The Effect Of Salinity On Enterocytozoon Hepatopenaei Infection In Penaeus Vannamei Under Experimental Conditions

Luis Fernando Aranguren (lfarangu@email.arizona.edu)
University of Arizona https://orcid.org/0000-0002-4126-9431
Faris Alghamdi
Ministry of Environment, Water and Agriculture
Katrien De Belder
University of Arizona
J. Lin
University of Arizona
Hung N. Mai
University of Arizona
Jasmine Millabas
University of Arizona
Y. Alrehaili
Ministry of Environment, Water and Agriculture
Anwer Alazwari
Minisytry of Environment, Water and Agriculture
Saif Algetham
Ministry of Environment, Water and Agriculture
Arun K. Dhar
University of Arizona

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Abstract

Background: *Enterocytozoon hepatopenaei* (EHP) is an enteric pathogen that affects *Penaeus vannamei* and *P. monodon* shrimp in many SE Asian countries. In the western hemisphere, EHP was reported for the first time in 2016 in farmed *P. vannamei* in Venezuela. Anecdotal evidence suggests that EHP is more prevalent in grow-out ponds where the salinity is high (>15 parts per thousand (ppt) compared to grow-out ponds with low salinities (<5 ppt). Considering that *P. vannamei* is an euryhaline species, we were interested in knowing if EHP can propagate in *P. vannamei* in low salinities.

Result: In this study, we described an experimental infection using fecal strings as a source inoculum. Specific Pathogen Free (SPF) *P. vannamei* were maintained at two different salinities including 2 ppt and 30 ppt, and continuously challenged using feces from known EHP-infected *P. vannamei* over a period of three weeks. The fecal strings, used as a source of EHP inocula in the challenges, was sufficient to elicit infection in shrimp maintained at both salinities. Infectivity of EHP in shrimp reared at 2 and 30 ppt salinities was confirmed by PCR and histopathology. The prevalence and the severity of EHP infection was higher at 30 ppt than 2 ppt.

Conclusion: The data suggest that fecal strings is a reliable method to conduct EHP challenges via fecal-oral route. EHP infection can occur at a salinity as low as 2 ppt, although the prevalence and the severity of EHP infection is higher at a salinity of 30 ppt compared to 2 ppt.

Background

Hepatopancreatic microsporidiosis (HPM) caused by *Enterocytozoon hepatopenaei* (EHP) is a disease that has been reported in several penaeid shrimp including *Penaeus monodon* and *P. vannamei* [1, 2]. EHP has been reported in different regions including Asian countries such as China, Indonesia, Malaysia, Vietnam, Thailand and India [3–6]. Recently, EHP has also been reported in the western hemisphere in Venezuela [7].

The main clinical signs of EHP are growth retardation [2, 8] that leads to increased size variability. Whitish fecal strings floating on the pond´s surface and the presence of shrimp displaying white discoloration of the gastro intestinal tract (GI tract) in these ponds have also been associated with EHP [3, 9]. In advanced stages of the disease, EHP-infected shrimp typically display soft shells, lethargy, reduced feed intake, an empty midgut and chronic mortalities. EHP is an intracellular microsporidium that cause lesions in the hepatopancreas (HP) tubule epithelial cells. EHP replicates within the cytoplasm of the affected cells. Histology of EHP-infected shrimp shows irregular/regular basophilic inclusion bodies within the cytoplasm with or without the presence of spores. Additional histological lesions include mild to severe sloughing of the tubular epithelial cells usually with presence of mature spores. Also, the presence of spores are observed in the lumen of the HP tubules and the GI tract [2, 9].

In countries where EHP has been reported, such as India, China, Vietnam, and Venezuela, shrimp farming is carried out in different environments including coastal marine areas, estuarine as well as inland areas.

For instance, in certain areas of Andhra Pradesh in India, the salinities in the grow-out ponds can fluctuate between 0-30 ppt with an average salinity of about 10 ppt, as the ponds are filled with borehole water mixed with estuarine water. In contrast, in some states in the western part of the country, such as Gujarat, salinities can vary between 30-44 ppt [10, 11]. In the western hemisphere in Venezuela, the shrimp farms are located in two areas. One of these areas is in the Maracaibo lake where the salinity range between 2-5 ppt. The other area is located in the northern part of the country where shrimp farms are located in the marine environment where salinity range between 20-40 ppt [12] . Interestingly, in both environments EHP has been reported. In these countries, the incidence of EHP seems to be higher in high salinity environments, but there is no study to support the possible relationship between salinity and presence of EHP. For this reason, the objective of this study was to compare the infectivity of EHP using fecal strings as inoculum in two different salinities under experimental conditions.

Results

Survival rates

The final survival at the end of the EHP challenge was high, ranging from 90-100% in both salinities in each of the two independent experimental challenges (Table 1). The survival percentage in the control treatments were 100% in both salinities in the two independent challenge experiments.

Table 1 Final survival in *Penaeus vannamei* challenged with fecal string as EHP inoculum in two independent experimental challenges at two different salinities. The data represented as Mean ± SD.

Experimental	Challenge Duration	Final survival (%)			
		2 ppt treatment	2 ppt control	30 ppt treatment	30 ppt control
1	20 days	95±7	100±0	100±0	100±0
2	26 days	90±0	100±0	90±7	100±0

Quantification of EHP in fecal strings by qPCR

Fecal strings were collected on a daily basis from the EHP-infected tanks. The daily fecal string samples were tested positive for EHP in both challenges. The average weight of fecal strings added to each tank was 1.17± 0.52 g. and 0.32±0.24 g. for the challenge #1 and #2, respectively.

The EHP copy number in the fecal strings used as inoculum was significantly higher in the challenge #2 (p<0.05). EHP copy number in the fecal string in challenge #1 was $1.6x10^3 \pm 2.1x10^3$ copies /ng DNA compared to $1.1x10^6 \pm 2.0x10^6$ copies /ng DNA in the inoculum of challenge #2 (Fig 1).

Prevalence of EHP by histopathology

The prevalence and severity of EHP in shrimp experimentally challenged using fecal strings as inocula in two independent challenges was assessed by H&E histology.

In both experimental challenges, the fecal strings used as inocula was able to provoke the diseases in SPF shrimp. The prevalence of EHP was of 25% in the challenge #1 vs. 64.3% in the challenge #2. The data confirms that fecal strings are a suitable method for experimental infection of EHP.

The prevalence of EHP at 2 ppt and 30 ppt salinities in the challenge #1 was 25%. In challenge #2, the prevalence of EHP at 2 ppt and 30 ppt was 33% and 87.5%, respectively. The degree of severity was also higher at salinity of 30 ppt in the second experiment (Table 2). In challenge #2, 50% of the EHP-infected population at 30 ppt displayed grade G3 (moderate to severe) and G4 (severe) lesions caused by EHP infection. The different grades of severity in this study are shown in Fig 2. Fig 2, panels A, B and C show tissue sections of HP at a low, medium and high magnification of HP of a healthy shrimp from the control tank without showing any histological lesions of EHP. In contrast, in panels D, E and F HP tissues displaying grade G1 of EHP infection are shown. A focalized region within the HP is observed (Fig 2D). The affected tubule shows the distinctive cytoplasmic inclusion bodies in the cytoplasm of the affected epithelial tubule cells that correspond to the uninucleate meront stage (Fig 2E -F). Fig 2, panels G, H, & I show grade G2 of EHP infection (low to moderate). A focal presence of EHP infection in few affected HP tubules epithelial cells are observed. Both meront stage and spore liberated into the lumen are observed (Fig 2I). Fig 2 J, K and L show a typical grade G3 of EHP infection. Multifocal lesions in HP tubules epithelial cells are observed (Fig 2J). In the affected tubules, the presence of both irregular multinucleated plasmodium and spores within the cytoplasm of the cuticular epithelial cells were observed (Fig 2L). Fig 2 M, N and O show the grade G4 EHP infection with multifocal tubules containing infected HP cells (Fig 2M). Both multinucleated plasmodium and spores within the cytoplasm of the affected cells as well as spores with lumina of the tubules was observed (Fig 2O).

Table 2 A summary of EHP histological findings of *P. vannamei* exposed to EHP-containing fecal strings in two independent challenges at two different salinities. G0-G4 indicates the degree of severity (Lightner, 1996).

Challenge	Salinity (2 ppt)		Salinity (30 ppt)		Combined data	
	Severity G0-G4 (number of positive / number of total shrimp	Prevalence (%)	Severity G0-G4 (number of positive / number of total shrimp	Prevalence (%)	Severity G0-G4 (number of positive / number of total shrimp	Prevalence (%)
#1	G4 (1/4) G0 (3/4)	25.0%	G1 (1/4) G0 (3/4)	25.0%	G4 (1/8) G1 (1/8) G0 (6/8)	2/8 (25.0%)
#2	G3 (1/6) G1 (1/6) G0 (4/6)	33.3%	G4 (3/8) G3 (1/8) G2 (2/8) G1 (1/8) G0 (1/8)	87.5%	G4 (3/14) G3 (2/14) G2 (2/14) G1 (2/14) G0 (5/14)	9/14 (64.3%)

Detection of EHP in hepatopancreas by PCR

The hepatopancreas of SPF shrimp challenged with fecal strings obtained from EHP-infected shrimp were positive to EHP by nested PCR in both tanks at both salinities. This confirms the presence of EHP in the treatment tanks from the challenge #1 and challenge #2. In both salinities, 2 ppt and 30 ppt, EHP was detected. The hepatopancreas tissue derived from animals reared at 2 and 30 ppt salinities in the negative control treatments tested negative for EHP by nested PCR.

Discussion

Since 2009, HPM caused by EHP emerged as an economically important disease being highly prevalent in some of the major shrimp producing countries in SE Asia, and more recently the pathogen has spread to Venezuela [13]. In SE-Asian countries, along with white spot disease (WSD) and acute hepatopancreatic necrosis (AHPND), HPM remains a major threat contributing to significant economic losses in grow-out ponds. *Penaeus vannamei* is an euryhaline species which is raised in a wide range of conditions including high salinities (30 ppt), estuarine environments (10-20 ppt), and low salinities (2 ppt) [14]. In this study, we investigated the prevalence and severity of EHP infection in two salinities, high salinity (30 ppt) and low salinity (2 ppt) under laboratory challenged conditions. The fecal strings, used as a source of EHP inocula, in the challenges was sufficient to cause disease to shrimp maintained at both salinities, as confirmed by histopathology and PCR. The results from this study provide a new EHP infection method through fecal strings via fecal-oral route. Previous studies have shown some routes of infection included cohabitation [15, 16], reverse gavage, and direct hepatopancreas injection with EHP-inoculum [16]. EHP lesions, including the presence of plasmodium in the cytoplasm of an infected cells and the mature spores within the cytoplasm or released spores in the lumen, were found by histopathology in EHP-challenged shrimp reared at both salinities. This unequivocally confirms EHP infection at a wide range of salinities as high as 2 to 30 ppt. When the initial inoculum used for the experimental challenge was low, i.e. $1x10^3$ copies of EHP/ ng of total HP DNA, the HPM prevalence was similar (i.e. 25%) irrespective of the salinities (See Table 1). However, the prevalence of EHP infection increased at 30 ppt salinity (87.5% prevalence) compared to 2 ppt salinity (33.3% prevalence) when the inoculum level was increased from $1x10^3$ to $1x10^6$ copies of EHP/ ng of total HP DNA in the challenge experiment (i.e. challenge #2). Dose-dependent challenge has been well documented for other shrimp pathogens such as AHPND and *Hepatobacter penaei* [9, 17]. In AHPND challenge tests, an infectious dose of $2.0x10^6$ CFU/ml is required to provoke high mortality (>90%) in immersion challenge method. In contrast, a lower dose ($2.0x10^4$ CFU/ml) does not cause mortality nor histological lesions in the challenged population [9]. In the present study, the inoculum with low copy number ($1.6x10^3$ copies/ng HP DNA) used in the challenge #1 caused mild infections in the challenged shrimp, whereas, severe infections (Grades G3 to G4) and a higher prevalence occurred in the challenge #2 when the EHP copy number in the inoculum was higher (i.e. $1x10^6$ copies/ng HP DNA).

The histological lesions in shrimp maintained at 30 ppt salinity were more severe. Moderate-severe grade of infection (G3-G4) were found in 50% of the shrimp affected. In contrast, only 16% of shrimp reared at salinity of 2 ppt showed grade G3 level of infection, according to the Lightner's scale [18]. The difference in the severity of EHP infection at two different salinities was probably due to the differential effect of salinity on spore germination. One of the critical phases in the spore germination is the increase of intra-spore osmotic pressure. It is possible that the difference in salinities led to a hypotonic environment at 2 ppt compared to hypertonic environment at 30 ppt. It is possible that the hypertonic solution enhances the germination of the spore by increasing the spore activation process. He et al [19] found a difference of eversion of the polar tube in different osmotic environments in *Encephalitozoon intestinalis*, an obligate intracellular microsporidium that causes gastrointestinal diseases in immunocompromised and immunocompetent people. Differences in polar tube germination associated to change in salinity have also been reported by other researchers. De Graff et al. showed an increase in germination of *Nosema apis* spores at 0.5 N NaCl concentration [20] and an increase of germination of *Nosema algerae* spores at 0.1 M NaCl concentration vs. 0.05 M NaCl [21, 22]. In our study, the NaCl concentration was about 0.5 M NaCl vs. 0.03 M at 30 ppt and 2 ppt, respectively. This difference could explain in part the effect on germination of EHP spores and resulting prevalence levels.

Hardness is another variable that was different in the two salinities used in this study and could have been a factor that affect the spore germination. The hardness in low salinity (2 ppt) was about 240 mg/L (City of Tucson https://www.tucsonaz.gov/water/water-quality-reports-and-publications), vs. the marine water artificially prepared at 30 ppt that was around 1575 mg/L (Crystal Sea, Marinex). It has been reported that calcium is an important second messenger that activates many cell events and calcium influx might be in part responsible for the activation of microsporidian spore discharge at higher salinities [23].

In grow-out ponds in some EHP endemic areas in Asia, the salinity conditions are found to vary widely. For example, in India there are some shrimp farming areas in high and low salinity, and the prevalence of EHP seems to be lower at lower salinities (below 5 ppt) as observed in Andhra Pradesh in 2019 (Aranguren et al., Unpublished data). Similar conditions were recorded in two major shrimp farming areas in Venezuela, i.e. Maracaibo lake where the salinities are around 4-6 ppt, and in Falcon state where the salinities varies from 36-40 ppt. In Venezuela, shrimp farming is not fully integrated, and the movement of nauplii and post-larvae between Falcon and Maracaibo lake area is a common practice. This suggests that EHP-infected PL or broodstock could have been moved between these two zones in a similar way. However, EHP has only been detected in the Falcon area where the salinities are high. In the Maracaibo's lake where the salinities are low, EHP has not been reported yet. One possibility that has limited the EHP infection could have been the difference in water salinity.

Conclusion

This study demonstrated that fecal strings from known EHP-infected shrimp could be used as a reliable source of inoculum to conduct EHP experimental infections via fecal-oral route. EHP infection can occur

at a low salinity (i.e. 2 ppt) although the prevalence and the severity of infection is higher at a salinity of 30 ppt. These findings have implications in disease management in EHP-endemic areas.

Methods

Shrimp

Specific pathogen free (SPF) *Penaeus vannamei* were obtained from a commercial vendor in the US. The bioassays were carried out in the Aquaculture Pathology Laboratory of The University of Arizona.

Enterocytozoon hepatopenaei bioassay

The EHP isolate used in this study was obtained from a SPF population of *P. vannamei* infected with EHP (Thailand isolate). Two independent EHP challenges were conducted. In both challenges, the pathogenicity of EHP in SPF juvenile shrimp maintained at two different salinities, 2 ppt and 30 ppt were compared. For each experimental challenge, four 90-L tanks were filled with artificial seawater (Crystal Sea Marinex, Baltimore, Maryland) for each corresponding salinity with two replicates for each salinity treatment. Temperature was adjusted at 25°C (±0.6), pH ranged from 7.5-8.0 and the salinity was adjusted by changing 3 parts of salinity every hour from 25 ppt (Initial salinity of the SPF population) down to 5 ppt. From 5 ppt to 2 ppt, each salinity part was changed every two hours. Ten (10) SPF *P. vannamei* (weights: 2.0-2.1 g) were stocked in each tank for the experimental infection, respectively. Two 90-L control tanks were set up for each salinity as negative control treatment. The experiments were repeated twice.

Inoculum preparation

Throughout the experiments, EHP-infected shrimp were fed daily at 5% of biomass. Every day, one hour after feeding, fecal strings were collected by siphoning. For the challenge #1, the fecal strings were pooled from three tanks with 5 shrimp per tank (mean weight 12.5 g). For the challenge #2, EHP inoculum was made by pooling fecal strings from one 1000 L tank with 60 EHP-infected shrimp (mean weight 9.0 g). The fecal samples were weighed and aliquoted in five equal parts. Four aliquots were used as inoculum for the experimental infection of each treatment tank. The remaining aliquot was preserved in 95% ethanol for EHP detection by PCR following a previously published protocol [4].

Survival was recorded daily from the start of the challenge. At the end of the challenge, shrimp samples from the challenge #1 and #2 were fixed in Davidson's (AFA) fixative [24]. Details of the number of samples is described in Table 2. The duration of the challenges were 20 and 26 days for the challenge #1 and #2, respectively. Pooled hepatopancreas tissue from 3-4 shrimp were collected from each tank and preserved in 95% ethanol for EHP detection by PCR.

Histopathology

The Davidson's alcohol-formalin-acetic acid (AFA)-fixed samples were processed, embedded in paraffin, and sectioned (5 µm thick) in accordance with standard methods [24] [18]. After staining with hematoxylin and eosin (H&E), the sections were analyzed by light microscopy. Severity grades of the EHP infection/lesion ranged from G0-G4 according to Lightner (1996) with G0 being absence of the disease and G4 being presence of severe lesions and advanced tissue destruction.

Conventional and quantitative PCR in detecting and quantifying EHP

EHP detection was carried out by a conventional nested PCR that targets the Spore Wall Protein 1 (SWP1) gene. The primers for the first step are: SWP 1F/1R (1F: 5'-TTG CAG AGT GTT GTT AAG GGT TT-3, 1R: 5'-CAC GAT GTG TCT TTG CAA TTT TC-3'). These primers generate a 514 bp amplicon. The primers for the nested step are: SWP 2F/2R (2F:5'-TTG GCG GCA CAA TTC TCA AAC A-3', 2R:5'-GCT GTT TGT CTC CAA CTG TAT TTG A 3') and generate a 148 bp amplicon. PCR profile was followed based on the published method [25]. For EHP quantification by real-time PCR, primers F:157 (5'-AGT AAA CTA TGC CGA CAA-3') and R:157 (5'-T TAA GCA GCA CAA TCC-3'), and a TaqMan probe (5-FAM-TCC TGG TAG TGT CCT TCC GT-TAMRA-3') were used following a previously published method [26].

Statistical analysis

The EHP copy number obtained from the quantification analysis was transformed to log base 10 value prior to carrying out statistical analysis using SPSS v16.0. A one-way ANOVA with Tukey's multiple comparison were performed and *P*-value <0.05 was considered as statistically significant.

Abbreviations

SE Asia: South East Asia; SWP: Spore Wall Protein; AFA: Alcohol-Formalin-Acetic acid; SPF: Specific Pathogen Free; ppt: part per thousand.

Declarations

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Author contributions

LF Aranguren conceptualized the study, wrote the manuscript, carried out the histopathology and provided full project oversight. F. Alghamdi, J. Lin, Y. Alrehaili, A. Alazwari, S. Algetham contributed in the bioassay, K. Debelder and J. Lin contributed with the DNA extractions and PCR. H.N Mai contributed with the experimental design, data analysis and confirmation of some test by PCR. A K. Dhar contribute with the review and editing of the manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Competing of interests

The authors declare that they have no competing interests.

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Figures

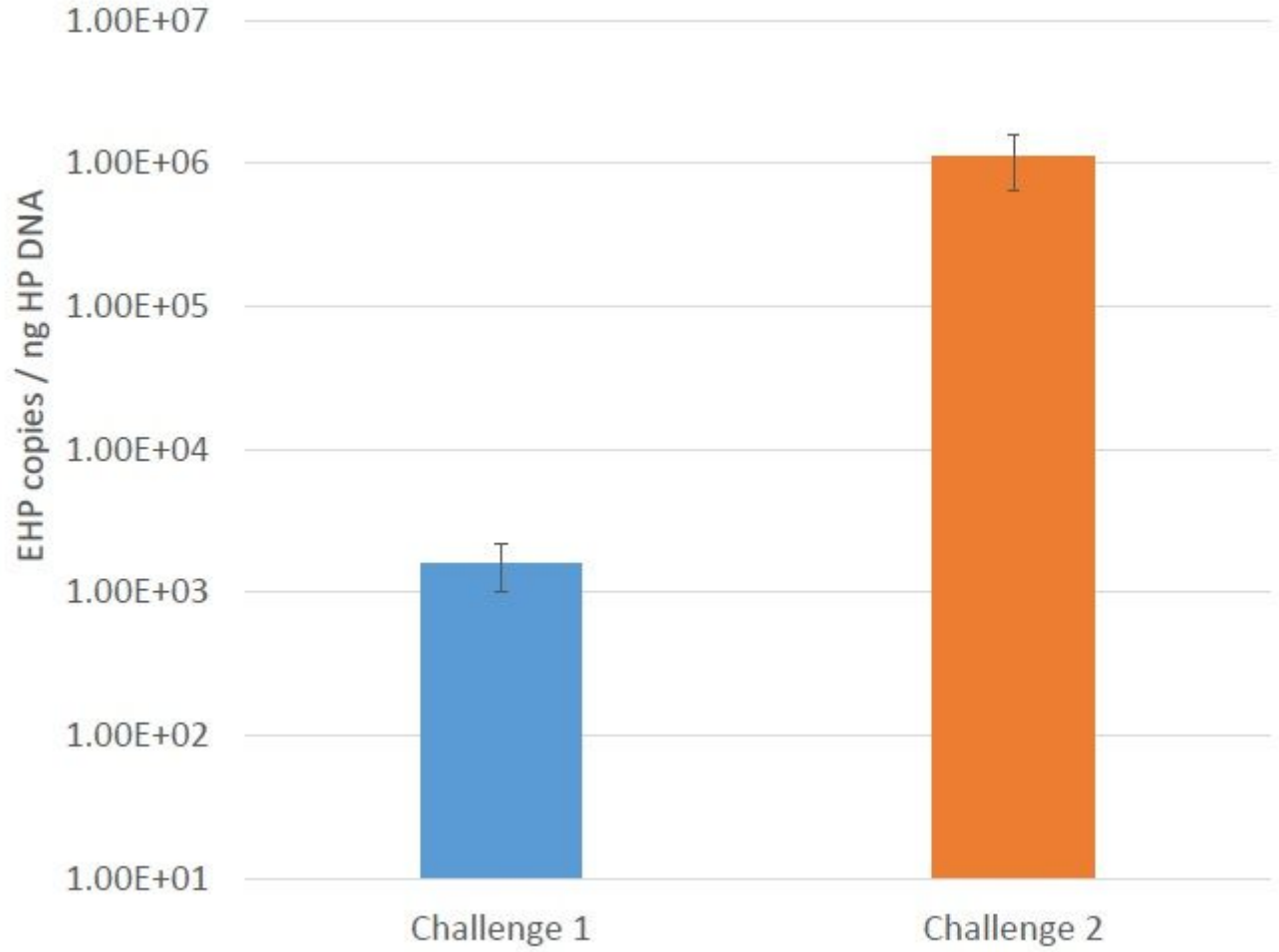

Figure 1

EHP copy number in fecal strings added daily to the tanks in challenge #1 and #2. Samples analyzed by Real time PCR. Data set is represented with the Mean±SE Letters a and b indicate significant differences ($p<0.05$).

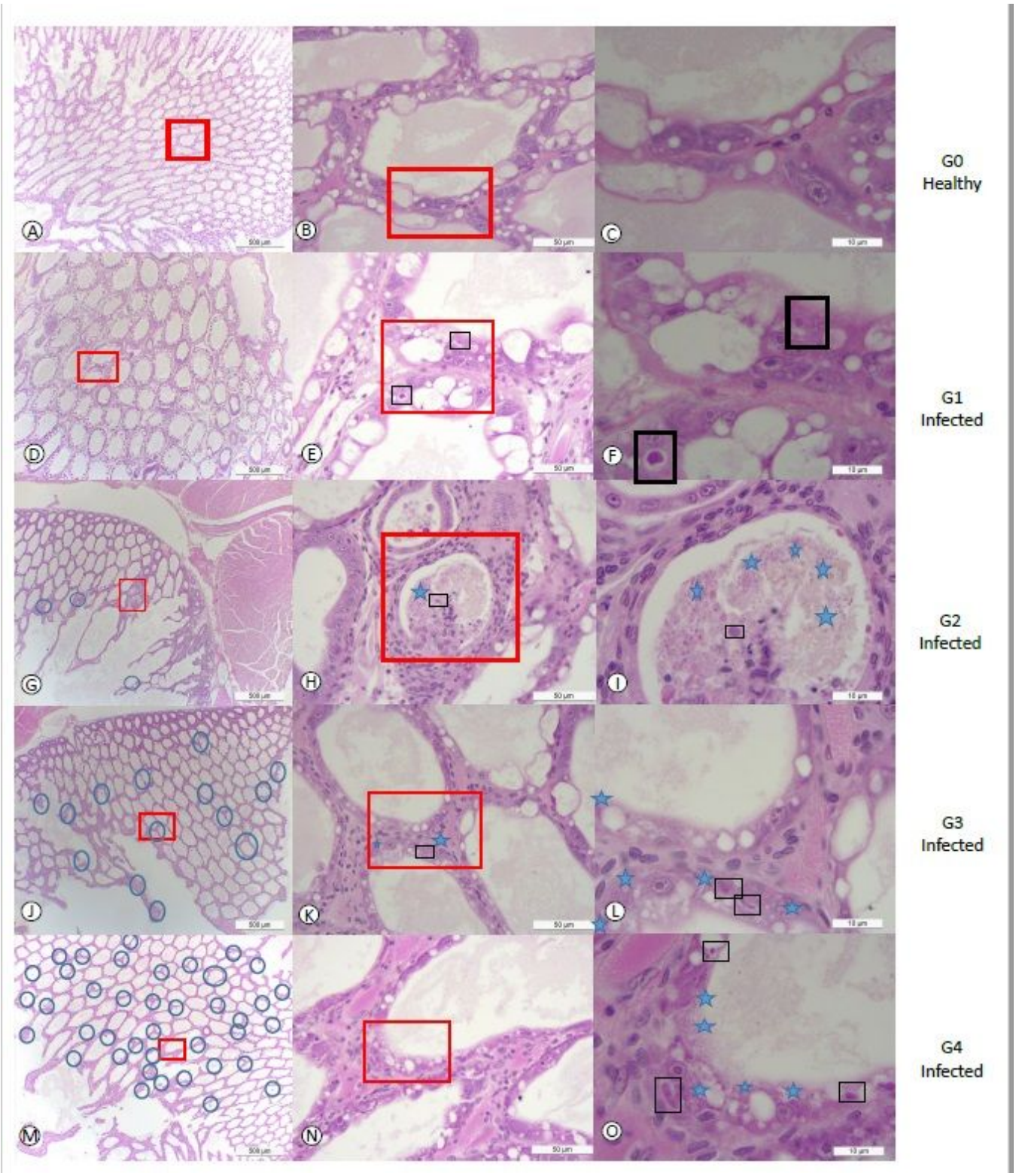

Figure 2

H&E (Mayer–Bennet hematoxylin and eosin-phloxine) staining of hepatopancreas tissue of Penaeus vannamei showing the presence of different stages of EHP infection A-C: grade 0; D-F: grade 1; G-I: grade 2; J-L: grade 3;. M-O: grade 4 of EHP infection. Mature spores are indicated by blue stars. Black square shows the typical regular-irregular plasmodium stage. Red square outlines regions that are magnified from the lower magnification. Scale bars are located in the inferior right part of each figure.