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The infection dynamics of PaV1 in the Caribbean spiny lobster Panulirus argus

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THE INFECTION DYNAMICS OF PAV1 IN THE CARIBBEAN SPINY LOBSTER *PANULIRUS ARGUS*

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DEDICATION

This work is dedicated to my parents, my wife and my son, who have supported me through everything.

谨以此论文献给一直以来支持我学业的父亲，母亲，爱妻和爱子.

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ABSTRACT

Panulirus argus Virus 1 (PaV1) is an emerging disease in Caribbean spiny lobster *Panulirus argus*. The virus was discovered coincidentally during a year of dramatic decline in total landings of the lobster. This virus is considered a threat to the lobster industry in the Florida Keys.

A sensitive and specific fluorescence *in situ* hybridization (FISH) assay was developed for diagnosis of PaV1 in the tissues of lobsters. The lower limit of detection using the 110-bp probe in a dot-blot hybridization for PaV1 DNA was 10 pg of cloned PaV1 template and 10 ng of genomic DNA extracted from hemolymph of diseased lobster. The probe specifically hybridized to PaV1-infected cells in all the tissues tested. The probe did not hybridize with host tissues of uninfected spiny lobsters, nor did it cross-react with other virus samples tested.

A primary culture of hemocytes was developed for *in vitro* study of PaV1. The modified Leibovitz L-15 medium supported the best survival of hemocytes in cultures. Hyalinocytes and semigranulocytes maintained higher viability (~ 80%) after 18 days when cultured separately. Hyalinocytes and semigranulocytes were susceptible to PaV1 *in vitro*. Cytopathic effects (CPE) were observed as early as 12 h post-inoculation, followed by cell debris and cellular exudates in inoculated cultures. This assay was further developed to assess viral load in hemolymph of diseased lobsters using a 50 % tissue culture infectious dose assay ($TCID_{50}$) based on CPE.

These techniques were applied to study the infection dynamics of the PaV1 virus in tissues of the lobsters over time-courses of experimentally induced infections with PaV1. The fixed phagocytes in the hepatopancreas were the initial site of PaV1 infection in spiny lobsters. Infection was subsequently observed in the hepatopancreas, gill, heart, hindgut, glial cells around the ventral nerves, as well as in the cuticular epidermis and foregut. As the disease progressed, the hepatopancreas became significantly altered, with hemal sinuses filled with massive amounts of cellular aggregates, including infected circulating hemocytes and infected spongy connective tissues. The virus caused significant decreases in total hemocyte density in early infections and significantly altered several constituents in the hemolymph serum of diseased lobsters, including: glucose, phosphorus, triglycerides, and lipase.

The results of this study facilitate our understanding of the pathogenesis of the PaV1 in the lobster host.

THE INFECTION DYNAMICS OF PAV1 IN THE CARIBBEAN SPINY LOBSTER *PANULIRUS ARGUS*

GENERAL INTRODUCTION

Viral disease in the Caribbean spiny lobster

The Caribbean spiny lobster, *Panulirus argus* (Crustacea: Decapoda: Palinuridae) (Fig. 1A) is widely distributed throughout the Caribbean basin and along the Atlantic Coast ranging from Brazil to Georgia, USA (Field and Butler, 1994). Spiny lobsters are important links in marine food webs, serving as major predators of various benthic species (e.g. snails, clams, and urchins) and important prey of large predators (e.g. sharks and finfish) (Lipcius and Eggleston, 2000).

The spiny lobster has a complex life cycle (Fig. 1B). It has five major life history stages, with adult, egg, phyllosoma larval stages, puerulus (or the post-larval stage) and juvenile stages (Phillips et al., 1980; Lipcius and Eggleston, 2000). The females bear eggs that hatch into phyllosoma in the spring and summer (Phillips and McWilliam, 1986). The planktonic larvae change into postlarvae after molting 11 times over 6-12 months. The postlarvae move onshore year around, settle in vegetation on shallow reef flats and metamorphose into the asocial early benthic juvenile stage (Lipcius and Eggleston, 2000). Once the juveniles reach approximately 15 mm in carapace length, they become social and take up refuge in crevices (Marx, 1986). Approximately two

years after settlement, lobsters mature and migrate seaward to reefs where mating and spawning occur (Forcucci et al., 1994).

The Caribbean spiny lobster supports a valuable commercial fishery in Florida estimated at >$30 million/year ($500 million Caribbean-wide – Cochrane and Chakalall, 2001). It also supports an important recreational fishery, which now accounts for 22% of the total catch (Harper, 1995; Butler, 2001). In Florida, for example, the commercial landings of Caribbean spiny lobster have varied between 4.3 million pounds and 7.9 million pounds per year from 1970 to 1999. In 1999, the total landings of the spiny lobster decreased and by 2001 they had dropped to 3.4 million pounds, the lowest reported landings since 1982, approximately 45% less than the historical average landings (FMRI, 2005; Muller et al., 1997).

In 1999 and 2000, a pathogenic virus *Panulirus argus* Virus 1 (PaV1) (Fig. 2A) was discovered in juvenile Caribbean spiny lobsters (Shields and Behringer, 2004). PaV1 is a large, non-enveloped, icosahedral, presumptive DNA virus with nucleocapsids ranging from 173 to 191 nm in diameter, and nucleoids approximately 118 ± 4 nm in diameter (Shields and Behringer, 2004). The virus primarily infects the small benthic juveniles (20 to 55 mm carapace length), with prevalence decreasing rapidly in larger sizes. The virus was prevalent throughout the Florida Keys with overall prevalences (among juveniles) ranging from 6% to 8%, with certain loci reaching as high as 37%. Because PaV1 is widespread in the Keys and highly pathogenic to juvenile spiny lobsters, Shields and Behringer (2004) speculated that it might be responsible for the recent declines in lobster populations since 1999.

PaV1 infects certain hemocytes (hyalinocytes and semi-granulocytes), and soft connective tissues in the hepatopancreas (digestive gland), hindgut (intestine), foregut (pyloric stomach), heart and elsewhere (Fig. 2B) (Shields and Behringer, 2004). However, the sites of early infection and the progression of PaV1 infection in the spiny lobster remain unknown. Heavily infected animals have characteristically milky hemolymph that does not clot (Shields and Behringer, 2004). This implies that there are pathological changes in the hemolymph such as an alteration in total hemocyte count (THC), differential hemocyte count (DHC), and serum constituents (total carbohydrate, total protein, hemocyanin, etc.) that are associated with viral load. Considering the catastrophic impact of shrimp viruses (see below) and their global spread, and the potential effect of PaV1 on the fishery for spiny lobsters, the development of efficient diagnostic tools and the assessment of the infection dynamics of the virus are keys to determining if the virus is a significant threat to the industry.

Viral diseases in other crustaceans

Prior to 2004, no naturally occurring viruses had been reported from lobsters (Shields and Behringer, 2004). However, over 30 viruses have been reported to infect crustaceans, primarily shrimp. Since Couch (1974a, b) described the first recognized crustacean virus, *Baculovirus penaei*, in *Penaeus duorarum* from the Gulf of Mexico, more than 20 viruses have been reported from penaeid shrimps (Brock and Lightner, 1990; Flegel, 1997; Lightner and Redman, 1998). At least 4 of these viruses are highly pathogenic and have severely damaged aquaculture stocks and, in some cases, fishery stocks of shrimps (Brock and Lightner, 1990; Evans et al., 2000; Flegel, 1997). Viruses

such as infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Lightner et al., 1983; Lu et al., 1989; Mari et al., 1993), Taura syndrome virus (TSV) (Lightner, 1995; Bonami et al., 1997; Mari et al, 1998), yellow head virus (YHV) (Wongteerasupaya et al., 1995, 1997; Lightner, 1999), and white spot syndrome virus (WSSV) (Inouye et al., 1994; Cai et al., 1995; Wongteerasupaya et al., 1996; Lo et al., 1996; Wang et al., 1998; Lightner, 1999), have severely impacted aquaculture production, causing catastrophic losses to the shrimp farming industries in Asia and America (Lightner, 1999). Further, WSSV was accidentally introduced into the Americas where it has caused widespread damage to native and cultured shrimp stocks (Lightner, 1999). These introductions were thought to have occurred from infected brood stock and possibly from infected frozen carcasses.

Viruses have been reported from other crustacean species; however, they have not received as much attention as the shrimp viruses, primarily because of the huge economic importance of the shrimp aquaculture industry. At least eight viruses have been reported from blue crab, *Callinectes sapidus* (see Shields and Overstreet, 2004 for review), of which four are known to be moderately or severely pathogenic. However, little is known about the effect of these viruses on blue crab populations other than their implication in occasional mortalities in short-term holding pens or shedding facilities (Johnson, 1983). Viruses have also been identified in several other crab species; five viruses have been identified in European shore crabs, *Carcinus maenas* and *Carcinus mediterraneus*, three from the crab, *Macropipus depurator*, one from the blue king crab *Paralithodes plathypus*, and one from the mud crab, *Rhithropanopeus harrisi* (see Brock and Lightner, 1990; Bonami and Lightner, 1991 for review). Several other viruses are also known from

diverse crustaceans including iridovirus infections in the ivory barnacle, *Balanus eburneus* (Leibovitz and Koulish, 1989), the pillbug, *Armadillidium vulgare* and the sow bug, *Porcellio dilatatus* (Federici, 1980).

PaV1 shares some properties with the herpes-like virus (bi-facies Virus, BFV) from the blue crab, *Callinectes sapidus* (Johnson 1978, 1988), the herpes-like virus in the mud crab, *Rhithropanopeus harrisi* (Payen and Bonami 1979), and the herpes-like virus from the blue king crab, *Paralithodes platypus* (Sparks and Morado, 1986). All of these virions are roughly similar in size, are icosahedral in shape, and are presumptive DNA viruses. PaV1 even caused similar pathologic changes as the BFV. Both viruses infect hemocytes and connective tissue cells in various tissues; cause reduction in the number of hemocytes, and a milky appearance of the hemolymph together with an abnormal clotting activity (Johnson, 1978, 1988; Shields and Behringer, 2004). However, PaV1 is unenveloped, large and does not form inclusion bodies in the nuclei of the infected cells (Shields and Behringer, 2004), distinguishing it from the Herpesviridae (Minson et al., 2000).

PaV1 is also similar to the iridoviruses such as irido-like virus (*Md*ILV) in the crab, *Macropipus depurator* (Montanie and Bonami, 1993) and the ivory barnacle, *Balanus eburneus* (Leibovitz and Koulish 1989), with respect to its size, shape, presumptive dsDNA, and lack of envelope. However, PaV1 virions are assembled entirely within the nucleus, whereas iridoviruses are assembled within the cytoplasm of host cells (Williams et al., 2000). As with most crustacean viruses, fundamental data (e.g. ultrastructure, DNA sequence, and capsid structure) necessary for the classification of PaV1 are lacking, thus its family assignment remains to be determined.

Application of *in situ* hybridization in study of viral diseases in crustaceans

In the past, diagnosis of viral infections in crustaceans relied upon clinical signs of disease, histological examination and electron microscopy (Bell and Lightner, 1988; Brock and Lightner, 1990; Johnson, 1995). However, these methods are laborious or time-consuming, or have other limitations, such as the difficulty of diagnosing disease from a large number of samples using electron microscopy. Sometimes similar pathological signs can be caused by a number of factors such as hypoxia, crowding, a sudden change in environmental factors, or even other pathogens, thus, reducing the capacity of certain diagnostic techniques to obtain a specific diagnosis (Lightner, 1988). In the past two decades, several molecular diagnostic methods have been developed as important diagnostic tools for viral pathogens of crustaceans. One such method is *in situ* hybridization (ISH), which detects specific types of pathogens in cells and tissues by hybridization of a labeled gene probe to a unique nucleic acid sequence (Singer et al., 1989).

ISH was initially developed to identify the genotype of human embryos and genomic constitution of human pre-implantation embryos (Sart and Choo, 1998; Andreeff and Pinkel, 1999; Darby, 2000). Because of the problems associated with radioactive probes and the time required for autoradiography, nonradioactive *in situ* hybridization is now the preferred method (Singer et al., 1989; Sart & Choo, 1998). The improved nonradioactive technique is essentially a 2-3 day procedure that involves the stable labeling of the nucleic acid probe, an overnight hybridization of probe onto target, post-hybridization washes followed by fluorescent or enzyme-immunochemistry for

hybrid molecule detection, and visualization of localized probes by fluorescent or light microscopy (Singer et al., 1989; Sart & Choo, 1998; Andreeff and Pinkel, 1999; Darby, 2000).

Lately, ISH has been applied to the diagnosis of various viral diseases in penaeid shrimp (Lightner and Redman, 1998). The first gene probe to be used to diagnose a viral disease in a crustacean was developed to diagnose IHHNV (Mari et al., 1993). Small DNA fragments (dsDNA) were selected from libraries of cloned fragments of IHHNV DNA, labeled with digoxigenin-11-dUTP (DIG) and applied to diagnosis of IHHNV in histological sections. This technique led to the development of the first commercial diagnostic kit for crustacean viruses named ShrimProbes^{TM} by DiagXotics (Wilton, CT, USA). Using specific gene probes, ISH has been subsequently applied to the diagnoses of several other crustacean viruses, such as *Baculovirus penaei* (BP) (Bruce et al, 1993, 1994), WSSV (Lo et al., 1997; Nunan and Lightner, 1997; Chang et al., 1998), HPV (Pantoja and Lightner, 2001; Phromjai et al., 2002) and gill associated virus (GAV) (Spann et al., 2003).

ISH is a sensitive and specific method to confirm infections associated with specific pathogens. A DIG-labeled DNA probe used in the diagnosis of *Baculovirus penaei* was capable of detecting the baculovirus well before the typical tetrahedral occlusion bodies (TOBs) were observable through routine tissue smears or histological examination (Bruce et al., 1993, 1994). The probes detected viral infections at 12-h post-infection, whereas H&E histology required a minimum of 24 h for detection. Similarly, Chang et al. (1996) detected WSSV-positive cells at 16-h post infection in the stomach, gill, cuticular epidermis and hepatopancreas the shrimp, *Penaeus monodon* using a

specific DIG-labeled DNA probe. More importantly, various mesodermally- and ectodermally-derived tissues, such as connective tissue, epithelia, nervous tissues and muscle, were also shown to be infected by the virus.

ISH has also been applied to diagnose pathogens in other marine organisms. A sensitive and specific DNA probe was developed and applied to diagnose the protozoan oyster pathogen *Haplosporidium nelsoni* (commonly called MSX) in the eastern oyster, *Crassostrea virginica*. The probe could detect 100 pg of cloned *H. nelsoni* rDNA and the presence of *H. nelsoni* in 1 μg of genomic DNA from an infected oyster (Stokes and Burreson, 1995). Lipart and Renault (2002) developed two DNA probes that were specific to oyster herpes virus in Pacific oysters, *Crassostrea gigas*; the probes were able to detect 50 pg of viral DNA in Southern blot hybridizations. Carnegie et al. (2003) designed a fluorescent *in situ* hybridization (FISH) assay to detect the parasite *Bonamia ostreae* in the flat oyster *Ostrea edulis*. The characteristic green, ring-shaped fluorescence was observed inside infected hemocytes, reflecting specific binding of the parasites distinguished from the host tissue background.

In this study, I developed a FISH assay to detect PaV1 infection in tissues of the Caribbean spiny lobster. With this technique, I can identify the major tissues or sites of initial viral infection (early tissue tropisms), and the infection dynamics of PaV1 in early stage of the disease.

Application of cell culture in the study of viral diseases in crustaceans

Crustacean cell lines are currently not available. However, in the past two decades, primary cell cultures have been obtained from various tissues and organs of crustaceans,

such as the lymphoid (oka) tissues (Tong and Miao, 1995; Hsu et al., 1995; Tapay et al., 1997; Chen and Wang, 1999; Kasornchandra et al., 1999; Owens and Smith, 1999; West et al., 1999; Itami et al., 1999; Wang et al., 2000; Lang et al., 2002; Assavalapsakul et al., 2003), embryonic tissues (Frerichs, 1996; Toullec et al., 1996; Fan and Wang, 2002), gonads (Luedeman and Lightner, 1992; Chen and Wang, 1999; Fraser and Hall, 1999; Owens and Smith, 1999; Lang et al., 2002; Maeda et al., 2004), heart (Tong and Miao, 1996; Chen and Wang, 1999; Owens and Smith, 1999), nerve tissues (Nadala et al., 1993; Owens and Smith, 1999; Gao et al., 2003), gut (Nadala et al., 1993), hepatopancreas (Owens and Smith, 1999) and hemolymph (Sano, 1998; Walton & Smith, 1999; Itami et al., 1999).

Of the tissues tested in primary culture, embryonic or larval tissues show promise for developing cell lines, as they contain undifferentiated and mitotically active cells. Therefore, the use of embryonic cells to establish long-term cultures and to obtain cell lines has been attempted in several crustacean species including freshwater prawn, *Macrobrachium rosenbergii* (Frerichs, 1996), and various penaeid shrimps (Toullec et al., 1996; Fan and Wang, 2002). Frerichs (1996) established subcultures of cells from the eggs of *M. rosenbergii* at 7-13 days post-fertilization. Cells were observed to proliferate in primary culture, but their passage into fresh medium resulted in the loss of adherence, cessation of cell multiplication and consequent failure to establish. Toullec (1996) also failed to obtain cultures from cells from the embryos of *Penaeus vannamei* and *P. indicus*. The cultures stopped at the 16-cell stage, and differentiated into three cells types, fibroblast-like cells, nerve-like cells and contractile cells. Fan and Wang (2002) tested two growth factors, insulin-like growth factor (IGF-II) and basic fibroblast growth fact

(bFGF) in primary cultures of embryonic tissue of *Penaeus chinensis*. They found that passage of primary cultures resulted in rapid proliferation and good adherence in the presence of IGF-II at 80 ng/ml and bFGF at 20 ng/ml. Cells maintained in subculture for up to 10 passages still had good cellular morphology and division rates. However, despite their efforts, only long-term primary cultures could be obtained.

Tissue culture is a standard tool employed in the diagnosis of viral pathogens of vertebrates, but it has not been fully developed for assessment of viral infection in invertebrates (Rinkevich, 1999; Toullec, 1999; Villena, 2003). Currently, only primary culture techniques have been developed for propagation and analysis of crustacean viruses. Lymphoid tissues are frequently applied in *in vitro* viral pathogenic studies, as these cells are often the targets for pathogenic viruses in shrimp (Lu et al., 1995; Tapay et al., 1997; Chen and Wang, 1999; Wang et al., 2000; Maeda et al. 2003; Assavalapsakul et al., 2003). Lu et al. (1995) developed a quantal assay for yellow head baculovirus (YBV) using primary cultures of shrimp lymphoid organ cells from two species of penaeid shrimp, *P. stylirostris* and *P. vannamei.* Visible cytopathic effects (CPE) appeared at 3 days post-inoculation. A gill suspension from YHV-infected shrimp was determined to have an infectious virus titer of $5 \times 10^{5.75}$ $TCID_{50}$ unit /ml. Tapay et al. (1997) used primary cultures of lymphoid organ to quantify a baculo-like virus isolated from *P. japonicus* and *P. stylirostris* using a $TCID_{50}$ assay. The virus caused cytopathic effects at 2 days post-infection; initially, the cells rounded up and finally detached from the culture vessels as the infection progressed.

Chen and Wang (1999) developed primary cultures of ovary, heart, lymphoid tissue and peripheral hemocytes from three species of penaeid shrimps *P. monodon*, *P.*

japonicus, and *P. penicillatus.* They found that lymphoid tissues were better for the formation of confluent cell monolayers. Lymphoid tissues and ovary were subcultured up to three times and were maintained for at least 20 days. At 5-7 days after inoculation with WSSV or YHV, significant CPE was observed in cell monolayers derived from the lymphoid organ. Virions of WSSV and YHV were observed in the nuclei and cytoplasm of cultured cells when examined by electron microscopy. Similar studies have also been carried out by Wang et al. (2000). CPE was first observed 2 days post-inoculation with WSSV filtrate. Hypertrophy of the nucleus, margination and diminution of nuclear chromatin was associated with WSSV infections.

The propagation profile of YHV was described using a primary culture of lymphoid organ and an *in vitro* quantal assay ($TCID_{50}$) (50% tissue culture infectious dose) (Assavalapsakul et al., 2003). Virus was detectable by PCR as early as 24 h post-inoculation. Maximal viral yields were reached by 4 days post-infection, approximately 24 h after the onset of the detectable cytopathic effects. The *in vitro* propagation of WSSV was studied in primary ovarian cultures from the kuruma shrimp *Marsupenaeus japonicus* (Maeda et al., 2004). WSSV caused marked cytopathic effect after 72 h post inoculation, followed by a rounding and detachment of most cells; the levels of WSSV in culture supernatant gradually increased during the period between 24 h and 120 h.

The PaV1 virus infects certain hemocytes and soft connective tissues (Shields and Behringer, 2004), and causes a characteristically milky hemolymph. This implies that hemocytes and soft connective tissues are potential target tissues for the *in vitro* study of the virus. Connective tissues have not been successfully obtained in culture. However, primary cultures of hemocytes have been obtained from the penaeid shrimp, *Penaeus*

japonicus (Sano, 1998; Itami et al., 1999) and two species of crab, *Liocarcinus depurator* and *Carcinus maeuas* (Walton and Smith, 1999). Sano (1998) cultured hemocytes from the kuruma shrimp *P. japonicus* and observed the unusual growth of pleomorphic cells *in vitro*. Itami et al. (1999) cultured granular hemocytes from *P. japonicus* for up to 10 days. Curiously, these hemocytes could not be infected by the penaeid rod-shaped DNA virus (RADV). Walton and Smith (1999) separated and collected hyaline hemocytes from the crabs, *Liocarcinus depurator* and *Carcinus maenas*. They were able to maintain these cells for up to 14 days with more than 70% viability in an optimized media.

In this study, I developed a primary culture of the hemocytes from the spiny lobster, *Panulirus argus* for studies on the *in vitro* propagation of PaV1. I assessed the utility of an *in vitro* quantal assay (Reed and Muench, 1938; Dee and Shuler, 1997; LaBarre and Lowy, 2001) based on induced cytopathic effects (CPE). A quantal assay can be used to quantify the viral load in hemolymph and other host tissues.

Hematological responses of crustaceans to viral infections

Although crustaceans do not possess an inducible immune system with a high degree of specificity and memory as in vertebrates, crustaceans do have efficient means to protect themselves against potential pathogens (Söderhäll and Cerenius, 1992; Roch, 1999). The external cuticle is an effective barrier that impedes the entry of infectious agents as well as protecting internal soft tissues from mechanical damage (Sugumaran, 2000). Once pathogens gain entry into the host, subsequent innate host responses are activated, including non-self recognition, phagocytosis, coagulation and encapsulation. This latter response is mediated by the prophenoloxidase (proPO) system (Johansson and

Söderhäll, 1989; Söderhäll and Cerenius, 1992; Kopácek et al., 1993; Vargas-Albores et al., 1996; Bachère, 2000; Lee and Söderhäl, 2001; Theopold et al., 2004; Jiravanichpaisal et al., 2006). Most host innate responses against pathogens involve a combination of cellular defenses (e.g. phagocytosis and encapsulation) as well as constitutive humoral molecules (e.g. lectins and antimicrobial peptides) (Söderhäll and Cerenius, 1992; Johansson and Söderhäll, 1989; Relf et al., 1999; Bachère, 2000; Marques and Barracco, 2000; Acharya et al., 2004; Alpuche et al., 2005; Kurtz, 2005; Jiravanichpaisal et al., 2006).

Hemocytes of crustaceans play a key role in host innate responses against foreign invasion (Söderhäll and Cerenius, 1992; Bachère, 2000; Jiravanichpaisal et al., 2006). Based on morphology, three types of circulating hemocytes are generally described in crustaceans: granulocytes, semi-granulocytes and hyalinocytes (Johnson, 1980; Bauchau, 1981; Johansson et al., 2000; Jiravanichpaisal et al., 2006). Semi-granulocytes are responsible for encapsulation and have a limited function in the storage of proPO system (Söderhäll and Cerenius, 1992; Johansson et al. 2000). This cell type is also capable of phagocytosis in several crustacean species (Hose et al., 1990). Granulocytes are the major storage cell in the proPO system and have a limited role in encapsulation (Hose and Martin, 1989; Hose et al., 1990; Söderhäll and Cerenius, 1992; Johnsson et al. 2000). There is no consensus about the function of hyalinocytes (or hyaline cells); hyalinocytes are capable of phagocytosis in freshwater crayfish (Söderhäll and Smith, 1983). However, hyalinocytes are not phagocytic in three other crustacean species; they play a significant role in clotting (Hose et al., 1990).

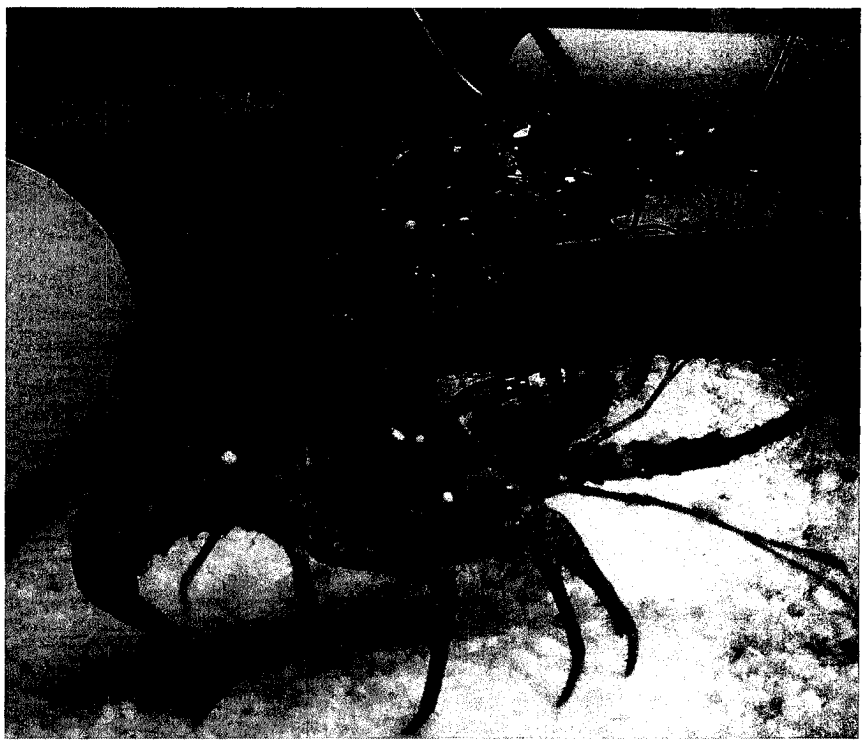
Hemocytes can be infected by several types of viruses in crustaceans. Granulocytes and semi-granulocytes are the targets for white spot syndrome virus (WSSV) infection in the shrimp *P. merguiensis*. Infection of these cell types was thought to seriously damage the immune system of the shrimp due to destruction of immune mediation from those hemocytes (Wang et al., 2002). Significant reductions in total hemocyte counts (THC) were observed in the shrimp *Penaeus indicus* infected with WSSV (Yoganandhan et al., 2003). WSSV can infect granulocytes and semigranulocytes of the crayfish *Pacifastacus leniusculus*, and semigranulocytes are more susceptible to the virus; while the proportion of granulocytes was significantly elevated from days 3 to 8 post-inoculation (Jiravanichpaisal et al., 2001). It is not clear whether the changes in hemocyte counts upon pathogen challenge can hamper the defense system of crustaceans (Jiravanichpaisal et al., 2006). In some cases, severe viral infection causes poor coagulation in the hemolymph of the blue crab *Callinectes sapidus* (Johnson 1976), the Caribbean spiny lobster *P. argus* (Shields and Behringer, 2004) and the shrimp *P. vannamei* (Song et al., 2003). However, it is not known how this lack of clotting ability otherwise affects the defensive responses of affected animals.

There have been a few studies on the biochemical changes that occur in the crustacean hosts with viral infection. Viral infections cause significant changes in biochemical and physiological parameters in hemolymph of crustaceans; and these may be associated with the host defense responses or they may result from pathological changes from infection. A significant decrease in hemocyanin content, and a significant increase in glucose and total carbohydrate levels occurs in the hemolymph of shrimp *P. indicus* infected with WSSV (Yoganandhan et al., 2003). Marked elevation in the

activities of transaminases, alanine transaminase (ALT) and aspartate transaminase (AST) has also been observed in hemolymph of the shrimp *P. indicus* infected with WSSV (Mohankumar and Ramasamy, 2006 a). When challenged with Taura syndrome virus (TSV), hemocyanin and clottable proteins decreased significantly in hemolymph of the shrimp *P. vanamei*, and the generation of intra-hemocytic superoxide anion, O_2^- and plasma proPO activity increased significantly (Song et al., 2003). A significant decrease in the activities of the antioxidant enzymes in the hemolymph of the shrimp *P. indicus* was observed with the progression of WSSV infection (Mohanhumar and Ramasamy, 2006 b). The study of these parameters in the hemolymph of crustaceans complements histopathological studies on the health status of crustacean host when challenged with viral pathogens.

Figure 1. A: Caribbean spiny lobster (*Panulirus argus*) collected from the Florida Keys. B: Life cycle of the Caribbean spiny lobster *Panulirus argus* (Lipcius and Eggleston, 2000).

A



B

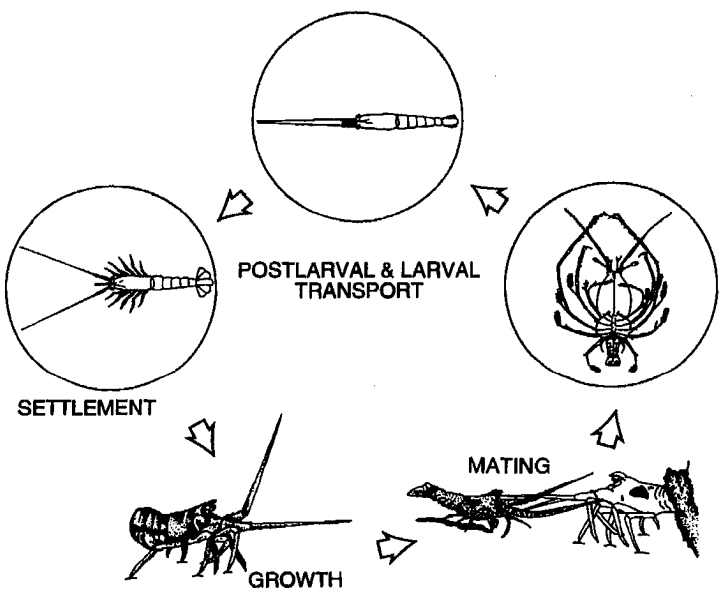
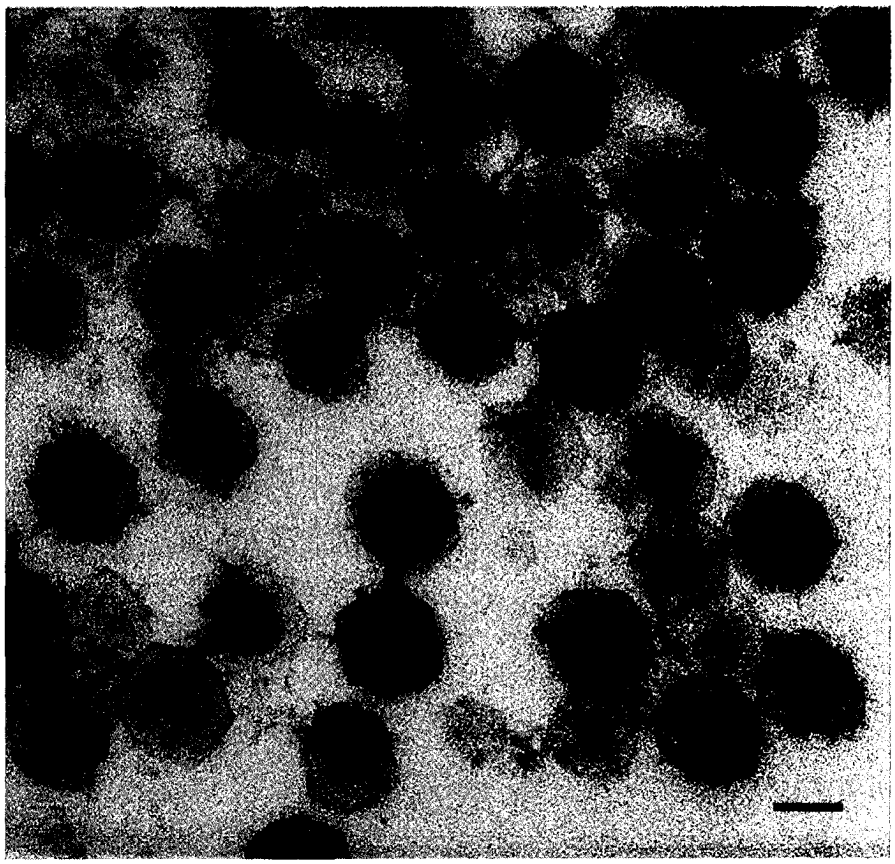
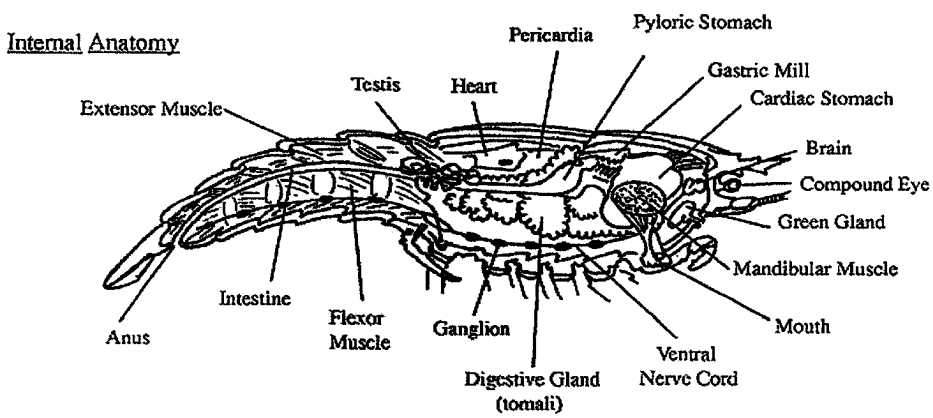


Figure 2. A: *Panulirus argus* Virus 1 (PaV1) in the hepatopancreas of a heavily infected spiny lobster. Scale bar = 100 μm. B: Internal anatomy of lobster. (http://www.maine.gov/dmr/rm/aquarium/teachers_guide/lobster_internal_anatomy.jpg)

A



B



GOALS AND OBJECTIVES

The overall goal of these studies was to determine the infection dynamics of *Panulirus argus* Virus 1 (PaV1) in the Caribbean spiny lobster, *Panulirus argus*. This was accomplished by examining the pathology and hematology of spiny lobsters experimentally infected with PaV1 using molecular, cell culture and histological techniques. Individual portions of this thesis were designed to address the following objectives:

Chapter 1.

To develop a fluorescence in situ hybridization (FISH) assay for diagnosis of PaV1 infections in tissues of lobsters.

Hypothesis: PaV1 has preferred target cells and specific tissue tropisms, which can be determined by histology and FISH.

Chapter 2.

1. To develop a primary culture of the hemocytes from the Caribbean spiny lobster *Panulirus argus*.

2. To assess the susceptibility of the primary culture of hemocytes from the spiny lobster to PaV1 infection.

3. To quantify infectious dose of PaV1 in hemolymph of spiny lobsters infected with PaV1.

Hypothesis: PaV1 is infectious *in vitro*, causing infection and detectable cytopathic effects (CPE) to cultured hemocytes.

Chapter 3.

To study the pathology and hematology of the Caribbean spiny lobsters over a time course of experimental infection by PaV1.

Hypothesis

1) PaV1 causes significant pathological changes in the spiny lobsters infected by PaV1 that can be determined by histology and FISH.

2) PaV1 infects the hemocytes causing demonstrable alterations to hemocyte subpopulations and hemolymph constituents that are related to the progression and severity of infection.

CHAPTER 1

Detection of *Panulirus argus* virus 1 (PaV1) in the Caribbean spiny lobster using fluorescence *in situ* hybridization (FISH)

Published:

Li, C., Shields, J.D., Small, H.J., Reece, K. S., Hartwig, C.L., Cooper, R.A., Ratzlaff, R.E., 2006. Diagnosis of *Panulirus argus* virus 1 (PaV1) in the Caribbean spiny lobster using fluorescence *in situ* hybridization. Diseases of Aquatic Organisms 72, 185-192.

MANUSCRIPT ABSTRACT

Panulirus argus virus 1 (PaV1) is the first virus known to be pathogenic to a wild lobster. It infects the Caribbean spiny lobster, *Panulirus argus* from the Florida Keys, and has a predilection for juveniles. The monitoring of the virus in wild populations and study of its behavior in the laboratory require the development of reliable diagnostic tools. A sensitive and specific fluorescence *in situ* hybridization (FISH) assay was developed for detection of PaV1. The lower detection limit using a 110-bp DNA probe in a dot-blot hybridization for PaV1 DNA was 10 pg of cloned template PaV1 DNA and 10 ng of genomic DNA extracted from hemolymph of diseased spiny lobster. The fluorescein (FITC)-labeled probe specifically hybridized to PaV1-infected cells in hepatopancreas, hindgut, gills, heart, foregut, and nerve tissues. FITC staining was observed around the inner periphery of the nuclear membrane, with lighter staining in a more dispersed pattern within the nucleus. The probe did not hybridize with host tissues of uninfected spiny lobsters, nor did it cross-react with the four other virus samples tested. This assay will facilitate our understanding of the pathogenesis of the viral disease and help in monitoring efforts directed at determining the prevalence of PaV1 in juvenile nurseries for the lobster.

INTRODUCTION

Panulirus argus Virus 1 (PaV1) causes disease in juvenile Caribbean spiny lobsters from the Florida Keys (Shields & Behringer 2004). It is a large, non-enveloped, icosahedral, presumptive DNA virus with nucleocapsids ranging from 173 to 191 nm in diameter, and nucleoids approximately 118 ± 4 nm in diameter. The virus infects certain hemocytes (hyalinocytes and semi-granulocytes) and spongy connective tissues (Shields & Behringer 2004). Infected cells have a characteristic appearance with emarginated condensed chromatin, hypertrophied nuclei and faint eosinophilic inclusions. Because PaV1 is widespread in the Florida Keys and is highly pathogenic to juvenile spiny lobsters, Shields and Behringer (2004) speculated that it may be responsible for recent declines in lobster populations since 1999. However, their study relied on histology and visual diagnosis, which may fail to identify low grade, latent or subclinical infections. Until now, there have been no molecular tools for diagnosis of PaV1 infections.

Diagnosis of viral infections in crustaceans has traditionally relied on clinical signs of disease, histological examination and electron microscopy (Bell & Lightner 1988, Brock & Lightner 1990, Johnson & Cassout 1995). Lately, more sensitive, specific and

rapid molecular techniques have been developed as important diagnostic tools for viral pathogens of crustaceans (e.g., Lightner & Redman 1998). One such method is *in situ* hybridization (ISH), which detects specific nucleic acid sequences in cells and tissues by hybridization of a labeled gene probe to a specific target nucleic acid sequence (Singer et al. 1989). ISH has been subsequently applied to diagnosis of several crustacean viruses, such as *Baculovirus penaei* (BP) (Bruce et al. 1993, 1994), white spot syndrome virus (WSSV) (Durand et al. 1996, Lo et al. 1997, Nunan & Lightner 1997, Chang et al. 1998), hepatopancreatic parvovirus (HPV) (Pantoja & Lightner 2001, Phromjai et al. 2002) and gill-associated virus (GAV) (Spann et al. 2003). ISH has also been applied to the diagnosis of several other pathogens of marine organisms (Stokes & Burreson 1995, Chang et al. 1996, Lo et al. 1997, Pantoja & Lightner 2001, Carnegie et al. 2003, Small et al. 2006). It is a useful tool to detect the presence of virions in infected tissues and determine tissue tropism of viral infections in hosts. Therefore, the objective of this study was to develop a fluorescence *in situ* hybridization (FISH) assay for the diagnosis of PaV1 infections in lobsters.

MATERIALS AND METHODS

Sample collection

Juvenile spiny lobsters, *Panulirus argus,* were collected from several sites located throughout the Florida Keys, USA. Tissue samples of hepatopancreas, hindgut, foregut, gill, heart, skin, nerve and in some cases ovary were dissected and fixed in 10 % neutral buffered formalin for approximately 48 h and then held in 70 % EtOH until further processing. Fixed tissues were dehydrated, embedded in paraffin and sectioned at 5 μm thickness on a rotary microtome. To verify the presence of the virus, sections were stained with hematoxylin and eosin (H&E) for histology (Humason 1979); infections were further confirmed by transmission electron microscopy (TEM) (Shields & Behringer 2004). Sections from the same tissue blocks were placed onto positively charged slides (Fisher Scientific) for fluorescence *in situ* hybridization (see below).

Fluorescent DNA probe synthesis

A 110-bp DNA probe was derived from a 177-bp DNA fragment (GenBank accession No. DQ465025) that putatively codes for a portion of the DNA polymerase from PaV1 (Robert Ratzlaff, unpublished data). The 110-bp DNA probe (PaV1 110) containing fluorescein-12-dUTP (fluorescein isothiocyanate, FITC) was synthesized

using a PCR Fluorescein Labeling Mix (Roche Applied Science). A plasmid vector (pCR 4-TOPO) containing the 177-bp DNA fragment was used as a template for probe synthesis. A specific primer set (PaV1 110F/R, generated with Invitrogen OligoPerfect™ Designer) was used to amplify and label a 110-bp fragment from the plasmid DNA containing the 177-bp insert. (See Table 1 for sequence of the 110-bp DNA probe and location of the PaV1 110 F/R primer set.) The polymerase chain reaction (PCR)-labeling reaction was performed according to the manufacturer's instructions (Roche Applied Science). Briefly, each PCR reaction contained the following: PCR buffer at a 1× concentration, 4 mM $MgCl_2$, 200 µM PCR Fluorescein Labeling Mix dNTP, 0.5 µM of each primer, 1 unit Taq DNA polymerase, 100 pg plasmid template, and distilled water (dH_2O) to a final volume of 100 µl. Thermocycling conditions were as follows: an initial denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 57.2°C for 30 seconds, and extension at 72°C for 90 seconds; followed by final extension at 72°C for 5 min. PCR products were purified using a QIAquick spin purification kit (Qiagen), and were visualized by agarose gel electrophoresis (2 %) with ethidium bromide staining. The amount of DNA was quantified using a Hoefer DyNA Quant200 Fluorometer.

To ensure that the PaV1 110F/R primer set was amplifying the correct domain of the viral 177-bp insert for synthesis of the 110-bp DNA probe, the PCR (above) was repeated with the Fluorescein labeling mix replaced with a standard dNTP mix (125 µM). The 110-bp product was visualized by agarose gel electrophoresis and excised from the gel using a sterile scalpel and purified using a

QIA-quick gel extraction kit (Qiagen). The amplicon was cloned using a TOPO TA Cloning Kit for Sequencing (Invitrogen) following the manufacturers protocols. Six clones were sequenced bidirectionally and analyzed using an ABI 3130 Prism Genetic Analyzer (Applied Biosystems) as in Dungan & Reece (2006). Sequences were compared to the original 177-bp fragment using the Clustal-W algorithm in the MacVector DNA sequence analysis package (Accelrys).

DNA probe sensitivity

The sensitivity of the probe was determined by dot-blot hybridization against a 10-fold serial dilution from 10 ng to 1 pg of plasmid DNA containing the PaV1 177-bp fragment. Additional controls consisted of 10 ng genomic DNA extracted from hemolymph of a healthy spiny lobster and 10 ng genomic DNA extracted from the hemolymph of a spiny lobster heavily infected with PaV1 (Infection was determined histologically). Genomic DNA was extracted using the DNeasy® Tissue kit according to the manufacturer's instructions (Animal blood protocol - Qiagen). Briefly, DNA solutions were denatured at 100°C for 10 min and transferred to ice for 5 min. The solution of denatured DNA was loaded onto a positively charged membrane (BrightStar®-Plus, Ambion) using a Bio-Rad Microfiltration Apparatus (Bio-Rad laboratories), and rinsed with 100 µl of 0.4 M NaOH. DNA was immobilized by UV crosslinking with a Stratalinker 1800 UV crosslinker (Stratagene). The membrane was placed in a sealed plastic bag containing pre-warmed (42°C) pre-hybridization solution (Sigma-Aldrich) and incubated for 30 min with gentle

agitation at room temperature (RT, 25°C). FITC-labeled probe was denatured as described above, diluted in hybridization buffer (Sigma-Aldrich) to a final concentration of 10 ng ml^{-1}, and incubated with membranes in a sealed plastic bag overnight at 42°C with gentle agitation. A series of stringency washes followed: 2× SSC (0.3 M NaCl, 30 mM Sodium Citrate; pH 7.0), 10 min, RT; 1× SSC, 10 min, RT and 0.1× SSC, 10 min, RT. The membrane was blocked for 30 min at RT with blocking buffer (Sigma-Aldrich), then incubated in anti-fluorescein alkaline phosphatase antibody (1:1000 diluted in blocking buffer) (Sigma-Aldrich) for 2 h with gentle agitation at RT. This was followed by removal of unbound antibody with two 15 min washes with TN buffer (0.1M Tris, 0.15 M NaCl, pH 7.5) and a 5 min wash with TNM buffer (0.1 M Tris, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5). The membrane was then incubated with BCIP/NBT liquid substrate solution (Sigma-Aldrich) for 2 h in a sealed plastic bag covered with foil. Color development was stopped with a 5-min TE buffer wash (10 mM Tris, 1mM EDTA, pH 7.5) and dH_2O for 5 min. The wet membrane was scanned with a Hewlett Packard Scanjet 3570c scanner for documentation.

Fluorescence *in situ* hybridization (FISH)

The FISH methodology was derived from published ISH protocols (Singer et al. 1989, Stokes & Burreson 1995, Darby 2000, Beatty et al. 2002). Sections were deparaffinized in xylene (5 min, 2×), rehydrated through a descending ethanol series: 100 % (5 min, 2×), 95 % (1 min, 2×), 70 % (1 min, 2×), and equilibrated in

phosphate-buffered saline (PBS; once for 5 min, once for 3 min). The sections were then digested with Proteinase K (100 μg ml^{-1} in PBS) for 15 min at 37°C, followed by a 5-min wash in 0.2 % glycine PBS solution to stop proteolysis, and incubated in 2× SSC for 10 min at room temperature. Slides were incubated in pre-hybridization buffer (4× SSC, 50 % formamide, 0.5 mg ml^{-1} Salmon sperm DNA, and 1 % fetal bovine serum) at 42°C for 45 min. After incubation, excess pre-hybridization buffer was carefully drained off, the area with tissue was outlined with a Frame-seal incubation chamber (MJ Research), aliquots of 50 μl of hybridization solution (50 % deionized formamide, 4× SSC, 0.5 % SDS, and 25 μg ml^{-1} DNA fluorescein probe) were added, and the slides sealed with a plastic cover slip. The slides were then placed in a thermal cycler for 3 min at 72°C and cooled on ice for 2 min. Slides were incubated in a humid chamber saturated with prehybridization buffer overnight at 42°C. The slides were then washed in 2× SSC (5 min), 1× SSC (5 min), PBS (10 min), air dried, mounted with anti-fading mounting medium (90 % glycerol, 0.1 m Tris-HCl, pH 8.0 and 2.3 % DABCO) and covered with glass coverslips. Clear fingernail polish was applied to the edges of the cover slips to prevent evaporation. Slides were examined using an Olympus BX51 microscope equipped with a FITC-Texas Red filter (U-MF2, Olympus), and images were captured with a Nikon DXM 1200 digital camera for comparison between matching sections stained with H&E.

To test the specificity of the probe, tissues with other viral infections were assessed. These included tissues with a herpes-like virus (HLV) from a blue king crab *Paralithodes platypus* obtained from Frank Morado (NOAA) (see Sparks & Morado

1986); lymphocystis disease virus (LDV) from a striped bass *Morone saxatilis*, obtained from Wolfgang Vogelbein (VIMS) (see Smail & Munro 2001 for review); Ostreid Herpesvirus 1 (OsHV-1) from an infected Pacific oyster *Crassostrea gigas,* obtained from Carolyn Friedman (Univ. Washington) (see Le Deuff & Renault 1999, Lipart & Renault 2002) and Intranuclear bacilliform virus (IBV) from an infected brown shrimp *Crangon crangon* from Grant Stentiford (CEFAS, UK) (Stentiford et al. 2004).

TEM

The hepatopancreas from an infected lobster was fixed for transmission electron microscopy (TEM) using 3 % glutaraldehyde (containing 0.2 M sodium cacodylate, 30 mg ml^{-1} NaCl, 20 ug ml^{-1} $CaCl_2$, pH 7.0) (Factor & Naar 1985). After fixation, tissues were washed 3 times in buffer and postfixed in 1 % osmium tetroxide in buffer. Samples were processed through an ethanol dehydration, *en bloc* stained with uranyl acetate, dehydrated further with propylene oxide, infiltrated through several changes of propylene oxide in various ratios with Spurr's resin, and finally embedded in Spurr's resin. Sections were cut on a Reichert-Jung ultramicrotome E, processed through a routine lead citrate stain, and observed with a Zeiss CEM-902 TEM.

RESULTS

DNA probe synthesis and sensitivity

The primer pair PaV1 110 F/R specifically amplified a single 110-bp fragment (Table 1) when using the plasmid containing the 177-bp DNA fragment as a template in the PCR labeling reaction. The 110-bp DNA probe sequence from 6 clones sequenced was 100 % identical to the corresponding region in the original 177-bp plasmid.

In dot-blot hybridizations (Fig. 1), the probe had a minimum sensitivity of 10 pg of the cloned plasmid DNA with the 177-bp insert. Additionally, the probe detected the presence of viral DNA from 10 ng of genomic DNA extracted from hemolymph of a PaV1 infected spiny lobster. A negative result was obtained when the probe was tested with genomic DNA extracted from the hemolymph of a healthy spiny lobster (Fig. 1, g).

Fluorescence *in situ* hybridization

The FITC-labeled probe hybridized to PaV1-infected cells in all tissues tested. The probe bound to those infected hemocytes and spongy connective tissue cells in or around the hepatopancreas, hindgut, foregut, gill, heart, skin, nerve and even ovary tissues (Fig. 2 A, B, C). The distribution of FITC-stained structures

inside infected cells matched the pathological changes caused by the viral infection when diagnosed by H & E staining (Fig. 3) and TEM (Fig. 4). Most FITC-stained foci were located around the inner periphery of the hypertrophied nuclear membrane, with a few dispersed throughout the inside of the nucleus.

The probe did not bind to the tissues of healthy spiny lobsters. No FITC-stained particles were present in tissues from healthy spiny lobsters. Only a weak brown/red background was observed (Fig. 2 D). The probe did not hybridize with HLV, OsHV-1, LDV, nor with IBV.

DISCUSSION

We have developed a FISH assay for the detection of the recently identified PaV1 virus from the Caribbean spiny lobster, *Panulirus argus,* using a sensitive and specific DNA probe. The probe detected 10 pg of plasmid DNA containing a 177-bp DNA fragment from PaV1 in a dot-blot hybridization. It could detect the presence of viral DNA in 10 ng genomic DNA extracted from the hemolymph of a diseased spiny lobster. The probe hybridized to PaV1-infected cells in all tissues tested by FISH. The specific binding of the 110-bp probe for PaV1 was visualized as ring-like green staining of infected cells, whereas only a brown or red background was observed in healthy tissues from uninfected spiny lobsters. This unique distribution pattern of the green staining fits the pattern observed in infected tissue with TEM. Most virions were diffusely distributed within the inner periphery of the hypertrophied nuclei of infected cells, and the probe specifically bound to complementary sequence of viral DNA in infected cells during *in situ* hybridization.

Traditional diagnostic tools such as histology or electron microscopy can not differentiate among certain etiologies. Occasionally, similar pathological signs can be caused by several factors including hypoxia, crowding, a sudden change in environmental factors, or even other pathogens, thus, reducing the capacity of certain diagnostic techniques to obtain a specific diagnosis (Lightner 1988). When

examined by TEM, PaV1 had properties similar to the Herpesviridae and the Iridoviridae (Shields & Behringer 2004). It even induces pathological changes similar to those caused by the herpes-like virus (Bi-Facies virus, BFV) from the blue crab, *Callinectes sapidus* (Johnson 1976, 1988; Shields & Behringer 2004). However, the 110-bp probe did not bind with the other viruses: OsHV (Le Deuff & Renault 1999, Lipart & Renault 2002), HLV (Sparks & Morado 1986), LDV (Smail & Munro 2001) and a virus outside these families, the bacilliform virus (Stentiford et al. 2004). Therefore, the specificity of the probe will facilitate its use in properly diagnosing PaV1 infections in lobsters.

In situ hybridization (ISH) has been applied to diagnose viral diseases in several crustaceans (Lightner & Redman 1998). A digoxigenin (DIG)-labeled DNA probe used in the diagnosis of *Baculovirus penaei* detected the baculovirus well before the typical tetrahedral occlusion bodies (TOBs) were observable in routine tissue smears or histological examinations (Bruce et al. 1993, 1994). The probe detected viral infections at 12-h post-infection, whereas H&E histology required a minimum of 24 h for detection. Similarly, Chang et al. (1996) detected WSSV-positive cells at 16-h post infection in the stomach, gill, cuticular epidermis and hepatopancreas of the shrimp *Penaeus monodon* using a specific DIG-labeled DNA probe. While we have not examined the infection dynamics of PaV1 over such short time periods, the specific binding of the 110-bp probe, coupled with the excitation sensitivity of FITC to fluorescence, should facilitate examining viral tropism over periods of a few days post inoculation.

Using H&E and FISH, we found infected cells in the ovaries of an infected lobster. Most of these cells were circulating hemocytes or spongy connective tissue cells; oocytes did not appear to be infected by the virus. Lo et al. (1997) reported that WSSV can infect oocytes in the ovary of the shrimp *Penaeus monodon*. However, infected oocytes were unable to develop into mature ova; therefore, WSSV was unlikely be transmitted to offspring. In our case, infected juvenile lobsters are not likely to survive to reproduce as they typically die within 30-80 d after infection (Shields & Behringer 2004). Further, whereas adults can become infected by PaV1, the prevalence in adults is extremely low (Shields & Behringer 2004); therefore, transovarial transmission is unlikely to play a major role in the spread of the virus. Given the sensitivity of the 110-bp PaV1 probe and its apparent specificity, this FISH assay is a powerful tool for detecting the presence of PaV1 virions in host tissues. With this technique we can identify the major tissues involved in infections and the initial sites of viral infection, investigate other hosts as reservoirs for the virus, and monitor disease prevalence in nursery populations of *P. argus* in the Caribbean Sea.

Table 1. Sequence of the 110-bp DNA probe from PaV1 and location of the PaV1 110 F/R primer set (bold).

1 **CTCGGTGTAT** **GGGTTTACGG** GGGTGACGAA AAAGGCCATC

41 GGCTTCGAAC CCGTCGCGGC GAGCATCACC GCCGTGGGGC

81 GACAGTCCGT **GCTGAAGGCG** **AAGAAACACT**

Figure 1. Dot blot hybridization with the 110-bp PaV1 probe. Left row of each dot blot a, b, c, d, e, f is 10 ng, 1ng, 100 pg, 10 pg, 1 pg, 0.1 pg of plasmid DNA containing the 177-bp fragment, respectively. Right row of dot blot, g is 10 ng genomic DNA from hemolymph of healthy lobster; h is 10 ng genomic DNA from hemolymph from lobster that was infected with PaV1.

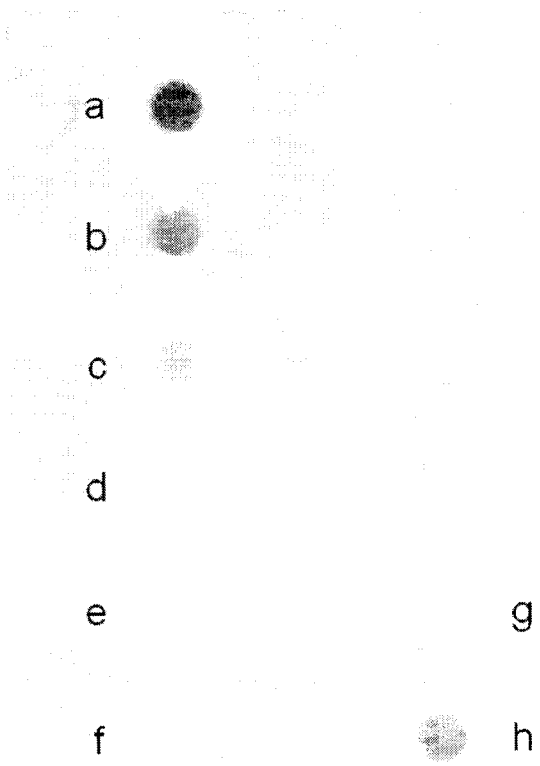


Figure 2. FISH using the PaV1 110-bp probe on histological sections of spiny lobster infected with PaV1 (scale bars = 50 μm). A: hepatopancreas, B: spongy connective tissue around foregut, C: ovary, D: hepatopancreas from a healthy spiny lobster. Green staining indicates specific binding of the PaV1 110-bp probe to viral nucleic acids in infected cells, brown or yellow signal indicates background.

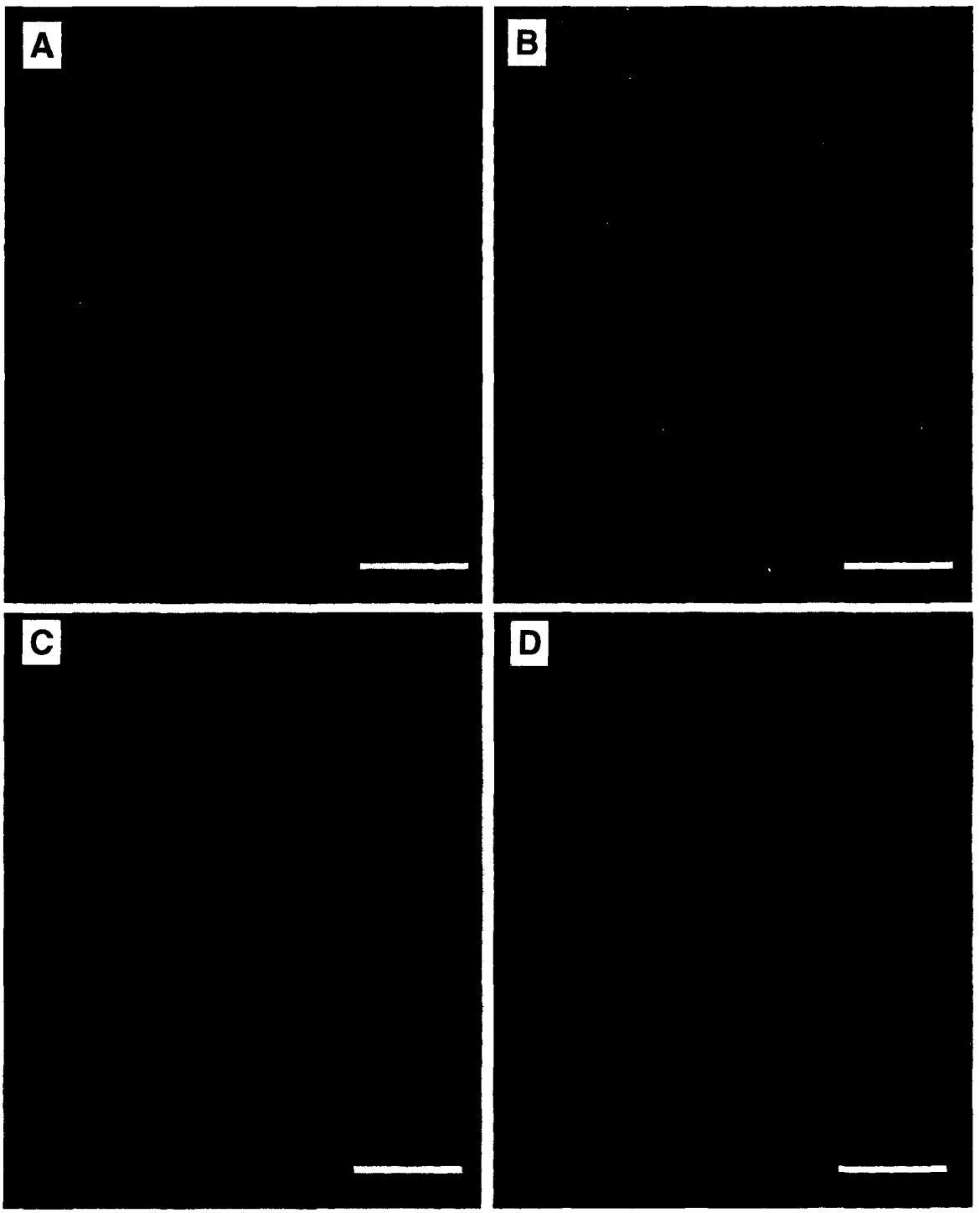


Figure 3. (A) FISH image of the hepatopancreas of a lobster infected with PaV1. Note the green staining of the virally infected hemocytes by the PaV1 110-bp probe (white arrows), scale bar = 20 μm. (B) H&E staining of the hepatopancreas of an infected lobster. Infected cells exhibit hypertrophied nuclei, and faint eosinophilic inclusions. Black arrows indicate infected hemocytes, scale bar = 20 μm.

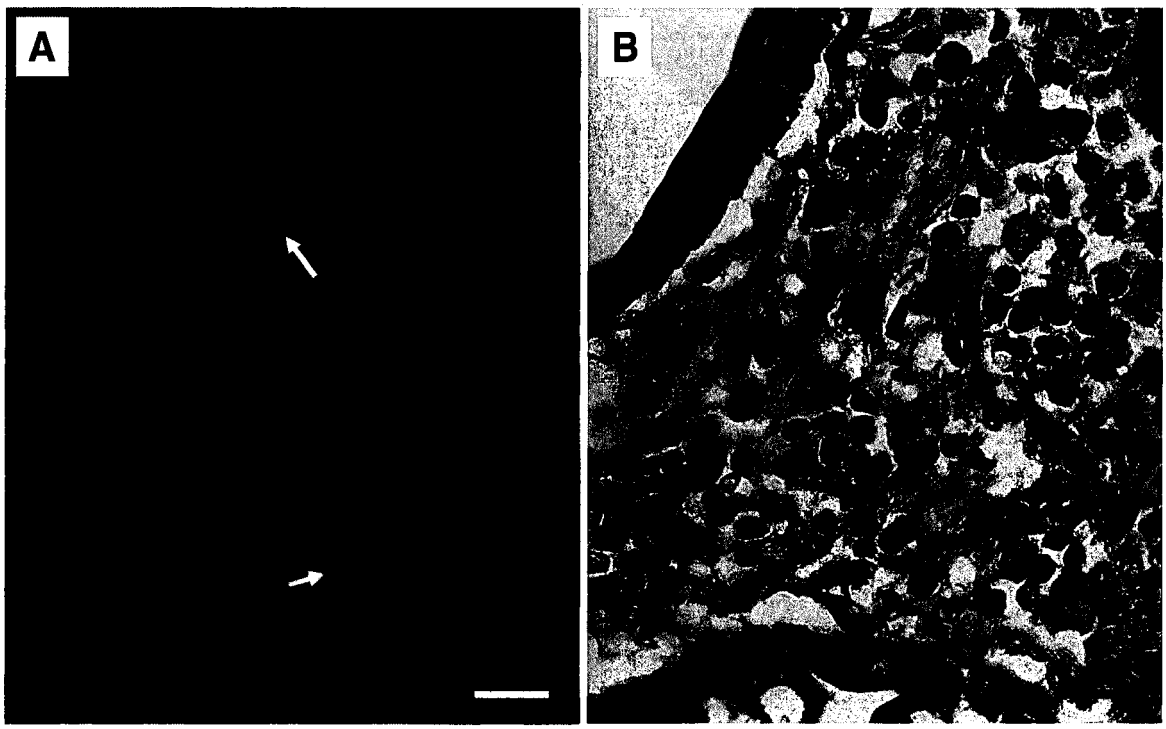
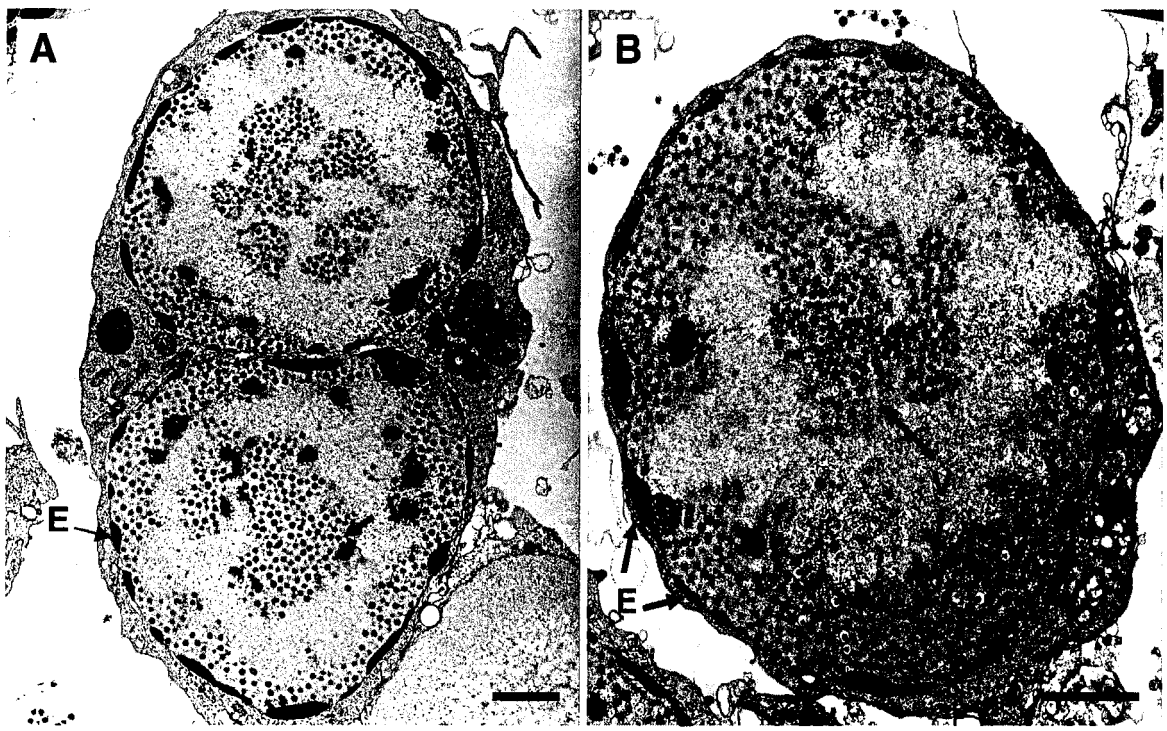


Figure 4. A, B. TEM of hepatopancreas from an infected lobster; virions (V) aggregated at inner periphery of the nuclear membrane, with a few dispersed inside the nucleus. Notice the condensed and emarginated chromatin (E), scale bars = 2 μm.



CHAPTER 2

Primary culture of hemocytes from the Caribbean spiny lobster, *Panulirus argus,* and their susceptibility to *Panulirus argus* Virus 1 (PaV1)

Published:

Li, C., Shields, J. D., 2007. Primary culture of hemocytes from the Caribbean spiny lobster, *Panulirus argus,* and their susceptibility to *Panulirus argus* Virus 1 (PaV1). Journal of Invertebrate Pathology 94, 48-55.

MANUSCRIPT ABSTRACT

Primary cultures of hemocytes from the Caribbean spiny lobster *Panulirus argus* were developed for studies on the *in vitro* propagation of *Panulirus argus* Virus 1 (PaV1). A modified Leibovitz L-15 medium supported the best survival of hemocytes in *in vitro* primary cultures. However, degradation of the cultures occurred rapidly in the presence of granulocytes. A Percoll step gradient was used to separate hemocytes into three subpopulations enriched in hyalinocytes, semigranulocytes, and granulocytes, respectively. When cultured separately, hyalinocytes and semigranulocytes maintained higher viability (~ 80%) after 18 days incubation compared with granulocytes, which degraded over 2-3 days. Susceptibility of the cell types was investigated in challenge studies with PaV1. Hyalinocytes and semigranulocytes were susceptible to PaV1. Cytopathic effects (CPE) were observed as early as 12 h post-inoculation, and as the infection progressed, CPE became more apparent, with cell debris and cellular exudates present in inoculated cultures. Cell lysis was noticeable within 24 hrs of infection. The presence of virus within cells was further confirmed by *in situ* hybridization using a specific DNA probe. The probe gave a unique staining pattern to cells infected with PaV1 24-h post inoculation. Cells in the control treatment were intact and negative to hybridization. This assay was further applied to the quantification of infectious virus in hemolymph using a modified 50% tissue culture infectious dose assay ($TCID_{50}$) based on

CPE. These tools will now allow the quantification of PaV1 using established culture-based methods.

INTRODUCTION

The Caribbean spiny lobster, *Panulirus argus,* is widely distributed throughout the Caribbean basin and along the Atlantic Coast ranging from Brazil to Georgia, USA. It supports one of the most valuable fisheries in the Caribbean. Recently, a pathogenic virus, *Panulirus argus* Virus 1(PaV1), was identified during field surveys of juvenile lobsters from the Florida Keys (Shields & Behringer, 2004). The virus infects the soft connective tissues, and two classes of hemocytes: hyalinocytes and semigranulocytes. The virus is highly pathogenic to juvenile spiny lobsters, which die within 30-80 days in experimentally induced infections (Shields and Behringer, 2004). Healthy lobsters are, however, able to detect diseased animals and avoid them (Behringer et al., 2006). Given its distribution throughout the Florida Keys and its relatively high prevalence in juvenile lobsters, PaV1 is thought to have significant potential to damage the fishery. Thus, it is critical to develop specific and sensitive diagnostic methods to better understand the pathogenesis of this viral pathogen.

Tissue culture is an important tool employed in the studies of viral pathogens of vertebrates, but it has not been fully developed for assessment of viral infection in invertebrates (Rinkevich, 1999; Toullec, 1999; Villena, 2003). At present there is no continuous culture of crustacean cell lines, however, primary culture of crustacean tissue has previously been developed for the diagnosis and *in vitro* proliferation of shrimp viruses (Chen and Wang, 1999; Fraser and Hall, 1999; Frerichs, 1996; Hsu et al., 1995;

Nadala et al., 1993; Rinkevich, 1999; Tapay et al., 1997; Toullec, 1996). Because PaV1 infects hyalinocytes, semigranulocytes, and soft connective tissues (Shields and Behringer, 2004), these hemocytes and soft connective tissues represent targets for the *in vitro* study of the virus. Connective tissues have not been successfully obtained in culture. However, primary cultures of hemocytes have been obtained from the penaeid shrimp, *Penaeus japonicus* (Itami et al., 1999; Sano, 1998) and two species of crab, *Liocarcinus depurator* and *Carcinus maenas* (Walton and Smith, 1999). The present study aimed to develop a primary culture of the hemocytes from the spiny lobster, *Panulirus argus*, and to assess the susceptibility of hemocytes to PaV1 in these cultures. The cell culture system with the virus was further developed into an *in vitro* assay for the quantification of virus in the hemolymph of infected lobsters.

MATERIALS AND METHODS

Experimental animals

Juvenile spiny lobsters, *Panulirus argus,* were collected from the Florida Keys, and housed in clean aquaria (salinity = 35 ± 1‰, temperature = 24 ± 1 °C) equipped with biological filters (Whisper) filled with crushed coral. Lobsters were fed with squid three times per week. Water quality was monitored weekly and water changes were made to ensure that various water quality parameters remained within acceptable limits: ammonia (0-0.3 ppm), nitrite (0-0.6 ppm), pH (7.4-8.4).

Analysis of *Panulirus argus* hemocytes

Hemolymph was drawn with a 27-ga syringe from the juncture between the base and ischium of the fifth walking leg. Prior to bleeding, the sample area was wiped with 70% ethanol. In most cases, hemolymph was collected into a syringe containing an equal volume of anticoagulant (0.45M NaCl, 0.1M glucose, 30mM sodium citrate, 26mM citric acid, 10mM EDTA; pH = 5.4; Söderhäll and Smith, 1983). Freshly collected hemocytes were examined with an Olympus BX51 microscope equipped with a U-UCD8 Universal condenser and Nomarski Differential Interference Contrast Filter. Hemocytes were categorized based on cell size, cell shape, and granularity (Söderhäll and Cerenius, 1992). Total hemocyte counts (THC) and differential hemocyte counts (DHC) were performed using a hemacytometer (Neubauer improved, Bright Line; two counts per lobster).

Optimization of culture media

Samples of collected hemolymph were centrifuged at 250 × g for 10 min at 4 °C (IEC Thermo Centra, with swinging bucket rotor), then resuspended in appropriate medium. Aliquots of 0.5 ml of the hemocyte suspension at densities of ~1 × 10^6 ml^{-1} were seeded into 24-well culture plates containing an additional 1.0 ml of culture medium per well. Plates were incubated in a Chamber (Lab-Line®) at 22 – 24 °C. Media were refreshed on day 2 and thereafter at 2-day intervals. Cultured cells were observed with an Olympus IX50 inverted microscope equipped with a Hoffman Modulation Contrast condenser. Images were taken with a Nikon DXM 1200 digital camera at days 1, 3, 5, 7, 9, and 11, prior to media refreshment. Cell viability was confirmed by the Trypan Blue exclusion method (Mascotti et al., 2000). Several commercial media were assessed for hemocyte viability: Leibovitz L-15 medium (L-15), Modified Leibovitz L-15 medium (ML-15: double strength components, supplemented with 0.6 g L^{-1} L-glutamine and 0.7 gL^{-1} glucose), Grace's insect medium, and RPMI-1640 medium. Each medium was adjusted to match the osmolarity of the hemolymph of the spiny lobster (1025 ± 6 mOsM kg^{-1}) by addition of NaCl. Penicillin (100 IU ml^{-1}) and streptomycin (100 μg ml^{-1}) were added to each culture medium to minimize potential bacterial contamination. In addition, some media were supplemented with 0%, 5%, 10%, or 15% charcoal-dextran-treated fetal bovine serum (FBS) for putative growth assessment. All media were sterilized by filtering through Nalgene® Disposable Filters (Pore size = 0.20 μm).

Primary culture of separated hemocytes

Hemocytes were separated by centrifugation using Percoll (Amersham Biosciences) in fine step-density gradients (1.110, 1.096, 1.091, 1.086, 1.082, 1.077, 1.072, 1.067, and 1.062 g ml^{-1} respectively) made with lobster physiological buffer (0.4M NaCl, 0.01M KCl, 0.01M Na_2HPO_4, 0.01M KH_2PO_4, $NaHCO_3$; pH = 7.8). Briefly, 1 ml of each density solution was carefully layered into the same15-ml centrifuge tube. Aliquots of 3 ml of the hemolymph-anticoagulant suspensions were gently layered on the top of the stacked gradients, then centrifuged at 400 × g for 30 min at 4 °C. Cell fractions were carefully aspirated into anticoagulant using Pasteur pipettes, and washed twice with culture media. Cell fractions were centrifuged at 250 × g for 10 min at 4 °C to remove residual Percoll prior to resuspension in fresh culture media. Separated hemocytes were cultured in the optimized media and assessed for cell viability as above.

Virus inoculation of cell cultures

The virus inoculum was extracted from the hemolymph of a heavily diseased lobster. Briefly, diseased hemolymph was mixed with 4-fold volume of ML-15 medium and homogenized with a homogenizer (Pyrex®, Corning Inc.) at 4 °C for 10 min, the homogenized mixture was centrifuged at 3000 × g for 10 min at 4° C, and the supernatant was filtered through 0.45-μm filter. Viral filtrates were serially diluted with ML-15 culture medium to 1:1, 1:10, 1:100 and 1:1000 and used as inoculum. Hemolymph from a healthy lobster was processed similarly and used as a control. Aliquots of 100 μl of inoculum were inoculated into 1 day old established hemocyte cultures in 24-well tissue culture plates (Falcon®, Becton Dickinson Labware), the plates were incubated at 22 – 24 °C for 4 days, and cultures were supplemented with new media

at 2-day intervals. The inoculated primary cultures were examined daily with an inverted microscope, and images were taken daily for assessment of cytopathic effects (CPE) and calculation of 50% tissue culture infectious dose ($TCID_{50}$) as in Reed and Muench (1938).

Diagnosis of *in vitro* infection of PaV1 by *in situ* hybridization (ISH)

For diagnosis of *in vitro* infections of PaV1, hemocytes were cultured and inoculated with 1:10 diluted inoculum in chamber slides (Lab-Tek®). On days 1, 2, 3 and 4 post inoculation, hemocytes cultured in chambers were fixed in 10% neutral buffered formalin for 10 min at room temperature, and then gently rinsed briefly with phosphate buffered saline (PBS) (8.0 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.44 g L^{-1} Na_2HPO_4, 0.24 g L^{-1} KH_2PO_4; pH 7.4). Infections were then prepared for *in situ* hybridization (ISH). Briefly, slides were permeabilized with 0.5% (v/v) Triton X-100 (in PBS) for 10 min, rinsed with PBS (5 min × 3), then digested with Proteinase K (100 μg ml^{-1} in PBS) for 15 min at 37 °C, followed by a 5-min wash in 0.2% (w/v) glycine PBS solution to stop proteolysis, and incubated in 2 × SSC (17.53 g L^{-1} NaCl, 8.82 g L^{-1} citric acid; pH 7.0) for 10 min at room temperature. Slides were incubated in pre-hybridization buffer (4 × SSC, 50% (v/v) formamide, 0.5 mg ml^{-1} Salmon sperm DNA, and 1% (v/v) fetal bovine serum) at 42° C for 45 min. After incubation, excess pre-hybridization buffer was carefully drained off, the area with tissue was outlined with a Frame-seal incubation chamber (MJ Research INC.), then aliquots of 50-μl of hybridization solution (50% de-ionised formamide; 4 × SSC; 0.5% (w/v) SDS; 25 μg ml^{-1} FITC-labeled DNA probe) (probe synthesis as in Li et al. 2006) were added within the area, sealed with plastic cover slip. Each slide was then placed into a thermal cycler for 3 min at 72° C, and cooled on ice for 2 min. Slides were incubated in a humid

chamber overnight at 42° C. The slides were then washed in 2 × SSC (2 x 5 min), 1 × SSC (2 × 5 min), PBS (10 min) and Maleic Acid Buffer (MAB: 100 mM maleic acid, 150 mM NaCl; pH 7.5) for 10 min. The slides were then incubated in blocking solution (1% (w/v) Blocking reagents in MAB, Roche). Anti-FITC alkaline phosphatase conjugate antibody (Sigma-Aldrich) was diluted 1:2000 in blocking solution and sections were incubated with the diluted antibody for 3 h at room temperature with gentle agitation. Unbound antibody was removed with two 5 min washes in buffer I (100 mM Tris, 150 mM NaCl, pH 7.5) and two 5 min washes in Buffer II (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5). The slides were then incubated with BCIP/NBT liquid substrate solution (Sigma-Aldrich) in dark for 2 h. The color reaction was stopped by washing in TE buffer (10 mM Tris, 1mM EDTA, pH 7.5) and dH_2O for 5 min, respectively. The slides were mounted with aqueous mounting medium (90 % glycerol, 0.1 m Tris-HCl, pH 8.0 and 2.3 % DABCO) and glass coverslips applied. Clear fingernail polish was applied to the edges of the cover slips to prevent evaporation. Slides were examined using an Olympus BX51 microscope, and images were captured with a Nikon DXM 1200 digital camera.

RESULTS

Hemocytes of the spiny lobster *Panulirus argus*

Three major hemocyte types were identified in the Caribbean spiny lobster: hyalinocytes, semigranulocytes, and granulocytes (Fig. 1). Hyalinocytes contained no or a few small granules, and were often found as a spindle or round shapes, ranging in size from 12 to 18 μm. Semigranulocytes contained many small and a few larger (>1 μm) granules, were morphologically variable from spindle, ovoid or round in shape, and ranged in size from 14 to 23 μm. Granulocytes contained many large (>1 μm) and a few small granules, were also variable in shape, and ranged from 17 to 28 μm.

The number of circulating hemocytes (total hemocyte count, THC) in the spiny lobster was around 8.0×10^6 cells ml^{-1}(range 6.68×10^6 - 9.58×10^6, n =16). Hyalinocytes (H) represented approximately 15% (15.2 ± 2.0%, n =16) of the total circulating hemocytes, semigranulocytes accounted for 58% (57.9 ± 3.7%, n =16), and granulocytes, 27% (26.9 ± 4.2%, n = 16).

Optimization of culture media

The lobster hemocytes survived but did not proliferate in each of the four culture media tested, and no mitosis was observed in any of the hemocyte cultures. After 1 day, hemocytes cultured in ML-15, L-15, Grace's media and RPMI-1640 maintained high viability, with >90% of the cells remaining alive (Fig. 2 A). However, over the next three

days, the viability of cells cultured in RPMI-1640 decreased to 25%, which was significantly lower than cells in the other media (Fig. 2 A). By day 8, most cells cultured in RPMI-1640 had died, whereas mean cell viability was 79.5 ± 4.7 % in ML-15 medium, 68.4 ± 7.2 % in Grace's medium, 45.8 ± 3.8 % in L-15 medium. By day 12, cell viability decreased to 43.0 ± 2.2 % in ML-15 medium, 32.4 ± 4.1 % in Grace's medium, and 26.4.0 ± 2.9 % in L-15 medium (Fig. 2 A).

Fetal bovine serum, a standard supplement in many cell culture systems, was not an effective additive and hampered cell viability in this study. Hemocytes cultured in the ML-15 medium supplemented with 5%, 10%, and 15% FBS, respectively, were consistently degraded, with viabilities of only 3 ~ 4% after 11 days in culture (Fig. 2 B). Hemocytes cultured in media without supplementation with FBS retained much higher viability (Fig 2A, B).

Primary cultures of separated hemocytes

In previous mixed hemocytes cultures, within 2-7 days, most of the granulocytes dehisced, releasing their contents into the culture media, potentially impacting the survival of other the cell types (Fig.3). In order to improve cell viabilities of cultured hemocytes, lobster hemocytes were separated into three distinct fractions using Percoll discontinuous gradients and cultured separately.

In the column of the Percoll step gradients, the fraction enriched with hyalinocytes was located between density gradients of 1.062 and 1.067 g ml^{-1}; in this fraction (H), hyalinocytes comprised more than 80% of the cells with semigranulocytes making up the rest. The fraction enriched with semigranulocytes was located between

density gradients of 1.067 and 1.072 g ml^{-1}. This semigranulocytes (SG) fraction was comprised of approximately 90% semigranulocytes, with 7% of hyalinocytes and 3% of granulocytes. The granulocytes (G) fraction accumulated in the interface between 1.082 and 1.086 g ml^{-1} gradients; over 94 % of the cells in this fraction were granulocytes, with only a small proportion (< 6 %) of semigranulocytes and no hyalinocytes (Fig. 4).

From the culture of unseparated hemocytes, cells maintained in ML-15 medium survived better than those in other media; therefore, ML-15 medium was selected as the medium for maintaining the separated cell types. Viability improved dramatically in hyalinocytes and semigranulocytes grown in the absence of granulocytes. Hyalinocytes and semigranulocytes survived up to 18 days with viabilities of 77.6 % (± 6.4%) and 79.1 (± 7.1%) respectively. Separated granulocytes died quickly, within 4 or 5 days (Fig. 5).

In vitro propagation of PaV1

Hemocytes from cultures inoculated with the virus showed cytopathic effects (CPE) after 12-h post-inoculation with 1:1 diluted PaVl inoculum. Initially, the affected cells changed from round or oval shapes to an irregular shape (Fig. 6A). As the infection progressed, the infected cells gradually shrank and became surrounded by exudates or cell debris (Fig. 6B). After 72 hrs, cell death was obvious, and inoculated cultures were comprised of few live cells and much cell debris (Fig. 6C).

The infection of PaV1 in cultured hemocytes was confirmed by *in situ* hybridization using a specific 110-bp DNA probe of Li et al. (2006). The unambiguous dark staining of PaV1 infected cells was observed after 24 h post-inoculation. No PaV1

positive signals were detected in cell cultures inoculated with hemolymph filtrates from a healthy lobster (Fig. 7).

By 48 h post inoculation, 23.45 % (±10) of the cells exposed to the 1:1-diluted inoculum had survived; over 50 % of cells exposed to greater dilutions of the inocula had survived, whereas more than 93 % of cells in the control groups had survived. By 72 h post inoculation, most cells that had been exposed to the virus had lysed in the 1:1 and 1:10 dilution groups; whereas most of the cells in control groups remained alive (~ 90 %) (Fig. 8). The amount of infectious virus in the undiluted inoculum was 4×10^3 $TCID_{50}$ ml^{-1} when calculated with the end point dilution assay (Reed and Muench, 1938) based on percentage of cells surviving at the end of the assay.

DISCUSSION

We have developed the primary culture of hemocytes from the Caribbean spiny lobster *Panulirus argus* for *in vitro* studies of *Panulirus argus* Virus 1(PaV1). Primary cultures of hyalinocytes and semigranulocytes were susceptible to PaV1, showing obvious cytopathic effects (CPE) within hours of exposure to a high dose of PaV1. Even though the cultured hyalinocytes and semigranulocytes were susceptible to PaV1, we could not develop a plaque assay for quantitative study of the virus, because lobster hemocytes do not undergo mitosis and no confluent cell layer could be formed; i.e., the cells formed dispersed monolayers. However, a CPE assay using an estimate of the 50% tissue culture infectious dose ($TCID_{50}$) method provided an alternative to determine viral titer (see Darling et al. 1998). Such assays have been successfully applied to quantify the infectious titer of several other crustacean viruses including yellow head baculovirus (Assavalapsakul et al., 2003; Lu et al., 1995) and non-occluded baculo-like virus (Tapay et al., 1997). The *in vitro* quantal assay based on CPE will facilitate better understanding of infection dynamics of PaV1 in the spiny lobster. However, because hemocytes do not multiply *in vitro*, the application is limited in its ability to grow large quantities of the virus for *in vitro* studies.

We demonstrated that the PaV1 virus infected *in vitro* cultured hyalinocytes and semigranulocytes using a specific DNA probe. The rapid infection and mortality of the hemocytes is interesting given that infections in juvenile lobsters last from 30-80 d

(Shields and Behringer, 2004). However, the dynamics within the host will no doubt be different due to immune defenses, stimulation of hematopoietic tissues, and other factors. In the lobster, the virus also infects the cells of the spongy connective tissues, and may cause different effects in these, however this tissue cannot as yet be successfully cultured.

Lymphoid and ovary tissues are often the targets for pathogenic viruses in shrimps; therefore, these cells are frequently used in *in vitro* studies of viral pathogenesis (Assavalapsakul et al., 2003; Chen and Wang, 1999; Lu et al., 1995; Maeda et al. 2004; Tapay et al., 1997; Wang et al., 2000). Comparatively limited efforts have been made to develop *in vitro* cultures of hemocytes from other crustacean species. Itami et al. (1999) cultured large granular hemocytes from *P. japonicus* for up to 10 days and inoculated them with penaeid rod-shaped DNA virus, however, no CPE was observed over the 10 day period of incubation. Walton and Smith (1999) separated and collected hyalinocytes from the crabs, *Liocarcinus depurator* and *Carcinus maenas*. They were able to maintain these cells for up to 14 days with more than 70% viability in an optimized L-15 medium. In our cultures, separated hyalinocytes and semigranulocytes survived up to 18 days with high viability (~ 80%), and maintained an even higher viability (> 90%) in the earlier period of culture.

A variety of culture media have been tested for the primary culture of crustacean tissues, these include Leibovitz L-15, Grace's insect medium, RPMI-1640, Medium 199, and several specifically formulated media (Luedeman and Lightner 1992; Nadala et al., 1993; Tong et al., 1996; Walton and Smith, 1999). Leibovitz L-15 and Grace's insect medium have been considered the best commercial medium for tissue culture of crustaceans (Luedeman and Lightner, 1992; Nadala et al., 1993; Walton and Smith, 1999).

In our study, Grace's insect medium and double strength Leibovitz L-15 medium (2 × L-15) supported cell survival in the first 6 days of culture for unseparated lobster hemocytes; however, the Modified L-15 medium yielded better results for longer term viability (> 80% by day 15). The higher concentration of glutamine and glucose in Grace's media and L-15 medium apparently benefited the survival of *in vitro* cultured hemocytes of the spiny lobster.

Fetal bovine serum (FBS) is often used as a supplement in crustacean and mollusk cell culture (Chen and Wang, 1999; Luedeman and Lightner 1992; Sano, 1998; Walton and Smith, 1999). In this study, fetal bovine serum (even treated with Charcoal-dextran absorption) degraded the viability in whole hemocyte cultures even at a low concentration of 5%. Semigranulocytes and granulocytes are sensitive to foreign particles, particularly glucans, lipopolysaccharides, and bacteria, and often lyse to release prophenoloxidase and other components involved in cytotoxicity and melanization pathways (Söderhäll and Cerenius, 1992). FBS presumably caused the granulocytes to dehisce and lyse, causing further deterioration of remaining cells. Other supplements, such as cell-free plasma or filtrates from homogenized tissues, that have been used to support microbial pathogens of crustaceans (Toullec, 1999), were not tested in this study due to their potential to induce cross reactions among individual lobsters.

Traditionally, crustacean hemocytes have been classified into three types of cells: hyalinocytes, semigranulocytes, and granulocytes, according to the number and size of granules they contain (Bauchau, 1980) and their biological function (Söderhäll and Smith, 1983). Hose et al. (1990) proposed a different classification based on morphology, cytochemistry, and studies of cell function, and suggested that hyalinocytes and

granulocytes represent two distinct cell lineages, with granulocytes representing a continuum of differentiation from the less mature small-granule hemocytes to the large-granule hemocytes. We found that granulocytes were distinctly different from the other two cell types in morphology, and that they were particularly sensitivity to the *in vitro* environment, surviving only a short period when compared with the other two subpopulations. In the initial period (2 to 3 days) of separated hemocyte cultures, some hyalinocytes became morphologically like semigranulocytes, whereas no transformation was observed between semigranulocytes and granulocytes. Although we retain the traditional classification of hemocyte types in the Caribbean spiny lobster, more work is needed to fully appreciate the functional and biochemical differences among cell types. Additional experiments with culturing techniques will facilitate further *in vitro* study of crustacean hemocytes.

Figure 1. Light microscopy of hemocytes from the spiny lobster, *Panulirus argus*: Hyalinocytes (H), semigranulocytes (SG), and granulocytes (G). Scale bar = 10 μm

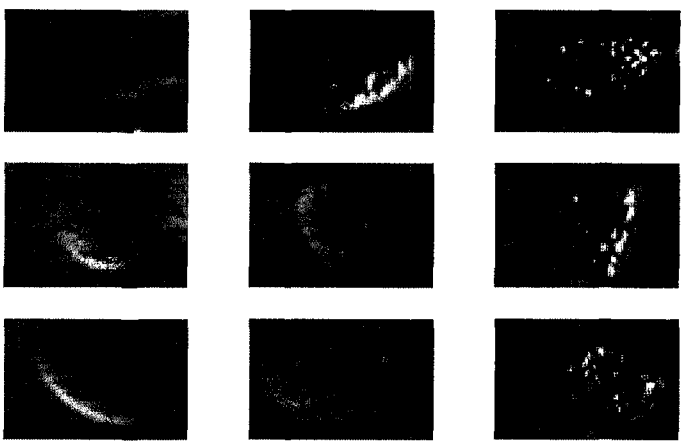
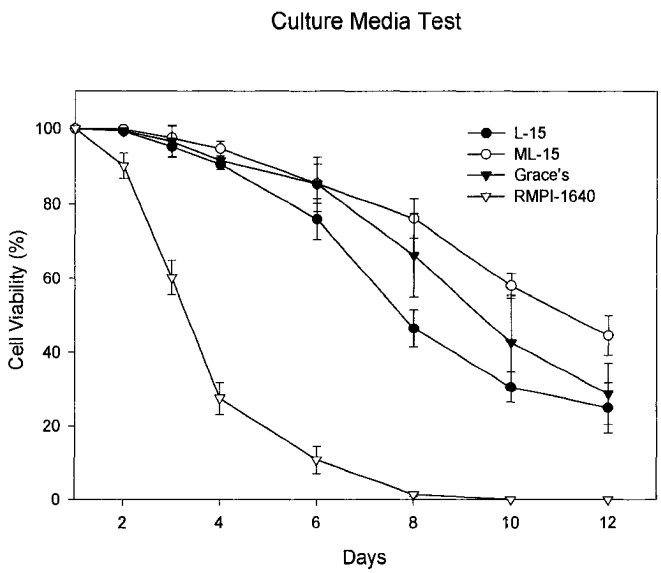


Figure 2. (A) Viability of unseparated hemocytes of *P. argus* cultured in L-15, ML-15, Grace's Insect medium, and RPMI-1640 medium. (B) Viability of unseparated hemocytes of *P. argus* cultured in ML-15 medium supplemented with 0%, 5%, 10%, and 15% FBS. Values are means ± STD (n=12).

A:



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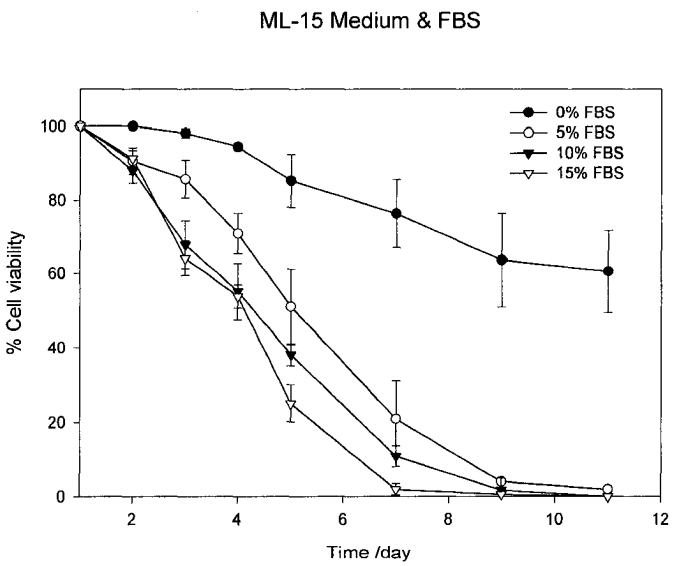


Figure 3. Light microscopy of unseparated hemocytes of the spiny lobster *P. argus* at 1st (A) and 5th (B) day in culture. Note those dehisced granulocyte (arrowheads). H: hyalinocytes; SG: semigranulocytes; G: granulocytes. Scale bars = 10 μm.

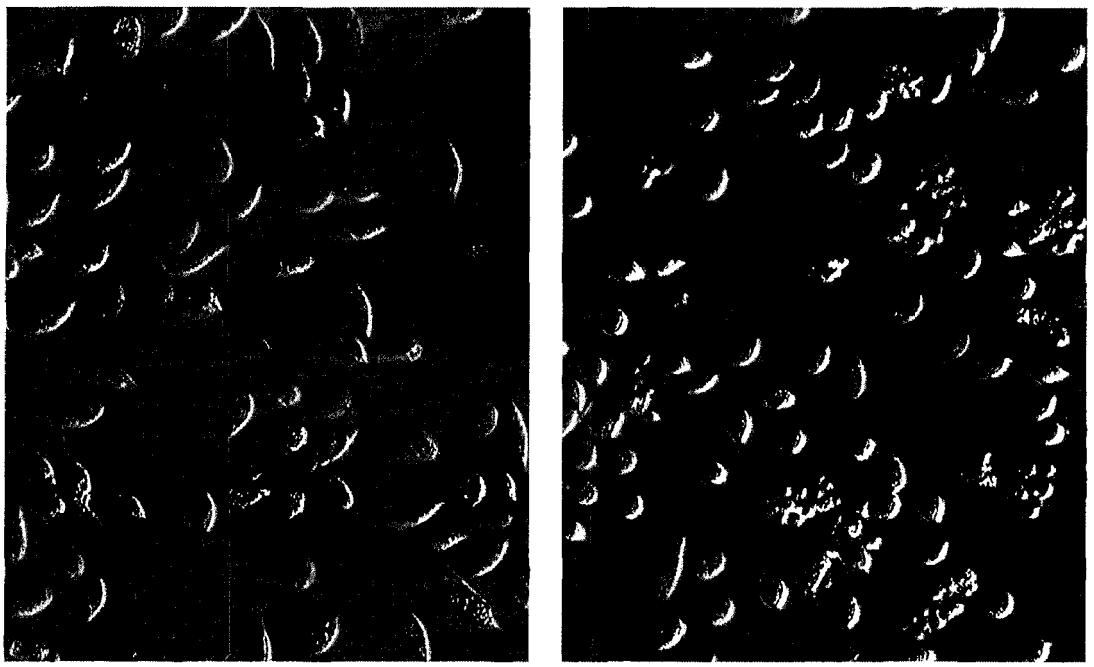


Figure 4. Light microscopy of *P. argus* hemocytes in fractions from Percoll discontinuous gradient separation: H: hyalinocytes, SG: semi-granulocytes, and G: granulocytes. Scale bars = 10 μm.

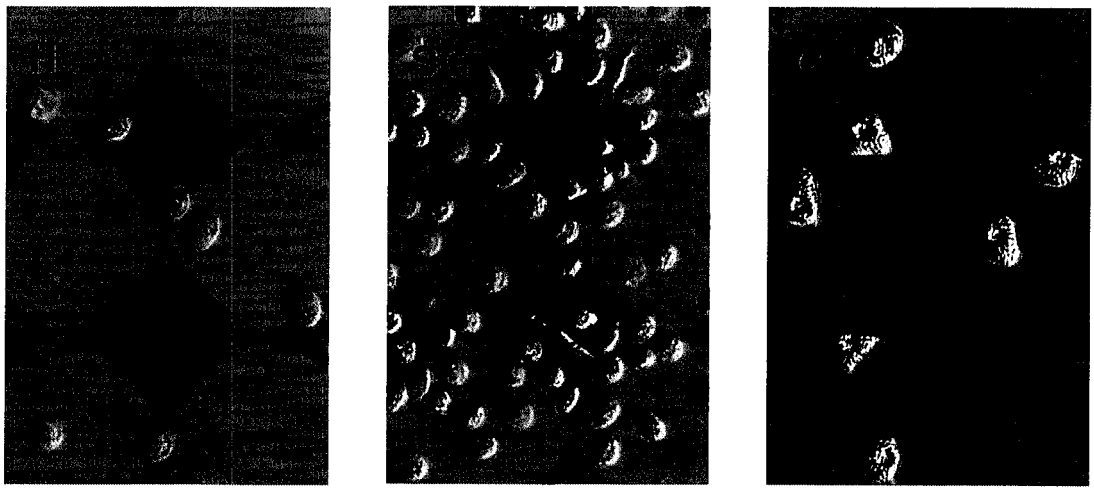


Figure 5. Cultures of separated hemocytes grown in ML-15 media. H: hyalinocytes; SG: semigranulocytes; G: granulocytes. Values are means ± STD (n=5).

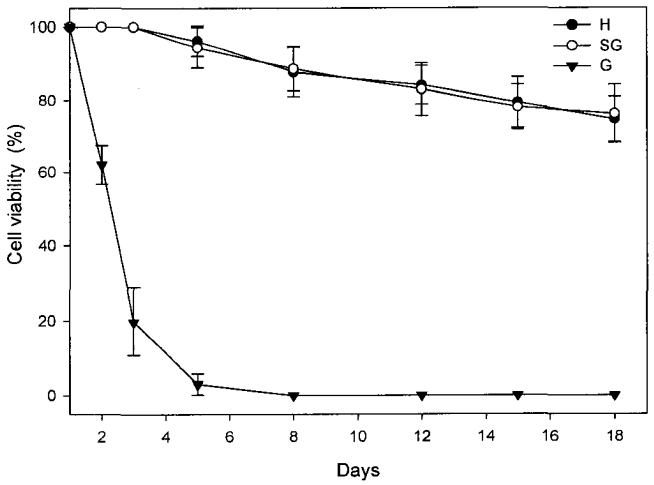


Figure 6. Cytopathic effects of cultured hemocytes infected with PaV1 inoculum (1:10 diluted). (A) 12 h post-inoculation with PaV1 filtrates, (B) 48 h post-inoculation, (C) 72 h post-inoculation. Note the morphological change (black arrow) and degradation (white arrow) of hemocytes when infected with PaV1. (D) Control culture at 72 h post-inoculated with hemolymph filtrates from a healthy lobster. Scale bars = 20 μm.

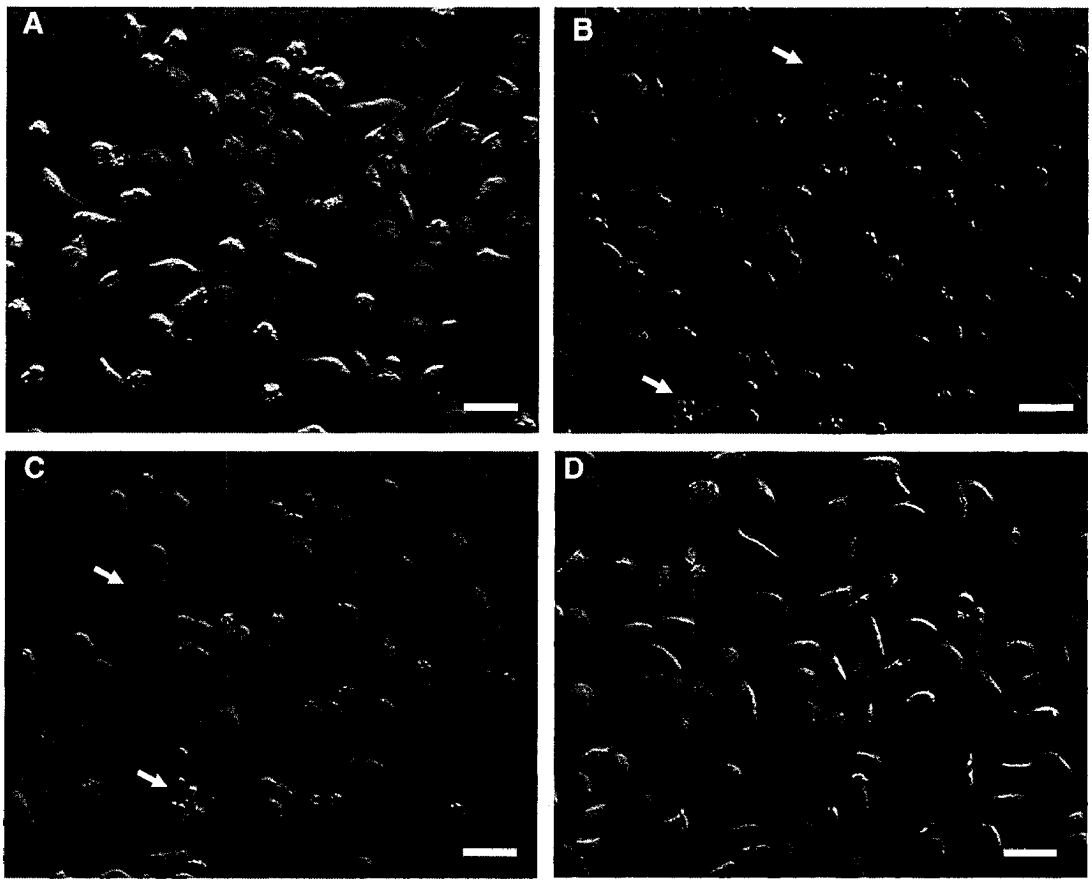


Figure 7. Detection of PaV1 in primary cultures of separated hemocytes (hyalinocytes and semigranulocytes) of *P. argus* by *in situ* hybridization. A: 24 h post-inoculation with hemolymph fitrates from a healthy lobster, B: 24 h post-inoculation with PaV1 filtrates; note the dark staining of infected cells (black arrows), and the debris of lysed cells (arrow heads). Transmitted light microscopy. Scale bars = 20 μm.

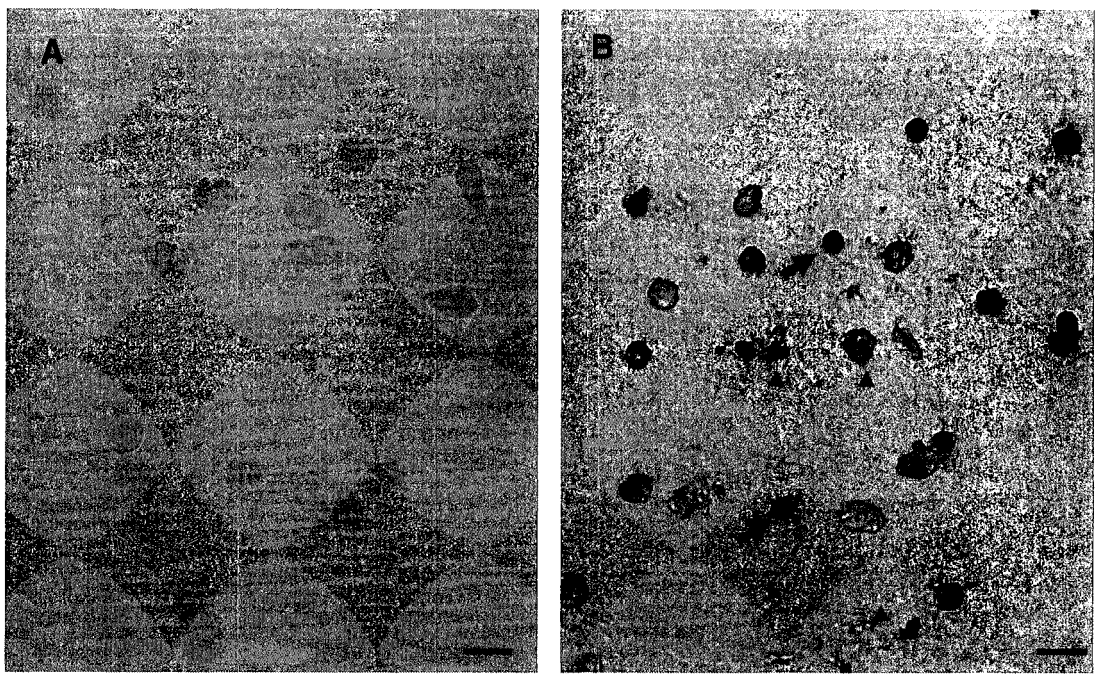
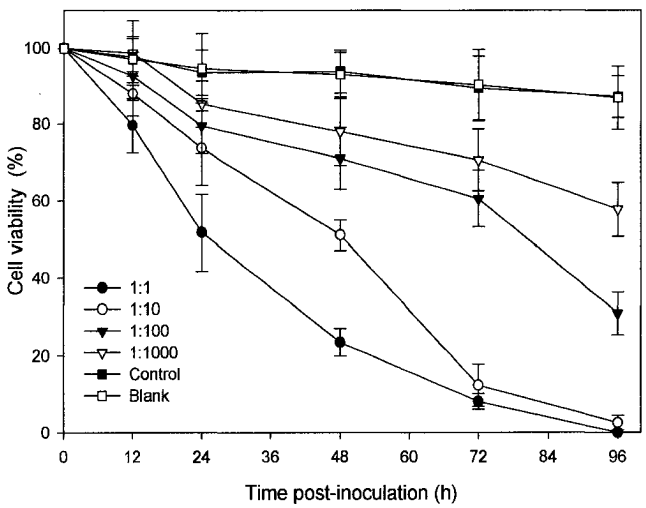


Figure 8. Survival of cultured hemocytes of *P. argus* inoculated with serially diluted viral inoculum. Survival was defined as (numbers of survived cells / initial amount of cells) × 100 % of each well. Values are means ± STD (n = 12).



CHAPTER 3

Pathology and hematology of the Caribbean spiny lobster experimentally infected with *Panulirus argus* virus 1 (PaV1)

Manuscript submitted to the journal of Virus Research:

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MANUSCRIPT ABSTRACT

We conducted a study of the histopathological and hematological response of the Caribbean spiny lobster to experimentally induced infections with PaV1. The fixed phagocytes in the hepatopancreas were the initial site of PaV1 infection in spiny lobsters. Fixed phagocytes were activated in early infections; however, as the disease progressed, the fixed phagocytes became infected and eventually lysed. Infected cells were subsequently observed in the hepatopancreas, gill, heart, hindgut, glial cells around the ventral nerves, as well as in the cuticular epidermis and foregut. In advanced infections, all of the spongy connective tissues were infected as were the glial cells around the optic nerves. The structure of the hepatopancreas was also significantly altered as the disease progressed. The hemal sinuses among the hepatopancreatic tubules filled with massive amounts of cellular aggregates, including infected circulating hemocytes and infected spongy connective tissues. Atrophy of the hepatopancreatic tubules occurred in the late stage of viral infection. The virus caused significant decreases in total hemocyte density in later stages of infection and significantly altered several constituents in the hemolymph serum of diseased lobsters, including: glucose, phosphorus, triglycerides, and lipase.

INTRODUCTION

The Caribbean spiny lobster *Panulirus argus* occurs throughout the Caribbean basin and Western Atlantic from Brazil to Bermuda (Holithius, 1991) and supports valuable commercial and recreational fisheries throughout its range (FAO, 2001, 2004; Harper, 1995). In Florida, for example, the commercial landings of Caribbean spiny lobster have varied between 4.3 million pounds and 7.9 million pounds per year from 1970 to 1999. In 1999, the total landings of the spiny lobster decreased and by 2001 they had dropped to 3.4 million pounds, the lowest reported landings since 1982, approximately 45% less than the historical average landings (FMRI, 2005; Muller et al., 1997). The recently identified pathogenic virus, *Panulirus argus* Virus 1 (PaV1), is thought to have contributed to the decline (Shields and Behringer, 2004).

PaV1 primarily infects benthic juvenile lobsters (20 to 55 mm carapace length, CL), with prevalence decreasing rapidly in relation to size (Shields and Behringer, 2004). The virus occurs throughout the Florida Keys, with the prevalences of visibly infected juveniles ranging from 6 % to 8 %, and reaching 37 % in some areas (Shields and Behringer, 2004). The virus is transmitted by contact, through food, and over short distances (< 1m) through the water (Behringer 2003; Butler *et al.* in review), but healthy lobsters can sense and avoid diseased lobsters and this may limit the spread of the virus in the lobster population (Behringer et al., 2006). PaV1 infects certain hemocytes and

spongy connective tissues in several tissues and organs (Shields and Behringer, 2004). However, the sites of early infection, the progression of PaV1 infection in the spiny lobster and the hematological response of the host to viral infection have not been examined. Therefore, we report the pathology and hematology of spiny lobster over a time course of experimental infection by PaV1.

MATERIALS AND METHODS

Caribbean spiny lobster *Panulirus argus*

Juvenile spiny lobsters, 25 to 50 mm carapace length (CL), were collected from the Florida Keys by hand using SCUBA. Lobsters with apparent signs of disease (Shields and Behringer, 2004) were held separately in 38 L glass aquaria with flow-through ambient seawater (salinity = 35 ± 1 ppt, temperature = 24 ± 1 ° C) equipped with biological filters (Whisper) filled with pre-conditioned crushed coral, and used as donors for inoculation trials. All other lobsters were randomly divided into groups (four to five animals) and housed separately in 76-L glass aquaria. Lobsters were acclimated for at least two weeks prior to inoculation trials. During experiments, lobsters were fed squid three times per week, and water changes were made as needed to ensure that water quality parameters remained within acceptable limits: ammonia (0-0.3 ppm), nitrite (0-0.6 ppm), pH (7.4-8.4), salinity (35 ±1 ppt) and temperature (23 ± 1 ° C).

Viral inocula

Viral inocula were extracted from hemolymph of heavily diseased lobsters, which were histologically confirmed to have heavy infections (Shields and Behringer, 2004). The infectious titer of the inocula was quantified later on frozen pooled sera

using a 50 % tissue culture infectious dose assay ($TCID_{50}$) from Li *et al.* (2007). Briefly, diseased hemolymph was mixed with an equal volume of citrate/EDTA anticoagulant (0.45M NaCl, 0.1M glucose, 30mM sodium citrate, 26mM citric acid, 10mM EDTA; pH = 5.4; Söderhäll and Smith, 1983) and homogenized with a glass homogenizer (Pyrex®, Corning Inc.) at 4 ° C for five minutes. The homogenate was then centrifuged at 3000 × g for 10 min at 4° C, and the supernatant filtered through a 0.45-μm filter. The viral filtrates were serially diluted with modified Leibovitz L-15 medium (ML-15) (Li et al., 2007) to 1:1, 1:10, 1:100 and 1:1000 and used as inocula.

Aliquots of 100 μl of inocula were inoculated separately into one-day old hemocyte cultures (semigranulocytes and hyalinocytes) in 48-well tissue culture plates (Costar®, Costar Corp.). The plates were incubated at 22 – 24 ° C for 96 h, and supplemented with fresh media at two-day intervals. The inoculated primary cultures were examined daily with an inverted microscope, and images were taken for assessment of cytopathic effects (CPE). $TCID_{50}$ was calculated at 96 h based on the end-point dilution assay of Reed and Muench (1938).

Experimental infection

Lobsters were inoculated through the arthrodial membrane at the juncture of the basis and ischium of the fifth walking leg. Two separate inoculation trials were conducted to examine the pathological response of the hosts over different time scales and viral dosages. **Trial I** was a short term infection study, where lobsters were dosed with the

virus, then necropsied over the course of fifteen days. **Trial II** was a long term infection study, where lobsters were inoculated with the virus, then necropsied at 10-day intervals over 77 days. Trials I and II were undertaken at different times with different viral doses (see below).

In Trial I, hemolymph samples from heavily infected donors were pooled, diluted with Citrate-EDTA anticoagulant (1:9) and used as inoculum (virus titer = 5.8×10^2 $TCID_{50}$ /ml). Aliquots of 100-µl of inocula were injected separately into 30 healthy lobsters using sterile 27-gauge needles. Inoculated animals were held together in groups of three to four animals per 76-L aquarium. Controls consisted of 18 animals injected with an equivalent volume of the mixture of the anticoagulant and hemolymph collected from healthy lobsters. Animals serving as controls were held together in groups of three to four animals per 76-L aquarium. At days 1, 3, 5, 7, 10, and15 post-inoculation (p.i.) five animals from the virus inoculated group and three animals from control group were randomly selected and processed for diseases assessment.

In Trial II, hemolymph from heavily infected donors (different donors than in Trial I) was pooled, then diluted with Citrate-EDTA anticoagulant (1:2) and used as inocula (virus titer = 1.2×10^3 $TCID_{50}$ /ml). Aliquots of 100-µl of inocula were injected separately into 50 healthy lobsters using sterile 27-gauge needles. Controls consisted of 30 lobsters injected with an equivalent volume of the anticoagulant. Inoculated and control animals were held separately in groups as detailed above. At roughly 10-day intervals until 80 days p.i., five lobsters from the experimental group and three lobsters from the control group were randomly selected, dissected and processed for histopathological assessment of PaV1 infection and blood chemistry analysis.

Hemolymph and other tissues were collected from each lobster and processed for histological and hematological analysis as described below.

Diagnosis of PaV1 infection

Several tissues were collected for histology from each dissected lobster, including hepatopancreas, hindgut, gill, heart, cuticle epidermis, nerve tissue, and in some cases, compound eyes and antennal gland. Tissues were fixed in Bouin's solution (Fisher) or Z-fix (Anatech Ltd.) for approximately 24 hours, rinsed with tap water for 45 min, then held in 70 % EtOH, processed through paraffin histology and stained with Harris hematoxylin and eosin Y (H&E) (Humason, 1979). Eyes were decalcified overnight in citrate-EDTA, cut in two and processed as above. All tissues were examined using an Olympus BX51 microscope and photographs were taken using a Nikon DXM1200 digital camera. When infections were ambiguous via normal histopathology, tissue samples were processed for diagnosis of viral infection using fluorescence *in situ* hybridization (FISH) as detailed in Li et al. (2006).

Biochemical and hematological analyses

Hemolymph was drawn with a 27-ga. syringe from the juncture between the basis and ischium of the fifth walking leg. Aliguots of 1 ml hemolymph of each animal were stored in an ultracold freezer (-80 °C) for biochemical analysis. Frozen hemolymph samples were thawed on ice. Serum was collected using a pipette, and then centrifuged at 3,000 ×g for 10 min at 4 °C (IEC Thermo Centra) to remove cell debris. Sera were then processed through an Olympus AU400 clinical chemistry analyzer (Olympus Americas,

Inc.) for blood chemistry analysis. Glucose, total protein, phosphorus, triglycerides, lipase, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALKP), gamma-glutamyl transpeptidase (GGT), calcium, sodium, potassium, and chloride were measured according to the manufacturer's manuals. Hemolymph collected from Trial II was immediately processed for total hemocyte counts (THC) and differential hemocyte counts (DHC). Briefly, THC and DHC were processed with a Neubauer hemacytometer using the Olympus BX51 microscope equipped with a Nomarski Differential Interference Contrast Filter. Hemocytes were categorized as hyalinocytes, semigranulocytes or granulocytes based on cell size, cell shape, and granularity as in Li and Shields (2007). Differences in biochemical and hematological constituents between control and experimental groups were examined statistically with SYSTAT (SYSTAT Software Inc.). One-way ANOVA was used to examine differences in measurement of biochemical and hematological constituents in lobsters with different disease categories (healthy, light-medium infection, and heavily infected) as determined by histological examination. The data were log transformed to meet parametric assumptions.

RESULTS

Disease status of experimental lobsters

The severity of viral disease in lobsters was rated as Category 0, 1, 2 or 3 based on pathological changes in all tissues examined (Table 1). Infected cells were characterized by hypertrophied nuclei, margination and condensation of peripheral chromatin and eosinophilic Cowdry-like inclusions. For each tissue, the level of infection was rated as 0, 1, 2, or 3 based on the number of infected cells per section examined microscopically at 400 ×: 0, no cells with observable pathology; 1, <10 infected cells/section; 2, 10 ~ 100 infected cells/section; 3, >100 infected cells/section.

The disease status of lobsters is summarized in Figure 1. In Trial I, only one lobster was lightly infected and another moderately infected by 15 day p.i. There was no observable pathology in any of the lobsters before that time. In Trial II, the virus was highly infectious, presumably because of the higher dose, and by day 10 p.i., 80 % of the lobsters were infected, including one with a moderate infection. By day 20 p.i., all lobsters inoculated with virus were infected; most (80 %) were moderately infected, and one was heavily infected. By day 30 p.i., 60 % of the inoculated lobsters were heavily infected and 40 % moderately infected. After 40 days p.i., all of the lobsters that were inoculated were heavily infected. In both trials, all but one of the control lobsters were healthy, the single infected control was consistent with the background level of the virus present in wild.

Progression of disease in experimentally inoculated animals from Trial I

In Trial I, infected cells were initially observed in the hepatopancreas of the two infected lobsters at day 15 p.i. Fixed phagocytes in the hepatopancreas appeared to be the primary cell associated with PaV1 infection in spiny lobsters. Significant alterations of the fixed phagocytes were observed in the hepatopancreas in relation to the progression of the infection (Fig. 2). Activated fixed phagocytes were significantly enlarged, with highly vacuolated cytoplasm and sparse granules (Fig. 2 B). As the infections progressed, fixed phagocytes were obviously infected by the virus (Fig. 2 C). In the hepatopancreas of the moderately infected lobster, the typical rosette structure of fixed phagocytes around the arterioles was no longer discernible. (Fig. 2 D). There were no overt pathological changes in other tissues during this early period of infection, except that granulomas were present in the antennal gland of the lightly infected lobster (Fig. 2 E). As no infected cells were observed in the antennal gland, this pathological changes may not be associated with PaV1 infection.

Total hemocyte counts (THCs) varied over time and between treatments. THCs in inoculated lobsters increased due to the stimulation of inoculation, and then decreased significantly thereafter (Fig. 3). By day 15 p.i., THCs of PaV1-inoculated lobsters were significantly lower than those in controls (ANOVA, $p<0.01$). THCs of lobsters from the control group varied in the first 3 to 4 days p.i. and then gradually recovered to the initial density. Relative changes among types of hemocytes were not significantly different between treatments (data not shown). Therefore, the absolute changes in THC were consistent between cell types with treatments. However,

granulocytes appeared to accumulate in the enlarged hemal spaces of the hepatopancreas of a lobster inoculated with PaV1 by day 15 p.i. (Fig. 2 F).

Progression of disease in tissues of experimental infected animals from Trial II

As expected, the progression of infection and disease varied between Trials (Fig. 1). The pathological changes in the tissues of lobsters with light or moderate infections were similar in both trials, but more animals were infected in Trial II.

Day 10 p.i.: Infected cells were observed in hepatopancreas, gill, heart, central nerve tissue and hindgut, but they were not observed in the cuticular epidermis and compound eyes (Table 2). The hepatopancreas of lightly infected animals maintained its normal architecture (Fig. 4 B). Many of the fixed phagocytes were infected, but the architecture of the supporting spongy connective tissue was unchanged (Fig. 2 C). Reserve inclusion (RI) cells were abundant in lobsters in the intermolt stage. No overt pathological changes were observed in other tissues in the initial stage of exposure, except that a few infected circulated hemocytes (<10 / section) were occasionally present.

Day 20 p.i.: The hepatopancreas, gill, heart and central nerve tissues were moderately infected by PaV1, with many infected cells (10 ~ 100 /section) present in these tissues (Table 2). A few infected cells were observed in the cuticular epidermis around the foregut, but no infected cells were observed in the tissues of the compound eye. Infected cells were easily identified in tissue sections stained with H&E. As infection progressed, the hemal sinuses within the hepatopancreas became filled with infected cells, including fixed phagocytes, hyalinocytes and semigranulocytes (Fig. 4 C).

Most fixed phagocytes were infected by the virus, and the rosette-like structure of the fixed phagocytes was apparently altered, with cross sections showing few normal cells in the rosette. There was no clear separation of the connective tissue cells supporting the arterioles and there was an apparent increase in spongy connective tissue cells (Fig. 4D).

Day 30 p.i.: The hepatopancreas, gill and central nerve tissue of diseased lobsters were heavily infected; and the spongy connective tissue in the myocardium of heart, hindgut and cuticular epidermis tissues was moderately infected. Spongy connective tissues appeared to proliferate around the tubules of the hepatopancreas in one heavily diseased lobster (Fig. 4 D). There were no obvious changes in the tissues of diseased animals by day 40 p.i. compared with those of day 30 p.i., except that infected cells occurred in the hemal sinuses of the optic nerve region in the eyes of diseased animals.

Day 50 p.i. and thereafter: There seems to be an abnormal proliferation of spongy connective tissues within the hepatopancreas and hindgut (Fig. 4 E, Fig. 5 A). However, the fibrous connective tissues showed no histological signs of viral infection (Fig. 5 B). The spongy connective tissues around the nerves were heavily infected (Fig. 5 C). In the hearts of heavily diseased animals, there were slight proliferations in the spongy connective tissues comprising the supportive, outer portion of the arterioles (Fig. 5 D). In lobsters chronically (> 60 days) infected by PaV1, the hemal spaces among the tubules of the hepatopancreas were markedly dilated; the tubules of the hepatopancreas were greatly atrophied and filled with large numbers of infected cells (Fig. 4 E, F). As the

disease progressed, RI cells were gradually depleted in the hepatopancreas of chronically infected lobsters.

Hemolymph serum chemistry of experimental animals

Values of the specific serum constituents were analyzed separately by trial using time course and by severity. Severity of the disease was significantly associated with changes in a few constituents, but the time course was not significantly associated with changes in serum constituents. That is, the sera from animals exhibiting light, moderate or heavy infections showed significant differences in the study, but there was no association with the time or length of infection. Only four constituents showed significant changes with severity of disease in Trial II (Table 3); whereas, there were no significant alterations in the constituents in Trial I because of the short span of time for the disease to develop (data not shown). Separate controls were used for each of the trials.

The changes in tissue constituents showed three patterns: (1) Absolute decreases in relation to severity: i.e., glucose levels in the hemolymph of infected lobsters decreased significantly in relation to severity, with healthy animals having the highest glucose levels, and infected animals having depleted glucose levels. (2) Fluctuations in relation to severity: i.e., phosphorus and triglyceride levels were significantly lower in lobsters with light or moderate infection, and were significantly higher in lobsters with heavy infections when compared to uninfected animals. (3) Dips in relation in severity: i.e., lipase was significantly lower in lobsters with light or moderate infections, and but not in lobsters with heavy infections when compared to controls. AST, ALKP, and serum

protein had similar trends as phosphorus with severity; however, no significant changes were observed due to the high variances. No significant differences were observed among other biochemical constituents (Table 3). These changes likely reflect tissue degradation and catabolism of the hepatopancreas in relation to the severity of disease.

DISCUSSION

PaV1 initially infects fixed phagocytes in the hepatopancreas. Along with circulating hemocytes, the fixed phagocytes in the hepatopancreas of decapod crustaceans play an important role in the cellular defenses by filtering foreign materials from the hemolymph (Factor, 1995; Factor et al., 2005; Johnson, 1980; 1987). Fixed phagocytes are the only cell type other than circulating hemocytes that phagocytize foreign particles in the hemolymph (Johnson, 1987). For example, fixed phagocytes apparently play a role in the phagocytosis and infection of BFV in the blue crab (Johnson, 1980), where virions aggregate around the degenerated cytoplasm of infected fixed phagocytes, and enclosed by the interrupted layer, a basal lamina surrounding fixed phagocytes (Johnson, 1980). Larger viruses are apparently recognized by the fixed phagocytes and removed from the hemolymph, sometimes accumulating within the interrupted layer. PaV1 is a relatively large virus at 187 nm in diameter (Shields and Behringer, 2004). Perhaps its large size facilitates its uptake by fixed phagocytes, which then, inadvertently, become infected. Podocytes in the gills are also known to be involved in the removal of small foreign particles from the hemolymph of decapod crustaceans (Johnson 1980, Hejkal and Gerba, 1981). However, there was no obvious infection of the podocytes by PaV1 (unpublished data).

The hepatopancreas was associated with the progression of the disease. In light infections, the fixed phagocytes in the hepatopancreas were initially infected, followed by adjacent spongy connective tissue cells and hemocytes. As infection progressed, the hemal sinuses within the hepatopancreas became filled with infected cells; and spongy connective tissues appeared proliferated within these sinuses. In heavy infections, the hepatopancreatic tubules were significantly altered, atrophying, and the hemal sinuses became filled with cellular aggregates. The atrophy of the hepatopancreas was apparent at both the gross and microscopic levels of observation. The spongy connective tissues and hemocytes in the other organs also became infected with PaV1, but the organs did not show gross alterations. These cellular aggregates associated with the infection appear to be comprised of spongy connective tissues and not infiltrates of hemocytes. Farley et al. (1972, 1978) reported the first invertebrate herpes-like virus from the oyster *Crassostrea virginica* and found massive cellular aggregates derived from hemocytes in the hemal sinuses and vascular tissues in advanced cases of infection. They speculated that the herpes-type viruses may have a proliferative component manifesting as cellular aggregates in diseased oysters. Similarly, lymphoproliferative disease is associated with herpes viruses in mammals, such as Epstein-Barr virus (EBV), which plays a primary role in the development of several types of B-lymphocyte malignancies in humans (Theate et al., 2002; Snow and Martinez, 2007).

PaV1 infects spongy connective tissue cells, fixed phagocytes, and circulating hyalinocytes and semigranulocytes, but not granulocytes (Shields and Behringer, 2004), nor fibrous connective tissue. These tissues are all developmentally derived

embryologically from mesoderm, and it is not unusual for viruses to have specific tropisms to particular developmental germ layers. At least six viruses primarily infect the hemocytes of crustaceans and other viruses can infect hemocytes as well as other tissues (Johnson, 1983; Brock and Lightner, 1990). For example, WSSV infects hemocytes and other tissues originating from both mesodermal and ectodermal germ layers (Wongteerasupaya et al., 1996; Chang et al., 1996; Lo et al., 1997). The bi-facies virus (BFV) from the blue crab *Callinectes sapidus* (Johnson, 1976, 1988) causes similar pathological changes as PaV1. However, no proliferation of tissues occurs in BFV infections.

In the later stages of exposure to PaV1 in Trial I, total hemocyte density decreased; however, the proportion of each type of cell in the hemolymph did not change, which is surprising because the granulocytes showed no histological signs of infection. Thus, one would expect to see a relative increase in granulocyte number relative to other cell types. We speculate that this is caused by a commensurate decline in granulocytes due to the presence of cellular aggregates interacting with the granulocytes in the tissues, which we observed in several virally inoculated animals. The circulating hemocytes of crustaceans play a key role in the host defense system against invasion of non-self particles (Bachère et al., 1995; Jiravanichpaisal et al., 2006; Ratcliffe et al., 1985; Roch, 1999; Smith and Söderhäll, 1983; Söderhäll and Cerenius, 1992; Söderhäll et al., 1986). However, the densities of circulating hemocytes vary upon challenge by different microorganisms. Taura syndrome virus (TSV) causes a significant decrease in THCs, with relative decreases in the hyalinocytes and granulocytes of infected Pacific white shrimp *Litopenaeus vannamei* (Song et al., 2003). White spot syndrome virus (WSSV)

infects the semigranulocytes and granulocytes of *Penaeus indicus* and causes significant decreases in THCs (Yoganandhan et al., 2003). However, WSSV did not cause a decline in THCs in infected freshwater crayfish *Pacifastacus leniusculus*, even though the semigranulocytes and granulocytes were susceptible to the virus (Jiravanichpaisal et al., 2001, 2006). The oomycete *Aphanomyces astaci* causes a decrease in THCs in the crayfish *P. leniusculus;* and the decline in hemocytes is thought to lower resistance of the crayfish to the pathogen (Persson et al., 1987). Interestingly, we have found no correlation between PaV1 and other diseases in the field or the laboratory (Shields and Behringer 2004; Shields unpublished data); therefore, it is not clear whether the loss of hemocytes negatively affects the innate defenses of the lobster host.

The analysis of biochemical constituents in hemolymph is an important assessment of tissue injury, overall health status, and immune function in crustaceans (Battison, 2006; Mohankumar and Ramasamy, 2006 a, b; Song et al., 2003; Wu et al., 2002; Yoganandhan et al., 2003). Glucose and total carbonhydrates in the hemolymph of penaeid shrimp *Penaeus indicus* increase significantly in WSSV infections (Yoganandhan et al., 2003). Similarily, activities of transaminases (ALT and AST) increased in the hemolymph, hepatopancreas, gills and muscles of *P. indicus* infected with WSSV (Mohankumar and Ramasamy, 2006 a). In lobsters infected with PaV1, concentrations of glucose, phosphorus, triglycerides, and lipase in the hemolymph differed from that of control lobsters. These constituents are involved in the short- and intermediate-term energy reserves of the lobster and their decline indicates the depletion of reserve inclusions (RIs) in RI cells. RI cells contain granules that are composed of polysaccharides, such as glycogen, and proteins such as hemocyanin (Johnson, 1980),

and their depletion is often associated with disease agents (Shields and Behringer, 2004, Stentiford and Shields, 2005), and is indicative of metabolic wasting or exhaustion (Shields and Behringer 2004). However, serum proteins and other constituents (e.g., AST, or ALKP) did not increase in response to infection. These enzymes are important markers of liver function in vertebrates, and they likely indicate a similar function in invertebrates. The changes in glucose, phosphorus, triglycerides and lipase likely reflect tissue degradation and catabolism of the hepatopancreas in relation to severity of the disease and support the hypothesis that metabolic exhaustion is the primary cause of death for infected lobsters.

We have presented the first study of the histopathological and hematological response of the spiny lobster to PaV1 over the time course of experimental viral infection. The results of this study facilitate our understanding of the pathogenesis of the PaV1 in the lobster host. PaV1 is widespread and highly pathogenic to spiny lobsters in Florida Keys (Shields and Behringer, 2004). Considering the catastrophic impact of viral diseases in penaeid shrimp (Cai et al., 1995; Inouye et al., 1994; Lightner, 1999; Lo et al., 1996; Wang et al., 1998; Wongteerasupaya et al., 1996), future emphasis should be placed on development of efficient diagnostic tools, effective control methods for applications in aquaculture, and understanding the transmission of PaV1 in nature.

Table 1. Categorization of the severity of PaV1 disease in the Caribbean spiny lobster *Panulirus argus*.

Category 0 Healthy	• No aberrant cells with hypertrophied nuclei, no peripheral chromatin nor eosinphilic inclusions • Hepatopancreas and other tissues appear normal • Fixed phagocytes appear normal, not activated
Category 1 Lightly infected	• A few infected cells (<10 per section) present in hepatopancreas or other organs • Hepatopancreas and other tissues appear normal • Fixed phagocytes in hepatopancreas activated or a few infected
Category 2 Moderately infected	• More infected cells (10 to 100 per section) present in hepatopancreas or other organs • Infected cells present in spongy connective tissue around midgut, heart or gills • Most fixed phagocytes activated or infected
Category 3 Heavily infected	• Interstitial spaces in hepatopancreas filled with numerous infected cells (> 100 per section) • Hepatopancreatic tubules atrophied • Many infected cells present in heart, and spongy connective tissue around midgut and other organs • Focal necrosis in heart • Morbid behavior

Table 2. Sequential progression of PaV1 in the tissues of spiny lobsters over the time course of experimental infection in Trial II. (Category of infection in each tissue = mean ± std., n = 5 per time period)

Days P.I.	Hepatopancreas	Gill	Heart	Central nerve tissue	Hindgut	Cuticular epidermis	Compound eyes
Day 10	1.4 ± 0.6	1.4 ± 0.9	1.2 ± 0.5	1.0 ± 0.7	0.4 ± 0.5	0.0 ± 0.0	0.0 ± 0.0
Day 20	2.0 ± 0.7	1.8 ± 0.4	1.6 ± 0.5	1.6 ± 0.5	1.0 ± 0.0	0.6 ± 0.5	0.0 ± 0.0
Day 30	2.6 ± 0.5	2.4 ± 0.9	2.0 ± 0.0	2.2 ± 0.8	1.8 ± 0.4	1.8 ± 0.8	0.0 ± 0.0
Day 40	2.8 ± 0.4	2.6 ± 0.5	3.0 ± 0.0	2.6 ± 0.5	2.0 ± 0.0	1.8 ± 0.5	1.0 ± 1.0
Day 50	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	2.4 ± 0.9	1.2 ± 1.3
Day 60	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	2.6 ± 0.5	1.6 ± 0.5
Day 77	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	2.0 ± 0.7

Table 3. Biochemical analysis of lobster serum compared with disease category (Each value is a mean ± SE).

Variable	Healthy (n = 13)	Light and Moderate (n = 11)	Heavily infected (n = 24)
Glucose (mg/dL)	$30.00 \pm 6.30^{a***}$	15.18 ± 2.46^{b}	13.00 ± 1.86^{b}
Creatinine (mg/dL)	0.23 ± 0.03	0.19 ± 0.04	0.22 ± 0.02
Phosphorus (mg/dL)	1.66 ± 0.16^{a}	1.61 ± 0.22^{a}	$2.25 \pm 0.18^{b***}$
Calcium (mg/dL)	46.85 ± 1.33	47.31 ± 1.76	48.08 ± 0.99
ALT (U/L)	1.54 ± 0.37	1.09 ± 0.37	1.46 ± 0.20
AST (U/L)	26.85 ± 4.73	25.00 ± 6.23	38.00 ± 4.98
ALKP (U/L)	383.23 ± 36.69	434.64 ± 66.07	479.08 ± 49.23
GGT (U/L)	0.31 ± 0.24	0.36 ± 0.15	0.79 ± 0.33
Triglycerides (mg/dL)	$2.92 \pm 0.64^{a**}$	$1.55 \pm 0.62^{b**}$	$4.54 \pm 0.78^{c**}$
Sodium (mEg/L)	474.62 ± 10.26	479.09 ± 8.39	473.13 ± 6.65
Potassium (mEg/L)	12.08 ± 0.61	10.82 ± 0.60	11.58 ± 0.40
Chloride (mEg/L)	459.23 ± 9.72	471.82 ± 8.35	463.33 ± 7.48
Lipase (U/L)	25.73 ± 16.55^{a}	$7.75 \pm 0.76^{b*}$	27.63 ± 7.84^{a}
Protein (g/dL)	4.04 ± 0.47	3.39 ± 0.49	4.03 ± 0.31
Refractometer (g/dL)	6.15 ± 0.64	5.15 ± 0.59	5.99 ± 0.42

Data were analysed using One-way ANOVA with significance limits of 0.01(***), 0.05 (**) or 0.08 (*). Values followed by different letter (a, b or c) were significantly different. Triglycerides were significant ($p<0.05$) when not transformed, but just over ($P<0.0603$) when transformed.

Figure 1. Infection status of spiny lobsters experimentally inoculated with PaV1 (Trial I: viral dose = 5.80×10^2 $TCID_{50}$ /ml; Trial I: viral dose = 1.20×10^3 $TCID_{50}$ /ml) (n = 5 per time period). Category 0 (C. 0): healthy; Category 1 (C. 1): lightly infected; Category 2 (C. 2): moderately infected; Category 3 (C. 3): heavily infected.

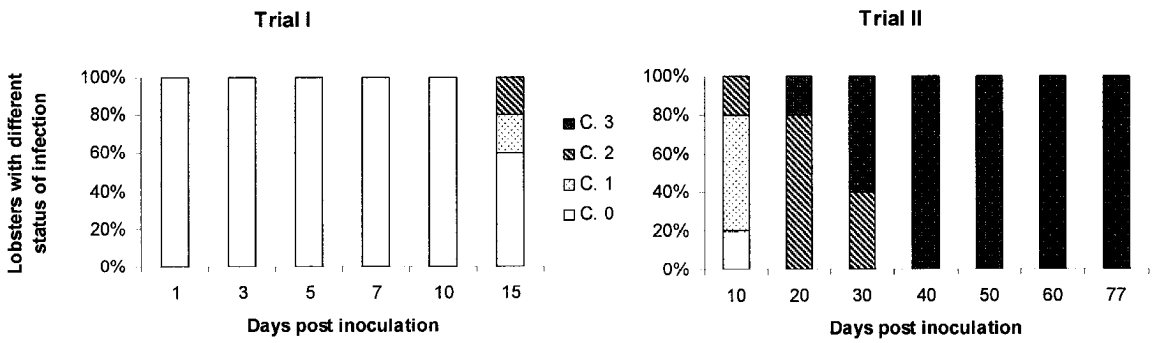


Figure 2. Pathological changes in the tissues of infected lobsters from Trial I (low dose, short term). A: resting fixed phagocytes surrounding an arteriole in the hepatopancreas of a healthy lobster, B: activated fixed phagocytes in a lightly infected lobster, C: infected fixed phagocytes (arrows) in the lightly infected lobster, D: infected fixed phagocytes (arrows) in a moderately infected lobster, E: granulomas in the antennal gland of the lightly infected lobster, F: accumulation of granulocytes in hemal sinuses of the hepatopancreas of the lightly infected lobster. Notice the alteration of fixed phagocytes in hemal sinuses of the hepatopancreas over a time course of PaV1 infection, and the change in cell densities surrounding the arterioles. FP: fixed phagocyte, A: arteriole, L: lumen of tubule, Gr: granuloma, G: granulocyte.

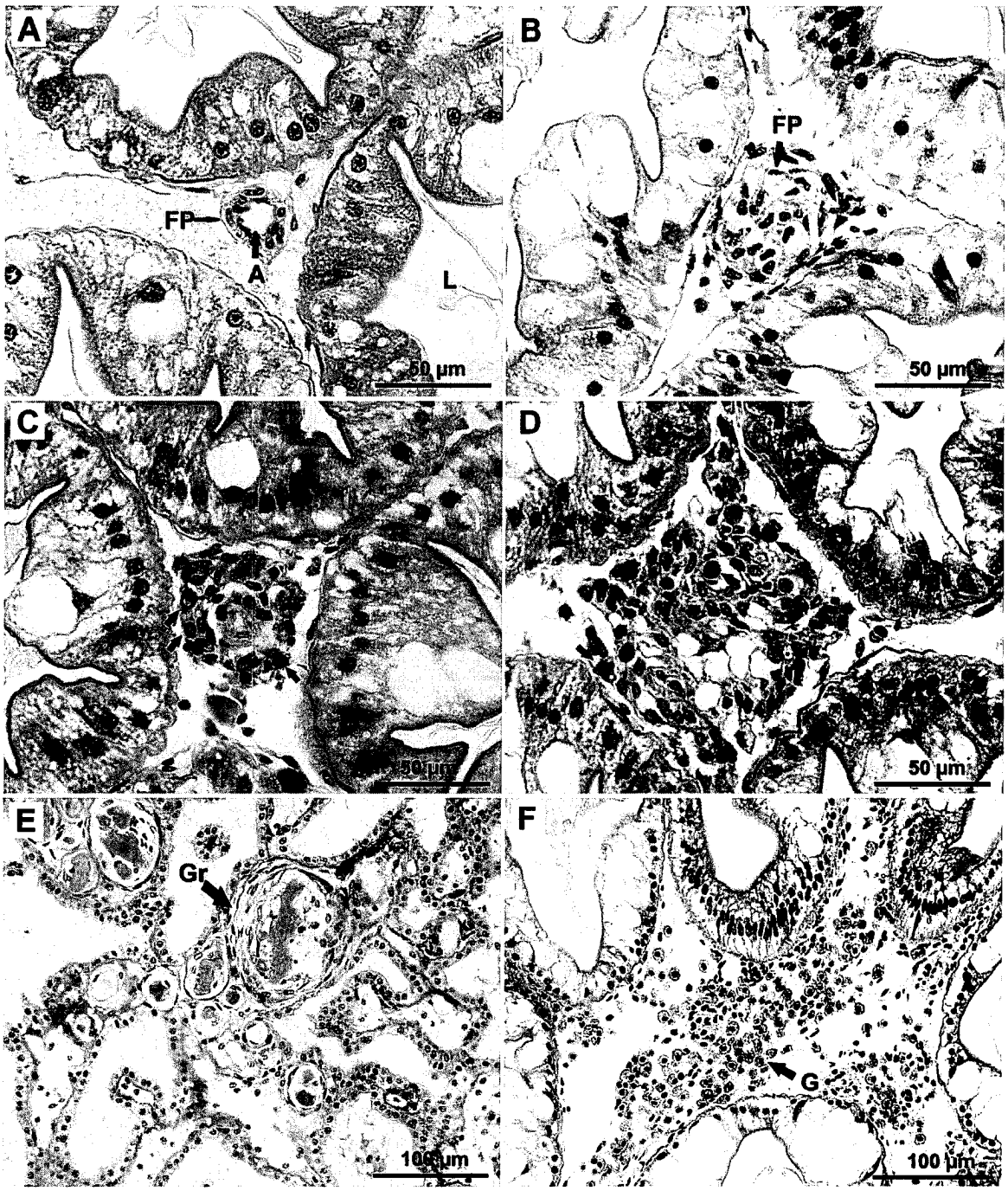


Figure 3. Total hemocyte counts (THC) of lobsters from control and inoculation group over early time course of infection (Trial I). The number of cells was log transformed. Sample size was three lobsters in control and five in experimental groups, bars = standard deviation.

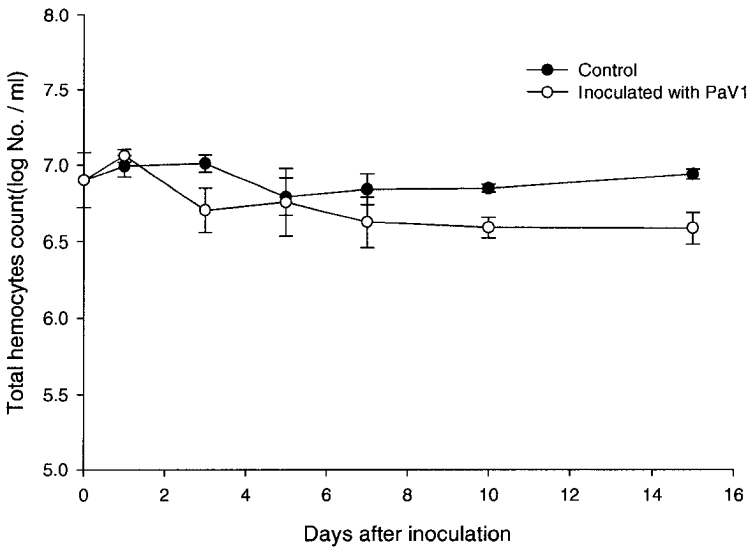


Figure 4. Pathological changes in the hepatopancreas of *P. argus* from Trial II (high dose, long term). A: healthy, B: light infection, C: moderate infection, D: heavy infection, E: chronic heavy infection, F: hemal space among the tubules of the hepatopancreas of a chronically infected lobster. Notice the accumulation of massive amount of cells in the hemal sinus (HS) among the tubules of the hepatopancreas in moderately and heavily diseased animals. Arrows indicated infected cells. L: lumen of tubule, RI: reserved inclusion cell, A: arteriole, SCT: spongy connective tissues.

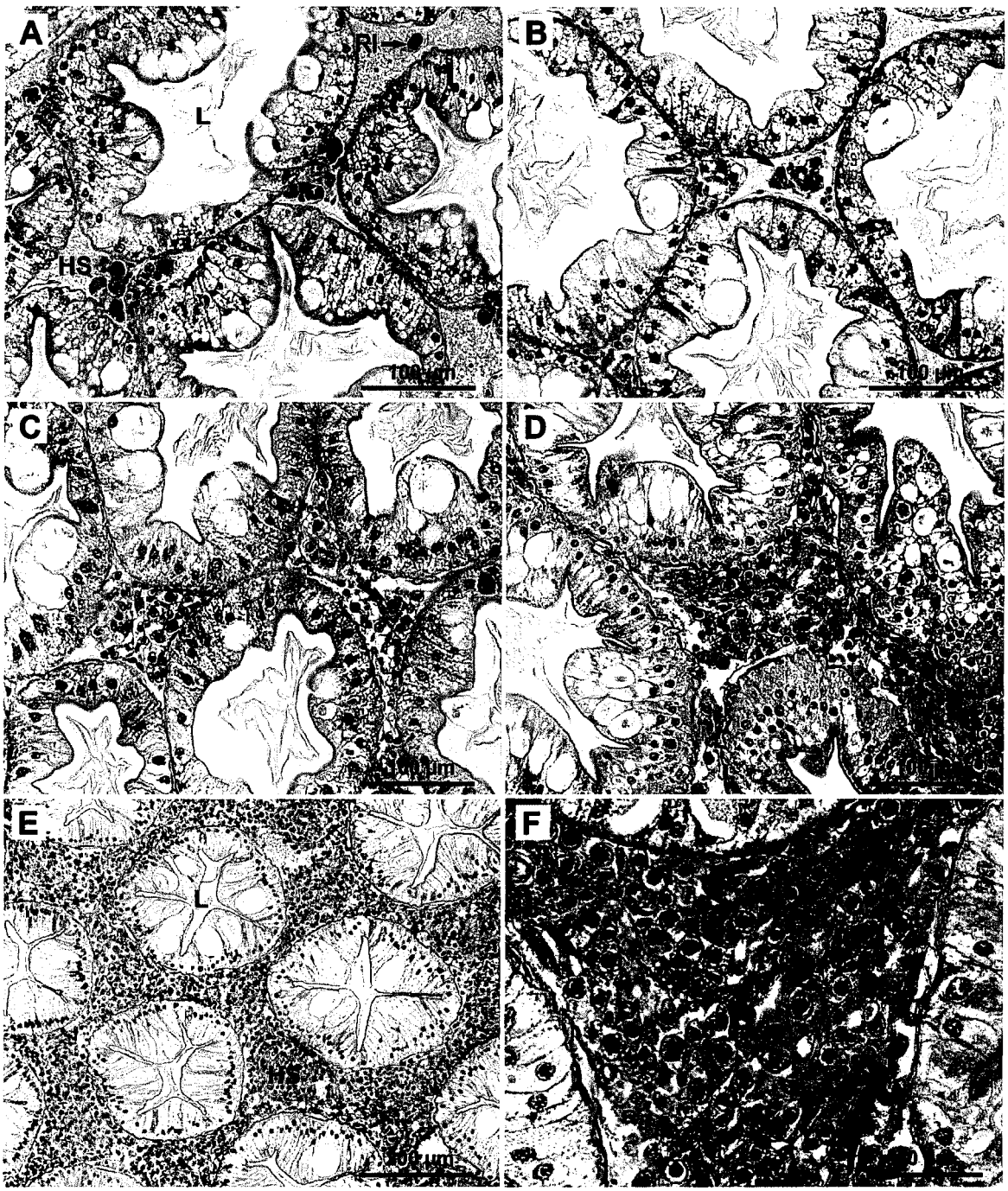
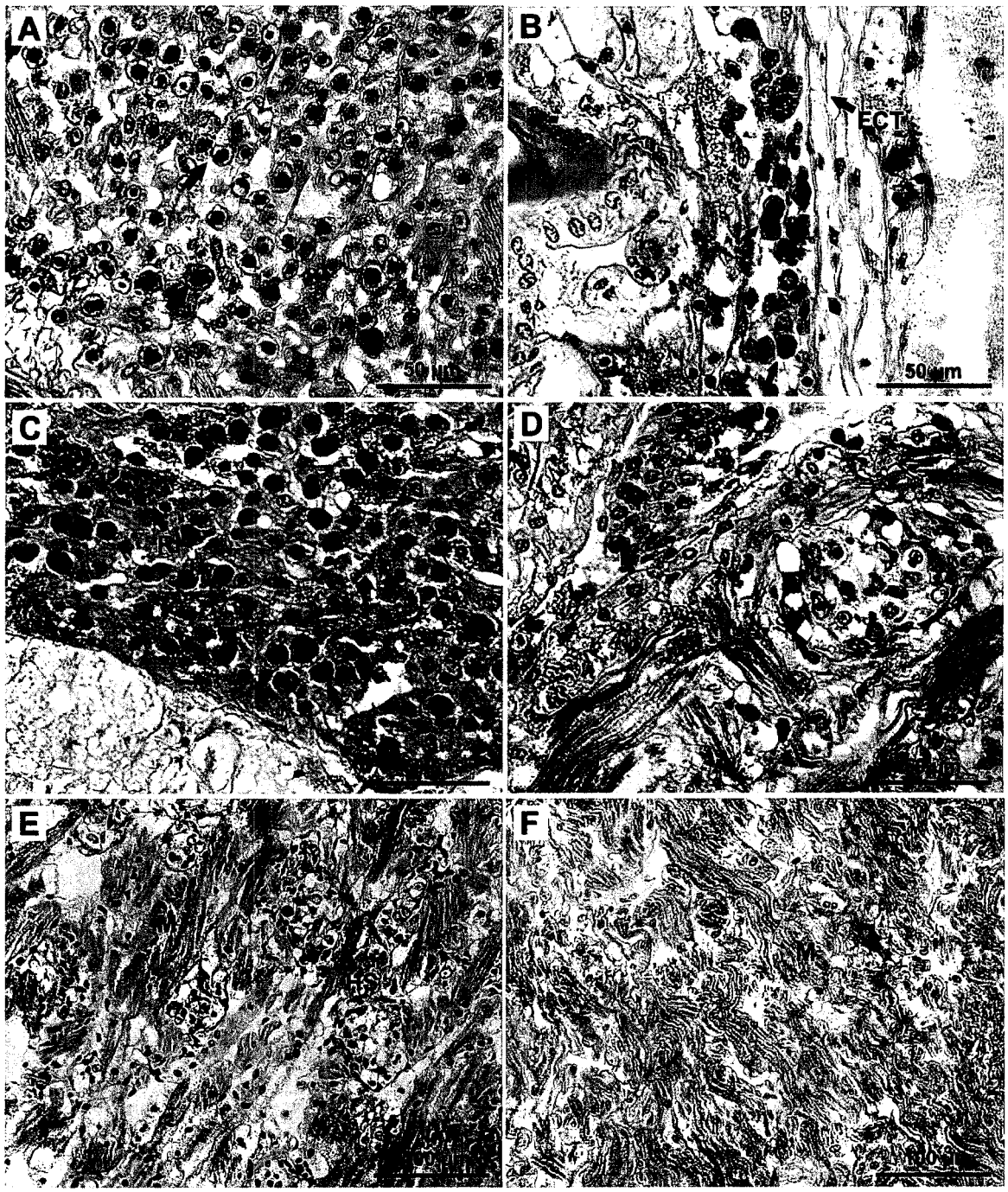


Figure 5. Pathological changes in the hindgut (A), gill (B), spongy connective tissues around nerve tissues (C) and heart (D, E, F). Infected cells (arrows) were present in the hemal sinuses of the heart, arteriole in the gill, and spongy connective tissues. Notice the significant changes in hemal sinuses (HS) in the heart tissues of heavily infected lobster. FCT: fibrous connective tissue, RI: reserve inclusion cell, M: myocardium, HS: hemal sinus.



GENERAL CONCLUSIONS

Chapter 1

1) A specific 110-bp probe was generated from a plasmid containing a 177-bp fragment of the virus using the primer pair PaV1 110 F/R. The probe was applied to diagnose PaV1 in tissues of the Caribbean spiny lobsters *Panulirus argus* using fluorescence *in situ* hybridization (FISH) assay.
2) In dot-blot hybridizations, the probe had a minimum sensitivity of 10 pg of cloned 177-bp plasmid DNA. The probe detected the presence of viral DNA from 10 ng of genomic DNA extracted from hemolymph of a PaV1 infected spiny lobster.
3) The fluorescein (FITC)-labeled probe specifically hybridized to PaV1-infected hemocytes and spongy connective tissue cells in or around the hepatopancreas, hindgut, foregut, gill, heart, skin, nerve and even ovary tissues. Most FITC-stained foci were located around the inner periphery of the hypertrophied nuclear membrane, with a few dispersed throughout the inside of the nucleus.

4) The probe did not hybridize with host tissues of uninfected spiny lobsters, nor did it cross-react with a herpes-like virus (HLV) from a blue king crab *Paralithodes platypus*, lymphocystis disease virus (LDV) from a striped bass *Morone saxatilis*, Ostreid Herpesvirus 1 (OsHV-1) from an infected Pacific oyster *Crassostrea gigas,* and Intranuclear bacilliform virus (IBV) from an infected brown shrimp *Crangon crangon.*

This specific and sensitive FISH assay provides a useful tool to investigate infections in tissues of lightly infected lobsters in our experimental trials. This probe can be used to detect the presence of virus in hemolymph of lobsters using dot-blot hybridization and to monitor the prevalence of PaV1 in nurseries of juvenile lobster.

Chapter 2

1) Three major hemocyte types - hyalinocytes, semigranulocytes, and granulocytes - were identified in the Caribbean spiny lobster based on cell size, cell shape, and granularity.
2) Lobster hemocytes were physically separated into three distinct subpopulations enriched in hyalinocytes, semigranulocytes, and granulocytes, respectively, using

Percoll discontinuous gradients centrifugation.

3) A modified Leibovitz L-15 medium supported the best survival of hemocytes in *in vitro* primary cultures. Fetal bovine serum was not an effective additive for survival of hemocytes *in vitro* in this study.
4) When hemocytes were cultured together, most of the granulocytes dehisced, releasing their contents into the culture media, negatively influencing the survival of other the cell types. When cultured separately, hyalinocytes and semigranulocytes maintained higher viability (~ 80%) over 18 days incubation compared with granulocytes, which degraded over 2-3 days.
5) Hyalinocytes and semigranulocytes cultured *in vitro* were susceptible to PaV1. Cytopathic effects (CPE) were observed as early as 12 h post-inoculation, and cell lysis was noticeable within 24 hrs of infection.
6) The presence of virus within cells was confirmed by *in situ* hybridization using the specific PaV1 110-bp DNA probe derived from Chapter 1. The unique staining of PaV1 infected cells was observed after 24 h post-inoculation.

The in vitro assay can be applied to quantify the dose of infectious virus in hemolymph using a 50% tissue culture infectious dose assay (TCID50) based on CPE. It may also be used in propagation of PaV1 *in vitro*, which is useful for isolation and purification of the virus. The *in vitro* culture of hemocytes will facilitate better characterization of different

hemocyte types of lobster, as well as cell lineage of hemocyte types in crustacean.

Chapter 3

1) The fixed phagocytes in the hepatopancreas were the initial site of PaV1 infection in spiny lobsters. Fixed phagocytes were activated in early infections and then became obviously infected as the disease progressed.
2) Infected cells, including hyalinocytes, semigranulocytes and spongy connective tissue cells, were subsequently observed in the hepatopancreas, gill, heart, hindgut, glial cells around the ventral nerves, as well as in the cuticular epidermis and foregut. In advanced infections, all of the spongy connective tissues were infected as were, the glial cells around the optic nerves.
3) The hepatopancreas of infected lobsters was associated with progression of the disease. As the infection progressed, the hemal sinuses within the hepatopancreas became filled with massive amounts of cellular aggregates, including infected circulating hemocytes and infected spongy connective tissues.
4) In lobsters chronically infected (> 60 days) by PaV1, the hemal spaces among the tubules of the hepatopancreas were markedly dilated. The tubules of the hepatopancreas were greatly atrophied.

5) Reserved inclusions (RI) cells were gradually depleted in the hepatopancreas of chronically infected lobsters. This depletion supports the contention that the cause of death in infected lobsters is metabolic exhaustion (Shields and Behringer, 2004).

6) The virus caused significant decreases in total hemocyte density in early infection; however, there was no significant change in the proportion of each type of cell in the hemolymph.

7) There were significant changes in concentrations of glucose, phosphorus, triglycerides, and lipase in the hemolymph of lobsters infected with PaV1. The changes in these parameters likely reflect tissue degradation and catabolism of the hepatopancreas in relation to severity, and support the hypothesis that metabolic exhaustion is the primary cause of death for infected lobsters.

The results of this study indicated that fixed phagocytes in the hepatopancreas of experimentally injected lobsters were the initial sites of PaV1 infection. I speculate that once PaV1 virions enter the open circulating hemolymph of the lobster, fixed phagocytes in the hepatopancreas and elsewhere phagocytize the viruses and remove them from the hemolymph. Because fixed phagocytes are susceptible to the virus or perhaps because of phagocytosis, the fixed phagocytes become infected by the virus and possibly trigger a series of host defense responses (e.g. accumulation of hemocytes) against the activated

and infected fixed phagocytes. However, hyalinocytes and semigranulocytes are also susceptible to the virus. Therefore, the virus may be spread via circulation of the hemolymph, and cause infection of those tissues originated from mesoderm (e.g. spongy connective tissues in hindgut and foregut, glial cells in nerve tissues). However, how the virus gains entry into the lobster host naturally and how the virus is spread in the lobster host with natural infections requires further study.

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