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Nutritional and immunological evaluation of juvenile spiny lobsters *Panulirus argus* (Latreille, 1804) (Decapoda: Achelata: Palinuridae) naturally infected with the PaV1 virus

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ABSTRACT

Panulirus argus virus 1 (PaV1) causes a long-lasting and systemic infection in the spiny lobster *Panulirus argus* (Latreille, 1804). Immunological, physiological, and nutritional indicators in the hemolymph and hepatopancreas were studied to investigate the effect of PaV1 on the spiny lobster. This included chymotrypsin, trypsin, glucosidase, cholesterol, acylglycerides, proteins, glycogen, hemagglutination, prophenoloxidase (proPO), phenoloxidase (PO), total hemocyte counts (THC), and hemocyte subpopulations. Spiny lobsters were sorted into uninfected, lightly, moderately, and severely infected groups based on the number of Cowdry Type A viral inclusions detected in their connective tissues. The results showed that the digestive enzymes chymotrypsin ($P < 0.001$), trypsin ($P < 0.001$), and glucosidase ($P < 0.001$) significantly decreased in the hepatopancreas as severity of PaV1 infection increased, denoting a loss of digestive efficiency. Cholesterol ($P < 0.001$), acylglycerids ($P < 0.001$), and protein ($P < 0.101$) values decreased in the hepatopancreas but increased in hemolymph. Glycogen from hepatopancreas decreased in moderately and severely infected lobsters. THC and the sub-populations of hyaline and semi-granular cells, as well as hemagglutination, were lower in the infected group than the uninfected group, but granular cells increased in the severely infected group. The proPO and PO enzymes had a 1:1 ratio in uninfected lobsters, but this balance disappeared in PaV1-infected lobsters, which showed an increase in proPO and a decrease in PO. Results from this study showed that the depletion of immunological, physiological, and nutritional indicators in lobsters infected with PaV1 included the deficiency of digestive efficiency, reduction of nutritional status, and decline in energy reserves.

Key Words: blood metabolites, digestive enzymes, fisheries, immunology, prophenoloxidase

INTRODUCTION

The Caribbean spiny lobster *P. argus* (Latreille, 1804) supports an economically valuable fishery throughout the Caribbean (Ehrhardt *et al.*, 2011). This species is currently affected by the pathogenic virus *Panulirus argus* Virus 1 (PaV1) (Shields & Behringer, 2004; Huchin-Mian *et al.*, 2008). PaV1 is a double stranded DNA virus that is icosahedral in shape (mean size ± SD 182 ± 9 nm). The virus is still unclassified but shares characteristics of the families Herpesviridae and Iridoviridae (Shields & Behringer, 2004). PaV1 was first detected in juvenile *P. argus* from the Florida Keys, USA (Shields & Behringer, 2004) but the virus has been reported throughout most of the Caribbean Sea (Huchin-Mian *et al.*, 2008;

Moss *et al.*, 2013). Juvenile lobsters are more susceptible to the virus than adults (Shields & Behringer, 2004; Huchin-Mian *et al.*, 2013). PaV1 produces a long-lasting infection characterized by a massive destruction of hyaline and semigranular hemocytes, along with structural damage to the hepatopancreas and production of eosinophilic Cowdry Type A intranuclear viral inclusions in connective tissues (Shields & Behringer, 2004; Li & Shields, 2007; Li *et al.*, 2008). The main clinical signs at the onset of the disease are a milky hemolymph that does not clot, lethargy, a reddish discoloration of the carapace, and suppression of molt reflected in fouling of the carapace by epibionts (Shields & Behringer, 2004; Lozano-Álvarez *et al.*, 2008). Glycogen reserves decline, and mortality occurs within 30–80 d in experimental infections (Li *et al.*, 2008).

Indicators of nutrient reserves such as lipids and hemolymph proteins are used for normal physiological functions and growth in healthy lobsters (Ciaramella *et al.*, 2014). Measurements of the nutritional condition of spiny lobsters have been used mainly in ecological and mariculture studies related to ontogeny and molt (Perera *et al.*, 2008a; Simon, 2009). Hemolymph serum-protein assay and the measurement of metabolites in plasma such as triglycerides, cholesterol, and total protein are widely used to assess nutritional condition in decapods, but their values are strongly influenced by molt stage. Three hepatopancreas-based indices (dry weight index, percent tissue dry weight, and relative weight) have also been used to determine nutritional condition in lobsters (inter-molt or pre-molt) (Briones-Fourzán *et al.*, 2003; Ciaramella *et al.*, 2014; Gutzler & Butler, 2017; Musgrove, 2001; Pascual-Jiménez *et al.*, 2012). Variations in nutritional and immunological indicators have been associated with disease in shrimps (Rodriguez & Le Moullac, 2000). The immune system and homeostasis in lobsters experimentally and naturally infected with PaV1 are highly compromised, and the measurement of their physiological and immunological indicators have contributed to understand the effects of the disease (Li *et al.*, 2008; Pascual-Jiménez *et al.*, 2012). Pascual-Jiménez *et al.* (2012), for example, reported preliminarily that some physiological and immunological variables were highly correlated to PaV1 infection in naturally infected juvenile *P. argus* and suggested that digestive enzymes might be altered because the hepatopancreas is atrophied during PaV1infection (Shields & Behringer, 2004; Li *et al.*, 2008).

There is nevertheless little information about the relationship between the disease and the nutritional condition assessed by measurement of digestive enzymes. The aim of this study was to describe the relationship between the PaV1 infection and the process of nutritional depletion through the measurement of several physiological, nutritional, and immunological parameters in naturally infected, wild juveniles of *P. argus*.

NHS and CPJ contributed equally to the research.

MATERIALS AND METHODS

Study area

Lobsters were collected in the Puerto Morelos reef lagoon, Mexico (20°51′N, 86°53′W). This lagoon is part of a coral reef system located on the northern Caribbean coast of the Yucatan Peninsula, where sea-related tourism activities are frequent. The reef lagoon is a nursery habitat for juvenile *P. argus* and PaV1 was first recorded there in 2000 (Lozano-Álvarez *et al.*, 2008; Huchin-Mian *et al.*, 2013).

Sampling and molt-stage determination

Juvenile *P. argus* were collected by SCUBA diving using a hand net. The lobsters were segregated in two containers on the boat, one for the "diseased group" (i.e., lobsters exhibiting macroscopic signs of PaV1; milky hemolymph and reddish discoloration of the carapace), and another one for the "healthy group" (i.e., lobsters with no signs of PaV1 infection) (Shields & Behringer, 2004). Lobsters were transported within 1 h of capture to the Reef Systems Academic Unit of the Universidad Nacional Autónoma de México at Puerto Morelos, where they were maintained in separate seawater tanks and maintained without food for 2 d before necropsy and tissue sampling (Pascual Jiménez *et al.*, 2012). Each lobster was measured with a Vernier caliper (carapace length ± 0.1 mm (CL) from between the rostral horns to the posterior edge of the carapace), weighed on a digital scale after blotting excess water (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). Only lobsters in intermolt were included in the study. Hemolymph was collected for immunological analysis and for estimation of metabolites concentration in plasma. A portion of the hepatopancreas, gills, and muscle tissues were collected and placed in cryovials in liquid nitrogen and further stored at –80 °C for biochemical analyses. A portion of hepatopancreas was fixed in Davidson´s solution (alcohol, formalin, and acetic acid) for histology, and a portion of hepatopancreas was fixed in 96% alcohol for polymerase chain reaction assays (PCR).

Grades of PaV1 infection in hepatopancreas

Histology. Tissue samples from the hepatopancreas were fixed for 48 h in Davidson's solution and processed by a routine histological method followed by hematoxylin and eosin (H&E) stains (Lightner, 1996). Stained sections were observed under a light microscope to examine histopathological changes. The severity of infection of each lobster was estimated based on the scale described by Li *et al.* (2008), with healthy (grade 0) hepatopancreas appearing normal, fixed phagocytes appearing normal, not activated, and no aberrant cells with hypertrophied nuclei, peripheral chromatin, or eosinophilic inclusions. Lightly infected (grade 1) hepatopancreas with few infected cells (< 10 per section), and appearing otherwise normal, fixed phagocytes in hepatopancreas activated or only a few infected. Moderately infected (grade 2) hepatopancreas with infected cells (10–100 per section), with most fixed phagocytes activated or infected. Heavily infected (grade 3) hepatopancreas with interstitial spaces filled with numerous infected cells (> 100 per section), hepatopancreatic tubules atrophied, and many infected cells present in spongy connective tissue.

Polymerase chain reaction. Genomic DNA was extracted from ~20 mg of hepatopancreas tissue using the Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA) following the protocol provided by the manufacturer. A tube containing only digestion reagents and double-distilled water was used as negative control for the DNA extraction procedure. For the PCR probe we used specific primers for PaV1 (45aF TTCCAGCCCAGGTACGTATC & 543aR AACAGATTTTCCAGCAGCGT) (Montgomery-Fullerton *et al.*, 2007) that amplify a region of 499 pb. PCR reactions were carried out in a total volume of 25 µl containing 2 µl of DNA, 0.33 µM of each primer, 2.5 mM of $MgCl_2$, 1.2× reaction, 0.4 mM dNTPs mixture (Promega), and 1 U of Taq DNA polymerase (Promega). Reactions were run at 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 electrophoresed on 2% agarose gels and bands were visualized using 0.1% ethidium bromide stain on a UV transilluminator. DNA from lobsters with a grade 3 infection in the hepatopancreas was used as positive controls, whereas sterile water and DNA from the hepatopancreas of adult lobsters diagnosed as negative to PaV1 were used as negative controls. The integrity of DNA was corroborated using the DNA control region of Actin (MH779998 *P. argus* alpha actin-like protein); with the primers 3ActF (TCATGAGGTGCGACATTGAT) & 3ActR (GAGCCAGAGCAGTGATTTCC) that amplify a region of 120 bp. All reactions were performed at a final volume of 25 µl

containing 2 µl of ADN, 0.33 µM of each specific primer (F and R), 2.5 mM of $MgCl_2$, 1.2 x reaction, 0.4 mM of the mixture of dNTPs (Promega), and 1 U of Taq DNA polymerase (Thermo Fisher, Waltham, MA, USA). All reactions were performed in a thermocycler (Bio-Rad, Philadelphia, PA, USA) at 95 °C for 10 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 S, and 72 °C for 1 min, with a final extension of 72 °C for 10 min.

Analysis of nutritional condition

The hepatopancreas was used as a source of digestive enzymes, protein, and metabolites. Tissues were homogenized with sterile water (pyrogen free) (Pisa®; Laboratorios Pisa, Mexico City, Mexico) at a 1:6 dilution (W:V), homogenates were centrifuged at 4 °C for 20 min at 16 000 *g* and the supernatant was used in the enzymes, protein, and metabolites assays. Glycogen analyses were undertaken on the hepatopancreas and muscle tissues.

Metabolite concentration. Glucose, cholesterol, and acylglycerids concentrations were determined using plasma and hepatopancreas extract and a clinical diagnostic reactive kit (Sera Pak Plus®; Bayer, Whippany, NJ, USA). Concentrations, as mg ml^{-1} or mg gr^{-1}, were calculated from a standard curve using the commercial standard. The protein content of hepatopancreas extracts and plasma were measured according to Bradford (1976) using bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) as the standard (Pascual-Jiménez *et al.*, 2012).

Analysis of digestive efficiency. The digestive efficiency was measured through two proteinase enzymes and two carbohydrases. The assays were run in triplicate and enzyme activities were expressed as specific level (activity), U mg $protein^{-1}$.

Trypsin-like metabolite was measured using a 96-well microplate aliquots of 5 µl of the enzyme extract that was mixed with 290 µl Tris buffer (100 mM, pH 8 at 60 °C), and 6 µl 100 mM N-benzoyl-DLarginine p-nitroanilide (BApNA). The substrate stock solutions of BApNA were prepared in dimethyl sulfoxide (DMSO). Absorbance was recorded at 405 nm in a Benchmark Plus spectrophotometer (Bio-Rad).

Chymotrypsin-like metabolite was measured with 100 mM Suc-Ala-Ala-Pro-Phe-p-nitroanilide (SApNA) aliquots of 5 µl of enzyme extract were mixed with Tris buffer (100 mM, pH 7 at 50 °C) in a 96-well microplate, and 6 µl of substrate solutions prepared in DMSO. Absorbance was recorded at 405 nm.

Amylase was measured using soluble starch (1.5% W:V) as substrate. The enzyme extract (20 µl), 80 µl starch prepared in 10 mM sodium phosphate buffer pH 5 at 60 °C, and 40 µl sterile water were incubated for 40 min at 60 °C. The reaction was stopped with 160 µl dinitosalicylic acid (DSA) and the microplate incubated for 5 min at 100 °C. The amylase concentrations were measured by calculating the reducing sugars released at 540 nm.

Glucosidase was measured with 120 mM *p*-nitrophenil D-glycopyranoside (PNPG) as substrate in a 96-well microplate. For this, 5 µl of enzyme extract were mixed with 145 µl sodium phosphate buffer (50 mM, pH 6 at 60 °C) and 6 µl of a substrate solution prepared in DMSO incubated at 60 °C for 60 min. The reaction was stopped with 145 µl of sodium carbonate. Absorbance was recorded at 405 nm.

Glycogen reserves in hepatopancreas and muscle

Glycogen was measured in 40 mg of hepatopancreas or muscle. Frozen tissue was homogenized individually with 5% trichloroacetic acid (TCA); homogenates were centrifuged for 6 min at 5000 g and 100 µl of the supernatants mixed with 95% ethanol and incubated at 37 °C for 3 h. The mixture was centrifuged for 15 min at 5000 g and the pellet dissolved in 20 µl of distilled water, 5% phenol, and sulfuric acid at 100 °C. Absorbance was recorded at 490 nm. Concentrations were recorded as mg g^{-1}, calculated from a commercial glucose standard reagent (1mg ml^{-1}) (Sera Pak Plus®).

Immunological analysis

Hemolymph sampling. The collection of hemolymph and all immunological analyses were performed following the protocol described by Pascual Jiménez *et al.* (2012). Hemolymph was collected from the pericardial sinus of each spiny lobster using a chilled syringe needle. The area of extraction was previously sterilized with 70% ethanol. Hemolymph was diluted (1:3) with chilled (8 °C) anticoagulant (350 mM NaCl, 10 mM KCl, 10 mM HEPES, 10 mM EDTA-Na2, pH 7.3, 850 mOsm kg^{-1}) (Söderhäll & Smith, 1983). The sample was then centrifuged at 800g for 5 min at 4 °C to separate the plasma, which was used to evaluate plasmatic metabolites, hemagglutination, and phenoloxidase (PO). The cellular pellet was washed with anticoagulant and centrifuged and subsequently resuspended with cacodylate buffer (10 mM cacodilic acid, 10 mM CaCl, pH 7.0) and centrifuged at 16 000g for 5 min at 4 °C. The supernatant was used to evaluate the PO level from degranulated hemocytes (see below).

Hemocytes count. Hemolymph (25 µl) was diluted 1:2 with Alsever solution (115 mM glucose, 30 mM sodium citrate, 10 mM EDTA, 338 mM NaCl) and 15% of formaldehyde (V:V) (Le Moullac *et al.* 1998). Samples were kept at 2−8 °C until subsequent analyses. Total hemocyte counts (THC) were read and characterized according to morphological characteristics in a Neubauer chamber. Samples were analyzed in duplicate.

Phenoloxidase determination. Phenoloxidase (PO) was measured by spectrophotometry to detect the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) (Ashida & Söderhäll, 1984).

To obtain the total phenoloxidase (POt), plasma or degranulated hemocytes were incubated with trypsin (1 mg ml^{-1}) to ensure the conversion of the precursor pro-phenoloxidase (proPO) into the active form, phenoloxidase (PO), in the presence of saturated L-DOPA (3 mg ml^{-1}) during an activation time of 10 min. Absorbance was measured at 490 nm for 30 s in an ELISA microplate reader (model 550; Bio-Rad). Results were expressed as the increment of 0.001 in optical density (OD).

In the same way, the active form (PO) was measured in degranulated hemocytes following the same protocol described above, but in this case, trypsin was not added to avoid the activation of the zymogen (inactivated form) proPO. Prophenoloxidase (proPO) was calculated only in degranulated hemocytes as the difference between total phenoloxidase (POt) and phenoloxidase (PO).

Hemagglutination assay. Hemagglutination was assayed using u-shaped microplates using plasma from individual lobsters. A 2-fold serial dilution starting with 50 µl of plasma and 50 µl of erythrocyte 2% prepared solution from human blood (type O+); erythrocytes were washed three times with 0.9% saline solution, centrifuged at 380 g at 25 °C for 5 min, and adjusted to a final volume of 2%. After 2 h of incubation at room temperature (26 ± 2 °C), hemagglutination was noted. Results were expressed as the inverse of the last dilution that showed a visible agglutination level.

Statistical analysis

We considered 23 immunological and nutritional variables. As the data did not meet the assumption of normality, separate

Kruskal-Wallis nonparametric analyses were used to test for statistically significant differences in each variable among the four groups of lobsters, followed by a test of comparisons of pairs between the medians of the different levels of infection using Infostat software ver. 11 (Di Rienzo *et al.*, 2010).

A discriminant analysis (DA) was used to identify the variables that distinguish the different levels of PaV1 infection in juvenile lobsters. We used nine variables that were measured in at least 65% of the lobsters. These included nutritional and digestive efficiency variables, but not immune variables. The DA was complemented by ANOVA between levels of PaV1 infection in juvenile lobster for each variable. A univariate analysis was constructed from a matrix standardized by the covariance between groups and the residual covariance matrix in the Infostat software. This allowed evaluation of the covariance matrices as a function of the group effect and the residual variance-covariance matrices obtained after discounting the group effect, allowing us to construct from the diagonals of these matrices the F-value, non-self-correlated, for each variable (Balzarini *et al.*, 2008). ANOVA and DA analyses were performed using Infostat software ver. 11 (Di Rienzo *et al.*, 2010).

RESULTS

Sampling

We collected 74 juveniles of *P. argus* (8.3–62.2 mm CL), of which 67 were in intermolt and were included in the study. In addition to the macroscopic signs of PaV1 infection, we used two other criteria to differentiate between diseased and healthy lobsters: the grade of infection of PaV1 by histology (Li *et al.*, 2008), where Cowdry Type A intranuclear inclusions (Fig. 1) were observed in 31 histological samples of hepatopancreas, and the detection of DNA of PaV1 by PCR (Montgomery-Fullerton *et al.*, 2007).

The healthy group consisted of 28 lobsters (mean ± SD 25.09 ± 8.81 mm CL). Histological observations exhibited the well-organized structure normally seen in *P. argus* (Fig. 1A), and these lobsters tested negative in the PCR assay. Eight lobsters (25.45 ± 15.84 mm CL) were categorized as lightly infected (Fig.1B). They did not have Cowdry Type A viral inclusions but tested positive to PaV1 by PCR (the molecular analysis showed amplification at the 499 bp region specific for PaV1). Six lobsters were categorized as moderately infected (22.45 ± 6.61 mm CL) (Fig. 1C) and 25 (25.79 ± 11.07 mm CL) were categorized as severely infected (Fig 1D). The moderately and severely infected groups were characterized by the presence of nuclear hypertrophy and condensed chromatin on the nuclear membrane in analyzed samples by histology and positive by PCR.

Analysis of nutritional condition

Metabolite concentration. The concentrations of proteins ($P < 0.101$), cholesterol ($P < 0.394$), and acylglycerides ($P < 0.997$) from the hepatopancreas were not significantly different among the four lobster groups. Proteins and cholesterol had a similar pattern, decreasing in lightly and moderately infected lobsters but increasing in severely infected lobsters. The mean values for glucose decreased significantly from 3.47 mg g^{-1} in the hepatopancreas of healthy

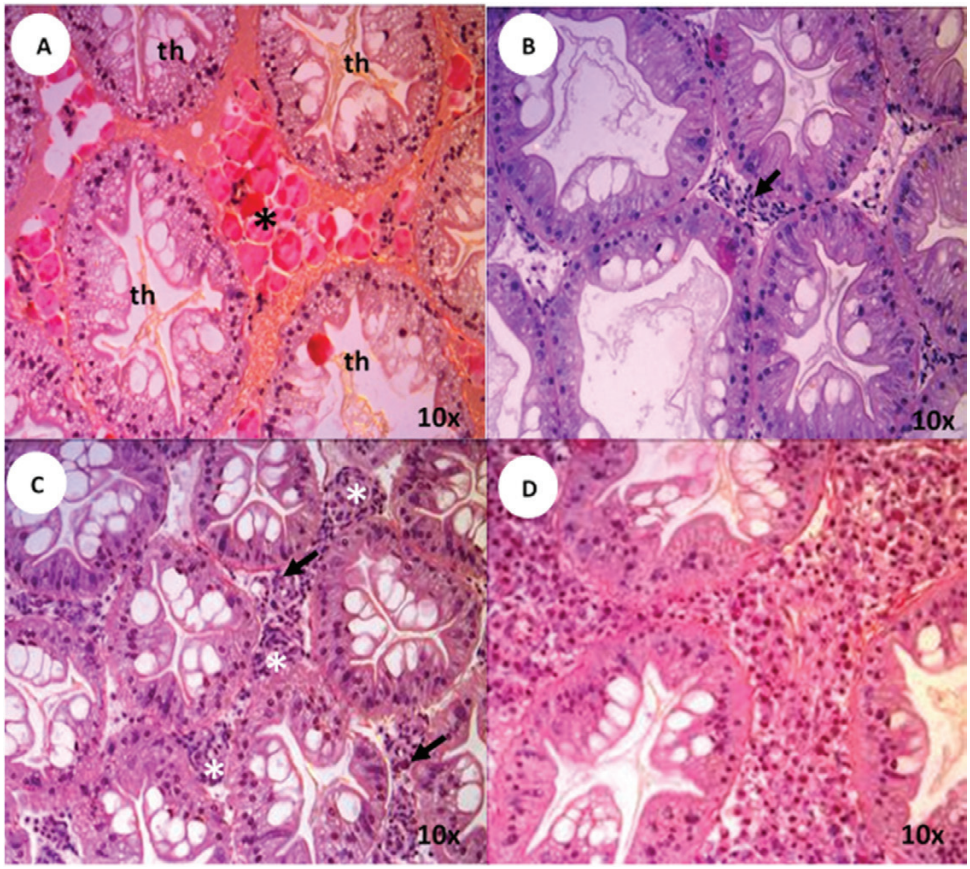

Figure 1. Noticeable reserve inclusion cells (asterisk) among the tubules of the hepatopancreas (th) in a healthy individual of the lobster *Panulirus argus* (**A**). Single-infected fixed phagocyte (arrow) surrounded by infiltrated hemocytes in a lightly infected lobster (**B**). Intranuclear inclusion bodies (arrows) in a moderately infected lobster. Notice the presence of nodules among the tubules of the hepatopancreas (asterisks) (**C**). Massive intranuclear inclusion bodies and changes in the spongy connective tissue of a heavily infected lobster (**D**). This figure is available in color at *Journal of Crustacean Biology* online.

juveniles to 1.85 mg g^{-1} in lightly infected lobsters, to 0.59 mg g^{-1} in moderately infected lobsters, and to 0.88 mg g^{-1} in severely infected lobsters ($P < 0.009$) (Table 1).

Plasma protein values were not affected by the level of infection, but glucose ($P = 0.001$), cholesterol ($P < 0.001$) and acylglycerides ($P < 0.001$) values differed significantly among the lobster groups. These metabolites displayed a similar pattern: the concentration increased in moderately infected lobsters. The values of cholesterol (1.38 mg ml^{-1}), for example, increased 4.5 times relative to healthy lobsters (0.18 mg ml^{-1}). Glucose concentration (0.75 mg ml^{-1}) was almost three times higher in severely infected lobsters than in healthy lobsters (0.28 mg ml^{-1}); cholesterol (1.38 mg ml^{-1}) increased 14 times than healthy lobsters (0.18 mg/ml), and acylglycerides (0.51 mg ml^{-1}) increased 4.4 times than healthy lobsters (0.012 mg ml^{-1}) (Table 1).

Analysis of digestive efficiency. The digestive efficiency was measured through the measurement of proteinases (trypsin and chymotrypsin) and carbohydrases. Trypsin and chymotrypsin showed a significant ($P < 0.001$) and progressive decrease in their level; both had 71% and 78% less, respectively, in severely infected lobsters compared to healthy lobsters. In contrast, the carbohydrases showed a different pattern. Glucosidase decreased 22% in moderately infected and 78% in heavily infected lobsters

Table 1. Individual Kruskal–Wallis (H) tests comparing 14 nutritional and digestive efficiency variables among four groups of the spiny lobster *Palinurus argus* differing in the severity of infection by PaV1 (Healthy:uninfected). Shown are the means, confidence intervals (between brackets), and the sample sizes (between parentheses). Means in a same row that do not share the same superscripts are significantly different at $P \leq 0.05$ (bold).

	Lobster group					
Variable	**Healthy**	**Lightly infected**	**Moderately infected**	**Severely infected**	**H**	***P***
Trypsin (U mg^{-1} protein)	5520 ^{c}	4829 ^{bc}	2809 ^{ab}	1564 ^{a}	29.9	**< 0.001**
	[2532–10072]	[2253–8635]	[120– 7456]	[37–4622]		
	(28)	(7)	(5)	(24)		
Chymotrypsin (U mg^{-1} protein)	22734 ^{b}	21548 ^{b}	17542 ^{b}	4896 ^{a}	34.4	**< 0.001**
	[6527–39568]	[13058–31864]	[1275–44440]	[271–14629]		
	(28)	(7)	(6)	(25)		
Amilase (U mg^{-1} protein)	0.0039	0.0036	0.0042	0.0029	6.6	0.085
	[0.0024–0.0049]	[0.0018–0.0055]	[0.0012–0.0068]	[0.0010–0.0040]		
	(25)	(7)	(5)	(25)		
Glucosidase (U mg^{-1} protein)	0.032 ^{bc}	0.035 ^{c}	0.022 ^{ab}	0.019 ^{a}	22.8	**< 0.001**
	[0.028–0.0036]	[0.030–0.040]	[0.019–0.026]	[0.016–0.023]		
	(26)	(7)	(5)	(25)		
Hepatopancreas protein (mg g^{-1})	35.6	26.6	28.1	35.1	6.2	0.101
	[16.6–58.8]	[15.1–33.4]	[5.7–40.7]	[24.1–45.2]		
	(28)	(7)	(6)	(25)		
Hepatopancreas glucose (mg g^{-1})	3.47 ^{c}	1.85 ^{bc}	0.56 ^{a}	0.88 ^{ab}	11.7	**0.009**
	[0.411–10.38]	[0.48–3.66]	[0.09–1.36]	[0.12–1.68]		
	(26)	(6)	(5)	(20)		
Hepatopancreas cholesterol (mg g^{-1})	0.56	0.40	0.44	0.61	3.0	0.394
	[0.13–0.99]	[0.35–0.43]	[0.26–0.65]	[0.30–1.09]		
	(25)	(4)	(4)	(21)		
Hepatopancreas acylglycerids (mg g^{-1})	1.51	1.35	1.45	1.44	0.1	0.997
	[0.42–2.93]	[0.97–1.69]	[0.76–2.01]	[0.56–3.52]		
	(25)	(4)	(4)	(19)		
Plasma protein (mg ml^{-1})	56.7	61.6	35.4	48.1	3.9	0.274
	[16.6–110.6]	[34–115.5]	[10.7–74.4]	[16–72.1]		
	(27)	(8)	(6)	(23)		
Plasma glucose (mg ml^{-1})	0.28 ^{a}	0.23 ^{a}	0.42 ^{ab}	0.75 ^{b}	15.5	**0.001**
	[0.13–0.49]	[0.18–0.3]	[0.15–1.08]	[0.09–1.34]		
	(26)	(3)	(6)	(20)		
Plasma cholesterol (mg ml^{-1})	0.18 ^{a}	0.08 ^{ab}	1.06 ^{ab}	1.38 ^{b}	16.7	**< 0.001**
	[0.03–1.76]	[0.06–0.1]	[0.05–2.8]	[0.02–3.21]		
	(19)	(2)	(3)	(16)		
Plasma acylglycerids (mg ml^{-1})	0.012 ^{a}	0.11 ^{a}	0.26 ^{a}	0.51 ^{b}	31.5	**< 0.001**
	[0.04–0.27]	[0.06–0.17]	[0.06–0.84]	[0.14–0.98]		
	(27)	(7)	(6)	(23)		
Hepatopancreas glycogen (mg g^{-1})	6.7 ^{b}	7.1 ^{b}	4.9 ^{ab}	2.9 ^{a}	21.8	**<0.001**
	[3.8–10.1]	[5.5–8.6]	[3.8–6.8]	[0.6–4.4]		
	(16)	(2)	(4)	(12)		
Muscle glycogen (mg g^{-1})	1.33	1.03	0.58	0.78	5.4	0.145
	[0.95–1.7]	[0.43–1.63]	[0.39–0.77]	[0.57–0.99]		
	(29)	(8)	(6)	(24)		

($P < 0.001$), whereas amylase did not exhibit significant changes with progression of the infection (Table 1).

Glycogen reserves. The concentration of glycogen in muscle and hepatopancreas decreased in lobsters with PaV1 infection; in the moderately infected group the values decreased around 30%, whereas severely infected lobsters lost at least half of their reserves. The decrease was significant in hepatopancreas ($P < 0.001$), but not in muscle ($P < 0.145$) (Table 2).

Immunological analysis

Total hemocytes count (THC) and hemocyte characterization. THC showed a wide variation even within the healthy group. The three types of hemocytes, hyaline, semigranular, and granular cells (Söderhäll & Cerenius, 1992; Johansson *et al.*, 2000) were observed. In the Hyaline cells were more abundant (50%) in the uninfected group, followed by semigranular (30%) and granular cells (20%). These values diminish in the lightly infected group following the same pattern observed in the healthy group. A shift was nevertheless observed in the moderately infected group; the values of hyaline and semi-granular cells decreased (40% and 20%, respectively), whereas the granular cells increased up to 40%. The proportion of semigranular cells decreased to 20% and of granular cells to 30% in the severely infected group (Table 2). The mean values of hyaline cell counts were not significantly different in the healthy group when compared to the severely infected group ($P < 0.072$), but the semigranular cell count was significantly lower (about 53% less, $P < 0.006$) in the severely infected group. The granular cell count ($P < 0.172$), did not differ significantly among groups, even though their counts were 60% higher in the moderately infected group and 37% higher in the severely infected group relative to the healthy group.

Phenoloxidase levels. Total phenoloxidase (POt) in hemolymph was measured in the plasma and in degranulated hemocytes. The highest level of this enzyme was found in the plasma. POt was 20% higher in the plasma of healthy lobsters than in degranulated hemocytes. POt levels in plasma increased significantly ($P < 0.003$) among the infected groups; the POt level in lightly infected lobsters was 70% higher than in the healthy lobsters, with PO remaining high throughout the PaV1 infection (Table 2).

POt increased 80% in the degranulated hemocytes of severely infected group compared to the healthy group. PO alone decreased 75% in severely infected group compared to the healthy group, whereas prophenoloxidase (proPO) increased significantly ($P < 0.02$) in lightly, moderately. and severely infected lobsters (Fig. 2, Table 2).

Hemagglutination assay. Hemagglutination levels increased in lightly infected lobsters (50% more than in the healthy group) but significantly decreased in moderately and severely infected lobsters ($P < 0.001$), with values falling even below those of healthy lobsters (Table 2).

Table 2. Individual Kruskal–Wallis (H) tests comparing nine immunological variables among four groups of the spiny lobster *Panilurus argus* differing in the severity of PaV1 infection (Healthy:uninfected). Shown are the means, confidence intervals (between square brackets), and sample sizes (between parentheses). Means in a same row that do not share the same superscripts are significantly different at $P \leq 0.05$ (bold).

	Lobster group					
Variable	**Healthy**	**Lightly infected**	**Moderately infected**	**Severely infected**	**H**	***P***
Total hemocytes ($\times 10^6$ cell ml^{-1})	10.5	9.9	8.3	7.3	3.5	0.321
	[3.7–23.1]	[3.4–22.6]	[3.8–17]	[3–13.8]		
	(28)	(8)	(6)	(24)		
Hyaline hemocytes ($\times 10^6$ cell ml^{-1})	5.8	4.5	3.6	3.5	7.0	0.072
	[1.7 11.8]	[1.7–10.1]	[1.5–7.5]	[1.1–7]		
	(27)	(8)	(6)	(24)		
Semigranular hemocytes ($\times 10^6$ cell ml^{-1})	3.4 [b]	3.7 [b]	2.0 [ab]	1.5 [a]	12.3	**0.006**
	[1.2–8.5]	[1–11.3]	[0.3–5]	[0.3–3.5]		
	(27)	(8)	(6)	(24)		
Granular hemocytes ($\times 10^6$ cell ml^{-1})	1.7	1.6	2.7	2.3	5.0	0.172
	[0.4–3.1]	[0.7–3.7]	[1.6–4.5]	[0.5–6.8]		
	(27)	(8)	(6)	(24)		
Total phenoloxidase activity on plasma (POt Pl) (490 nm)	0.63 [a]	1.33 [b]	1.17 [ab]	1.12 [a]	13.6	**0.004**
	[0.001–1.39]	[1.26–1.39]	[1.04–1.37]	[0.94–1.33]		
	(19)	(4)	5)	(15)		
Total phenoloxidase activity on degranulated (POt DH)	0.52	0.58	0.47	0.78	4.7	0.194
(490 nm)	[0.09–1.02]	[0.34–0.87]	[0.05–0.70]	[0.17–1.14]		
	(25)	(6)	(3)	(13)		
Phenoloxidase activity on degranulated (PODh) (490 nm)	0.26	0.02	0.07	0.12	2.9	0.412
	[0.013–0.39	[0.021–0.027]	[0.006–0.18]	[0.01–0.28]		
	(25)	(6)	(4)	(13)		
Prophenoloxidase activity on degranulated (proPODh)	0.27 [a]	0.56 [b]	0.46 [ab]	0.65 [b]	9.8	**0.021**
(490 nm)	[0.14–0.38]	[0.40–0.72]	[0.04–0.87]	[0.41– 0.9]		
	(25)	(6)	(3)	(13)		
Hemagglutination activity	76 [bc]	112 [c]	59 [ab]	45 [a]	14.6	**0.001**
	[32–128]	[64–128]	[8–128]	[16–64]		
	(25)	(8)	(6)	(21)		

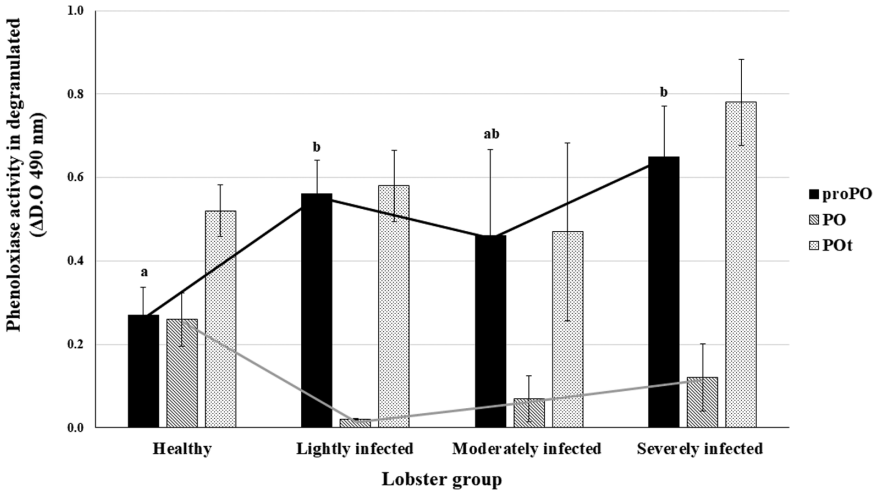

Figure 2. Phenoloxidase levels in degranulated hemocytes of the spiny lobster *Panulirus argus* infected with the PaV1 virus. Prophenoloxidase (proPO), phenoloxidase (PO), and phenoloxidase total (POt) in healthy, lightly infected, moderately infected, and severely infected lobsters. Bars with mean and standard error (SE); different superscripts are significantly different at $\alpha = 0.05$.

Multivariate analysis

The model included nine discriminant variables measured in lobsters from the four grades of PaV1 infection; both proteinases, glucosidase, all the metabolites, and proteins from plasma, as well as glycogen and glucose from hepatopancreas were the most important parameters. The first two canonical axes explained 98.67% of the variance (first axis 90.74%; second axis 7.93%) and the table cross classification had a total error of 23%, mostly for healthy and moderately infected lobsters, whereas the misclassification error for heavily infected lobsters was 0% (Fig. 3).

DISCUSSION

PaV1 has become a threat to spiny lobsters because the viral infection is characterized by massive destruction of hyaline and semigranular hemocytes along with atrophy and structural change to the hepatopancreas (Shields & Behringer 2004; Li & Shields 2007; Li *et al.* 2008).

Hemolymph components are used as indicators of physiological condition in crustaceans (Ciaramella *et al.*, 2014). Four metabolites (cholesterol, acylglycerids from plasma, and glucose from both plasma and hepatopancreas) emerged in our study as important discriminant variables associated with PaV1 infection.

As in our results, Li *et al.* (2008) found that the levels of these metabolites were lower in laboratory-infected *P. argus* than in uninfected lobsters. Triglycerides are primary metabolic reserves and their values are used as a condition index because of their importance in transportation and storage of energy (Ocampo *et al.*, 2003). Cholesterol is the main component of cellular membranes and a precursor of vitamin D. It participates in the synthesis of some steroid hormones and β-ecdysone, necessary in the molt cycle (Ciaramella *et al.*, 2014). The decrease of cholesterol and triglyceride values in the hepatopancreas and their subsequent rise in plasma could cause the milky appearance of the hemolymph in lobsters infected with PaV1. This "milky hemolymph" is more evident in severely infected lobsters (Shields & Behringer, 2004; Lozano-Álvarez *et al.*, 2008; Huchin-Mian *et al.*, 2013). This condition has also been identified as a major macroscopic sign associated with the presence of other pathogens such as rickettsia-like organisms (RLO) in other species of lobsters and shrimps, producing the so-called "milky hemolymph syndrome" or MHS (Nunan *et al.*, 2010). This syndrome is also characterized by the presence of lipoproteins and fatty tissues in the hemolymph (Yehezkel *et al.*, 2000).

Glucose values in the severely infected (diseased) lobsters we studied were low in hepatopancreas and high in hemolymph. Pascual-Jiménez *et al.* (2012) also found high values of glucose in hemolymph in severely infected lobsters, but in contrast to our findings, Li *et al.* (2008) found that glucose levels decreased in the serum of experimentally infected *P. argus*. These contrasting results can be reconciled by taking into consideration that crustacean hemocytes contain high levels of glucose, amino acids, and proteins, is the reason why some authors recommend the use of plasma rather than serum to avoid the interference caused by the cytoplasmic components of lysed hemocytes (Johnstoni & Davies, 1972; Dall, 1974; Pascual-Jiménez *et al.*, 2012).

The damage to hepatopancreas and muscle tissues in our heavily infected lobsters may have caused the release of glucose and carbohydrates into the hemolymph (see Yoganandhan *et al.*, 2003), potentially explaining the high values of glucose found in their hemolymph. Moreover, the glycogen values in the hepatopancreas and muscle tissues also decreased. The nutritional reserves or reserve inclusions (RI) in the hepatopancreas decrease and fade in advanced stages of PaV1 infection evaluated by histology (Li *et al.*, 2008). In our study, glycogen was an important discriminant variable associated with the degrees of PaV1 infection (Fig. 3).

The presence of vacuoles in the hepatopancreas of crustaceans with significant levels of glycogen and lipids indicates active absorption of nutrients, whereas the mobilization of energetic reserves has been related to periods of starvation, molting, and reproduction (Sousa & Petriella, 2001). In post-molt shrimps, glycogen from the hepatopancreas is accumulated in the epidermis as a source of N-acetylglucosamine, which is a precursor in the synthesis of the chitin of the new endocuticle (Rosas *et al.*, 2001). The loss of these reserves might be associated with the suppression of molting in PaV1-infected lobsters (Shields & Behringer, 2004; Lozano-Álvarez *et al.*, 2008).

The lobsters infected with PaV1 that we studied exhibited a significant decrease in their digestive enzymatic level. Yet, despite the importance of carbohydrates in metabolism (as a source of direct metabolic energy, for the synthesis of chitin, and for the synthesis of nucleic acids), the enzymatic activity of carbohydrases (e.g., amylase, glucosidase, cellulase, chitinase) in lobsters we analyzed was low (see Johnston *et al.*, 2003; Perera *et al.*, 2008b). Glucosidase declined significantly only in the last stage of PaV1 infection (severely infected lobsters; grade 3 infection). In contrast, the level of proteolytic enzymes was high, showing that dietary protein is more important than carbohydrate, reflecting the carnivorous habits of lobsters (Perera *et al.*, 2008b). Two of the main

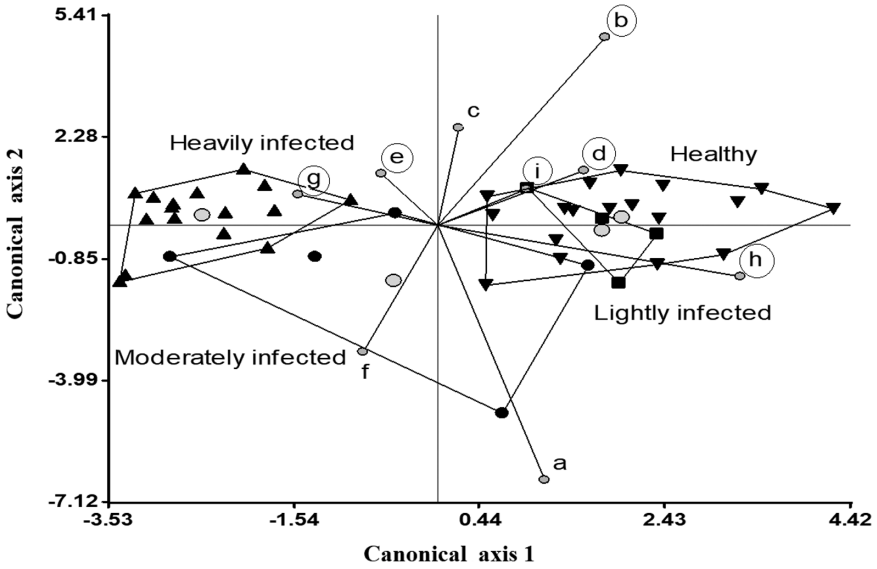

Figure 3. Discriminant analysis of metabolites concentrations in four groups of the spiny lobster *Panulirus argus* with different grades of severity of PaV1 infection. ▼ Healthy lobsters, ■ lightly infected lobsters, ● moderately infected lobsters and ▲ heavily infected lobsters. (a) Chymotrypsin, (b) trypsin, (c) glycosidase, (d) plasma proteins, (e) plasma glucose, (f) plasma cholesterol, (g) plasma acylglycerids, (h) hepatopancreas glycogen, (i) hepatopancreas glucose. Circled letters refer to the variables having more influence on axis 1.

endoproteinases from the hepatopancreas, trypsin and chimotrypsin, decreased significantly as the infection progressed. Severely infected lobsters had between 70% and 80% less proteinases compared to uninfected individuals. The activity of these two main digestive proteinases has been related to the absence of molting in shrimps (Fernández Gimenez *et al.*, 2001), but to our knowledge this is the first report of a decrease of these digestive proteinases related to a viral infection in *P. argus*. This is relevant because PaV1 causes a long-lasting infection that affects the physiology of its host as infection progresses, and even if lobsters feed, their hepatopancreas produce only a third of these digestive enzymes.

The immune system of crustaceans relies on hemocytes to ensure efficient defense responses against pathogens due to their participation in recognition, phagocytosis, melanization, cytotoxicity, synthesis, and exocytosis of bioactive molecules (Jiravanichpaisal *et al.*, 2006a; Rao *et al.*, 2015). In infected lobsters, total hemocyte (THC) and hyaline and semigranular cell counts decreased progressively as the severity of the infection increased. Li & Shields (2007) reported the susceptibility of hyaline and semigranular cells to PaV1 infection after *in vitro* exposure to PaV1 of cultured hemocytes, whereas granular cells were apparently uninfected (Li *et al.*, 2008). The counts of granular cells found herein increased as severity of infection increased (see Li *et al.*, 2008; Pascual-Jiménez *et al.*, 2012). Total counts of granular cells in lobsters with advanced stages of infection were higher than in healthy and lightly infected lobsters, suggesting a compensatory effect (see Jiravanichpaisal *et al.*, 2006b). A similar response was observed in the freshwater crayfish *Pacifastacus leniusculus* (Dana, 1852) infected with the WSSV virus, in which semigranular cells showed a greater susceptibility to the virus than granular cells (Jiravanichpaisal *et al.*, 2006b).

The observed decrease of hyaline and semigranular cells might be related to the lack of hemolymph coagulation that is exacerbated in heavily PaV1 infections (see Shields & Behringer, 2004; Lozano-Alvarez *et al.*, 2008). Aono & Mori (1996) described that during the coagulation process in the spiny lobster *Panulirus japonicus* (Von Siebold, 1824), the cytolysis of hyaline and semigranular cells releases the enzyme transglutaminase. The transglutaminase levels in these cells is four times greater than in granular cells and, once released, the enzyme catalyzes the gelation of the plasma. The reduction of hyaline and semigranular cells in severely infected lobsters would reduce the activity of this enzyme necessary in the coagulation process. This lack of coagulation could become irreversible in moderate infection, when the decrease of hyaline and semi-granular cells is more evident. Additional is needed to address this observation.

Our results showed that hemagglutination level was higher in lightly infected lobsters than in healthy lobsters, then decreased significantly as PaV1 infection progressed. This pattern was consistent with results of Pascual Jiménez *et al.* (2012), who concluded that this process can be attributed to lectins. The potential sources of hemagglutinins are the hemocytes and the hepatopancreas (Marques *et al.*, 2000; Pais *et al.*, 2007), which are also the main targets of PaV1. The modulation of hemolymph hemagglutinin levels in crustaceans as a response to viral infection has been reported in the giant freshwater prawn *Macrobrachium rosenbergii* (De Man, 1879), a species that is highly tolerant to WSSV; in experimental infections, an increase in the hemagglutination level was reported at 48 h post infection (Pais *et al.*, 2007). In contrast, in the blue crab *Callinectes sapidus* Rathbun, 1896, the parasitic dinoflagellate *Hematodinium perezi* Chatton & Poisson, 1931 can cause mortality in their natural environment by metabolic exhaustion, but the hemagglutination values were not affected by infection (Shields *et al.*, 2003). In this sense, the hemagglutination response depends on lectins that function as opsonins, tagging particles for subsequent agglutination or phagocytosis and, in some instances, have antimicrobial activity, as some invertebrate lectins are essential components of the proPO pathway (Söderhäll & Smith, 1983). Additional work needs to be done to evaluate the biochemical profile of lectins and hemagglutinin values when addressing innate immune response in different models of infection.

Circulating hemocytes rely on the proPO pathway activating system to achieve pathogen entrapment and wound healing (Cerenius & Söderhäll, 2018). This system is involved in cytotoxic reactions, cell-adhesion encapsulation, and phagocytosis (Bowden, 2016). It is activated by lipopolysaccharides (LPS), β-glucans, or peptidoglycans, causing exocytosis of granular cells and formation of the active form; phenoloxidase (PO), an enzyme that initiates melanization (Söderhäll & Smith, 1983). In *P. argus* ProPhenol (ProPO) activating enzyme and its inhibitor, named panulirin (a trypsine inhibitor) are found in the hemocytes (Perdomo-Morales *et al.*, 2007, 2013). whereas in *P. interruptus* (Randall, 1840), most of

the proPO activity occurs in the plasma (Hernández-López *et al.*, 2003).

In *P. argus* the activity of the phenoloxidase (PO) enzyme occurs in plasma and in hemocytes by degranulation (Perdomo-Morales *et al.*, 2007). PO level was measured in both (hemocytes and plasma) and we observed that the total level of PO (POt) was higher in plasma than in degranulated hemocytes. The precursor ProPO became active in degranulated hemocytes after the addition of exogenous trypsin. In this sense, in healthy lobsters the ProPO-PO system is balanced, but in PaV1-infected lobsters the ProPO-PO system showed a different pattern. In lightly infected lobsters, ProPO was higher than in healthy lobsters but formation of PO occurred in small amounts. In moderately and severely infected lobsters, the formation of PO increased one half of that observed in healthy lobsters, but the ProPO was always higher than in healthy lobsters. These data are relevant because at the onset of the disease the POt from plasma and degranulated hemocytes remained high, but the PO was very low in degranulated cells. It is therefore important to address if the virus is suppressing this function in lobsters. In shrimp infected with WSSV, for example, a novel virus protein (WSSV164) suppressed the activation of proPO, suggesting an infection route of WSSV by interfering with the activation of the ProPO cascade in shrimp (Sangsuriya *et al.*, 2018).

The activation cascade for PO is a highly regulated system and several pathogens can suppress melanization in their host by various molecular mechanisms. The *Microplitis demolitor* bracovirus produces a protein that blocks activation of the PO cascade via prophenoloxidase-activating proteinase (PAP) inhibition (Beck & Strand, 2007). The proPO system in the hemocytes of in the crayfish *Pacifastacus leniusculus* experimentally infected with WSSV was affected the protein kinase C pathway was also affected during the replication of the virus within the cells (Jiravanichpaisal *et al.*, 2006b). The downregulation of proPO has been attributed to the ability of the virus to employ novel ways to regulate the activation of the proPO cascade to evade host defenses with the probable use of pathogen-related peptides (PRPs) (Pais *et al.*, 2007). Although we were not able to show an evasive response of the virus, our data show that the ProPO-PO ratio remained balanced in degranulated hemocytes of uninfected lobsters, whereas during PaV1 infection, the proPO-PO increased but the PO decreased, making it important to investigate if PaV1 interferes with the activation of proPO into PO.

Our results validate through the measurement of nutritional, physiological, and immunological indicators that PaV1 compromises the physiology and immune system of infected. The structural damage of the hepatopancreas leads to a systemic imbalance and the reduction of the activity of the digestive enzymes, reflecting a lack of digestive efficiency. Even if infected lobsters feed, they are unable to absorb nutrients (as during starvation), leading to a deficient nutritional condition and loss of energetic resources that were evidenced by the decrease of metabolic reserves via glycogen in the hepatopancreas.

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