Vol. 100: 113–124, 2012
doi: 10.3354/dao02497

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published August 27

Contribution to DAO Special 6 'Disease effects on lobster fisheries, ecology, and culture'

OPEN ACCESS

Physiological and immunological characterization of Caribbean spiny lobsters *Panulirus argus* naturally infected with *Panulirus argus* Virus 1 (PaV1)

Cristina Pascual Jiménez[1,*], Juan Pablo Huchin-Mian[2], Nuno Simões[1], Patricia Briones-Fourzán[3], Enrique Lozano-Álvarez[3], Ariadna Sánchez Arteaga[1], Juan Antonio Pérez-Vega[2], Raúl Simá-Álvarez[2], Carlos Rosas Vazquez[1], Rossanna Rodríguez-Canul[2]

[1]Universidad Nacional Autónoma de México, Facultad de Ciencias, Unidad Académica Sisal, Puerto de Abrigo Sisal S/N. Yucatán 97355, Mexico

[2]Laboratorio de Inmunología y Biología Molecular. Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Unidad Mérida, Km. 6 Antigua Carretera a Progreso, CORDEMEX, Mérida, Yucatán 97310, Mexico

[3]Universidad Nacional Autónoma de México, Instituto de Ciencias del Mar y Limnología, Unidad Académica de Sistemas Arrecifales Puerto Morelos. Prol. Av. Niños Héroes s/n, domicilio conocido, Puerto Morelos, Quintana Roo 77580, Mexico

ABSTRACT: The present study compares 13 physiological and immunological variables between a group of healthy *Panulirus argus* lobsters and a group of lobsters naturally infected with *Panulirus argus* Virus 1 (PaV1). Viral infection was determined through histopathology and PCR. Ten of the 13 variables differed significantly between the 2 groups. Using these variables, a principal component analysis yielded 2 separate clusters: one corresponding to the healthy group and the other corresponding to the infected group. In particular, infected lobsters exhibited significantly lower levels of osmotic pressure, total hemocyte counts, plasmatic proteins, and total phenoloxidase (PO) activity in plasma, as well as significantly higher levels of cholesterol and acylglycerides. These features are consistent with metabolic wasting, hyperlipidemia, and presumed immune suppression. Infection with PaV1 appears to increase the susceptibility of lobsters to some other opportunistic pathogens, as 61.1% of infected lobsters presented infestations of ciliate epibionts (*Epystilis* and *Zoothamniun*) in the gill chamber compared with 11.5% lobsters in the healthy group. Infected lobsters also showed significantly higher levels of total PO activity in degranulated hemocytes and trypsin inhibitor activity, potentially indicating activation of immune response by the PO system during the systemic infection with PaV1.

KEY WORDS: Phenoloxidase · Hemocyte · *Panulirus argus* · Immunology · Hemolymph component

INTRODUCTION

The Caribbean spiny lobster *Panulirus argus* (Latreille, 1804) supports valuable commercial fisheries through the wider Caribbean region (Holthuis 1991). Since the last decade, this species has been affected by a highly pathogenic virus known as *Panulirus argus* Virus 1 (PaV1) (Behringer et al. 2011). PaV1 was first detected in juveniles of *P. argus* from the Florida Keys (Shields & Behringer 2004), and then in juvenile lobsters from the Caribbean coast of Mexico (Huchin-Mian et al. 2008). Currently, PaV1 appears to be widespread throughout the Caribbean (Butler et al. 2008, Huchin-Mian et al. 2009, Cruz Quintana et al. 2011) and, given its high level of pathogenicity, this virus is considered a potential

*Email: pascual.cristina@gmail.com

threat to local lobster fisheries in the Caribbean region (Butler et al. 2008, Lozano-Álvarez et al. 2008, Briones-Fourzán et al. 2009, Shields 2011).

Field studies have shown that the prevalence of lobsters visibly infected with PaV1 tends to decline as lobster sizes increase, being higher in early benthic (15–25 mm carapace length [CL]) and large juveniles (25–45 mm CL) than in sub-adults (45–80 mm CL) and adults (>80 to <200 mm CL) (Shields & Behringer 2004, Butler et al. 2008, Lozano-Álvarez et al. 2008). Nevertheless, Huchin-Mian et al. (2009) detected PaV1 in frozen tails of subadult–adult lobsters from Belize, and Cruz Quintana et al. (2011) confirmed PaV1 infection in adult lobsters from Cuba by histology and PCR of hemolymph and tissues.

Macroscopic signs of PaV1 infection are a milky hemolymph that lacks the ability to clot, a reddish discoloration of the clear marks on the carapace, lethargy and, occasionally, fouling of carapace by epibionts indicating suppression of molt (Shields & Behringer 2004, Lozano-Álvarez et al. 2008). In particular, the change in aspect of the hemolymph, from clear or bluish to a milky-white color, is one of the major clinical signs of infection and is used for diagnosis of PaV1 (Shields & Behringer 2004, Behringer et al. 2011). In laboratory experiments, PaV1 was transmitted to healthy lobsters via inoculation, prolonged contact with infected lobsters, ingestion of infected tissue, and over short distances in the water (Butler et al. 2008). Observations of virus-infected hemocytes in the hemal sinuses of the ovary of female lobsters (Cruz Quintana et al. 2011) and the presence of PaV1-infected postlarvae of *Panulirus argus* in the Florida Keys (Moss et al. 2012) suggest the possibility of vertical transmission, but this has yet to be confirmed.

The primary sites of infection by PaV1 are the fixed phagocytes in the hepatopancreas and the hyalinocytes and semigranulocytes in the hemolymph, followed by proliferation of infective cells in the soft connective tissue around the hepatopancreatic tubules (Shields & Behringer 2004, Li et al. 2008). In heavily infected lobsters, viral particles can be found in the spongy connective tissue surrounding most organs, in the gills, heart and hindgut, the glial cells around the optic nerves, and the cuticular epidermis and foregut, with the hepatopancreas showing marked atrophy (Li et al. 2008). Experimental infection with PaV1, which can cause up to 100% of mortality in juvenile *Panulirus argus* (Shields & Behringer 2004), causes a significant decrease in total hemocyte counts and severe alterations of some constituents in the hemolymph, such as glucose, phosphate, and triglycerides (Li et al. 2008). Behringer et al. (2008) found that wild lobsters exhibiting macroscopic signs of PaV1 infection had a significantly lower concentration of hemolymph serum proteins, indicating poor nutritional condition.

The pathogenesis and prognosis of the PaV1 disease has been well addressed, primarily through experimental infections in *Panulirus argus*, which have also been important for the development of molecular techniques to detect PaV1 (Li et al. 2006, Montgomery-Fullerton et al. 2007). However, little is known about the host response during natural infections with PaV1. Therefore, the objective of this study was to compare some physiological and immunological variables between healthy lobsters and lobsters naturally infected with PaV1.

MATERIALS AND METHODS

Lobster sampling

Spiny lobsters were collected in June 2006 from the Puerto Morelos reef lagoon, located on the Caribbean coast of Mexico (20° 51′ N, 86° 53′ W). Over a 3 d period we searched, while on SCUBA, for lobsters exhibiting macroscopic signs of PaV1 infection (initially categorized as 'infected') and lobsters not exhibiting these signs (initially categorized as 'healthy'). In total, we collected 21 infected lobsters (17.9–50.3 mm CL) and 35 healthy lobsters (38.2–72.3 mm CL). It is important to clarify that this collection of lobsters does not reflect local macroscopic prevalence of PaV1 because the sampling was directed rather than random.

Healthy and infected lobsters were transported to the laboratory in separate containers with cooled, aerated seawater and then placed into 2 separate holding tanks (3 m in diameter, 80 cm in depth, with ~5.7 m^3 of seawater, salinity of 35 PSU) subjected to a flow-through seawater system with an exchange rate of 300% per day. Lobsters were held and kept unfed for 2 d prior to analyses. Before extracting hemolymph for laboratory analyses, lobsters were placed in aerated seawater 5°C lower than the temperature of the water of the holding tanks to reduce their metabolic activity and to minimize handling effects on hemolymph components (Rosas et al. 2007).

Hemolymph sampling

Hemolymph (350–1500 µl) was collected from the pericardial sinus using a chilled syringe needle in-

jected through the transparent membrane between the cephalothorax and the abdomen. The area of extraction was previously sterilized with 70% ethanol and dried with a paper towel. To avoid clotting, hemolymph samples were placed on parafilm over a flat freeze container (Pascual et al. 2006). Sub-samples were taken immediately to perform laboratory tests.

Plasma and degranulated hemocyte preparation

Hemolymph was diluted (1:3) with pre-chilled (8°C) anticoagulant according to Hernández-López et al. (2003), who titrated the appropriate concentration of NaCl to avoid lysis of hemocytes in *Panulirus argus* (350 mM NaCl, 10 mM KCl, 10 mM HEPES, 10 mM EDTA-Na_2, pH 7.3, 850 mOsm kg^{-1}). The sample was then centrifuged at 800 × *g* for 5 min at 4°C to separate the plasma, which was used to evaluate plasmatic metabolites, phenoloxidase (PO) activity, hemagglutination activity, and trypsin-inhibitory activity. The cellular pellet from each sample was washed with anticoagulant and centrifuged as described above. Then, the cellular pellet was re-suspended several times with cacodylate buffer (10 mM cacodilic acid, 10 mM CaCl, pH 7.0) in equal volume (hemolymph plus anticoagulant) and centrifuged at 16 000 × *g* for 5 min at 4°C. The supernatant was used to evaluate the PO activity from degranulated hemocytes.

Biochemical and physiological variables

Osmotic pressure. To measure the osmotic pressure, we placed 20 µl of hemolymph in a micro-osmometer (3MO Plus, Advanced Micro-osmometer). Results are expressed as mOsm kg^{-1} (Lignot et al. 1999).

Oxyhemocyanin concentration. The hemocyanin was measured by placing 20 µl of hemolymph diluted in 980 µl of distilled water in a 10-mm cuvette. The absorbance was measured at 335 nm (UV-SENSE; SLM AMINCO Mod DW). Hemocyanin concentration was calculated using an extinction coefficient of 17.26 calculated on the basis of the functional subunit of 74 kDa (Chen & Cheng 1993a,b).

Plasmatic metabolites. We used chromogenic kits adapted in an ELISA microplate format to detect the following metabolites: glucose (Bayer Sera pack Plus B 014509-01), acylglycerides (Bayer Sera pack Plus B 01451-01), and cholesterol (Advia B 01-4124-01). We adjusted the volume of plasma using 50 µl of plasma for glucose, 40 µl for acylglycerides, and 80 µl for cholesterol, and added 200 µl of the appropriate reagent to each sample. The determination of protein concentration was also performed in an ELISA microplate format, diluting 5 µl of plasma in 1000 µl of sterile water, and then taking 10 µl of this solution and mixing it with 200 µl of the commercial solution (Biorad Protein assay 500-0006) according to Bradford (1976). Bovine serum albumin was used as a standard. Absorbance values were recorded in an ELISA microplate reader (Biorad model 550) and the concentrations (mg ml^{-1}) were calculated using standard curves considering the dilution factor for each sample.

Immunological tests

To avoid immune system activation by endotoxins, all glassware was washed with Etoxa-clean prior to use and solutions were prepared using pyrogen-free water.

Hemocyte counts. A sample of 25 µl of hemolymph was diluted 1:2 with Alsever solution (115 mM glucose, 30 mM sodium citrate, 10 mM EDTA-Na_2, 338 mM NaCl) and 10% of formaldehyde (v/v) according to Le Moullac et al. (1998). Samples were kept at 2–8°C until further analyses. Hemocytes were counted in a Neubauer chamber. Samples from each lobster were analyzed in duplicate, yielding a minimum area count of 0.04 mm^3.

Hemagglutination activity. Human blood (type O+) was obtained from a local blood bank. Prior to use, the erythrocytes were washed 3 times with 0.9% saline solution, centrifuged at 380 × *g* at 25°C for 5 min, and then adjusted to a final volume of 2%. Hemagglutination activity in lobster plasma was assayed in microtiter U plates by a 2-fold serial dilution, i.e. 50 µl of plasma were diluted twice in saline solution and then mixed with an equal volume of erythrocytes solution. After 2 h of incubation at room temperature (26 ± 2°C), hemagglutination was observed. Results were expressed as the inverse of the last dilution showing visible agglutination activity.

Total PO activity. Total PO activity was measured by spectrophotometry in an ELISA microplate format to detect the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) (Ashida & Söderhall 1984, Hernández-López et al. 1996). The technique was adjusted for *Panulirus argus*. Briefly, 100 µl of plasma or degranulated hemocytes were incubated for 10 min at room temperature with 100 µl

of trypsin (1 mg ml^{-1}) to transform the proPO into PO. Then, 150 µl of L-DOPA (3 mg ml^{-1}; Sigma D9628) was added. Absorbance was measured at 490 nm for 30 s in an ELISA microplate reader (Biorad model 550). Results were expressed as the increment of 0.001 in optical density.

Trypsin inhibitory capacity. The technique of Le Moullac et al. (1998) to measure trypsin inhibitory capacity was adjusted for *Panulirus argus*. Briefly, 50 µl of bovine pancreatic trypsin (0.2 mg ml^{-1}; Sigma T 8003) in 0.1 M Tris buffer (pH 8) were incubated with 130 µl plasma for 10 min at 25°C. Then, 50 µl of a low-molecular weight substrate, *N*-benzoyl-D/L-arginine *p*-nitroanilide (54 mg ml^{-1}; Sigma B4875) was added. Every minute for 5 min, protease activity was measured by hydrolysis of the substrate at 405 nm. Trypsin inhibitory capacity was defined as the percentage of decrease in protease activity per minute. As a control, plasma was substituted with Tris buffer (pH 7.3), representing 0% of inhibition.

Determination of molt stage and hepatosomatic index

Immediately after hemolymph sampling, each lobster was measured (CL; mm) and weighed (total body mass; g), and its molt stage was determined by microscopic observation of the degree of setal development and epidermal retraction in the tip of one pleopod (Lyle & MacDonald 1983). The digestive gland was then excised and weighed to obtain the hepatosomatic index (mass of the digestive gland/body mass × 100).

Polymerase chain reaction

To test for the presence of PaV1 in lobsters, a PCR (first and second step) was performed according to Montgomery-Fullerton et al. (2007). A portion of hepatopancreas and gills was individually fixed in 70% ethanol. DNA was extracted from ~25 mg of hepatopancreas with the Wizard genomic DNA purification kit (Promega) following the manufacturer's protocol. Specific primers for PaV1 (45aF: TTC CAG CCC AGG TAC GTA TC; and 543aR: AAC AGA TTT TCC AGC AGC GT) that amplify a region of 499 bp (Montgomery-Fullerton et al. 2007). All PCR reactions were carried out in a total volume of 25 µl containing ~32.5 ng of DNA, 0.33 µM of each primer, 2.5 mM of $MgCl_2$, 1.2× reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100), 0.4 mM dNTPs mixture (Promega), and 2.5 U of *Taq* DNA polymerase (BioLabs). The PCR reactions were run on a thermal cycler (TECHNE TC-312). The cycling conditions were 94°C for 10 min, followed by 30 cycles of 94°C for 30 s, 63°C for 30 s, and 72°C for 1 min, with a final extension of 72°C for 10 min. PCR products were run in a 2% agarose gel electrophoresis with a 100-bp DNA ladder. Bands were visualized using 0.1% ethidium bromide stain on a UV documentation system (MiniBis Pro). In all cases, ultrapure water and tissue from non-infected lobsters were used as negative controls, and DNA from a heavily infected lobster was used as a positive control (Huchin-Mian et al. 2008, 2009). All PCR analyses were performed in triplicate.

Histology

To determine the severity of PaV1 infection, sample portions of gills, hepatopancreas, and muscle of 16 infected lobsters and 28 healthy lobsters were fixed in Davidson's AFA fixative (alcohol, formalin, and acetic acid), processed, embedded, sectioned (thickness: 5–6 µm), and stained with haematoxylin and eosin (H&E) or Pinkerton's stain following routine histological protocols (Bell & Lightner 1988). Sections were analyzed by standard light microscopy to determine histopathological changes. The degree of severity was determined according to Li et al. (2008) and Cruz-Quintana et al. (2011), from non-infected lobsters (grade 0) to heavily infected lobsters (grade 3). However, as there were few lightly and moderately infected lobsters in our samples, we grouped these lobsters into one category (grades 1–2; Table 1).

We examined the secondary branchial lamellae (right side) of healthy and PaV1-infected lobsters under a light microscope (40×) for the presence of epibionts and compared the proportion of lobsters harboring branchial epibionts between the 2 groups of lobsters with a 2-tailed Fisher's exact test. We further quantified the epibionts present in the interlamellar space of one gill filament (right side) from each lobster.

Statistical analysis

Only lobsters in inter-molt were used for the statistical analyses, which were performed in 2 steps. In the first step, we determined which variables differed significantly between healthy and infected lob-

Table 1. Grades of severity of *Panulirus argus* virus 1 (PaV1) infection in *Panulirus argus* lobsters from the Puerto Morelos reef lagoon, Mexico

Severity	PCR result and histopathological lesions	Lobsters (n)
Grade 0 Healthy (no infection)	PCR negative No aberrant cells with hypertrophied nuclei, no peripheral chromatin or eosinophilic inclusions Hepatopancreas and other tissues appear normal	26
Grades 1–2 Lightly to moderately infected	PCR positive Fixed phagocytes activated or infected Some infected cells present in hepatopancreas or other organs (1–100 per section) Moderate hemocytic infiltrate Moderate obstruction of hemal sinuses	15
Grade 3 Heavily infected	PCR positive Hepatopancreatic tubules atrophied Many infected cells present in spongy connective tissue around hepatopancreas and other organs (>100 per section) Presence of nodulations or granulomas	8

sters (i.e. those that exhibited macroscopic signs of infection and/or tested positive for PaV1 by histology and PCR) by subjecting each of the 13 variables to a Student's *t*-test, assuming unequal variances, or a normalized Mann-Whitney test. Given that 11 of the 13 variables were measured in the hemolymph of the same subjects, the α-level was adjusted to 0.005 to account for the experimentwise error rate. To compare each variable between healthy and infected lobsters, we used data from all individuals for which that variable was measured. Because of missing values for some variables, the sample size was not the same for each variable comparison.

In the second step, we performed a principal component analysis (PCA) to model the hematological data (Dove et al. 2005). We used only the 10 variables that differed significantly between the 2 groups of lobsters (see 'Results') to examine their interrelationships as well as their individual contribution to the description of the 2 health conditions (healthy vs. infected). Because the data of variables differed widely in scale and dispersion, each variable used in the PCA was standardized by subtracting the overall average from each observation and dividing by the standard deviation (Gotelli & Ellison 2004). Histological and PCR analyses revealed that 2 lobsters initially categorized as healthy (no macroscopic signs of PaV1 infection) were actually lightly infected, and these lobsters were re-categorized into the infected group (grade 1; Table 1) for PCA analysis. Of the 44 lobsters evaluated for the 10 selected variables for the PCA, 19 had missing values in 1 or 2 variables (yielding a total of 24 empty cells), representing 5.4% of the 440 cells in the data matrix. Three variables (hemagglutination activity, cholesterol, and acylglicerides) contained 70% of the missing values (a total of 17 empty cells), whereas 2 variables (plasmatic protein concentration and number of epibionts) had no missing values. Each missing value was replaced with the average value of the corresponding variable for either the healthy or infected groups (Table 1). All statistical analyses were performed using Minitab Statistical Software (ver. 15.1).

RESULTS

Histology and PCR

Of the 35 lobsters initially categorized as healthy, 7 were in pre-molt or post-molt stages and 28 were in inter-molt. In these 28 lobsters, histological observations of the hepatopancreas, gills, and spongy connective tissue exhibited the well-organized structure normally seen in *Panulirus argus* (Li et al. 2008). However, 2 of these lobsters tested positive by PCR to PaV1 and this was confirmed by DNA sequencing; therefore, they were reclassified as lightly infected (grade 1; Table 1). The remaining healthy lobsters were not positive for PaV1 by PCR.

In contrast, all lobsters exhibiting macroscopic signs of PaV1 infection showed amplification at the 499 bp region, and the DNA sequence confirmed the presence of PaV1 (Montgomery-Fullerton et al. 2007). However, in 5 of the 21 lobsters initially categorized as infected, the molt stage was not determined; therefore, data from those 5 lobsters were not included in the statistical analyses. Histopatho-

Table 2. Individual tests comparing 13 immunological and physiological variables between healthy and infected lobsters *Panulirus argus* (infected: testing positive for PaV1 by histology and PCR). The first 10 variables were compared with independent Student's *t*-tests, assuming unequal variances (hence the variable degrees of freedom), and the last 3 variables were compared with normalized Mann-Whitney tests adjusted for ties. The α-level was adjusted to 0.005. PO: phenoloxidase; OD_{490}: optical density at 490 nm; IQ: inter-quartile

Variable	Condition	n	Mean	SE	*t*	df	p
Hemocytes (cells mm^{-3})	Healthy	26	14018	1117	5.29	36	<0.0005
	Infected	23	7141	664			
PO in degranulated hemocytes (OD_{490})	Healthy	26	0.317	0.020	–3.23	20	0.0021
	Infected	16	0.484	0.048			
Trypsin inhibitory activity (%)	Healthy	26	59.3	3.2	3.60	38	0.0004
	Infected	16	74.9	2.9			
Hemocyanin (mM)	Healthy	26	0.675	0.048	–0.29	31	0.3869
	Infected	16	0.698	0.062			
Osmotic pressure (mOsm kg^{-1})	Healthy	26	1212	4	3.17	37	0.0015
	Infected	16	1194	4			
Proteins (mg ml^{-1})	Healthy	26	73.7	9.5	2.85	39	0.0034
	Infected	16	37.2	8.5			
Glucose (mg ml^{-1})	Healthy	26	1.49	0.16	–0.96	27	0.1728
	Infected	16	1.78	0.25			
Acylglycerides (mg ml^{-1})	Healthy	26	0.560	0.061	–6.20	16	<0.0005
	Infected	16	2.620	0.330			
Cholesterol (mg ml^{-1})	Healthy	26	0.182	0.024	–4.57	15	<0.0005
	Infected	16	2.230	0.450			
Hepatosomatic index	Healthy	26	2.784	0.12	1.31	47	0.0982
	Infected	23	3.071	0.16			
			Median	IQ range	*Z*		
Branchial epibionts (N)	Healthy	26	0	0–0	–3.452		0.0006
	Infected	18	2.5	0–7.8			
Plasmatic PO (OD_{490})	Healthy	26	1.191	1.144–1.278	3.986		<0.0005
	Infected	18	0.923	0.130–1.079			
Hemagglutination (titer)	Healthy	26	16	16–32	4.953		<0.0005
	Infected	18	2	2–4			

logical analyses performed in 18 infected lobsters (including the 2 recategorized as infected) showed that 10 of these lobsters were lightly to moderately infected (grades 1–2), and 8 were heavily infected (grade 3; see Table 1). 61.1% of diseased lobsters (11/18) presented infestations of stalked ciliates (*Epistylis* sp.) on their secondary branchial lamellae, compared with 11.5% (3/26) of healthy lobsters. These percentages were significantly different (Fisher's exact test, $p < 0.001$).

Physiological and immunological condition

Healthy lobsters showed significantly higher levels (all $p < 0.005$; Table 2) of 5 of the 13 physiological and immunological variables: total hemocyte counts (Fig. 1B), total PO activity in plasma (Fig. 1D), hemagglutination activity (Fig. 1E), osmotic pressure (Fig. 1H), and plasmatic protein concentration (Fig. 1I, Table 2). Infected lobsters showed significantly higher levels of 5 other variables (Table 2): number of branchial epibionts in the interlamellar space of the gill filament (Fig. 1A), total phenoloxidase activity from degranulated hemocytes (Fig. 1C), trypsin inhibitory activity (Fig. 1F), acylglycerides (Fig. 1K) and cholesterol concentration (Fig. 1L). The 3 remaining variables—glucose concentration (Fig. 1J), hemocyanin concentration (Fig. 1G), and hepatosomatic index (not shown)—did not differ significantly between healthy and infected lobsters (Table 2).

Multivariate analysis

The PCA using the 10 physiological and immunological variables that differed between healthy and infected lobsters yielded 2 separate clusters along the first component axis, one corresponding to the healthy group and the other corresponding to the infected group, with both clusters showing a greater dispersion along the second component axis (Fig. 2).

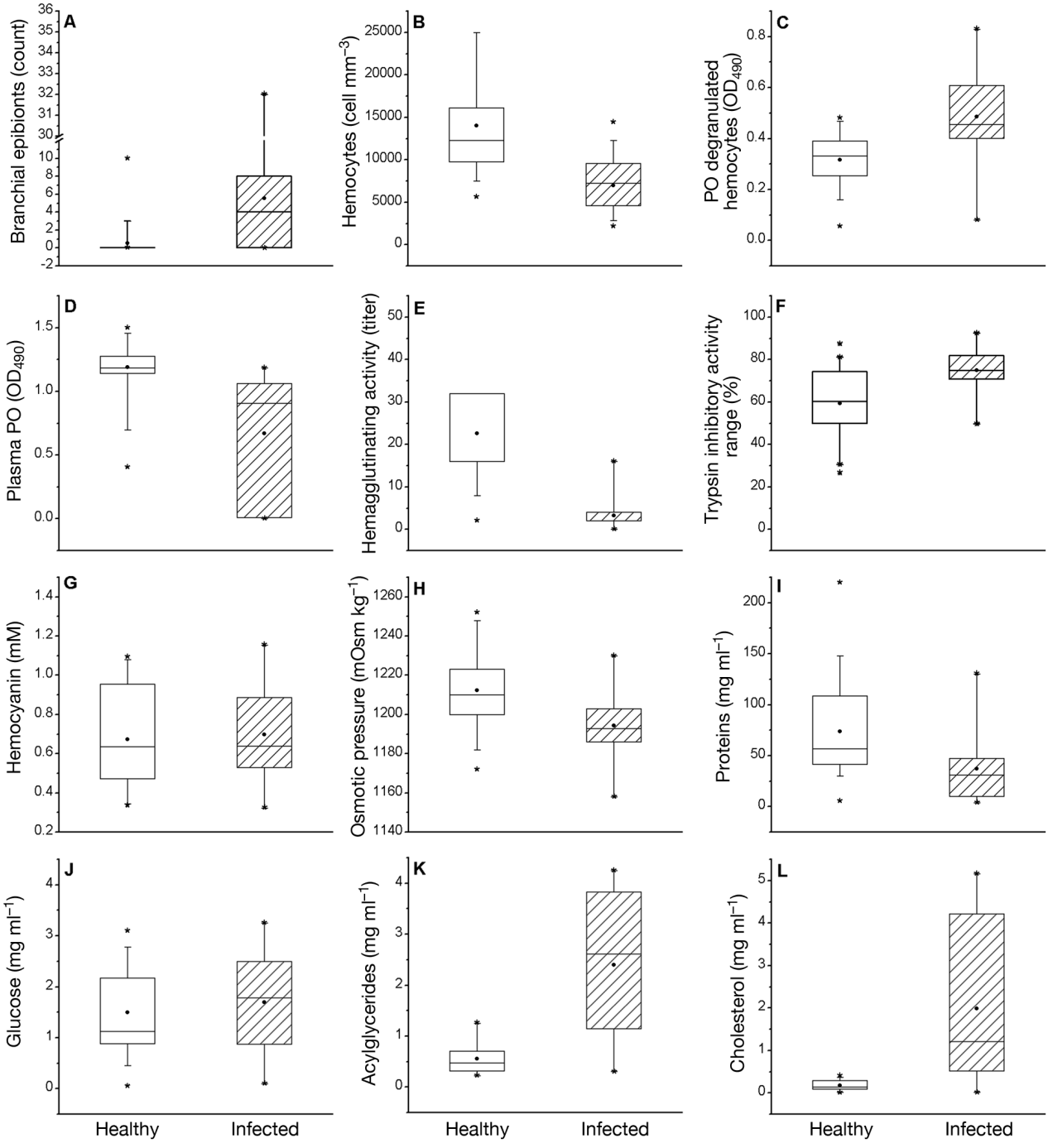

Fig. 1. *Panulirus argus*. Immunological and physiological variables from healthy and infected lobsters (i.e. lobsters that tested positive for PaV1 by histology and PCR). Horizontal line: median; box: 25th and 75th percentiles; whiskers: 5th and 95th percentiles; black dots: arithmetic mean; stars: outliers. PO: phenoloxidase; OD_{490}: optical density at 490 nm

The separation of the 2 clusters across the first component axis was mainly due to 2 groups of variables (Fig. 3). One group consisted of the 5 variables that showed higher values in infected lobsters (total PO activity from degranulated hemocytes, trypsin inhibitory activity, cholesterols, acylglycerides, and counts of branchial epibionts). The other group consisted of the 5 variables that showed higher values in healthy lobsters (osmotic pressure, hemocyte counts, total phenoloxidase activity in plasma, hemagglutination activity, and protein concentration; Fig. 3). The immunological

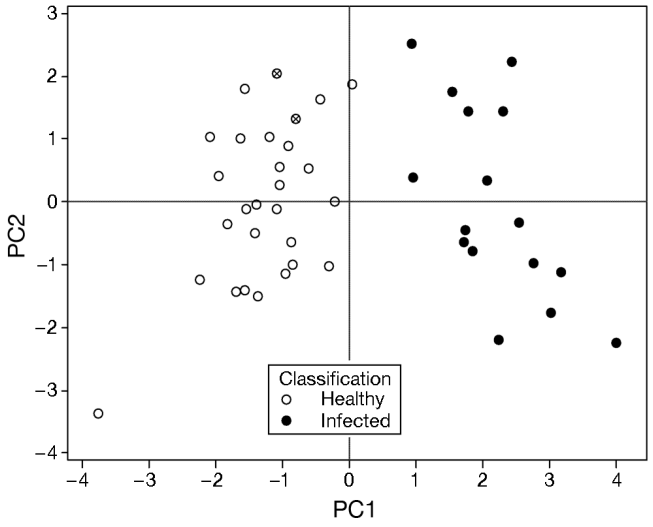

Fig. 2. Principal components analysis based on 10 physiological and immunological variables from *Panulirus argus* diagnosed as healthy (○) and infected with PaV1 (●) through initial observation and later confirmed with histopathology and PCR. Two lobsters initially classified as healthy (⊗) were reclassified as infected after histological and PCR analyses confirmed the presence of PaV1 in these lobsters

response variables that differed more markedly between infected and healthy lobsters (i.e. PO, trypsin inhibitory activity, hemagglutination activity, and hemocyte count; see Fig. 1) contributed more to the separation of the clusters. The physiological variables, particularly the concentration of plasmatic lipids (cholesterol and acylglycaerides), reinforced this separation. In contrast, the separation

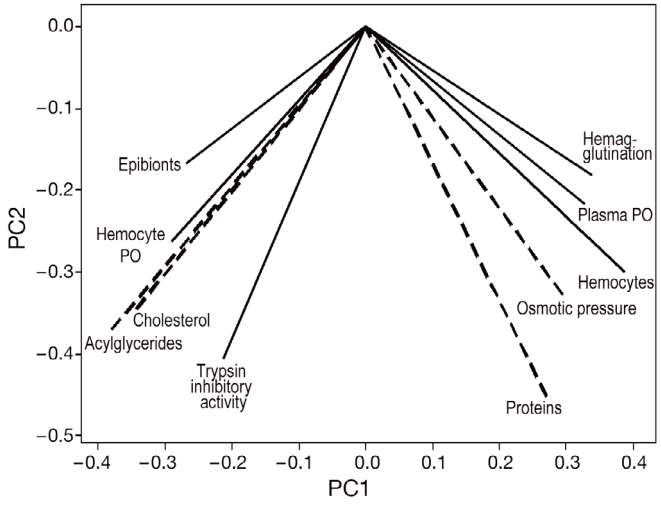

Fig. 3. Principal component analysis with immunological and physiological variables from lobsters *Panulirus argus* diagnosed as healthy and infected with PaV1. Solid lines represent physiological variables, and dashed lines represent immunological variables. PO: phenoloxidase

along the second component axis could not be assigned to a particular group of variables. However, the vertical dispersion pattern appeared to be mainly driven by the physiological variables (plasmatic lipids, proteins, and osmotic pressure) in conjunction with trypsin inhibitory activity (Fig. 3). Interestingly, the 2 lobsters that were initially categorized as healthy but were further found to be lightly infected with PaV1 were classified by the PCA in the 'healthy' group (see Fig. 2) because their physiological and immunological variables did not differ from those of truly healthy lobsters, which could reflect either the early stage of infection shown by these lobsters (Behringer et al. 2006) or some type of immunity to PaV1. These results underline the limitation of clinical diagnosis in accurately classifying lobsters lightly infected with PaV1.

DISCUSSION

The present study provides a baseline of physiological and immunological variables in *Panulirus argus* naturally infected with PaV1. Ten of the 13 hematological variables examined differed significantly between PaV1-infected and healthy lobsters. The PCA using these 10 variables yielded 2 separate clusters along the first component axis: one corresponding to the healthy group and the other corresponding to the infected group.

Consistent with previous findings in both naturally (Shields & Behringer 2004) and experimentally infected lobsters (Li et al. 2008), we found that heavily infected lobsters showed an increase of spongy connective tissue in the intertubular spaces of the hepatopancreas and distortion of the hemal sinus. Structural alterations in these ducts can cause malfunction in processes such as absorption and storage of ingested nutrients (Johnston et al. 1998, Vogt et al. 1989). Blood constituents are widely used to determine nutritional condition because there are direct relationships between nutritional characteristics of food, hepatopancreatic enzyme activity, and blood metabolites (Cuzon et al. 2001, Gaxiola et al. 2005, Pascual et al. 2006). Li et al. (2008) observed that as severity of PaV1-infection increased, glucose in the hemolymph decreased but protein concentration was not affected. In contrast, we found that plasmatic glucose was not affected by PaV1 infection and that infected lobsters showed significantly lower levels of plasmatic proteins. These apparently contradictory results probably reflect the different substrates used

for the respective analyses. Li et al. (2008) performed their analyses using hemolymph lysates. These lysates, in addition to plasmatic metabolites, contain cytoplasmatic components of the hemocytes that include high levels of glucose, amino acids, and proteins (Johnston & Davies 1972, Dall 1974). In contrast, we performed our analyses using plasma to avoid the interference associated with variations in total hemocyte counts by PaV1 infection.

Reserve inclusion cells, which contain polysaccharides, abound in the hepatopancreas of healthy lobsters but are absent or very rare in the hepatopancreas of PaV1-infected lobsters (Shields & Behringer 2004, Li et al. 2008). Therefore, the similar levels of plasmatic glucose that we found between healthy and infected lobsters may be explained by the release of polysaccharides because of degradation of reserve inclusion cells in infected lobsters. In contrast, infected lobsters showed lower values of plasmatic proteins, an indication of strong tissue degradation, catabolism of hepatopancreatic cells, and/or metabolic impairment to extract nutrients from food. In decapod crustaceans, starvation decreases total serum protein concentration because, in the absence of food, one of the main mechanisms used to draw energy is the catabolism of the free amino acids and lipids (Comoglio et al. 2004). Thus, Behringer et al. (2008) observed that, relative to healthy lobsters, wild lobsters infected with PaV1 had a significantly lower index of hemolymph refraction (which provides an estimate of serum protein concentration), indicating poor physiological condition. Although hemocyanin is one of the main protein components in the hemolymph of the crustaceans, we found that hemocyanin levels did not appear to be affected by PaV1 infection. However, it is possible that the turbidity of the milky hemolymph of infected lobsters may have altered the spectrophotometric evaluation of this molecule.

Hyperlipidemia (a significant increase in serum lipids, mainly cholesterol and triacylglycerides) can result in a milky aspect of the hemolymph or blood in many animals, including humans (Rupprecht et al. 2001, Barbaro 2003). For example, Johnson et al. (1981) and Stewart (1984) reported milky hemolymph and lipid material in the hemolymph of clawed lobsters *Homarus americanus* infected with *Aerococcus viridans* (gaffkemia). Li et al. (2008) found significantly higher levels of triglycerides in heavily infected lobsters that had been experimentally inoculated with PaV1. We found that the plasmatic concentration of acylglycerides and cholesterol in lobsters naturally infected with PaV1 was, on average, 400% higher than in healthy lobsters, indicating that hyperlipidemia is an important feature of the PaV1 disease.

According to the PCA analysis, total hemocyte count, hemagglutination activity, and total PO activity in plasma were positively associated with the healthy lobster group. Crustacean hemolymph coagulation is initiated by the release of transglutaminase from the hemocytes, which polymerizes the hemolymph-clotting protein into a stabilized gel (Martin et al. 1991). In shrimps, this clotting protein is synthesized in the sub-cuticular epidermis and in the heart (Cheng et al. 2008). The inability to coagulate of the hemolymph of PaV1-infected lobsters reflects the distinct predilection of PaV1 for hyalinocytes and semigranulocytes, as well as the ability of the virus to infect the main tissues where the synthesis of the clotting protein occurs (Shields & Behringer 2004, Li et al. 2008).

The lower hemagglutination activity in infected lobsters probably reflects their lower hemocyte count. In crayfish *Macrobrachium rosenbergii* (Pais et al. 2007) and clawed lobsters *Homarus americanus* (Cornick & Stewart 1978), hemagglutinins or lectins appear to be involved in immune response. The source of hemolymph lectins remains to be determined, but the hemocytes and/or the hepatopancreas have been identified as potential sources of hemagglutinins (Pais et al. 2007). As these are also the main targets of PaV1, the mechanism of pathogen recognition in infected lobsters would become more impaired as the disease progresses, potentially increasing their susceptibility to opportunistic pathogens. We observed infestation of gills by ciliates in 5 times as many infected lobsters (61.1%) as healthy lobsters (11.5%). However, it is unclear whether this result reflects immune suppression caused by PaV1 and/or an inability of PaV1-infected lobsters to molt and groom. Further studies are needed to examine whether these and other potentially opportunistic pathogens increase mortality rates in PaV1-infected *Panulirus argus* (Nunan et al. 2010).

The presence of nodules in different tissues is associated with viral infections (Munday et al. 2003, Stentiford & Feist 2005). We observed nodules in the gills, hepatopancreas, and muscle of lobsters heavily infected with PaV1. Some of these nodules were covered with melanin pigment, which could be produced by PaV1 infection. Melanization implies the activation of the proPO system by microbial components and hence is considered to be a mechanism against pathogens (Agius & Roberts 2003, Hur et al. 2006). For example, Stentiford & Feist (2005) found melanized nodules in the hepatopancreas of *Cran-*

gon crangon shrimps infected with intranuclear bacilliformus virus. In our study, infected lobsters exhibited a significantly higher activity of total PO in degranulated hemocytes and of trypsin inhibitor, which could be indicative of the activation of immune response by the PO system during the natural process of PaV1 infection. This mechanism may represent an immunological compensation, potentially explaining the long period of the PaV1 infectious process (>80 d, Li et al. 2008) and suggesting that the survival of some lobsters to this process may be due to an adaptive response. This sort of immunology response to overcome viral infection has already been observed in *Macrobrachium rosenbergii* infected with white spot syndrome virus (Sarathi et al. 2008).

In summary, the present study provides insight into the physiological and immunological responses of *Panulirus argus* lobsters naturally infected with PaV1. In particular, we determined several hematological variables that are strongly altered by PaV1 infection, but many questions about the infectious process in natural conditions remain unanswered. For example, although viral infections can cause catastrophic mortalities in cultured crustaceans (Lightner 2011, Bonami & Sri Widada 2011), their effects on wild populations are far less understood (e.g. Bonami & Zhang 2011). The presence of PaV1 has been confirmed in wild populations of *P. argus* from Florida, Belize, Mexico, the Virgin Islands, and Cuba (reviewed in Behringer et al. 2011), but the prevalence of infected lobsters in specific areas remains unknown and the effects of PaV1 on population parameters of lobsters are poorly understood, if at all (Shields 2011). As *P. argus* comprises one of the most important fisheries in the Caribbean region, it is essential to continue the research on important issues such as virus characterization, geographical variants of the virus and host responses, the role of the immune system on the susceptibility/resistance to infection by lobsters of different sizes, and the mechanisms of PaV1 dispersion in nature.

Acknowledgements. We thank C. Barradas-Ortiz, F. Negrete-Soto (UNAM-UASA Puerto Morelos), and R. Arana Guardia (UNAM-UMDI-Sisal) for their invaluable technical support. M. Mascaró-Miquelajaúregui provided statistical assistance. This study was partially financed by CONACyT (project 131815) and through external services performed at the Laboratory of Immunology and Molecular Biology, CINVESTAV IPN Unidad Mérida (reference number: A3329). Special thanks are given to CONACyT for providing a PhD studentship to J.P.H.M. (grant 166083). A permit to collect lobsters was issued by CONAPESCA-Mexico.

LITERATURE CITED

Agius C, Roberts RJ (2003) Melano-macrophage centres and their role in fish pathology. J Fish Dis 26:499–509

Ashida M, Söderhall K (1984) The prophenoloxidase activating system in crayfish. Comp Biochem Physiol B 77: 21–26

Barbaro G (2003) Pathogenesis of HIV-associated heart disease. AIDS 17:S12–S20

Behringer DC, Butler MJ, Shields JD (2006) Avoidance of disease by social lobsters. Nature 441:421

Behringer DC, Butler MJ IV, Shields JD (2008) Ecological and physiological effects of PaV1 infection on the Caribbean spiny lobster (*Panulirus argus* Latreille). J Exp Mar Biol Ecol 359:26–33

Behringer DC, Butler MJ IV, Shields JD, Moss J (2011) Review of *Panulirus argus* virus 1—a decade after its discovery. Dis Aquat Org 94:153–160

Bell TA, Lightner DV (1988) A handbook of normal penaeid shrimp histology. World Aquaculture Society, Baton Rouge, LA

Bonami JR, Sri Widada J (2011) Viral diseases of the giant fresh water prawn *Macrobrachium rosenbergii*: a review. J Invertebr Pathol 106:131–142

Bonami JR, Zhang S (2011) Viral diseases in commercially exploited crabs: a review. J Invertebr Pathol 106:6–17

Bradford MM (1976) A refined and sensitive method for the quantification of microgram quantities of protein utilizing the principal protein-dye binding. Anal Biochem 72: 248–254

Briones-Fourzán P, Baeza-Martínez K, Lozano-Álvarez E (2009) Nutritional indices of juvenile Caribbean spiny lobsters in a Mexican reef lagoon: are changes over a 10-year span related to the emergence of *Panulirus argus* Virus 1 (PaV1)? J Exp Mar Biol Ecol 370:82–88

Butler MJ, Behringer DC, Shields JD (2008) Transmission of *Panulirus argus* virus 1 (PaV1) and its effect on the survival of juvenile Caribbean spiny lobster. Dis Aquat Org 79:173–182

Chen C, Cheng SY (1993a) Hemolymph PCO_2, hemocyanin, protein level and urea excretions of *Penaeus monodon* exposed to ambient ammonia. Aquat Toxicol 27:281–292

Chen C, Cheng SY (1993b) Studies on hemocyanin and hemolymph protein levels of *Penaeus japonicus* based on sex, size, and moulting cycle. Comp Biochem Physiol B 106:293–296

Cheng W, Tsai IN, Huang GJ, Chiang PC, Cheng CH, Yeh MS (2008) Cloning and characterization of hemolymph clottable proteins of kuruma prawn (*Marsupenaeus japonicus*) and white shrimp (*Litopenaeus vannamei*). Dev Comp Immunol 32:265–274

Comoglio L, Gaxiola G, Roque A, Cuzon G, Amin O (2004) The effect of starvation on refeeding, digestive enzyme activity, oxygen consumption and ammonia excretion in juvenile white shrimp *Litopenaeus vannamei*. J Shellfish Res 23:243–249

Cornick JW, Stewart JE (1978) Lobster (*Homarus americanus*) hemocytes: classification, differential counts, and associated agglutinin activity. J Invertebr Pathol 31: 194–203

Cruz-Quintana Y, Rodríguez Canul R, Vidal Martínez VM (2011) First evidence of *Panulirus argus* Virus 1 (PaV1) in spiny lobster from Cuba and a clinical estimation of its prevalence. Dis Aquat Org 93:141–147

Cuzon G, Rosas C, Gaxiola G, Taboada G, Van Wormhoudt A (2001) Effect of dietary carbohydrates on gluconeogenesis in premolt *Litopenaerus stylirostris* juveniles and preadults. J Shellfish Res 20:931–937

Dall W (1974) Indices of nutritional state in the western rock lobster, *Panulirus longipes* (Milne Edwards). I. Blood and tissue constituents and water content. J Exp Mar Biol Ecol 16:167–180

Dove ADM, Sokolowski MS, Bartlett SL, Bowser PR (2005) Spatio-temporal variation in serum chemistry of the lobster, *Homarus americanus* Milne-Edwards. J Fish Dis 28:663–675

Gaxiola G, Cuzon G, Garcia T, Taboada E and others (2005) Factorial effects of salinity, dietary carbohydrates and moult cycle on digestive carbohydrases and hexokinases in *Litopenaeus vannamei* (Boone, 1931). Comp Biochem Physiol A 140:29–39

Gotelli NJ, Ellison AM (2004) A primer of ecological statistics. Sinauer Associates, Sunderland, MA

Hernández-López J, Gollas-Galván T, Vargas-Albores F (1996) Activation of the prophenoloxidase system of brown shrimp (*Penaeus californiensis* Holmes). Comp Biochem Physiol C 13:61–66

Hernández-López J, Gollas-Galván T, Gómez-Jiménez S, Portillo-Clark G, Vargas-Albores F (2003) In the spiny lobster (*Panulirus interruptus*) the prophenoloxidase is located in plasma, not in haemocytes. Fish Shellfish Immunol 14:105–114

Holthuis LB (1991) Marine lobsters of the world: an annotated and illustrated catalogue of species of interest to fisheries known to date. FAO Fish Synop no. 125, Vol. 13. FAO, Rome

Huchin-Mian JP, Rodríguez-Canul R, Arias-Bañuelos E, Simá-Álvarez R, Pérez-Vega JA, Briones-Fourzán P, Lozano-Álvarez E (2008) Presence of *Panulirus argus* Virus 1 (PaV1) in juvenile spiny lobsters *Panulirus argus* from the Caribbean coast of Mexico. Dis Aquat Org 79:153–156

Huchin-Mian JP, Briones-Fourzán P, Simá-Álvarez R, Cruz-Quintana Y and others (2009) Detection of *Panulirus argus* Virus 1 (PaV1) in exported frozen tails of subadult–adult Caribbean spiny lobster *Panulirus argus.* Dis Aquat Org 86:159–162

Hur JW, Woo SR, Jo JH, Park IS (2006) Effects of starvation on kidney melano-macrophage centre in olive flounder, *Paralichthys olivaceus* (Temminck and Schlegel). Aquac Res 37:821–825

Johnston MA, Davies PS (1972) Carbohydrates of the hepatopancreas and blood tissues of *Carcinus.* Comp Biochem Physiol B 41:433–443

Johnston PT, Stewart JE, Arie B (1981) Histopathology of *Aerococcus viridans* var. *homari* infection (Gafkemia) in the lobster, *Homarus americanus*, and a comparison with histological reactions to a gram-negative species, *Pseudomonas perolens*. J Invertebr Pathol 38:127–148

Johnston D, Alexander C, Yellowhees D (1998) Epithelial cytology and function in the digestive gland of *Thenus orientalis* (Decapoda, Scyllaridae). J Crustac Biol 18: 271–278

Le Moullac G, Soyez C, Saulnier D, Ansquer D, Avarre J, Levy P (1998) Effect of hypoxic stress on the immune response and the resistance to vibriosis of the shrimp *Penaeus.* Fish Shellfish Immunol 8:621–629

Li C, Shields JD, Small HJ, Reece KS, Hartwing CL, Cooper RA, Ratzlaff RE (2006) Detection of *Panulirus argus* Virus 1 (PaV1) in the Caribbean spiny lobster using fluorescence *in situ* hybridization (FISH). Dis Aquat Org 72: 185–192

Li C, Shields JD, Ratzlaff RE, Butler MJ (2008) Pathology and hematology of the Caribbean spiny lobster experimentally infected with *Panulirus argus* Virus 1 (PaV1). Virus Res 132:104–113

Lightner DV (2011) Virus diseases of farmed shrimp in the Western Hemisphere (the Americas): a review. J Invertebr Pathol 106:110–130

Lignot JH, Cochard JC, Soyez C, Lamaire P, Charmantier G (1999) Osmoregulatory capacity according to nutritional status, molt stage and body weight in *Penaeus stylirostris.* Aquaculture 170:79–92

Lozano-Álvarez E, Briones-Fourzán P, Ramírez-Estévez A, Placencia-Sánchez D, Huchin-Mian JP, Rodríguez-Canul R (2008) Prevalence of *Panulirus argus* virus 1 (PaV1) and habitation patterns of healthy and diseased Caribbean spiny lobsters in shelter-limited habitats. Dis Aquat Org 80:95–104

Lyle WG, MacDonald CD (1983) Molt stage determination in the Hawaiian spiny lobster *Panulirus marginatus.* J Crustac Biol 3:208–216

Martin GG, Hose JE, Omori S, Chong C, Hoodbhoy T, McKrell N (1991) Localization and roles of coagulogen and transglutaminase in hemolymph coagulation in decapod crustaceans. Comp Biochem Physiol B 100: 517–522

Montgomery-Fullerton MM, Cooper RA, Kauffman KM, Shields JD, Ratzlaff RE (2007) Detection of *Panulirus argus* Virus 1 in Caribbean spiny lobsters. Dis Aquat Org 76:1–6

Moss J, Butler MJ IV, Behringer DC, Shields JD (2012) Genetic diversity of the Caribbean spiny lobster virus, *Panulirus argus* virus 1 (PaV1), and the discovery of PaV1 in lobster postlarvae. Aquat Biol 14:223–232

Munday BL, Sawada Y, Cribb T, Hayward C (2003) Diseases of tunas, *Thunnus* spp. J Fish Dis 26:187–206

Nunan LM, Poulos BT, Navarro S, Redman RM, Lightner DV (2010) Milky hemolymph syndrome (MHS) in spiny lobsters, penaeid shrimp and crabs. Dis Aquat Org 91: 105–112

Pais R, Shekar M, Karunasagar I (2007) Hemagglutinating activity and electrophoretic pattern of hemolymph serum proteins of *Penaeus monodon* and *Macrobrachium rosenbergii* to white spot syndrome virus injections. Aquaculture 270:529–534

Pascual C, Sánchez A, Zenteno E, Cuzon G and others (2006) Biochemical, physiological, and immunological changes during starvation in juveniles of *Litopenaeus vannamei.* Aquaculture 251:416–419

Rosas C, Cooper EL, Pascual C, Brito R, Gelabert R, Moreno T, Sánchez A (2007) Physiological and immunological conditions of wild populations of *Farfantepenaeus duorarum* from the Campeche Sound (Crustacea, Penaeidae). Mar Biol 152:929–938

Rupprecht HJ, Blankenberg S, Bickel C, Rippin G and others (2001) Impact of viral and bacterial infectious burden on long-term prognosis in patients with coronary artery disease. Circulation 104:25–31

Sarathi M, Nazeer Basha A, Ravi M, Venkatesan C, Senthil Kumar B, Sahul Hameed AS (2008) Clearance of white spot syndrome virus (WSSV) and immunological changes in experimentally WSSV-injected *Macrobrachium rosenbergii.* Fish Shellfish Immunol 25:222–230

Shields JD (2011) Diseases of spiny lobsters: a review. J Invertebr Pathol 106:79–91

Shields JD, Behringer DC Jr (2004) A new pathogenic virus in the Caribbean spiny lobster *Panulirus argus* from the Florida Keys. Dis Aquat Org 59:109–118

Stentiford GD, Feist SW (2005) A histopathological survey of shore crab (*Carcinus maenas*) and brown shrimp (*Crangon crangon*) from six estuaries in the United Kingdom. J Invertebr Pathol 88:136–146

Stewart JE (1984) Lobster disease. Helgol Meeresunters 37: 243–254

Vogt G, Stöcker W, Storch V, Zwilling R (1989) Biosynthesis of *Astacus* protease, a digestive enzyme from crayfish. Histochem Cell Biol 91:373–381

Editorial responsibility: Donald Behringer, Gainesville, Florida, USA

Submitted: September 1, 2011; Accepted: June 27, 2012
Proofs received from author(s): August 22, 2012