

PREVALENCE OF THE MAJOR DISEASES IN *Penaeus vannamei* FARMED OF SINALOA MEXICO

Prevalencia De Las Principales Enfermedades En *Penaeus Vannamei* De Cultivo En Sinaloa, Mexico

María Soledad Morales-Covarrubias^{1*}, Noemí García-Aguilar¹, María del Carmen Bolan-Mejía¹ and Oscar Guillermo Zamora-García²

¹Research Center in Food and Development A.C., Mazatlán Unit in Aquaculture and Environmental Management in Mazatlán, Av. Sábalo Cerritos S/N, Mazatlán, 82100, Sinaloa, México. *Corresponding author. Email: marisol@ciad.mx. Orcid.org/0000-0001-6230-3718 Tel: +526699898700 Ext. 275; Fax: +526699898701 ²Postgraduate program in Marine Sciences and Limnology, Mazatlán Academic Unit, National Autonomous University of Mexico, Mazatlán, Sinaloa, México.

ABSTRACT

The aim of this study was to determine the prevalence and severity of the diseases: acute hepatopancreatic necrosis disease (AHPND), necrotizing hepatopancreatitis (NHPB) caused by intracellular bacterium (*Hepatobacter penaei*), septic hepatopancreatic necrosis (NHPS), and the white spot syndrome virus (WSSV) in farmed shrimp during two productive cycles (spring and summer) in the years (yrs) 2016 and 2017. From 360 studied shrimps, 90% showed external characteristics of apparently healthy animals, while only 10% of the specimens presented red coloration, soft cuticle, and muscular opacity. AHPND was the disease with the higher average prevalence for all the studied zones and seasons, with a prevalence of 53.33% in 2016 and 60.16% in 2017, followed by NHPS with 36.66% in 2016 and 42.83% in 2017. The incidence of WSSV was the lowest compared to the other diseases studied in this work, with 16.16% in 2016 and 20% in 2017; NHPB was only observed in summer with an average prevalence of 16.16% for both yrs. The northern area of Mexico registered the highest rates of mortality associated to these diseases, excluding WSSV, which resulted in being the most prevalent disease in the southern area (although such difference it is not statistically significant). The specimens diagnosed with AHPND exhibit severe damage in hepatopancreas as well as cellular detachment in the intestinal proximity. The organisms with NHPS showed multifocal cellular detachment in hepatopancreas and bacteria growth in heart, gills, lymphoid organ, connective tissue and muscles. The physicochemical variables considered for this study did not show significant variability among the zones and seasons analyzed, however, any alteration on these parameters might cause stress in the shrimps impairing the immune response against opportunistic pathogens. The presence of bacterial and viral diseases identified in this work is likely related to a low quality of the post-larvae, or an inadequate farming environment.

Key words: Diseases; AHPND; WSSV; NHPS; NHPB; shrimp

RESUMEN

El objetivo del presente estudio fue determinar la prevalencia y severidad de las enfermedades Necrosis Aguda del Hepatopáncreas (AHPND), Necrosis del Hepatopáncreas (NHPB) causado por bacterias intracelulares (*Hepatobacter penaei*), bacteriosis (NHPS) y el virus de la mancha blanca (WSSV), en camarones de cultivo durante dos ciclos (primavera y verano) en los años 2016 y 2017. Los resultados de los 360 camarones colectados mostraron características externas de animales aparentemente sanos (90%) y 10% con coloración rojiza, cutícula suave y opacidad muscular. AHPND fue la enfermedad con mayor prevalencia promedio para todas las zonas y temporadas con 53,3% en 2016 y 60,6% en 2017, seguida de NHPS con 36,66% en 2016 y 42,3% en 2017. La prevalencia de WSSV fue baja respecto a las demás con 16,16% en 2016 y 20% en 2017. NHPB se presentó en verano con prevalencias de 16,6 para 2016 y 2017. La zona norte presentó la mayor prevalencia de las enfermedades excluyendo a WSSV, misma que obtuvo una prevalencia mayor en la zona sur que en las demás zonas (sin embargo, dicha diferencia no es estadísticamente significativa). Los organismos con AHPND presentaron daños severos en hepatopáncreas con desprendimiento celular de la región proximal al intestino y NHPS con desprendimiento celular multifocal en hepatopáncreas y presencia de colonias de bacterias en corazón, branquias, órgano linfóide, tejido conectivo, músculo y ciegos hepáticos. Los parámetros fisicoquímicos en este trabajo no tuvieron diferencias significativas entre zonas en cada temporada, sin embargo, la alteración de alguno de ellos ocasiona estrés en los camarones, reduciendo la actividad anti-patógenos oportunistas. La presencia de las enfermedades bacterianas y virales identificadas en este trabajo probablemente estuvieron relacionadas con la calidad de la postlarva, o por un inadecuado ambiente de cultivo, propiciado por acumulación de materia orgánica

Palabras clave: Enfermedades; AHPND; WSSV; NHPS; NHPB; camarones

INTRODUCTION

Shrimp farming industry in Mexico generates significant revenues and employment on the States where it is carried out and has now become a vital development axis in rural areas [7] Nevertheless, diseases such as white spot syndrome virus (WSSV), septic hepatopancreatic necrosis (NHPS), hepatopancreatic necrosis (NHPB) caused by intracellular bacterium (*Hepatobacter penaei*), and acute hepatopancreatic necrosis (AHPND) have become the most common pathologies affecting the health of farmed shrimp and causing high mortalities in many shrimp farms.

Semi-intensive and intensive shrimp farms in Nayarit, México, suffered from massive mortalities of shrimps within the first 20 days (d) of farming in early April 2013. Almost simultaneously, the same problem was observed all around the Northwest area of Mexico (Sinaloa, Sonora y Nayarit), which is the most productive area of farmed shrimp in Mexico (90% of the total production). At present, high early-mortality rates have occurred in Chiapas, Tamaulipas, Yucatán, Colima, and Baja California (Valle de Mexicali); the latter two farm shrimp on low salinity water systems [18].

The principal characteristic of these disease outbreaks was AHPND with no visible infectious agents in the necrotic tissue. The AHPND diagnosis was carried out in a presumptive way using several techniques: findings in the production ponds (clinical signs); wet mount (wet analysis), from which three growing stages were determined (initial, acute and final phases); confirmation through histopathologic study (initial, acute and final phases); and by polymerase chain reaction (PCR) on infected shrimps individually until defining each bacterial taxonomy [18, 23].

Within the last three years there has been a steady increase in farming densities, while the correct management of the physicochemical variables has been decreasing [2]. This situation has promoted the spread of diseases with moderate to severe mortality that directly affects the rentability of the shrimp farming systems. Several strategies have been implemented to minimize the impact of high mortality caused by AHPND in Mexican shrimp farms: introduction of domesticated and genetically modified strains of white shrimp *Penaeus vannamei*; introduction of maternity techniques in shrimp farm; two-phase intensive production; closed systems; probiotic, prebiotic, and natural medicine; low farming density; and improvement of soil and water quality.

The aim of this study was to determine the prevalence and severity of the diseases: acute hepatopancreatic necrosis (AHPND), hepatopancreatic necrosis (NHPB) caused by intracellular bacterium (*Hepatobacter penaei*), septic hepatopancreatic necrosis (NHPS), and the white spot syndrome virus (WSSV) in farmed shrimp during two productive cycles (spring and summer) in 2016 and 2017 yrs.

MATERIALS AND METHODS

Area of study

The study was conducted in the State of Sinaloa, located in the northwest of Mexico (27° 02' - 22° 29' W; 105° 23' - 109° 28' N), using three regions: North, Guasave; Center, Navolato; and South, Mazatlán (FIG. 1).



FIGURE 1. GEOGRAPHICAL DISTRIBUTION OF STUDY LOCATIONS:

NORTH (N), CENTER (C), AND SOUTH (S), IN SINALOA, MEXICO

During the yrs 2016 and 2017, the study was carried out in six shrimp farms operating under a semi-intensive farming scheme with a density of 15 organisms/meters (m). A sampling location was selected if at least one farm in each region had previously reported cases of AHPND with an incident rate between 10 and 20%, severity grade of 1 and 2, and mortality rate of at least 15%. The sampling locations were selected using the diseases reports from 2014 and 2015 [16-18].

Sample collection

Sample collection was conducted two times per yrs (during May and September) in each of the selected farms (6 per yrs; 30 shrimps were randomly selected, totaling 360 samples in two yrs). The average weights of the organisms were between 2.12 ± 1 grame (g), for the first sampling and $15.38 \pm 3g$ for the second, in both yrs.

Capture of sample organisms were performed in four different spots, independent from the inlet and outlet gates of pond, using throw nets of 2 m in diameter. The collected shrimps were transported to the laboratory in plastic containers with aeration and filled with water from the sampled pond. The samples were physically inspected to detect any possible external alterations such as body coloring; melanization; deformation and necrosis of cuticle, gills, and appendixes; since these symptoms commonly indicate the presence of pathologies.

Preservation and sampling processing for histology

Organisms were injected with Davidson solution (Alcohol-Formaldehyde-Acetic Acid; AFA as recommended by Bell & Lightner [1], deposited in glass jars (250 and 500 milliliters (mL)) and kept them immersed for 48 hours (h). Subsequently, the samples were washed with regular water and preserved in 70% ethanol to be histologically analyzed using the Bell & Lightner [1] and Lightner [12]. General histopathological diagnosis to detect alteration in different organs and tissues of the sample organisms were performed using the protocols described by Bell & Lightner [1], Lightner [12], Tran *et al.* [25], and Morales-Covarrubias *et al.* [18]. A microscope with transmitted light (Olympus BX 60-Japan), using 4x, 10x, 40x, X60 and 100x objectives and a digital camera (Infinity 5.0 – Olympus Japan) mounted on the microscope were used for visual examination and image recording.

Bacteriology analysis

Organisms were disinfected with 70% ethanol; the exoskeleton was then removed while stomach and hepatopancreas were dissected. Samples were weighed (BOECO-FA-2204-Germany) and homogenized in a 2.5% NaCl sterile solution (1000 microliters (μ L)). Subsequently, 100 μ L homogenized samples were taken and deposited in 900 μ L 2.5% NaCl solution. From the latter solution a 100 μ L sample was deposited in thiosulfate agar – citrate – bile salts – sucrose (TCBS-Merck) and Marine Agar (AM-DIFCO), incubated for 24 h at 30 °C for total and green colony counting. The green colonies obtained from the TCBS plates were then transferred to CHROMagar™ Vibrio (chromogen medium for detection and isolation of *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae*) and incubated (VWR-Scientific-1516 USA) for 24 h at 30°C.

WSSV analysis

Nested polymerase chain reaction (Nested PCR) [12] was performed on gills and muscle fixed with 70% ethanol. The test

was carried out using an IQ 2000™ commercial kit for WSSV analysis (PCR TECH) for detecting DNA fragments of the WSSV's genome. The test uses a semi-quantitative direct PCR which diagnoses four infection levels: no infection, slight infection (20 DNA copies), moderate infection (200 DNA copies), and severe infection (2000 DNA copies). To validate the results, the test includes an internal control that identifies shrimp actin and a positive standard that is quantifiable according to the severity of the infection.

Statistical analysis

Disease prevalence

The prevalence determination of the four diseases were performed through estimation of the percentage of infected organisms in each of the sampling zones (three regions: North, Guasave; Center, Navolato; and South, Mazatlán), yrs (2016 and 2017) and season (spring and summer).

The statistic difference between zones and seasons of the yrs were evaluated through the χ^2 test. Moreover, the independence regarding the prevalence of both variables were evaluated using 3D-contingency tables (Microsoft Excel®). χ^2 homogeneity test was performed to evaluate the statistical differences between the prevalence of disease per zone. When no statistical difference was found, the average prevalence was used, for each disease under this condition, to have a comparing figure between seasons and other yrs.

Effect of water physicochemical variables on the prevalence of different diseases

A collinearity evaluation of the water physicochemical variables was carried out prior the assessment of the possible effects of these variables on the prevalence of the disease. The evaluation assumed that the variables change depending on the season on which are measured. Pearson correlation tests were conducted

TABLE I
PREVALENCE (%) OF ACUTE HEPATOPANCREATIC NECROSIS (AHPND), HEPATOPANCREATITIS NECROSIS CAUSED BY INTRACELLULAR BACTERIA (NHPB), SEPTIC HEPATOPANCREATIC NECROSIS (NHPS), AND THE WHITE SPOT SYNDROME VIRUS (WSSV) ON THE 3 ZONES AND SAMPLING SEASON DURING 2016 AND 2017

| | | Spring | Summer | Spring | Summer | Spring | Summer |
|------|-------|--------|--------|--------|--------|--------|--------|
| 2016 | AHPND | 60 | 43 | 97 | 30 | 43 | 47 |
| | NHPS | 23 | 70 | 20 | 43 | 27 | 37 |
| | WSSV | 13 | 20 | 7 | 20 | 10 | 27 |
| | NHPB | 0 | 50 | 0 | 30 | 0 | 17 |
| 2017 | AHPND | 50 | 97 | 67 | 47 | 50 | 50 |
| | NHPS | 40 | 50 | 50 | 27 | 50 | 40 |
| | WSSV | 10 | 13 | 27 | 23 | 27 | 20 |
| | NHPB | 0 | 50 | 0 | 27 | 0 | 20 |

[26].

Interaction between diseases

The interaction between diseases were evaluated using contingency tables (1x2) for all the possible combination of diseases, obtaining the coincidence percentage of presence for more than one disease in the same organism.

RESULTS AND DISCUSSION

From 360 analysed organisms, 90% showed external signs of apparently healthy animals, while 10% presented red colour, soft cuticle, and muscular opacity. The prevalence of the four major diseases was obtained for every zone, y and sampling season (spring and summer) as shown in TABLE I. The alterations description of the organs and tissues through histology was made in a decreasing order related to the medium value of the prevalence of each disease.

Bacteriology and histopathology analysis

Acute hepatopancreatic necrosis (AHPND), was observed on all three sampling zones con 53.33% of prevalence in 2016 and 60.16% in 2017. The infected organisms (degree 1 and 2) exhibited vermiform structures in the hepatopancreas lumen tubules (FIG. 2-A); cellular detachment of the hepatopancreatic tubules, from proximal to distal areas, revealing the tubular conduits (FIG. 2-B); and elongation of epithelial cells (FIG. 2-C) cells with picnotic nuclei. Bacteriologic assessment of the organisms with AHPND, whose infection was confirmed through PCR and histology, revealed 1×10^6 colony forming units (CFU) in the stomach, and 1×10^4 CFU in hepatopancreas.

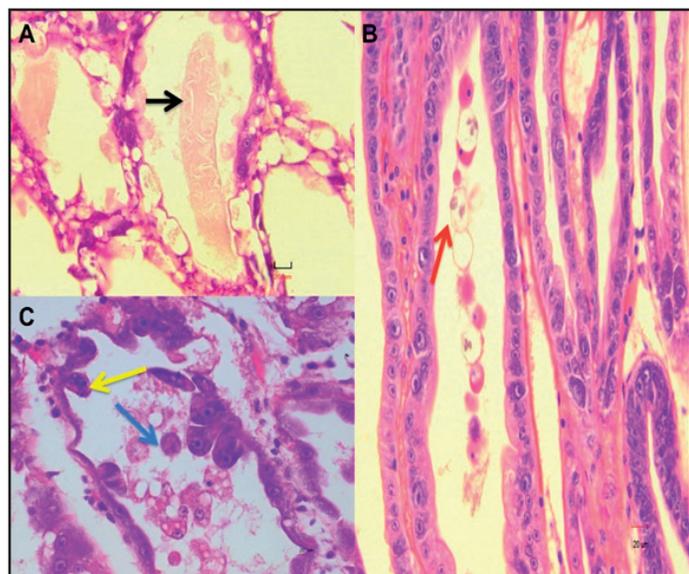


FIGURE 2. *Penaeus vannamei* HISTOLOGICAL SECTIONS OF HEPATOPANCREAS WITH AHPND THE VERMIFORM STRUCTURES ((A-BLACK ARROW) IN

THE HEPATOPANCREAS LUMEN TUBULES. CELLULAR DETACHMENT OF THE HEPATOPANCREATIC TUBULES ((B-BLUE AND C-RED ARROWS) FROM PROXIMAL TO DISTAL AREAS, REVEALING THE TUBULAR CONDUITS AND ELONGATION OF EPITHELIAL CELLS (C-YELLOW ARROW). STAINING METHOD: HEMATOXYLIN-EOSIN. SCALE BAR = 20 μm

Septic hepatopancreatic necrosis (NHPS) was detected in the three sampling zones and with an average prevalence of 36.66% in 2016 and 42.85 in 2017. The detected organisms were associated with one or two species of bacteria (*Vibrio parahaemolyticus* and *Vibrio harveyi*) in the hemolymph and hepatopancreas (1×10^5 UFC). The samples exhibited infiltration in haemocytes (FIGS. 3A – blue arrow), clustered in the center of haemocytic nodule (FIGS. 3A and 3B – black and red arrows), and infiltrations in the haemolymph (FIG. 3A-blue arrow); causing lumen's hypertrophy, melanization, necrosis, moderate to severe atrophy of the hepatopancreatic tubules (FIG. 3A, white arrow), and cellular detachment. Moreover, the hemocytes infiltration and hemocytes nodule formation in the presence of bacterial colonies were also spotted in heart, gills, connective tissue (FIG. 3C, yellow arrow), lymphoid organ (FIG. 3D, black arrow), muscle, and blind liver tissue with severity degrees of 2 and 3.

The white spot syndrome virus (WSSV) showed relatively low prevalence, 16.16% in 2016 and 20% in 2017 (TABLE I) according to PCR analysis (FIG. 4). WSSV was observed forming inclusion bodies (Cowdry Type C) in eosinophilic hypertrophied cells of the stomach epithelium, cuticular epithelium (FIG. 5), and in gills, all with severity degree 1 and 2.

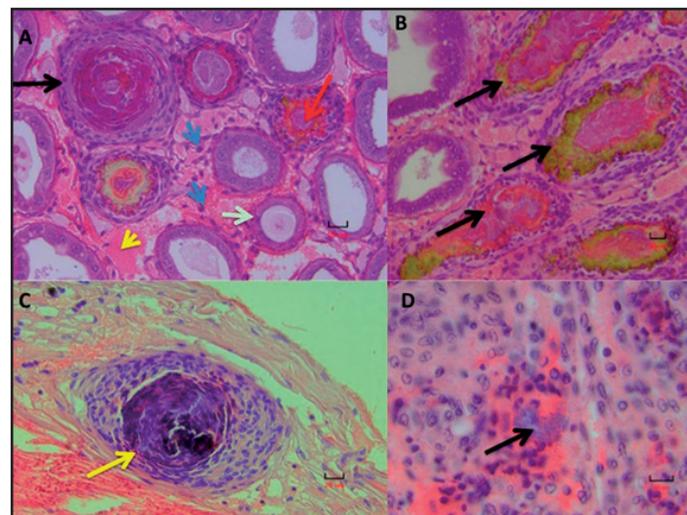


FIGURE 3. HISTOLOGICAL SECTIONS OF HEPATOPANCREAS (A AND B), CONNECTIVE TISSUE (B) AND LYMPHOID ORGAN (D) OF *Penaeus vannamei* WITH HEPATOPANCREATIC NECROSIS CAUSED BY BACTERIA (NHPS), SHOWING MELANIZED HAEMOCYTES NODULES WITH BACTERIAL CLUS-

TERS IN THE CENTER AREA (A, B AND C; BLACK, RED AND YELLOW ARROWS); HAEMOCYTES INFILTRATION (A, BLUE ARROW) AND HAEMOLYMPH (A, YELLOW ARROW); HEPATOPANCREATIC TUBULES ATROPHY (A, WHITE ARROW); CLUSTERS OF BACTERIA (D, BLACK ARROW). STAINING METHOD: HEMATOXYLIN-EOSIN. SCALE BAR = 10 μ m (D) And 20 μ m (A,B and C)

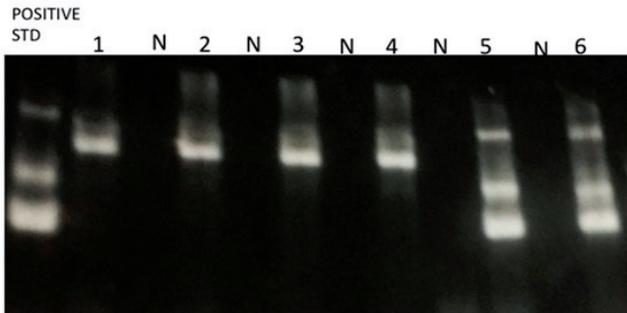


FIGURE 4. WSSV INFECTION LEVELS OBTAINED FROM NESTED PCR ANALYSIS (IQ 2000™). POSITIVE STANDARD (2000 COPIES), N: NEGATIVE. LINES: 1-3: NEGATIVE SAMPLES; 5-6: POSITIVE SAMPLES (2000 COPIES)

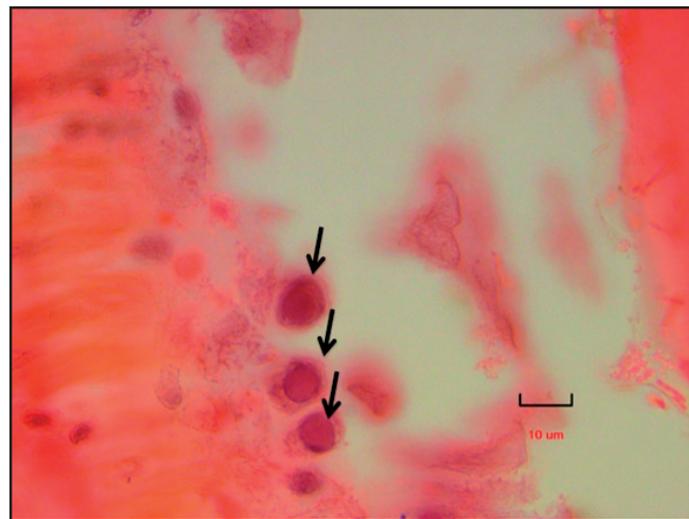


FIGURE 5. *Penaeus vannamei* HISTOLOGICAL SECTIONS OF CUTICULAR EPITHELIUM WITH INCLUSION BODIES (ARROWS) IN HYPERTROPHIED EOSINOPHILIC (DEGREE 1) NUCLEI. STAINING METHOD: HEMATOXYLIN-EOSIN. SCALE BAR = 10 μ m

Hepatopancreatic necrosis (NHPB) resulted in an average prevalence of 16.16% in 2016 and 2017, only during summer. The analyzed organisms presented tubular deformation, lumen hypertrophy, hemocytic infiltration, melanization, necrosis, intracellular bacteria, cellular detachment, and hemocytic encapsulation surrounding the atrophied tubules of the hepatopancreas, all with severity degree 1 and 3 (FIG. 6).

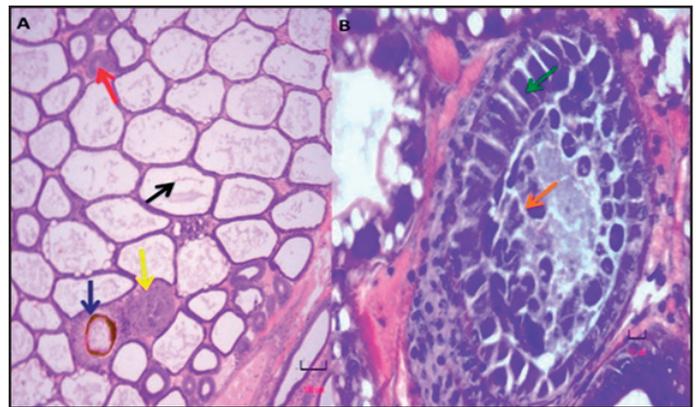


FIGURE 6. *Penaeus vannamei* HISTOLOGICAL SECTIONS OF HEPATOPANCREAS WITH TUBULAR ATROPHY (A: RED ARROW), LUMEN HYPERTROPHY (A: BLACK ARROW); INTERCELLULAR BACTERIA IN THE TUBULES CELLS CYTOPLASM (B: GREEN ARROW), INTERCELLULAR BACTERIA IN THE TUBULAR LUMEN (B: ORANGE ARROW); MELANIZED HEMOCYTIC NODULES (A: BLUE ARROW), AND HEMOCYTIC ENCAPSULATION SURROUNDING THE ATROPHIED TUBULES OF THE HEPATOPANCREAS (A: YELLOW ARROW) STAINING METHOD: HEMATOXYLIN-EOSIN. SCALE BAR = 10 μ m (b) AND 10 μ m (a)

Statistical analysis

The mutual independence test between the zone and the sampling season for each disease per y is summarized in TABLE II.

The hypotheses contrasting this test was:

TABLE II
Summary of the mutual independence test results that prove the relationship between the sampling zone and season for each disease per year

| Disease | Year | Test result (p value: χ^2) | Interpretation |
|---------|------|----------------------------------|----------------|
| AHPND | 2016 | 0.00003 | Rejects H_0 |
| | 2017 | 0.0015 | Rejects H_0 |
| NHPS | 2016 | 0.0024 | Rejects H_0 |
| | 2017 | 0.6248 | Accepts H_0 |
| WSSV | 2016 | 0.5253 | Accepts H_0 |
| | 2016 | 0.7107 | Accepts H_0 |

H_0 : There is independence between the sampling zone and season concerning the presence or absence of a determined disease during the sampled yrs.

H_1 : There is dependence between the sampling zone and season concerning the presence or absence of a determined disease during the sampled yrs.

TABLE III

Summary of the measured physiochemical variables on the farming ponds for each sampled zone, season and yrs

| Year | Zone Variable | North | | Center | | South | |
|------|------------------|--------|--------|--------|--------|--------|--------|
| | | Spring | Summer | Spring | Summer | Spring | Summer |
| 2016 | Temperature (°C) | 27 | 31 | 29 | 30 | 29 | 30 |
| | Salinity | 36 | 39 | 38 | 38 | 35 | 39 |
| | O2 (mg/l) | 6 | 5 | 7 | 6 | 7 | 6 |
| | PH | 7.5 | 8 | 7 | 8.5 | 7 | 9 |
| | Ammonia | 1 | 1.5 | 1 | 2 | 1 | 2 |
| 2017 | Temperature (°C) | 27 | 30 | 29 | 30 | 29 | 30 |
| | Salinity | 35 | 39 | 38 | 39 | 38 | 39 |
| | O2 (mg/l) | 7 | 6 | 7 | 6 | 7 | 6 |
| | PH | 7 | 8.5 | 7 | 8.5 | 8 | 8.5 |
| | Ammonia | 1 | 2 | 1 | 2 | 1 | 2 |

However, in the case of NHP, the evaluation did not follow the rules above mentioned, because the prevalence of this disease was null in spring; therefore the mutual independence was assessed between the sampling year and zone directly, showing that both variables are independent ($P > 0.05$). Although there was no significant difference between sampled y it existed between the sampling zones; the northern zone showed the higher average prevalence (25%), followed by the center (15%) and southern zone (9%).

In those cases where the H_0 about the mutual independence was rejected, a partial evaluation was performed to measure the degree of interaction between the presence of the diseases and the sampled zone or season. In all the cases where the null hypothesis was accepted, a sum was made with all the data and later contrasted through contingency tables (2x2) to evaluate the differences between yrs.

AHPND

The interaction tests between variables (zones, sampling season, and presence/absence of AHPND) rejected the H_0 ($P < 0.05$), implying the existence of the interaction between the causal variables and at least one of the variables that is being responsible for the variation of the presence/absence of the disease in each yrs

In 2016, there was no evidence of significant differences between the three sampling zones ($P > 0.05$) in the homogeneity test. Significant differences were only found for the sampling season, with the average prevalence of this disease being higher in spring (67%) than in summer (40%)

Contrary to the observations in the previous yrs in 2017 significant differences were found ($P > 0.05$) between sampling zones; the northern zone resulted with a higher prevalence (73.5%), fol-

lowed by the central zone (57%) and southern zone (50%). No significant evidence was found ($P = 0.2018$) after evaluating the AHPND prevalence rate in the two yrs of the study, regardless of the zone or season sampled.

NHPS

The interaction tests between variables (zones, sampling season, and presence/absence of NHPS) rejected the H_0 of mutual independence ($P < 0.05$).

In 2016, there was no evidence of significant differences between the three sampling zones ($P > 0.05$) in the homogeneity test. Significant differences were only found for the sampling season, with the average prevalence of this disease being higher in summer (50%) than in spring (23%).

Despite not having found significant differences between zones for this disease, the northern zone showed a higher prevalence of NHPS in 2017 (47%), compared to the central (32%) and southern (32%) zones.

-Interaction evidence was not found during 2017 between the variables (sampling zone and season), thus accepting the mutual independence hypothesis. Moreover, there were no meaningful differences observed between zones, season ($P > 0.05$) or sampled yrs for this disease.

WSSV

In 2016, WSSV showed no evidence of significant variation between zones (north: 17%; central: 13% and south: 18%), but between seasons of that yrs, showing increasing prevalence in the summer (spring 10% - summer 22%). However, in 2017, not enough evidence was obtained to statistically sustain seasonal variations (spring 21% - summer 19%). Additionally, the

homogeneity degree between zones remained still (north: 12%; center: 25% and south: 24; $P=0.7094$). Differences between yrs were not observed ($P=0.3375$).

Physiochemical variables of ponds

The most relevant measurements of the physiochemical variables of the farming ponds are shown in TABLE III.

The discrete nature of the values measured hinders the possibility of their inclusion in the calculation of the disease prevalence variation as causal factors. Moreover, the values showed high collinearity and, in this regard, the variation of all variables greatly depend upon the season of the yrs (spring/summer), including the average weight of the organisms, which is logically a time-dependent variable.

The TABLE IV summaries the values of the Pearson correlation.

Interaction of diseases

TABLE IV
COLLINEARITY ANALYSIS OF THE PHYSIOCHEMICAL VARIABLES AND THE SAMPLING SEASON (r =PEARSON CORRELATION COEFFICIENT)

| Variables | r |
|---------------------------|------|
| Season vs. Average weight | 1.00 |
| Season vs. Ammonia | 0.96 |
| Season vs. PH | 0.88 |
| Season vs. Oxygen | 0.80 |
| Season vs. Temperature | 0.79 |
| Season vs. Salinity | 0.73 |

The 85% of the total analyzed organisms exhibited at least one disease, while 55% of the organisms were affected with at least two diseases. It is difficult to establish a hypothesis about the causal relationship between one diseases with another. However, the percentage of concurrent incidence of two diseases was calculated (TABLE V).

TABLE V
PERCENTAGE (%) OF THE SIMULTANEOUS PRESENCE OF 2 DISEASES IN THE SAME ORGANISM

| Diagnosis 1 | Diagnosis 2 | % Coincidence |
|-------------|-------------|---------------|
| AHPND | NHPS | 19.72 |
| NHPS | WWSV | 9.44 |
| NHPB | NHPS | 8.89 |
| AHPND | NHPB | 7.78 |
| AHPND | WWSV | 6.94 |
| NHPB | WWSV | 2.50 |

AHPND and NHPS were the diseases with more prevalence in all the studied areas. Moreover, AHPND had the most significant impact on shrimp's health, judging by visible alterations in hepatopancreas through histopathology analysis. Severe stress and physical injuries exhibited by the shrimps, under unfavorable environmental conditions for farming, promote the organisms' susceptibility to some pathogens [14, 22]. Furthermore, the primary infection with AHPND probably weakened the organisms and made them more susceptible to the other infections reviewed in this study.

AHPND exhibited vermiform structures in the initial stage of the disease which is associated with the rupture of the membrane of the hepatopancreatic tubules [24]. Although this has not been reported previously for this disease in Asia [25], matches with the initial stage symptoms reported for this disease in Mexico [18, 23].

The results of the interaction of bacterial diseases are comparable with the reported by Lightner [13] in which different *Vibrios* are present in all marine crustaceans and become opportunistic pathogens when the natural defense of the host organism is suppressed or compromised. The stress derived from inadequate farming conditions is usually one of the most important factors that favor the development of disease in aquatic organisms, among which the presence of bacterial diseases [9, 13].

The members of the genus *Vibrio* have been associated with mortality of penaeid shrimps in several countries: *V. harvey*, *V. alginolyticu*, *V. parahaemolyticus*, and *V. mimicus* are some of the reported species linked to significant damage in all the stages of the farming of shrimp [10, 11, 13], *V. harvey*, *V. parahaemolyticus* and *Vibrio spp* have been reported in the hemolymph of apparently healthy shrimp, with an average load lower than 1×10^3 CFU without causing mortality [10].

The increase in nutrient availability after disinfection in combination with a destabilized and impoverished microbial community favors the growth of bacteria [3]. Considering that a type of *Vibrio* causes AHPND, this practice promotes the proliferation of the agent that causes AHPND in the pond instead of preventing its development. The same issue was also observed with luminescent vibriosis during the first 10-45 d after storing the shrimp postlarvae in the growth ponds. The disease outbreak was preceded by a substantial increase in the number of opportunistic vibrios in the pond [11]. The rise occurred after the disinfection of the pond and was linked to a disturbed microbial community and the presence of nutrients [3, 21].

Although there were no significant differences in the physiochemical variables measured, the toxicity generated by the nitrogenated compounds and unsuitable temperatures for shrimp culture induce stress in the organisms, reducing appetite, greater body's energy expenditure, deficiency in metabolic processes and lower activity against opportunistic pathogens [6].

The presence of WSS in the studied zones indicated severity degrees of 1 and 2, without apparently compromising the health of the affected shrimp and discarding the direct association with the low recorded survival rate. Regarding the lower prevalence of infection observed, it is probably due to better sanitary control in the larvae production laboratories, given that intensive sanitary campaigns have been promoted across the Country, and viral analyzes are currently carried out on the total number of broodstocks, which did not happen in the past. It is also possible that the low prevalence is due to an increase in AHPND, reported for the first time in 2013, causing mortalities of 100%.

Infections caused by intracellular or similar organisms in wild or farmed shrimp have been described [5]. However, the severe pathologies or epizootics associated specifically with NHPB, are only documented in Mexico, USA, Central and South America from 1986 until recent yrs [4, 8, 19]. The development of epizootics by NHPB has been reported in Texas (USA), Peru, Venezuela, Ecuador, Costa Rica, Mexico, Honduras, Nicaragua, Brazil, and Panama when there are prolonged periods of high temperatures (>29 -30C), together with high salinities (20-40gL⁻¹). NHP can go unnoticed for days and suddenly become an out-of-control event [12, 15, 20].

CONCLUSIONS

The 85% of all the organisms analyzed showed at least one disease and 55% at least two diseases. The disease with the highest prevalence on average for all the zones and seasons was AHPND with 53% in 2016 and 60.16% in 2017. NHPS was the second most prevalent with 36.66% in 2016 and 42.83% in 2017.

The prevalence of WSSV was relatively low compared to the rest of the diseases; 16.16% in 2016 and 20% in 2017. NHPB was only observed during the summer with a prevalence of 16.16% for both yrs.

The northern zone was the most affected since it had the highest prevalence of diseases excluding WSSV, which had a higher prevalence in the southern zone than in the other zones (however, this difference is not statistically significant).

The organisms with AHPND presented severe damage in hepatopancreas with cellular detachment of the proximal intestinal region. While the organisms with NHPS showed multifocal cellular detachment in hepatopancreas and presence of bacterial colonies in the hearth, gills, lymphoid organ, connective tissue, muscle and lymphoid organ, connective tissue, muscles, and blind liver spots.

The presence of bacterial and viral diseases identified in this study are considered to be related to the susceptibility of each organism or an inadequate culture environment, caused by physiochemical variables and organic matter accumulation; or by the alteration of the ecosystem caused by the usual practice of disinfection of ponds to eliminate potential pathogens or their carriers before depositing shrimp for their growth.

FUNDING INFORMATION

This work was partially funded by CIAD grant 2017710219-MCMS.

CONFLICTS OF INTEREST

The authors of this paper deny any financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

BIBLIOGRAPHIC REFERENCES

- [1] BELL, T.A.; LIGHTNER, D.V. A handbook of normal penaeid shrimp histology. **World Aquac. Soc.** Pp 1-114. 1988.
- [2] BOYD, C.E. Consideraciones Sobre La Calidad Del Agua y Suelo. En: Cultivos de Camarón. Métodos para mejorar la camaricultura en Centroamérica. Centro Investigación de Ecosistemas Acuáticos (Nicaragua). 1^{ra} Ed. Pp1-30. 2001.
- [3] BRATVOLD, D; LU, J; BROWDY, C.L. Disinfection, microbial community establishment and shrimp production in a prototype biosecure pond. **J. World Aquac. Soc.** 30: 422-432. 1999.
- [4] BRINES, B; ARANGUREN, L.F; SALAZAR, M. Fecal samples as DNA source for the diagnosis of necrotizing hepatopancreatitis (NHP) in *Penaeus vannamei* broodstock. **Dis. Aquat. Org.** 55:69-73. 2003.
- [5] BROCK, J.A; NAKAGAWA, L.K.; HAYASHI, T.; TERUYA, S.; VAN CAMPEN, H. Hepatopancreatic rickettsial infection of the penaeid shrimp, *Penaeus marginatus* (Randall) from Hawaii. **J. Fish Dis.** 9: 353-355. 1986.
- [6] CUI, Y; REN, X; LI, J; ZHAI, Q; FENG, Y; XU, Y. Effects of ammonia-N stress on metabolic and immune function via the neuroendocrine system in *Litopenaeus vannamei*. **Fish Shellfish Immun.** 64: 270-275. 2017.
- [7] DEL RÍO-SALAS, M.L; MARTÍNEZ-DURAZO, A; JARAMARINI, M. E. La acuicultura y su impacto en la zona costera del Golfo de California. **Rev. Cien. Biol. Salud. Biotec.** 3: 37-46. 2016.
- [8] FRELIER, P.F; SIS, R.F; BELL, T.A; LEWIS, D.H. Microscope and ultrastructural studies of necrotizing hepatopancreatitis in Pacific white shrimp (*Penaeus vannamei*) cultured in Texas. **Vet. Pathol.** 29:269-277. 1992.
- [9] GNANA-SELVAM, D; MUJEEB-RAHIMAN, K.M; MOHAMED-HATHA, A.A. An investigation into occasional white spot syndrome virus outbreak in traditional Paddy cum Prawn fields in India. **Sci. World J.** 2012: 1-11. 2012.
- [10] GÓMEZ-GIL, B; TRON-MAYEN, L; ROQUE, A; TURNBULL,

- J.F; INGLIS, V; GUERRA-FLORES, A.L. Species of *Vibrio* isolated from hepatopancreas, haemolymph and digestive tract of a population of healthy juveniles *Litopenaeus vannamei*. **Aquac.** 163:1-9. 1988
- [11] LAVILLA-PITOGO, C.R; LEAÑO, E.M; PANER, M.G. Mortalities of pond-cultured juvenile shrimp, *Penaeus monodon*, associated with dominance of luminescent *vibrios* in the rearing environment. **Aquac.** 164: 337–349. 1998
- [12] LIGHTNER, D. V. Diagnostic procedures for diseases in shrimp. A Handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp. **World Aquac. Soc.** Pp 1-220. 1996.
- [13] LIGHTNER, D.V; BELL, T.A; REDMAN, R.M; MOHNEY, L.L; NATIVIDAD, J.M; RUKYANI, A; POERNOMO, A. A review of some major diseases of economic significance in penaeid prawns/shrimp of the Americas and Indopacific. In: Shariff, M.; Subasinghe, RP; Arthur, JR (Eds.). **Diseases in Asian Aquaculture.** I. Fish Health Section. Asian Fisheries Society. Manila, Philippines. Pp. 57-80. 1992.
- [14] LIU, C.H.; CHEN, J.C. Effect of ammonia on the immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus*. **Fish Shellfish. Immunol** 16:321–334. 2004.
- [15] LIU, K. Histopathological study of the bacterial induced hepatopancreatitis of cultured shrimp (*Penaeus monodon Fabricius*). **Fish. Dis. Res.** 9:34-41. 1989
- [16] MORALES-COVARRUBIAS, M.S. Evaluación sanitaria de granjas camaronícolas con eventos de mortalidades asociados a la necrosis del hepatopáncreas en la región noroeste de México. Informe técnico final. CIAD-INAPESCA. Pp 1-15. 2014.
- [17] MORALES-COVARRUBIAS, M.S. Evaluación sanitaria preliminar de granjas camaronícolas con eventos de mortalidades asociados a la necrosis del hepatopáncreas en la región noroeste de México. Informe técnico final. CIAD-INAPESCA. Pp 1-10. 2015
- [18] MORALES-COVARRUBIAS, M.S; GARCÍA-AGUILAR, N; BOLAN-MEJÍA, M.C; PUELLO-CRUZ, A.C. Evaluation of medicinal plants and colloidal silver efficiency against *Vibrio parahaemolyticus* infection in *Litopenaeus vannamei* cultured at low salinity. **Dis. Aquat. Org.** 122: 57–65. 2016.
- [19] MORALES-COVARRUBIAS, M.S; RUIZ-LUNA, A; PEREIRA, MOURA-LEMUS, A; SOLIS-MONTIEL, V.T; CONROY, G. Prevalencia de enfermedades de camarón blanco (*Litopenaeus vannamei*) cultivado en ocho regiones de Latinoamérica. **Rev. Cientif. FCV-LUZ / XXI** (5): 434-446.2011.
- [20] MORALES-COVARRUBIAS, M.S; OSUNA-DUARTE, A.G; GARCIA-GASCA, A; LIGHTNER, D.V; MOTA-URBINA, J.C. Prevalence of necrotizing hepatopancreatitis in female broodstock of *Penaeus vannamei* with unilateral eyestalk ablation and hormone injection. **J.Aquat. Anim. Health.** 18:19-25. 2006.
- [21] NATRAH, F.M.I; BOSSIER, P; SORGELOOS, P; YUSOFF, F.M; DEFOIRDT, T. Significance of microalgal-bacterial interactions for aquaculture. **Rev. Aquac.** 6: 48–61. 2014.
- [22] PRAYITNO, S.B; LATCHFORD, J.W. Experimental infections of crustaceans with luminous bacteria related to Photobacterium and *Vibrio*. Effect of salinity and pH on infectiosity. **Aquac.** 132:105–112. 1995.
- [23] SOTO-RODRIGUEZ, S.A; GOMEZ-GIL, B; LOZANO-OLVERA, R; BETANCOURT-LOZANO, M; MORALES-COVARRUBIAS, M.S. Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (*Litopenaeus vannamei*) in Northwestern Mexico. **Appl. Environ. Microbiol.** 81 (5): 1689–1699. 2015.
- [24] SRIURAIRATANA, S; BOONYAWIWAT, V; GANGNONNGIW, W; LAOSUTTHIPONG, C; HIRANCHAN, J; FLEGEL, T.W. White Feces Syndrome of Shrimp Arises from transformation, sloughing and aggregation of hepatopancreatic microvilli into vermiform bodies superficially resembling gregarines. **PLoS ONE** 9(6): e99170. 2014.
- [25] TRAN, L; NUNAN, L; REDMAN, R.M; MOHNEY, L.L; PANTOJA, C.R; FITZSIMMONS, K; LIGHTNER, D.V. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. **J. Dis. Aquat. Organ.** 105: 45–55. 2013.
- [26] ZUUR, A; IENO, E.N; WALKER, N; SAVELIEV, A. A; SMITH-GRAHAM, M. Limitations of linear regression applied on ecological data. **Mixed Effects Models and Extensions in Ecology with R.** Vol. 53. Pp 11-33. 2009.