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# Study: White Feces Syndrome in shrimp can be caused by more than one pathogen

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By Luis Fernando Aranguren Caro, Ph.D. , Hung N. Mai, Ph.D. , Roberto Cruz-Florez, Ph.D. , Frances Laureen Agcalao Marcos , Rod Russel R. Alenton, Ph.D. and Arun K. Dhar, Ph.D.

## **Aquaculture Pathology Laboratory finds conclusive evidence that EHP is not the only pathogen associated with the presence of White Feces Syndrome in *Litopenaeus vannamei***

The shrimp gut microbiota is fundamental to the host's nutrition, growth, pathogen resistance and maintenance of the internal steady state or homeostasis. Feeding characteristics of shrimp, such as actively grazing and cannibalism, make the host vulnerable to pathogen invasion. Colonization by alternative microorganisms destabilize the intestinal microbiota, which leads to infections and co-infections in a taxonomically diverse gastrointestinal (GI) tract.

Although there is limited information on specific immune mechanisms provided by the shrimp gut microbiota, there is evidence of its interplay with the digestive activities that directly affect shrimp



Cephalothorax (head + thorax) of Pacific white shrimp (*Litopenaeus vannamei*) displaying white discoloration of the gastrointestinal tract. Results of this study demonstrated for the first time the experimental reproduction of WFS under laboratory conditions when shrimp are concurrently infected with EHP and *V. parahaemolyticus*.

growth and severity of diseases. Likewise, new emerging diseases were found to target the shrimp hepatopancreas [digestive gland] and causing growth inhibition, size disparity, loss of appetite and chronic mortality. Recently identified shrimp diseases that exhibit these clinical signs are the hepatopancreatic microsporidiosis (HPM) caused by *Enterocytozoon hepatopenaei* (EHP) and White Feces Syndrome (WFS), whose causative agent or agents remain unidentified.

HPM is a disease of the hepatopancreas in penaeid shrimp caused by several species of microsporidians [spore-forming, unicellular parasites] including EHP. This microsporidian species has been found in several Asian countries such as China, Vietnam, Malaysia, Indonesia, Thailand, and India. Recently, EHP has been reported in the Western Hemisphere in South America. The main clinical sign of EHP at the farm level is shrimp growth retardation, leading to an increased size variability and feed conversion rate (FCR). In advanced stages of the disease, EHP-infected shrimp typically display soft shells and chronic mortalities. EHP has been reported in several species of farmed penaeid shrimp, including black tiger prawn *P. monodon*, *L. vannamei* and *P. stylirostris*. EHP is an intracellular parasite that proliferates within the cytoplasm of the affected tubule epithelial cells in the hepatopancreas.

At the farm level, WFS manifests as white fecal strings floating along the surface water in grow-out ponds. Shrimp in these ponds display unusual clinical signs such as poor growth rate, size disparity, gastrointestinal tract with yellowish to whitish discoloration, softshell, and chronic mortalities. Although an association between EHP and WFS has long been suggested, no studies have successfully reproduced this syndrome in a controlled laboratory setting. Even when severe EHP infections occur, WFS does not always manifest, and these observations suggests that WFS is the result of an interplay of more than one microorganism and another causative agent(s) are yet to be identified. A strong association between WFS and EHP was reported in EHP-endemic regions in Southeast Asia and Venezuela. In these cases, WFS and EHP were detected along another opportunistic pathogen, *Vibrio* spp., that causes Septic Hepatopancreatic Necrosis (SHPN).



(<http://info.globalseafood.org/goal-2022-save-the-date>).

This article – adapted and summarized from the **original publication** (<https://doi.org/10.1371/journal.pone.0261289>). (Aranguren, L.F. et al. 2021. Experimental reproduction of White Feces Syndrome in whiteleg shrimp, *Penaeus vannamei*. *PLoS ONE* 16(12): e0261289) – reports on our study to isolate and identify a strain of *Vibrio parahaemolyticus* as the missing link between EHP and WFS. Co-infecting EHP-infected shrimp by an immersion challenge with a unique strain of *V. parahaemolyticus* that was isolated from gut of a shrimp (*L. vannamei*) displaying WFS allowed us to provide the first experimental evidence of WFS reproduction. To our knowledge, this is the first demonstration of the reproduction of WFS under controlled laboratory conditions using two unique pathogens.

## Study setup

Specific pathogen free (SPF) *L. vannamei* were obtained from the Oceanic Institute (Oahu, Hawaii). For at least two consecutive years, this population has tested negative by PCR for all OIE-listed pathogens as well as OIE non-listed pathogens, including EHP. The bioassay was carried out in the Aquaculture Pathology Laboratory of the University of Arizona. All procedures for the sampling and euthanasia of shrimp were performed following the guidelines established by the American Veterinary Medical Association.

For detailed information on the experimental design and animal husbandry; isolation of *Vibrio parahaemolyticus* from *L. vannamei* shrimp displaying WFS and inoculum preparation; experimental challenge to reproduce WFS; histopathology and in-situ hybridization; PCR and quantitative PCR for detection of *V. parahaemolyticus* and quantification of EHP; and statistical analyses, refer to the original publication.

## Results and discussion

Results of our study describe for the first time the experimental reproduction of WFS in shrimp, *L. vannamei* pre-infected with EHP (primary pathogen) and challenged with a particular *V. parahaemolyticus* (secondary pathogen) isolated from the GI tract of a shrimp displaying WFS. Our data provides empirical support of a strong association of EHP with WFS as recorded in different regions of the world. In Indonesia, from 2016 to 2018, and in Venezuela in 2019, we observed shrimp with clinical signs of WFS displayed histological lesions typical of EHP and SHPN. Our histological

findings revealed a similar scenario, as shrimp experiencing clinical manifestations of WFS under laboratory-challenged conditions also displayed histological lesions that are reminiscent of EHP and SHPN pathology.

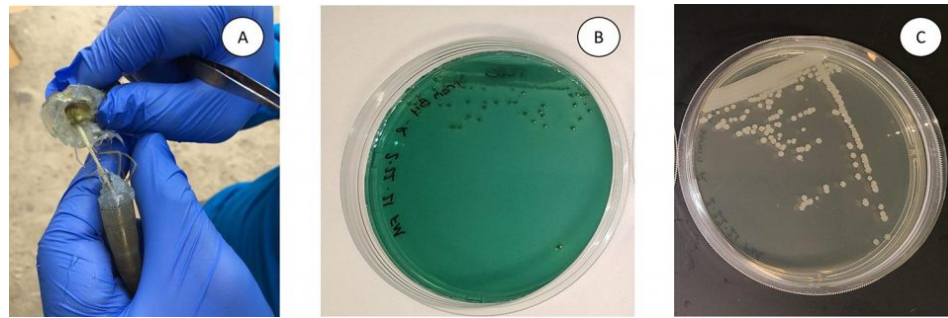


Fig. 1: Isolation of *Vibrio parahaemolyticus* from *Litopenaeus vannamei* GI-tract displaying WFS. A juvenile *L. vannamei* shrimp displaying white gastrointestinal (GI) tract, a clinical manifestation of WFS. A section of the GI tract was plated on a TCBS plate (a). CFU Sucrose negative indicating *Vibrio parahaemolyticus* growth in TCBS plate (b). Growth of *V. parahaemolyticus* in TSA+2.5% NaCl medium (c).

## Aranguren, WFS, Table 1

Trial	Tank	Group	Initial number of animals	Final number of animals	Final survival (%)	CV%	WFS
1	1	SPF negative control	7	6	85.7	21.4	No
1	2	SPF + <i>V. parahaemolyticus</i>	7	6	85.7	27.6	No
1	3	EHP positive control	8	8	100	41.6	No
1	4	EHP + <i>V. parahaemolyticus</i>	7	4	57.1	40	Yes
2	1	SPF negative control	10	9	90	12	No
2	2	SPF + <i>V. parahaemolyticus</i>	10	8	80	10.1	No
2	3	EHP positive control	9	4	44.4	30.8	No
2	4	EHP + <i>V. parahaemolyticus</i>	8	2	25	40.7	Yes

Table 1. Experimental design and comparative survival of shrimp pre-infected with EHP and challenged with *V. parahaemolyticus* (WFS isolate) in two independent trials.



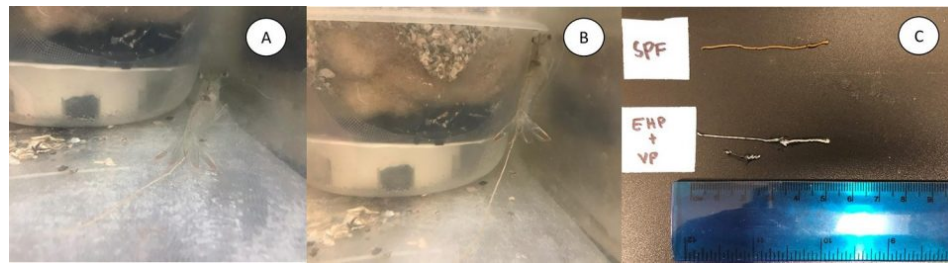


Fig. 2: Successful reproduction of WFS in *Litopenaeus vannamei* under a laboratory experimental condition. Shrimp displaying clinical signs of White Feces Syndrome (WFS) in shrimp that were pre-infected with EHP and challenged with *Vibrio parahaemolyticus* via immersion route. White to yellowish colored fecal strings are observed in animals in different treatments. The black arrow indicates white fecal string adhering to the anal opening in EHP infected shrimp that were challenged with *V. parahaemolyticus*. Adapted from the original.

The White Feces Syndrome has proven to have one of the most elusive etiologies of any of the shrimp disease that have been characterized to date. It has been more than a decade since the first formal report of EHP-infected shrimp showing WFS was published, although anecdotal evidence suggests WFS has been observed in shrimp grow-out ponds prior to this publication. Despite the presence of WFS over a decade, the etiology [causation] of the syndrome remains inconclusive.

There are also several publications with contradictory claims with respect to the role of EHP in WFS. For example, some authors reported WFS is not caused by EHP while others found a strong evidence of a clear link between EHP and WFS. There is one report claiming *Vibrio cholerae* as the etiologic agent of WFS, although this finding has not been confirmed by other researchers from anywhere else despite the fact WFS is an emergent problem worldwide.

Several other studies aimed to investigate the WFS phenomenon have also found some association between WFS and different etiologies. Fecal strings containing vermiform structures called aggregated transformed microvilli (ATM) has been reported as one of the causative agents of **WFS in Thailand** (<https://doi.org/10.1371/journal.pone.0099170>). WFS appears to be correlated with coinfection with EHP and other opportunistic *Vibrio* spp. that **cause SHPN** (<https://doi.org/10.1016/j.aquaculture.2016.12.038>). In India, running mortality syndrome (RMS) has been reported in shrimp displaying WFS and SHPN. Researchers studying microbiome in WFS affected shrimp reported a significant bacterial increase in *Candidatus Bacilloplasma* and *Phascolarctobacterium* and decreases in *Paracoccus* and *Lactococcus* may contribute to **WFS in shrimp** (<https://doi.org/10.1007/s00253-018-8855-2>). Heavy gregarine [intestinal parasites] infestation, vibriosis and hemocytic enteritis, some specific bacterial taxon or “pathobiome” [groups of species associated with a host that, when present, reduce host strength under proper environmental conditions], and different **environmental conditions** (<https://doi.org/10.1016/j.jip.2020.107458>), in a grow-out pond have been proposed to be the **contributing factors** (<https://doi.org/10.1038/s41598-020-68891-6>) for causing WFS.

While studies to reproduce WFS in a laboratory setting have been attempted by several authors, few have been successful in consistently reproducing this syndrome and determining the etiology. **Huang et al.** (<https://doi.org/10.1186/s40168-020-00802-3>), recently found that transplantation of the intestinal

microbiota of shrimp displaying WFS to healthy shrimp leads to similar clinical manifestations as in WFS. They proposed WFS is caused by dysbiosis [microbial imbalance] in intestinal microbiota and this complex disease could be caused by particular pathogens. While a non-infectious etiology is worth further investigation, we cannot disregard the fact that WFS has been consistently associated with particular pathogens notably EHP. Furthermore, the constant co-occurrence of certain patterns of the **bacterial communities** (<https://doi.org/10.1038/s41598-020-68891-6>) in shrimp that present WFS lead us to hypothesize that WFS is the result of the synergetic effects of two pathogens with EHP possibly acting as a primary pathogen and an uncharacterized bacteria belonging to the family Vibrionaceae acting as a secondary pathogen.

Under laboratory challenge experiments, we were able to confirm the presence of white fecal strings only in shrimp that were pre-infected with EHP and subsequently exposed to the *V. parahaemolyticus* isolate. This demonstrates that, in order to induce clinical manifestations of WFS, both EHP and this particular *V. parahaemolyticus* isolate are required. Shrimp exposed only to EHP or *V. parahaemolyticus* did not develop WFS. Therefore, WFS manifestation involves a unique combination of two pathogens that had not been observed previously for any infectious disease of shrimp known to date.

## Aranguren, WFS, Table 2

Trial	Treatments	Clinical signs	Number of samples	EHP % detection by H&E histology	SHPN % detection by H&E histology	EHP detection by in situ hybridization	EHP detection by real time PCR
1	SPF negative control	No WFS	6	0	0	0	
1	SPF + <i>V. parahaemolyticus</i>	No WFS	6	0	0	0	
1	EHP positive control	No WFS	8	100	37.5	100	
1	EHP + <i>V. parahaemolyticus</i>	WFS	6	100	83.3	100	
2	SPF negative control	No WFS	6	0	0	NT	
2	SPF + <i>V. parahaemolyticus</i>	No WFS	6	0	0	NT	
2	EHP positive control	No WFS	5	100	60	NT	
2	EHP + <i>V. parahaemolyticus</i>	WFS	5	100	60	NT	

Table 2. A summary of histopathology and PCR data of *Penaeus vannamei* pre-infected with EHP and challenged with *Vibrio* sp. NT: not tested.

Typically, in a shrimp grow-out pond, the cultured population starts showing the presence of EHP first. Later on, during the grow-out cycle, shrimp start developing white discoloration of the GI tract, and floating white fecal strings start appearing in the pond. By H&E [one of the main stains to study tissues] histology, we observed a similar sequential order in cellular pathology during the infection process. A primary pathogen, in this case EHP, caused the typical histological lesions and later on affected shrimp display SHPN caused by opportunistic *V. parahaemolyticus* that was used to challenge the EHP-infected shrimp. The histopathology image in Fig. 3D clearly displays co-infection of EHP as represented by a typical plasmodium inclusion and bacteria in the same HP tubule. This establishes the role of a primary pathogen like EHP to accentuate the impact of opportunistic bacteria like *Vibrio* sp. resulting WFS.

Fig. 3: Histopathology analysis of hepatopancreas tissue. H&E (Mayer–Bennet hematoxylin and eosin-phloxine) staining of hepatopancreas tissue of *Litopenaeus vannamei* from four experimental groups: SPF negative control (A), SPF+ *V. parahaemolyticus* (B), EHP positive control (C), and EHP+ *V. parahaemolyticus* (D). Presence of cytoplasmic inclusion bodies (black arrows) that correspond to a plasmodium stage and spores (arrowhead) typical of EHP infection are shown. The scale bars of 200 µm (A), 50 µm (B) and 10 µm (C-D) are indicated. Adapted from the original.

Shrimp in the treatment group EHP+*V. parahaemolyticus* in both of our trials displayed a high size disparity, which is higher or comparable to shrimp infected with EHP or *V. parahaemolyticus* alone (Table 1). A similar finding was also reported by Liu et al. (2015. Development of real-time PCR assay for detection of microsporidian *Enterocytozoon hepatopenaei* and detection in shrimp samples under different. *Prog Fish Sci.* 2015.). These authors reported the impact of EHP on size variation of shrimp in a grow-out farm when the pathogen load was found to be  $>10^3$  copies/ng total DNA in hepatopancreatic tissue. In our study, the mean EHP load in shrimp displaying clinical signs of WFS was  $1.4 \times 10^7$  copies/ng of extracted DNA, which is higher from what Liu et al. (2015) mentioned is required to observe high size variation. In previous studies, shrimp displaying WFS show a copy number in the order of  $\sim 10^6$  copies/ng of extracted **hepatopancreas DNA** (<https://doi.org/10.3354/dao03522>).

The reproduction of WFS in shrimp infected with EHP and challenged with *V. parahaemolyticus* is very likely due to the synergistic actions of the microsporidian and a particular bacterial strain on hepatopancreatic tubules. As mentioned previously, Huang and colleagues suggested that WFS is caused by intestinal microbiota dysbiosis and this proposition bears some similarities to our finding. Our observations during the early stage of EHP infection in shrimp displaying WFS include high lipid content in the hepatopancreatic tubule but some hepatopancreas cells being heavily infected. However, at this point, there is no obvious cellular shrimp immune response such as inflammation or hemocytic congestion. Later, as the infection progresses, EHP-infected epithelial cells in the HP tubule slough and expose the underlying basal membrane of the tubule to opportunistic bacteria that rapidly colonizes the affected tubules.

Bacterial colonization then leads to a cell response of the host resulting in hemocytic inflammation, subsequent melanization [pigmentation] and further sloughing. There is a moderate to severe atrophy of the hepatopancreas tubule cells leading to a partial or total dysfunction of the hepatopancreas. In a healthy shrimp, the bulk of the feces generated upon digestion of feed consists of residual vacuoles from hepatopancreas cells, which involve a major process for packaging and removing waste products from the digestive system. In EHP-infected shrimp, when the cluster of hepatopancreas cells are released from the tubule to the GI tract, the combination of lipids, digested/undigested feed, EHP and the bacterial mass will contribute with the formation of a whitish fecal string. It is possible, due to the presence of lipids in the feces, that the fecal mass becomes lighter compared to feces from a healthy shrimp and floats on the water surface water instead of sinking to the pond bottom.

Alterations involving the gastrointestinal tract, where a sign of the disease is whitish stools, are common to all animals including humans. In humans, whitish stools are caused by changes in the gut microbiome or by particular pathogens such as *Cryptosporidium* spp. This might also be the case for shrimp WFS, where the disease – and therefore the particular signs – are caused by two distinct etiologies. Our study clearly shows that the two pathogens induce white feces in shrimp and decisively shows that EHP plays a central role in the development of this syndrome, as our research group proposed in **previous research** (<https://www.globalseafood.org/advocate/white-feces-syndrome-shrimp-predictor-ehp/>).

Our study provides conclusive evidence that EHP is not the only pathogen associated with the presence of White Feces Syndrome. In Thailand, some studies have shown the association of WFS in shrimp population with an increase of *Vibrio* spp. where the intestines in shrimp, with and without WFS, were analyzed. An increase of intestinal *Vibrio* population from a normal shrimp with  $1.8 \times 10^7$  CFU [colony forming units]/g population versus WFS-shrimp with  $6.1 \times 10^7$  CFU/g. Some of these *Vibrio* spp found in those samples were *V. parahaemolyticus*, *V. vulnificus* and *V. damsela*. This corroborates our identification of a strain of *V. parahaemolyticus* as the unidentified pathogen co-infecting with EHP on shrimp manifesting WFS. However, it is possible that other *Vibrio* species in addition to *V. parahaemolyticus* could induce WFS.



## Conclusions

EHP is a primary enteric pathogen of penaeid shrimp. Infection with EHP in association with a particular strain of *V. parahaemolyticus* leads to the development of WFS. These findings also corroborate and provide an explanation as to why WFS is manifested in areas where EHP and *V. parahaemolyticus* are also endemic.

## Authors

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**LUIS FERNANDO ARANGUREN CARO, PH.D.**

Corresponding author  
Aquaculture Pathology Laboratory  
School of Animal and Comparative Biomedical Sciences  
The University of Arizona  
Tucson, Arizona, USA

[lfarangu@email.arizona.edu](mailto:lfarangu@email.arizona.edu) (<mailto:lfarangu@email.arizona.edu>).



**HUNG N. MAI, PH.D.**

Aquaculture Pathology Laboratory  
School of Animal and Comparative Biomedical Sciences  
The University of Arizona  
Tucson, Arizona, USA



**ROBERTO CRUZ-FLOREZ, PH.D.**

Ensenada Center for Scientific Research and Higher Education (CICESE)  
Baja California, Mexico



**FRANCES LAUREEN AGCALAO MARCOS**

Aquaculture Pathology Laboratory  
School of Animal and Comparative Biomedical Sciences  
The University of Arizona  
Tucson, Arizona, USA



**ROD RUSSEL R. ALENTON, PH.D.**

Aquaculture Pathology Laboratory  
School of Animal and Comparative Biomedical Sciences  
The University of Arizona  
Tucson, Arizona, USA



**ARUN K. DHAR, PH.D.**

Aquaculture Pathology Laboratory  
School of Animal and Comparative Biomedical Sciences  
The University of Arizona  
Tucson, Arizona, USA

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