Provided for non-commercial research and education use. Not for reproduction, distribution or commercial use.



This article was published in an Elsevier journal. The attached copy is furnished to the author for non-commercial research and education use, including for instruction at the author's institution, sharing with colleagues and providing to institution administration.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright



Available online at www.sciencedirect.com





Virus Research 132 (2008) 104-113

www.elsevier.com/locate/virusres

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

Caiwen Li^a, Jeffrey D. Shields^{a,*}, Robert E. Ratzlaff^b, Mark J. Butler^b

^a Virginia Institute of Marine Science, The College of William and Mary, P.O. Box 1346, Gloucester Point, VA 23062, USA ^b Department of Biological Sciences, Old Dominion University, Norfolk, VA 23529, USA

Received 15 May 2007; received in revised form 1 November 2007; accepted 10 November 2007 Available online 4 January 2008

Abstract

We examined the histopathological and hematological response of the Caribbean spiny lobster to experimentally induced infections with *Panulirus argus* Virus 1 (PaV1). The fixed phagocytes in the hepatopancreas were the primary sites 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, and in the cuticular epidermis and foregut. In advanced infections, spongy connective tissues were heavily infected, as were glial cells around the optic nerves. The structure of the hepatopancreas was 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 spongy connective tissues. Atrophy of the hepatopancreatic tubules occurred in the late stage of viral infection. The virus caused significant decreases in total hemocyte counts and significantly altered several constituents in the hemolymph lysates of diseased lobsters, including: glucose, phosphorus, and triglycerides. © 2007 Elsevier B.V. All rights reserved.

Keywords: Histopathology; Tissue proliferation; Hemocyte; Blood chemistry; Virus; Crustacea

1. Introduction

The Caribbean spiny lobster *Panulirus argus* occurs throughout the Caribbean basin and Western Atlantic from Brazil to Bermuda (Holthuis, 1991) and supports valuable commercial and recreational fisheries throughout its range (Harper, 1995; FAO, 2001, 2004). 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 (Muller et al., 1997; FMRI, 2005). The recently identified pathogenic virus, *Panulirus argus* Virus 1 (PaV1), may have contributed to the decline (Shields and Behringer, 2004).

PaV1 primarily infects benthic juvenile lobsters (20–55 mm carapace length, CL), with prevalence decreasing rapidly in relation to increasing 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 (<1 m) through the water (Behringer, 2003; Butler et al., unpubl. data). However, 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. Herein, we report the pathology and hematology of spiny lobster over a time course of experimental infection by PaV1.

2. Materials and methods

2.1. Caribbean spiny lobster P. argus

Juvenile spiny lobsters, 25–50 mm carapace length (CL), were collected from the Florida Keys by hand using SCUBA.

^{*} Corresponding author. Tel.: +1 804 684 7128; fax: +1 804 684 7186. *E-mail address:* jeff@vims.edu (J.D. Shields).

^{0168-1702/\$ -} see front matter © 2007 Elsevier B.V. All rights reserved. doi:10.1016/j.virusres.2007.11.005

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 2 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).

2.2. 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 (TCID₅₀) from Li and Shields (2007). Briefly, diseased hemolymph was mixed with an equal volume of citrate/EDTA anticoagulant (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA; pH 5.4; Söderhäll and Smith, 1983) and homogenized with a glass homogenizer (Pyrex[®], Corning Inc.) at 4 °C for 5 min. The homogenate was then centrifuged at $3000 \times g$ 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) to 1:1, 1:10, 1:100 and 1:1000 and used as inocula. Aliquots of 100 µl of inocula were inoculated separately into 1-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 medium at 2days intervals. The inoculated primary cultures were examined daily with an inverted microscope, and images were taken for assessment of cytopathic effects (CPE). TCID₅₀ was calculated at 96 h based on the end-point dilution assay of Reed and Muench (1938) as described in Li and Shields (2007).

2.3. 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 time course of 15 days. **Trial II** was a long-term infection study, where lobsters were dosed with the virus, then necropsied at 10day intervals over almost 80 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 TCID₅₀/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, and 15 post-inoculation (p.i.) five animals from the virus inoculated group and three animals from control group were randomly selected and processed for disease 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 TCID₅₀/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 ~10-days 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.

2.4. 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 h, rinsed with tap water for 45 min, then held in 70% EtOH, processed through paraffin 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) (Li et al., 2006).

2.5. 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. Aliquots of 1 ml hemolymph of each animal were stored in ultracold freezer ($-80 \,^{\circ}$ C) for biochemical analysis. Frozen hemolymph samples were thawed on ice, the lysate serum was collected using a pipette, and then centrifuged at $3000 \times g$ for 10 min at $4 \,^{\circ}$ 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), gammaglutamyl transpeptidase (GGT), calcium, sodium, potassium, and chloride were measured according to the manufacturer's manual. 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 an 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 (Li and Shields, 2007). Differences in biochemical and hematological constituents between control and experimental groups were examined statistically with SYSTAT (SYSTAT Software Inc.). Multivariate ANOVA was used to examine differences in 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 examined for parametric assumptions and were tested in both the raw and log-transformed state.

3. Results

3.1. 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, 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 $400 \times : 0$, no cells with observable pathology; (1) <10 infected cells/section; (2) 10–100 infected cells/section; (3) >100 infected cells/section.

In Trial I, only one lobster was lightly infected and another moderately infected by 15 days 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. The pathological changes in the tissues of lobsters with light or moderate infections were similar in both trials, but more animals were confirmed to be infected in Trial II. 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 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.

3.2. Progression of disease in experimental 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 were the primary cells 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. 1). Activated fixed phagocytes were significantly enlarged, with highly vacuolated cytoplasm and sparse granules (Fig. 1B). As infection progressed, fixed phagocytes were obviously infected by the virus (Fig. 1C). In the hepatopancreas of the lobster moderately infected by PaV1, the typical rosette structure of the fixed phagocytes around the arterioles was no longer discernible (Fig. 1D). 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. 1E). However, no infected cells were observed in the antennal gland.

Total hemocyte counts (THCs) varied over time and between treatments (Fig. 2). Infected lobsters had significantly lower hemocyte counts than uninfected lobsters (2-way ANOVA, df = 1, 29, p < 0.0005). However, there was a highly significant downward trend in the total hemocyte counts in both treatments over time that probably resulted from the stress of handling and the inoculation (2-way ANOVA, df = 5, 29, p < 0.002). Further, there was a nearly significant statistical interaction between treatment and sample day, partly because the infected ani-

Table 1

Categorization of the severity PaV1 disease in the Caribbean spiny lobster Panulirus argus

Category 0 Healthy	 No aberrant cells with hypertrophied nuclei, no peripheral chromatin or 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 (10–100 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

C. Li et al. / Virus Research 132 (2008) 104–113



Fig. 1. Pathological changes in the tissues of PaV1-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.

mals had significantly lower hemocyte counts over time than did the uninfected animals, and partly because of the fluctuation in hemocyte counts on days 1 and 3 (2-way ANOVA, df = 5, 29, p < 0.051). Nonetheless, infected animals had significantly lower hemocyte counts after day 7 when compared to the controls. Relative changes among types of hemocytes were not significantly different between treatments (data not shown). Therefore, the absolute changes in THC were consistent among 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. 1F).

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

Day 10 p.i.: Infected cells were observed in hepatopancreas, gill, heart, central nerve tissue and hindgut; they were not observed in the cuticular epidermis and compound eyes (Table 2). The hepatopancreas of lightly infected animals maintained its normal architecture (Fig. 3B). Many fixed phagocytes were infected, although the architecture of the supporting spongy connective tissue was unchanged (Fig. 1C). Reserve inclusion (RI) cells were abundant in lobsters in the intermolt stage. No overt pathological changes were observed in other tissues in the



Fig. 2. Total hemocyte counts (THC) of lobsters from control and inoculation group over early time course of infection with PaV1 (Trial I). The number of cells was log transformed. Sample size was three lobsters in control and five in experimental groups, bars = standard deviation.

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 ($10 \sim 100$ /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, hemal sinuses within the hepatopancreas became filled with infected cells, including fixed phagocytes, hyalinocytes and semigranulocytes (Fig. 3C). 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. 1D).

Day 30 p.i.: The hepatopancreas, gill and central nerve tissue of diseased lobsters were heavily infected. The spongy connective tissue in the myocardium of heart, hindgut and cuticular epidermis tissues were moderately infected. Spongy connective tissues had begun to aggregate around the tubules of the hepatopancreas in one heavily diseased lobster (Fig. 3D). 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: Abnormal cellular aggregates of spongy connective tissues were observed within the hepatopancreas and hindgut (Fig. 3E, Fig. 4A). However, the fibrous connective tissues showed no histological signs of viral infection (Fig. 4B). The spongy connective tissues around the nerves were heavily infected (Fig. 4C). In the hearts of heavily diseased animals, cellular aggregates of the spongy connective tissues were observed around the supportive, outer portion of the arterioles (Fig. 4D). In lobsters chronically (>60 days) infected by PaV1, the hemal spaces among the tubules of the hepatopancreas were greatly atrophied and filled with large numbers of infected connective tissue cells (Fig. 3E,F). As the disease progressed, RI cells were gradually depleted in the hepatopancreas of chronically infected lobsters.

3.4. Hemolymph serum chemistry of experimental animals

Values of the specific serum constituents were analyzed by MANOVA. As dependent variables, the hemolymph parameters showed a nearly significant trend with infection status (MANOVA, Wilks' lambda = 0.323, $F_{28,60} = 1.629$, p = 0.057). However, three hemolymph parameters varied with infection status when analyzed separately. These were glucose (univariate $F_{2,43} = 7.911$, p = 0.001) and triglycerides (univariate $F_{2,43} = 3.409$, p = 0.042), with a nearly significant trend in phosphorus (univariate $F_{2,43} = 3.061$, p = 0.057). Two canonical variables were produced in the canonical discriminant analysis of infection status and hemolymph parameters; the first variable had a higher correlation with infection status (r=0.743) than did the second variable (r = 0.529). Four hemolymph variables (glucose, phosphorus, AST and triglycerides) were significantly correlated with the two canonical variables. Glucose (0.546)had a large positive loading with the first canonical variable, whereas the other variables were small and negative (<-0.108), indicating that changes in glucose contributed most to the overall effect, and that the other variables had weak negative correlations with the first canonical variable. Triglycerides (0.609), phosphorus (0.499) and AST (0.371) had large positive loadings with the second canonical variable, but glucose (0.052) contributed little to it, indicating that their changes were in the same direction with respect to the second canonical variable.

Table 2

Sequential progression of PaV1 in the tissues of the spiny lobsters over the time course of experimental infection in Trial II

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

(Category of infection in each tissue = mean \pm std., n = 5 per time period).

C. Li et al. / Virus Research 132 (2008) 104–113



Fig. 3. 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. Note the proliferation of cells in the hemal sinus (HS) among the tubules of the hepatopancreas in moderately and heavily diseased animals. L: lumen of tubule, RI: reserve inclusion cell, A: arteriole, SCT: spongy connective tissues.

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 in each trial.

The changes in tissue constituents showed two 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. AST, ALKP, and serum protein had similar trends as phospho-

rus with severity; however, no significant changes were observed due to the high variances, even when log transformed. No significant differences were observed among other biochemical constituents (Table 3).

4. Discussion

PaV1 has an apparent early predilection for fixed phagocytes in the hepatopancreas of spiny lobsters. Along with circulating hemocytes, the fixed phagocytes in the hepatopancreas of decapod crustaceans play an important role in the cellular defenses by removing foreign materials from the hemolymph (Johnson, 1980, 1987; Factor, 1995; Factor et al., 2005). Fixed phagoC. Li et al. / Virus Research 132 (2008) 104-113



Fig. 4. Pathological changes in the hindgut (A), gill (B), spongy connective tissues around nerve tissues (C) and heart (D, E). Uninfected heart (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.

cytes 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 bi-facies virus (BFV) in the blue crab *Callinectes sapidus* (Johnson, 1980), where virions aggregate around the degenerated cytoplasm of infected fixed phagocytes, and are enclosed by the interrupted layer, a basal lamina surrounding fixed phagocytes (Johnson, 1980). Larger viruses are apparently recognized by 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 become infected. Podocytes in the gills are also 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).

Pathology in 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 massive amount of cellular aggregates including infected circulating hemocytes and spongy connective tissue cells. In heavy infections, the hepatopancreatic tubules were significantly altered, atrophying, and the hemal sinuses became filled with cellular aggregates. Atrophy of the hepatopancreas

C. Li et al. / Virus Research 132 (2008) 104-113

r · · · · · · · · · · · · · · · · · · ·									
Variable	Healthy $(n = 13)$	Light and moderate $(n = 11)$	Heavily infected $(n = 24)$	F	р				
Glucose (mg/dL)	$30.0 \pm 6.3^{a^{***}}$	15.2 ± 2.5^{b}	13.0 ± 1.9^{b}	7.91	0.001				
Creatinine (mg/dL)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.27	0.762				
Phosphorus (mg/dL)	$1.7 \pm 0.2^{\mathrm{a}}$	1.6 ± 0.2^{a}	$2.2 \pm 0.2^{b^*}$	3.06	0.057				
Calcium (mg/dL)	46.8 ± 1.3	47.3 ± 1.8	48.1 ± 1.0	0.09	0.911				
ALT (U/L)	1.5 ± 0.4	1.1 ± 0.4	1.5 ± 0.2	0.88	0.422				
AST (U/L)	26.8 ± 4.7	25.0 ± 6.2	38.0 ± 5.0	1.46	0.244				
ALKP (U/L)	383.2 ± 36.7	434.6 ± 66.1	479.1 ± 49.2	0.62	0.541				
GGT (U/L)	0.3 ± 0.2	0.4 ± 0.2	0.8 ± 0.3	0.64	0.534				
Triglycerides (mg/dL)	$2.9 \pm 0.6^{a^{**}}$	$1.5 \pm 0.6^{b^{**}}$	$4.5 \pm 0.8^{c^{**}}$	3.41	0.042				
Sodium (mEg/L)	474.6 ± 10.3	479.1 ± 8.4	473.1 ± 6.6	0.12	0.885				
Potassium (mEg/L)	12.1 ± 0.6	10.8 ± 0.6	11.6 ± 0.4	1.89	0.163				
Chloride (mEg/L)	459.2 ± 9.7	471.8 ± 8.4	463.3 ± 7.5	0.41	0.668				
Lipase (U/L)	25.7 ± 16.5	7.8 ± 0.8	27.6 ± 7.8	0.99	0.380				
Refractometer (g/dL)	6.1 ± 0.6	5.2 ± 0.6	6.0 ± 0.4	0.83	0.445				
Protein (g/dL)	4.0 ± 0.5	3.4 ± 0.5	4.0 ± 0.3	ND	ND				

Table 3 Biochemical analysis of lobster serum compared with disease category infected with PaV1 (Each value is a mean \pm S.E.)

Data were analysed using MANOVA with significance limits of univariate ANOVAs at 0.01 (***), 0.05 (**) or 0.10 (*). Values followed by different letter (a, b or c) were significantly different. ND: not determined.

F = the F statistic for the univariate ANOVA of health status on each variable, p = probability for the univariate ANOVA.

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 appear to be comprised of spongy connective tissues and not infiltrates of hemocytes. Farley et al. (1972) and Farley (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 might 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-cell malignancies in humans (Theate et al., 2002; Snow and Martinez, 2007). The cellular aggregates observed in PaV1 infections may be proliferative in nature, but that was not determined in this study.

PaV1 infects spongy connective tissue cells, fixed phagocytes, and circulating hyalinocytes and semigranulocytes, but not granulocytes (Shields and Behringer, 2004), or fibrous connective tissue. These tissues are all developmentally derived 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 and other tissues (Johnson, 1983; Brock and Lightner, 1990). For example, White spot syndrome virus (WSSV) infects hemocytes and other tissues originating from both mesodermal and ectodermal germ layers (Chang et al., 1996; Wongteerasupaya et al., 1996; Lo et al., 1997). Specifically, WSSV infects semigranulocytes and granulocytes, but not hyalinocytes (Wang et al., 2002). The BFV from the blue crab C. sapidus (Johnson, 1976, 1988) causes similar pathological changes as PaV1. However, no aggregation of spongy connective tissues occurs in BFV infections.

In the later stages of exposure to PaV1, total hemocyte density decreased; however, the proportion of each type of cell in the hemolymph did not change. This 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 was observed in only one infected animal. The circulating hemocytes of crustaceans play a key role in the host defense system against invasion of non-self particles (Söderhäll and Smith, 1983; Ratcliffe et al., 1985; Söderhäll et al., 1986; Söderhäll and Cerenius, 1992; Bachère et al., 1995; Roch, 1999; Jiravanichpaisal et al., 2006). However, the densities of circulating hemocytes vary upon challenge by different microorganisms. Taura syndrome virus (TSV) causes significant decrease in THCs, with relative decreases in the hyalinocytes and granulocytes of infected Pacific white shrimp Penaeus vannamei¹ (Song et al., 2003). WSSV infects the semigranulocytes and granulocytes of Penaeus indicus and causes significant decreases in THCs (Yoganandhan et al., 2003). However, WSSV does not cause a decline in THCs in infected freshwater crayfish Pacifastacus leniusculus, even though the semigranulocytes and granulocytes are 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 could 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 (Wu et al., 2002; Song et al., 2003; Yoganandhan et al., 2003; Battison, 2006; Mohankumar

¹ Here we follow the revision of *Penaeus* proposed by Flegel (2007).

and Ramasamy, 2006a,b). Glucose and total carbonhydrates in the hemolymph of penaeid shrimp Penaeus indicus increased significantly in WSSV infections (Yoganandhan et al., 2003). Similarly, activities of transaminases (ALT and AST) increased in the hemolymph, hepatopancreas, gills and muscles of P. indicus infected with WSSV (Mohankumar and Ramasamy, 2006a). In lobsters infected with PaV1, concentrations of glucose and triglycerides in the hemolymph differed from that of control lobsters, and phosphorus showed a potential change with infection. These constituents are involved in the shortand intermediate-term energy reserves of the lobster and their decline indicates the depletion of reserve inclusions (RIs) in RI cells. RI cells contain polysaccharides such as glycogen and proteins such as hemocyanin (Johnson, 1980). The depletion of this cell type 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, albeit AST showed some correlation to infection. These enzymes are important markers of liver function in vertebrates, and they may have 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 viral infection 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 (Shields and Behringer, 2004). Considering the catastrophic impact of viral diseases in penaeid shrimp (Inouye et al., 1994; Cai et al., 1995; Lo et al., 1996; Wongteerasupaya et al., 1996; Wang et al., 1998; Lightner, 1999), 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.

Acknowledgements

This work was supported by NOAA, Saltonstall-Kennedy Program Grant No. NA17FD2366 and NSF Biological Oceanography Program Grants # OCE-0136894 and OCE-0452805. We thank Don Behringer, Butler's ODU field crew, and the staff of KML for help with sample collection. Kersten Wheeler provided skilled histological support. This is VIMS Contribution No. 2860.

References

- Battison, A., 2006. Tissue distribution and hemolymph activity of six enzymes in the American lobster (*Homarus americanus*): potential markers of tissue injury. J. Shellfish Res. 25, 553–560.
- Behringer, D.C. Jr., 2003. The ecological ramifications of disease and density on juvenile spiny lobster, *Panulirus argus*. Ph.D. Dissertation, Old Dominion University.
- Behringer, D.C., Butler, M.J., Shields, J.D., 2006. Avoidance of disease by social lobsters. Nature 441, 421.
- Brock, J.A., Lightner, D.V., 1990. Diseases of Crustacea: diseases caused by microorganisms. In: Kinne, O. (Ed.), Diseases of Marine Animals, vol. III. Biologische Anstalt Helgoland, Hamburg, pp. 245–325.
- Cai, S., Huang, J., Wang, C., Song, X., Sun, X., Yu, J., Zhang, Y., Yang, C., 1995. Epidemiological studies on the explosive epidemic disease of prawn in 1993–94. J. Fish. China 19, 112–117.
- Chang, P.S., Lo, C.F., Wang, Y.C., Kou, G.H., 1996. Identification of white spot syndrome associated baculovirus (WSBV) target organs in the shrimp *Penaeus monodon* by in situ hybridization. Dis. Aquat. Org. 27, 131–139.
- Factor, J.R., 1995. Biology of the Lobster *Homarus americanus*. Academic Press Inc., San Diego, CA, USA.
- Factor, J.R., Orban, K., Szarowski, D.H., Lin, G., Larocca, T., Becker, A., Kapusta, K.J., 2005. A method for assessing removal of foreign particles from the blood by fixed phagocytes of the American lobster, *Homarus americanus*. J. Shellfish Res. 24, 713–717.
- FAO, 2001. Report of the Workshop on Management of the Caribbean Spiny Lobster (Panulirus argus) Fisheries in the Area of the Western Central Atlantic Fishery Commission. Merida, Mexico, 4–8 September 2000. FAO Fisheries Report. No. 643. Rome, pp. 66.
- FAO, 2004. Fisheries Global Information System, Factsheet for *Panulirus argus*. Website: http://www.fao.org/figis/servlet/species?fid=3445.
- Farley, C.A., 1978. Viruses and viruslike lesions in marine mollusks. Mar. Fish. Rev. 40, 18–20.
- Farley, C.A., Banfield, W.G., Kasnic, G., Foster, W.S., 1972. Oyster herpes-type virus. Science 178, 759–760.
- Flegel, T.W., 2007. The right to refuse revision in the genus *Penaeus*. Aquaculture 264, 2–8.
- FMRI, 2005. A Summary of Recreational Spiny Lobster Landings and Effort in Florida. http://marineeconomics.noaa.gov/SocmonFK/ FMRI_Lobster_Fact.pdf.
- Harper, D. E., 1995. The 1995 spiny lobster update of trends in landings, CPUE, and size of harvested lobster. National Marine Fishery Service Report No. MIA-94/95-47, Miami, Florida.
- Hejkal, T.W., Gerba, C.P., 1981. Uptake and survival of enteric viruses in blue crab, Callinectes sapidus. Appl. Environ. Microbiol. 41, 207–211.
- Holthuis, L.B., 1991. Marine lobsters of the world. An annotated and illustrated catalogue of species of interest to fisheries known to date. FAO Fisheries Synopsis, vol. 125, no. 13. FAO, Rome, p. 292.
- Humason, G.L., 1979. Animal Tissue Techniques, fourth ed. W.H. Freeman and Company, San Francisco.
- Inouye, K., Miwa, S., Oseko, N., Nakano, H., Kimura, T., 1994. Mass mortalities of cultured kuruma shrimp *Penaeus japonicus* in Japan in 1993: electron microscopic evidence of the causative virus. Fish Pathol. 29, 149–158.
- Jiravanichpaisal, P., Bangyeekhun, E., Söderhäll, K., Söderhäll, I., 2001. Experimental infection of white spot syndrome virus in freshwater crayfish *Pacifastacus leniusculus*. Dis. Aquat. Org. 47, 151–157.
- Jiravanichpaisal, P., Sricharoen, S., Söderhäll, I., Söderhäll, K., 2006. White spot syndrome virus (WSSV) interaction with crayfish haemocytes. Fish Shellfish Immunol. 20, 718–727.
- Johnson, P.T., 1976. A herpes-like virus from the blue crab, *Callinectes sapidus*. J. Invertebr. Path. 27, 419–420.
- Johnson, P.T., 1980. Histology of the Blue Crab, *Callinectes sapidus*. Praeger Publishers, New York, USA.
- Johnson, P.T., 1983. Diseases caused by viruses, rickttsiae, bacteria and fungi. In: Provenzano, A.J. (Ed.), The Biology of Crustacean, Vol 6. Academic Press, New York, pp. 1–78.
- Johnson, P.T., 1987. A review of fixed phagocytic and pinocytotic cells of decapod crustaceans, with remarks on hemocytes. Dev. Comp. Immunol. 11, 679–704.

Bachère, E., Mialhe, E., Rodriguez, J., 1995. Identification of defense effectors in hemolymph of crustaceans with particular reference to the shrimp *Penaeus japonicus* (Bate): prospectus and applications. Fish Shellfish Immunol. 5, 597–612.

- Johnson, P.T., 1988. Development and morphology of an unusual nuclear virus of the blue crab, *Callinectes sapidus*. Dis. Aquat. Org. 4, 67–75.
- 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). J. Invertebr. Pathol. 94, 48–55.
- 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. Dis. Aquat. Org. 72, 185–192.
- Lightner, D.V., 1999. The penaeid shrimp viruses TSV, IHHNV, WSSV and YHV: Current status in the Americas, available diagnostic methods, and management strategies. J. Appl. Aquac. 9, 27–52.
- Lo, C.F., Ho, C.H., Peng, S.E., 1996. White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. Dis. Aquat. Org. 27, 215–225.
- Lo, C.F., Ho, C.H., Chen, C.H., Liu, K.F., Chiu, Y.L., Yeh, P.Y., Peng, S.E., Hsu, H.C., Liu, H.C., Chang, C.F., Wang, C.H., Kou, G.H., 1997. Detection and tissue tropism of white spot syndrome baculovirus (WSBV) in cultured brooders of *Penaeus monodon* with a special emphasis on reproductive organs. Dis. Aquat. Org. 30, 53–72.
- Mohankumar, K., Ramasamy, P., 2006a. Activities of membrane bound phosphates, transaminases and mitochondrial enzymes in white spot syndrome virus infected tissues of *Fenneropenaeus indicus*. Virus. Res. 118, 130–135.
- Mohankumar, K., Ramasamy, P., 2006b. White spot syndrome virus infections decrease the activity of antioxidant enzymes in *Fenneropenaeus indicus*. Virus. Res. 115, 69–75.
- Muller, R.G., Hunt, J.H., Matthews, T.R., Sharp, W.C., 1997. Evaluation of effort reduction in the Florida Keys spiny lobster, *Panulirus argus*, fishery using an age-structured population analysis. Mar. Freshw. Res. 48, 1045–1058.
- Persson, M., Cerenius, L., Söderhäll, K., 1987. The influence of the hemocyte number on the resistance of freshwater crayfish, Pacifastacus leniusculus to parasitic fungus *Aphanomyces astaci*. J. Fish Dis. 10, 471–477.
- Ratcliffe, N.A., Rowley, A.F., Fitzgeral, S.W., 1985. Invertebrate immunity: basic concepts and recent advances. Int. Rev. Cytol. 97, 183–349.
- Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent end points. Am. J. Hyg. 27, 493–497.
- Roch, P., 1999. Defense mechanisms and disease prevention in farmed marine invertebrates. Aquaculture 172, 125–145.
- Shields, J.D., Behringer, D.C., 2004. A pathogenic herpes-like virus in the Caribbean spiny lobster, *Panulirus argus*, from the Florida Key. Dis. Aquat. Org. 59, 109–118.

- Snow, A.L., Martinez, O.M., 2007. Epstein-Barr virus: evasive maneuvers in the development of PTLD. Am. J. Transplant. 7, 271–277.
- Söderhäll, K., Cerenius, L., 1992. Crustacean immunity. Annu. Rev. Fish. Dis. 2, 3–23.
- Söderhäll, K., Smith, V.J., 1983. Separation of the haemocyte populations of *Carcinus meanas* and other marine decapods, and prophenoloxidase distribution. Dev. Comp. Immunol. 7, 229–239.
- Söderhäll, K., Smith, V.J., Johansson, M.W., 1986. Exocytosis and uptake of bacteria by isolated haemocyte populations of crustaceans: evidence for cellular co-operation in the defence reactions of arthropods. Cell Tissue Res. 245, 43–49.
- Song, Y.L., Yu, C.I., Lien, T.W., Huang, C.C., Lin, M.N., 2003. Hemolymph parameters of Pacific white shrimp (*Litopenaeus vannamei*) infected with Taura syndrome virus. Fish Shellfish Immunol. 14, 317–331.
- Stentiford, G.D., Shields, J.D., 2005. A review of the parasitic dinoflagellates *Hematodinium* species and *Hematodinium*-like infections in marine crustaceans. Dis. Aquat. Org. 66, 47–70.
- Theate, I., Michaux, L., Dardenne, S., Guiot, Y., Brière, J., Emile, F.J., Fabiani, B., Detry, R., Gaulard, P., 2002. Epstein-Barr virus-associated lymphoproliferative disease occurring in a patient with sarcoidosis treated by methotrexate and methylprednisolone. Eur. J. Haematol. 69 (Suppl. 4), 248–253.
- Wang, Y.C., Lo, C.F., Chang, P.S., Kou, G.H., 1998. Experimental infection of white spot baculovirus in some cultured and wild decapods in Taiwan. Aquaculture 164, 221–231.
- Wang, T.T., Liu, W., Seah, J.N., Lam, C.S., Xiang, J.H., Korzh, V., Kwang, J., 2002. White sport syndrome virus (WSSV) infects specific hemocytes of the shrimp *Penaeus merguiensis*. Dis. Aquat. Org. 52, 249–259.
- Wongteerasupaya, C., Wongwisansri, S., Boonsaeng, V., Payim, P., Nash, G.L., Withyachumnarnkul, B., Flegel, T.W., 1996. A non-occluded, system baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn, *Penaeus monodon*. Dis. Aquat. Org. 21, 69–77.
- Wu, J.L., Nishioka, T., Mori, K., Nishizawa, T., Muroga, K., 2002. A timecourse study on the resistance of *Penaeus japonicus* induced by artificial infection with white spot syndrome virus. Fish Shellfish Immunol. 13, 391–403.
- Yoganandhan, K., Thirupathi, S., Sahul Hameed, A.S., 2003. Biochemical, physiological and hematological changes in white spot syndrome virus-infected shrimp, *Penaeus indicus*. Aquaculture 221, 1–11.