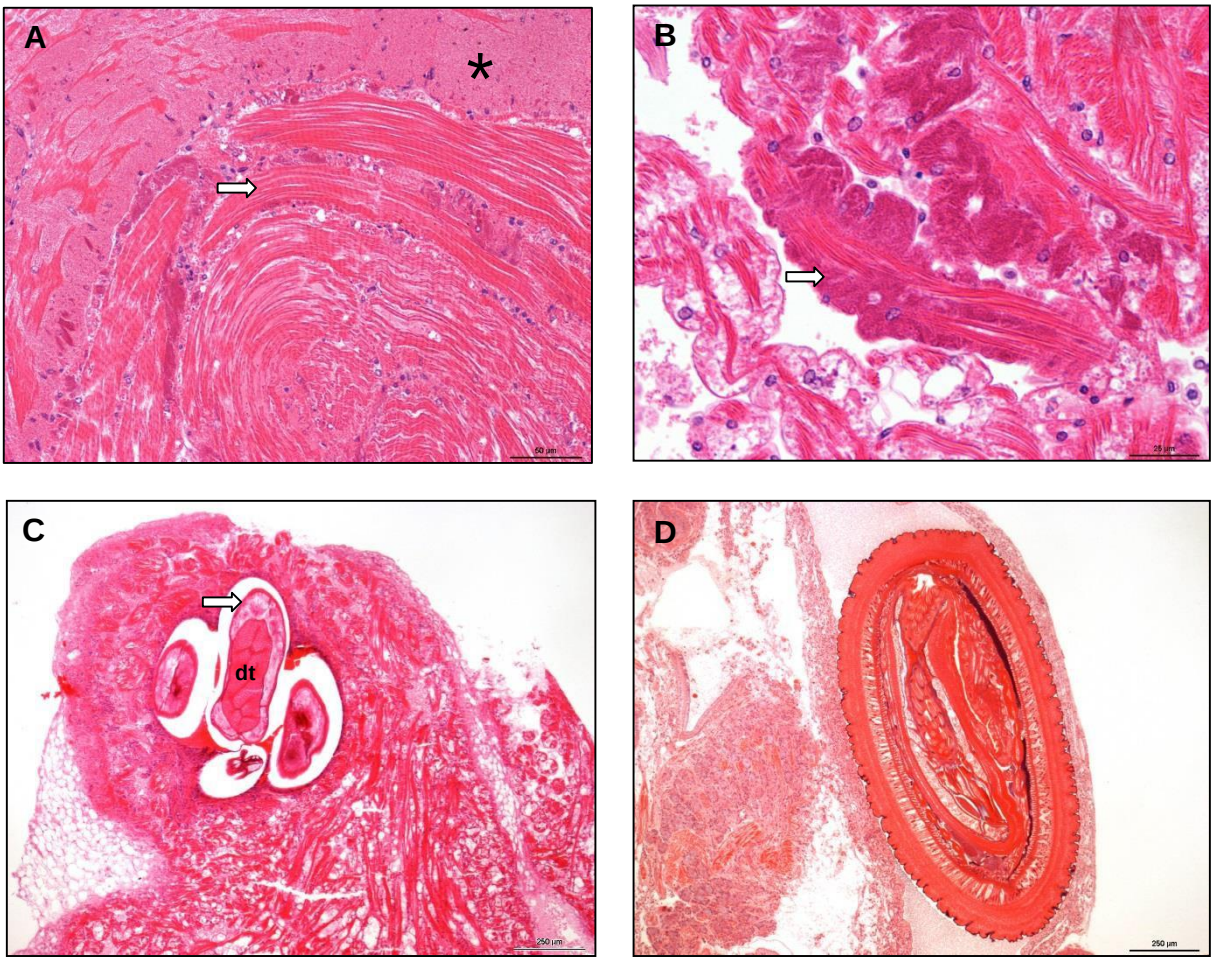


Figure 4. Pathogen descriptions. A. *Ameson pulvis* infection of skeletal muscle, muscle fibres (arrow) are gradually replaced with parasite (*) as the infection progresses. Scale bar = 50µm. B. *Ameson pulvis* infection of heart muscle of shore crab. Early infection shows the microsporidian replicating within the sarcolemma of muscle fibres (arrow). Scale bar = 25µm. C. Juvenile nematodes shown infecting the heart tissue, surrounded by a cuticle (arrow) and containing a clear digestive tract (dt). Scale bar = 250µm. D. Acanthocephalan parasite, *Profilicollis botulus.* Scale bar = 250µm. All images H&E Stain.

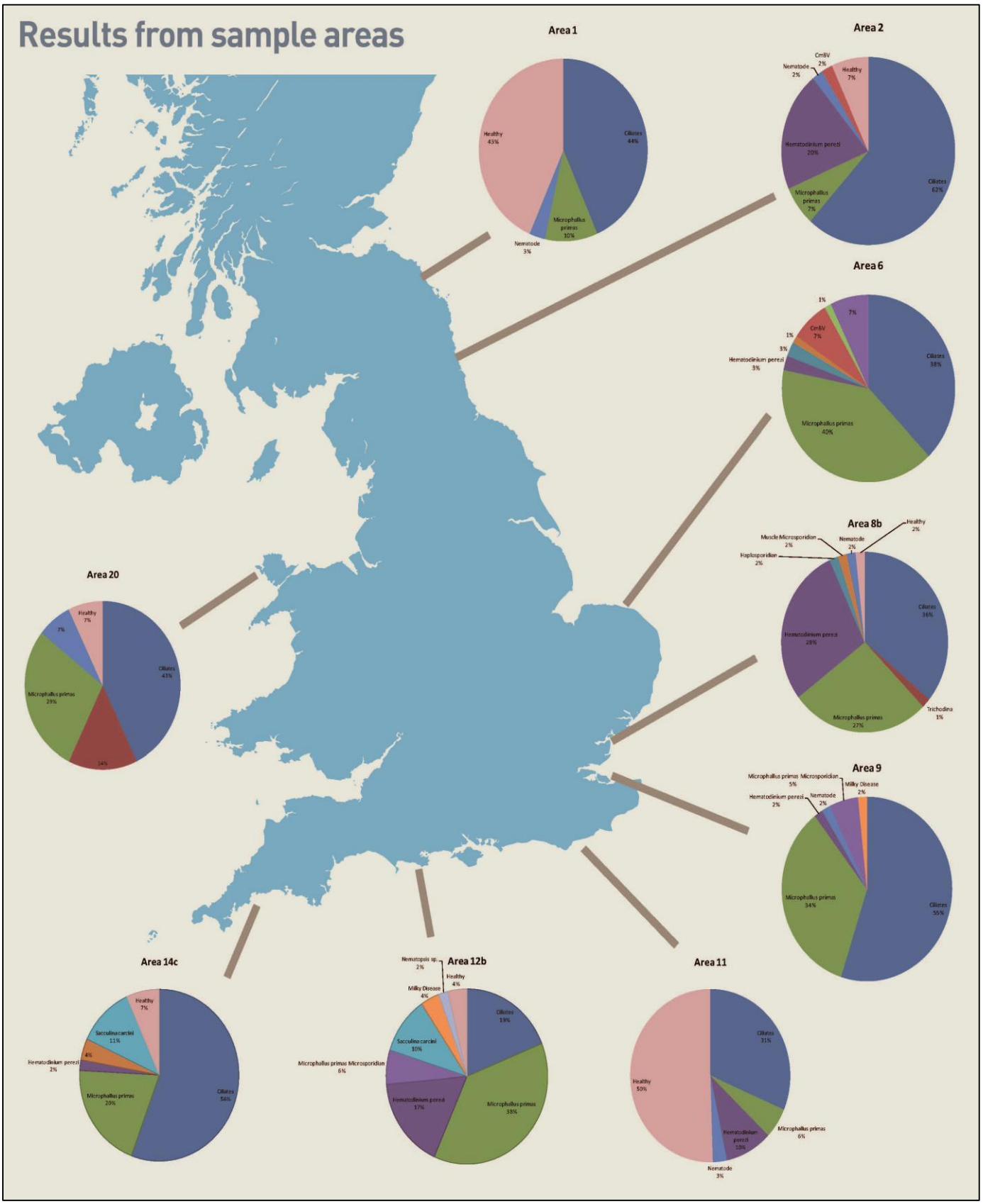


Figure 5. Map showing sampling locations around the UK and pathogens present at each location. Pink - Healthy, Mauve - Ciliates, Green - *Microphallus primas*, Light purple - *Microphallus primas* microsporidian, Purple - *Hematodinium perezi*, Blue - *Sacculina carcini*, Orange - Milky disease, Turquoise - *Haplosporidium littoralis*, Red - *Carcinus maenas* bacilliform virus (CmBV), Dark red - Trichodina, Pale mauve - *Nematopsis* sp., Dark orange – *Ameson pulvis*.

8.3.3 Herpes-like Virus (HLV) in *Carcinus maenas*

Tissues sampled from infected shore crabs displayed enlarged nuclei within haemocytes, haematopoietic tissues and connective tissues which contained a distinct eosinophillic inclusion body (Figure 6). Some of these infected haemocyte cells contained two enlarged nuclei, both of which contained eosinophillic inclusions (Figure 6). Infected connective tissues and haemocytes appeared to be systemic throughout the crabs.

TEM revealed that infected nuclei contained hexagonal particles within a granular viroplasm (Figure 7). The hexagonal particles appeared to contain an electron dense brick shaped core which was surrounded by an electron dense sphere within a capsid (inner envelope), this capsid then being encapsulated within an envelope (outer envelope). Ultrastructurally this virus appears very similar to Bi-Facies virus that was described by Johnson (1978; 1988b) infecting the blue crab, *C. sapidus*.

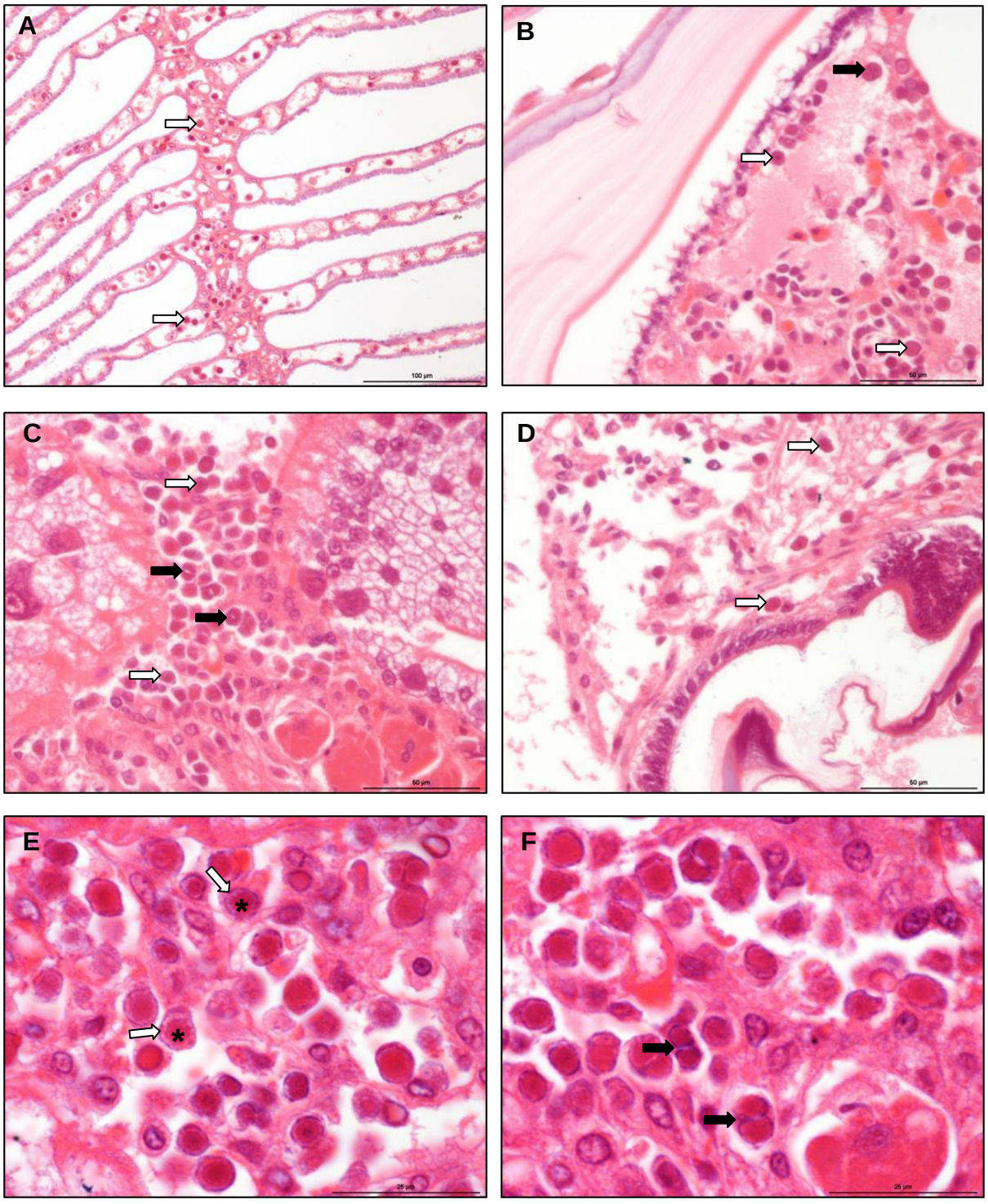


Figure 6. Herpes like virus infection of *Carcinus maenas*. A. HLV infected nuclei (arrow) within the gill filaments. Scale bar = 100µm. B. HLV infected connective tissues (white arrows) present underneath the cuticular epithelium, some cells contained two nuclei (black arrow). Scale bar = 50µm. C. HLV infected haemocytes (white arrows) within the haemal spaces of the hepatopancreas, some cells contained two nuclei (black arrows). Scale bar = 50µm. D. HLV infected connective tissues (arrows) surrounding the gut. Scale bar = 50µm. E. Higher power magnification of HLV infected haemocytes, note the distinct eosinophillic inclusion body (*) within the enlarged nuclei with marginalised chromatin (arrows). Scale bar = 25µm. F. HLV infected haemocytes; some cells appeared to contain two nuclei (black arrows), both of which were enlarged with eosinophillic inclusions. Scale bar = 25µm. All images H&E Stain.

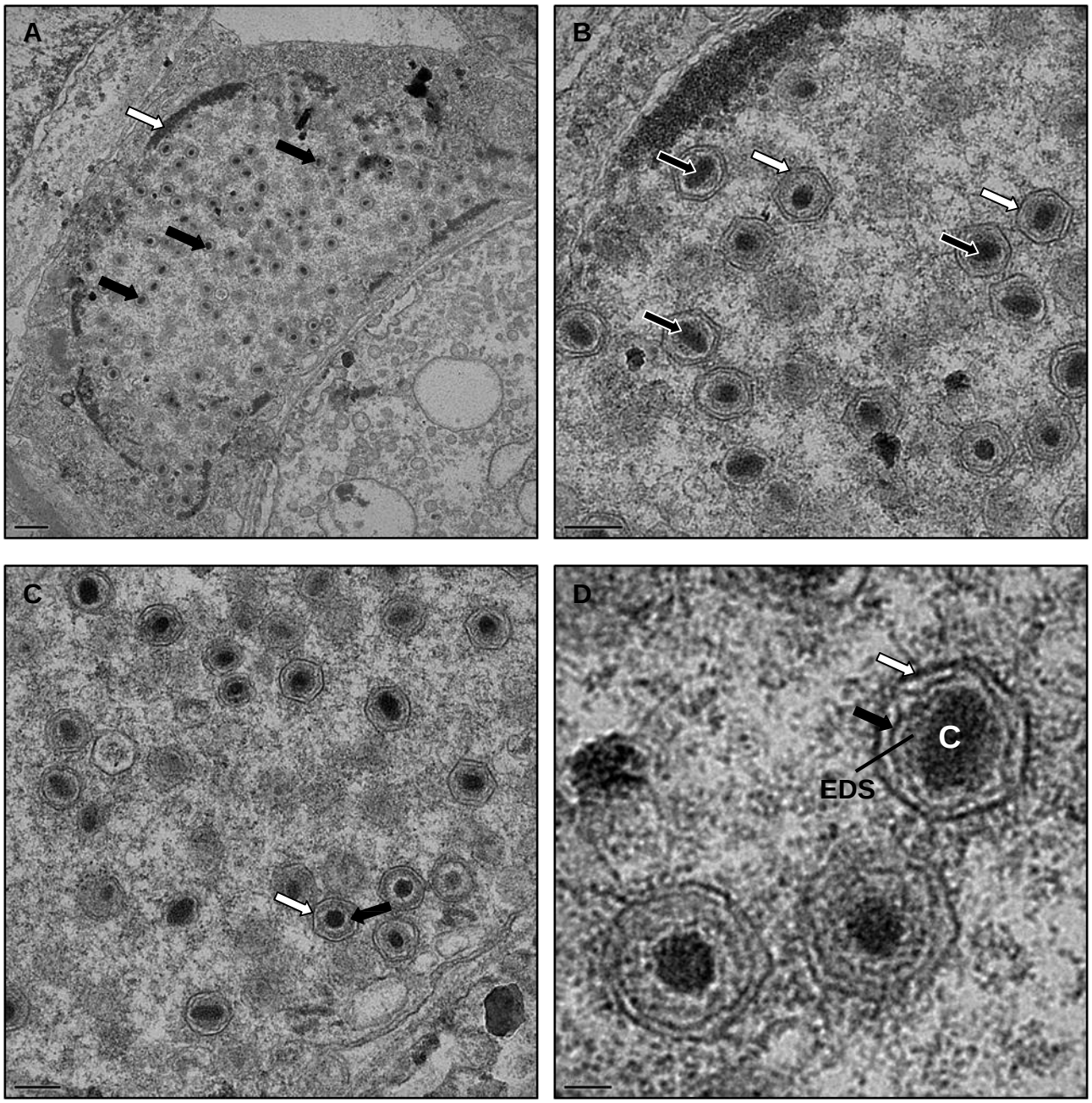


Figure 7. TEM of Herpes like virus infection of *Carcinus maenas*. A. Enlarged nuclei containing marginalised chromatin (white arrow) and HLV particles (black arrows) at various stages of development. Scale bar = 250µm. B. Viral particles displaying brick shaped core (black arrows) surrounded by an electron dense sphere and encapsulated within a hexagonal envelope (white arrows). Scale bar = 100µm. C. Cross-sectioned viral particles, clearly displays the electron dense sphere within the capsid (black arrow) surrounded by an envelope (white arrow). Scale bar = 100µm. D. HLV particles consisting of an electron dense core (C) surrounded by an electron dense sphere (EDS) which is enclosed in a capsid (black arrow) and surrounded by an envelope (white arrow). Scale bar = 50µm.

Crabs injected with HLV homogenate were sampled at day 7, day 14 and day 21. No mortalities were observed outside of these sampling points. Figure 8 displays the pathogen profile present in the crabs at each sampling point for each of the pathogens identified during histological screening of samples. A high prevalence of *Microphallus primas* and a low prevalence of *Ameson pulvis* were evident in samples taken at each time point. *Hematodinium perezi* infected samples were only seen at day 7. 25% of crabs sampled at day 7 displayed signs of infection with HLV; this reduced slightly by day 14 to 20% of crabs sampled showing signs of infection. At the end of the study, day 21, 30% of crabs sampled displayed pathology suggesting infection with HLV. Interestingly milky disease was seen in crabs sampled on day 7 and day 21 only. It should be noted co-infections with multiple pathogens present within tissues of some crabs were evident at each time point.

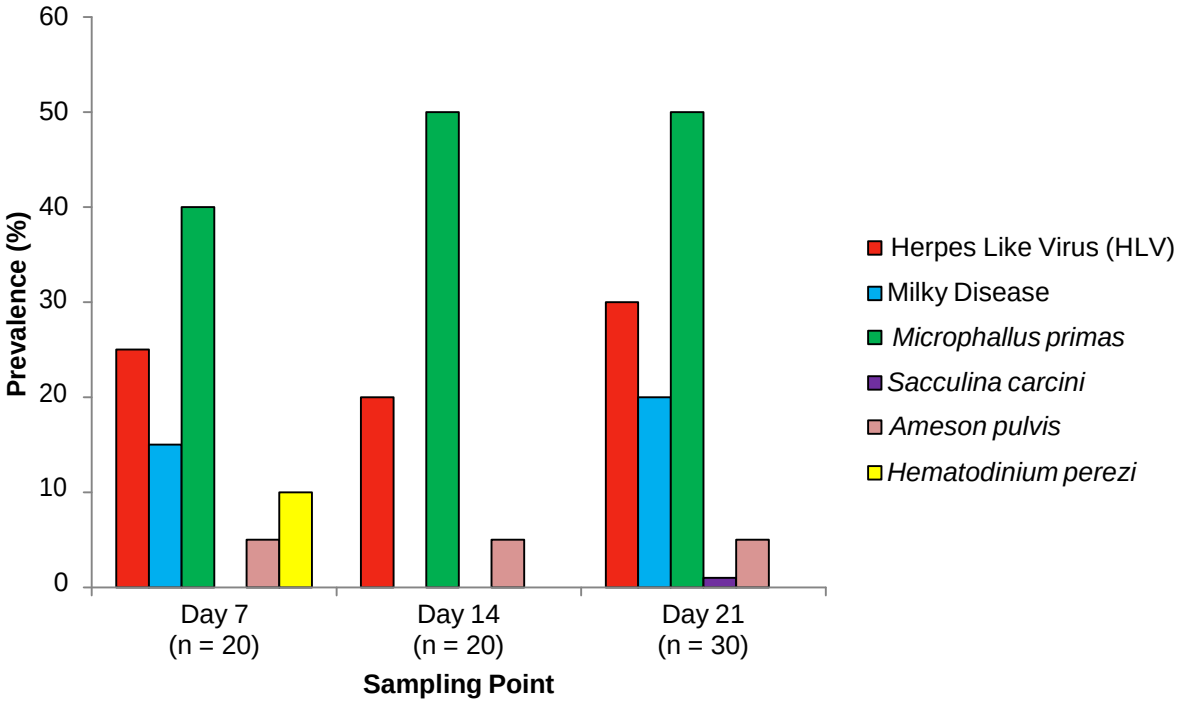


Figure 8. Pathogen prevalence present within shore crab tissues at each sampling point for each of the pathogens identified during histological screen (day 7 (n = 20 crabs), day 14 (n = 20 crabs) and day 21 (n = 30 crabs)).

8.3.5 454 Sequence read processing

In total, 116,000 raw 454 reads were obtained. After clipping of the primer sequences and removal of sequences less than 50bp in length, 87,370 reads remained. The

length of the pre-processed reads ranged from 50 to 752 bp, with a mean of 313.61 ± 133.04 bp. Assembly of these reads resulted in the construction of 2,790 contigs, ranging from 100 - 37,257 bp in length (mean sequence length of 702.93 ± 840.61 bp) and an N50 (the contig length where half of the assembly is represented by contigs of this size or longer) of 847 bp.

The majority of the annotated contigs represented bacterial sequences, in particular *Vibrio* species (see Figures 9 and 10).

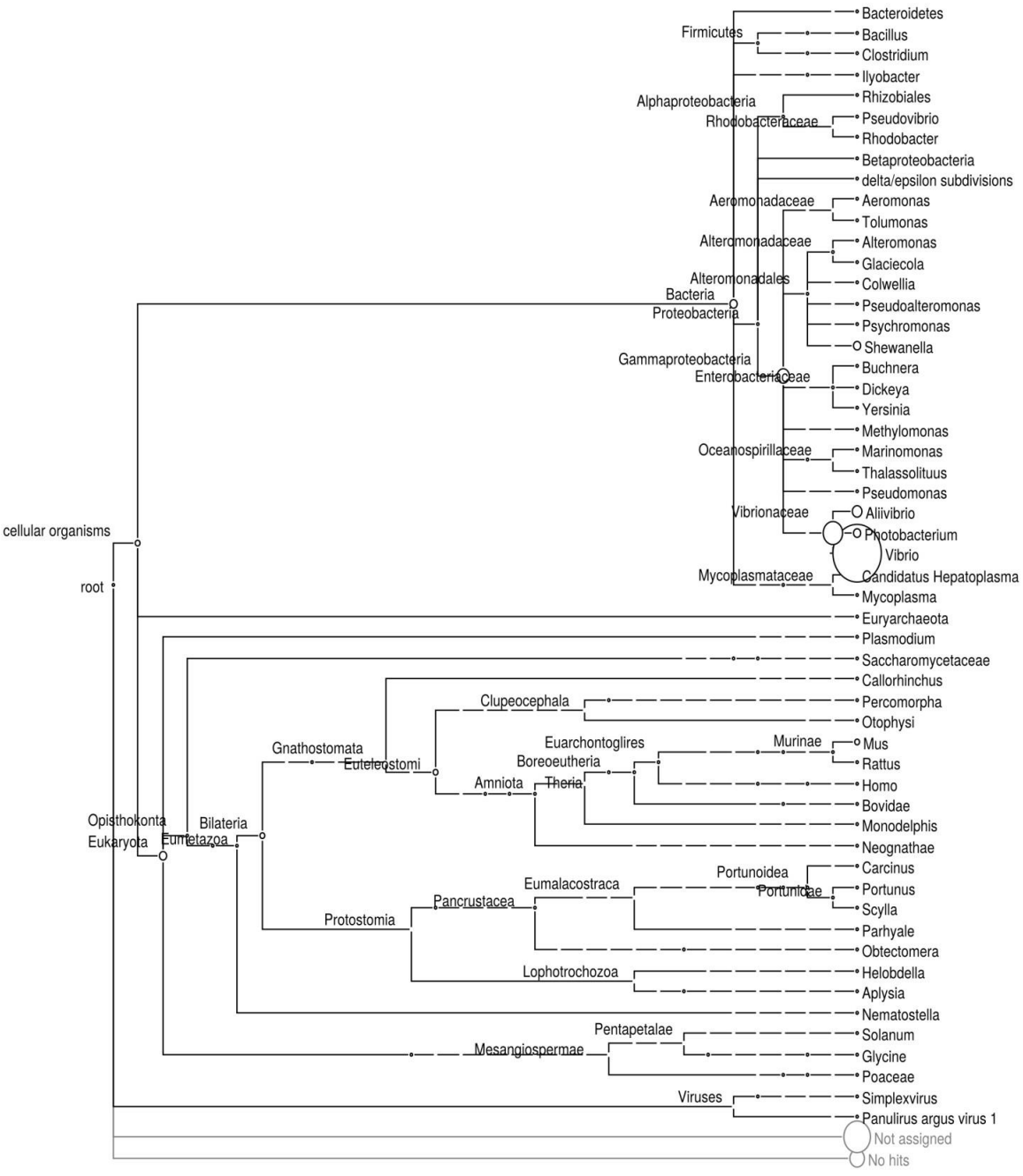


Figure 9. Taxonomic representation of the assembled contigs based on Blast results. Circle size is relative to the number of contigs that represent each taxon.

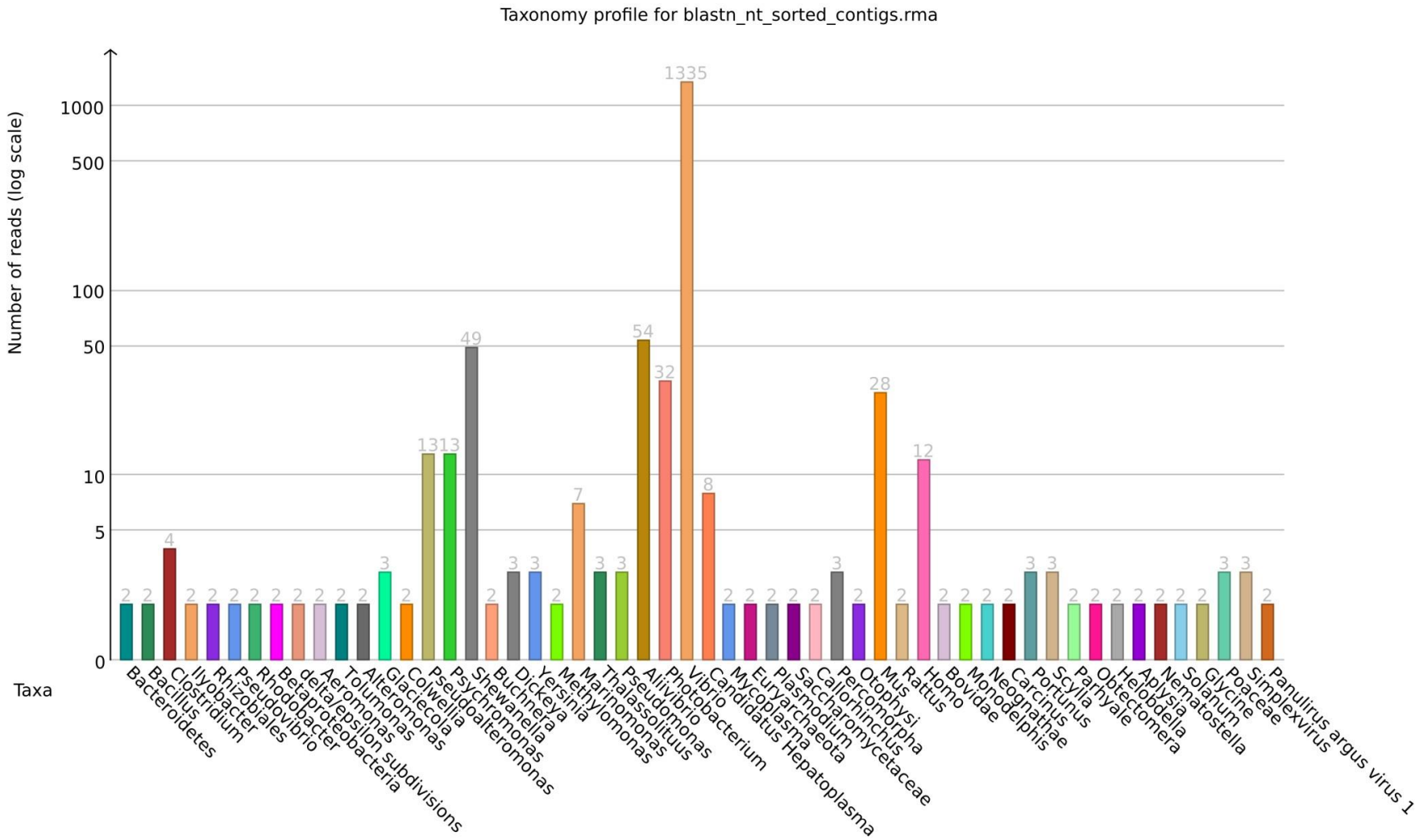
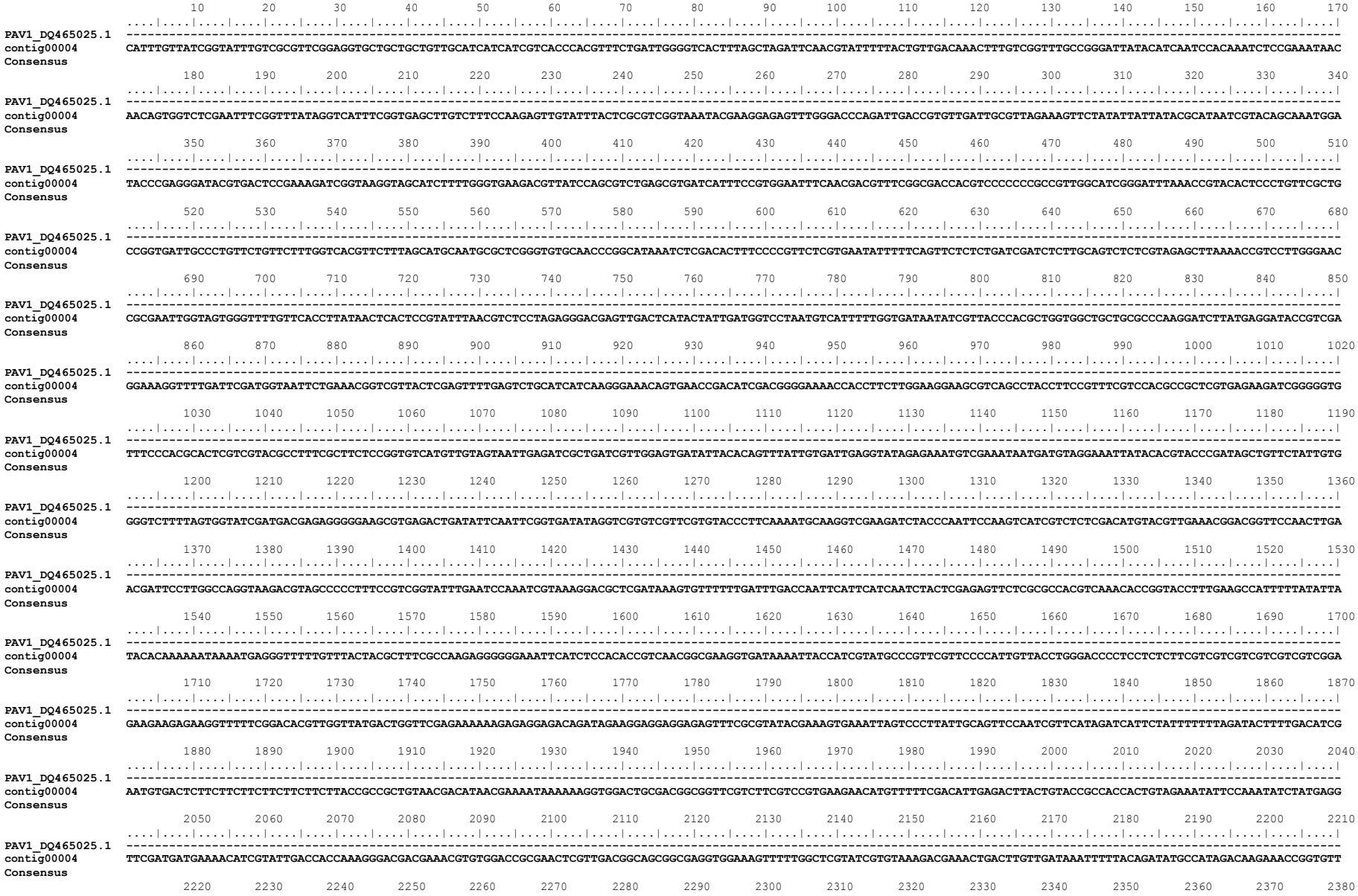


Figure 10. Taxonomy profile for the assembled contigs. Results are ranked on “Genus” and the number of contigs representing each taxon are displayed above the bars. The majority of the contigs represented bacterial sequences, in particular *Vibrio* species, 1335 reads.

Two contigs showed high homology to *Panulirus argus* virus 1 sequences in the NCBI Nucleotide Database. Contig00004, a sequence of 4,767 bp in length and represented by 239 reads, contained a region that was highly similar (78.53%) to the 177 bp *Panulirus argus* virus 1 DNA-directed DNA polymerase sequence (Genbank Accession Number DQ465025.1; Figure 11). The second contig, Contig00343 (1,152 bp and represented by 39 reads) showed high homology (72.15%) to a 892 bp *Panulirus argus* virus 1 genomic sequence (Genbank Accession Number EF206313.1; Figure 12).


10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170
PAV1_DQ465025.1 ----------
contig00004 CATTTGTTATCGGTATTTGTCGCGTTCGGAGGTGCTGCTGCTGTTGCATCATCATCGTCACCCACGTTTCTGATTGGGGTCACTTTAGCTAGATTCAACGTATTTTTACTGTTGACAAACTTTGTCGGTTTGCCGGGATTATACATCAATCCACAAATCTCCGAAATAAC
Consensus
180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340
PAV1_DQ465025.1 ----------
contig00004 AACAGTGGTCTCGAATTTCGGTTTATAGGTCATTTCGGTGAGCTTGTCTTTCCAAGAGTTGTATTTACTCGCGTCGGTAAATACGAAGGAGAGTTTGGGACCCAGATTGACCGTGTTGATTGCGTTAGAAAGTTCTATATTATTATACGCATAATCGTACAGCAAATGGA
Consensus
350 360 370 380 390 400 410 420 430 440 450 460 470 480 490 500 510
PAV1_DQ465025.1 ----------
contig00004 TACCCGAGGGATACGTGACTCCGAAAGATCGGTAAGGTAGCATCTTTTGGGTGAAGACGTTATCCAGCGTCTGAGCGTGATCATTTCCGTGGAATTTCAACGACGTTTCGGCGACCACGTCCCCCCCGCCGTTGGCATCGGGATTTAAACCGTACACTCCCTGTTCGCTG
Consensus
520 530 540 550 560 570 580 590 600 610 620 630 640 650 660 670 680
PAV1_DQ465025.1 ----------
contig00004 CCGGTGATTGCCCTGTTCTGTTCTTTGGTCACGTTCTTTAGCATGCAATGCGCTCGGGTGTGCAACCCGGCATAAATCTCGACACTTTCCCCGTTCTCGTGAATATTTTTCAGTTCTCTCTGATCGATCTCTTGCAGTCTCTCGTAGAGCTTAAAACCGTCCTTGGGAAC
Consensus
690 700 710 720 730 740 750 760 770 780 790 800 810 820 830 840 850
PAV1_DQ465025.1 ----------
contig00004 CGCGAATTGGTAGTGGGTTTTGTTCACCTTATAACTCACTCCGTATTTAACGTCTCCTAGAGGGACGAGTTGACTCATACTATTGATGGTCCTAATGTCATTTTTGGTGATAATATCGTTACCCACGCTGGTGGCTGCTGCGCCCAAGGATCTTATGAGGATACCGTCGA
Consensus
860 870 880 890 900 910 920 930 940 950 960 970 980 990 1000 1010 1020
PAV1_DQ465025.1 ----------
contig00004 GGAAAGGTTTTGATTCGATGGTAATTCTGAAACGGTCGTTACTCGAGTTTTGAGTCTGCATCATCAAGGGAAACAGTGAACCGACATCGACGGGGAAAACCACCTTCTTGGAAGGAAGCGTCAGCCTACCTTCCGTTTCGTCCACGCCGCTCGTGAGAAGATCGGGGGTG
Consensus
1030 1040 1050 1060 1070 1080 1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190
PAV1_DQ465025.1 ----------
contig00004 TTTCCCACGCACTCGTCGTACGCCTTTCGCTTCTCCGGTGTCATGTTGTAGTAATTGAGATCGCTGATCGTTGGAGTGATATTACACAGTTTATTGTGATTGAGGTATAGAGAAATGTCGAAATAATGATGTAGGAAATTATACACGTACCCGATAGCTGTTCTATTGTG
Consensus
1200 1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320 1330 1340 1350 1360
PAV1_DQ465025.1 ----------
contig00004 GGGTCTTTTAGTGGTATCGATGACGAGAGGGGGAAGCGTGAGACTGATATTCAATTCGGTGATATAGGTCGTGTCGTTCGTGTACCCTTCAAAATGCAAGGTCGAAGATCTACCCAATTCCAAGTCATCGTCTCTCGACATGTACGTTGAAACGGACGGTTCCAACTTGA
Consensus
1370 1380 1390 1400 1410 1420 1430 1440 1450 1460 1470 1480 1490 1500 1510 1520 1530
PAV1_DQ465025.1 ----------
contig00004 ACGATTCCTTGGCCAGGTAAGACGTAGCCCCCTTTCCGTCGGTATTTGAATCCAAATCGTAAAGGACGCTCGATAAAGTGTTTTTTGATTTGACCAATTCATTCATCAATCTACTCGAGAGTTCTCGCGCCACGTCAAACACCGGTACCTTTGAAGCCATTTTTATATTA
Consensus
1540 1550 1560 1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680 1690 1700
PAV1_DQ465025.1 ----------
contig00004 TACACAAAAAATAAAATGAGGGTTTTTGTTTACTACGCTTTCGCCAAGAGGGGGGAAATTCATCTCCACACCGTCAACGGCGAAGGTGATAAAATTACCATCGTATGCCCGTTCGTTCCCCATTGTTACCTGGGACCCCTCCTCTCTTCGTCGTCGTCGTCGTCGTCGGA
Consensus
1710 1720 1730 1740 1750 1760 1770 1780 1790 1800 1810 1820 1830 1840 1850 1860 1870
PAV1_DQ465025.1 ----------
contig00004 GAAGAAGAGAAGGTTTTTCGGACACGTTGGTTATGACTGGTTCGAGAAAAAAAGAGAGGAGACAGATAGAAGGAGGAGGAGAGTTTCGCGTATACGAAAGTGAAATTAGTCCCTTATTGCAGTTCCAATCGTTCATAGATCATTCTATTTTTTTAGATACTTTTGACATCG
Consensus
1880 1890 1900 1910 1920 1930 1940 1950 1960 1970 1980 1990 2000 2010 2020 2030 2040
PAV1_DQ465025.1 ----------
contig00004 AATGTGACTCTTCTTCTTCTTCTTCTTACCGCCGCTGTAACGACATAACGAAAATAAAAAAGGTGGACTGCGACGGCGGTTCGTCTTCGTCCGTGAAGAACATGTTTTTCGACATTGAGACTTACTGTACCGCCACCACTGTAGAAATATTCCAAATATCTATGAGG
Consensus
2050 2060 2070 2080 2090 2100 2110 2120 2130 2140 2150 2160 2170 2180 2190 2200 2210
PAV1_DQ465025.1 ----------
contig00004 TTCGATGATGAAAACATCGTATTGACCACCAAAGGGACGACGAAACGTGTGGACCGCGAACTCGTTGACGGCAGCGGCGAGGTGGAAAGTTTTTGGCTCGTATCGTGTAAAGACGAAACTGACTTGTTGATAAATTTTTACAGATATGCCATAGACAAGAAACCGGTGTT
Consensus
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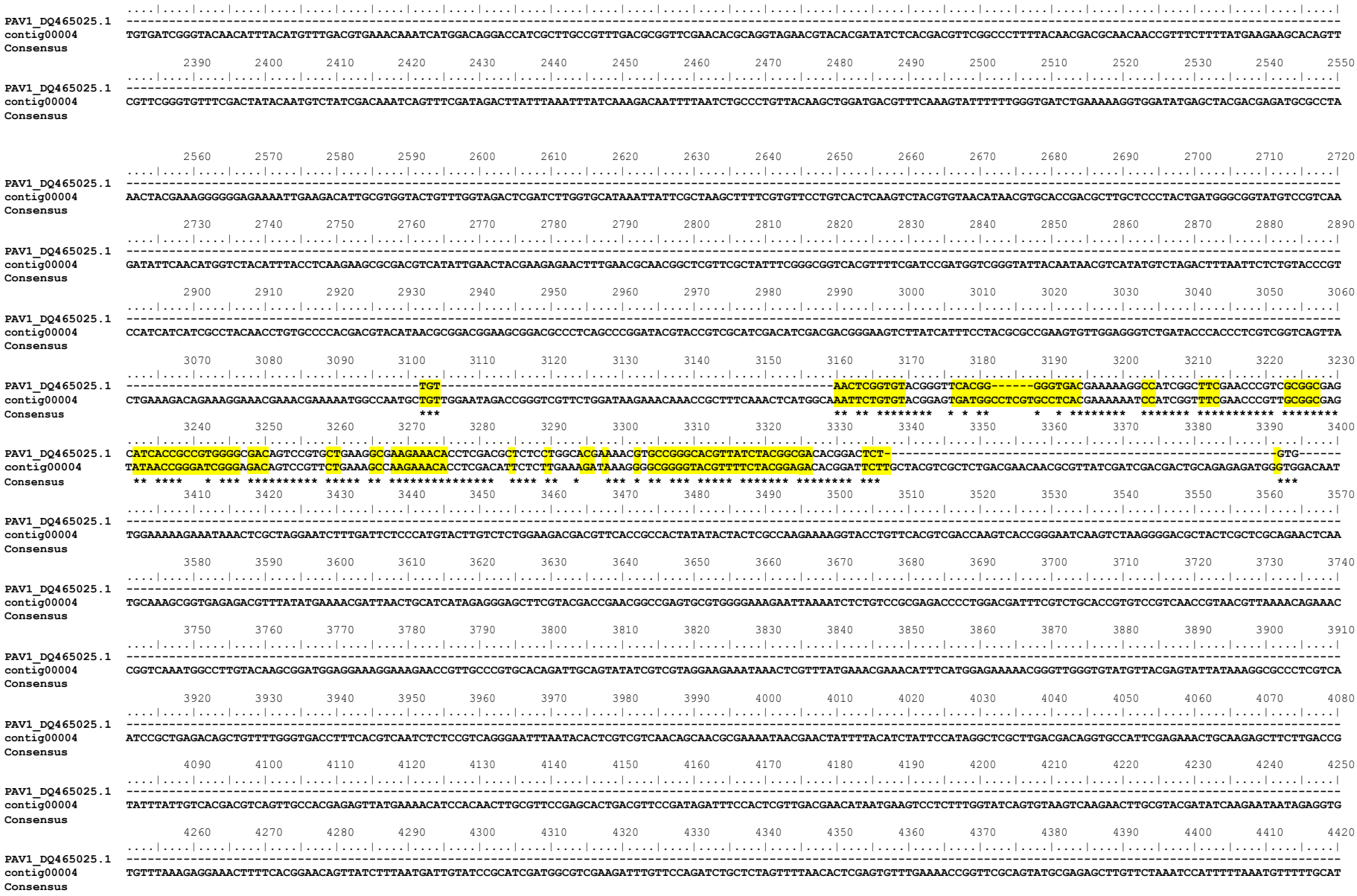
PAV1_DQ465025.1
contig00004
Consensus

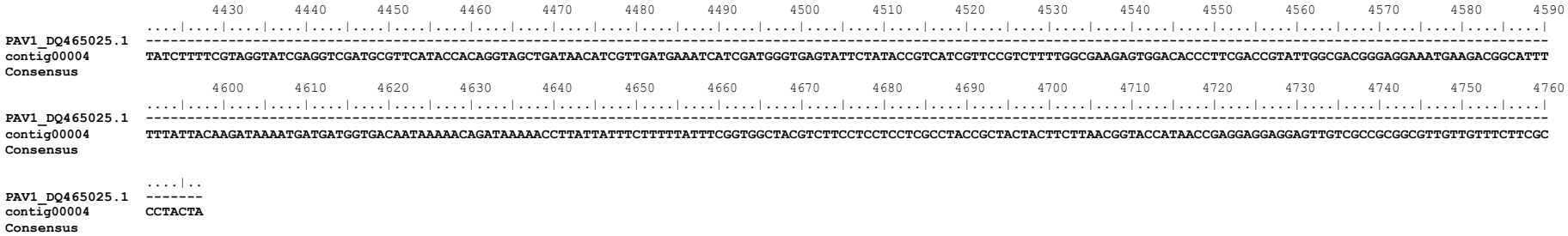

Figure 11. Multiple sequence alignment of contig00004 and the 177bp sequence of *Panulirus argus* virus 1 DNA-directed DNA polymerase (DQ465025.1) that was found in the NCBI Nucleotide Database. Conserved nucleotides are highlighted in yellow.

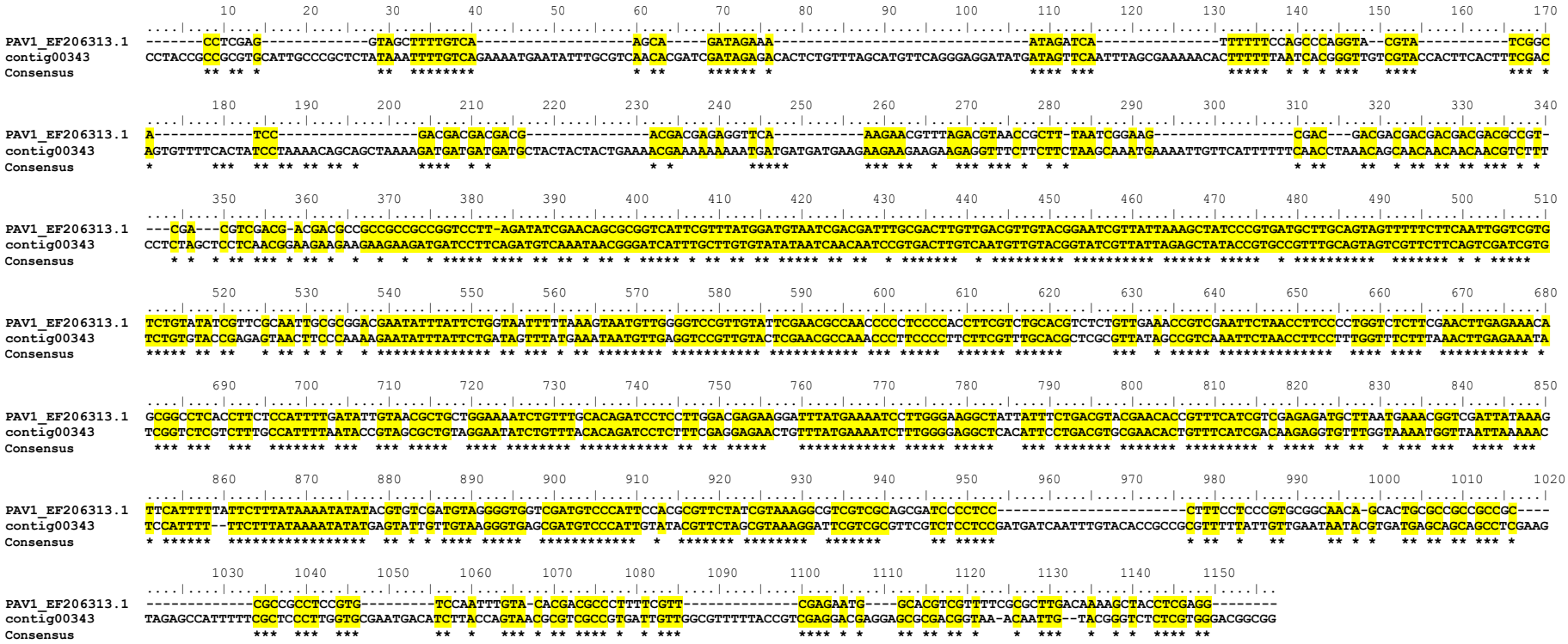

Figure 12. Multiple sequence alignment of contig00343 and the 892bp sequence of *Panulirus argus* virus 1 genomic sequence (DQ465025.1) that was found in the NCBI Nucleotide Database. Conserved nucleotides are highlighted in yellow.

8.4 Discussion

8.4.1 athogens of the shore crab in UK waters

A wide range of viral, bacterial, protistan and metazoan pathogens were identified in UK populations of *C. maenas* with prevelance and profile differing considerably between sites. The highest prevalence of healthy crabs (i.e. crabs with no obvious signs of pathology or pathogens within histological sections) was on the South coast with 50% of shore crabs sampled at Rye Bay showing no signs of pathology or pathogens in tissues sampled. In contrast shore crabs sampled from the east coast, Blakeney Harbour, Norfolk, showed no healthy crabs in the animals sampled. Ciliates and *Microphallus primas* were ubiquitously present at all sites sampled. *Hematodinium perezi* was identified at 7 sites along the south and east coasts, CmBV were present at 2 sites along the east coast. The muscle microsporidian *Ameson pulvis* was present at 3 sites along the south and east coasts and Milky disease was also present at 2 sites in the south east. *Nematopsis* sp. was only found at 1 site. It should be noted co-infections with multiple pathogens present within tissues of some crabs were evident at all sites sampled. These differences may be partly due to the variation in sampling protocols as shown in Figure 1, some crabs were caught using pots or drop nets which would mean that these crabs are actively feeding and fit enough to climb into nets. In contrast shore line collection is non discriminatory with all crabs that were found being sampled, this may explain why there appears to be a higher number of fitter crabs healthier crabs, i.e. less disease being present, in the offshore collected crabs when compared to shoreline collected crabs. Variation between the pathogens present varied according to sampling site as well as the sampling method. This may be due to the differences in habitats between the locations and the biodiversity of species present at each of the sites. *Microphallus primas* is a parasitic trematode with a complex life cycle involving more than one host, crabs acting as second intermediate host, snails usually being primary host with birds as definitive host. The full lifecycle of some of the pathogens such as *Haplosporidium littoralis* and *Hematodinium* sp. are unknown and it is possible that there are more than one host for these pathogens. If this is the case then the alternative hosts would also need to be present within that location to enable transmission and this may account for why pathogens are present in some areas and not others.

All crabs sampled were shown to be WSSV free via PCR analysis. The use of *C. maenas* in any surveillance programmes given their low susceptibility to the virus is an interesting choice. Initially it would seem most suitable to choose a highly susceptible species for any surveillance, crayfish (*Pacifastacus leniusculus* or *Austropotomobius pallipes*) for example. Crayfish mortality events from rivers and lakes are frequently reported implying that it is possible to witness when there is a problem within these environments. Crayfish are highly susceptible to the virus and will die within 10 days following initial exposure (Bateman *et al*., 2012a). As highlighted in chapter 5 crayfish are at most risk from infection with anglers being known to use commodity product as bait. It should be noted here though that it is frequently assumed that most crayfish mortalities are the result of crayfish plague infections, diagnostics are not applied in the correct manner to determine this. Should crayfish be used in surveillance programmes and disease events thoroughly investigated, any potential WSD outbreak in the freshwater environment should be easily identified. In contrast mortalities within the marine environment are extremely difficult, if not impossible, to monitor. Witnessing large mortality events in this environment is extremely unlikely, first signs of potential issues would most likely be a decline in the fisheries. The shore crab may actually prove to be a beneficial species in order to determine presence of WSSV in the marine environment. Unlike other species, due to the low susceptibility of the shore crab it would be unlikely that large numbers of mortalities would occur in this species and this species is likely to live with the virus for a longer period than, say, edible crabs (*Cancer pagurus*) or lobsters (*Homarus gammarus*). *C. maenas* can be found in most coastal locations around the UK so are easily attainable and they are not fished within the UK meaning that any surveillance programme would not interfere with any fishery and that they are a much cheaper product to use. However they have been shown to possess viral infections such as B virus which could be closely related to WSSV and may result in false positive results which could mean trade restrictions are put in place when they are not needed. Unfortunately B virus was not re-isolated during this study, which meant that a comparison of this virus to WSSV was not possible. It is still important that this virus is re-isolated and comparisons made to fully understand any potential similarities between these two viruses. The hunt for B virus will continue, should funding allow suggest that crabs are sampled from the type location of B virus and that *C.*

mediterraneus are also sampled in the hope of finding B2 virus, another virus that is potentially very similar to WSSV.

8.4.2 LV in Carcinus maenas

This study provides the first description of herpes like viral infection in *Carcinus maenas*. The virus was first seen during the stress study in chapter 4 and was initially assumed to be B virus infection. However TEM revealed the presence of hexagonal viral particles within the enlarged nuclei which possessed a distinct brick shaped core surrounded by an electron dense sphere and displayed an inner and outer envelope, extremely similar to that described infecting *C. sapidus* in America. The virus most closely resembled particles described in the Herpes family so was named HLV until full characterisation could be completed. Prevalence of HLV infection was based upon histological observation of prepared tissues; this meant that the crabs used in the passage study could not be determined to be free from the virus at the start of the study therefore it is possible that asymptomatic carriers of the virus may have been included in the experiment and subsequently expressed the disease. In order to determine this crabs should have been randomly selected to enter a treated (HLV injected) and an untreated (saline injected) group, it would then have been possible to show differences between the groups. This study did not use a control saline injected group due to limited tank space at the time of the exposure so it was not possible to determine whether crabs already had the virus before injection. However shoreline surveys indicated a 2% prevalence of the virus at the time of the exposure (Lauren Hall, personal communication). Following exposure a 30% prevalence was present suggesting that the virus could be transmitted between individuals after exposure via injection. Should this study be repeated it would probably be advisable to take a blood smear at day 0 and screen for the virus to determine infection prior to the start of any passage studies.

Transmission of HLV may be affected by host (age, molt stage, injuries, and pathogen load) or environmental conditions (temperature, water quality) that are known to influence the disease dynamics of other crustacean diseases (Bateman *et al*., 2011; Bateman and Stentiford, 2008; Messick and Shields, 2000; Shields, 1992; Shields and Wood, 1993; Stentiford *et al*., 2001). Presence of the virus does appear to be correlated to increasing temperature as it was first identified following a temperature

stress study and the prevalence in shoreline collected samples appears to increase as the summer temperature increases. Further work is needed to confirm this theory.

Throughout all the studies completed during this thesis it has been observed that shore crabs often show a white haemolymph, this can be evident after collection and prior to studies beginning or after the completion of studies. It has been shown to be caused by a rickettsia like organism (RLO) and the condition is known as milky disease (Eddy *et al*., 2007). Work at Cefas has shown that the RLO is sometimes associated with presence of HLV (data not shown). It is possible that the virus clears the circulating haemocytes from the blood and leaves the individuals open to secondary bacterial infections, the haemolymph becoming cloudy as described for milky disease. A similar phenomenon is reported for PaV1, in the late stages of PaV1 the normally bluish tinted transparent haemolymph is reported to become chalky white with cellular debris (Butler *et al*., 2008). It is possible that PaV1 also clears circulating haemocytes leaving the lobsters open to secondary infections; further work would be needed to confirm this theory in both crabs and lobsters with the two virus infections. It is not known whether this virus affects other species or is acquired from another species from the same habitat. Further work would be needed to establish this.

A sample of homogenised crab tissue known to be heavily infected with the HLV particles were prepared and submitted for 454 sequencing. Sequences were analysed to see if viral genes could be identified to try to classify this virus. Unfortunately the data showed a lot of bacterial contamination highlighting that the virus isolation had not been optimised. However if we consider the theory that the virus may clear circulating haemocytes and leave the crab susceptible to secondary bacterial infections this may be expected. In order to study the virus in detail methods to separate virus from host tissues would need to be trialled and developed, ideally you would need lots of infected tissues which could then be homogenised and separated via the use of various gradients, i.e. sucrose and caesium chloride gradients. Bands within these gradients could then be examined via negative stain under the TEM to determine where the viral particles collected within the gradients. These bands would then be prepared and submitted for sequencing and should lower the levels of bacterial contamination.

However the results did provide enough information to highlight similarities between HLA and PaV1. Two contigs with high similarity to PaV1 were identified, confirming the ultrastructural observations, it had been highlighted that HLV virions were similar to those described during PaV1 infections. Unfortunately only 2 sequences for PaV1 are present in NCBI database so full analysis and comparison has not been possible. The full genome for PaV1 has been sequenced however this data has not been published as yet. It is likely that these two viruses are closely related and will belong to the same virus family and possibly genus. This work will continue in collaboration with the researchers that discovered PaV1.

8.5 Conclusion

Although not isolated during these studies the re-isolation of B virus is vital in order to classify this virus and to enable a full comparison to WSSV to be completed. Work will continue to try and find this virus. The discovery of a novel virus that appears to mimic the pathology seen during WSD infections highlights the importance of being able to fully characterise virus infections when they are identified. Few viruses from marine invertebrates have been assigned to a particular family of viruses with certainty because biochemical, biophysical and immunological data are incomplete or lacking. This lack of characterisation has been due essentially to the lack of cell cultures and to the difficulties in producing viruses in wild animals. Viruses have so far been tentatively assigned to families based upon morphological and developmental characteristics and the location within the cell. There is a need to complete full characterisations and harmonise the naming of new viruses using International Committee on Taxonomy of Viruses (ICTV) guidelines. Development of techniques will assist with these full classifications and improve diagnostic capabilities.

Chapter 9.

General Discussion

In 2006 European Directive, EC Council Directive 2006/88/EC listed three crustacean diseases, White Spot Disease (WSD), Yellowhead disease (YHD) and Taura syndrome (TS). This was the first time crustacean diseases had been listed within European legislation, recognising the global importance of these viral pathogens, pathogens which had caused massive economic losses in shrimp farming regions. More importantly the listing of these diseases recognised the potential for these viruses to occur in non-farmed hosts, either as passive, latent or disease causing agents. WSD was listed as a 'non-exotic' disease within Europe following evidence that identified the presence of this virus within some countries within the European Union (Stentiford and Lightner, 2011). One of the duties of Member States under this Directive is that they are required to declare a national status for WSD; categories ranging from Category I (free from disease) to Category V (infected). In order to declare disease freedom, Member States may be required to undertake an epidemiologically-rigorous surveillance programme which utilises appropriate diagnostic tests designed to detect WSSV if present. The new listing of WSD in European legislation exposed a number of knowledge gaps that prevented risk assessors from accurately defining risks of exposure of temperate species to these pathogens. Work completed during this thesis aimed to answer some of these questions and concentrated upon the susceptibility of European crustaceans to WSD, highlighting the potential risks of an outbreak of this virus occurring within the UK.

A review of the literature on DNA viruses that affect crustaceans highlighted that very little research had been carried out on the susceptibility of European species to WSD, despite its demonstrable ability to establish in new hosts and the fact that there had been relatively free movement of this viral agent around the globe. Chapter 2 investigated the susceptibility of seven European crustacean species from both marine (*Cancer pagurus*, *Carcinus maenas*, *Eriocheir sinensis*, *Homarus gammarus*, *Nephrops norvegicus*) and freshwater environments (*Austropotomobius pallipes*, *Pascifasctacus leniusculus*). Exposure trials based upon natural (feeding) and artificial (intra-muscular injection) routes of exposure

to WSSV revealed universal susceptibility to WSSV infection in these hosts but also that relative susceptibility (measured in terms of temporal mortality and pathogenic manifestation of disease) varied significantly between species. In all species, WSSV associated pathology was most pronounced within the cuticular epithelium of the gill, suggesting gill was the most suitable tissue to be used in any potential surveillance programmes. Viability of the agent was confirmed via passage bioassay of infected host tissue to Specific Pathogen Free (SPF) *L. vannamei*, results confirming that *C. pagurus*, *C. maenas*, *E. sinensis*, *H.gammarus*, *N. norvegicus*, *P. leniusculus* and *A. pallipes* were considered susceptible to infection with WSSV and that the virus was able to replicate and remain virulent within these species. If the survival curves of each species exposed to WSSV via injection were compared on a single plot they formed three groupings (Chapter 2, Figure 7). Group A contained an estuarine species (*E. sinensis*), no mortalities had occurred in this species despite high levels of pathology being present within these samples. Group B contained the marine species (*C. pagurus, H. gammarus, N. norvegicus* and *C. maenas*) which showed a steady mortality over the course of the study and Group C contained the freshwater species (*P. leniusculus* and *A. pallipes*) which were deemed highly susceptible to infection, showing higher levels of mortality after WSSV injection earlier in the challenge than marine species. It is interesting to note that the level of susceptibility may alter depending upon the habitat of the host species and that the species exposed from these environments grouped together when mortality was compared.

Despite becoming infected with WSSV following exposure, the European shore crab (*Carcinus maenas*) appeared most resistant to the development of WSD and could be considered as an asymptomatic carrier of the virus. In *C. maenas,* pathology was evident within the tissues after injection but at a much lower severity to that of the other species. It was extremely difficult to identify areas of pathology within this species with fewer infected nuclei being evident within the cuticular epithelium of the gill and heart tissues. The aims of chapter 3 (long term exposure) and 4 (temperature stress) were to determine the true susceptibility of *C. maenas* to WSSV.

We knew from previous studies that the edible crab and shore crab were susceptible to infection although at varying levels (Bateman *et al*., 2012a). Edible crabs (*C. pagurus*) and shore crabs (*C. maenas*) were fed with WSSV-infected shrimp tissues and crabs were then observed in tanks for 3 months; the aim being to determine whether WSSV would remain in the tissues of edible crabs and shore crabs as a persistent infection or whether these crabs would clear the viral infection from their systems over time. Crabs were sampled for histology and PCR and tissues used in a bioassay with specific pathogen free (SPF) *L. vannamei* to identify whether the crabs could act as vectors for dispersal of the disease in the event of an outbreak in Europe. At the end of the study only two shore crabs and three edible crabs were PCR positive for WSSV. Edible crabs showed signs of replication via histology but only within a few crabs; these crabs also testing positive for WSSV via PCR. However, when these tissues were homogenised and inoculated into SPF shrimp no mortalities or pathology was observed and shrimp tissues were PCR negative for the virus. There were no signs of infection in tissue sampled from shore crabs despite the tissues being shown to be PCR positive for the virus suggesting that the virus may be present as a persistent infection within the shore crab tissues; the virus replicating at low levels without causing any detrimental effects on the host. It was also possible that the shore crab was capable of eradicating the virus from its tissues, and could explain why no signs of infection were seen in the majority of the samples. However, the mechanism to enable this is unknown and further work is needed in this area. The results of this long term feed study suggest that although the virus was transferred from infected feed to the crab, the virus was not passaged from crab to a susceptible host and so indicates that the infected shore and edible crab carcasses pose a limited risk 3 months after initial, single exposure to WSSV-infected material. However, whilst it is not clear whether the virus was present but below level of detection in the shrimp tissues, further studies including larger sample numbers and longer term exposures of shrimp are needed to confirm that there was absolutely no passage.

The aim of chapter 4 was to determine whether the shore crab was truly resistant to WSSV infection and show whether WSD could be expressed after the crab was exposed to a stressor. Using temperature as the stressor, individual *C.*

maenas were injected with WSSV and then exposed to varying temperature stress conditions. Assessment of total viral load per mg of crab tissue and histopathology from gill, connective tissues and heart suggested that crabs exhibited different disease development between individuals and this did not appear to be affected by the different temperature or “stress” conditions. Some crabs appeared to reduce the level of virus present in tissues i.e. final copy number was below that initially inoculated at day 0. Other crabs appeared to show replication of WSSV within the tissues with the final viral loading being above that initially inoculated at day 0 (Chapter 4, Figure 6). However, this distribution of viral reduction or viral replication occurred both crabs maintained at 20°C, and those which were exposed to an increasing temperature. Crabs could be divided into two groups according to the differences in development of the disease (histopathology) and infection (viral load). One group termed high responders (HR) developed increased viral loading and related pathology in target tissues whilst the second group (low responders, LR) exhibited lower viral loading and related pathology. HR and LR animals were present in both the ‘stressed’ (elevated temperature) and control (fixed temperature) exposure groups suggesting that these individuals in these groups would either replicate the virus or not, irrespective of the different temperature conditions in which they were maintained. It is important to note that this stress study would not have been possible with any other temperate marine species previously tested, edible crab (*Cancer pagurus*), nephrops (*Nephrops norvegicus*) and lobster (*Homarus gammarus*) since all were shown to be moderately susceptible to this virus and would have died within 10 days of exposure to WSSV and maintenance at 20°C (Bateman *et al*., 2012a). This again highlights the unique way in which *C. maenas* individuals react to this virus when compared to other species.

The natural variation that appears to be seen within shore crabs sampled direct from the wild is in contrast to the situation seen within shrimp farms. Shrimp in ponds have usually been stocked for grow-on and farmers want to ensure they achieve the most profitable results possible so choose their stock carefully. These shrimp have been cultivated and bred over time to ensure they are SPF and grow rapidly to enhance yields. Through this refinement there is likely to have been a narrowing of the gene pool in order to select for these beneficial traits (Moss *et*

al., 2012). However in this process they have also become less capable of surviving a disease outbreak, (the premise being that animals from the same genetic stock similarly susceptible to the same disease conditions). In nature the spread of pathogens among marine species occurs in numerous ways including ingestion of diseased tissue, contact with diseased individuals, transmission through the water and via alternate hosts (Morado *et al*., 2012; Shields, 2012). All of these routes are likely to occur similarly within shrimp ponds. However in shrimp ponds the stocking density is much higher than that which would be naturally present (leading to relatively stressed conditions). When combined with stock homogeneity, ideal scenarios for pathogen transmission and disease are created (Flegel, 2012; Lightner *et al*., 2012; Shields, 2013). This could explain in part why diseases such as WSSV have such a devastating effect in cultured conditions but do not appear to cause the same level of mortality within wild populations despite being shown to be present (Baumgartner *et al*., 2009; Chen *et al*., 2000; Hasson *et al*., 2006; Macías-Rodríguez *et al*., 2014). Results presented in this thesis appear to conclude that the stocking of shrimp ponds with animals derived from genetically heterogeneous stocks may lead to reduced disease burden and increased yield in the global industry.

To further investigate WSD susceptibility in shore crabs, viral replication of shore crabs (a low susceptibility species) was compared with a highly susceptible species (*L. vannamei*). Both species were exposed to WSSV and samples were taken at set sampling points throughout a 48 hour period for comparison. This study revealed that the viral loading in shrimp tissues increased over the 48 hour period whereas the viral loading in shore crabs decreased over the same time period. The shore crab appeared to be able to limit the viral replication process in the first 48 hours of infection when compared to shrimp indicating that viral replication appeared to be limited by host response. The reduction in viral loading in shore crab implies control of viral replication in this host during the very early stages of infection. This study again highlighted that the shore crab, a low susceptibility species responds to WSSV infection in a different manner to that of a highly susceptible species.

WSSV strains with smaller genomes have been found to be the fittest and most virulent forms of the virus (Marks *et al*., 2005). Comparison of WSSV genome

sequences revealed three variable nucleotide tandem repeat (VNTR) loci on the genome open reading frame (ORF) which contained repeat units (RUs), the number of RUs corresponding to the size of the genome. It has been suggested that genomic variations seen in the number of RUs present in ORF 94 may result from host selection pressures with the host playing a role in the selection of which WSSV genotype replicates within that individual. The aim of chapter 6 was to determine whether there were any differences in the viral types present after passage through different hosts, a highly susceptible host such as signal crayfish (*Pacifastacus leniusculus*) and a low susceptible host the shore crab (*Carcinus maenas*). This study was conducted using the DNA extracts from ten signal crayfish and ten shore crabs that had been exposed to WSSV via injection described in Chapter 2. In addition, it was vital that the composition of the initial inoculums was determined to establish which genotypes were present before passage as both species had been inoculated with the same initial inoculums. Extracts were analysed using ORF 94 primer sets and the products cloned, these clones were sequenced and analysis of these clones was then conducted to determine whether there were any differences in the viral types between a highly susceptible and a low susceptible species after passage. Although results from this pilot study are limited, they may suggest subtle differences between the frequency of RU types present in the initial inoculum and those that are present following passage through different hosts. Until now VNTR analysis has been used to determine geographic spread of the virus and to determine where outbreaks were likely to have originated (Dieu *et al*., 2004; Dieu *et al*., 2010; Marks *et al*., 2004). This work shows that the VNTR analysis can be used to obtain information regarding influences of the host themselves upon the genotype of the viral population that replicates within an individual, or species. However, the the mechanism by which the host may influence this viral population is currently unknown.

C. maenas has been shown to possess other viral infections, two of which, B virus and Rod shaped virus of *Carcinus maenas* (RV-CM) have been tentatively listed within the same genus as WSSV (Vlak *et al*., 2005). WSSV is currently the only member of the *Nimaviridae* genus, a situation that is acknowledged to be unusual by the International Committee on Taxonomy of Viruses (ICTV). This is

likely to change as new strains and isolates are discovered. B virus and RV-CM (along with a few other viruses from different crab species, B2 and Baculo-B) have been reported to be extremely similar in terms of size, shape and morphogenesis to that reported for WSSV. In fact, these viruses appear morphologically indistinguishable and it has been suggested that they may be ancestral forms of WSSV (Bonami and Zhang, 2011). However, despite their scientific importance, the taxonomic position of these viruses has never been defined and the tentative classification by ICTV has now been removed due to lack of evidence (Lo *et al*., 2012).

Similar to WSSV infection, histology of B virus and Rod shaped virus of *Carcinus maenas* (RV-CM) infection revealed hypertrophied nuclei with marginalized chromatin within the haemocytes and connective tissues of shore crabs (Bazin *et al*., 1974; Johnson, 1988). TEM of B virus and RV-CM showed viral particles within these enlarged nuclei appearing in parallel arrays. Virions were often associated with vesicles of various sizes within the nucleus, and were rod-shaped although sometimes appeared curved or ‘u’ shaped (Johnson, 1988). Of most interest was the coiling of the capsid material within both WSSV and B virus particles. One of the key characteristic features of WSSV is that the virions possess a very distinctive capsid layer giving the DNA core a cross-hatched or striated appearance; the capsid is described as being composed of rings of subunits in a stacked series (Vlak *et al*., 2005). This feature of WSSV has only been described in one other pathogen - B virus infecting *Carcinus* spp. (Bazin *et al*., 1974; Bonami and Zhang, 2011). During trials with WSSV detailed in this thesis, TEM of viral particles showed differences to classic WSSV ultrastructure in that while un-enveloped nucleocapsid material appeared identical to that observed for WSSV in other hosts, the assembled viral particles differed. The nucleocapsid within these viral particles appeared curved or ‘u’ shaped within the envelope, similar to that described in RV-CM virus infections (Johnson, 1988). The crab that displayed these particles was sampled from a WSSV study and tissues had been shown to be PCR positive for WSSV so it was assumed that they were WSSV particles and not B virus. There was no way of confirming this since this crab had been exposed to WSSV, however this pathology had not been seen during extensive surveys of shore crabs sampled direct from the shoreline

or in shore crabs which had been kept in tanks but not exposed to WSSV so it is likely that the pathology present here was WSSV and not B virus.

Considering *C. meanas* displayed differential susceptibility to WSSV when compared to other species and that there appeared to be evidence in the literature suggesting that a similar viral infection may occur in this species, could this exposure to a similar virus provide the shore crab with some kind of protection against WSSV? Crustacea have been shown to possess a form of innate immunity to certain viruses which is developed over time following exposure to the virus, for example Yellowhead Virus (YHV) (Flegel, 2007). A similar response was also seen following initial exposure of farmed shrimp to WSSV - with apparent toleration of the virus with time (Flegel, 2007). It is conceivable that shore crab have developed strategies for dealing with WSSV infections via previous exposure to a virus that was genetically similar to WSSV. This relative resistance could provide a heritable innate form of immunity that differs between specific familial lines within wild populations. Such a phenomenon would explain the differences in viral replication and pathology observed between HR and LR groups of *C. maenas* studied in this thesis.

To investigate this theory a survey of *C. maenas* from around the UK was carried out in order to attempt to re-isolate B virus and complete the necessary comparisons between this virus and WSSV. As previously highlighted, in order to declare disease freedom for WSSV, Member States within the EU will be required to undertake an epidemiologically-rigorous surveillance programme which utilises appropriate diagnostic tests designed to detect WSSV if present (Stentiford *et al*. 2011). The shore crab is a ubiquitous species around Europe and may well be chosen as the species of choice for such surveillance programmes. The potential of finding B virus during any surveillance programmes highlighted the need to re-isolate this virus to identify any potential similarities between B virus and WSSV, both histologically and genetically.

There is evidence to suggest that non-infectious sequences of viral origin can become inserted into host genomes and that these inserts could lead to false positive results with standard PCR tests (Tang and Lightner, 2006; Tang *et al*., 2007a; Tang *et al*., 2007b). Could crustaceans, in particular the shore crab,

integrate viral sequences into their genome in order to possess some kind of protection from future infections? It has been suggested that random integration of viral genome fragments into the host genome could lead to antisense RNA transcripts which are capable of suppressing propagation of the same virus within host tissues (Flegel, 2009b). These sequences provide protection via RNA interference (RNAi) pathways which have been demonstrated in plants, shrimp and insects (Kumria *et al*., 1998; Lu *et al*., 2004; Robalino *et al*., 2005; Saleh *et al*., 2009; Su *et al*., 2008). The host RNAi mechanism is thought to produce immune-specific RNA that binds with viral mRNA to suppress viral propagation, leading to low-level active infections whereby the host exhibits no sign of disease but may remain infected and infectious for naïve hosts (Flegel, 2009b). It is possible that the shore crab may have viral gene inserts within its genome from such a previous exposure to B virus and may be producing miRNA which could limit the WSSV replication within shore crab tissues. Following this work, the possibility of viral gene inserts and presence of miRNA is currently being investigated via an ongoing project between the Cefas and the University of Exeter, UK. It is important to resolve this issue and determine whether WSSV viral inserts are possible and if so present in shore crabs in order to prevent false positive results in WSSV surveys of wild stocks.

The survey identified a wide range of viral, bacterial, protistan and metazoan pathogens and found that the apparent prevalence and pathogen profile differed considerably between sites. This was not totally unexpected as the crabs in the different sampling locations will be exposed to different environmental conditions such as differing substrates and temperature fluctuations. In addition there will be natural differences in biodiversity between the sites which would in turn account for differences in diet, and subsequent pathogen exposure. B virus was not re-discovered during the survey. However, another viral infection was identified in shore crab tissues which similarly mimicked the pathology observed during B virus and WSSV infections. This thesis provides the first description of a herpes like viral (HLV) infection in *C. maenas*. Initially assumed to be B virus infection, TEM revealed the presence of hexagonal viral particles within the enlarged nuclei which possessed a distinct brick shaped core surrounded by an electron dense sphere and displayed an inner and outer envelope. The virus most

closely resembled particles described in the Herpes family so was named HLV until full characterisation could be completed. Throughout of the experimental studies described in this thesis, it has been observed that shore crabs often exhibit a whitening/opacity of the haemolymph, often apparent soon after collection or during captivity. The clinical sign has previously been associated with a rickettsia like organism (RLO) and the condition is known as 'Milky Disease' (Eddy *et al*., 2007). Work at Cefas has shown that the RLO is sometimes associated with presence of HLV (data not shown). It is hypothesised here that HLV infection may be a precursor to Milky Disease, with infection of circulating haemocytes leading to anaemia and subsequent secondary bacterial infections by the RLO. However, further work is needed to confirm this theory.

In this thesis, a sample of homogenised crab tissue known to be heavily infected with the HLV particles were prepared and submitted for 454 sequencing. Despite being heavily contaminated with bacteria it was possible to obtain reads that indicate that the HLV virus contained within the sample may be closely related to *Panulirus argus* virus 1 (PaV1) described infecting juvenile spiny lobsters in the Caribbean (Shields and Behringer, 2004). Ultrastructurally HLV also appeared to be extremely similar to the Bifacies virus which was described infecting *C. sapidus* in America (Johnson, 1988), and to PaV1 (Shields and Behringer, 2004). Sequence data suggests that HLV and PaV1 are closely related and will likely belong to the same virus family and possibly genus. Bifacies virus may also fall into this virus family but the virus would need to be re-isolated in order to complete this comparison. Work to compare HLV and PaV1 will continue in collaboration with the researchers that discovered PaV1, the aim being to fully characterise both viruses.

The discovery of a novel virus that appears to mimic the pathology seen during WSD infections highlights the importance of being able to fully characterise virus infections when they are identified. Few viruses from marine invertebrates have been assigned to a particular family of viruses with certainty because biochemical, biophysical and immunological data are incomplete or lacking. This lack of characterisation has been due essentially to the lack of cell cultures and to the difficulties in producing viruses in wild animals. Viruses have so far been tentatively assigned to families based upon morphological and developmental

characteristics and the location within the cell. There is a need to complete full characterisations and harmonise the naming of new viruses using International Committee on Taxonomy of Viruses (ICTV) guidelines. Development of techniques will assist with these full classifications and improve diagnostic capabilities and is an area I will pursue following the completion of this thesis.

A significant finding of this thesis related to the survey of commodity products covered in Chapter 5. The EU imports a large volume of raw frozen or cooked frozen commodity products ($500m in 2005), much of which originates in WSD positive regions. This study confirmed the presence of WSSV-contaminated products within supermarket commodity shrimp imported for human consumption, demonstrating that frozen commodity shrimp is a route of entry for WSSV into European Member States. Commodity product is not covered or controlled by Directive 2006/88/EC as this product is intended for human consumption. However anecdotal evidence (online forums, angling magazines) suggests that the use of raw, frozen shrimp as angling bait has become common practice due to the availability of the product (easily available in supermarkets and angling shops) and the low competitive price of these products when compared with other angling baits. Experimental trials described in chapter 5 showed how this virus is viable and that the commodity products were infective and could passage the infection to naive crustaceans that are known to be susceptible to the virus (in this case *Homarus gammarus*).

Central to any national import risk assessment for commodity products contaminated with pathogens such as WSSV, is an assessment of the likelihood for a pathogen to establish in naïve susceptible hosts if the pathogen is released into waters in the importing nation. This study has highlighted that the use of these products as bait is an extremely high risk practice; it is known that crayfish are particularly susceptible to WSSV and are most likely to be the crustacean species which are exposed to these commodity products that are used as angling bait in the rivers and lakes. The use of these products as bait may need to be banned or otherwise controlled in order to limit the risk posed by this practice. This could be achieved via better education of anglers and the banning of sale of these products in angling shops. In addition further work is needed to investigate the potential import of other viral diseases such as Taura Syndrome and

Yellowhead disease which are also listed within the European Directive as exotic diseases to Europe but are also likely to be present within these products.

Finally materials and observations gathered through the course of these studies have been used to identify and describe novel pathogens, *Ameson pulvis* (Stentiford *et al*., 2013b) and *Paramikrocytos canceri* (Hartikainen *et al*., 2014), see appendix.

Chapter 10.

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Appendix

11.1 Common and corresponding Latin names of crustacean species

Common Name	Latin name
Crabs	
Edible crab	*Cancer pagurus*
Shore crab	*Carcinus maenas*
Mitten crab	*Eriocheir sinensis*
Spider crab	*Maia squinado*
Blue crab	*Cal/inectes sapidus*
Mud crab	*Scylla serratus*
Green crab	*Carcinus mediterraneus*
King crab	*Paralithodes platypus*
Mud crab	*Rhithropanpeus harrisii*
Lobsters	
Nephrops	*Nephrops n01vegicus*
European lobster	*Homarus gammarus*
Caribbean spiny lobster	*Panulirus argus*
Crayfish	
White claw crayfish	*Austropotomobius pal/ipes*
Signal crayfish	*Pascifastacus /eniuscu/us*
Red claw crayfish	*Cherax quadricarinatus*
Common yabby	*Cherax destructor*
Noble crayfish	*Astacus astacus*
Shrimp	
White leaaed shrimp	*Litopenaeus vannamei*
Kuruma shrimp	*Marsupenaeus japonicus*
Northern brown shrimp	*Penaeus aztecus*
Northern pink shrimp	*Penaeus duorarum*
Tiger shrimp	*Penaeus monodon*
Western blue shrimp	*Penaeus sty/irostris*
Banana prawn	*Peneaus merguiensis*
Indian prawn	*Penaeus indicus*
Chinese white shrimp	*Fenneropenaeus chinensis IPenaeus chinensis*
Brown shrimp	*Crangon crangon*
Pink shrimp	*Panda/us montagui*
Common prawn	*Pa/eamon serratus*
Rock shrimp	*Palaemon e/egans*
Krill	*Acetes erythraeus*
Titi shrimp	*Protrachypene precipua*

11.2 2% Sterile saline solution

2g NaCl diluted in 1OOml distilled water.

Solution was dispensed into appropriately sized glass bottles and autoclaved (Priorclave autoclave, London UK) at 121C for 15mins.

11.3 Davidson's Seawater Fixative

Stock Solution

Filtered sea water	3340ml
95% Ethanol	3330ml
36 - 40% Formaldehyde	2220ml
Glycerin	111Oml

Working Solution

Stock solution	720ml
Glacial acetic acid	80ml

11.4 Davidson's Freshwater Fixative

To be used for fixation of freshwater crustacean samples, for example crayfish

Stock Solution

Distilled water	3350ml
95% Ethanol	3300ml
36 - 40% Formaldehyde	2200ml

Working Solution

Stock solution	885ml
Glacial Acetic Acid	115ml

11.5 Peloris Wax Infiltration Protocol

Solution	Time Temperature 287

70% Industrial Methylated Spirit	30 mins	Ambient
90% Industrial Methylated Spirit	30 mins	Ambient
Absolute Industrial Methylated Spirit	30 mins	Ambient
Absolute Industrial Methylated Spirit	30 mins	Ambient
Absolute Industrial Methylated Spirit	30 mins	Ambient
Sub X-Clearing Agent	40 mins	Ambient
Sub X-Clearing Agent	40 mins	Ambient
Sub X-Clearing Agent	40 mins	Ambient
Wax formula R	45 mins	60°C
Wax formula R	45 mins	60°C
Wax formula R	50 mins	60°C

11.6 Publications arising from work during this thesis

11.6.1 Bateman, K.S., Tew, I., French, C., Hicks, R.J., Martin, P., Munro, J., Stentiford, G.D. (2012) Susceptibility to infection and pathogenicity of White Spot Disease (WSD) in non-model crustacean host taxa from temperate regions. Journal of Invertebrate Pathology, 110, 340-351.

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Susceptibility to infection and pathogenicity of White Spot Disease (WSD) in non-model crustacean host taxa from temperate regions

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A B S T R A C T

Despite almost two decades since its discovery, White Spot Disease (WSD) caused by White Spot Syndrome Virus (WSSV) is still considered the most significant known pathogen impacting the sustainability and growth of the global penacid shrimp farming industry. Although most commonly associated with penaeid shrimp farmed in tropical regions, the virus is also able to infect, cause disease and kill a wide range of other decapod crustacean hosts from temperate regions, including lobsters, crabs, crayfish and shrimp. For this reason, WSSV has recently been listed in European Community Council Directive 2006/88. Using principles laid down by the European Food Safety Authority (EFSA) we applied an array of diagnostic approaches *to* provide a definitive statement on the susceptibility *to* White Spot Syndrome Virus (WSSV) infection in seven ecologically or economically important crustacean species from Europe. We chose four marine species: *Cancer pagurus*, *Homarus gammarus*, *Nephrops 11orvegiws* and *Carci1111s 111ae11as;* one estuarine species. *Erioc/reir si11e11sis* and two freshwater species, *A11stropota111obi11s pallipes* and *Pacifastacus le11i11sc11l11s.* Exposure trials based upon natural (feeding) and artificial (intra-muscular injection) routes of exposure to WSSV revealed universal susceptibility to *WSSV* infection in these hosts. However, the relative degree of susceptibility {measured by progression of infection to disease, and mortality) varied significantly between host species. In some instances (Type 1 hosts), pathogenesis mimicked that observed in penaeid shrimp hosts whereas in other examples (Types 2 and 3 hosts), infection did not readily progress to disease, even though hosts were considered as infected and susceptible according *to* accepted principles. Results arising from challenge studies are discussed in relation to the potential risk posed to non-target hosts by the inadvertent introduction of WSSV to European waters via trade. Furthermore, we highlight the potential for susceptible but relatively resistant hosts to serve as models to investigate natural mitigation strategies against WSSV in these hosts. We speculate that these non-model hosts may offer a unique insight into viral handling in crustaceans.

1. lntrnduction

Since initial outbreaks of White Spot Syndrome Virus (WSSV) infections in Asian shrimp farms during the early 1990s, the disease associated with infection in farmed penaeids (White Spot Disease, WSD) has been responsible for massive economic losses in both the western and eastern hemispheres (Lightner, 2011). The name of the disease refers to the clinical signs (white spots) reported to occur in relatively few of the species known to be susceptible to infection. Other signs of WSD include lethargy, sudden reduction of food consumption, red discolouration of body and appendages and a loose cuticle. Patent WSD results in rapid, high mortalities, affecting up to 100% of the exposed stock within 3-10 days of initial signs (Lightner, 1996). Numerous ;iuthors have described the epidemiology of the disease in individual farms, farm clusters and geographic regions (Lotz, 1997; Corsin et al., 2002) while others have detailed the prevailing practice of so-called 'emergency harvest' as a mechanism to prevent total crop loss following initial discovery of diseased animals (Turnbull et al., 2005). Overall, despite almost two decades since its discovery, WSD is still considered the most significant known pathogen impacting the sustlinability and growth of the global penaeid shrimp fanning industry (Stentiford et al., 2009, 2010; Lightner, 2011).

In recognition of its significance, WSD has been listed as a notifi 1ble disease by the OIE for over a decade (OIE, 2009). More recently, WSD has also been listed in regional legislation within the European Union (EU) via its inclusion in European Directive 2006/88/EC (on animal health requirements for aquaculture

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animals and products thereof, and on the prevention and control of certain diseases in aquatic animals). This legislation provides guidelines for EU Member States with regard designation of their national status for specific listed diseases (including WSD) and the measures that should be applied to ensure that any outbreak of a listed disease is handled appropriately. Furthermore, it stipulates the requirements that must be met by importing nations (outside of the EU) wishing to trade susceptible live aquatic animals and their products to Member States within the EU, and to Member States wishing to trade susceptible species and products to other EU Member States. The overall aim is one of improved biosecurity for the listed globally significant pathogens within the European Union (Stentiford et al., 2010).

Since the issue of susceptibility of hosts to a particular disease is a key component of any limitation to the trading in that host or its products, recent work by the European Food Safety Authority (EFSA) has attempted to define 'susceptibility' (with regards to the listing of hosts as such in EC Directive 2006/88/EC). In terms of the listed crustacean diseases (including WSD), this work has recently been reviewed by Stentiford et al. (2009). Here, the available published and grey literature (for WSD) was assessed against four objective susceptibility criteria: (A) Evidence of replication or growth of the organism: (B) Presence of a viable organism: (C) Presence of specific clinico-pathological changes; and (D) Specific location of the pathogen within the host. Using this approach, and despite the fact that EC Directive 2006/88/EC currently lists 'all decapod crustaceans' (>20,000 extant species) as potentially susceptible to WSD, scientific data were available to support designation as 'susceptible' in just 67 species. Numerous other aquatic hosts including rotifcrs, bivalves, polychaete worms, non-decapod crustaceans and some aquatic insects were considered as mechanical vectors for WSSV (Stentiford et al., 2009). The review does however indicate a particularly wide spectrum of WSD-susceptibility across host taxa within the Decapoda, essentially spanning the two major sub-orders (Dendrobranchiata and Pleocycmata), and numerous families within each of these (Stentiford et al., 2009).

Current guidelines for the methodological approach to diagnosis of WSD are based upon the detection of viral nucleic acids and the manifestation of disease in penaeid shrimp hosts (OIE, 2009). In these hosts, WSSV infection occurs in all tissues of mesodermal and ectodermal origin (e.g. gills, lymphoid organ, cuticular epithelium, sub-cuticular connective tissues). Infected nuclei become hypertrophied with marginalized chromatin, and contain inclusion bodies that stain intensely eosinophilic in early stage infection and basophilic in more advanced infection (Lightner, 1996). Virions measure 120–150 nm in diameter and 270–290 nm in length, possess a tail-like projection and a striated nucleocapsid (Wang et al., 1995; Vlak et al., 2005), features similar to the currently unclassified 13 and 132 viruses from portunid crabs (Bonami and Zhang, 2011). In contrast to the penaeids, relatively little information exists on the pathogenesis and outcome of WSD in non-penaeid decapod species (including crabs, lobsters and crayfish). Furthermore, there is a paucity of information on the utility of the recommended confirmatory diagnostic tests for diagnosis of *WSSV* infection and disease in these non-model hosts (OIE, 2009). Such information is now deemed vital given the global distribution of *WSSV* and its significant potential for interaction with novel host species residing in farmed and natural settings, and in locations distant from those traditionally associated with its presence (Baumgartner et al., 2009). As highlighted by Stentiford et al. (2010) and Stenliford and Lightner (2011), following the listing of WSD as a 'non-exotic' disease in EC Directive 2006/88, European Member States are required to declare a national status for WSD: categories ranging from Category I (free from disease) to Category *V* (infected). In order to do so, Member States may be required to undertake a suiveillance programme which utilises appropriate diagnostic tests designed to detect *WSSV* if present. In this context, an improved understanding of host susceptibility, the pathogenesis of WSD in non-model (but susceptible) hosts, the manifestation of disease under European ambient conditions, and the suitability of current diagnostic methodologies for its detection, are urgently required.

Several authors have provided useful information on this topic by demonstrating susceptibility to WSD in certain decapod crustacean species found in European marine habitats (Corbel et al., 2001) and in freshwater crayfish maintained at ambient temperatures found within European regional waterways (Jiravanichpaisal et al., 2001: Du et al., 2008). In the current study, we have extended this knowledge base by utilising an array of diagnostic approaches to provide a definitive statement on the susceptibility to *WSSV* infection in seven ecologically or economically important European crustacean species from freshwater, estuarine and marine habitats, based on the principles laid down by the European Food Safety Authority (EFSA) and outlined by Stentiford et al. (2009). Furthermore, we describe the histopathological manifestation of WSD in these non-model hosts and compare this to disease progression within (model) penaeid hosts (Pantoja and Lightner, 2003). Exposure trials based upon natural (feeding) and artificial (intra-muscular injection) routes of exposure to WSSV revealed universal susceptibility to WSSV infection in these hosts but also that relative susceptibility (measured in terms of temporal mortality and pathogenic manifestation of disease) varied significantly between species. These results are discussed in relation to the problems inherent in defining absolute susceptibility or non-susceptibility to particular pathogenic agents in crustacean hosts. The data presented herein provides further indication that WSSV has the potential to impact upon non-penaeid crustacean host species residing in a wide range of aquatic habitats and at temperatures considered sub-optimal for the replication of the virus *per se* (Vidal et al., 2001: Guan et al., 2003; Jiravanichpaisal et al., 2004: Rahman et al., 2007a, 2007b: Sanchez-Paz, 2010).

2. Materials and methods

2.1. Criteria for assessing susceptibility

Susceptibility to *WSSV* infection was tested in seven non-model decapod species commonly found in European marine, estuarine and freshwater habitats. Susceptibility was assessed using criteria developed by the European Food Safety Authority (EFSA) and summarised by Stentiford et al. (2009). For WSD, the specific characteristics utilised to test the four criteria (replication, viability, pathology and location) are given in Table 1. These characteristics were assessed in each of the non-model decapod species following their exposure to WSSV.

2.2. Collection of samples and husbandry

Samples were collected from natural sources as follows: a total of 18 edible crabs (Cancer pagurus) and 18 European lobsters (Homarus gammarus) were captured using baited pots in the Weymouth and Portland area of the English Channel, United Kingdom (50°32'50"N 002°11'00"W). Sixty Norway lobsters (*Nephrops norvegicus*) were captured on the *RV* Cefas Endeavour from the North Sea fishery (54°08'566"N 002°35'019"E) using a Granton trawl and transported to the laboratory in tanks with a running sea water supply. Fifteen White claw crayfish (Austropotamobius *pallipes*), were sourced from a breeding facility in Stainforth (Near Settle) Yorkshire, United Kingdom prior to transportation to the laboratory in a small amount of freshwater with an air supply. Sixty Signal crayfish (*Pacifastacus leniusculus*) were sourced from

Table 1
Specific techniques and characteristics applicable to support criteria A-D for White Spot Disease. Recreated and adapted from Stentiford et al. (2009).

	A. Replication	B. Viability	C. Pathology	D. Location
WSD	Presence of characteristic intranuclear inclusion bodies Presence of virions In inclusions bodies by TEM Positive labelling of inclusion bodies by ISH or IFAT Serial passage from individual to SPF individual	Passage bioassay to a SPF susceptible host	Eosinophilic inclusions within nuclei of target organs and tissues	Cells of tissues of ectodermic and endodermic origin

a crayfish farm in Kent, United Kingdom and transported back to the laboratory overnight. Sixty Chinese Mitten Crab (E1ioc/1eirsi11-*e11sis*) were captured using baited pots from the River Thames, London, United Kingdom (51°27'12"N, 00°00'44"E) and transported back to the laboratory. Sixty shore crabs (*Carci11us 111ae11as*) were collected from the shoreline at Newton's Cove, Weymouth, UK (50°34'N, 02°22'W) or using drop-nets in Weymouth Harbour (50°36.G'N, 02°27'W).

All experimental trials were performed within the biosecure exotic disease facility at the Cefas Weymouth laboratory and utilised local. filtered and UV treated water. Day length was set at 14 h/day, night was at 10 h with a 30 min fade to simulate dusk and dawn. Flow rate was set a 3-4 l/min and for marine species, salinity during the experimental period remained constant at 35ppt. Temperature was regulated according to the experimental conditions required. All animals utilised in experimental challenge trials appeared externally healthy. Species-specific challenges using WSSV were carried out independently (no multi-species challenges). Animals were transferred into custom-made compartments within large trough tanks, with individuals separated by tank divisions to prevent conflict but sharing the same water supply. Water temperatures in all tanks was held constant at 20 °C for *C. 11ae11as, E.* si11e11sis, *A. pallipes* and *P. /e11iusculus,* and 16 °C for *N. 1101vegicus.* The different challenge temperature utilised for these species was chosen to reflect the maximal summer temperatures likely experienced by these species in Europe. For *C. pagurus* and *H. gammarus,* chelipeds were banded to prevent conflict prior to their transfer to two large tanks (11 = 6 per tank) and three medium tanks which had been divided in half (n = 2 per tank). Water temperatures in all tanks was held constant at 20 °C for *C. pagurus* and at 15 °C for *H. ga111111arus.* Once again, ambient temperatures were chosen to reflect maximal summer temperatures likely experienced by these species in Europe. All animals were acclimatised to these exposure trial condition temperatures for a minimum of 1 week before trials commenced.

2.3. *Preparation of viral* i11oculu111 and *c/1al/e11ge trials*

Viral inoculates of WSSV were obtained from the OIE reference laboratory for White Spot Syndrome Virus (WSSV) at the University of Arizona, USA. The OIE isolate of WSSV (UAZ 00-173B) was generated in *Litope11ae11s vllmiamei* (Holthuis, 1980) from an original outbreak of WSD in *Fe1111erope11em1s cl1i11e11sis* (Holthuis, 1980) in China in 1995. Subsequent passage of this isolate into Specific Pathogen Free (SPF) *L. va1111a111ei* held at the Cefas Weymouth laboratory have demonstrated continued virulence of this isolate (data not reported here). All challenges reported utilised WSSV-infected *L. va1111amei* carcasses generated within the Cefas Weymouth laboratory. As such, WSSV-infected shrimp carcasses were prepared by injection of the UAZ 00-173B isolate into SPF *L. vannamei* obtained from the Centre for Sustainable Aquaculture Research (CSAR) at the University of Swansea, United Kingdom. Individual *L. var11ia111ei* were inoculated via intra-muscular injection of the diluted viral homogenate at a rate of 10 µl g^{-1} shrimp weight. Following incubation, dead and moribund shrimp were removed from the experimental tanks and infection with WSSV was confirmed using histopathology, transmission electron microscopy (TEM) and PCR as appropriate (see below for techniques). Remaining tissues were stored at −80 °C until required. Confirmed infected and uninfected (sham-injected) carcasses were used to prepare inoculums and feed rations for challenge studies using the non-model host species. Infected and uninfected shrimp carcasses were macerated in isolated conditions using a sterile razor blade prior to homogenisation in sterile saline (4 ml of saline per gram of minced tissue) using a blender until tissues were liquefied. The homogenate was centrifuged at 5000g for 20 min at 4 °C to pellet solid debris prior to the supernatant being diluted 1:20 with sterile saline and filtered to form the inoculums for the injection studies. For feeding trials, confirmed infected and uninfected carcasses were macerated into approximately 2-3 mm^3 blocks using a sterile razor blade immediately prior to feeding.

For all exposure trials, a similar protocol was followed. Group 1 (negative control feed) animals were fed with a single ration of confirmed SPF shrimp tissue at approximately 5% bodyweight on Day 0 of each trial. Group 2 (WSSV positive feed) animals were fed with a single ration of confirmed WSSV-infected (but otherwise SPF) shrimp tissue at a ratio of approximately 5% bodyweight on Day 0 of each trial. Group 3 (WSSV positive injection) animals were injected with a single dose of the diluted WSSV homogenate (see above) at a rate of 10 µl g^{-1} wet body weight, on Day 0 of each trial. Since the key objective of this study was to investigate the potential for WSSV infection via a confirmed natural route (i.e. feeding), and the fact that we do not consider direct injection as a natural route of infection for WSSV (see Stentiford et al., 2009), Group 3 served purely to introduce WSSV into hosts for the investigation of histopathological progression of disease in non-model decapods. As such, a negative control (sham-injected) group was not included in the trials. Thereafter, samples in all tanks were fed on squid tissues at a ratio of approximately 3-4% wet body weight day^{-1} for the remainder of the trial period. Tanks were observed regularly throughout daylight hours. Dead and terminally morbid samples were removed from each tank and dissected. At the end of each challenge trial (Day 10), and for moribund animals sampled within the trial, su1living animals were chilled on ice for 30 min prior to dissection. As standard, gill, epidermis, hepatopancreas, heart, gonad, nerve and muscle were placed into histological cassettes and fixed immediately in the appropriate fixative. Davidson's fixative made with seawater (Davidson's seawater) was used for tissues from marine species and Davidson's fixative made with tap water (Freshwater Davidson's) was used for all freshwater crayfish tissues. For molecular analyses, gill, epidermis and hepatopancreas samples were removed and placed into tubes containing 100% ethanol. For electron microscopy, gill, epidermis, hepatopancreas and heart tissues were fixed in 2.5% Glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for EM. The remaining tissues and carcasses were frozen at −80 °C for storage.

2.4. Histology and transmission electron microscopy

For histology, fixation was allowed to proceed for 24 h before samples were transferred to 70% industrial methylated spirit. Fixed samples were processed to wax in a vacuum infiltration processor using standard protocols. Sections were cut at a thickness of 3-5 Lm on a rotary microtome and mounted onto glass slides before staining with haematoxylin and eosin (H&E) and Feulgen stains. Stained sections were analysed by light microscopy (Nikon Eclipse E800) and digital images and measurements were taken using the LuciaTM Screen Measurement System (Nikon, UK). For electron microscopy, tissues were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature and rinsed in 0.1 M sodium cacodylate buffer (pH 7.4). Tissues were post-fixed for 1 h in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Samples were washed in three changes of0.1 M sodium cacodylate buffer before dehydration through a graded acetone series. Samples were embedded in Agar 100 epoxy(Agar Scientific, Agar 100 pre-mix kit medium) and polymerised overnight at 60 °C in an oven. Semi-thin (1-2 Lm) sections were stained with Toluidine Blue for viewing with a light microscope to identify suitable target areas. Ultrathin sections (70-90 nm) of these areas were mounted on uncoated copper grids and stained with 2% aqueous uranyl acetate and Reynolds' lead citrate (Reynolds, 1963). Grids were examined using a jEOLJ EM 1210 transmission electron microscope and digital images captured using a Gatan Erlangshen ES500W camera and Gatan Digital Micrograph™ software.

2.5. DNA extraction a11d PCR

Genomic DNA was extracted from tissues using a High Pure PCR Template Preparation Kit (Roche Diagnostics) following the manufacturer's protocols. Ethanol-prese1ved tissues were soaked in molecular grade H_2O prior to DNA extraction, to remove trace ethanol. DNA was eluted in 100 µl elution buffer and quantified using a NanoDrop -1000 (Thermo Fisher Scientific). WSSV-infccted and specific pathogen free (SPF) shrimp tissue samples were obtained from the OIE reference laboratory in Arizona and DNA was extracted as above. These were used as positive and negative control material for WSSV in subsequent molecular diagnostic assays.To ensure that amplifiable DNA was present in all extracted samples, crustacean genomic DNAs were assessed using the universal small subunit ribosomal RNA (SSU-rRNA) gene primers 16S-A (5'-AACCTGGTTGATCCfGCCAGT-3') and 16S-B (5'-GATCCrfCTGCAGGTTCACCTAC-3') (Medlin et al., 1988) with an expected amplification product of approximately 1800 bp. Each 20 LI reaction contained 1 x Green GoTaq Flexi Buffer (Prornega), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 1 LM of each primer, 1 unit *Taq* polymerase, and 1 LI genomic DNA (20-50 ng total). Amplifications were performed with an initial denaturation temperature of 94 °C for 4 min, followed by 35 cycles at 94 °c for 30 s, 45 °C for 30 s, 65 °C for 2 min, with a final elongation step at 65 °C for 5 min. Following amplification, 5 LI of each PCR product were analysed by agarose gel electrophoresis (1.5% w/v), stained with ethidium bromide, and viewed under a UV light source. Images were captured with a Gel Doc 2000 (Bio Rad) imaging system.

WSSV-infection status of tissues was confirmed using the nested PCR assay of Lo et al. (1996) with minor modifications (Ms. Bonnie Poulos, University of Arizona, personal communication). First, a product of 1447 bp was amplified using the primer pair 14GF1 (5'-ACrACJ'AACITCAGCCl'ATCI'AG-3') and 146R1 (5'-TAATGC GGGTGTAATGTTCTTACGA-3'), followed by an approximate 941 bp product in the nested reaction using prim er pair 14GF2 (5'-GTAACTCCCCCTJ'CCATCTCCA 3') and 146R2 (5'TACGGCAGCfG CTGCACCT-TGT-3').For the first round of amplification (primer pair 146F1/146R1) each 25 LI PCR reaction contained the following: 10 mM Tris-HCI (pH 8.4), 50 m M I<Cl, 2 mM $MgCl_2$, 200 LM of each dNTP, 0.31 LM of each primer, 2.5 units *Taq* polymerase, and 1 µl genomic DNA (20-50 ng total). Amplifications were performed with an initial denaturation temperature of 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s, with a final elongation step at 72 °C for 2 min. Reaction conditions and reagent concentrations were the same for the second round of amplification using the 14GF2/146R2 primer pair, however 0.5 LI of the first round of amplification was used as a template in place of genomic DNA. Following amplification, 1O LI samples of the second round PCR product were analysed by agarose gel electrophoresis as described above. WSSV-challenged tissue samples that were negative by PCR were re-analysed for a second time by the nested WSSV PCR assay to confirm the result.

2.6. Sequencing

PCR amplification products generated in non-target decapod host species exposed to WSSV via feeding of WSSV-infected shrimp tissues were sequenced for confirmation of their identity. Reactions were analysed on an ABI 3130 Avant Genetic Analyser. The final product was compared to known sequences using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) to determine phylogenetic homology. This is in line with OIE confirmato1y diagnostic technique for WSSV (Claydon et al., 2004).

2.7. Bioassay of 11011-target decapod tissues to L va11t1amei

Following PCR confirmation of the presence of amplicons of WSSV in tissues of WSSV-exposed non-target decapods, the infectivity of these tissues (from each test species) was tested via bioassay exposure to a known WSSV susceptible species *L.* vannamei). Tissues from each test species were homogenised using the aforementioned approach for shrimp. Control innoculum was also prepared for each test species using confirmed WSSV PCR negative samples. Inoculate was diluted 1:20 using sterile saline and filtered prior to intramuscular injection of SPF shrimp (10 LI g^{-1} body weight). Pre-trials utilising inoculate generated from C *111aenas* revealed that dilution to 1:40 was required to prevent rapid death of shrimp following injection (a condition previously termed 'protein shock' by Lightner, 1996).Shrimp exposed to confirmed-WSSV PCR positive and negative inoculate were obse1ved for a period of 5 days. A total of 14 tanks, containing 5 shrimp per tank were maintained at a water temperature of 24 °C and observed regularly throughout daylight hours. Dead and terminally moribund animals were removed from each tank and sampled as above. At the end of the challenge period (Day 5), all surviving shrimp were sampled. Pleopods were removed from each shrimp and fixed in 100% ethanol for WSSV detection by PCR (as above). The remaining shrimp carcass was injected with Davidson's seawater fixative and shrimp placed whole into the same fixative for histology (as above).

3. Results

3.1. Fulfilling criteria for susceptibility

Using the EFSA criteria for susceptibility (see Table 1), replication of the agent, pathology, and topogr,1phic location of the pathogen within the host, were demonstrated for all non-model host species via the presence of WSSV virions within intranuclear inclusion bodies in tissues of mesodermal and ectodermal origin (see section on pathogenicity below). Furthermore, viability of the agent (within the non-model host) was confirmed via passage bioassay of infected host tissue to SPF *L. vanna111ei.* In this case, the majority of SPF shrimp exposed to WSSV positive inoculate arising

Table 2
Implementation of EFSA criteria A-D for exposure of non-model crustacean hosts to WSD. Table shows criteria that were fulfilled for each species and exposure route.

Replication	A Viability	B. Pathology	C. Location	D.
Cancer pagunis				
Group 2 – WSSV positive feed				
Group 3 – WSSV positive injection				
Carcinus maenas				
Group 2 – WSSV positive feed				
Group 3 – WSSV positive injection				
Ilomanis gammanis				
Group 2 – \VSSV positive feed				
Group 3 – WSSV positive injection				
Neplirops norvegicus				
Group 2 – \VSSV positive feed				
Group 3 – \VSSV positive injection				
frioch elr sinensis				
Group 2 – \VSSV positive feed				
Group 3 – \VSSV positive injection				
Austropotamobius paiiipes				
Group 2 – WSSV positive feed				
Group 3 – \VSSV positive injection				
Pacifastacus leniusculus				
Group 2 – \VSSV positive feed				
Group 3 – \VSSV positive injection				

from non-model hosts died within 3 days post-injection. All mortalities and all remaining shrimp culled at the end of the challenge period (5 days) were PCR positive for *WSSV.* All shrimp showed pathognomonic signs of WSD infection via histology according to Lightner (1996) (data not shown). Despite the universal fulfilment of the EFSA WSD susceptibility criteria by the non-model hosts, there were considerable differences between pathological outcomes in the respective species, particularly when the mode of virus dose delivery is considered (Table 2). All non-model species, apart from *C. 111ae11as* and *A. pallipes* fulfilled all four susceptibility criteria via feeding and injection. In the case of *C. 111ae11as,* replication was not observed in target tissues from animals fed with *WSSV,* however when tissues from *C. mae11as* formed the basis of the inoculate for bioassay to SPF *L. var111a111ei,* characteristic signs of WSD, and mortalities occurred in shrimp. This finding is suggestive of infection in *C. maenas* with viral loading below that required to cause pathology in target tissues of this species, at least within the time frame of the study. Replication in injected *A. pallipes* could not be confirmed due to a rapid, non-specific mortality following injection of the inoculate. Replication was demonstrated however following feeding.

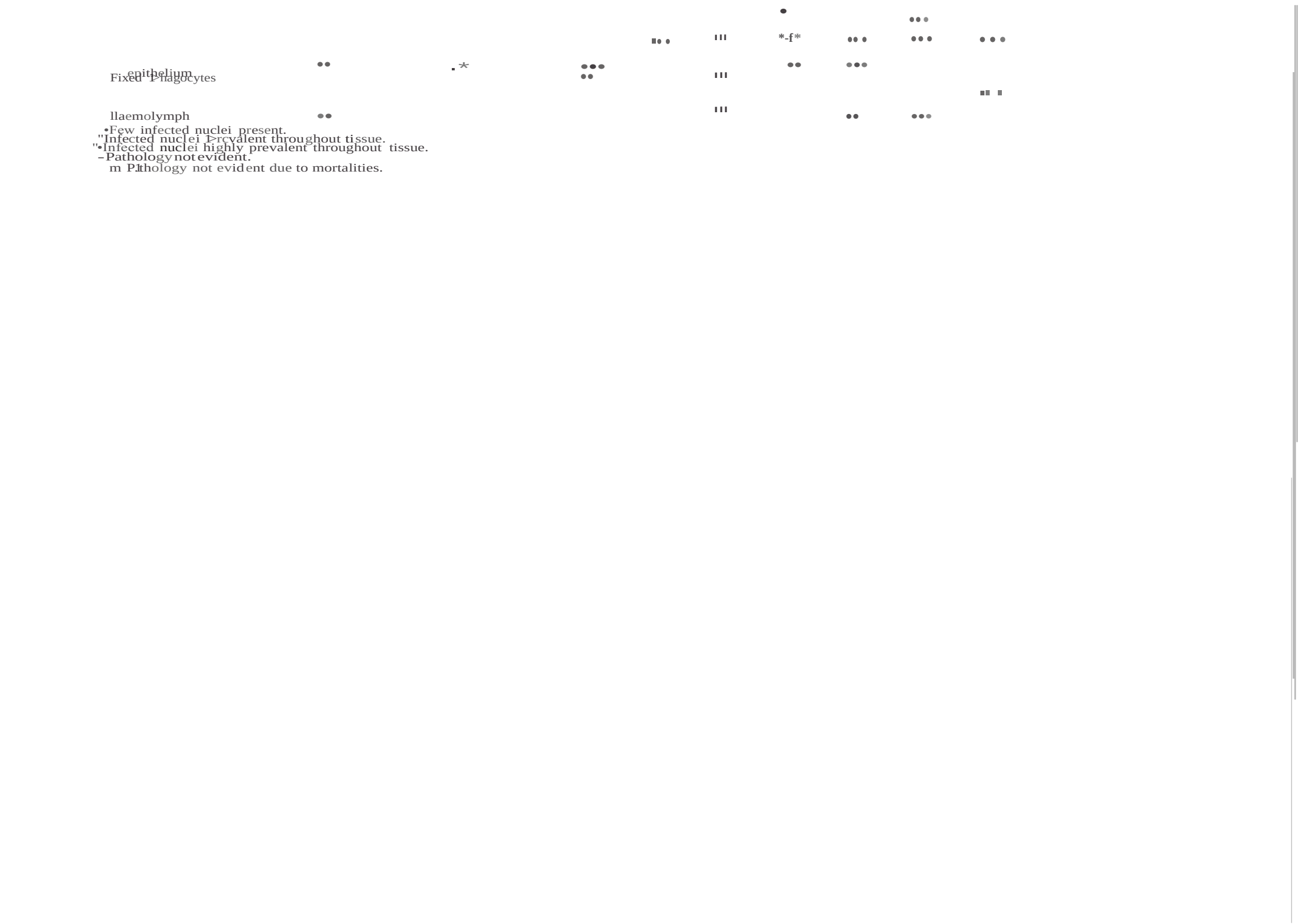
3.2. Pat/1oge11icity

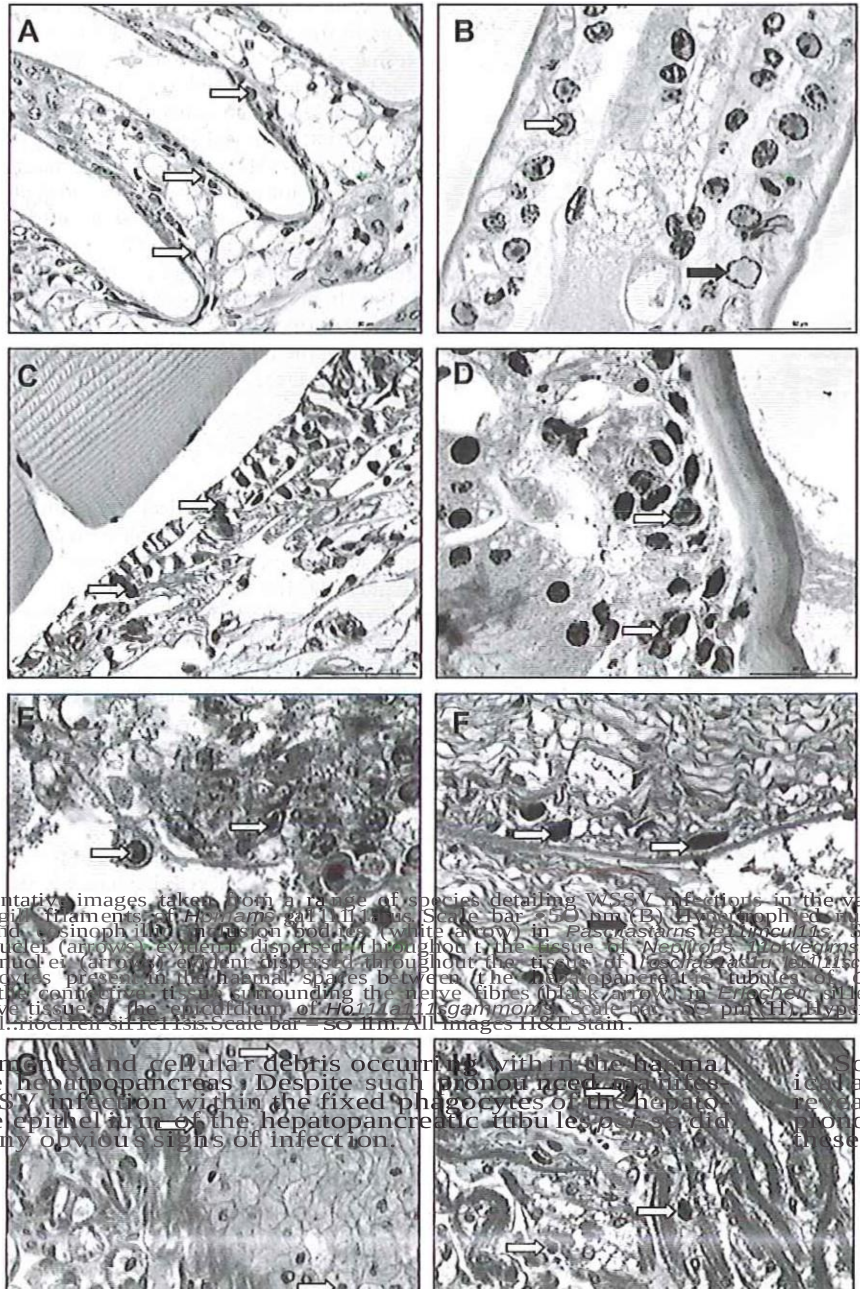
A summary of pathological observations in *WSSV* infected non-model hosts is provided in Table 3. In all species, WSSV-associated pathology was most pronounced within the cuticular epithelium of the gills (Fig. 1A). WSSV-infected nuclei were enlarged with marginalized chromatin, and often contained a distinct eosinophillic inclusion body (Fig. 1B). Infected nuclei were also identified within other regions of the cuticular epithelium (Fig. 1C and D). Within the heart, the nuclei of spongy connective tissue cells of the epicardium displayed characteristic WSSV inclusions (Fig. 1E) as did nuclei in the region of the sarcolemma surrounding the muscle fibres of the myocardium (Fig. 1E). Haemocytes and connective tissues, particularly surrounding the haemal vessels and nerves also showed pronounced signs of infection (Fig. 1F). The germinal epithelium surrounding the oocytes (Fig. 2A and B) and the connective tissues of the testes also displayed characteristic signs *of WSSV* infection in some species (Fig. 2C and D).

The lymphoid organ (LO), present in members of the Superfamily Penaeoidea (including members of the genus *litopenaeus)* was absent from the non-model hosts considered in this study. The characteristic pathologies of the LO previously associated with *WSSV* infection in penaeid shrimp, was mimicked by a similar manifestation in fixed phagocytes, particularly within the haemal sinusoids of the hepatopancreas. Fixed phagocytes within the haemal spaces displayed enlarged nuclei containing eosinophilic inclusions and marginated chromatin (Fig. 2E and F). In advanced stages of disease, infected cells appeared to degenerate, with remnant

Table 3
White Spot Disease pathology shown in differing tissue types for each species of non-model crustacean hosts after exposure via feeding (Group 2) and injection (Group 3).

	Cancer *pagw* us		*Vomall/ S* ganunams		*Nepllrops* norvegicus		*A11s1ropoto111obi11s pallipes*		*Pacifastacus* leniusculus		*Eriocliеir sl11e11sls*		*Cai* cinus *maenas*	
	Group 2	Group 3	Group 2	Group 3	Group 2	Group 3	Group 2	Group 3	Group 2	Group 3	Group 2	Group 3	Group 2	Group 3
Gill		●●●		●●●	●	●● ●	●● ●	m	●●●	●●●	●●●	●●●		
Heart		●	●	●●		●●	●	m	··		●● ●	●●		
Ovary		●●		●●		●●	●	m					N/A	N/A
		●●		●●		●●	●●	m	*					
		··		··					●●●	·■■	●●●			
							●	m	●		●●	●●		
Testis							●●●					●●●		
Nerve	N/A	N/A												
Connective tissue								m						
Cuticular							··		●●			●●●		


epithelium
Fixed Phagocytes
llaemolymph
•Few infected nuclei present.
"Infected nuclei Prevalent throughout tissue.
"•Infected nuclei highly prevalent throughout tissue.
–Pathology not evident.
m Pathology not evident due to mortalities.



Flg. 1. Representative images taken from a range of species detailing WSSV infections in the various tissues sampled. (A) Infected nuclei (arrows) can l>e observed scattered throughout the gill filaments of *Homams ga111111arus*. Scale bar = SO pm (B) Hypertrophied nuclei can be found within the gill filaments displaying marginalized chromatin (black arrow) and eosinophilic inclusion bodies (white arrow) in *Pasclrastarns le11imcul11s*. Scale bar = SO 1101. (C) Cuticular epithelium showed signs of infection with hypertrophied nuclei (arrows) evident dispersed throughout the tissue of *Neplirops 11orvegirns*. Scale bar – SO jlm. (D) Cuticular epithelium showed signs of infection with hypertrophied nuclei (arrows) evident dispersed throughout the tissue of *l'osclrastac1u le1l11sc11l11S*. Scale bar = SO 11111. (E) Infected nuclei present within the haemocytes (arrows), haemocytes present in the haemal spaces between the hepatopancreatic tubules of *Ca11cer pagums*. Scale bar 25 11m. (F) Infected nuclei (white arrows) were evident within the connective tissue surrounding the nerve fibres (black arrow) in *Eriocheir* si1eJ1Sis. Scale bar • 50 11111. (G) Infected nuclei (irrows) were present within the spongy connective tissue of the epicdfdium of *Ho111a111sgammoms*. Scale bar SO pm (H) Hypertrophied nuclei (arrows) can also be observed infecting the sarcolemma of the he.ut muscle in l..riocl1eir si11e11sis. Scale bar = SO 11m. All images H&E stain.

nuclear fragments and cellular debris occurring within the haemal spaces of the hepatpopancreas. Despite such pronounced manifestation of WSSV infection within the fixed phagocytes of the hepatopancreas, the epithelium of the hepatopancreatic tubules *per se* did not display any obvious signs of infection.

Some exceptions to this gener.11 pattern were observed. Histological assessment of tissues and organs from WSSV-injected *A. pallipes* revealed an apparent widespread apoptosis of haemocytes, and a pronounced bacteraemia of the hacmolymph in most samples. In these cases, 100% mortality occurred within 48 h of injection and

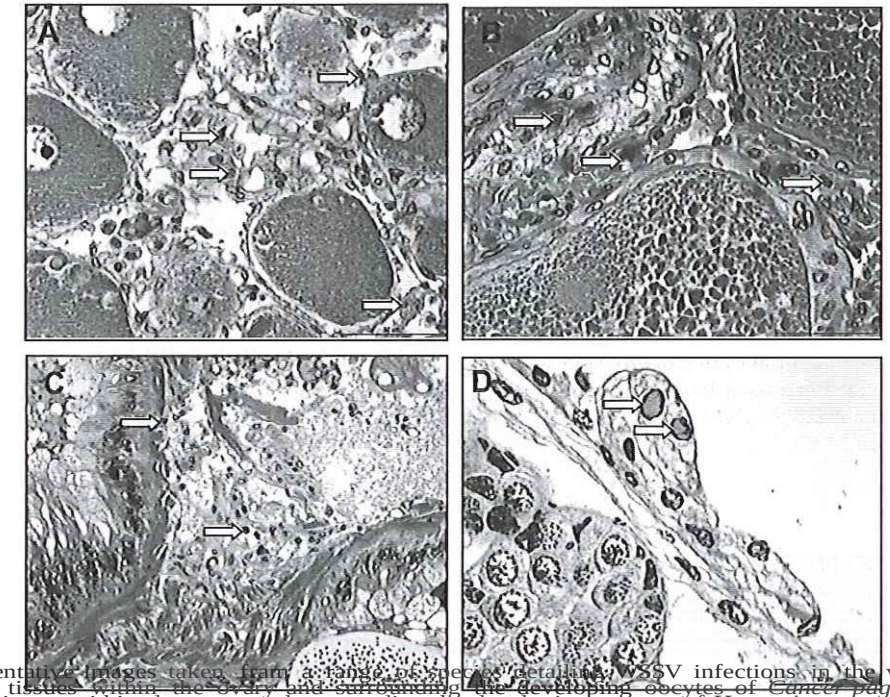


Fig. 2. Representative images taken from a range of species detailing WSSV infections in the various tissues sampled. (A) Infected nuclei (arrows) were present within the connective tissues within the ovary and surrounding the developing oocytes of *Cancer pagunis*. Scale bar SO µm. (B) Infected nuclei (arrows) were present within the connective tissues within the ovary and surrounding the developing oocytes of *Homanis gammarus*. Scale bar = SO µm. (C) Infected nuclei (arrows) were present within the connective tissues surrounding the testes of *Eriocheir sinensis*. Scale bar 10011m. (D) Infected nuclei (arrows) were present within the connective tissues surrounding the testes of *Pascifastaws leniuswlus*. Scale bar 125 in 11. (E) Fixed phagocyte cells from *Pascifastaws leniuswlus* displaying signs of infection, nuclei containing marginalized chromatin and an eosinophillic inclusion (white arrow). Areas of apoptotic cells (black arrows) were also evident within these cells. (F) Fixed phagocyte cells displayed signs of infection in *Austropotamobius pallipes*, nuclei containing marginalized chromatin and an eosinophillic inclusion (arrow). Scale bdr = SO 11m. All images H&E stain.

despite all samples displaying positivity for WSSV at time of death (positive PCR results), it is conceivable that this may have resulted from detection of residual inoculum rather than virus present within infected host cells. In *C. maenas*, WSSV-associated lesions were observed within the tissues of the WSSV-injected group but at a much lower severity than that seen in the other non-model species. In such cases, very few nuclei containing WSSV-associated inclusions were observed. No pathology was observed in *C. maenas* fed with WSSV contaminated feed (see Table 3).

All non-model host species were sourced from wild populations in the United Kingdom. Since these hosts were potentially infested with other pathogens at the start of each trial, it is important to consider the effect that these may have had on the outcome of challenge trials. In addition to pathologies associated with infection by WSSV, other conditions recorded at the termination of challenge trials included: Pink Crab Disease (Stentiford et al., 2002) caused by the dinoflagellate *Hematodinium* sp. in 27% of *C. pagurus*; parasitic copepod *Nicathoë astaci* in 50% of *H. gammarus*; *Austropotamobius pallipes* Bacilliform Virus (*ApBV*) (Edgerton et al., 2002) in 60% of *A. pallipes*; the microsporidian *Hepatospora* eriocheir (Stentiford et al., 2011) in 51% of *E. sinensis*. *C. maenas* were infected by a range of pathogens including the digenean *Microphallus primus* (Saville and Irwin, 2005), the parasitic rhizocephalan *Sacculina carcini* (Boschma, 1955), bacterial 'Milky disease' (Eddy et al., 2007), a novel microsporidian infection of the musculature, and *Hematodinium perezi* (Chatton and Poisson, 1931). In the majority of challenge studies, underlying infections did not cause significant mortalities in control groups. However, an unidentified bacterial infection caused mortality in 12% of control *P. leniusculus*. In addition, the WSSV-fed *C. maenas* group displayed 7% mortality which upon histological examination was apparently due to a pronounced microsporidian infection of the musculature rather than to WSD.

Transmission electron microscopy (TEM) and PCR followed by sequencing are recognised as confirmatory tools for diagnosis of WSSV infection according to the OIE (2009). TEM of gill from each non-model species used in the current study revealed the presence of WSSV virions within hypertrophied nuclei of infected cells. Nuclei were enlarged with marginalised chromatin and contained

virions at varying stages of development (Fig. 3A). Fully formed virions were ovoid to elliptical in shape and contained an electron dense nucleocapsid surrounded by a trilaminar envelope (Fig. 3B and C). The dimensions of virions varied according to stage of development but measurements of fully formed virions were consistent with those previously described for *WSSV*. During patent WSD in *E. sillenisis*, paracrystalline arrays of *WSSV* virions were observed at the periphery of infected nuclei (Fig. 30). Un-enveloped nucleocapsid material was present within the nuclei of some hosts this displayed the characteristic stacked sub-units of the *WSSV* genome (Fig. 3E). Interestingly, viral particles present within infected nuclei of *C. maenas* tissues were morphologically distinct from characteristic *WSSV* virions, although un-enveloped nucleocapsid material also appeared identical to that observed in *WSSV*. The nucleocapsid within intact viral particles in various stages of development in *C. maenas* appeared curved or 'u' shaped within the viral envelope (Fig. 3F).

3.3. PCR and sequencing

PCR analysis of gill tissues from the WSSV-injected sample group of all species revealed universal positivity for *WSSV*. PCR analysis of gill tissues from *WSSV-fed* groups of the different non-model host species revealed vaiying proportions of WSSV positive samples as follows: 33% *C.* pagurus, 20% *C. maenas* 17% *H. gammarus*, 65% *N. 1w1vegicus*, 10% *E. sillenisis*, 40% A *pallipes* and 5% *P.* /eniuscu/us. For confirmation of identity, a single *WSSV* PCR-positive sample from each of test species was sequenced. All sequenced samples showed between 99.6% and 99.8% similarity to the *WSSV* isolate utilised for challenge (OIE isolate of *WSSV* UAZ 00-1738).

These results confirm that the non-model species tested in this study were considered susceptible to infection with *WSSV* and that the virus is able to replicate and remain virulent within these species.

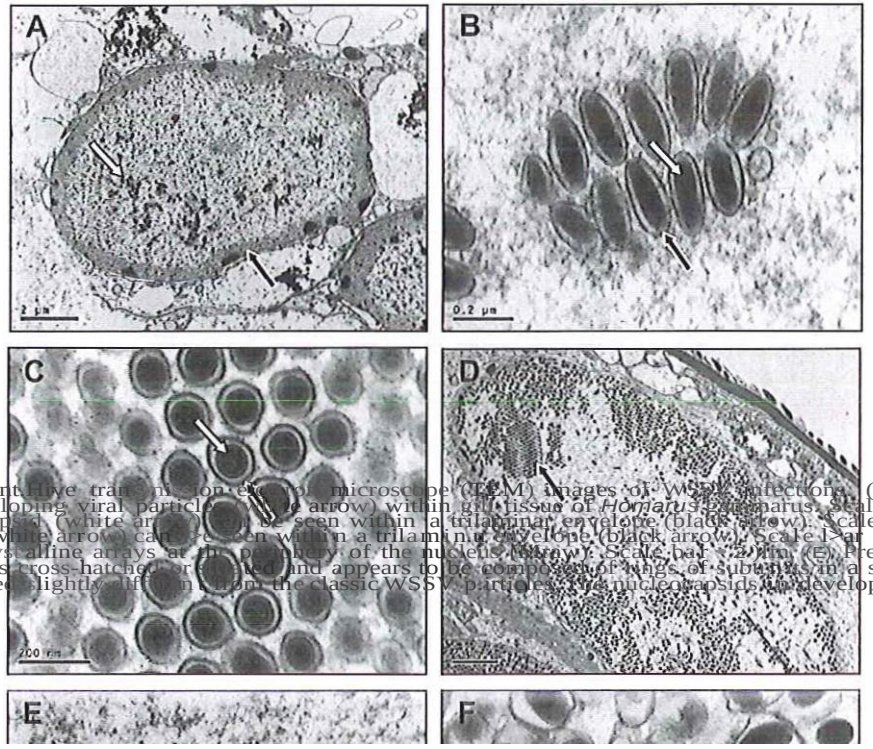


Fig. 3. Representative transmission electron microscope (TEM) images of WSSV infections. (A) Infected nuclei displaying marginalised host chromatin (black arrow) containing developing viral particles (white arrow) within gill tissue of *Homarus gammarus*. Scale bar = 2 µm. (B) Longitudinal cross section of WSSV virions, ovoid electron dense nucleocapsid (white arrow) can be seen within a trilaminar envelope (black arrow). Scale bar = 0.2 µm. (C) Transverse cross section of *WSSV* virions, electron dense nucleocapsid (white arrow) can be seen within a trilaminar envelope (black arrow). Scale bar 200 nm. (D) Heavily infected nucleus from *Eriocheir sinensis*, virions can be seen in paracrystalline arrays at the periphery of the nucleus (arrow). Scale bar = 2 µm. (E) Presumptive nucleocapsid material within the nucleus prior to envelopment, material appears cross-hatched or fibrillated and appears to be composed of rings of subunits in a stacked series (arrow). Scale bar = 0.2 µm. (F) Virion particles from *Carcinus maenas* appeared slightly different from the classic WSSV particles. The nucleocapsids in developing particles appeared slightly curved or 'u' shaped within the envelope. Scale bar = 200 nm.

4. Discussion

4.1. Fulfilling t/1e criteria for susceptibility

This study has demonstrated universal susceptibility to *WSSV* infection in a range of European decapod crustaceans, exposed to *WSSV* via a natural route (feeding). Furthermore, we show that *WSSV* can replicate and cause disease within European crustacean species at ambient water temperatures encountered in European waterways. Despite universal susceptibility, the manifestation of disease associated with prior infection with *WSSV* differs considerably between species: freshwater crayfish undergoing more rapid mortality and development of pathognomonic signs than most of the marine species tested. Despite becoming infected with *WSSV* following exposure, the European shore crab (*C. mae11as)* appeared most recalcitrant to the development of disease and may be considered as an asymptomatic carrier under those conditions utilised in the CLtrrent trials. Stentiford et al. (2009) list all of the species which have been shown by experimental and natural exposures to be susceptible to WSD according to EFSA guidelines. In this review, susceptibility criteria were partially fulfilled for several European species. Corbel et al. (2001) demonstrated replication and some evidence for topological location of the pathogen during exposure trials of C*pagurus* and C *111ae11as*. Extension of such work in the current study has shown that *WSSV* can replicate, cause pathology and that the virus remains viable {by bioassay), thereby fulfilling all four EFSA categories for susceptibility. There have also been several reports of susceptibility to *WSSV* in species of freshwater crayfish Uiravanichpaisal et al., 2001: Huang et al., 2001: Edgerton, 2004; Baumgartner et al., 2009; Soowannayan and Phanthura, 2011). *P.* leniuscu/us was listed by Stentiford et al. (2009) as fulfilling three of the four categories (replication, pathology and location) but not viability. Our studies provide confirmatory evidence of the latter by demonstrating viable passage to SPF *L. va1111amei.* In summary, we provide definite evidence for susceptibility to *WSSV* infection in *//.gammarus, C pagurus, C. maenas, N.* 1101vegicus, *E.* sinensis, *P.* /eniuscu/us and A *pallipes.* This is the first description of susceptibility to *WSSV* infection in *N. 1101vegicus, E. si11emis* and A *pal/ipes.*

4.2. Pathog110111011ic signs of WSSV infection it1 11011-mode/ crustacean llosts

Studies with pcnaeid shrimp have shown that WSSV targets crustacean tissues of ectodermal and endodermal origin. It is most commonly detected in the cuticular epithelium, particularly associated with the gill and other appendages, or in the epithelial layer surrounding the stomach (Lightner, 1996). Despite the wide susceptible host range to *WSSV,* few studies have focussed on the pathognomonic signs of infection (and disease) in these species. In the current study, no definitive external signs (such as white spots) were associated with infection or disease in non-model hosts. *WSSV* infection and WSD could however be detected using histology in a manner similar to that previously described for penaeid shrimp {Wongteerasupaya et al., 1995; Lightner, 1996; Lo ct al., 1997). Characteristic eosinophillic inclusion bodies were observed within the hypertrophic nuclei of *WSSV* infected hosts cells. In penaeids, such eosinophillic bodies are usually obse1ved in early stage *WSSV* infection, these staining more basophilic as disease progresses (Lightner, 1996). Although this indicates that the pathology within individual cells of WSSV-infected non-model hosts were at a relatively early stage, longer term exposme trials may have led to a higher frequency of basophilic types as obse1ved in penaeid hosts. Fed animals displayed fewer WSSV-infected nuclei per tissue section, this most likely due to the lower viral loading of single feed dose compared to those directly injected with viral inoculate.

WSSV inclusions were most evident in epithelial cells of the gills and other regions of sub-cuticulum, within haemocytes, connective tissue cells, fixed phagocytes, heart myofibrils and spongy pericardia! cells. The absence of a lymphoid organ in hosts other than members of the Superfamily Penaeoidea (including the genus *Litopenaeus)* (Rusaini and Owens, 2010) prevent this organ being used as a sentinel for viral infection in non-model hosts. However, characteristic pathognomonic signs comparative to those obse1ved within penaeids during WSD (Lightner, 1996), were seen in the fixed phagocytes of the hepatopancreas of non-model hosts, suggesting that this system is progressively degraded during pathogenesis of *WSSV* infection in non-model hosts. It is likely therefore that the fixed phagocyte system provides a comparative target (to the lymphoid organ) for *WSSV* in non-model crustaceans. Infection severity within specific tissues varied considerably from species to species though in the majority of cases, the tissues of the gill provided a reliable means to diagnose infection via histology. Although considerable variation in pathology was observed between fed and injected groups for some species, in the freshwater crayfish (*P. /e11i11sc11/11s, A. pallipes)* and Chinese mitten crab *(E.* sinensis) significant pathology was obsetved, even in fed animals, during the short duration of exposure trials. These results are suggestive of more rapid pathogenesis of *WSSV* infection in these species. In some instances, WSSV-infected host nuclei were evident within the supporting cells and other connective tissue cells surrounding the oocytes and spermatozoa, a feature previously highlighted in penaeid shrimp by Lo et al. (1997). Whether this reflects an ability for vertical transmission in non-model crustacean hosts remains to be demonstrated.

Whilst PCR (and sequencing) was utilised to confirm the presence of *WSSV* in non-model crustacean hosts, it should be noted that PCR alone is not adequate to confirm host susceptibility *per se.* Since the technique simply detects the presence or absence of elements of the viral genome, its sole use cannot be applied to unequivocally demonstrate host cell infection by a given pathogen. For this reason, PCR data is not utilised to assess host susceptibility according to EFSA (see Stentiford et al., 2009).

4.3. The concept of relative susceptibility

Whilst adding to the remarkable list of species susceptible to *WSSV* infection (Stentiford et al., 2009), we have highlighted a significant variation in pathogenesis in the different non-model species investigated in this study. By considering this data alongside the mortality rates observed during the period of specific trials we have defined three broad categories of relative susceptibility to *WSSV* infection and disease in crustacean hosts {Table 4). In such a way, the concept of 'susceptibility' to infection and disease in crustaceans can be considered both at the species level. but also at the level of the individual, with factors such as exposure dose, moult status, physiological condition and the presence of pre-existing pathogen infections disease likely playing a part in progression of infection to disease, and the propensity for mortality to occur. We have shown that some species such as the freshwater crayfish species *P. /e11i11sc11/11s* and *A. pal/ipes* display characteristic pathognomonic signs and undergo rapid mortality following single-dose feeding of WSSV-contaminated shrimp carcasses. In this context, they are classified alongside penaeid shrimp as 'highly susceptible' to *WSSV* infection and its disease, WSD. In contrast, *C. 111ae11as* does not display widespread pathognomonic signs, nor does it undergo significant mortalities when exposed to *WSSV,* either via feeding or direct injection. This species is therefore considered to display 'low susceptibility' to WSD (despite being able to be infected with *WSSV* via both feeding and direct injection). Low susceptible hosts,

Table 4
White Spot Disease susceptibility categories and classification for European crustaceans.

	Mortdlity	Pathology	Species
Type 1 – high	High mortality in both injected and fed exposures	Classic white spot pathology obvious in tissues from both fed and injected exposures	Penaeid shrimp, *Aust ropotamobius paltipes*, *Pacifastacus leniusculus*, *Erioc/Jeir si11e11sis*
Type 2 – medium	High mortality in injected exposure, little or no mortal ity in fed exposure	Classic white spot pathology obvious in tissues from injected exposure. Little or no pathology evident in fed exposure	*Homanis gammonis* , *Nepl1rops noiveg icus*, *Cancer pagurus*
Type *3* – low	Low *level* mortality in both injected and fed exposures	Lillie or no pathology evident in either injected or fed exposures	*Carclnus maenas*

which presumably also harbou r low viral titres, can however passage sufficient doses to cause disease and mortality in high ly susceptible hosts (such as the SPF *L vamramei* utilised here). In this context, they are considered as asymptomatic carriers for WSSV. Further work is curren tly undetway withi n our laboratoiy to investigate the potential for initiation of disease in low susceptible hosts (carriers) duri ng ambient stressor events.

4.4. T/1e basis of differential susceptibility to WSSV

One species, C. *maenas,* clearly showed a lower suscepti bilily lo WSSV when com pared to other Eu ropea n decapod species; this supports the fi ndings of Corbel et al. (2001). WSSV has been classified with in a new genus *Wlrispovirus* in the family *Nimaviridae* and was na med White Spot Syndrome Vi rus 1 by the ICTV (Mayo, 2002a. 2002b; Vlak et al.. 2005). It is currently the only member of this genus bu t Vlak et al. (2005) state that as this is a newly recognised fa mily the species orga nisation may change once existing a nd new isolates are d iscovered. Vlak et al. (2005) also tentatively list so-called 8 virus from C. *maenas*, 82 virus and), (tau) virus from Carcinus *mediterraneus*, and 8aculo-A and 8aculo-8 viruses from *Cal/inectes sapid11s* as potential members of the genus. The 8, 82, and 8aculo-8 viruses are extremely similar in terms of size, shape and morphogenesis to that reported from WSSV; in fact these vi ruses appear morphologically i nd istinguishable a nd it has been suggested that they may in fact be ancestral forms of WSSV (8ona mi and Zhang, 2011). Anecdota l evidence suggests that du ri ng initia l outbreaks of WSSV in Asian penaeid fa rms, broodstock shrimp were fed wi th carcasses of portu nid crabs (Prof. Grace Lo, Pers. Com m.). It is conceivable therefore that the virus now plagui ng the global penaeid shrimp farmi ng i ndu stry may have its origi ns i n crabs. As such, these ancestral hosts may well possess genome-based mechanisms to deal with vi ruses such as WSSV. In this context, h istorical exposu re of *Carcinus* spp. to 8/02 virus, which is simila r if not identica l to WSSV at least at morphological level, may have inferred inherited recalcitrance to WSSV. Further work is now requ ired to rediscover 8/82 vi rus and to perform comparative phylogenetic analysis against WSSV from penaeid hosts.

4.5. Predicting consequence for European crustacean stocks

The European crustacean industiy centres on com modity i mports(e.g. frozen tropica l sh rimp) and on wild fisheries, with li mi ted aqu,1Culture production of crustaceans (Stcntiford et al., 20 I 0). Wild fisheries for ma rine crustaceans are considered key resou rces in the Eu ropean a rea with total fishery production of crustaceans from Eu ropean waters totalling 120,000Mt i n 2008 (www.fao.org/figis). Im port of com mod ity 1Products has been highlighted as a possible route of introduction of WSSV to new locations (Nu nan et al., 1998; Durand et al.. 2000; Ba teman et al., 2012). In some regions, shrimp i mported for huma n consumption are d iverted for use as angling bai ts; this identified as a significa nt risk factor for the introduction of viral crustacean pathogens (see 8iosecu rity Australia, 2006). In this study we have demonstrated susceptibility of several European decapods to WSSV-i nfection following a single meal of contami nated feed and fu rther, that the vi rus remains viable for passage to other susceptible hosts. Significantly, this was even shown to occur when the donor host d isplayed show no chMacteristic signs of infection or d isease. Further work is needed to assess the fu ll risk of imported commod ity product to European crustacean species, pa rticularly due to anecdotal' in formation rela ting to the use or raw shrimp commod ity as a ngling bait i n UK freshwa ter and marine envi ron ments.

Al though we have shown that Europea n species are susceptible to WSD it is not known whether infection would lead to transmission, establishment, disease and elevated mortality in exposed populations. Chapman *et* al. (2004) comment that severe i nfections are rarely seen in the wild and suggest that this may be due to limited monitori ng of such populations, the elevated vulnerability of diseased individuals to predation, the swift progression of d isease, or i n fact to the ra rity of the event occurri ng. Chang ct al. (2001) have shown that WSSV is present within wild populations of bl ue crabs (C. sapidus) along the coast of the USA but that since these populations do not appear to be symptomatic for WSD, it was suggested they instead act as rcsc1voi r hosts. Wu et al.(200 I) showed that high cu mu lative mortal i ties, plus extended periods of mortality due to WSSV infection were associated wi th can nibalism. We have shown that the virus is viable after a single feed so the li keli hood is that the ca rcasses will be consumed and infect ion passed on, it is likely that multiple feeds of WSSV infected tissues will result i n an increased viral loading and increased levels of mortality.

I lowcvcr, it is important to note thal WSSV has previously been shown to be carried i n shrimp populations at low i ntensities in low-stress cu lture conditions wi thout mortality events occu rri ng (Tsai ct al., 1999).It is possi ble therefore that even highly susceptible species may be able to tolerate infection while conditions are favourable, but may succumb to the disease when sub-optimal conditions occur. Disease outbreaks on shrimp farms are known to be induced by stressors such as rapid change i n sali nity and d rop in temperature (Vidal et al.. 2001; Granja et a!., 2003; Guan et al., 2003). Corteel et al. (2009) have shown that shrimp were more susceptible to WSD i nfection via i mmersion after molti ng tha n in the period before molting and tha t woundi ng aids infection.I low th is latent disease or 'carrier' status wi ll alter during stressfu l events such as mou l ting, reprodu ction and d isease with non-penaeid species is u nclear and requ ires further stud ies. Longer term stud ies lhan those presented here are necessary to add ress the issue of whether low-level infections in wild (non-model) crustacea ns would persist as such for extended periods or whether stressors cause progression of infection to disease at the individual and popula t ion levels.

Although most commonly associated with disease in tropical shrimp, one of the first reports of WSSV described mortalities of Morsupenoeus joponicus in water temperatures of 19 °C (Takahashi et al., 1994). This water temperature is experienced within the Southern European region over several seasons and even in Northern Europe during summer months. Due to its lower temperature tolerance, Mo. joponicus is farmed in Southern Europe (Stentiford and Lightner, 2011). Although temperature has been shown to affect the replication of WSSV and the expression of WSD in penaeid shrimp and crayfish (both undergoing reduced mortality rates and lower viral loading in both hypothermic and hyperthermic conditions), disease develops rapidly when infected animals are returned to optimal conditions for the pathogen (Vidal et al., 2001; Granja et al., 2003, 2006; jiravanichpaisal ct al., 2004; Du et al., 2006, 2008; Rahman et al., 2006, 2007a, 2007b). These findings suggest that susceptible hosts exposed to *WSSV* at sub-optimal temperatures for viral replication may not develop disease but that disease (and mortality) may occur when conditions become more favourable for viral replication. During this time however, they may setve as a resetvoir to spread the virus to other naive hosts. Transmission from asymptomatic carrier status hosts to other hosts of the same species or disease susceptibility (e.g. low, medium, high) will be required to assess the true risk of WSSV to wild decapod crustacean populations following introduction of the pathogen. This data is critical in assessing the consequence of WSSV infection to European crustacean populations.

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11.6.2 Bateman, K.S., Munro, J., Uglow, B., Stentiford, G.D. (2012) Susceptibility of juvenile European lobster *(Homarus gammarus)* fed high- and low-dose White Spot Syndrome Virus (WSSV) infected shrimp products. Diseases of Aquatic Organisms, 100, 169-184.

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Susceptibility of juvenile European lobster *Homarus gammarus* to shrimp products infected with high and low doses of white spot syndrome virus

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ABSTRACT: White spot syndrom e virus (WSSV) is the most important pathogen known to aff ect the sustainability and growth of the global penaeid shrimp farming industry. Although most commonly associated with penaeid shrimp farmed in warm waters, WSSV is also able to infect, cause disease in and kill a wide range of other decapod crustaceans, including lobsters, from temperate regions. In 2005, the European Union imported US$500 million worth of raw frozen or cooked frozen commodity products, much of which originated in regions positive for white spot disease (WSD). The presence of WSSV within the UK food market was verified by means of nested PCR performed on samples collected from a small-scale survey of supermark et commodity shrimp. Passage trials usi ng inoc11h1m derived from commodity shrimp from supermarkets and delivered by injection to specific pathogen-free Pacific white shrimp *Litopenaeus vannamei* led to rapid mortality and pathognomonic signs of WSD in the shrimp, demonstrating that WSSV present within commodity shrimp was viable. We exposed a representative European decapod crustacean, the European lobster Homarns gammarns, to a single f eeding of WSSV-positive, supermarket-derived commodity shrimp, and to positive control material *(L. vamwmei* infected with a high dose of WSSV). These trials demonstrated that lobsters fed positive control (high dose) frozen raw products succumbed to WSD and displayed pathognomonic signs associated with the disease as determined by means of histology and transmission electron microscopy. Lobsters f ed WSSV-positive, supermarket-derived commodity shrimp (low dose) did not succumb to WSD (no mortality or pathognomonic signs of WSD) but demonstra ted a low level or latent infection via PCR. This study confirms susceptibility of H. gammarns to WSSV via single f eedings of previously frozen raw shrimp prod ucts obtained directly from supermarkets.

KEY WORDS: White spot syndrome virus · WSSV · Commodity · Transmission · Risk assessment

INTRODUCTION

White spot disease (WSD) caused by white spot syndrom e virus (WSSV) has caused devastating losses in the global shrimp farming indust ry since its discovery in the 1990s. WSD is estimated to have caused at least US$10 billion in losses to the penaeid shrimp production systems since 1993 (Stentiford et al. 2012). Cult ivation of marine shrimp accounts for a large propor tion of global aquatic food production. In 2007, global shrimp production was over 3 million t, with a value of US$13 billion (www.fao.org/fishery/statistics/global-aquaculture-production/en). An n ually, the European Union (EU) imports around

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US$500 million worth of farmed shrimp commodity products, the majority of which are raw frozen or cooked frozen penaeid shrimp (Stentiford et al. 2010). Owing to the wide global distribution of WSSV, large proportions of this product originate from regions in which WSSV is endemic (Lightner 2003, OIE Collaborating Centre for Information on Aquatic Diseases www.cefas.defra.gov.uk/idaad/).

Aquaculture production of crustaceans is limited within the EU and only accounts for around 200 t yr^{-1} (www.fao.org/figis). Conversely, the total fishery production of crustaceans from European waters totalled almost 400 000 t in 2004, with a large majority of this comprising marine prawns (ca. 200 000 t), lobsters (ca. 60000 t) and crabs (ca. 85 000 t). In freshwaters, capture fisheries are solely composed of crayfish (ca. 6000 t). As a result, wild fisheries for marine crustaceans are considered key resources in the European maritime area, and in many countries (such as the UK) they rank above several important finfish species in terms of production quantity and value. Despite the high economic value of this fishery the exposure and consequent risks to wild populations of imported commodity products that may inadvertently enter the aquatic environment are largely unknown.

The European Commission (EC) Directive 2006/88 (on animal health requirements for aquaculture animals and products thereof and on prevention of and control of certain animal diseases; http://ec.europa.eu/fisheries/cfp/aquaculture/facts/index_en.htm) lists 3 notifiable crustacean diseases, all caused by viral pathogens and generally associated with farmed penaeid shrimp from tropical and subtropical regions. These are WSD, caused by WSSV, yellowhead disease, caused by yellowhead virus (YHV), and Tama syndrome, caused by Taura syndrome virus (TSV). All have caused severe economic losses in the global shrimp farming industry owing lo their transmissible nature, their potential for socio-economic impact and their likelihood for spread via the international trade of animals and animal products. The listing of these diseases implies that their detection in crustaceans from European waters would be subject to compulsory control measures and formally recognises the potential for diseases traditionally associated with tropical penaeid shrimp to have an impact upon the sustainability of commercially and ecologically significant crustacean populations in the European aquatic network. YHV and TSV are listed as exotic to the EU, whereas WSSV is listed as non-exotic (Stentiford et al. 2010, Stenliford & Lightner 2011). In the European context, WSSV is considered to be the most significant threat owing to its wide host range, pathogenesis in temperate conditions and its potential for rapid spread (Stentiford et al. 2009, 2010).

EC Directive 2006/88 requires health certification for import of commodity products unless these products are destined for further processing, packaged in 'retail sale' packages and labelled in accordance with EC Regulation 853/2004. Therefore, products (live or frozen) imported directly for human consumption are not covered by the directive and do not need to originate from areas designated free from listed pathogens, even when imported to confirmed 'disease free' member states. Nunan et al. (1998) and Hasson et al. (2006) demonstrated that frozen commodity shrimp imported to the USA for human consumption tested positive for WSSV by PCR. Previous transmission trials with conunodity shrinlp have shown that sufficient viable virus remains in frozen commodity products to induce mortality of Pacific white shrimp *Litopenaeus vannamei* (Durand et al. 2000, Hasson et al. 2006). Oidtmann & Stentiford (2011, p. 479) highlighted the risk of these commodity products to naive crustacean populations by stating '...if such shrimp were introduced into a country free from the pathogen, and crustaceans in the receiving country were exposed to infected tissues per os, there is a considerable risk that such exposed crustaceans may become infected and the infection established in domestic populations of crustaceans'.

Stenliford & Lightner (2011) reported the presence of WSSV within European shrinlp farms in 2000 after shrinlp on these farms were exposed to WSSV-infected shrimp carcasses; however, the virus has not been discovered in wild European crustacean populations to date. WSSV can be successfully transmitted to a range of hosts from European marine and fresh waters. These include the marine crab species *Liocarcinus depurator* and Necorn *puber,* the commercially significant marine erab *Cc?nccr pagurns* and freshwater crayfish of the genera *Astac11s* and *Pacifastacus* (Corbel et al. 2001, Jiravanichpaisal et al. 2001, 2004). These authors and others noted the unusually large host range for this virus, its potential for infection of freshwater, brackish and marine species and the potential sensitivity of naive European crustaceans to WSSV. These studies also demonstrated the high potential for spread to hosts from European waters, particularly from imported shrimp products and from imported brood stock or larvae for new cultivation ventures in the region and beyond. Purthermore, they demonstrated that temperature may affect host susceptibility and WSSV pathogenicity (Jiravanichpaisal ct al. 2004).

In the present study, inlported fresh and frozen shrimp products were screened for the presence of WSSV nucleic acid. Imported commodity products were also tested for the potential to act as a source for transmission of WSSV to a known WSSV-susceplible host, *Litopenaeus vannamei*, and an important species in the European crustacean fishery, the European lobster Homarns *gammarus*. Juvenile H. gcimmcirns obtained from a commercial hatchery in the UK were exposed to known high-dose (high WSSV viral load) feed at 2 temperatures to determine initial susceptibility of this species. In addition, juvenile *H.* ganunarus were fed high-dose WSSV-infected *L. vannamei* carcasses and low-dose (low WSSV viral load), supermarket-derived commodity product. Viral loading in feed was assessed before feeding using quantitative PCR. The susceptibility of juvenile lobsters to WSSV was assessed using histopathology, transmission electron microscopy (TEM) and nested PCR assays specific for WSSV.

MATERIALS AND METHODS

Market sampling and passage conditions

Prozen uncooked shrimp from various global production regions (Table 1, location as defined on the package) were purchased from supermarkets and from a large fish market in the UK. Products were tested for the presence of WSSV using a nested PCR assay recommended by the Office International des Epizooties (OIE 2009) with minor modifications, as described below. Products displaying positivity via PCR were used for production of inoculates and feeds for subsequent passage trials. All passage trials were conducted within the biosecure exotic diseases facility at the Centre for Environment, Fisheries and Aquaculture Science (Cefas) laboratory in Weymouth, UK, and used local, filtered and UV treated seawater. Day length was set at 14 h, night was at 10 h with a 30 min fade to sinrnlate dusk and dawn. Temperature was regulated according to the experimental conditions required for *Litopenaeus* vcinnamei (Holthuis 1980) and for *Honwrus gammarus* as appropriate.

Viability of WSSV in commodity products

Commodity shrimp from Ecuador and Vietnam that had been confirmed positive for WSSV via nested PCR were macerated using a sterile razor blade prior lo homogenisation in sterile saline (4 ml of saline per gram of minced tissue) using a blender until tissues were liquefied. The homogenate was centrifuged at 5000 x *g* for 20 min at 4°C lo pellet solid debris prior lo the supernatant being diluted 1:20 with sterile saline and filtered (0.45 pm) to form the inoculum. Individual specific pathogen free (SPP) *Litopenaeus vannamei* (approxinlately 5 g in weight) obtained from the Centre for Sustainable Aquaculture Research at the University of Swansea, UK, were inoculated via intramuscular injection of the diluted viral homogenates al a rate of 10 pl g^{-1} shrimp weight. Water temperature was held constant at 24°C. Shrimp were monitored throughout the day for 5 d, and dead and moribund shrimp were removed

Table 1. Commodity shrimp tested for presence of white spot syndrome virus (WSSV) by nested PCR

Species	Origin	Source and description	Nested PCR (% positive)
Litope1lc1e11s vdnnamei	Ecuador	Supermarket; headless, shell off	65
L. Vd/J/Jc11llei	Honduras	Supermarket; headless, shell off	80
Penc1eus *monodon*	Indonesia	Supermarket; headless, shell of f	0
L. vc111nc1111ei	Thailand	Supermarket; headless, shell off	5
P. monoclon	Thailand	Supermarket; headless, shell off	0
P. monoclon	Vietnam	Supermarket; headless, shell off	100
P. monoclon	Bangladesh	Market; whole animal	0
P. monoc/011	Bangladesh	Market; whole animal	0
P. monoc/011	Bangladesh	Market; headless, shell on	0
L. vam1nmei	Brazil	Market; whole animal	0
L. vannamei	China	Market; headless, shell on	0
P.111011oc/011	India	Market; whole animal	0
P.monoclon	Indonesia	Market; whole animal	0
L. vannamei	Indonesia	Markel; whole animal	0
Farfc111tepenc1e11s notinlis	Senegal	Markel; headless, shell on	0
P. monoclon	Vietnam	Market; headless, shell on	20

from the experimental tanks; pleopods were fixed in ethanol for molecular analysis, gills were taken for TEM and the carcass was fixed whole in Davidson's seawater fixative for histopathological confirmation of WSD.

Preparation of WSSV positive low- and high-dose feeds

For preparation of high-dose feeds, viral inoculates of WSSV were obtained from the OIE reference laboratory at the University of Arizona (UAZ), USA. The OIE isolate of WSSV (UAZ 00-173B) was generated in *Litopenaeus vannamei* from an original outbreak in *Fenneropenaeus chinensis* (Holthuis 1980) in China in 1995. Subsequent passages of this isolate into naive *L. vannamei* held at the Cefas Weymouth laboratory have demonstrated continued pronounced virulence of this isolate (data not reported here). High-dose WSSV-infected shrimp carcasses were prepared by direct injection of the OIE isolate into SPF *L. vannamei* as detailed above. The viral loading in high-dose feeds was assessed using quantitative PCR (qPCR). Abdominal tissues from *L. vannamei* that were confirmed positive for WSSV via histology and nested PCR were macerated into approximately 2 to 3 mm blocks using a sterile razor blade. For preparation of low-close feeds, individual supermarket-derived shrimp (abdominal section) originating from Ecuador, Vietnam and Honduras, and confirmed positive for WSSV via nested PCR, were macerated. The viral loading in low-dose feeds was assessed using qPCR.

Lobster feeding trials with low- and high-dose products

Juvenile *Homarus gammarus* were obtained from the National Lobster Hatchery in Padstow, Cornwall, UK. Lobsters were at Moult Stage 4 and were approximately 2 mo of age. To prevent conflict, juvenile lobsters were housed individually in custom-made 'Orkney pots' that were suspended in the upper water column of each 30 l experimental tank. The pots contained a perforated base to allow for water circulation.

In Trial 1, water temperature was held constant at 15°C. Lobsters in Tank 1 (n = 20) received a single ration (~0.05 g) of high-close WSSV-infected shrimp tissue on Day 0 and a further ration on Day 7. Lobsters in Tank 2 (n = 20) received a single ration (~0.05 g) of uninfected shrimp tissue on Day 0 and another on Day 7. Between these times lobsters were fed on 3 mm Royal Oyster pellets at a ration of approximately 3 to 4% body weight (bw) d^{-1} for 10 d.

In Trial 2, water temperature was held constant at 22°C. Lobsters in Tank 1 (n = 20) received a single ration (0.05 g) of high-dose WSSV-infected shrimp tissue on Day 0 and a further ration on Day 7. Lobsters in Tank 2 (n = 20) received a single ration (0.05 g) of uninfected shrimp tissue on Day 0 and another on Day 7. Thereafter, lobsters in both tanks were fed on 3 mm Royal Oyster pellets at a ration of approximately 3 to 4% bw d^{-1} for 10 d.

In Trial 3, water temperature was held constant at 20°C. On Day 0 lobsters in all tanks (n = 20 for each tank) received a single ration of feed (0.05 g). Lobsters in Tank 1 received high-close WSSV-infected shrimp tissue, those in Tank 2 received uninfected shrimp tissue, those in Tank 3 received low-close commodity shrimp originating from Ecuador, those in Tank 4 received low-dose commodity shrimp originating from Vietnam and those in Tank 5 received low-dose commodity shrimp originating from Honduras. Thereafter, lobsters in all tanks were fed on 3 mm Royal Oyster pellets at a ration of approximately 3 to 4% bw d^{-1} for 12 d.

In all trials, tanks were observed regularly throughout daylight hours. Dead and moribund animals were removed from each tank. Cheliped samples from all dead, terminally morbid and live (at the end of the trial period) animals were fixed in 100% ethanol for PCR. Remaining carcasses were prepared for histology and, selectively, for TEM. Juvenile lobsters were prepared for histology by making an incision along each side of the carapace, placing whole animals into histological cassettes and fixing them immediately in Davidson's seawater fixative. From selected juvenile lobsters, the abdomen was removed from the cephalothorax and the carapace opened as described for histology. The abdomen was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature; cuticular epithelium and gills were dissected out of these fixed animals and processed for TEM.

Histology

For all tissues, fixation was allowed to proceed for 24 h before samples were transferred to 70% industrial methylated spirit. Fixed samples were dehydrated and infiltrated with paraffin wax in a vacuum infiltration processor using standard protocols. Sections were cut at a thickness of 3 to 5 µm on a rotary microtome and were mounted onto glass slides

before staining with haematoxylin and eosin (H&E) and Feulgen stains. Stained sections were analysed by light microscopy (Nikon Eclipse E800) and digital images and measurements wer e taken using the Lucia'•M Screen Measurement System (Nikon UK).

Transm ission electron m icroscopy

Tissues were f ixed in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buff er (pH 7.4) for 2 h at room temperat ur e and rinsed in 0.1 M sodium cacodylate buff er (pH 7.4). Tissues were post-fixed for 1 h in 1% osmium tetroxide in 0.1M sodium cacodylate buff er. Samples were washed in 3 changes of 0.1 M sodium cacodylate buff er before dehydration through a graded acetone series. Samples were embedded in Agar 100 epoxy (Agar 100 pre-mix kit medium, Agar Scientific) and polymerised overnight at 60°C in an oven. Semi-thin (1 to 2 µm) sections were stained with toluidine blue for viewing under a light microscope to identif y suitable target areas. Ultrathin sections (70 to 90 nm) of these areas were mounted on uncoated copper grids and stained with 2 % aqueous uranyl acetate and Reynolds' lead citrate (Reynolds 1963). Grids were examined using a JEOL JEM 1210 transmission electron microscope and digital images captured using a Gatan Erlangshen ES500W camera and Gatan Digital Micrographrn software.

Detection of WSSV in commodity shrimp via PCR and qPCR

Total shrimp DNA was extracted from the com - mod ity product (tail muscle) using the High Pure PCR Template Preparat ion Kit (Roche Diagnostics) following the manuf acturer's protocol for the isolat ion of nucl eic acids f rom mammalian t issue. The quality and quantity of the DNA was determined with a Na nocl rop spectrophotomet er (Thermo Pisher Scientific). For use in the qPCR, prior to analysis, total DNA was standardised at 20 ng µl^{-1} to remove variations in vira l load owing to variation in tissue weight or extraction efficiency between samples.

Nested PCR for high-dose fed lobsters

WSSV DNA presence was assessed by using the nested PCR assay of Lo et al. (1996) with mi nor modifications (13. Poulos, UAZ, pers. comm.). Pirst, a product of 1447 bp was amplif ied using the primer pair 146Fl (5'-ACT ACT AAC TIC AGC CTA TCT AG-3') and 146R1 (5'-TAA TGC GGG TGT AAT GTT CTT ACG A -3'), followed by an approximate 941 bp product in the nested reaction using prim er pair 146F2 (5'-GTA ACT CCC CCT TCC ATC TCC A-3') and 146R2 (5'TAC GGC AGC TGC TGC ACC TTG T-3'). For the first round of amplif ication (primer pair 146F1/146R1) each 25 µl PCR reaction contained the following: 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 200 µM of each dNTP, 0.31 µM of each prin1er, 2.5 U *Taq* polymerase and 1 µl genomic DNA (20 to 50 ng total). Amplif ications were performed wiU1an initial denaturation temperature of 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, 62°C for 30 s and 72°C for 30 s, with a final elongation step at 72°C for 2 min. Reaction conditions and reagent concentrations were the same for the second round of amplification using the 146F2/146R2 prin1er pair; however, 0.5 µl of the first round of amplif ication was used as a template in place of genomic DNA. Amplif ied products were resolved on a 2 % (w/v) agarose/TAE (40 mM Tris-acetate, pH 7.2, 1mM EDTA) gel containing 1.0 lg 1111^{-1} ethidium bronlide and visualised under UV irradiation.

Nested l'CR for commodity product and low-dose f eed lobsters

The initial PCR met hod described above was modified to include an internal cont rol to ensure f alse negatives did not occur without displaying any noticeable reduction in sensitivit y (data not shown). To ensure validity of each sample, an internal control was included in each PCR sample. The internal control, consisting of a single primer set 5'-GTG GAC ATC CGT AAC CAC CTG TAC G-3' (forward) and 5'-CTC CTG CIT GCT GAT CCA CAT CTG C-3' (reverse), amplif ied a 201 bp product specific towards beta-acti n, which is found in all eukaryotic cells. Both outer and inner nested PCR was performed in a 50 µl reaction volume containing 0.15 pM of primers, 0.25 pM dNTPs, 1.25 U GoTaq (Promega), 2.5 mM $MgCl_2$, 5x buff er and 2.5 µl of template. Amplification was performed as above.

qPCR

In individual shrimp demonstrating positivity for WSSV via nested PCR, and to ensure suitability for f ur ther utilisation for the feeding trial to naive *Homarns* gammarns, a DNA fragment of 69 bp representing the upstream primer plus the probe and the

downstream primer was cloned into the pGEM-T easy vector (Promega) and then transformed into JM109 competent cells (Promega) following the manufacturer's instructions. The plasmid was extracted using QIAprep spin Miniprep Kit (Qiagen) and the concentration was determined using a Nanodrop spectrophotometer. The copy number of the plasmid DNA containing the 69 bp insert was estimated and a series of dilutions were made to use as standards. The primers and probe used for the quantification of WSSV in study were developed by Durand & Lightner (2002). The primers were WSS1011F (5'-TGG TCC CGT CCT CAT CTC AG-3') and WSS1079R (5'-GCT GCC TTG CCG GAA ATT A-3'). The TaqMan probe (5'-AGC CAT GAA GAA TGC CGT CTA TCA CAC A-3') was synthesized and labeled with the fluorescent dyes 5-carboxyfluroscein (FAM) on the 5' end and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end. The TaqMan assay used was adapted from Durand & Lightner (2002). Briefly, 100 ng of total DNA was added to TaqMan Universal mastermix (Applied Biosystems) containing 0.3 µM of each primer and 0.15 µM of TaqMan probe in a final volume of 25 µl. Amplification and detection were performed using an ABI Biosystems TaqMan machine. The reaction mix was subjected to an initial temperature of 50°C for 2 min, then 1 cycle at 95°C for 10 min, 50 cycles at 95°C for 15 s and 60°C for 1 min. Quantification of the number of WSSV copies in samples to be used for feeding trials were determined by measuring C_T values and using the standard curve to determine the initial copy number per nanogram total DNA. Each unknown sample was analysed in triplicate and the mean calculated.

Sequencing

Selected reactions were analysed on an ABI 3130 Avant Genetic Analyser to confirm the specificity of the PCR. The final product was compared with known sequences using Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) to determine phylogenetic homology. This is in line with the OIE confirmatory technique for WSSV (Claydon et al. 2004).

RESULTS

Commodity shrimp product screening for WSSV

The prevalence of WSSV in commodity shrimp imported into the UK for human consumption ranged from 0 to 100 %. Of the batches of shrimp purchased from supermarkets, 66 % (4/6) tested positive for WSSV, and the within-bag prevalence ranged from 0 to 100%. Of the shrimp samples purchased from fish markets, 10% (1/10) were also positive for WSSV (Table 1).

Viability of WSSV in commodity shrimp products

Litopenaeus vannamei injected with homogenised WSSV positive commodity shrimp from either Ecuador or Vietnam experienced 100 and 40% mortality, respectively, within 3 d post-injection (Fig. 1). Nested PCR analysis (Fig. 2) indicated that all shrimp injected with commodity inoculate from Ecuador were positive for WSSV, and 4 of these animals displayed infection detectable in the first round of PCR, indicating a pronounced disease status (Fig. 2A). Shrimp injected with commodity inoculate from Vietnam were similarly all positive for WSSV (Fig. 2B), but only weak bands were present in the second round of PCR, suggestive of a lower level infection. Histological examination of these shrimp demonstrated the characteristic pathology associated with WSSV infection (Fig. 3); shrimp injected wilh commodity inoculate from Ecuador displayed signs of advanced WSD, whilst shrimp injected with commodity inoculate from Vietnam displayed lower grade, albeit characteristic, lesions associated with WSSV infection. Shrimp displaying advanced WSD identified from histopathology were further selected for TEM analysis. Electron microscopy revealed large

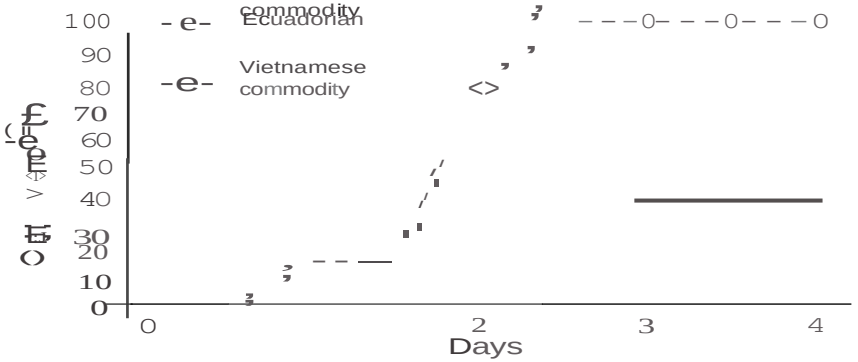

Fig. 1. *Litopenileus vm11rnmei*. Mortality dntn of shrimp exposed to WSSV

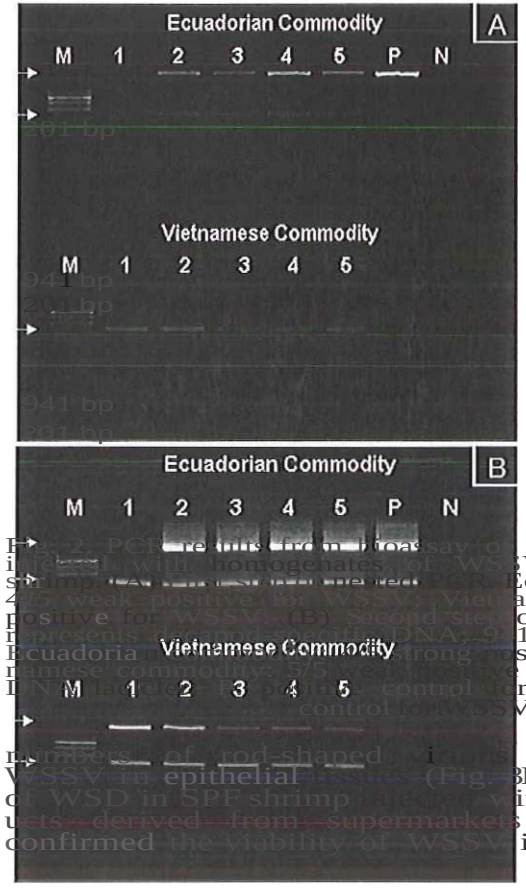

Fig. 2. PCR assay of *Litopenaeus vannamei* infected with homogenates of WSSV-infected commodity shrimp. (A) First step of nested PCR. Ecuadorian commodity: 4/5 weak positive for WSSV. Vietnamese commodity: *0/5* positive for WSSV. (B) Second step of nested PCR. 201 bp represents host control DNA. 941 bp represents WSSV. Ecuadorian commodity: 5/5 strong positive for WSSV; Vietnamese commodity: 5/5 positive for WSSV. M: 100 bp DNA ladder; P: positive control for WSSV; N: negative control for WSSV

numbers of rod-shaped virions synonymous with WSSV in epithelial tissues (Fig. 3E,F). Manifestation of WSD in SPF shrimp fed with commodity products derived from supermarkets and fish markets confirmed the viability of WSSV in such products.

Viral loading in low- and high-dose feeds

The WSSV-infected shrimp used in the high-dose challenge had an equivalent viral loading of 3.65×10^5 copies ng^{-1} total DNA. The supermarket-derived commodity shrimp used in the low-dose feed challenge had equivalent viral loadings of 5.16×10^2, 4.68×10^1 and 1.04×10^2 copies ng^{-1} total DNA in the Honduran, Ecuadorian and Vietnamese shrimp, respectively.

Lobster feeding trials: high-dose products

Cumulative mortality of WSSV-fed lobsters in Trial 1 (15°C) reached 5% on Day 1 and 10% by Day 10 of the trial. In Trial 2 (22°C), cumulative mortality of WSSV-fed lobsters reached 40% by Day 3 and 55% by Day 6. No further mortalities occurred between Day 6 and Day 10. Cumulative mortalities in control tanks in Trials 1 and 2 reached 15% by the end of the 10 d trial (Fig. 4).

Two moribund or recently dead lobsters in Trial 1 (15°C) and 8 in Trial 2 (22°C) displayed histopathological lesions typical of WSD in other crustacean species, including penaeids. WSD-associated lesions were most apparent in the gill, haemopoietic centres, haemocytes, connective tissues, stomach epithelium and particularly the cuticular epithelium. Cellular changes in each of these target sites included nuclear hypertrophy, margination of chromatin at the nuclear periphery and the presence of a single basophilic to strongly cosinophilic inclusion that occupied a large proportion of the host nucleoplasm. In some cases, this inclusion was separated from the nuclear periphery by an unstained zone (Fig. 5A-E). Feulgen-positive staining of affected nuclei demonstrated the DNA composition of the inclusion and of the darkly staining nuclear periphery (Fig. SF). The connective tissues and cuticular epithelium of the lin1bs appeared to be particularly heavily affected with an apparent loss of tissue mass in these regions and the presence of necrotic cellular debris and remnant WSSV inclusions. The hepatopancreas, midgut and skeletal muscle of infected lobsters appeared to be unaffected. Of those lobsters that were fed WSSV-infected material and survived the experimental period, none exhibited the pathologies described above.

Semi-thin sections (Fig. 6A) and TEM sections (Fig. 6B-F) of lobsters displaying the aforementioned pathologies confirmed the presence of intranuclear inclusions and oval-shaped viral particles, typical of WSSV, within epithelial cell nuclei. Viral particles were elliptical to short-rod shaped in longitudinal sections and round in transverse sections (Fig. 6C,D). Virions consisted of an electron dense capsid, bound by a clear envelope, surrounded by a double membrane. Virions at various stages of development and apparent assembly could be observed embedded within a granular matrix that corresponded to a viral

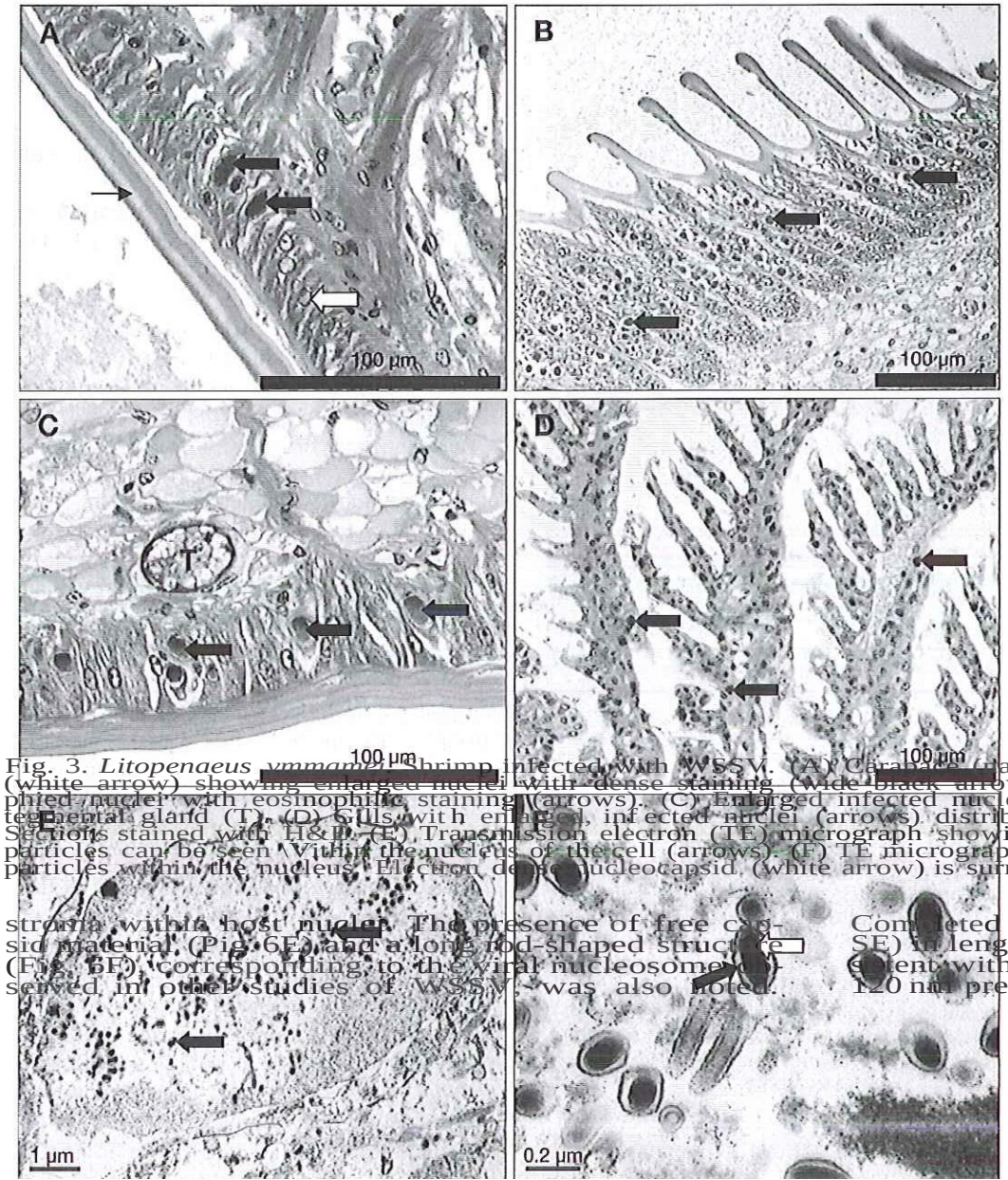

Fig. 3. *Litopenaeus vannamei* Shrimp infected with WSSV. (A) Carapace (narrow black arrow) and columnar epithelial cells (white arrow) showing enlarged nuclei with dense staining (wide black arrows). (B) Stomach epithelium showing hypertrophied nuclei with eosinophilic staining (arrows). (C) Enlarged infected nuclei (arrows) within epithelial cells, also showing tegmental gland (T). (D) Gills with enlarged, infected nuclei (arrows) distributed throughout the cuticular epithelium. (A-D) Sections stained with H&E. (E) Transmission electron (TE) micrograph showing infected cell within the gills. Numerous viral particles can be seen within the nucleus of the cell (arrows). (F) TE micrograph of longitudinal and transverse sections of viral particles within the nucleus. Electron dense nucleocapsid (white arrow) is surrounded by a trilaminar envelope (black arrow)

stroma within host nuclei. The presence of free capsid material (Fig. 6E) and a long rod-shaped structure (Fig. 6F) corresponding to the viral nucleosome observed in other studies of WSSV was also noted. Completed virions measured 280 ± 5.5 nm (mean ± SE) in length and 116 ± 1.4 nm in width (n = 30), consistent with measurements of 270 to 300 nm x 90 to 120 nm previously described for WSSV.

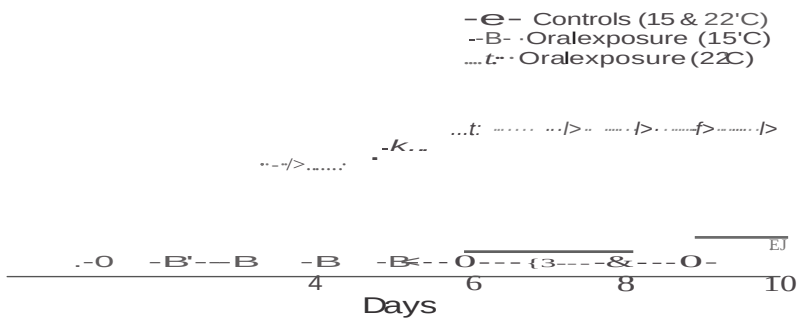

Fig. 4. *Homarus gammarus*. Mortality data of lobsters held nt different temperatures nfter oral exposme to WSSV

Lobster cheliped tissues used for DNA extraction and nested PCR yielded high quality host genomic DNA as indicated by a characteristic 1800 bp amplification product using the universal SSU rRNA gene primers. All WSSV-challenged lobsters (n = 18) held at 15°C were positive for WSSV by nested PCR. Of the WSSV-challenged lobsters held at 22°C, 80% (16/20) were positive for WSSV by nested PCR. No WSSV-positive results were obtained from the negative control group samples.

Lobster feeding trials: low-dose products

Cumulative mortality of lobsters in all treatment tanks varied between groups. Lobsters fed the positive control diet (high-dose WSSV-infected *litopenaeus vannamei*) underwent 41% mortality while lobsters in the negative control tank (fed with SPF *L. vd11namei*) underwent 17% mortality. All negalive r.ontrol lohsters tlrnt died during the experiment were undergoing moult at the time of death. Mortality rates in lobsters fed low-dose, supermarket-derived commodity shrimp were 0, 20 and 22% for the feed prepared from Honduran, Ecuadorian and Vietnamese shrimp, respectively. Histological examination of low-dose fed lobsters did not reveal any of the characteristic signs of WSD as observed in penaeid shrimp or in high-dose fed lobsters. However, characteristic signs of WSD were observed once more in the positive control high-dose fed lobsters, particularly in the antenna! gland and gill epithelium (Fig. 7). TEM of tissues from positive control lobsters once again revealed the rod-shaped viral particles typical of WSSV within the nucleus of infected cells (Fig. 7G,H). Despite the lack of cellular pathology associated with WSD, nested PCR carried out on samples from the low-dose study revealed that almost 100% (16 of 17) of lobsters fed positive control shrimp were positive for WSSV (Fig. BA). In addition, 70% of the lobsters fed supermarket-derived commodity shrimp from Honduras displayed positivity in the second round of PCR (sub-patent infection) whilst lobsters feel with supermarket-derived commodity shrin1p from Ecuador and Vietnam displayed 30 and 45% positivity for WSSV in the second round of PCR, respectively (Fig. 8B-D). Sequencing of the nested PCR amplicon from one lobster in each of the treatment tanks followed by analysis using BLAST confirmed with al least 99% homology that the amplicons were from WSSV (Genl3ank accession AF332093.1) in all cases tested.

DISCUSSION

As expected from similar surveys in the USA and Australia, this small-scale survey of imported penaeid shrimp commodity products from UK supermarkets and fish markets has revealed an apparent widespread prevalence of WSSV-contaminated products. This study demonstrated that frozen commodity shrin1p is a route of entry for WSSV into European member states. We have also demonstrated that the WSSV contaminuted shrimp tissues cunlain viable virions that can be transmitted to naive susceptible hosts via injection into *Litopenae11s vannamei* and by feeding these shrimp to Homarus *gammarus*. Despite the limitations of the survey design, the underlying within-batch prevalence of the WSSV was relatively high, though inindividual components of these batches (e.g. single abdominal sections), the virus was present at a relatively low viral titre and was certainly lower than positive control material generated by passage bioassays to shrimp within our laboratory. The present study has also revealed relativel y h igh batch prevalence for WSSV in supermarket-derived commodity shrimp tested using PCR. The prevalence was apparently lower in shrimp sampled from a large fish market, possibly owing to the larger size of specimens obtained from this location. Albeit limited, these preliminary results demonstrate a potentially large variation in the presence of WSSV in batches of commodity shrimp between type of importer and country of origin as demonstrated in Table 1. For example, the batch of shrimp imported from Vietnam for sale in supermarkets had a within-batch prevalence of 100% while shrimp imported from the same country but sold at the

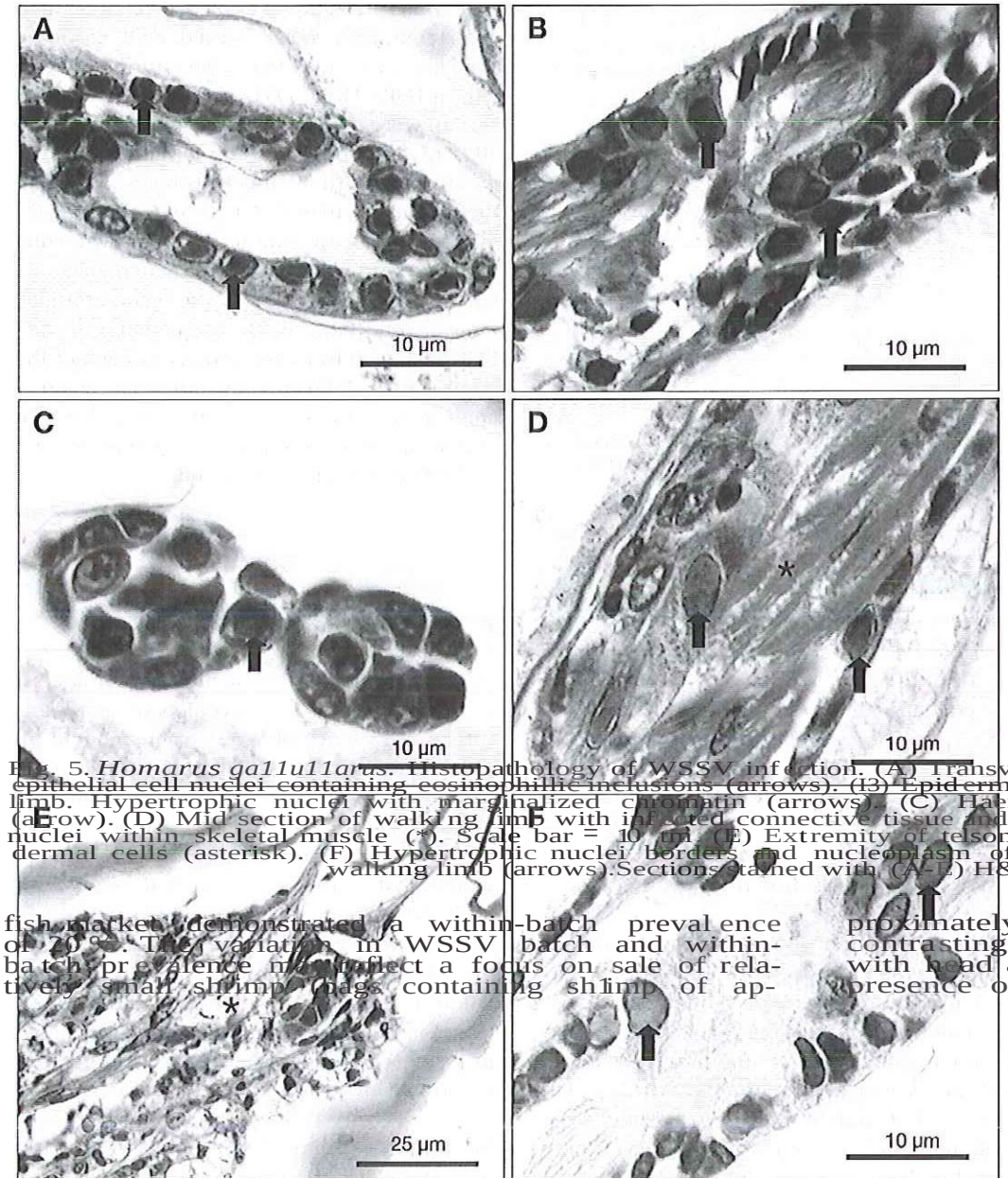

Fig. 5. *Homarus gammarus*. Histopathology of WSSV infection. (A) Transverse section of gill secondary lamellae. Infected epithelial cell nuclei containing eosinophillic inclusions (arrows). (B) Epidermis and connective tissue at extremity of walking limb. Hypertrophic nuclei with marginalized chromatin (arrows). (C) Haemopoeitic cluster with infected germinal cells (arrow). (D) Mid section of walking limb with infected connective tissue and epidermal cells (arrow) but absence of infected nuclei within skeletal muscle (*). Scale bar = 10 µm. (E) Extremity of telson containing infected connective tissue and epidermal cells (asterisk). (F) Hypertrophic nuclei borders and nucleoplasm of epidermal and connective tissue cells within walking limb (arrows). Sections stained with (A-E) H&E or (F) Feulgen stain

fish markets demonstrated a within-batch prevalence of 20%. The variation in WSSV batch and within-batch prevalence may reflect a focus on sale of relatively small shrimp bags containing shrimp of approximately 10 g original size) by supermarkets and a contrasting focus on larger, whole animals (15 to 20 g) with head and shell on at fish markets. Whether the presence of smaller animals in batches from super-

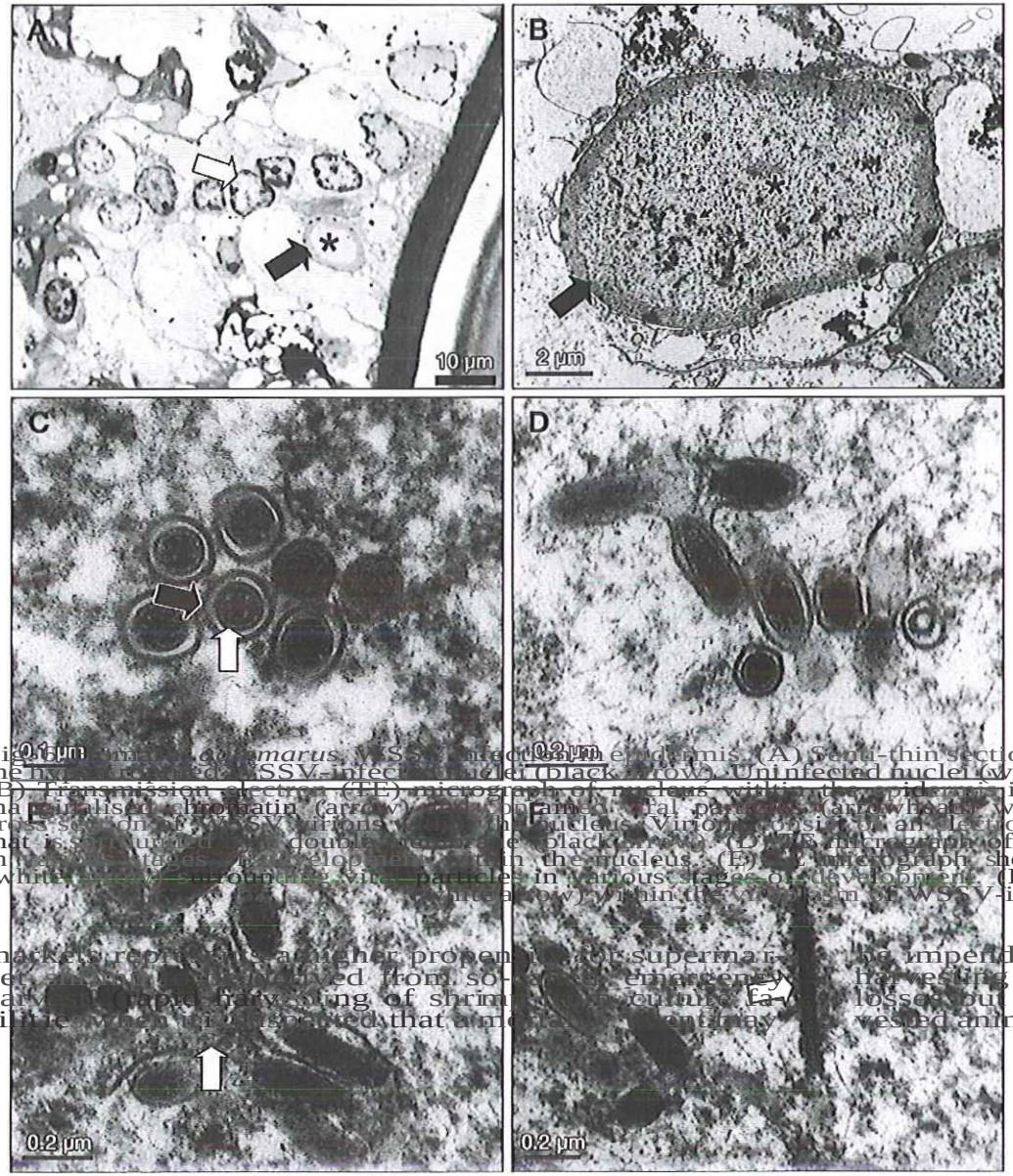

Fig. 1. *Homarus gammarus*. WSSV infection in the epidermis. (A) Semi-thin section of epiderntis. Viroplasm (*) can be seen within the hypertrophied WSSV-infected nuclei (black arrow). Uninfected nuclei (wltite arrow) are also present. Toluidine blue stain. (B) Transmission electron (TE) micrograph of nucleus within the epidermis infected with WSSV. Nuclei were enlarged with marginalised chromatin (arrow) and contained viral particles (arrowheads) within the viroplasm (*). (C) TE micrograph of a cross section of WSSV virions within the nucleus. Virion consist of an electron-dense capsid within an envelope (white arrow) that is surrounded by a double membrane (black arrow). (D) TE micrograph of longitudinal and cross sections of WSSV virions in various stages of development within the nucleus. (E) TE micrograph showing capsid material free within the viroplasm (white arrow) surrounding virus particles in various stages of development. (F) TE micrograph of long rod-shaped structure (white arrow) within the viroplasm of WSSV-infectecl nucleus

mackerel reported a higher propensity for supermarket shopping impending), remains to be ascertained. The rapid ket [illegible] harvested from so- [illegible] emergency harvesting of animals essen t ially averts financial hardship (rapid) harvesting of shrim [illegible] culture farms losses but may lead to higher viral loading in harcilitie [illegible] reported that a [illegible] ent may [illegible] vested animals (Flegel 2009).

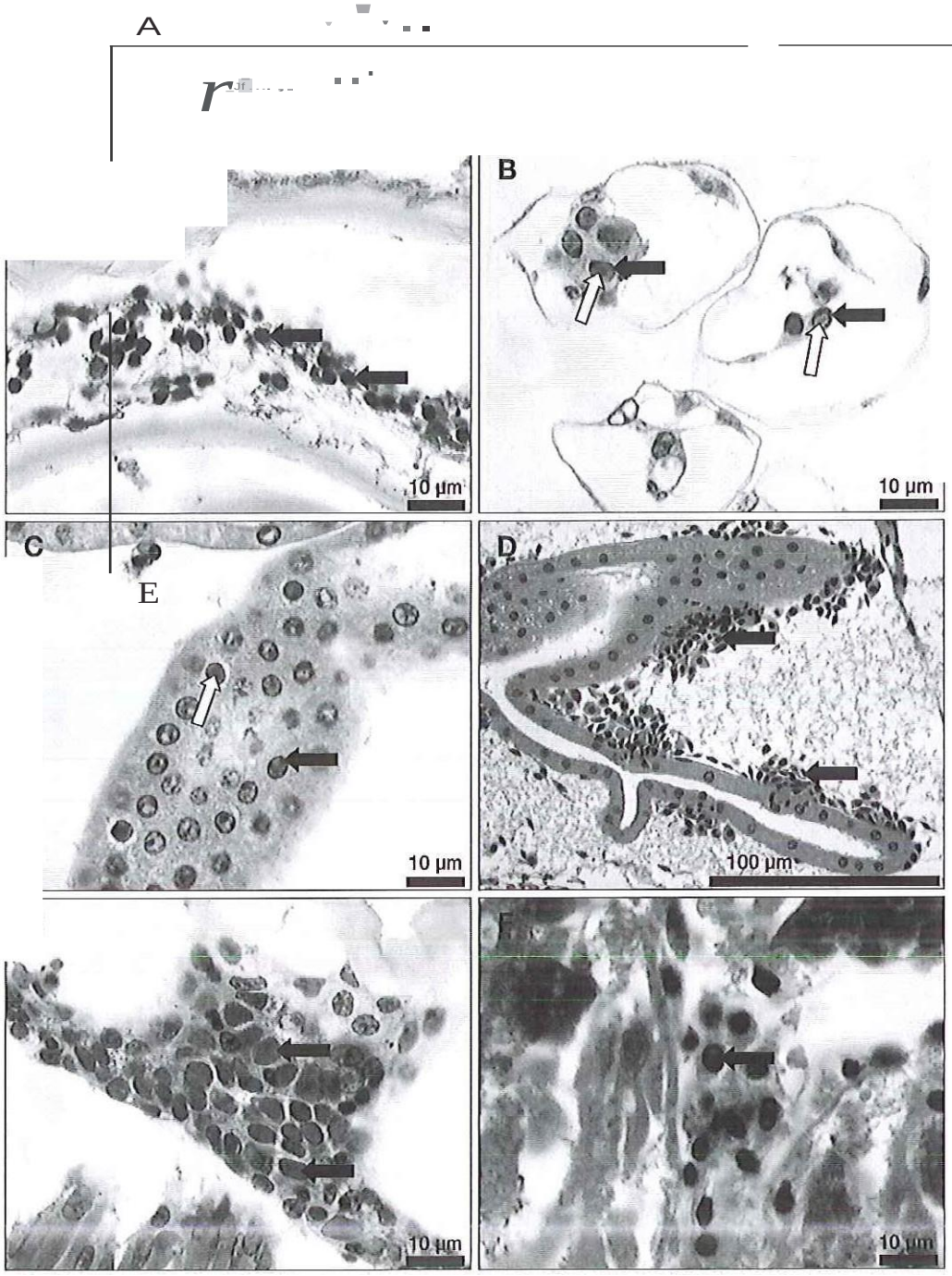
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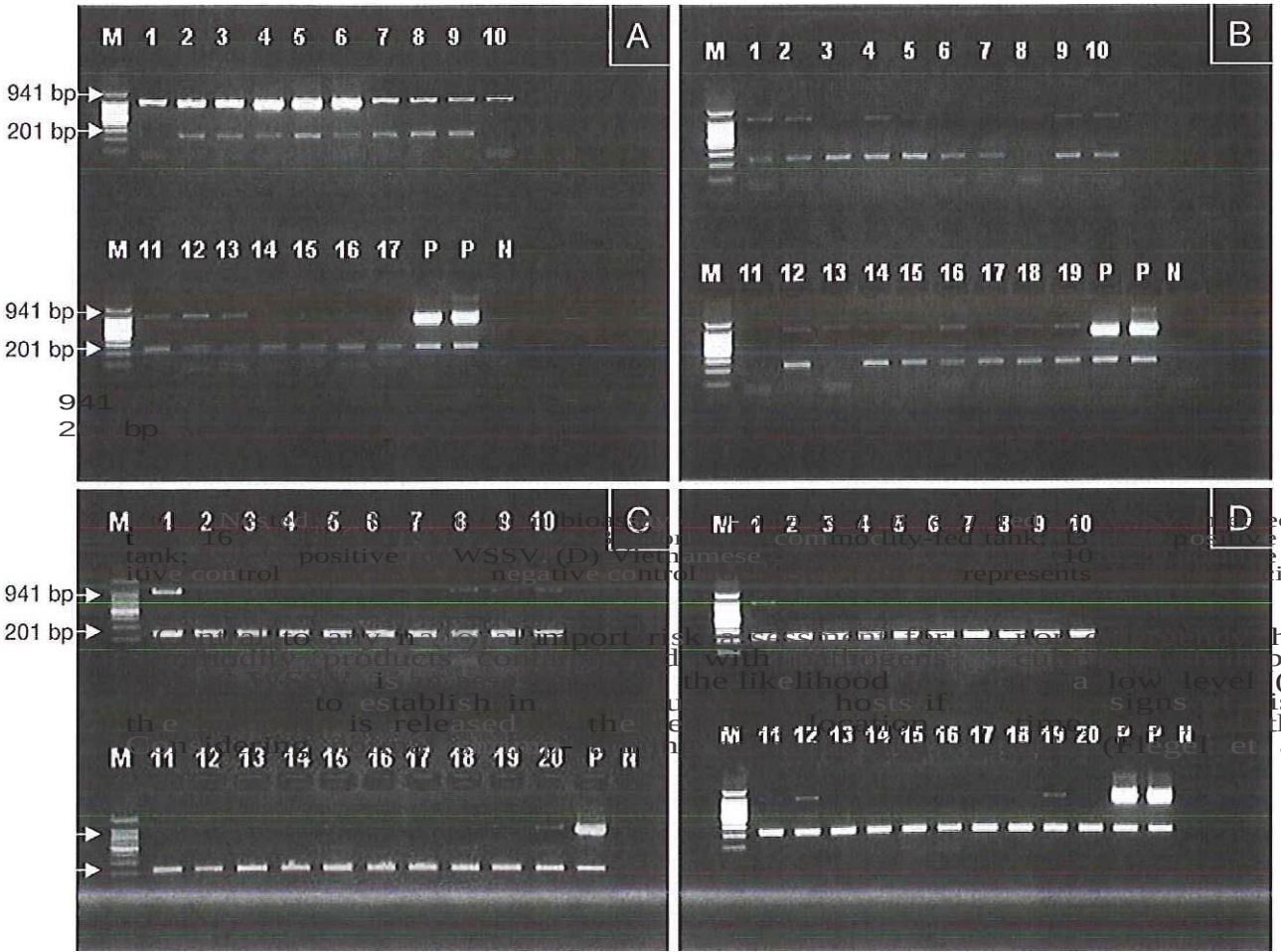
Fig. 7. *Homarus gammarus*. Lobster infected with WSSV. (A) Carapace (narrow black arrow) and epithelial cells with marginated chromatin and eosinophilic staining (wide black arrows). (B) Transverse sections of secondary gill filaments showing marginated chromatin (black arrows) and eosinophillic inclusions (white arrows) within nuclei. (C) Antenna! gland showing infected nuclei with marginated chromatin (black arrow) and eosinophillic inclusions (white arrow). {D) Antenn gland with haemocyte aggregations (arrows). (E) Haematopoietic tissue with numerous enlarged nuclei showing marginated chromatin and dense, uniform staining (arrows). {F) Connective tissues with infected nuclei displaying dense unifom1 staining and marginated chromatin (arrow). (A-F) Sections stained with H&E. (G) Transmission electron (TE) micrograph of infected cell within the gills. Numerous viral particles can be seen within the nucleus of the cell (arrows). {H) TE micrograph of viral particles within the nucleus. Electron-dense nucleocapsid (white arro\v) is surrounded by a trilaminar envelope {black arrow)



ed commodity shrimp. (A) Positive control
e for WSSV. (C) Ecuadorian commodity-fed
e for WSSV. M: 100 bp DNA ladder; P: pos-
ific DNA; 941 bp represents WSSV DNA

harvest), it has been reported that
o can carry viruses (such as WSSV)
(latent) in fection without showing
isease for relatively long periods of
hat rearing conditions are favour-
al. 2004). However, a change in

physiological conditions, often due to environmental changes, can allow for rapid replication of the virus and the onset of disease. Whilst the loacling of WSSV in commodity shrin1p products analysed in the present study was apparently low in most cases, suggesting latent or sub-patent infection in those hosts, the virus remained viable and was able infect and kill SPF *Litopenaeus vannamei* within a few days of exposure (albeit by a non-natural route of transmission). However, feeding trials in which lobsters were fed with the same low-dose commodity products also demonstrated that passage of WSSV could occur via this natural route and that infection could establish, albeit in latent form, in naive hosts (lobsters) held at temperatures experienced within Europe. Since infection progressed rapidly to disease in lobsters fed with high-dose products (positive control). particularly at higher temperatures, the limiting factor in the rapid appearance of WSD in lobsters is therefore the initial dose; a low-level infectious dose establishes latent infection and a high-level dose progresses more rapidly to disease. Fundamental studies are now required to assess the potential for latent infections to progress to a disease state and cause mortality in lobsters retained at European temperatures. Such data are vital for accurate consequence assessment following release and establishment of WSSV infections in wild populations.

Results from the high-dose WSSV feed trial confirm the susceptibility of juvenile European lobsters to WSSV. Lobsters were fed with WSSV-infected carcasses of *Litopenaeus vannamei* and were shown to succumb to the disease within the time course of the experiment (10 d). However, not all of the lobsters fed WSSV-positive commodity shrimp products became infected with the virus. This could be due to a number of reasons. Firstly, although the lobsters were proviclecl with the same sized ration originating from the same individual shrimp (per treatment tank). some animals did not consume all of the food provided. In addition, since the shrimp were not homogenised prior to feeding but rather, fed as blocks of tissue, the concentration of virus may also have varied in specific meals (Soto el al. 2001). However, the result may also indicate an inherent variation in susceptibility to viral infection between individuals of the same species. The concept of 'susceptibility' in hosts should therefore not only be considered at the species level but also at the level of the individual within that species; this is particularly important in animals that display non-continuous growth and that must moult al relatively short intervals. Differential pathogen prevalence related to the crustacean moult cycle has previously been demonstrated in field studies (Stentiford et al. 2001). In such cases, differential susceptibility probably relates directly to immune suppression around the time of ecdysis and in relation to other stressful events (Le Moullac & Haffner 2000). Further, it is clear that European species such as Homarns *gammarns* can become infected with WSSV following ingestion of a single contaminated meal, though factors such as viral loading and host age or condition appear to be key variables in assessing the likelihood of progression from an infection to a disease state. We have also shown that in *H. gammarus*, the development of WSD and associated cumulative mortality is enhanced when animals are held at 22°C rather than 15°C. Temperature can also affect the development of WSD in penaeid shrin1p and in crayfish, causing reduced mortality rates and lower viral loads when hosts are retained in both hypothermic and hyperthermic conditions. Interestingly, in both scenarios disease cleveloped rapidly when infected individuals were returned to optinrnl conditions for viral replication (Vidal et al. 2001, Granja et al. 2003, 2006, Jiravanichpaisal et al. 2004, Uu et al. 2006, 2008, Rahman et al. 2006, 2007a,b). Whether infection would continue in a latent state in lobsters retained at 15°C for longer periods of time, or would progress to disease via exposure of infected hosts to external stressors, will govern the potential for WSSV to sustain a presence in natural aquatic environments of temperate regions.

Only one naturally occurring virus infection has been documented in lobsters. *Parwlirus argus* virus 1 (PaVl) infects the spiny lobster species *P. argus* from the Florida Keys, USA, and the Caribbean (Shields & Behringer 2004). To elate, no viral infections have been clescribed from homarid lobsters. Whilst this may be due to a general lack of comprehensive disease surveys or n samplin g bias away from juvenile life stages (as suggested in studies on crabs by Bateman & Stentiford 2008 and Bateman et al. 2011), it also suggests some inherent non-susceptibility to viruses in this group. The latter is now apparently unlikely owing to the rapid pathogenesis of WSSV in juvenile *Homarus gammarus* demonstrated during this study. Pathogenicity of WSSV has also been investigated in the spiny lobster species *P. versicolor, P. penicilla tus, P.* on1c1tus, *P. longipes, P. Jwmarns* and *P. polyphagous* (Chang et al. 1998, Rajenclran et al. 1999, Musthaq el al. 2006). Chang et al. (1998) reported that lobsters fed WSSV-

contaminated feed did not succumb (although WSSV was detected within their tissues). while Rajendran et al. (1999) provided some evidence that WSSV infection via feeding and ingestion could result in mortalities of *P. polyphagous* and *P. omatus.* However, in a follow-up study by Musthaq et al. (2006). *P. lwmarus* and *P. ornatus* feel WSSV-contaminated feed did not die, while those that were injected with WSSV did. These studies demonstrate the likelihood that spiny and homarid lobsters are susceptible to viral infections and that investigations into the presence of natural viral infections in field-caught lobsters, particularly juvenile life stages, may prove fruitful.

In terms of import risk assessment for the trading of shrimp commodity products, the risks associated with viral release through shrinp packaging and reprocessing plants in importing countries has previously been highlighted (Nunan et al. 1998, Durand et al. 2000). Shrimp inported for human consumption can also be diverted into alternative uses. The use of raw, frozen shrimp products as angling bait has been identified as a relevant risk for introduction of viral crustacean pathogens in Australia (Biosecurily Australia 2008). Furthermore, there is anecdotal evidence (e.g. via online forums) that this practice is also relatively commonplace in the UK (and probably other parts of Europe). A recent questionnaire sent to a subset of the UK angling community (thought to exceed 4 million individuals) suggested that up to 7% of these may use frozen shrinp products as bait (B. Oidtmann pers. comm.). This increased use appears to be directly associated with the current price competitiveness between frozen, imported shrimp commodity and other types of specialist angling bait, as well as the ease of purchase through supermarkets.

Since per os feeding represents a realistic route of entry for pathogens (such as WSSV), further work is now required to investigate the likelihood for pathogen transmission between latently infected lobster conspecifics, and also between infected lobsters and other non-penaeid decapod hosts. Only when such studies are carried out will it be possible to determine the potential for the establishment of WSSV in wild populations of decapods residing in aquatic habitats of temperate regions. Further work is also required to assess the potential for differential risk associated with commodity inported from particular regions, particularly where approaches to within-country biosecurily or emergency harvesting of infected shrimp ponds are likely to generate products with high disease status.

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Plastic parasites: Extreme dimorphism creates a taxonomic conundrum in the phylum Microsporidia *

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ABSTRACT

In this paper, we combine field observations of highly statistically significant co-occurrence with histopathological, ultrastructural and molecular phylogenetic analyses, to provide evidence for extreme morphological plasticity in a microsporidium parasite infecting the musculature of marine crabs. The parasite appears to alternate between lineages that culminate in production of either bizarre needle-like spores in the peripheral sarcoplasm of heart and skeletal muscle fibres (reminiscent of *Nadelspora ca11ceri* infecting *Ca11cer 111agister)* or alternatively, Ameson-like spores with pronounced surf.lee projections, in the skeletal muscles (as for *A111eso11 pulvis,* previously described infecting *Carci1111s 111ae11as).* Both lineages occur in direct contact with the cytoplasm of host muscle cells and can exist simultaneously within the same cell. Pathological data appears to reveal a remarkable shift in morphology during pathogenic remodelling of host tissues. Sequence analysis of multiple clones derived from amplification of the ssrRNA gene from infected regions of the heart and skeletal muscles appear to confirm the genetic identity of the two lineages. Furthermore, derived ssrRNA gene sequences are more similar (>99%) to *N. cancen* than to the coparasite *A111eso11 micllae/is* infecting *Cal/i11ectes* sapidus (93%). Although molecular phylogenetic data support transfer of A pu/vis into the genus *Nadelspora,* the expansion in the generic description required to include such widely divergent characteristics is so significant as to be unfeasible within the current taxonomic framework of the phylum Microspoiidia. At present, it is preferable to propose that the parasite infecting *C. 111ae11as* forms a clade with other morphologically diverse but phylogenetically and ecologically similar muscle-infecting microsporidians from marine crustacean hosts. Given the strong evidence for significant plasticity in morphology amongst members of the phylum Microsporidia, novel approaches to phylogeny, based predominantly upon the informed use of molecular sequence data, are now deemed a necessity.

1. Introduction

The phylum Microsporidia consists of obligate intracellular parasites thought to be derived from the Fungi (Edling et al., 1996; Keeling and Doolittle, 1996; James et al., 2006). They are known to parasitise most of the major phyla of invertebrates and all five classes of vertebrates (Canning and Vavra, 2000). The higher level taxonomy of the phylum, largely based upon morphological characteristics of parasite life stages has been increasingly challenged by the utilisation of nucleic acid-based approaches to phylogeny. Recent reviews have shown that some of the pathogen characters used to generate the accepted taxonomy of the group are in fact relatively 'plastic' or are homoplasious. As such, over-reliance on taxonomic characters based upon morphological features alone have allowed numerous contradictions in the classification of the phylum (Vossbrinck and Debrunner-Vossbrinck, 2005). These contradictions have been amply demonstrated when combined approaches to taxonomy (using morphological and nucleic acid-based phylogeny) have been utilised simultaneously for the description of novel taxa (e.g. Brown and Adamson, 2006; Stentiford et al., 2010, 2011).

In their review, Canning and Vavra (2000) stated that the basic character of the microsporidian life cycle (i.e. alternation between merogonic and sporogonic development in host cells) is shared amongst all described taxa within the Microsporidia. However, the developmental spectrum inherent within these two phases is considerable. Some taxa display a rather simple life-cycle in which a single morphological spore type is produced (mono-morphic) from a solely unikaryotic lineage of intermediates. In others, two or more spore types (polymorphic), either monokaryotic, diplokaryotic or both are produced. In these instances, polymorphic spore

** Nucleotide sequence data reported in this paper are available in GenBank under accession number KC465966.

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types (and their associated intermediate stages) can occur within single host species or in different host taxa. The majority of published reports of spore polymorphism within the Microsporidia are from infections of (mainly dipteran) insect hosts. The most complete life-cycle descriptions are from representative genera within the family Ambylosporidae, and in particular from the type genus *Amby/ospora.* In *Ambylospora,* since different spore types (and ka1yotypic lineages) occur in different host taxa (e.g. *Ambyls-pora californica* in insects and aquatic crustaceans, Kelling and Lipa, 1960), the genus is considered to possess some of the most complex (known) life cycles within the phylum (Becnel and Andreadis, 1999; Vossbrinck et al., 2004). In other genera within this family, polymorphic spore development can occur within either a single life stage of a single host taxon (e.g. *Hazardia milleri* in the dipteran *Cu/ex pipiens,* Hazard and Fukuda, 1974), or in subsequent life stages of a single host taxon (e.g. *Culicospora magna* in larval and adult stages of *C. pipiens.* Kudo, 1920). The phylogenetic relationship of numerous genera grouped by these complex life-cycle traits into the family Ambylosporidae has been outlined in studies by Baker et al. (1998) and by Vossbrinck et al. (2004). In the case of these mainly mosquito-infecting genera, it has been proposed that due to their apparent early phylogenetic divergence, such complexity represents a relatively primitive trait among the so-called 'higher' Microsporidia (Baker et al., 1997). This would further imply that simpler life cycles (e.g. one host, fewer sporulation sequences) observed in genera such as the monoka1yotic/monomorphic *Endoreticu/atus,* the diploka1yotic/monomorphic *Nosema,* and even the monoka1yotic-diploka1yotic/dimorphic *Vairimorplia* result from loss of various life cycle features over evolutionary time. In the latter case, the alternation of *Vairiomorplia necatrix* between developmental stage and spore ka1yotype status is influenced by environmental conditions (eg. a switch from diplokaryotic and disporoblastic sporogony to monokaryotic and octosporoblastic sporogony in the lepidopteran host *Pseuda/etia unipunctata* occurs when external temperatures drop below 25 °C, Kramer, 1965).

Until recently the majority of observed cases considered to possess relatively high life cycle complexity (i.e. polymorphic, multi-host) have been obse1ved in microsporidians proposed to be of freshwater origin (Class Aquasporidia) (according to the classification system proposed by Vossbrinck and Debruner-Vossbrinck (2005)). However, some microsporidians of presumably marine origin (Class Marinosporidia) are also known to exhibit true dimorphism (i.e. the alternation of katyostatus and spore morphology). These include infections by the xenoma-forming microsporid ium *Spraguea /oplr ii* in the nervous tissue of the European monkfish *(Lopll ius* piscatorius). In this case, the monokaryotic development cycle in the outer xenoma precedes the diploka ryotic cycle in the inner xenoma. Both lead to morphologically and karyotypically distinct spore types (Weissenherg, 1976). In a recent discovery, Nylund et al. (2010) described a microsporidian with a life-cycle of similar complexity to that of the dipteran-infecting members of the Ambylospidae. Here, *Desmozoort lepeop/ 1t/1erii* (Freeman and Sommerville 2009) *(=Paranuc/eospora t/1eridio11* Nylund et al., 2010) undergoes a multi-host, polymorphic life cycle in which the parasitic copepod crustacean *lepeophtlr eirus* salmonis and the Atlantic salmon *(Sa/mo salar)* are implicated. Development within the salmon is diploka ryotic and can be both intra-cytoplasmic {Cycle 1) and intra-nuclear (Cycle 2) (the latter a trait currently observed only in members of the Enterocytozoonidae). Within the copepod, infection of several cell types is intra-cytoplasmic, with diplokaryotic merogony preceding monokatyotic sporogony. Recent work from our laboratory has placed further emphasis on the potential for multi-trophic transmission (and therefore, complex life-cycles) in members of the family Enterocytozoonidae by demonstrating a close phylogenetic relationship between the crab parasites *Enterospora ca11ceri* and fish- or mammal-infecting genera within this family (Stentiford et al.. 2007, 2011). In this way, it may be proposed that the potential for life-cycle complexity appears to characterise the phylum Microsporidia, rather than specific subdivisions thereof.

In light of these life-cycle complexities in individual microsporidian taxa, and since the erection of most extant taxa preceded the standardised usage of nucleic-acid based phylogenetic approaches, inherent errors in the current taxonomy of the phylum are now well accepted. A serendipitous discovery by our laboratory has highlighted this issue in its simplest form by demonstrating the potential for extreme dimorphism in a microsporidian parasite infecting a single cell type of a single species of marine crab (the common European shore crab Carcinus *maenas).* In this case, the parasite alternates between a diplokatyotic lineage in which unusual needle-like spores are produced (Nadelspora-like), and a monokatyotic lineage that culminates in the production of an oval spore with pronounced surface projections (Ameson-like).The latter forms the basis for the taxonomic description of Arneson *pu/vis* by Vivares and Sprague {1979) in this crab host. Both life-cycle sequences occur in direct contact with the cytoplasm of host muscle cells and can occur within the same cell simultaneously. Inclusion of the microsporidians, *Nade/spora canceri* (from the marine crab *Cartcer magister,* Olson et al., 1994) and *Ameson micltaelis* (from the marine crab *Callinectes* sapidus, Weidner, 1970) in previously published phylogenetic assemblages based upon partial sequences of the ssrRNA gene have demonstrated (although not previously discussed) a very close relationship between these two parasite genera, despite the fact that their described spore morphology and developmental cycle are very different (e.g. Vossbrinck and Debrunner-Vossbrinck, 2005; Stentiford et al., 2010). The discove1y reported herein provides evidence that the morphologically divergent genera, *Ameso11* and *Nadelspora,* both previously described infecting the musculature of marine crabs, are potentially life-cycle variants of the same taxon. Furthermore, they appear to reside within a clade with other morphologically diverse but phylogenetically and ecologically similar muscle-infecting microsporidians from marine crustacean hosts. The current study highlights the life-cycle plasticity in A *pulvis* from *C. maenas* (Vivares and Sprague, 1979; Vivares, 1980; Vavra et al., 1981) and proposes a similar potential in *Nade/spora-like* and Arneson-like infections of other commercially significant decapod crustaceans.

2. Materials and methods

2.1. Sampling, Itisco/ogy and transmission electron microscopy

European shore crabs, C. *maenas* (11 = 162) were collected as part of ongoing surveys by shoreline sampling at Newton's Cove, Weymouth, UK (50°34' N, 2°22' W) between 2008 and 2009. Following transfer to the laboratory, crabs were anaesthetised by chilling to 4 °(and processed for histology, electron microscopy and molecular biology. For histology, the hepatopancreas. gill, gonad, central nerve ganglia, heart and body musculature were removed. Excised samples were placed immediately into Davidson's seawater fixative (Hopwood, 1996). In several cases, the musculature of the heart and skeletal muscles appeared relatively opaque. Fixation was allowed to proceed for 24 h before samples were transferred to 70% industrial methylated spirit. Fixed samples were processed to wax in a vacuum infiltration processor using standard protocols. Sections were cut at a thickness of 3-5 1111 on a rota1y microtome and mounted onto glass slides before staining with H&E. Stained sections were analysed by light microscopy (Nikon Eclipse E800) digital images and measurements were taken using the LuciaTI • Screen Measurement System (Nikon, UK).

For electron microscopy, regions of opaque and apparently normal skeletal and heart muscles were removed and small blocks of tissue (2 mm^3) were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature. Fixed tissue samples were rinsed in 0.1 M sodium cacodylate buffer (pH 7.4) and post-fixed for 1 h in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Specimens were washed in three changes of 0.1 M sodium cacodylate buffer before dehydration through a graded acetone series. Specimens were embedded in epoxy resin 812 (Agar Scientific-pre-mix kit 812 (Agar Scientific, UI<) and polymerised overnight at 60 •C in an oven. Semi-thin (1-2 1m) sections were stained with Toluidine Blue for viewing with a light microscope to identify suitable target areas. Ultrathin sections (70-90 nm) of these areas were mounted on uncoated copper grids and stained with uranyl acetate and Reynolds' lead citrate (Reynolds, 1963). Grids were examined using a JEOL JEM 1210 transmission electron microscope and digital images captured using a Gatan Erlangshen ES500W camera and Gatan Digital Micrographn.[1] software.

2.2. DNA extraction, PCR and sequencing

Samples of opaque and apparently normal skeletal and heart muscles corresponding to those regions sampled for histology and electron microscopy were removed and processed for the partial sequencing of the ssrRNA gene. Dissected muscle samples were preserved in 100% ethanol at -20 •C until DNA extraction. Tissue samples were weighed and added to Lysing Matrix D FastPrep® tubes. They were diluted in G2 buffer and proteinase I< (Qiagen, UI<). Following disruption of the tissues using the Fast Prep cell disrupter, homogenates were incubated at 56 •C overnight. Volumes equivalent to 5 mg of tissue were removed and DNA was extracted using the QIAGEN EZI DNA Tissue l<it and the BioRobot® EZl.

A generic primer set for the microsporidium ssrRNA (Tourtip et al., 2009) was already in use in the laboratory, however it was noted that there were significant mismatches at the 3'end of the MFI primer compared with of the Ameson-like microsporidia that would limit its use in a study of this type. By alignment of published sequence data available for *N. cancer i* and *A. mic/welis,* it was possible to modify the MFl primer to ensure that it was suitable to amplify the small subunit of both previously described *Nadelspora* and Arneson taxa that were considered to be present in the sample.

PCRs were performed in a 50 11 reaction volume consisting of 1x GoTaq flexi buffer (Promega, UI<), 2.5 mM $MgCl_2$, 1 mM dNTP mix, 50 1>rnol of the modified MF I primer (MICROEC F, 5'-CCGGAGAGGGCGCM TTTA GAGA-3') and the MR1 primer (Tourtip et al., 2009), 1.25 units of GoTaq® DNA Polymerase (Promega, UI<) and 2.5 pl of the purified DNA. The reaction mix was overlaid with mineral oil and after an initial denaturing step (5 min at 95 °C) was subjected to 35 temperature cycles (1 min at 95 •C, 1 min at 55 •C and 1 min at 72 •C) in a Peltier PTC-225 thermal cycler followed by a final extension step of 10 min at 72 •C. PCR products were visualised on 1.5% agarose gels stained with ethidium bromide. PCR products were purified using the Freeze N' Squeeze DNA purification system (Anachem, UI<) and ligated into the pGEM[to]-T Easy Vector Sysytem (Promega). Following transformation of *Escher ichia coli* JMl 09 cells and growth on Lysogeny Broth agar (Sigma, UI<), the M13 forward and M13 reverse primers (Promega) were used to amplify across the multi-cloning site of plasmid extracted from individual colonies. PCRs were performed in a 50 ii reaction volume consisting of 1x GoTaq flexi buffer, 2.5 mM $MgCl_2$, 1 mM dNTP mix, 50 pmol each of the primers M 13 Forward and M 13 Reverse, and 125 units of GoTaq«> DNA Polymerase. The amplification products were purified using a MinElute kit and sequenced using both M 13 Forward and M13 Reverse primers. Sequences were analysed using the Sequencher software (Gene Codes Corporation, Ann Arbor, MI, USA). The resulting consensus sequences were submitted to GenBank under accession number I<C465966 to be assigned. The ssrRNA gene sequence was obtained from a total of 158 clones, representing both heart (11 = 76) and muscle (11 = 82) samples from two animals shown to contain high levels of both spore types by microscopic examination.

In addition to the *Nade/spora*- and Ameson-specific primer set described above, an analysis of the samples was also undertaken using the universal 530F/580R primer set (Docker et al., 1997). The amplifications, cloning and sequencing protocols were the same as above. A partial ssrRNA gene sequence was obtained from a total of 43 clones, representing both heart (11 a 24) and skeletal muscle (11 = 19) samples from the same animals described above.

2.3. Plly/oge11etic analysis

Multiple sequence alignments and phylogenetic analysis were performed using the partial ssrRNA gene sequences from 22 representative microsporidium species including the partial ssrRNA sequence for *N. canceri* (AY958070) and *A. michae/is* (L15741). The partial ssrRNA sequence from T/1e/o/Ja11ia *so/e11opsae* (AF031538) was used as an outgroup. Multiple alignments were performed using Clustal W (Thompson et al., 1997) using the following Clustal parameters: a gap opening penalty of 15, and gap extension penalty of 6.66. Phylogenetic analyses were conducted using MEGA version 4 (Tamura et al., 2007). The neighbour-joining tree was constructed using a maximum composite likelihood model and the robustness of the tree was tested using 1,000 bootstrap replicates.

3. Results

3.1. Histopatlwlogy

A total of 12 out of 162 (7.4%) *C. 111ae11as* collected over the study period displayed distinct signs of disease, involving progressive colonisation of the skeletal musculature of the host by a pathogen, specifically a progressive invasion of the peripheral and underlying sarcoplasm of heart and skeletal muscle fibres by two distinct forms of an intracellular pathogen. Early infections involved expansion of the peripheral sarcoplasm of individual skeletal fibres and particularly heart myofibres by dense basophilic colonies, the features of which were difficult to ascertain by light microscopy (Fig. 1A). In some crabs, the peripheral sarcopl,Ism of the majority of heart and skeletal myofibres contained these colonies although the myofibrillar components remained unaffected (Fig. IB and C). In skeletal muscle, the dense basophilic colonies appeared to be associated with apparent liquefaction of myofibrillar components of the muscle fibres. Fibres were subsequently colonised by masses of small oval cells. Oval cells appeared to be in direct contact with the liquefied musculature but were still contained within the confines of the muscle fibre sarcolemma (Fig. 1E). Late stage infection within skeletal muscle was characterised by progressive replacement of myofibres by masses of oval cells whilst the dense basophilic colonies observed in early infections became scarce (Fig. IF). Eventually, the majority of skeletal muscle fibres were replaced by oval cells, these corresponding to the opaque muscle fibres obse1ved upon dissection. Oval cells were not observed within heart myofibres, even during advanced disease. Based upon the simultaneous occurrence of pathology in the heart and skeletal musculature in all 12 crabs and an absence of pathology in either organ in all remaining crabs sampled (11 a 150), a 2 x 2 contingency table analysed with Fisher's exact test indicates a highly significant

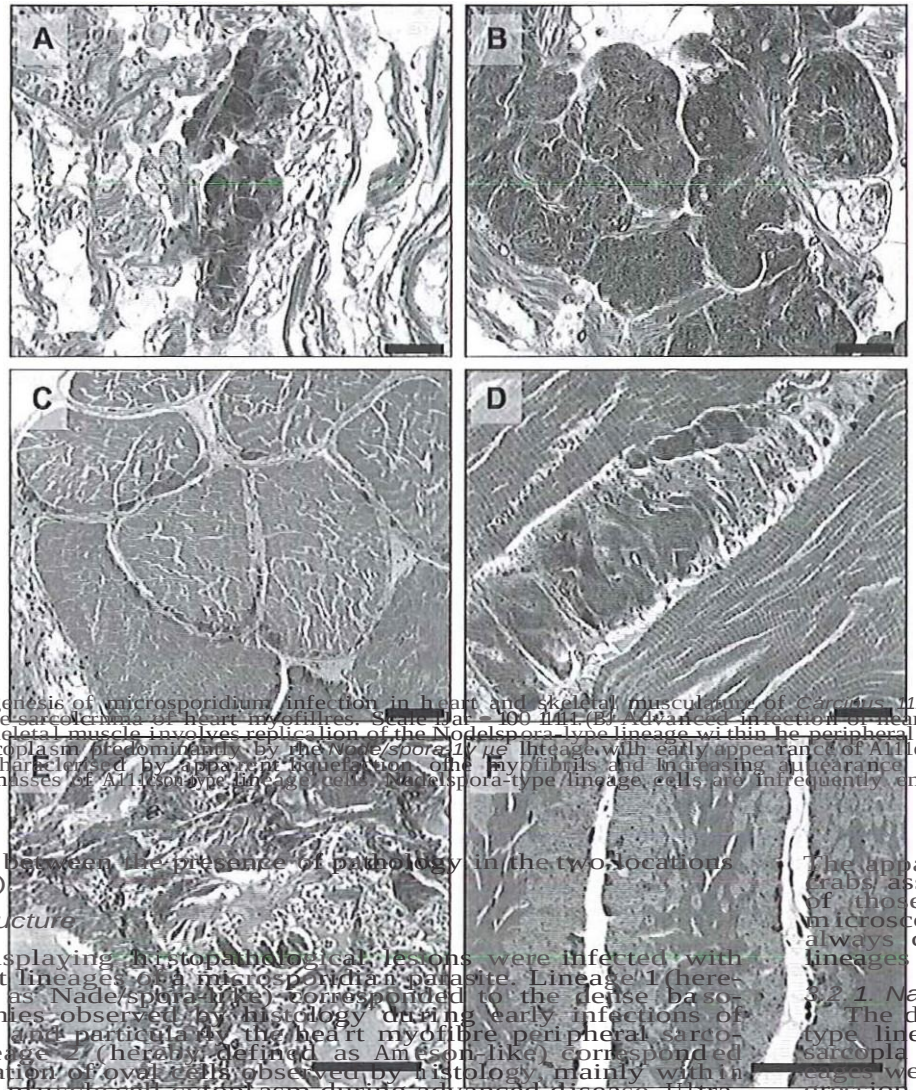

Fig. 1. Pathogenesis of microsporidium infection in heart and skeletal musculature of *Carcinus maenas*. (A) Early infection, predominantly by the *Nadelspora-type* lineage confined to the sarcoplasm of heart myofibres. Scale bar = 100 μm. (B) Advanced infection of heart myofibres by the *Nadelspora-type* lineage. Scale bar = 50 μm. (C) Early infection of skeletal muscle involves replication of the Nadelspora-type lineage within the peripheral sarcoplasm of the muscle fibres. Scale bar = 100 μm. (D) Expansion of the peripheral sarcoplasm predominantly by the *Nadelspora-type* lineage with early appearance of Arneson-type lineage cells. Scale bar = 50 μm. (E) Invasive stage within skeletal musculature characterised by apparent liquefaction of the myofibrils and increasing appearance of Arneson-type lineage cells. Scale bar = 50 μm. (F) Population of the myofibre by masses of Arneson-type lineage cells. Nadelspora-type lineage cells are infrequently encountered during the later stage of disease. Scale bar = 100 μm. All H&E histology.

association between the presence of pathology in the two locations ($P < 0.0001$).

3.2. Ultrastructure

Crabs displaying histopathological lesions were infected with two distinct lineages of a microsporidian parasite. Lineage 1 (hereby defined as Nadelspora-like) corresponded to the dense basophilic colonies observed by histology during early infections of the skeletal and particularly the heart myofibre peripheral sarcoplasm. Lineage 2 (hereby defined as Arneson-like) corresponded to the formation of oval cells observed by histology, mainly within the skeletal muscle cell cytoplasm during advanced disease. Ultrastructural observations of crab musculature at different stages of infection revealed a complex series of life stages that culminated in the production of either *Nadelspora*- or Arneson-type spore types.

The apparent co-occurrence of both lineages in all of the infected crabs assessed with histopathology ($n = 12/12$), and the majority of those additionally assessed using transmission electron microscopy ($n = 4/5$) provides strong evidence that both lineages always co-occur in infected crabs. The ultrastructure of both lineages is described in Sections 3.2.1 and 3.2.2.

3.2.1. Nadelspora-type lineage

The description of merogony and sporogony in the *Nadelspora*-type lineage is based upon observed life stages in the peripheral sarcoplasm of infected muscle fibres. Only *Nadelspora-type* lineages were observed within the heart. Merogony involved the progression of diplokaryotic meronts to quadri-nucleate (two diplokaryotic nuclear sets) and octo-nucleate (four diplokaryotic nuclear sets) plasmodia. All developmental stages occurred in direct contact with the sarcoplasm of host muscle cells (Fig. 2A-C,

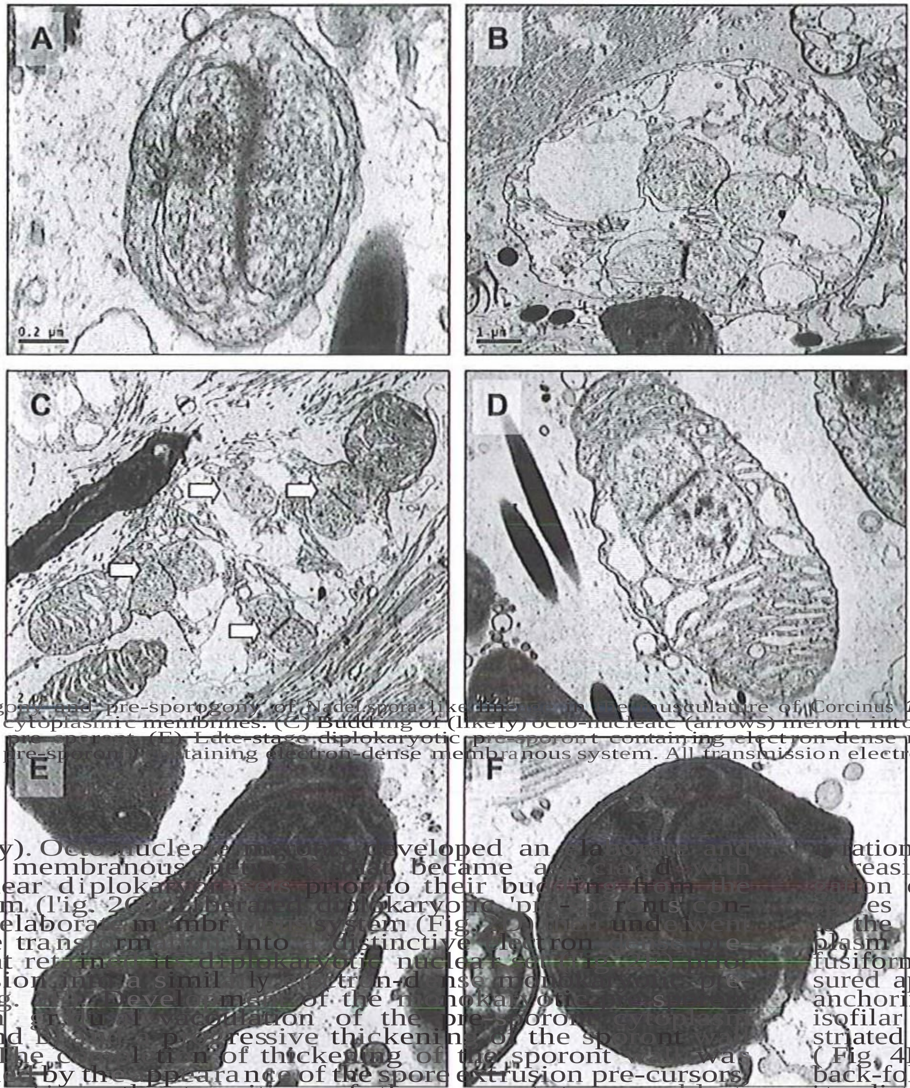

Fig. 2. Merogony and pre-sporogony of Nadelspora-like parasite in the musculature of Corcinus *111ae11as*. (A) Diplokaryotic meront. (B) Tetra-nucleate meront containing vacuoles and cytoplasmic membrines. (C) Budding of (likely) octo-nucleate (arrows) meront into membrane-rich diplokaryotic pre-sporonts.(0) Early stage membrane-rich diplokaryotic pre-sporont. (E) Ldte-stage diplokaryotic pre-sporont containing electron-dense membranous system. (F) Unikaryotic pre-sporont (product of division of diplokaryotic pre-sporont) containing electron-dense membranous system. All transmission electron microscopy.

respectively). Octo-nucleate meronts developed an elaborate and distinctive membranous network that became associated with single nuclear diplokaryon sets prior to their budding from the plasmodium (l'ig. 2C). Liberated diplokaryotic 'pre-sporonts' containing an elaborate membranous system (Fig. 2D) then underwent progressive transformation into a distinctive electron dense pre-sporont that retained its diplokaryotic nuclear set (Fig. 2E) prior to its division into a similarly electron-dense monokaryotic pre-sporont (l'ig. 2F). Development of the monokaryotic sporont involved a gradual vacuolation of the pre-sporont cytoplasm (Fig. 3A and B) and a progressive thickening of the sporont wall (Fig. 3C). The completion of thickening of the sporont wall was accompanied by the appearance of the spore extrusion pre-cursors, thereby marking the transition of the sporont to sporoblast. Monokaryotic sporoblasts were initially club-shaped (Fig. 30) but gradually became elongated and were seen to contain recognisable elements of the spore-extrusion apparatus (Fig. 3E and insel). Maturation of the sporoblast to the mature spore was determined by increasing electron density of the sporoblast cytoplasm and elongation of the cell to produce a fusiform spore (Fig. 31'). Mature spores often formed closely opposed linear arrays corresponding to the dense basophilic colonies observed in the peripheral sarcoplasm of heart myofibres via histopathology (Fig. 4A). Individual fusiform spores fixed for transmission electron microscopy measured approximately 6 x 0.3 1111 in size and contained a distinct anchoring disk at the apical end of an uncoiled and predominantly isofilar polar filament (Fig. 4B and C). The polar filament was non-striated in its apical region (Fig. 4B) and striated in its distal region (Fig. 4D). Transverse sections of spore clusters reveal a potential for back-folding and some terminal tapering of the polar filament in its distal region (Fig. 4E). The fusiform spore was surrounded by an electron-lucent endospore overlaid with an electron-dense exospore (Fig. 4C). Minor crenulations at the exosporc surface were observed in some preparations (l'ig. 413 and C). Other features of tile

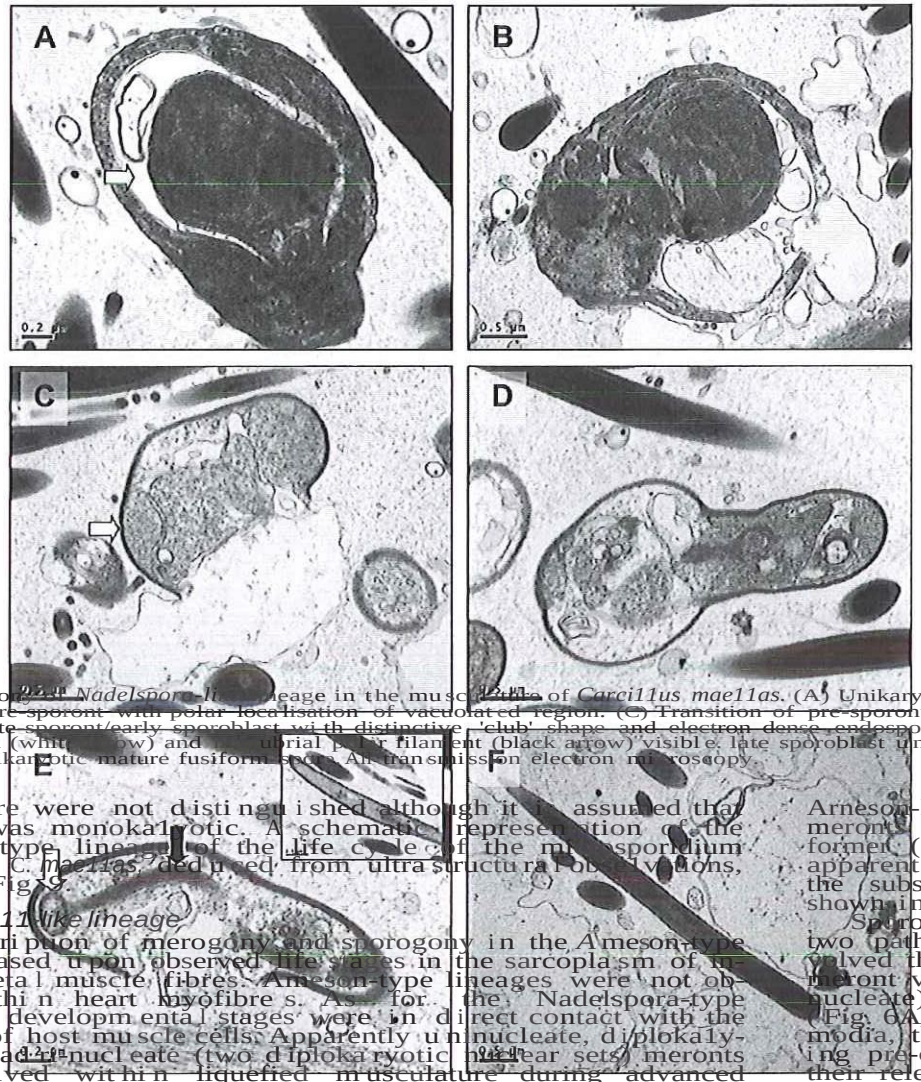

Fig. 3. Sporogony of *Nadelsporα-like* lineage in the muscle cells of *Carcinus maenas*. (A) Unikaryotic pre-sporont with early development of vacuolated region (arrow). (B) Unikaryotic pre-sporont with polar localisation of vacuolated region. (C) Transition of pre-sporont to unikaryotic sporont involving thickening of endospore (arrow). (D) Unikaryotic late sporont/early sporoblast with distinctive 'club' shape and electron dense endospore. (E) Unikaryotic early sporoblast with pre-cursors of the terminal anchoring disk (white arrow) and tubular polar filament (black arrow) visible. late sporoblast undergoes lengthening to presume the form of the mature fusiform spore (inset). (F) Unikaryotic mature fusiform spore. All transmission electron microscopy.

mature spore were not distinguished although it is assumed that the spore was monokaryotic. A schematic representation of the Nadelsporn-type lineage of the life cycle of the microsporidium infection of *C. maenas*, deduced from ultrastructural observations, is given in Fig. 4.

3.2.2. Ameson-like lineage

The description of merogony and sporogony in the Ameson-type lineage is based upon observed life stages in the sarcoplasm of infected skeletal muscle fibres. Ameson-type lineages were not observed within heart myofibres. As for the Nadelspora-type lineage, all developmental stages were in direct contact with the cytoplasm of host muscle cells. Apparently uninucleate, diplokaryotic and quadrinucleate (two diplokaryotic nuclear sets) meronts were observed within liquefied musculature during advanced infection (i.e. involving large proportions of the skeletal musculature). Maturation of meronts was defined by a decrease in the nuclear:cytoplasm ratio, bulging of the outer nuclear membrane and an increase in the volume of the electron lucent cytoplasm. Ameson-like meronts could be distinguished from *Nadelspora-like* meronts by the absence of elaborate membranous systems in the former (Fig. 5A-E). An infrequently observed stage involving apparent plasmotomy of a large multi-nucleate plasmodium and the (subsequent generation of the aforesaid meront variants is shown in Fig. 5F.

Sporogony of the Ameson-type lineage appeared to occur via two pathways. The first (hereby termed 'random sporogony') involved the progressive thickening of the endospore of the different meront variants (unikaryotic, diplokaryotic and potentially multinucleate) to form sporonts with apparently different nuclear status (Fig. 6A-C). Where this was shown to occur in multi-nucleate plasmodia, the resultant sporonts progressed to sporoblasts (containing pre-cursors to the spore extrusion apparatus), even prior to their release from the plasmodium (Fig. 6D). Maturing sporoblasts were defined by the gradual alignment of spore extrusion precursors to their final position (as defined in the mature spore) and the appearance of distinctive villous protrusions from the surface of the developing exospore (Fig. 6F). A second type of sporogony

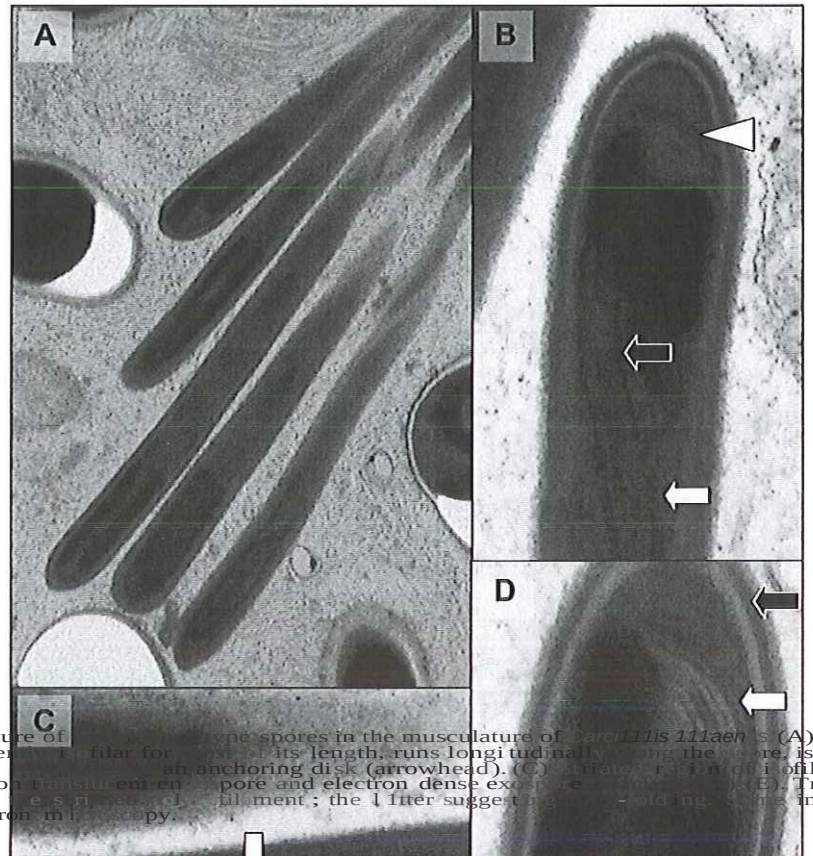

Fig. 4. Ultrastructure of [illegible] type spores in the musculature of *Carcinus maenas*. (A) Spore arrays in direct contact with the host sarcolemmal. (B) The uncoiled polar filament is apparent [illegible] for most of its length, runs longitudinally along the spore, is striated in its posterior region (white arrow) and smooth in its anterior region (black arrow) and terminates in an anchoring disk (arrowhead). (C) Striated region of isofilar polar filament (arrow). (D) Terminal anchoring disk (white arrow) closely opposed to electron translucent endospore and electron dense exospore. [illegible] Transverse sections of spores showing one profile (white arrow), or two (black arrow) profiles of the striated filament; the latter suggesting coiling. Also indication of terminal tapering of the polar filament is evident (arrowhead). All transmission electron microscopy.

(hereby termed chain sporogony) characterised by the formation of precursors (the plasmodium) trophonts in sporont chains containing most four isolated nuclei (Fig. 7A and B). Budding of individual unikaryotic pre-sporoblasts (Fig. 7C) led to the formation of early (Fig. 7D) and maturing (Fig. 7E) sporoblasts the latter containing a distribution of organelles characteristic of the mature spore and the [illegible] protrusions on the surface of the exospore. Individual [illegible] shaped spores fixed for transmission electron microscopy, measured approximately 1.2 × 1.0 μm in size, contained an anchoring disk (Fig. 8A and B) at the apical end of an isofilar polar filament. The polar filament was coiled approximately six times in a linear manner and diverted into the centre of the spore in a distal region (Fig. 8A). The polar filament passed through a distinctive polaroplast with an electron-dense outer layer and a laminar inner region (Fig. 8A). The oval spore was surrounded by a thick electron-lucent endospore and an electron-dense exospore containing distinctive surface projections (Fig. 8A and C). Although not observed, it is assumed that the Ameson-type lineage spore is monokaryotic. Arrays of Nadelsporn-type lineage spores were occasionally observed amongst predominantly Ameson-type life stages (Fig. 8D). A schematic representation of the Ameson-type lineage of the life cycle of the microsporidium infection of *C. maenas*, deduced from ultrastructural observations, is given in Fig. 9.

3.3. Molecular phylogeny

Phylogenetic analyses were based upon two infected crabs displaying only *Nadelspora*-like life stages in the musculature of the heart and predominantly Ameson-like life stages within the skele-

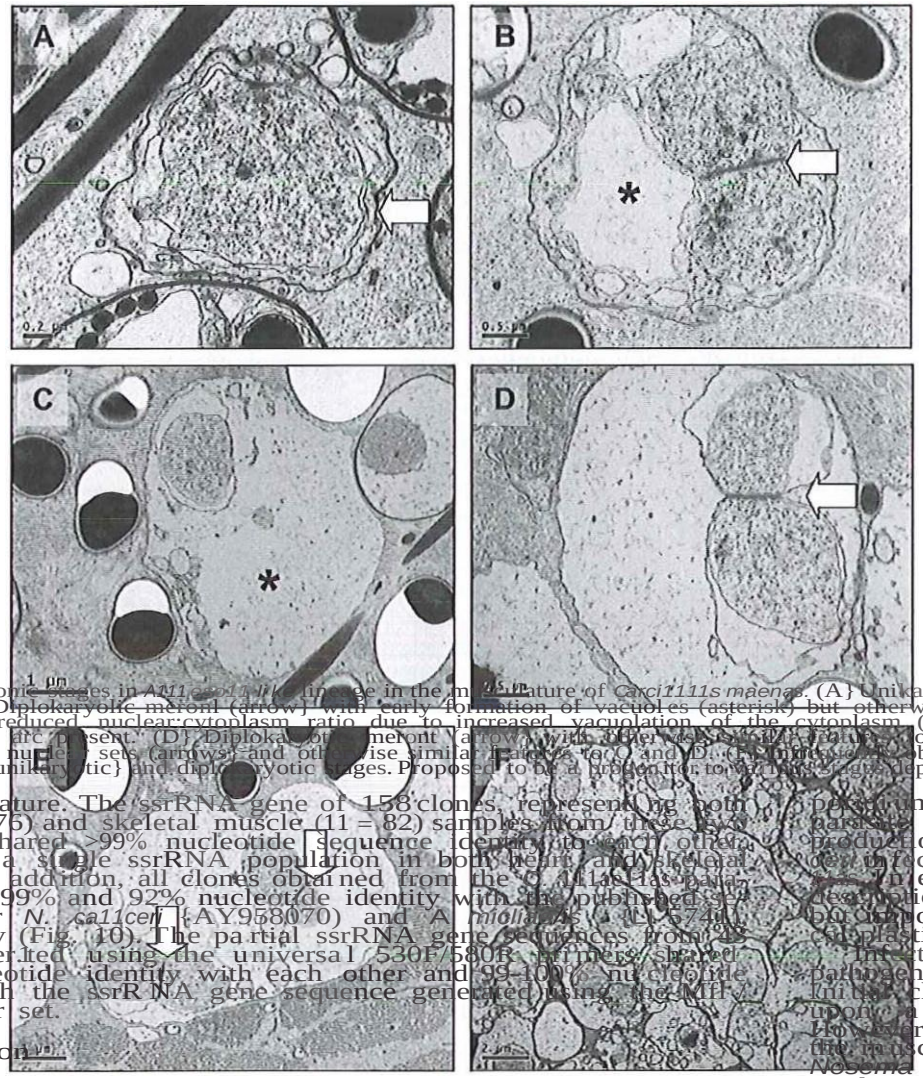

Fig. 5. Merogonic stages in *A111ae11as* lineage in the musculature of *Carci1111s maenas*. (A} Unikaryotic meront with high nuclear:cytoplasm ratio and simple plasmalemma (arrow}. (B} Diplokaryolic meronl (arrow) with early formation of vacuoles (asterisk) but otherwise similar cytological features to A. (C} Unlkaryotic meront showing significantly reduced nuclear:cytoplasm ratio due to increased vacuolation of the cytoplasm (asterisk}. Infrequent cytoplasmic membrane structures and a simple plasmalemma are present. (D} Diplokaryotic meront (arrow) with other features similar to B. (El Apparently quadranucleale meronl plasmodia containing two diplokMyotic nuclei sets (arrows) and otherwise similar features to C and D. (F} Unidentified observed stage apparently involving the fomMtion of uninucleate, multinucleale (but unikaryotic} and diplokaryotic stages. Proposed to be a progenitor to various stages depicted in A-E and Fig. GA-E. All transmission electron microscopy.

tal muscu lature. The ssrRNA gene of 158 clones, representing both heart (11 = 76) and skeletal muscle (11 = 82) samples from three [illegible] animals shared >99% nucleotide sequence identity to each other, suggesting a single ssrRNA population in both heart and skeletal muscle samples. In addition, all clones obtained from the *C. 111ae11as* parasite shared 99% and 92% nucleotide identity with the published sequences for *N. ca11cell* (AY958070) and *A. miali* (AF274401), respectively (Fig. 10). The partial ssrRNA gene sequences from 48 clones generated using the universal 530F/580R primers shared >99% nucleotide identity with each other and 99-100% nucleotide identity with the ssrRNA gene sequence generated using the MiFl/Mrl primer set.

4. Discussion

Combined field observations of highly statistically significant co-occurrence, combined with histopathological, ultrastructural and molecular phylogenetic analyses, have been used to provide strong evidence for extreme morphological plasticity in a microsporidian parasite infecting the musculature of marine crabs. The parasite appears to alternate between lineages that culminate in production of either bizarre fusiform spores (reminiscent of *N. ca11cell* infecting *C. magister)* or alternatively, *A. pulvis* infecting *C.* 111ae11as. In essence therefore, the current study extends the taxonomic description of *A. pulvis* (to include the alternative spore sequence) but importantly, highlights the potential for extreme morphological plasticity in the Microsporidia.

Infection of the musculature of *C. 111ae11as* by a microsporidian pathogen was described over 100 ye.us ago (Perez, 1905a,b). Initial classification of the pathogen as *Nosema pulvis* was based upon a presumed similarity to congeners within *Nosema*. However, this was superseded when a similar pathogen within the musculature of the American blue crab *(C. sapidus)* (initially *Nosema mic/raelis)* was shown to undergo sporoblast production in chains. This discovery led to removal of the blue crab pathogen from the genus *Nosema* and designation of a new genus (Ameson) in which the blue crab pathogen was erected as the type species (A 111iclrae/is). At the same time, the pathogen from *C. 111ae11as,*

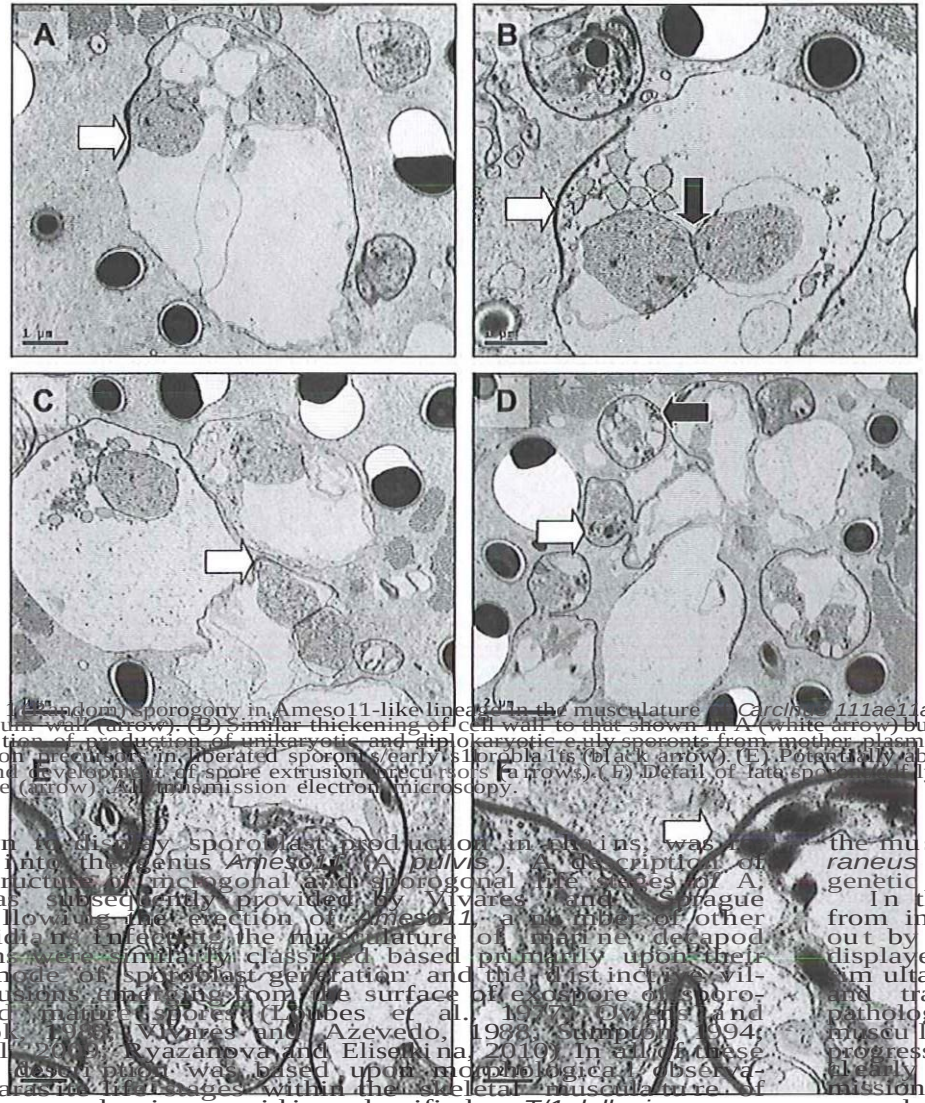

Fig. G. Type 1 (random) sporogony in Ameso11-like linear in the musculature of *Carcinus 111ae11as*.(A) Early sporogony of apparent bi-nucleate stage involving thickening of the plasmodium wall (arrow). (B) Similar thickening of cell wall to that shown in A (white arrow) but involving life stage with diplokaryotic nuclear set (black arrow). (C and D) Combination of production of unikaryotic and diplokaryotic early sporonts from mother plasmodium. Note thickening of cell walls (white arrows) and early formation of spore extrusion apparatus in liberated sporonts/early sporoblasts (black arrow). (E) Potentially aberrant liberated bi-nucleate sporont/early sporoblast with isolated nuclei (asterisks) and development of spore extrusion apparatus (arrows). (F) Detail of late sporont/early sporoblast wall showing initial development of villous protrusions from the exosposre (arrow). All transmission electron microscopy.

also shown to display sporoblast production in plasmodia; was initially classified into the genus *Ameso11a* (*A. pulvis*). A description of the ultrastructure of merogonal and sporogonal life stages of *A. p11/vis* was subsequently provided by Vivares and Sprague (1979). Following the erection of *Ameso11a* and a number of other microsporidian infections of the musculature of marine decapod crustaceans were primarily classified based primarily upon their apparent mode of sporoblast generation and the distinctive villous protrusions emerging from the surface of exospore of sporoblasts and mature spores (Toubes et al., 1977; Overstreet and Glazebrook, 1979; Vivares and Azevedo, 1986; Shields, 1994; Kiryu et al., 2004; Ryazanova and Elisenkina, 2010). In addition these cases, the description was based upon morphological observations of parasite life stages within the skeletal musculature of the host. A second microsporidian, classified as *T/1elollania maenadis* based upon the presence of a 'pansporoblast' and distinct spore morphology from *A pulvis,* was also described infecting the musculature of *C. 111ae11as* and its congener *Carcinus mediterraneus* from France (Vivares, 1980; Vavra et al., 1981). No phylogenetic information is available for this pathogen.

In the current study, multiple organs and tissues were sampled from individual *C. mae11as* as part of routine field surveys carried out by our laboratory. Upon dissection, a low percentage of crabs displayed lethargy, white-opaque skeletal musculature with simultaneous opacity within the heart myofibres. Histopathology and transmission electron microscopy revealed a distinctive pathology, consistent with infection by *A. pulvis,* within the skeletal musculature. However, the heart myofibres of the same crabs were progressively replaced with basophilic colonies which could not be clearly classified as a microsporidian pathogen by histology. Transmission electron microscopy of heart myofibres from these crabs revealed a bizarre form of microsporidian pathogen, consistent with the previous description of *N. carceri* in Dungeness crabs (*C. 111agister)* captured from the US Pacific coast (Olson et al., 1994).

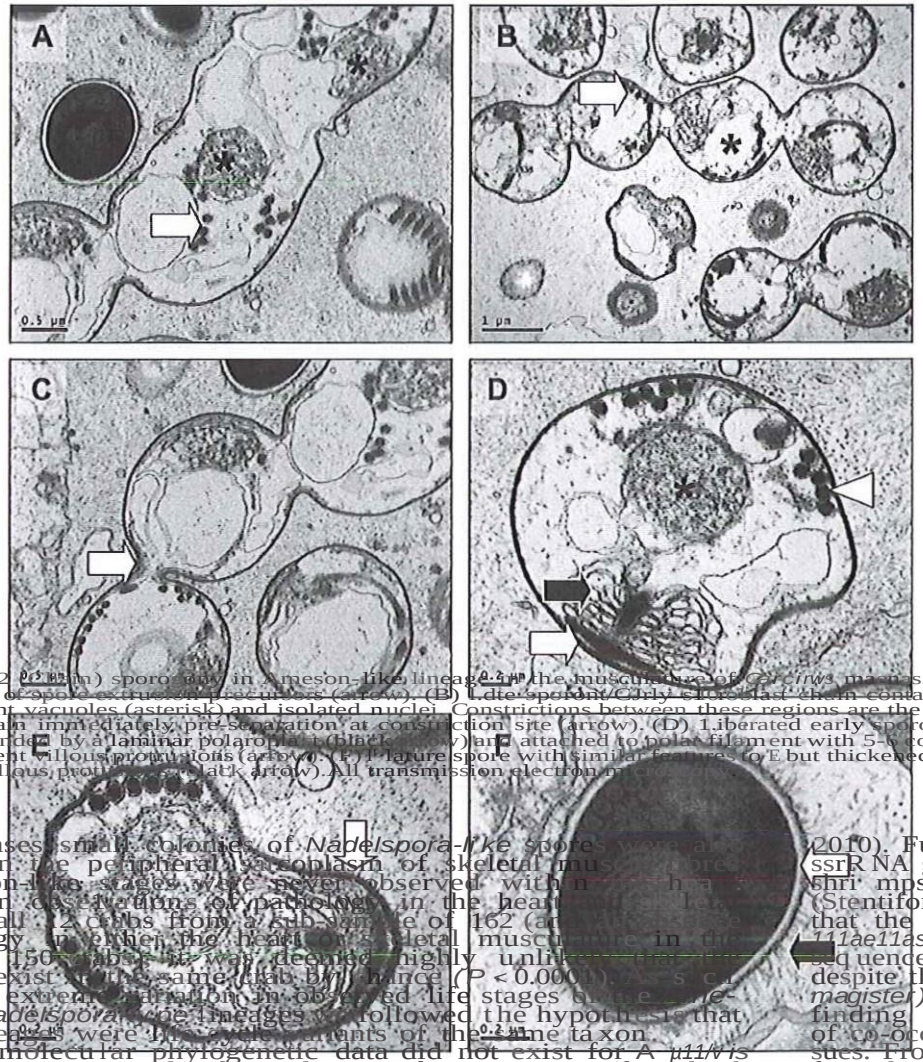

Fig. 7. Type 2 (0.2 µm) sporogony in Ameson-like lineage in the musculature of *Carcinus maenas*. (A) Elongated sporont containing isolated nuclei (asterisk) and early development of spore extrusion precursors (arrow). (B) Late sporont/early sporoblast chain containing four distinct regions housing spore extrusion precursors (arrow), electron lucent vacuoles (asterisk) and isolated nuclei. Constrictions between these regions are the sites of future separation of discrete sporoblasts. (C) Late sporont/early sporoblast chain immediately pre-separation at constriction site (arrow). (D) Liberated early sporoblast with isolated nucleus (asterisk), flattened anchoring disk (white arrow) surrounded by a laminar polaroplast (black arrow) and attached to polar filament with 5–6 coils (arrowhead). (E) Late stage sporoblast with similar features to D but more prominent villous protrusions (arrow). (F) Mature spore with similar features to E but thickened endospore (white arrow), electron dense spore cytoplasm (asterisk) and prominent villous protrusions (black arrow). All transmission electron micrographs.

In some cases, small colonies of *Nadelspora-like* spores were also observed in the peripheral sarcoplasm of skeletal muscle precursors but Ameson-like stages were never observed within such tissues. Based upon observations of pathology in the heart and skeletal muscle of all 12 crabs from a sub-sample of 162 (approximately 7%) of pathology in either the heart or skeletal musculature in the remaining 150 crabs, it was deemed highly unlikely that the two types exist within the same crab by chance ($P < 0.000$). As such, despite the extensive variation in observed life stages between Ameson- and *Nadelspora*-like lineages we followed the hypothesis that the two lineages were life cycle variants of the same taxon.

Whilst molecular phylogenetic data did not exist for *A. µ11/vis* prior to the current study, the inclusion of *A. mic/1aelis* (for which ssrRNA sequence data was available, Zhu et al., 1993) in previous phylogenetic analyses of the Microsporidia showed that it grouped most closely with other microsporidians infecting marine animals (Vossbrinck and Debrunner-Vossbrinck, 2005; Stentiford et al., 2010). Furthermore, its closest relatives (at least based upon ssrRNA gene sequences) were *Perezia 11e/so11i*, a parasite of penaeid shrimps, and particularly, *N. ca11ceri*, infecting cancrid crabs (Stentiford et al., 2010). In the current study, we have demonstrated that the microsporidian infecting the musculature of European C *111ae11as* (=A. *µu/vis*) is more similar (>99% based upon ssrRNA gene sequence) to *N. canccri* than it is to its congener *A micliae/is* – this despite the former being an infection of a different crab species (C *magister*) from North American waters (Olson et al., 1994). The finding would seem remarkable were it not for our observation of co-occurring needle-like spores within infected *C. 111ae11as* tissues. From the data presented herein, it seems most likely, therefore, that previous descriptions of *A. p11/vis* in *C. mae11as* failed to detect needle-like spores in infected specimens, perhaps due to the fact that samples of heart tissues (where the needle-like forms predominate) were not analysed. Despite this, an 'unidentified stage' which at the time could not be confidently aligned with

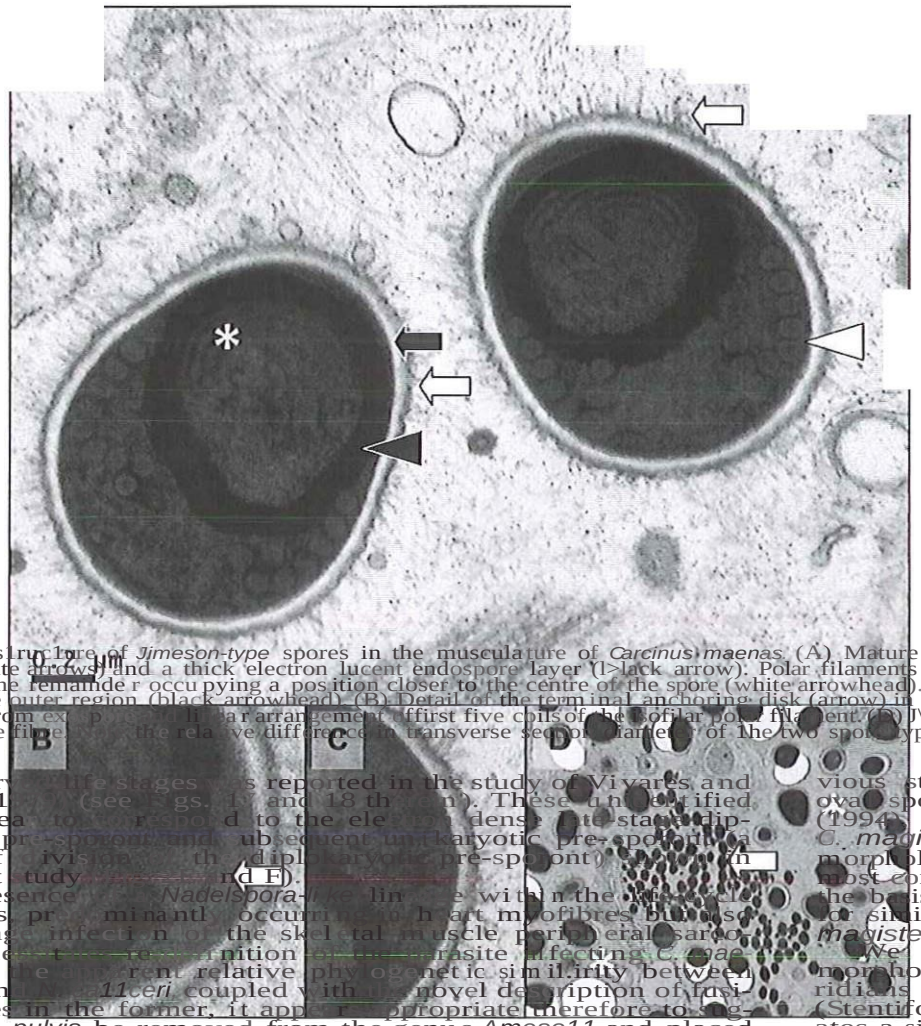

Fig. 8. Ultrastructure of *Jimeson-type* spores in the musculature of *Carcinus maenas*. (A) Mature spores display characteristic villous projections from the surface of the exospore (white arrows) and a thick electron lucent endospore layer (black arrow). Polar filaments are arranged in 8-9 isofilar coils with five of these occurring in a linear fashion and the remainder occupying a position closer to the centre of the spore (white arrowhead). A distinctive polaroplast contains a laminar inner region (asterisk) and an electron dense outer region (black arrowhead). (B) Detail of the terminal anchoring disk (arrow) in close proximity to the inner surface of the endospore. (C) Detail of villous projections from exospore. (D) Isofilar arrangement of first five coils of the isofilar polar filament. (E) Jimeson-type (asterisk) and *Nadelspora-type* spores (arrow) occupying the striated muscle fibre. (F) The relative difference in transverse section of filaments of the two spore types. All transmission electron microscopy.

other observed life stages as reported in the study of Vivares and Sprague (1980) (see Figs. 17 and 18 therein). These unidentified stages appear to correspond to the electron dense proto-stage, diplokaryotic pre-sporont and subsequent uninucleate pre-sporont (a product of division of the diplokaryotic pre-sporont) observed in the current study (Fig. 7D and E).

The presence of *Nadelspora*-like lineage within the host muscle of *A pulvis* predominantly occurring in heart myofibres, but also in early stage infection of the skeletal muscle peripheral sarcoplasm, necessitates redefinition of the parasite infecting *C. maenas*. Given the current relative phylogenetic similarity between *A pulvis* and *Ameso11 ceri* coupled with the novel description of fusiform spores in the former, it appears appropriate therefore to suggest that *A pulvis* be removed from the genus *Ameso11* and placed within the genus *Nadelspora*. However, considering the taxonomic description both of the genus *Nade/spora* and its type species *(N. ca11ceri)* provided by Olson et al. (1994), this would require a significant redefinition (essentially to accommodate parasites with the potential to produce oval spores with villous protrusions, at least for part of their life cycle). Despite a brief mention of a previous study which noted 'an undescribed microsporidium with oval spores' in the type description of *Nadelspora* by Olson et al. (1994), no further descriptions of microsporidium infections in *C. magister* have been made. However, the description of extreme morphological plasticity in the parasite from *C. maenas* (with almost complete identity to *N. ca11ceri*, both in morphology and on the basis of ssrRNA gene sequence) provides at least the potential for similar morphological plasticity in *N. canceri* infections of *C. magister*.

We have previously discussed the potential for considerable morphological variation in crustacean muscle-infecting microsporidians shown to be similarly related via phylogenetic analyses (Stentiford et al., 2010). Furthermore, such plasticity in form creates a significant issue when attempting to classify novel microsporidians. For example, the closely related taxa, *Tl1e/olw11ia butleri* and *Myospom metanephrops*, form sporophorous vesicles within myofibres (Brown and Adamson, 2006) or develop in direct contact with the host sarcoplasm, respectively (Stentiford et al., 2010). We utilised these observations to suggest that the crustacean musculature does not appear to impose any particular limita-

Arneson-li ke lineage **Nade/spora-l i ke lineage**

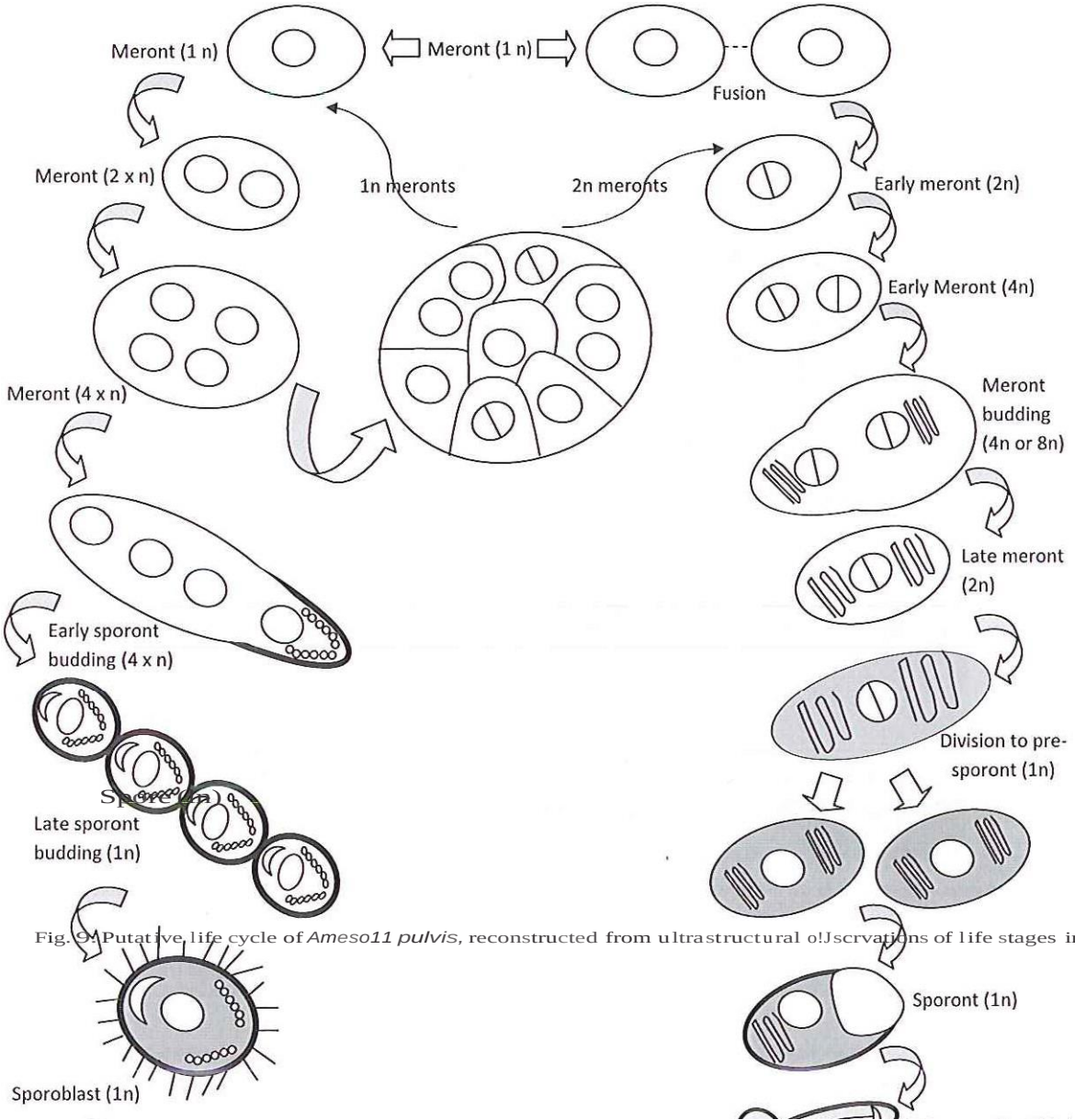

Fig. 9. Putative life cycle of *Ameso11 pulvis*, reconstructed from ultrastructural o!Jscrvations of life stages in heart and skeletal musculatu re of *Carci1111s mae11as*.

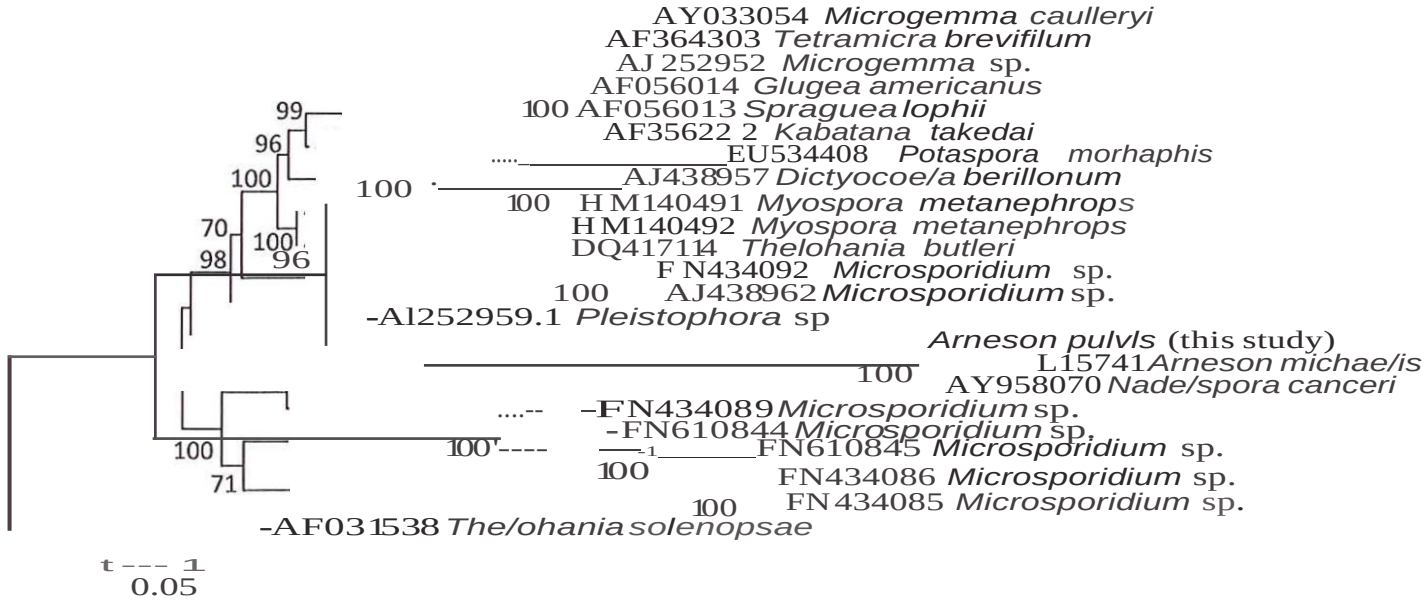

Fig. 10. Neighbour-joining tree based on a 891 nucleotide partial ssrRNA gene sequence. The ssrRNA gene sequence from *Thelolia11ia solenopsae* (Af03I538) was used as an outgroup. The phylogenetic analysis was performed using MEGA vcrsion 3.1. Analysis was done on 1,000 bootstrapped data sets and values >70X arc shown on the tree. The scale bar represents substitutions per nucleotide site.

tions on the potential for development of such widely divergent characters in this parasite group. Given the fact that *Ameso11, Nade/spora* and *Perezia are* also closely related at the small subunit gene level, but morphologically diverse, we proposed that a family (Myosporidae) be erected to contain these pathogens, despite the fact that a strict morphological definition of the family, a requirement of the International Code for Zoological Nomenclature (ICZN), would be impossible (Stentiford et al., 201O). The current study, describing the co-occurrence of a microsporidian with extremely divergent life stage characteristics, within individuals of a single host species, supports the notion that morphological plasticity defines at least this group of pathogens within the phylum Microsporidia. For this reason, and abiding by rules of the ICZN, it is not possible here to redefine either the genus *Ameso11*, or the genus *Nadelspora* to satisfactorily accommodate the parasite in C. *mae11as*. The issue is further confounded by a description of another microsporidian infecting the musculature of C. *mae11as (T. 111ae11adis)* in which spores form within a 'pansporoblastic membrane'. Given the observations of extreme plasticity presented here for A *pu/vis,* it is interesting to speculate whether this potential for plasticity may also extend to the formation of such a 'pansporoblast membrane' in A)JU/vis (and therefore additionally consuming the description of *T. 111ac11adis* in the musculature of C. *111ae11as* and C. *111editerra11eus* into this taxon). Taken together, the information presented herein further supllorts the concept that morphological variI11ces are the rule rather than the exception within the Marinosporidia (Vossbrincl< and Debrunner-Vossbrinck, 2005). In addition, although limitations may exist in exclusive use of the ssrRNA gene for phylogenetic analyses, it is certainly apparent that descriptions based purely on morphological criteria are no longer feasible for this parasite group.

The rationale for the extreme plasticity shown by *A. pulvis* is not known. However, the Nodelspora-like spores apparently appeared earliest in the infection process and were found exclusively within heart myofibres or within the peripheral sarcoplasm of skeletal muscle fibres. In many instances, the needle-like spores formed arrays which appeared to align and displace individual myofibrils of the muscle fibre. As the infection became more severe within the skeletal muscles, liquefaction of the peripheral fibres occurred; this coincided with a decrease in the presence of *Nade/spora-like* spores and a preponderance of Ameso11-like spores (and their precursors). The progressive colonisation of the skeletal muscle mass was then almost solely due to Ameson-like spores. Whilst these observations were made on individual crabs (rather than at time points following experimental infection), the progression from *Nade/spora-* to Ameson-like life stages appeared to be consistent. It is tempting to suggest therefore that the Nac/e/sporn-like lineage occurs following initial invasion of the peripheral sarcoplasm with the *Ameso11-* like lineage predominating later in the infection process. Since Amesou-like spores are far more abundant in crabs displaying advanced stage disease, it is presumed also that this spore type is involved in transmission to new hosts. In this way, a switch between Ameso11-like spore and a Nadels)Jorn-lineage meront would elucidate the transition between the two distinct lineages (see Fig. 9). Standard infection trials with isolated spore types or studies on the 'secretome' of *Nadc/spora-like* and 'Amesón-like life stages, and the effect that these excreted products may have on the remodelling of host muscle fibres, may offer considerable insight into

the invasion processes employed by this pathogen and the Microsporidia more generally (see van Ooij et al., 2008; Choi et al., 2010). The convenient anatomical separation of only *Nadelspora-like* lineages (in the heart) and predominantly Ameson-like lineages (in late stage infection of the skeletal muscle) may assist in the collection of these discrete life stages.

In conclusion, we have provided epidemiological, pathological, morphological and molecular evidence that a parasite described over a century ago in a common species of European crustacean appears to undergo a remarkable shift in morphology during pathogenic remodelling of host tissues. In addition to the known lineage of the life-cycle fulfilling the taxonomic criteria for the genus *Ameson*, an alternative lineage, which results in the production of bizarre fusiform spores, places it within the genus *Nadelspora*.

Although molecular phylogenetic data is supportive of the latter, the significant expansion in taxonomic description required to include these widely divergent lineages is not feasible within the current taxonomic framework of the phylum Microsporidia.

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Report

Mikrocytids Are a Broadly Distributed and Divergent Radiation of Parasites in Aquatic Invertebrates

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Summary

Microcell parasites have independently evolved in several eukaryotic lineages and are increasingly recognized as important and emerging pathogens of diverse hosts, including species of economic importance subject to international legislation concerning the trading of aquatic animals [1-3]. The microcell *Mikrocytos mackini* causes Denman Island disease of oysters and represents one of the most genetically divergent eukaryotes known. *Mikrocytos* has remained an isolated lineage with a limited distribution. We investigated two emerging diseases of juvenile crabs and oysters from the UK using massively parallel sequencing and targeted primer approaches to reveal that their causative agents are highly divergent lineages related to *M. mackini* (*Paramikrocytos canceri*) n. gen. et n. sp. and *M. mimicus* sp. nov., respectively). We demonstrate a major new globally distributed parasite radiation (Mikrocytida ord. nov.) with phylogenetic affinities to the commercially important haplosporidian parasites of invertebrates. Mikrocytids have eluded detection because of their small size, intracellular habit, and extreme sequence divergence. *P. cancer*/ was frequently detected in a range of shoreline invertebrates, demonstrating that these newly recognized parasites are in fact common, diverse, and widespread and should be considered when assessing the risks of aquaculture activities, invasive species spread, and movements of ballast water and sediments with associated invertebrates.

Results and Discussion

In 2011 a "haplosporidian-like" microcell infection was reported at high prevalence in juvenile European edible crab *Cancer pagurus* in Weymouth, UK (4), and recently a similar parasite was reported in the same host from south Wales, UK [5]. The development of unicellular stages into plasmodia and the presence of cytoplasmic inclusions suggested an affinity with asporous haplosporidians of decapod crustaceans (2, 6) (Figure 1). However, our phylogenetic analyses robustly grouped the parasite as a sister to *Mikrocytos mackini* (Figures 2 and 3), which was previously known as an orphan lineage with one very close relative, *Mikrocytos* sp. BC (HM563061) (7), very recently described as *M. boweri* (KF297352/3) (CL. Abbott, personal communication). Our finding of a highly divergent relative commonly infecting a commercially significant crustacean was unexpected as *Mikrocytos* was known only from bivalve molluscs.

The novel microcell induces hypertrophy of the antennal gland and bladder of reproductively immature crabs (Figure 1A). Infections comprise unicellular and plasmodial parasite stages within the epithelial cells of infected glandular tubules (Figures 1B-1D). As all attempts to sequence the small subunit (SSU) rDNA from this microcell using existing pathogen assays and an extensive range of PCR primer combinations failed, a high-throughput next-generation sequencing (NGS) approach (Illumina MiSeq) was used to sequence total DNA extracted from a heavily infected juvenile crab antennal gland, from which SSU sequences of only a decapod and a highly divergent eukaryote were recovered. The complete parasite SSU-ITS1-5.8S-ITS2-LSU (LSU, large subunit) rDNA was assembled and verified by Sanger sequencing. The SSU rDNA sequence was only 68% similar to that of *M. mackini* and was more dissimilar to all other eukaryotes, a level of sequence dissimilarity equivalent to phylum-level differences or above in many other eukaryotic lineages. Lineage-specific in situ hybridization (ISH) was then used to demonstrate that this sequence type derived from the observed microcells within crab tissues (Figure 1B). We thus describe a new taxon, *Paramikrocytos canceri* n. gen. et n. sp., on the basis of its phylogenetic affinity to (but with high divergence from) the genus *Mikrocytos*, different host range, ability to form plasmodia, and relative abundance of putative mitochondria-related organelles (MROs) (Figures 1F and 1G; see the Supplemental Results, Section 1, available online). The putative MROs in *P. canceri* are double-membrane-bounded organelles of ~0.5 μm diameter. Their presence contrasts with ultrastructural observations in *M. mackini*, in which obvious MROs were not recorded [8], even though genomic signatures suggest their presence [9]. Further work is required to determine the identity of MRO-like organelles in *P. canceri*.

Paramikrocytos canceri Is Frequently Detected in a Wide Range of Invertebrates

After the discovery that *M. mackini* and *P. canceri* are sister lineages, we designed a mikrocytid PCR assay and screened 511 marine, freshwater, and soil environmental DNA/cDNA (eDNA) samples from the UK, continental Europe, South Africa, Panama, and Borneo and 425 organismal samples from eight invertebrate phyla for mikrocytid sequences. The most intensive sampling was conducted at two sites, one with high *P. canceri* prevalence in *C. pagurus* (a rocky shore at Newton's Cove in Dorset, UK (35 ppt salinity; 50°34'N, 2°22'W)), and a nearby brackish site with few *C. pagurus*

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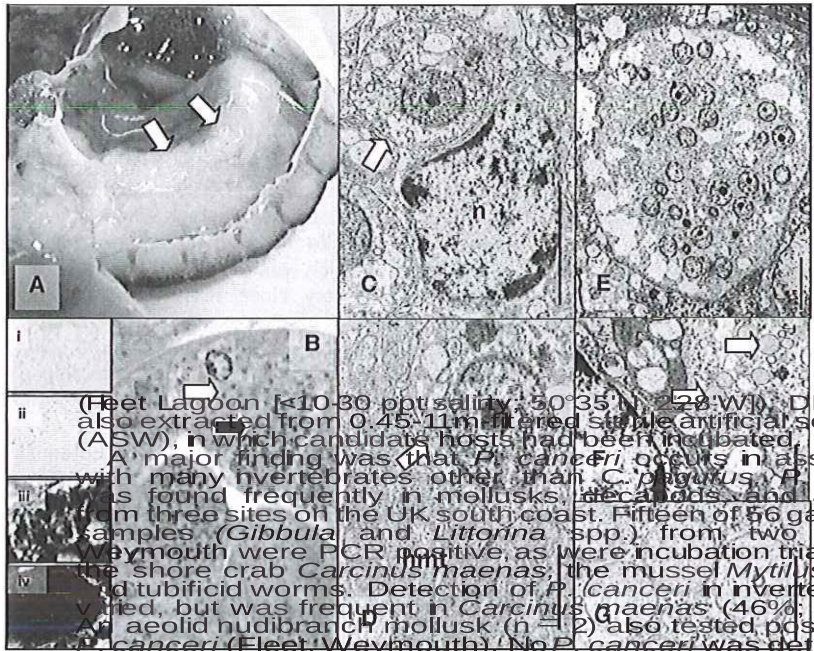

Figure 1. *Paramikrocytos canceri* Infection of the Edible Crab, *Cancer pagurus*

Hypertrophy of the antennal gland (A, arrows) is associated with colonization of the epithelium by masses of uninucleate (B, white arrow) and plasmodial (B, black arrow) life stages of the parasite. Both life stages are detected using in situ hybridization (B, inset; i, no probe I, uninfected tissue with probe; Iii, uninucleate forrn with probe; iii, plasmodium with probe). Uninucleate life stages occur in direct contact with the host cell cytoplasm and can displace organelles (see nucleus, n, in C) and are often closely opposed to host mitochondria (hmt in D, opposed to parasite, arrow). Plasmodial life stages (E) containing many nuclei and an abundance of putative mitochondria-related organelles are shown (arrows in F and higher power in G). Scale bars represent C31in1 (C), 111m (0), 511m (E), 111m (F) and 500 nm (G). Additional figures are provided In the Supplemental Results, Section 1, Figure S1.

(Fleet Lagoon [<10-30 ppt salinity; 50°35'N, 2°28'W]). DNA was also extracted from 0.45-11m-filtered sterile artificial seawater (ASW), in which candidate hosts had been incubated.

A major finding was that *P. canceri* occurs in association with many invertebrates other than *C. pagurus*. *P. canceri* was found frequently in mollusks, decapods, and annelids from three sites on the UK south coast. Fifteen of 56 gastropod samples *(Gibbula* and *Littorina* spp.) from two sites in Weymouth were PCR positive, as were incubation trials using the shore crab *Carcinus maenas*, the mussel *Mytilus edulis*, and tubificid worms. Detection of *P. canceri* in invertebrates varied, but was frequent in *Carcinus maenas* (46%; n = 24). An aeolid nudibranch mollusk (n = 2) also tested positive for *P. canceri* (Fleet, Weymouth). No *P. canceri* was detected in zoeae and megalopae of *C. pagurus* or in other decapod larvae in a plankton sample from the eastern English Channel coast, suggesting that infection of *C. pagurus* by *P. canceri* may occur after the larvae have settled in the littoral zone. The presence of *P. canceri* in littoral habitats close to the plankton tow site has not yet been confirmed.

In contrast to the invertebrate tissue and incubation water samples, *P. canceri* was not detected in any environmental sample (eDNAs). This was surprising, given (1) the high prevalence of parasite infection in crabs at the sampling site, (2) the large volume of the planktonic samples (200l), and (3) that the uninucleate and plasrnodial forms of *P. canceri* are excreted copiously in the urine via the crabs' antenna! glands [4]. The reasons for this nondetection are unknown; perhaps the parasite cells rely on trophic transmission and degrade soon after excretion from the antenna! gland. The wide range of invertebrates in which *P. canceri* was detected would certainly support such a trophic transmission route via incidental consumption during grazing and detritus feeding. In any case, this pattern is in clear contrast to haplosporidian invertebrate parasite SSU types from the same sites, which amplify from many of the same eDNA samples using very similar methods (1OJ.

Mikrocytid Diversity Revealed by PCR Screening

The mikrocytid PCR assay also revealed a divergent radiation of other novel mikrocytid lineages. One SSU type from filtered water from the brackish Fleet Lagoon (Weymouth, UK; marked "a" in Figure 2) was highly distinct from other known and novel mikrocytids. Two further related SSU types originated from the slightly brackish lagoon of Rondevlei Nature Reserve (Cape Town, South Africa) and from sediment associated with mangroves near Sandakan, Borneo. These are sister to two further novel lineages from eDNA samples, one from a freshwater forest stream in Panama and the other from a freshwater lake near Reading, UK. This clade (marked "b" in Figure 2) therefore comprises separate lineages from freshwater and brackish environments, providing the first evidence that mikrocytids have radiated in a wide diversity of aquatic habitats. Other novel SSU types were detected only in DNA extracted from organisms, one from the trochid gastropod *Gibbula umbilicalis*, another from *Cancer pagurus* incubation trials, and another from several individuals of tidepool shrimp from the Pembrokeshire coast, Wales (marked "c" in Figure 2).

During the course of our study, a novel pathogen was received for investigation by the UK Centre for Environment, Fisheries and Aquaculture Science from an intertidal Pacific oyster *(Crassostrea gigas)* farm on the North Norfolk coast (Brancaster), UK. The causative agent was initially suspected to be the first UK record of *M. mackini* due to its superficially similar gross pathology (green pustules within the adductor muscles) and morphology. However, histology and transmission electron microscopy (TEM) analyses revealed distinct pathological responses and ultrastructure (Supplemental Results, Section 2). SSU phylogenies robustly grouped this parasite in the same clade as *M. mackini* and *M. boweri*, but the sequence was only 79% similar to *M. mackini* (Figure 2). The standard assays for *M. mackini* listed by the World Organization for Animal Health (OIE) did not detect it in infected oyster tissue. This pathogen is the first incidence of a *Mikrocytos* infection in Pacific oysters cultured in Europe and represents a new potential threat to the commercial mollusk industry. We describe it here as *Microcytos mimicus* n. sp. (Supplemental Results, Section 2).

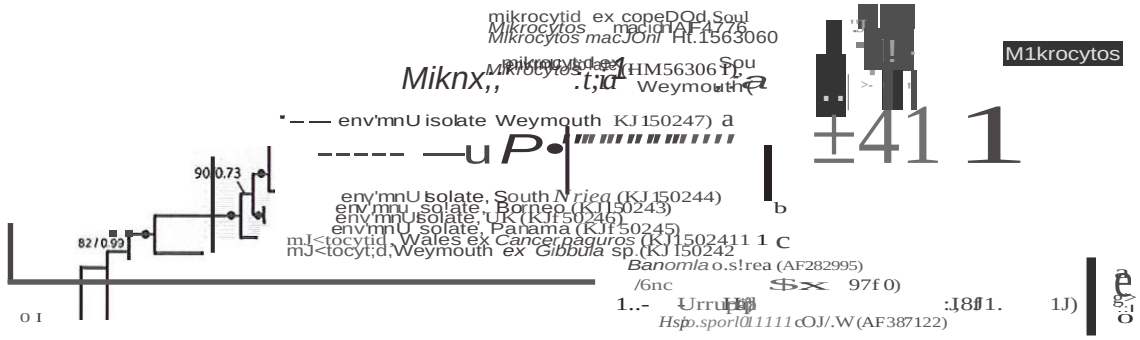

Figure 2. Novel Hosts and Geographic Range of Microcytids Revealed by Targeted Lineage-Specific PCR of the Approximately 480 bp Variable Regions V5 to V7 of the SSU rONA Letters a-<: Idate novel mikrocytid lneages detected In this study, andlmages Indicate the range of hostsh which *P. cancerl* was detected by PCR. The full range of samples tested Is pro·vided h the Supplemental Results, Section 3, Table S1. The maximum-ikelihood topology Is shown. Dark circles hdicate bootstrap support values >90 and Bayesian posterior probabilities >99, and htermediate support values are mapped on the tree and branches with bootstrap support values <80 and/or posterior probabilities <90 were collapsed.

M. mackini itself, which causes disease and mortalities in several economically important oysters (11), has so far only been found along the west coast of North America (C.L. Abbott, personal communication). A very closely related lineage referred to as *"Mikrocytos* sp.BC" (HM563061), originally found in Pacific oysters (Crassostrea *gigas)* [7], has now also been found causing pathology in Olympia oysters *(Ostrea lurida)* In British Columbia, Canada, from which it is described as *M. boweri* (C.L. Abbott, personal communication). Mikrocytids with an identical SSU variable region 4 (V4) sequence to *M. boweri* have been detected in edible oysters (0.*edu/is)* in Nova Scotia, Canada (after transport and quarantine in France) (12); in C. *gigas* from the north coast of the Yellow Sea, China (13); and in *Donax trunculus* in France (14). Other Mkrocytos-like parasites have been detected in *Donax trunculus* in France (14) and O. *lurida* in San Francisco Bay, California (15). We detected the *M. mackinl* SSU type (AF477623, HM563060) only once in our study, from copepods sampled from a gyre in the southern Atlantic Ocean (Supplemental Results, Section 3, Table S1) and a sequence type very similar to *M. boweri* from filtered water samples from Newton's Cove (Figure 2). It is therefore clear that environmental and organismal samples provide complementary information, suggesting that wider and more diverse sampling would reveal higher diversity.

Mikrocytids Are Not Represented in Public Sequence Databases

Environmental sequencing approaches are facilitating a massive increase in knowledge of microbal eukaryote diversity. Despite this, no sequences similar to any mikrocytid lineage were found in any publicly available NGS data sets on Camera (16) or on NCBI GenBank portals. Their absence from even large-scale amplicon data sets can be explained by their extremely divergent SSU rDNA, eluding "universal" eukaryotic PCR primers. Therefore the use of targeted primers on host-associated material clearly has huge potential for expanding our knowledge of exceptional and enigmatic groups.

In this study, PCR-free metagenomic shotgun sequencing was initially required to determine the SSU rDNA sequences of *P. canceri* from infected crab tissue. Mikrocytids have been overlooked because of their very small size, intracellular habit, lack of spore stages (all conspiring toward faint staining via histology), and extreme sequence divergence [9, 12]. Therefore, highly targeted PCR and PCR-free approaches are likely to become powerful tools offering insights into the ecology of divergent lineages currently not afforded by traditional parasitological methods.

The Phylogenetic Position of Mlkrocytids

The extreme divergence of mikrocytlds precluded molecular phylogenetic placement until a recent 119-gene phylogenomic analysis [9] showed that *M. mackini* most likely groups within the nonfilosan Cercozoa, possibly within a clade including *Gromia* and *Filoreta* (incorrectly *"Corallomyxa"* [17]). However, *M. mackini* was very long branched on the multigene tree, and its specific placement within nonfilosan Cercozoa was unresolved [9]. We judged that a similar analysis including *Paramikrocytos* would not be more informative and that a better way to increase phylogenetic resolution was to increase taxon sampling in this region of the tree. We chose four genes (SSU and LSU rDNA, *hsp90* and *{J-tubulin,* assembled from MiSeq parasite-host metagenomes or generated using lineage-specific primers), which allowed us to add new gene data for some related lineages: *Filoreta marina* (LSU rDNA), the plasmodiophorid *Spongospora* sp. (SSU and LSU rDNA), and *Haplosporidium littora/is* (SSU and LSU rDNA, *hsp90,* and *{J-tubulin).*

Our phylogenetic analyses group mikrocytids as sister to Haplosporida (represented by *H. littoralis* and *Bonamia* ostreae) within a clade also including *Gromia* and *Filoreta* (Figure 3). This is consistent with morphobgical analyses, which show more affinities with haplosporidia than any other microcell truca [2-4].

Bayesian posterior probabilities for these groupings are mrucimal, but bootstrap support is low (64% and 53%, respectively) and only marginally higher when the relatively divergent sequence of the foraminiferan is removed (75% and 83%, respectively). Given the high sequence divergence of mikrocytids (and to some extent haplosporidia), our main concern was to ascertain that the tree topology we obtained was not the result of long-branch attraction artifacts. The four genes analyzed (plus polyubiquitin) were therefore screened for known and novel clade-specific genetic signatures, which can provide robust phylogenetic signal without calculating molecular phylogenies. Such signatures are considered highly unlikely to occur several times independently in unrelated lineages and are therefore a powerful means of confirming evolutionary relationships when statistical support from molecular phylogenies is weak or long-branch attraction

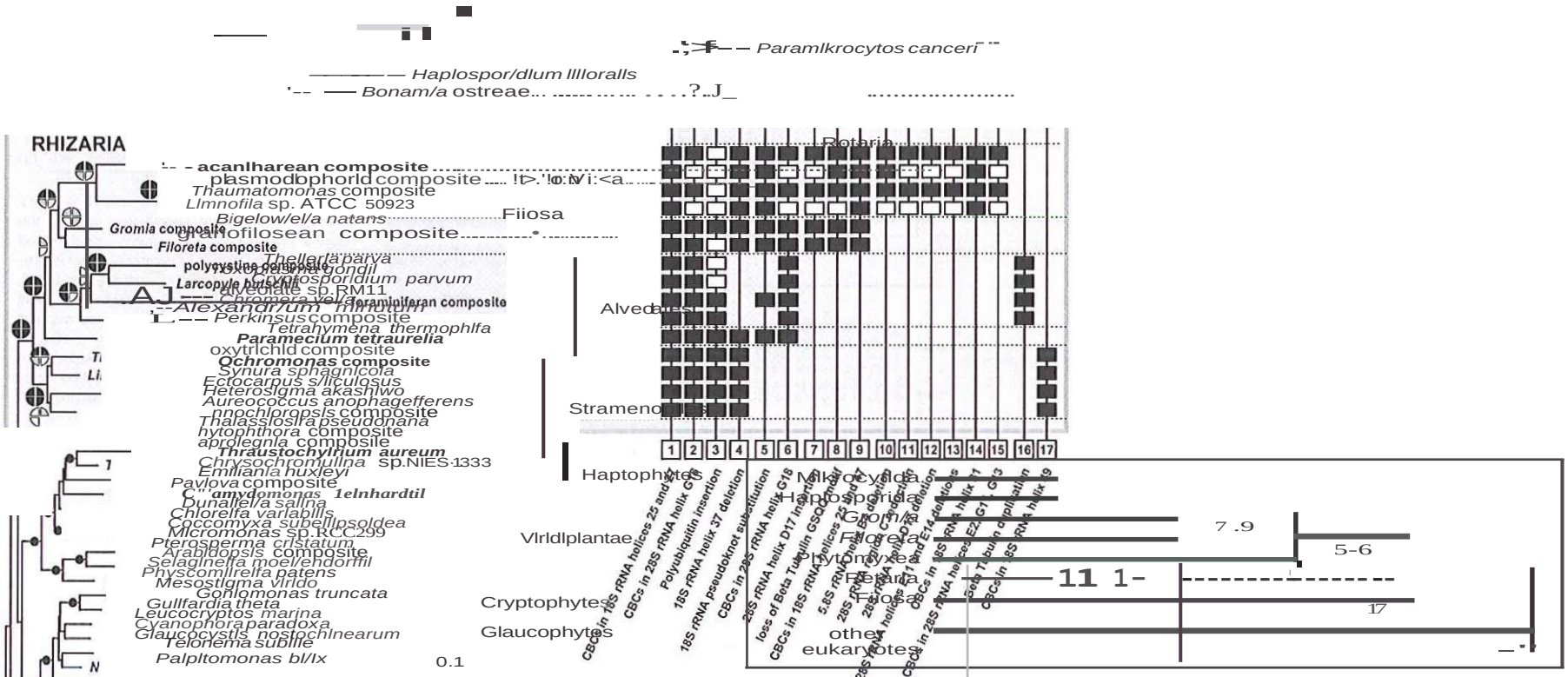

Figure 3. Phylogenetic Placement of Mikrocylids within Rhlzaria, Based on Concatenated Sequences of SSU and LSU rDNA, *Hsp90,* and *(J-tubulin,* Highlighting Known and Newly Identified clade-Specific Genetic Signatures that Corroborate the Tree Topology

The maximum-likelihood trees shown. All branch lengths are drawn to scale, except In Miktocytida, where they were reduced to half *ot* their actual size. With Rhlzarla, circles at Internal nodes indicate Bayesian posterior probabilities (upper half) and ML bootstap support values alter 1,000 replicates (lower half) for analyses performed without (eft half) and with (right half) the foraminiferan composite sequence. The following color codes used: black, 0.99 or 95%; gray, 0.90 or 75%; and white, 0.75 or 50%, respectively. In Rhlzaria, only nodes with support values <!.0.99 or 95% are h ghlghted (black dots). Seventeen genetic signatures supporting internal relationships within Rhlzarla are sted on the let! (CBCs, complementary base changes; SSU and LSU rDNA hel x numbering are explained n the Supplemental Results, Section 4). Black rectangles highlight taxa in which the gnature has been found, and white rectangles ndicate that the signature is expected to be present but the gene sequences missing for that taxon. The nsert shows a schematic summary *ot* relationships within Rhlzaria as evidenced by our phylogenetic analyses and the seventeen genetic signatures. The exact position of Retaria remains uncerta n due to the absence of some Rhlzaria and nonfilosan signatures h radiolarians and foram nlferans (dashed lines), but It does not pertain to the posilion of Mikrocy1da as a sister taxon to Haplosporida. The sequence signature details are provided in the Supplemental Results, Section 4.

artifacts are suspected (18). Not all individual substitutions were considered for SSU and LSU rDNA (but note that these contribute statistical support to the relationships shown h Figure 3). We mostly focused on sequence signatures affecting a whole stem or hairpin or on indels that could not be included in the sequence mask used for the phylogenetic analyses. These genetic signatures are summarized n Figure 3 and detailed h the Supplemental Results, Section 4; they are concordant with the concatenated gene phylogeny. h particular, they support the existence of a clade containing *Filoreta, Gromia,* rnikrocytids and haplosporidia, with the latter two as sisters.

We group *M. mackini, M. mimicus,* and *P. canceri* in a novel family (Mikrocytidae) and order (Mikrocytida), and our phylogenetic analyses support their inclusion in class Ascetosporea together with Haplosporida.

Conclusions

Evidence of ten distinct mikrocytid lineages including at least two genera associated with three invertebrate phyla (Arthropoda, Annelida, and Mollusca) on four continents and in both hemispheres greatly increases the known diversity of mikrocytids. Although the mikrocytids are mostly marine, we reveal a

new radiation in freshwater and brackish habitats. A more extensive and diverse sampling, particularly by PCR-based methods, is likely to reveal even greater diversity and distribution of these parasites. To facilitate future reference, we define a novel order (Mikrocytida) and family (Mikrocytidae) to include the lineages discovered here. Highly targeted PCR primer approaches for disease monitoring and risk assessment are areas of growing interest [10, 19, 20], and we show here that this approach can also reveal novel diversity and distribution patterns of endoparasites, including the identification of putative hosts and vectors.

Experimental Procedures

Histological Examination

Thirty juvenile edible crabs (*Cancer pagurus,* carapace width <70 mm) were collected each month, between May 2008 and April 2009, from the shoreline at Newton's Cove, Weymouth, UK (50°34'N, 2°22'W). All sampled crabs appeared externally normal. Crabs were anesthetized by chilling on ice prior to dissection of the hepatopancreas, gills, heart, midgut, antennal gland, gonad, and skeletal muscles from the abdomen, cephalothorax, and claw for histological examination. Thirty-five cultured Pacific oysters (*Crassostrea gigas*) (collected in May 2013) were prepared for histopathology by fixing a steak through the animal that encompassed the main tissues in Davidson's sea water fixative. Samples for molecular biology were preserved in molecular-grade ethanol, while samples for ultrastructural studies were preserved in glutaraldehyde. Details of methods are provided in the Supplemental Experimental Procedures.

Identification of *P. canceri* via NGS Sequencing and FISH

As PCR consistently failed to amplify parasite from the heavily infected *C. pagurus* antennal gland (~80% of the tissue estimated to be parasite), 50 ng of genomic DNA was prepared using Nextera method (Illumina) and sequenced for 250 cycles in each direction on a MiSeq platform (Illumina) at the Natural History Museum, London. The same approach was used for a genomic DNA extraction from a *Haplosporidium littoralis*-infected *Carcinus maenas* hepatopancreas, tagged and sequenced on 50% of a MiSeq run. Blastn searches against GenBank were used to identify SSU rDNA sequences, allowing identification of both the parasite species.

Blastx searches using ~500 bp previously sequenced seeds of target genes from *M. mackini* or haplosporidian representatives were used to identify *P. canceri* and *H. littoralis* gene orthologs for phylogenetic analyses. The blast results were assembled into contigs where possible and used as search seeds that were extended in mira 3.4 [21,22]. For confirmation of the assemblies, open reading frames were identified and alignments were assessed manually. In addition, the SSU and LSU rDNA sequences of *P. canceri* assembled from the MiSeq data were confirmed via PCR amplification and Sanger sequencing. A lineage-specific FISH assay was designed to confirm that the SSU sequence obtained corresponded with the histology results (see the Supplemental Experimental Procedures).

Collection of Invertebrates and Environmental Samples

Intertidal invertebrates and associated sediment were collected from a wide variety of sites and tissues fixed in ethanol. Size fractions of plankton material from the water column were collected by filtering large volumes of water (200 l) sequentially through a series of mesh sizes (55, 20, and 0.45–1 mm). Some invertebrates were roughly surface cleaned and incubated in small volumes of ASW for 1–2 hr, after which 50–100 ml of the incubation ASW was filtered onto 0.45–1 mm filters. A full list of samples screened is given in the Supplemental Results, Section 3, Table S1.

Environmental and Host Screens by PCR

A nested primer set mik451F/mik1511R in the first round and mik868F/mik1340R was used to screen environmental and host DNA for mikrocytid infections. The expected fragment size was 480 bp (based on *P. canceri* sequence) from the second-round PCR and spanned the variable V5 to V7 regions of the SSU rDNA. Sequences generated with the second-round primers were used in the analyses of mikrocytid diversity (Figure 2). Details of DNA extraction methods, primers, and PCR assays to detect mikrocytids are given in the Supplemental Experimental Procedures.

Taxon and Gene Choice for Phylogenetic Analyses

Alignments of SSU and LSU rDNA, *Hsp90,* and *{J-tubulin* genes were constructed for available Rhizaria and suitable outgroups based on the phylogenetic position of Rhizaria. Gene choice was based on good phylogenetic signal for eukaryote phylogeny, maximal taxon sampling within nonfibrous Cercozoa, and presence of known clade-specific genetic signatures within Rhizaria. *{J-tubulin* has undergone a clade-specific duplication in Retaria [23, 24], and we checked whether this duplication *may* be present in *P. canceri* and/or *H. littoralis.* No evidence of more than one version of *{J-tubulin* was found in either taxon, but a sequence signature that supports the clade containing *Fiforeta,* Gromia, Haplosporida, and Mikrocytida was detected. Hsp90 and SSU and LSU rDNA are generally informative genes for eukaryote phylogeny. Phylogenetic methods are detailed in the Supplemental Experimental Procedures.

Accession Numbers

Sequences generated in this study were submitted to GenBank under accession numbers KJ150289–KJ150293, KJ150241–KJ150251, and KJ572223–KJ572226.

Supplemental Information

Supplemental Information includes Supplemental Results, Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.02.033.

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