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RESEARCH ARTICLE

Experimental Infection of *Enterocytozoon hepatopenaei* (EHP) by Water and Sediment Transfer Between Pacific White Shrimp (*Penaeus vannamei*) and Green Mud Crab (*Scylla paramamosain*)

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A B S T R A C T

Enterocytozoon hepatopenaei (EHP) is an important shrimp pathogen, causing growth retardation syndrome which leads to substantial economic losses worldwide. In this study, we examined the possibility of EHP transmission between Pacific white shrimp (*Penaeus vannamei*) and green mud crabs (*Scylla paramamosain*), a common benthic species in shrimp culture environments. Naturally infected shrimp (with EHP loads ranging from 10^2 to 10^6 copies/µL) were used as donors for EHP transmission to EHP-free crabs and shrimp through water and sediment transfer. The recipient shrimp became EHP-positive 7 days post-exposure (dpe) (with EHP loads ranging from 10^1 to 10^4 copies/µL). Histopathological examination confirmed EHP spores in the hepatopancreatic cells of the recipient shrimp at 7 and 14 dpe. The recipient crabs were EHP-positive after 14 dpe (EHP loads between 10^1 and 10^2 copies/µL) and the crabs could transmit EHP back to the recipient shrimp (EHP loads ranging from 10^1 to 10^2 copies/µL) 14 dpe *via* the same route. Although the crabs tested positive for EHP through PCR and qPCR, no histopathological change was observed. The present study suggests that green mud crabs may act as a mechanical vector for EHP transmission, providing information to enhance biosecurity protocols in shrimp farms to reduce the risk of EHP contamination.

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INTRODUCTION

Enterocytozoon hepatopenaei (EHP) is one of the most important causes of growth retardation syndrome in cultured shrimp nowadays. The infection of EHP is also known as hepatopancreatic microsporidiosis (HPM). An initial report of EHP was published in 2004 from cultured black tiger shrimp (*Penaeus monodon*) in Thailand)Chayaburakul *et al.*, (2004. EHP has been reported in most of the important cultured shrimp and prawn, such as Pacific white shrimp (*Penaeus vannamei*), kuruma prawn (*Penaeus japonicus*), and Indian prawn (*Penaeus indicus*) (Naveen *et al.*, 2025). Several major-shrimp producing countries has been affected by this problem, such as Thailand) Chayaburakul *et al.*, (2004, India (Rajendran *et al.*, 2016), Indonesia (Desrina *et al.*, 2020), Korea (Kim *et al.*, 2022), Malaysia (Wan Sajiri *et al.*, 2023), and China (Dewangan *et al.*, 2023). Even though EHP does not cause high mortality, economic loss from EHP infection is severe, not only from wasting resources due to slow growing shrimp, but also the impairing host immunity that leads to secondary viral or bacterial infections) Rajendran *et al.*, 2016; Aranguren *et al.*, (2017. Moreover, HPM is reported to be associated with white feces syndrome (WFS). EHP infects and replicates in hepatopancreatic cells causing cell damage and detachment. The sloughing cells are excreted into the lumen of hepatopancreases thereby exposing the basement membrane. This situation is

prone to opportunistic infection by *Vibrio* bacteria inhabiting in the gut, and creating pathobiome conditions for WFS)Caro *et al.*, 2020; Piamsomboon and Han, 2022; Subash *et al.*, (2023. In Thailand and India, estimated economic losses from EHP were 76.4 and 573.03 million USD, respectively (Shinn *et al.*, 2018; Patil *et al.*, 2021). To our best knowledge, specific treatment against EHP is not yet available.

EHP has been classified as a fungus. It is an obligate intracellular parasite sorted within the phylum *Microsporidia*, and family *Enterocytozoonidae*. Fecal-oral is the main route of infection for this infection. EHP spores are shed with sloughing tubular cells through feces and contaminates the culture environment, including soil, sediment, and water (Salachan *et al.*, 2017; Chaijarasphong *et al.*, 2021). EHP spores are highly resilient, and they can persist in water without a host for more than 10 days)Pattarayingsakul *et al.*, (2021, and can survive across a wide range of salinities, from 0 to 30 ppt (Caro *et al.*, 2021; Jang *et al.*, 2022). It is difficult to eradicate EHP spores from the culture environment; therefore, precise biosecurity measures are necessary to control EHP contamination in shrimp culture systems.

The impacts of EHP are only reported in penaeid shrimp. For other crustacean or mollusk related to shrimp culture, they are only reported as carriers for this pathogen (WOAH, 2022). Mani *et al.* (2022) conducted EHP challenge *via* oral and intramuscular injection routes in several species of marine crabs, including orange mud crab (*Scylla olivacea*), giant mud crab (*Scylla serrata*), flower crab (*Portunus pelagicus*), Atlantic ghost crab (*Ocypode quadrata*), and red-spotted swimming crab (*Portunus sanguinolentus*). The results showed that these crabs could be infected with EHP without any signs of disease. Furthermore, Mondal *et al.* ((2023 reported the detection of EHP by PCR assay in natural *S. serrata* found in the coastal region of India. This information implies the possibility of inter-species transmission, which poses a significant risk to shrimp culture. Even though research on EHP has been conducted, the information regarding the transmission pathways of EHP through water and sediment between shrimp and crabs is still limited.

Mud crabs, also known as mangrove crabs, belong to the genus *Scylla* and are classified into four species, including *S. serrata*, *S. tranquebarica*, *S. paramamosain*, and *S. olivacea*. These species are inhabitants of mangroves and estuaries areas. Also, the crabs hold significant value for the aquaculture industry in the Indo-Pacific region (Ma *et al.*, 2010). Green mud crab (*S. paramamosain*) is distributed along the coastlines of the South China Sea and down to the Java Sea. It has become an economic specie cultured in the coastal areas of Southeast Asian countries, including Thailand (Shelley and Lovatelli, 2011). *S. paramamosain* is among the most common crab species, accounting for approximately 40% of the population found in the estuarine habitats surrounding major shrimp farming areas of Thailand (Sodsuk *et al.*, 2009; Kunsook *et al.*, 2022; DoF, 2023). Moreover, *S. paramamosain* is sometimes stocked in polyculture ponds with *P. vannamei* in Vietnam (Nguyen *et al.*, 2023). These scenarios may increase the risk of disease transmission through contamination in water or cohabitation. Therefore, the objective of this study was to investigate the possibility of EHP transmission between *P. vannamei* and *S. paramamosain*, through focusing on transmission pathways involving water and sediment. Particularly, our study explores the dynamics of EHP transmission from shrimp to shrimp, shrimp to green mud crabs, and back to shrimp using the cohabitation challenge model.

MATERIALS AND METHODS

Animal preparation: This study was carried out under the ethical guidelines of the Chulalongkorn University Animal Care and Use Committee (CU-ACUC: Approval No. 2431054). A group of 40 Pacific white shrimp (*P. vannamei*) with growth retardation syndrome was evaluated for EHP infection at Chulalongkorn University. Five shrimp from each population were screened for EHP using fresh hepatopancreas (HP) mount preparation and, examined at 1000X microscopy and confirmed with qPCR assay (Liu *et al.*, 2018). Populations with more than 30 spores per high-power field and EHP copy numbers above 10^4 copies/µL were selected as donors. Then, 85 specific-pathogen-free (SPF) *P. vannamei* from GAP-approved farms were confirmed EHP-free using qPCR assay and designated as recipients. Twenty green mud crabs were provided by the Chanthaburi Coastal Aquaculture Research and Development Center, Department of Fisheries, Thailand.

Experimental groups: During a 14-day acclimatization, donor shrimp (1.5±0.3g) were stocked in two 90-liter tanks (S0). Eighty recipient shrimp (0.5±0.2g) were equally divided into four aquaria: one negative control (S3) and three SPF-recipient groups (S1, S2, and S4; n=20 per tank). All tanks were maintained with 16 ppt artificial seawater, 4–7mg/L dissolved oxygen, <0.5 mg/L ammonia and nitrite levels, and temperature at 28–30°C. To minimize aggressive behavior between the crabs, lower stocking density was applied. A total of 20 crabs (25±5g) were divided into four 100-liter tanks (n=5 per tank), containing 15-liter seawater sufficient to cover the carapace of each crab. Experimental groups were assigned as negative control (C4), and triplicate challenge tanks (C1-C3). Water conditions were maintained as the shrimp tanks. The experimental design is illustrated in Fig. 1.

Water and sediment transmission challenge protocol

Intra-species transmission: This trial included a donor group (S0), a recipient positive control (S1), an EHP-negative control (S3), and a recipient negative control (S4). For 14 days, 80% of water in the S1 tanks was replaced daily with water from S0 tanks, while S4 tanks received SPF water from S3 tanks. Shrimp were euthanized with an anesthetic overdose (Aquanes®, Better Pharma, Thailand) for HP collection. Half of the shrimp in S1 were sampled at 7 days post-exposure (dpe), with the remainder at 14 dpe; all S4 shrimp were collected at 14 dpe. Histopathological samples were analyzed for EHP presence using nested PCR (Jaroenlak *et al.*, 2016), qPCR (Liu *et al.*, 2018), and histopathology (Srisuwatanasagul *et al.*, 2018).

Inter-species transmission: This trial involved donors (S0), the EHP-negative control (S3), an SPF-recipient group (S2), and green mud crabs (C1-C4). Each day,

water in the C1-C3 crab tanks was replaced with water from S0 tanks, while C4 received from S3. To determine the duration required for infection, crabs HP from each replicate tank were collected on 2-time points, 7 and 14 dpe, and tested with nested PCR and qPCR. In addition, a pooled feces sample from the C3 tank was screened for EHP positive using PCR assay. Following EHP confirmation from C2 and C3 tanks, C3 crabs were rinsed with sterile water before being transferred to new tanks to prevent surface contamination. Water and sediment from C3 were transferred to S2 for 14 days, following the previous sampling protocol. The shrimp from the S2 tanks were sampled on day 21 and 28 of the experiment (7 and 14 dpe), while the crabs from the C3 tanks were sampled on day 28 of the experiment.

Hepatopancreas DNA extraction and PCR assay: Genomic DNA was extracted from 25 mg of HP tissue using the NucleoSpin® Tissue kit (MACHEREY-NAGEL, Germany) following standard protocols, with samples achieving a 260/280 absorbance ratio ≥1.8. DNA concentration was set to 50 ng/µL and stored at −20°C. EHP detection was performed with nested PCR (Jaroenlak *et al.*, 2016), yielding 514 bp and 148 bp products. The first PCR step used primers SWP 1F and SWP 1R, while the second step used SWP 2F and SWP 2R. Products were analyzed by agarose gel electrophoresis under UV light. The qPCR assay (Liu *et al.*, 2018) used primers F157 and R157 in a TaqMan-based reaction on a CFX96 system (BIO-Rad, CA, USA). All primer sequences and procedures are described in Table 1.

Histopathological assay: Briefly, histopathology samples were fixed in Davidson's AFA solution. The samples were then sent to the Department of Anatomy, Faculty of Veterinary Science, Chulalongkorn University. An automatic tissue processor (Tissue-Tek VIP 5 Jr., Sakura, Japan) was used to process the tissue. Then, the processed samples were embedded in paraffin blocks. The samples were subsequently sectioned into 4µm thick slices using a microtome (Shandon, Anglia Scientific Instruments Ltd., UK) and mounted on slides for H&E staining. Selected slides were then digitized using a slide scanner (3DHISTECH), and photomicrographs were analyzed with CaseViewer software (3DHISTECH) (Srisuwatanasagul *et al.*, 2018).

RESULTS

The transmission challenge of EHP between Pacific white shrimp (*P. vannamei*) and the green mud crab (*S. paramamosain*) was evaluated through a laboratory bioassay. Clinical signs or mortality were not observed in all the challenged groups throughout the experiment.

Confirmation of EHP transmission by PCR assay

Intra-species transmission: EHP was tested negative in all shrimp from the negative control group (S3) and the negative recipient control groups (C4 and S4). All donor shrimp (S0) remained EHP-positive by the end of the experiment (Table 2), with EHP copy numbers in hepatopancreas ranging from 10^2 to 10^6 copies/µL. In the shrimp-positive control group (S1), 8 out of 10 shrimp were EHP-positive at 7 dpe, and all shrimp tested positive by 14 dpe. The EHP copy numbers ranged from 10^1 to 10^5 copies/µL at 7 dpe, and from 10^1 to 10^3 copies/µL at 14 dpe (Table 2).

Inter-species transmission: In the inter-species transmission study, all hepatopancreas of green mud crabs showed no pathological change in gross examination (Fig 2). All crabs in the C1 group tested negative for EHP at 7 dpe. However, 2 out of 5 crabs from the C2 group tested positive for EHP at 14 dpe. As a result, the C3 group was designated as the donor crab group, and by the end of the trial (14 days after water and sediment transfer back to shrimp), 3 out of 5 crabs in this group were EHP-positive.

Table 1: shows DNA sequence of primer and PCR procedure used in this study

	Primers name	sequence	Procedure
First step nested PCR	SWP 1F	5'-TTGCAGAGTGTTGTTAAGGGTTT-3'	Initial denaturation step at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 45 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes.
	SWP 1R	5'-CACGATGTGTCTTTGCAATTTTC-3'	
Second step nested PCR	SWP 2F	5'-TTGGCGGCACAATTCTCAAACA-3'	Initial denaturation step at 95°C for 5 minutes, followed by 20 cycles of denaturation at 95°C for 30 seconds, annealing at 64°C for 45 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes.
	SWP 2R	5'-GCTGTTTGTCTCCAACTGTATTTGA-3'	
qPCR	F157	5'-AGTAAACTATGCCGACAA-3'	Initial denaturation at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 5 seconds and extension at 60°C for 30 seconds
	R157	5'-AATTAAGCAGCACAATCC-3'	

Table 2: shows nested PCR and qPCR result from both intra-species and Inter-species transmission challenge.

Groups	Sampling days (dpe)	Nested PCR (positive samples)	qPCR (copies/ 50 ng HP DNA)
Donors			
S0 (Donor shrimp)	14	20/20	5.8×10^2 - 6.5×10^6
C3 (Donor crab)	28	3/5	8.2×10^1 - 7.1×10^2
Recipients			
S1 (Shrimp recipient I)	7	8/10	3.8×10^1 - 8.4×10^4
S1 (Shrimp recipient I)	14	10/10	2.2×10^1 - 6.9×10^2
C1 (Crab recipient I)	7	0/5	ND
C2 (Crab recipient II)	14	2/5	7.3×10^1-6.5×10^2
S2 (Shrimp recipient II)	7	0/10	ND
S2 (Shrimp recipient II)	14	6/10	6.1×10^1-5.1×10^4
Negative controls			
S3(Shrimp negative control)	-	ND	ND
S4 (Shrimp recipient control)	14	0/20	ND
C4 (Crab recipient control)	14	0/5	ND

dpe: day post exposure; ND: not determined.

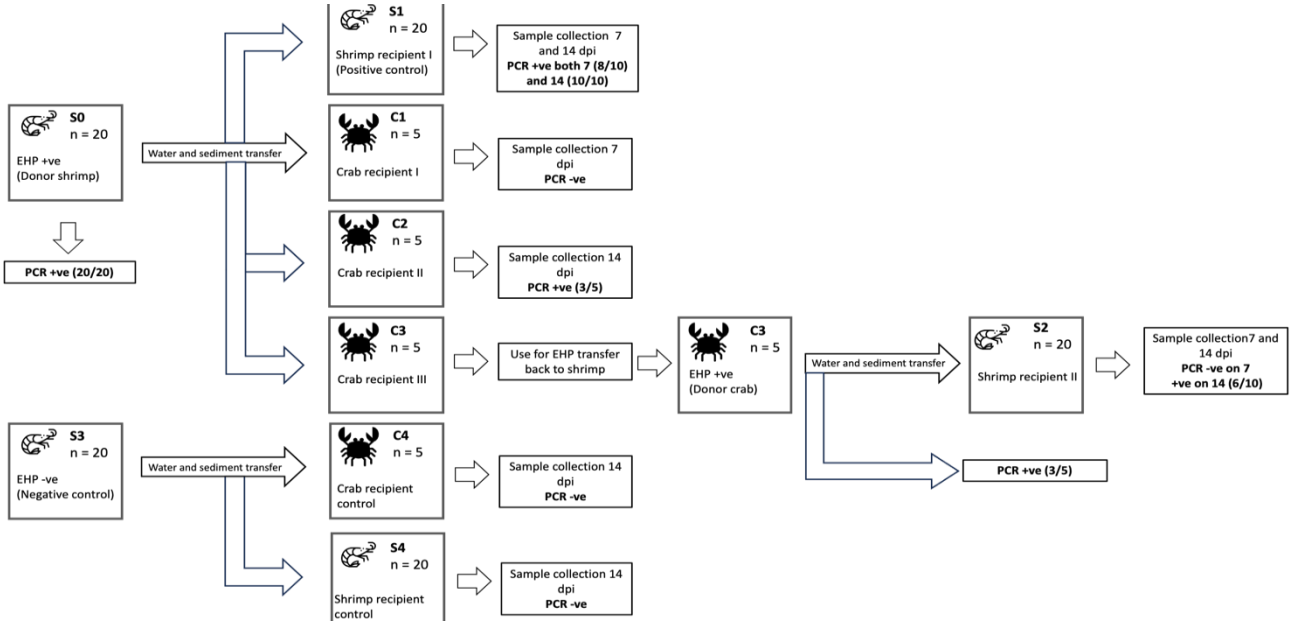

Fig. 1: Experimental design of EHP transmission challenge study.

Fig. 2: Hepatopancreas of green mud crab (*S. paramamosain*).

For the shrimp recipient group (S2), all shrimp tested negative for EHP at 7 dpe. By 14 dpe, however, nested PCR results showed that 6 out of 10 shrimp were EHP-positive, with copy numbers ranging from 10^1 to 10^5 copies/µL (Table 2). The nested PCR and qPCR results from both the intra-species and inter-species transmission challenge experiments are summarized in Table 2.

Histopathological assay: Histopathological analysis of EHP-positive shrimp revealed various stages of EHP development in hepatopancreatic tubular cells at 7 and 14 dpe, including early plasmodia, as well as branched and late plasmodia within the HP tubules (Fig. 3). In contrast, histopathological analysis of EHP-positive crabs showed a normal hepatopancreatic structure, with no EHP spores was observed (Fig. 4).

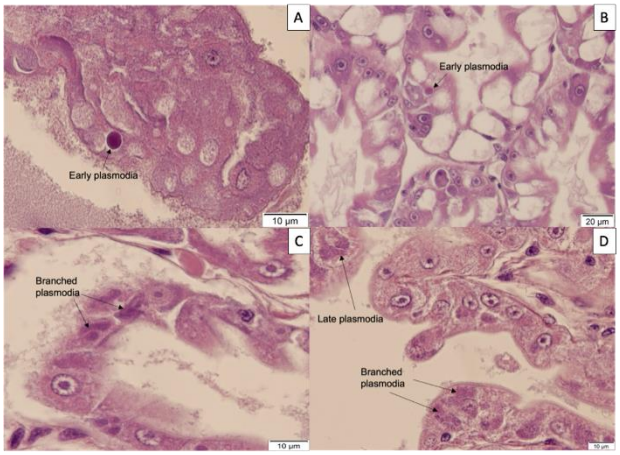

Fig. 3: Histopathological changes in EHP-infected recipient shrimp. Various stages of the microsporidian infection are shown, including early plasmodia on day 7 (A) and day 14 (B), as well as branched and late-stage plasmodia in the hepatopancreas on day 7 (C) and day 14 (D).

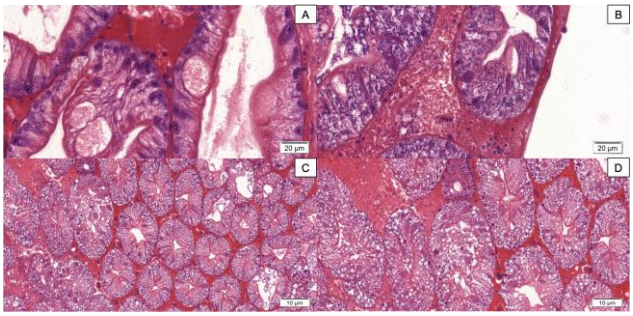

Fig. 4: Histopathological analysis of EHP-infected recipient crab group on day 7 (A) and day 14 (B), and the shrimp negative control group on day 7 (C) and day 14 (D). The images show normal HP structures with no EHP spores observed in any of the samples.

DISCUSSION

The majority of shrimp farms are located near coastal areas. They are often exposed to various marine crustaceans, particularly many species of wild crabs and shrimp. Those coastal animals may act as biological or mechanical vectors

for several shrimp diseases, harboring the pathogens inside their body without pathological effects (Lo *et al.*, 1996; Kanchanaphum *et al.*, 1998). Free roaming behavior of those animals leads them into shrimp ponds due to the availability of food sources. In addition, pathogens are constantly released into the water that is pumped into a farm, increasing the risk of disease occurrence. EHP is among the shrimp pathogens that can persist in several hosts. The present study investigated the potential for EHP transmission between Pacific white shrimp (*P. vannamei*) and green mud crab (*S. paramamosain*) using a laboratory bioassay. Our findings suggested that green mud crabs may act as a mechanical vector for EHP transmission without sustaining any pathological changes or clinical signs.

The fecal-oral route is the main transmission route for EHP. The slough hepatopancreatic cells with EHP spore called aggregated transformed microvilli (ATM) are excreted into the lumen of the gastrointestinal tract and mixed with fecal matter (Salachan *et al.*, 2017; Mai *et al.*, 2020; Pattarayingsakul *et al.*, 2021). Interestingly, our findings indicated that the EHP copy numbers in the recipient positive controls were lower than in the donor shrimp. This is because shrimp were kept under experimental conditions with low stocking density and optimal water quality parameters. Therefore, EHP cannot effectively replicate in a less stressful environment, unlike those in culture ponds. The impact of EHP on shrimp's health is related to the EHP copy number. Caro *et al.* (2023) described that reduced disease severity is associated with lower EHP copy number in HP tissue. This discrepancy may be attributed to the longer infection duration and different culture conditions of the donor shrimp compared to the recipients. A positive correlation was identified between EHP infection and several factors, such as stocking density and high concentrations of ammonia and nitrite in ponds (Nkuba *et al.*, 2021; Geetha *et al.*, 2022). Therefore, good aquaculture practices, such as reducing stocking density and maintaining good water quality, especially dissolved oxygen and nitrogen waste, are recommended methods to reduce stress and control EHP infection in shrimp farming systems (Chaijarasphong *et al.*, 2021). Moreover, the immune system of healthy shrimp reared under optimal conditions can limit EHP replication and mitigate the impact of this pathogen. Although the recipient shrimp showed a lower EHP copy number in this study, infection from crabs was confirmed through the presence of early plasmodia observed in the hepatopancreas *via* histopathology.

Several species of aquatic inhabitants in shrimp culture environments were investigated for their potential role as either mechanical or biological carriers of EHP (Mondal *et al.*, 2023; Wan Sajiri *et al.*, 2023; Guo *et al.*, 2024). For instance, false mussels (*Mytilopsis leucophaeata*) were identified as mechanical carriers of EHP. Although these mussels did not show any clinical signs, cohabitation with infected mussels for 10 days resulted in 37.5% of the exposed naïve shrimp were tested positive for EHP using PCR assay (Munkongwongsiri *et al.*, 2022). Similarly, studies on dragonfly nymphs (*Ischnura senegalensis* and *Pantala flavescens*) showed that shrimp could become infected after oral ingestion or cohabitation with EHP-infected nymphs (Dewangan *et al.*, 2023). In a study involving Indian marine crabs (*S. olivacea*, *S. serrata*, *P. pelagicus*, *O. quadrata*, and *P. sanguinolentus*), EHP was introduced *via* intramuscular injection and oral feeding. Histopathological examination of the hepatopancreas of these crabs at 5 dpe revealed immature spores with no pathological changes. PCR results from feces and hemolymph were positive at 5 dpe but turned negative at 10, 25, and 50 dpe (Mani *et al.*, 2022).

The histopathological examination of challenged crabs showed normal hepatopancreatic tubular cells, and no EHP-related structure was observed. Our results support the study of Mani *et al.* (2022), showing that EHP did not infect and multiply in the hepatopancreas cells of the crabs, indicating the role of crabs as mechanical carriers of EHP. Various shrimp pathogens can be carried by several species of mud crab (Somboonna *et al.*, 2010; Saravanan *et al.*, 2021). However, while EHP transmission has been studied in *S. serrata* and *S. olivacea* (Mani *et al.*, 2022; Mondal *et al.*, 2023), it has not yet been examined in *S. paramamosain*. This is the initial report demonstrating that *S. paramamosain* can harbor EHP and subsequently transmit the pathogen to Pacific white shrimp. Practically, crabs in the environment can receive EHP spore *via* wastewater from shrimp farms. Crabs can migrate to other areas or into other culture ponds and shed EHP spores into the water particularly those farms without proper water treatment protocols and biosecurity structures that prevent those marine benthic animals from entering the ponds. Although biosecurity measures in several intensive shrimp farms are strictly implemented, such as using chemical disinfectants (15ppm potassium permanganate for 15 minutes, 40ppm of 65% active chlorine for 15 minutes, or 2.5% sodium hydroxide) to eliminate EHP spores during pond preparation or equipment disinfection (Aldama-Cano *et al.*, 2018; Newman, 2018). These methods cannot prevent crabs or other aquatic animals from entering the ponds and spreading the disease. Thus, strategies to eliminate and manage these vectors during cultivation should be considered in shrimp aquaculture.

Conclusions: The present study demonstrates that EHP can be transmitted between Pacific white shrimp (*P. vannamei*) and green mud crabs (*S. paramamosain*) through water and sediment transfer. qPCR assay was used to determine the quantities of EHP that were transmitted between hosts. Our results confirmed that asymptomatic crabs may act as a mechanical vector, posing a biosecurity risk for shrimp aquaculture. This crab specie is a native inhabitant of coastal areas of Thailand where major shrimp farms are located. The findings suggest enhanced management practices to control EHP spread, thereby supporting sustainable shrimp farming practices.

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Authors contribution: PT contributed to the project through conceptualization, data curation, formal analysis, and the preparation of the original draft. OC supported the project through data curation, formal analysis, and original draft writing, while CS was involved in the investigation and drafting. AC and NS provided essential resources for the investigation. SS contributed to data curation,

validation, and manuscript review and editing. HJ and BK focused on data curation and formal analysis. JH and PP were involved in conceptualization, funding acquisition, project administration, supervision, and manuscript review and editing.

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